A randomized equivalence study evaluating the efficacy of two commercially available teat sealants in dairy cows

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Abstract

The use of internal teat sealants is common in the U.S. to prevent new intramammary infections during the dry period. The objective of this study was to compare the efficacy of a new internal teat sealant containing bismuth subnitrate (SO[a]) to the first product introduced to the U.S. dairy industry (ORB[b]). The hypothesis was completed using a multi-site randomized, positively controlled equivalence evaluation of new intramammary infection risk difference over the dry period. At dry-off, milk samples were collected for culture and then cloxacillin benzathine[c] was administered, followed by the randomly assigned internal teat sealant (SO = 404 cows; ORB = 418 cows). After calving, repeat milk samples were collected. The effect of treatment on quarter-level new intramammary infection risk, cured intramammary infection risk, and risk of presence of intramammary infection post-calving was determined using generalized linear mixed models. The effects of treatment on cow-level outcomes, including incidence of clinical mastitis, culling and death, as well as performance in early lactation based on test-day milk production and somatic cell count, were also evaluated. The dry period new intramammary infection adjusted risk difference (SO minus ORB) was -1.60% (95% CI -5.62, 2.42). Final models demonstrated that there was no difference in risk rates of quarter-level outcomes between treatment groups. Analysis of cow-level factors, including clinical mastitis, culling and death rate within the first 120 DIM, also revealed no differences. The results of this study indicate that SO was equivalent to ORB for dry period new intramammary infection risk when utilizing blanket dry-cow therapy.

Key words: dry cow therapy, intramammary infections, bovine mastitis, teat sealant

Introduction

Subclinical intramammary infection (IMI) at dry-off is a known risk factor for development of clinical mastitis during the subsequent lactation.[1] Subclinical IMI at dry-off (DO) or during the dry period can also contribute to decreased production and milk quality during the subsequent lactation.[2] Formation of a keratin plug is the natural defense of the udder to physically block the entry of pathogens through the streak canal, however, the length of time required for keratin plug formation after DO varies widely between individuals, with some teats taking up to 6 weeks to develop the keratin plug.[3] This leaves the gland at risk to acquire a new IMI during the dry period. The use of an internal teat sealant (ITS) containing bismuth subnitrate at DO is one method to mitigate the risk of acquiring a new IMI during the dry period.[4,5]

The first ITS product on the U.S. market (ORB[b]) has been shown to be effective at preventing new IMI during the dry period when used with an intramammary dry cow antimicrobial.[6] Development of alternative ITS products has created a competitive market which benefits dairy producers. However, clinical evaluation is imperative to ensure that the efficacy of these new products meets or exceeds that of currently available products. The objective of this study was to compare an alternative ITS containing bismuth subnitrate (SO[a]) to ORB in a randomized, positively controlled equivalence trial evaluating quarter-level IMI dynamics during the dry period and cow-level health events during the first 120 days in milk (DIM). Our hypothesis was that SO would be equivalent to ORB in efficacy at preventing new intramammary infections in the subsequent lactation.

Materials and methods

Study design, population and enrollment

This multi-state study was conducted by Iowa State University (ISU) and the University of Minnesota (UMN). Prior to initiation of the study, study protocols were approved by ISU and UMNs' respective Institutional Animal Care and Use Committee's (ISU protocol number 21-068; UMN protocol number 2102-38826A). The study was carried out at 2 commercial dairy farms in Iowa and 4 commercial dairy farms in Minnesota from May to September 2021. To be eligible for enrollment in the study, cows were required to have an expected dry period of 30 to 90 days, at least 3 functional quarters, a body condition score > 2.0 out of 5.0, and have a lameness score < 4 out of 5. Cows that farm management had designated for culling before 120 days into the subsequent lactation were ineligible for enrollment. Finally, cows were excluded if they had received any antimicrobial treatment within 14 days prior to DO. All dairies practiced routine pre- and post-milking teat disinfection as part of their milking routine. Eligible animals were identified the week before DO and were randomized by farm

into 1 of 2 treatment groups (SO or ORB). To assure approximately equal enrollment between groups each week and on each farm, randomization was blocked within every 6 cows to be dried off, by using the "rand ()" function in a spreadsheet program.[d]

Study technicians collecting milk samples and applying treatments were not blinded to treatment, as they needed to know treatment assignment to treat animals correctly. However, farm care staff and laboratory personnel responsible for culturing milk samples were blinded to treatment.

Study treatments

Cows were dried off once per week on each study farm. On the day of DO, study personnel traveled to farms to enroll cows. Prior to DO, farm records were verified that no cows had been treated with antimicrobials, and animals were evaluated to ensure that they still met enrollment criteria. Prior to milking, study personnel collected aseptic, duplicate, quarter-level DO milk samples following National Mastitis Council guidelines for sample collection.[7] After milking machine detachment, all cows were administered 500 mg of cloxacillin benzathine[c] per quarter followed by their assigned sealant (SO or ORB). Post-milking teat dip was applied per farm protocol and cows were moved to their respective dry-cow facilities. Dry-period management took place per each individual farm's protocol.

Microbiological techniques

Following sample collection, milk samples were immediately placed on ice and transported to each university's respective research laboratory where samples were frozen at -20°C for at least 24 hours prior to culture. Afterward, 1 of each set of the duplicate quarter samples was submitted to the respective investigator's veterinary diagnostic laboratory for routine aerobic culture per laboratory protocols for each respective institution, with colony counts recorded for each isolate. The veterinary diagnostic laboratories use standardized procedures for culture set up and confirmation of growth that has been used on previous studies by 2 of the authors (PG and SG).[8] Briefly, culture procedures were performed after allowing the milk to warm to room temperature. Milk was transmitted onto agar media using a 0.01 mL calibrated loop. Agar plates were incubated at 37°C for 48 h and interpreted by an experienced lab technician. Following bacterial isolation, MALDI[e] was used for speciation of microbes. Isolation of a single colony of bacteria from a quarter was indicative of an IMI, with the following exceptions: Non-aureus *Staphylococcus* species required at least 2 colonies (≥ 200 cfu/mL) to be considered infected, while *Bacillus* spp. isolates needed 5 or greater colonies (≥ 500 cfu/mL) to be considered infected. Quarters producing these pathogens at lower levels were reclassified as "No Growth" to improve specificity.[9] If culture determined that the first sample was contaminated, the second sample from that quarter was submitted for culture. Quarters were classified as contaminated if 3 or more bacterial isolates with different morphological features were recovered from both quarter samples collected at the same timepoint.[8]

All milk samples with microbiological growth underwent matrix assisted laser desorption/ionization-time of flight mass spectrometry (MALDI) to determine bacterial identity, with each diagnostic laboratory utilizing the same MALDI library. To ascertain bacterial identification, if the MALDI reported confidence level was > 2.0 the identification was reported at the species level, while if the confidence level was 1.8 to 2, only genus level identification was recorded. If the MALDI confidence level was < 1.8, traditional identification methods (colony morphology, catalase reaction, gram stain and cytology) were used to determine bacterial identification.

To simplify pathogen evaluation, pathogens were broken into the following categories: *Staphylococcus aureus*; **Group A** – Non-aureus staphylococci (NAS: *S. chromogenes, S. haemolyticus, S. hyicus, S. sciuri, S. simulans, S. xylosus, S. saprophyticus* and all other *Staphylococcus* species); **Group B** – Strep group – containing the streptococci and *Streptococcus*-like species (*Enterococcus* sp., *S. dysgalactiae*, *S. uberis*, all other *Streptococcus* species, and *Lactococcus* sp.); **Group C** – Other Gram positives (*Bacillus* sp., *Corynebacterium* sp., *Micrococcus* sp.); **Group D** – Other Gram positives (Gram-positive cocci and Gram-positive rods); **Group E** – Coliforms (*Escherichia coli*, *Klebsiella pneumoniae* and all *Serratia* sp.); **Group F** – Other Gram negatives (*Acinetobacter* sp. and other Gram-negative [non-coliform] organisms); and yeast.

Dry period and post-calving follow-up

Within 14 days of parturition, duplicate, aseptic, quarter-level post-calving (PC) milk samples were collected by study personnel for culture. Samples were handled similarly to the pre-dry-off samples for determination of IMI status. Health events for enrolled cows were identified by farm personnel and tracked in herd record software, which was monitored during the dry period and for 120 days PC. Monthly DHIA test day milk production and somatic cell count data were also captured through 120 days in milk.

Statistical analysis

Sample size calculation

Sample size was determined based on an equivalence hypothesis evaluating the effect of ITS on new intramammary (NIMI) risk over the dry period. Treatments were assigned at the cow level, with an a *priori* NIMI margin of equivalence (Δ) established at 5%, α = 2.5%, and power of 80%. We assumed that 20% of quarters would develop a NIMI over the dry period[10] and the equivalence margin was selected based on previous work.[11] Based on these assumptions, we estimated that 1,320 quarters (330 cows) would be required for each treatment. These estimates were inflated 1.2 times to account for clustering within the data and to account for some anticipated missed samples, contaminated samples, and loss to follow up post-calving. The final sample size was determined to be 1,600 quarters (400 cows) per treatment group.

Data management

All cow- and quarter-level treatment and laboratory data were captured in spreadsheets[d] housed within a cloud-based data management and sharing environment[f]. Milk yield and $\log_e$ SCC were generated through monthly DHIA testing. Disease or health event date (clinical mastitis, culling and death) data were captured from the farms' electronic data management systems.

Data merging and cleaning, as well as data analyses, were performed using commercial statistical software[g] using the packages *lme4, nlme, car, emmeans, multcomp, stats* and *survival*. The data sets and analyses can be downloaded at: **https://github.com/gustavossvet/ISU-Gorden_equivalence_study.** Continuous variables were evaluated visually for normality

using quantile-quantile plots. Somatic cell count data was not normally distributed, so these were $\log_e$ transformed for analysis using the equation: $\log_e$ (cells per mL/1,000 + 1).

Individual cows were retrospectively excluded from all analyses if their dry period was outside the 30-to-90-day range, or if they received a parenteral or intramammary antimicrobial treatment between calving and collection of the PC samples. Quarters that failed to have a determined IMI status (e.g., due to missed or contaminated samples) were excluded from prevalence evaluations for that time point and all risk evaluations. Additionally, if the PC sample was not collected within 14 days after parturition, these quarters were excluded from further evaluation.

Effect of treatment group on quarter-level IMI dynamics

A comparison of culture results from DO and PC milk samples was used to evaluate dry period IMI dynamics. Per Rowe et al.,[8] a cured intramammary infection (CIMI) was defined as a quarter with an IMI at dry-off and either no growth or the presence of a different IMI pathogen at the PC sampling. A NIMI was defined as either a quarter with no growth at dry-off and a positive PC culture result, or a positive culture result at dry-off and different species-level pathogen present at the PC sampling. Isolates were matched at the genus-level if species was not able to be determined.

A generalized linear mixed model (logistic regression) was used to build a multivariable model to assess the differences between treatments for each explanatory variable (IMI at enrollment, IMI at 1-14 DIM, CIMI risk and NIMI risk) while controlling for other factors. Potential cow-level confounders evaluated in all models included parity at dry-off, milk yield (lb) at the last DHIA test-day before enrollment, $\log_e$ SCC at the last DHIA test-day before enrollment, and DIM at the PC sample.[11] Models accounted for clustering of quarters and cows by including random intercepts of cows and herds.

The multivariable models were built including all potential cow-level associated factors. Before entering the potential controlling variables in the model, variables that were highly correlated to each other, as determined by having a Pearson or Kendall's tau correlation coefficient > 0.7, were removed, and only 1 variable was used to assess confounding. In these instances, the most suitable variable was chosen based on biological plausibility for each model. Confounders were then added to the model one by one and retained in the final model as fixed effects if they were significant. Interaction terms with Wald tests at $P < 0.05$ were retained in the final model, and potential confounders were removed from the model one at a time. If removal changed the effect estimate by more than 10%, the covariate was added back to the model.[12]

An equivalence analysis was conducted to evaluate effect of ITS treatment on quarter-level dry period NIMI risk with an a priori margin of equivalence (Δ) established at ± 5%. The null hypothesis tested was that risk of NIMI for SO was either ≤ -5% or ≥ +5% risk of NIMI for ORB (risk difference = SO NIMI risk minus ORB NIMI risk). To conduct the hypothesis test, a 2-sided, 95% confidence interval for the risk difference was used. If the upper and lower limits of the 95% confidence interval are within the boundaries of equivalence (Δ), SO would be equivalent to ORB. No superiority tests were conducted. Since the reporting of the risk difference is more appropriate for equivalence trials, the risk difference and confidence intervals were obtained from the output of the generalized linear mixed models (odds ratio) and converted into risk ratio using the formula:

$$\text{Risk difference} = \frac{\text{Odds ratio}}{1 - Rc + (Rc \times \text{Odds ratio})}$$

where Rc = adjusted risk of the reference group (ORB). This method was used to obtain the risk ratio since the odds ratio will overestimate risk when risk is greater than 10%. This equation modifies the outcome to better approximate the actual risk.[13]

Effect of treatment group on cow-level outcomes

The effect of treatment group on milk yield and loge SCC were analyzed using repeated measures models. A linear mixed model was constructed, with cow and herd as random intercepts to account for the clustering of tests within cows, and cows within herds. A compound symmetry correlation structure was used. To assess the differences over time, the time in lactation was added into the model at 6 levels by DIM (level 1 = 1 to 20 DIM, 2 = 21 to 40 DIM, 3 = 41 to 60 DIM, 4 = 61 to 80 DIM, 5 = 81 to 100 DIM, 6 = 101 to 120 DIM). To assess the differences between treatment over time, an interaction term between treatment and time was added in the model. Procedures for model building and addressing potential confounders were the same as those utilized when evaluating quarter-level IMI dynamics. Least square means were used to compare differences between treatment groups.

Clinical mastitis, culling and death events before 120 DIM were assessed using generalized linear mixed models (logistic regression) for each individual outcome. Procedures for model building to address potential confounders were the same as those utilized when evaluating quarter-level IMI dynamics. Clinical mastitis cases were defined as the first case of mastitis noted by farm personnel from 0 to 120 DIM. The analysis followed the same process described above. In addition, survival analysis was conducted to determine the effect of ITS treatment on the time to outcome (clinical mastitis, culling and death during the first 120 DIM) on separate models. Cows that died or were culled before calving were excluded (left-censored) from this measurement. Cows were right-censored if they developed clinical mastitis, at culling, death or when they reached 120 DIM. Kaplan-Meier survival curves were generated and tested with the log-rank test to compare the association between treatment groups, and hazard ratios (HR) were estimated using Cox proportional hazards regression. Clustering of cows within herds was mitigated using a robust sandwich estimator within these estimates. Schoenfeld residuals were compared over time in order to assess proportional hazards assumptions for each covariate using the Schoenfeld test. The final Cox proportional hazards models for the effect of treatment group on cow-level outcomes were produced using the same potential confounders and model building strategies that were applied to evaluate quarter-level IMI dynamics.

Results

Demographics

Descriptive demographics of the herds involved in the study and the cows enrolled are noted in Table 1. The number of cows enrolled per dairy varied from 29 to 221, and the herd size of study farms varied from 779 to 2886 cows. Bulk tank SCC at the outset of the study was between 163,000 and 501,000 cells/mL. A total of 848 cows (ORB = 428; SO = 420) were initially randomized; however, 26 cows (13 from each randomization

Table 1: Comparison of study herds for herd- and cow-level characteristics at dry off.

	Farm A	Farm B	Farm C	Farm D	Farm E	Farm F	Overall
Cows enrolled	203	221	125	38	29	206	822
State	IA	IA	MN	MN	MN	MN	
Predominant breed	Jersey	Crossbred	Holstein	Holstein	Jersey	Holstein	
Milking herd size	1916	1950	2886	2559	779	1735	
Bulk tank SCC (x1,000 cells/mL)	179	163	417	438	501	217	
Dry cow bedding system	Sand	Composted bedded pack	Manure solids	Sawdust	Sawdust	Sand	
Lactating cow bedding system	Sand	Sand/ composted bedded pack	Manure solids	Manure solids	Sawdust	Sand	
Treatment group allocation, no. (%)							
ORB[1]	100 (49%)	112 (51%)	65 (52%)	20 (53%)	14 (48%)	107 (52%)	418 (51%)
SO[2]	103 (51%)	109 (49%)	60 (48%)	18 (47%)	15 (52%)	99 (48%)	404 (49%)
Mean (SD) SCC at last test (log cells × 10^3)	2.6 (1.3)	2.3 (1.5)	3.1 (1.7)	2.9 (1.5)	2.0 (1.6)	2.6 (1.8)	2.6 (1.6)
Mean (SD) milk yield at last test (lb/d)	52.4 (12.6)	64.2 (12.7)	71.7 (19.7)	70.1 (22.6)	53.7 (9.6)	78.7 (18.4)	65.9 (18.7)
Crude prevalence (95% CI) of IMM[3] infection at dry off	23.6% (20.4, 26.6%)	24.9% (21.8, 28.0%)	46.6% (41.7, 51.5%)	27.4% (19.4, 35.4%)	39.8% (30.0, 49.6%)	43.6% (39.8, 47.4%)	32.8% (31.0, 34.5%)
Parity at enrollment, no. (%)							
1	94 (46.3)	66 (29.9)	51 (40.8)	12 (31.6)	10 (34.5)	71 (34.5)	304 (37.0)
2	32 (15.8)	73 (33.0)	35 (28.0)	14 (36.8)	9 (31.0)	56 (27.2)	219 (26.6)
3	37 (18.2)	39 (17.6)	17 (13.6)	5 (13.2)	5 (17.2)	44 (23.3)	151 (18.4)
4	26 (12.8)	24 (10.9)	17 (13.6)	5 (13.2)	4 (13.8)	23 (11.7)	100 (12.2)
≥5	14 (7.9)	19 (8.6)	5 (4.0)	2 (5.3)	1 (3.4)	7 (3.4)	48 (5.8)

[1] ORB = Orbeseal (Zoetis, Parsippany, NJ)
[2] SO = ShutOut (Merck & Co., Inc., Rahway, NJ)
[3] IMM = Intramammary

assignment) were not enrolled because they failed to meet the inclusion criteria on the day of dry-off. On the day of enrollment, 3 cows that were initially randomized to receive SO inadvertently received ORB. These cows were left in the final analysis in the ORB group, following an on-treatment approach.[14] At dry-off, 22 quarters from ORB-assigned cows were non-functional, while 27 SO-assigned quarters were non-functional. As a result, 418 cows (1,650 quarters) were enrolled in the ORB group and 404 cows (1,589 quarters) were enrolled in the SO group. Figure 1 outlines cow- and quarter-level exclusions at each phase of the study minus contaminated quarters. Table 2 summarizes measured demographics based on treatment groups as treated at DO and demonstrates that randomization effectively balanced treatment groups against potential confounders. After DO procedures, no adverse events related to either treatment were noted.

Loss to follow-up

Figure 1 details the loss to follow-up for each treatment group throughout the study. Pre-calving exclusion criteria included culling due to a short dry period, a long dry period, abortion or death, with 16 cows excluded during the dry period (ORB = 13 cows; SO = 3 cows). Retrospective evaluation of DO culture results indicated 457 quarters out of 3,239 (14.1%; ORB = 223, SO = 234) were contaminated. After calving, 304 quarters (ORB = 142, SO = 162) were not collected within 14 days of calving and 466 quarters of the 2,871 remaining quarters (16.2%; ORB = 225, SO = 241) were contaminated on the PC samples, leaving 2,085 quarters (ORB = 1,072, SO = 1,013) eligible for risk evaluation. The overall level of contaminated quarters was 15.2% and was approximately equally distributed by state (IA = 13.8%, MN = 16.5%). Animal exclusions during the 14-day PC period that resulted in missed sampling occurred due to culling for dystocia, metritis, hypocalcemia, hospitalization and death. The final number of cows at risk for mastitis and death or culling in the

Table 2: Comparison of treatment groups for cow-level characteristics at dry off.

	ORB[1] n = 418	SO[2] n = 404	Overall n = 822
Mean (SD) milk yield at last test (lb/d)	66.4 (18.4)	65.5 (19.0)	65.9 (18.7)
Mean (SD) $\log_e$ SCC at last test	2.6 (1.6)	2.6 (1.5)	2.6 (1.6)
Crude prevalence (95% CI) of IMM[3] infection at dry off	33.5% (31.1, 36.0%)	32.0% (29.5, 34.5%)	32.8% (31.0, 34.5%)
Parity at enrollment, no. (%)			
1	147 (35.2)	157 (38.9)	304 (37.0)
2	113 (27.0)	106 (26.2)	219 (26.6)
3	70 (16.7)	81 (20.0)	151 (18.4)
4	61 (14.6)	39 (9.7)	100 (12.2)
≥ 5	27 (6.5)	21 (5.2)	48 (5.8)

[1] ORB = Orbeseal (Zoetis, Parsippany, NJ)
[2] SO = ShutOut (Merck & Co., Inc., Rahway, NJ)
[3] IMM = Intramammary

first 120 DIM were 405 for ORB and 401 for SO. The 120-day follow up was not completed by 52 ORB-treated cows (42 culled, 10 died) and 42 SO-treated cows (31 culled, 11 died).

Culture results at dry-off and post-calving

The grouping of culture results from isolates at DO is shown in Figure 2a and the grouping at PC is shown in Figure 2b. The isolates represented in the figures encompassed 93.3% (851/912) of all pathogens identified in DO cultures and 93.2% (714/766) of pathogens isolated from PC cultures. The remaining bacteria isolated represented a mixture of minor species, each isolated in low numbers.

At DO, the prevalence of infection was 32.8% (912 of 2,782 quarters). Bacteria from the groups containing non-aureus *Staph* (groups A) and Other gram-positives (group C) were isolated from the highest percentage of quarters, 13.6% (378/2,782) and 8.6% (240/2,782), respectively. Bacteria from other groups were isolated at much lower levels, the Strep group (Group B) represented 1.6% of all quarters sampled at dry-off (59/2,782), Misc. gram-positives (Group D) infected 4.0% of quarters (110/2,782), coliforms (Group E) infected just 0.4% of quarters (10/2,782), and Other gram-negatives (Group F) represented 1.5% of quarters sampled (41/2,782). *Staphylococcus aureus* was identified in 0.3% of quarters sampled at DO (9/2,782 quarters), while 3 species of yeast (*Candida krusei, Candida tropicalis* and one unspecified yeast) were identified in 0.14% of quarters (4/2,782 quarters). The most common species isolated was *Staphylococcus chromogenes* at 8.16% of DO samples (227/2,782).

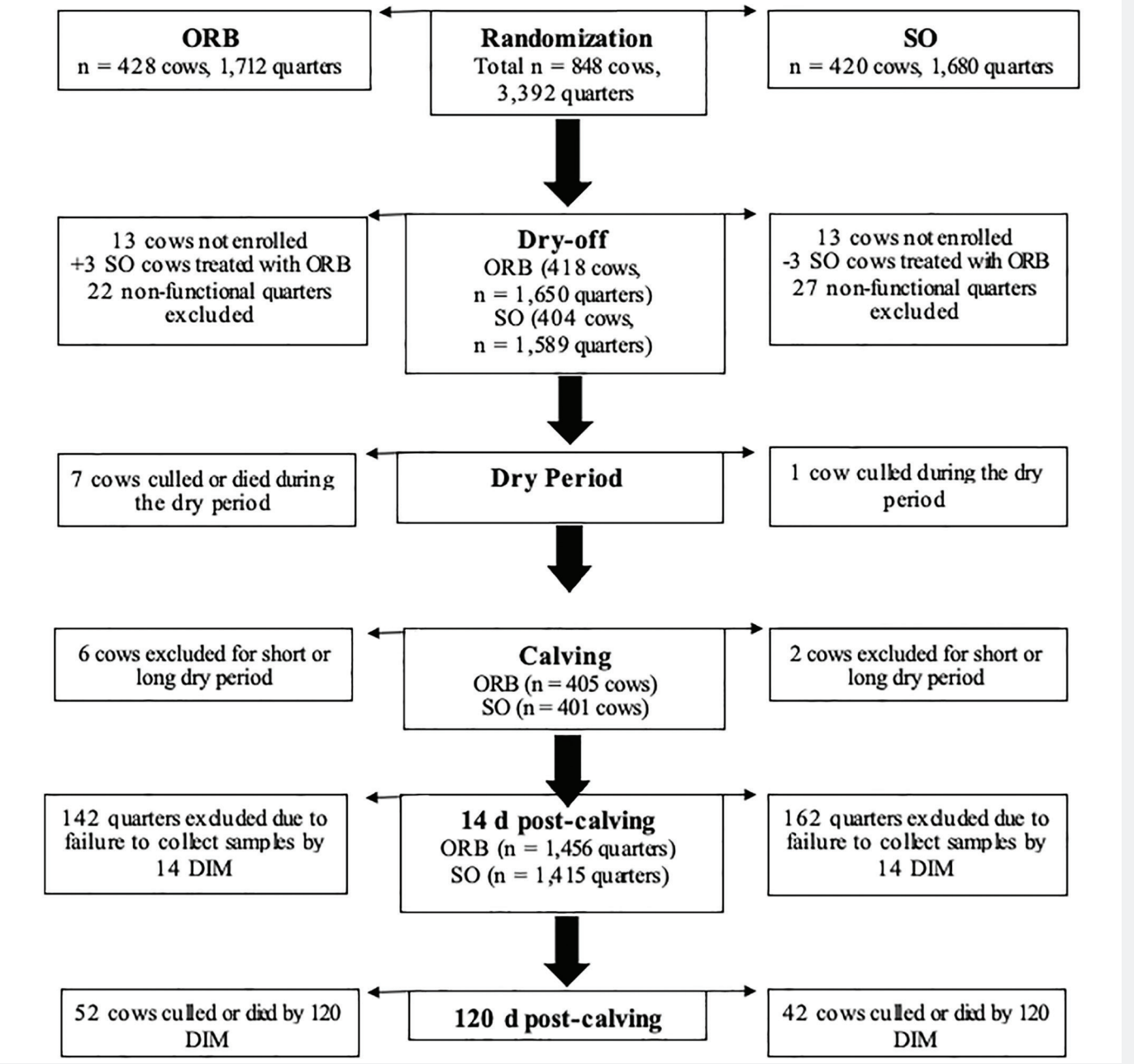
Culture of PC samples found the non-aureus *Staph* group pathogens to be the most prevalent, representing 14.2% of quarters sampled (342/2,405). The Other Gram-positive group (C) and Misc. Gram positives (D) were present in similar amounts, with 7.2% (172/2,405) and 5.6% (135/2,405) of isolates, respectively. The Other Gram-negative group (F) followed at 1.3% (31/2,405), with the *Strep* group (B) representing 1.1% (26/2,405), and the Coliform group (E) at 0.2% of pathogens identified (6/2,405). *Staphylococcus aureus* was isolated from 2 quarters at PC sampling and no yeast were identified. The most prevalent pathogens identified on PC sampling were *Staphylococcus xylosus/saprophyticus* and *Staphylococcus chromogenes,* found in 4.5% (108/2,405) and 4.1% (99/2,405) of samples, respectively.

Effect of treatment group on quarter-level outcomes

Quarter-level crude IMI prevalence for all cows at DO was 32.8% (912/2,782, 95% CI 31.0, 34.5%) with approximately equal crude prevalence between ORB quarters (33.5%; 475/1,416, 95% CI 31.1, 36.0%) and SO quarters (32.0%; 437/1,366, 95% CI 29.5, 34.5%). Crude prevalence IMI at DO by farm ranged from 23.6 to 46.6% and was similar between treatment groups (Table 1), except for Farm E which had a crude prevalence of 28.3% and 53.3% within the ORB and SO groups, respectively (data not shown). This farm had the least number of animals enrolled at 29. The final outputs of the generalized linear mixed models are shown in Table 3. The adjusted risk for IMI at DO was 31.79% for ORB quarters versus 30.08% for SO (risk difference = -1.71, CI -5.68, 2.26). Final models for IMI at Dry Off (IMI at DO) included random intercepts for cow, herd and cow within herd. The only fixed-effect covariate included in the final model due to evidence for confounding when using the 10% change in estimate approach was lactation. The combined ICC accounting for clustering of cows within herds and for herds was 0.14.

Quarter-level crude prevalence of IMI for all cows at PC sampling (1 to 14 DIM) was 31.8% (766/2,405, 95% CI 30.0, 33.7). Similar crude prevalence levels were determined between ORB quarters (32.6%, 402/1,231, 95% CI 30.1, 35.3) and SO quarters (31.0%, 364/1174, 95% CI 28.4, 33.7). Across farms, the crude prevalence of IMI at PC ranged from 16.7 to 54.4% with similar prevalence between treatment groups by farm, except for Farm E which had a crude prevalence of 41.1% and

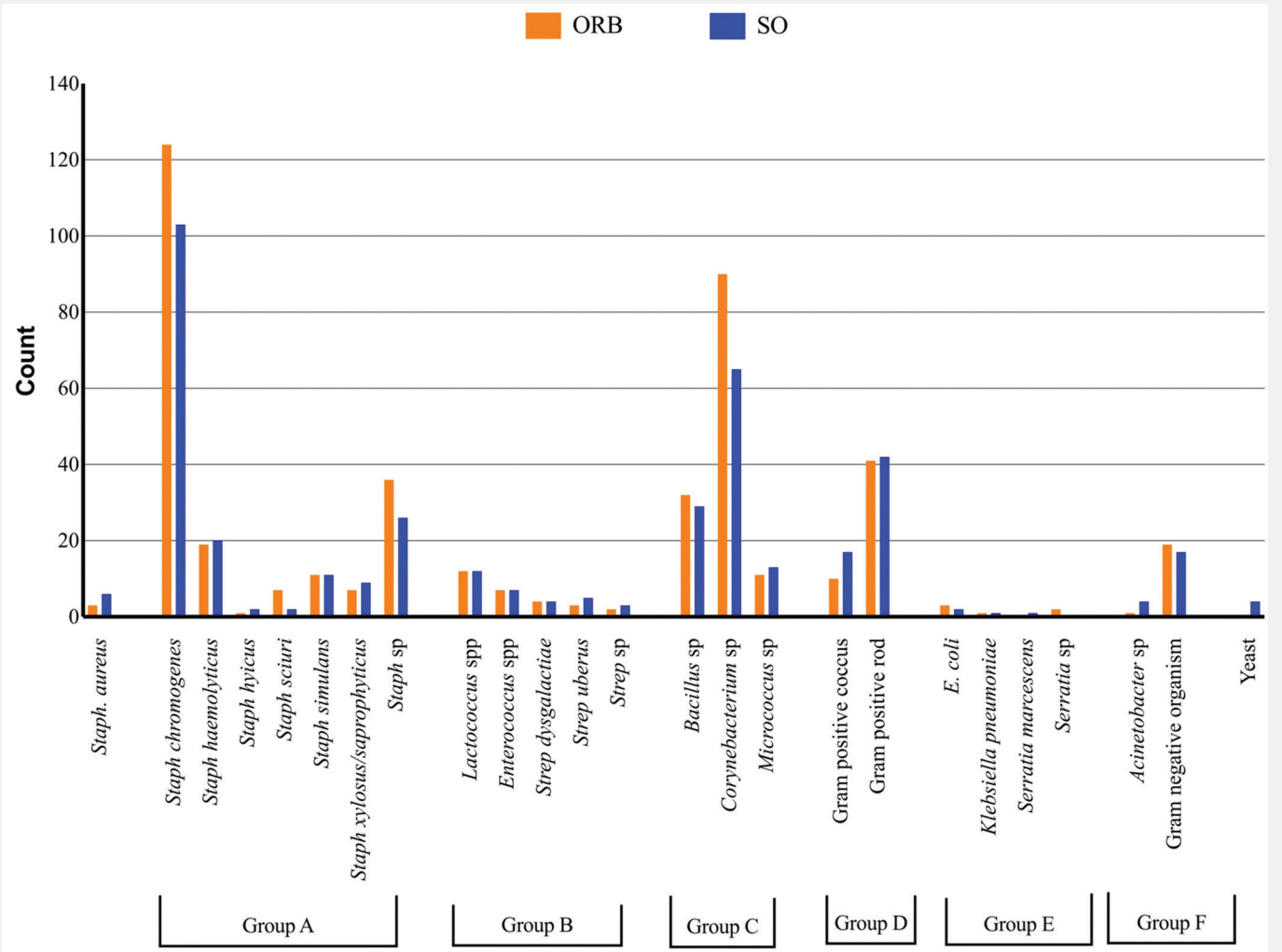
Figure 1: Loss to follow-up summary for cows for cows treated with one of two internal teat sealants after application of a dry cow antimicrobial. Contaminated quarter counts are not included.



[1] ORB = Orbeseal (Zoetis, Parsippany, NJ)

[2] SO = ShutOut (Merck & Co., Inc., Rahway, NJ)

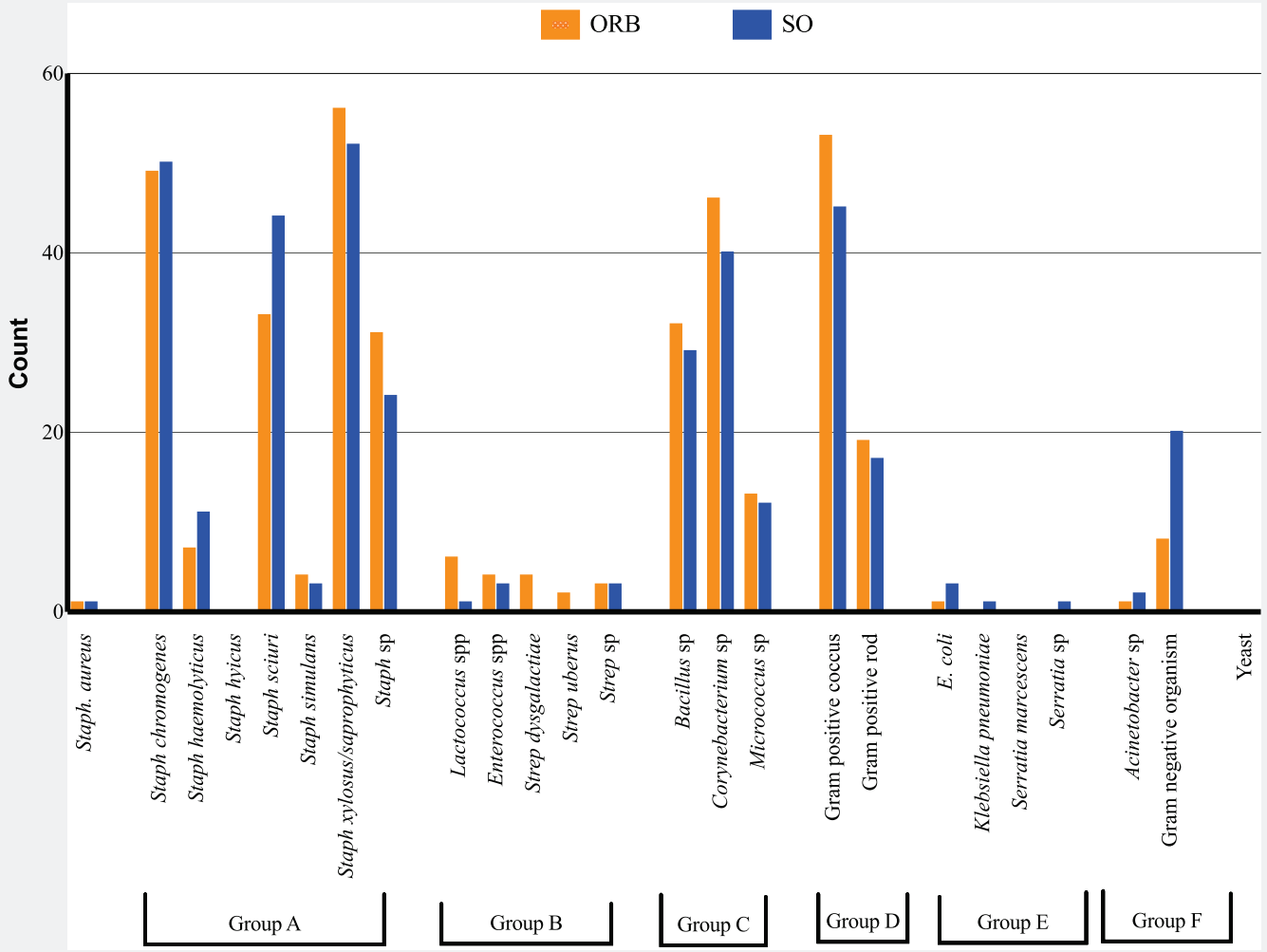
Figure 2a: Intramammary pathogens* identified at dry-off sampling for cows treated with an internal teat sealant at dry off.[1]



[1] ORB = Orbeseal (Zoetis, Parsippany, NJ)

[2] SO = ShutOut (Merck & Co., Inc., Rahway, NJ)

Figure 2b: Intramammary pathogens* identified at post-calving sampling for cows treated with an internal teat sealant at dry off.[1]



* Significant pathogens identified at dry off and post-calving sampling *Staphylococcus aureus*,

Group A – Non-aureus staphylococci (*S. chromogenes, S. haemolyticus, S. hyicus, S. sciuri, S. simulans, S. xylosus/saprophyticus* and all other *Staphylococcus* species),

Group B – Streptococci & *Streptococcus*-like species (*Lactococcus sp., Enterococcus sp., S. dysgalactiae, S. uberis* and all other *Streptococcus* species),

Group C – Other gram positives (*Bacillus* sp., *Corynebacterium* sp., *Micrococcus* sp.)

Group D – Gram-positive cocci & Gram-positive rods

Group E – Coliforms (*Escherichia coli, Klebsiella pneumoniae* and all *Serratia* sp.)

Group F – Other Gram negatives (*Acinetobacter* sp. and Gram-negative organisms)

Yeast

[1] ORB = Orbeseal (Zoetis, Parsippany, NJ) and SO = ShutOut (Merck & Co., Inc., Rahway, NJ)

23.6% in the ORB and SO group, respectively (data not shown). The final generalized linear mixed model determined that the adjusted IMI risk at PC was 35.46% for ORB and 32.89% for SO (risk difference = -2.57, 95% CI -6.64, 1.50) (Table 3). Final models included random intercepts for cow and herd with no fixed-effect covariates included in the final model as evidence for confounding was not present. The combined ICC accounting for clustering of cows within herds and for herds was 0.20. The clustering variation of quarter within cows was assessed but no evidence was found to support an association and was excluded from the final model.

Crude risk of cure for the entire study population was 96.3% (674/700, CI 94.6, 97.4), with 95.9% (350/365, CI 93.3, 97.5) of ORB quarters and 96.7% (324/335, CI 94.2, 98.2) of SO quarters attaining a cure during the dry period. Crude cure risk by farm ranged from 93.7 to 97.8% and was similar between treatment groups for all farms (data not shown). The final generalized linear mixed model determined that the adjusted cure risk was 95.89% for ORB and 96.72% for SO (risk difference = 0.83, 95% CI -1.96, 3.62%) (Table 3). The final model for CIMI risk included random intercepts for cow and herd, and no fixed-effect covariates were included in the final model as evidence for confounding was not present. The clustering variation of quarter within cows was assessed but no evidence was found, and this was excluded from the final model.

Crude NIMI risk was 30.6% (638/2085, CI 28.7, 32.6%), with 31.0% (332/1072, CI 28.3, 33.8%) of ORB treated quarters and 30.2% (306/1013, CI 27.5, 33.1%) of SO quarters obtaining a NIMI during the dry period. Across farms, the NIMI risk ranged from 17.2 to 54.4% and did not differ considerably by treatment within farm, except for farm D which had a NIMI risk of 47.7% for the ORB group and 60.9% for the SO group and farm E with a NIMI risk of 43.4% for the ORB group and 24.4% for the SO group (data not shown). The final generalized linear mixed model determined that the adjusted NIMI risk was 33.11% for ORB and 31.51% for SO (risk difference = -1.60%, 95% CI of RD -5.62, 2.42) (Table 3). The final model for NIMI risk included random intercepts of cow and treatment as fixed-effect and no covariates. The combined ICC accounting for clustering of cows within herds and for herds was 0.22. As the NIMI risk difference indicated that the NIMI risk for SO was 1.60% lower than for ORB and the upper margin of the 95% confidence interval for risk difference was below the upper margin of equivalence (Δ) of +5%, SO was determined to be equivalent to ORB (Figure 3).

Effect of treatment group on cow-level outcomes in the first 120 days in milk

Figure 4a and Table 4 illustrate milk yield (in lb) for each treatment group stratified by DIM in lactation for the first 120 DIM. Least square means for milk yield was 91.15 lb for the ORB group versus 90.35 lb for the SO group. A repeated measures model was utilized to evaluate this data with the fixed effects of treatment and DIM by level included and no additional covariates. Based on this model, no difference was identified between treatment groups (milk yield difference = -0.80 lb, CI -3.55, 1.95) and treatment by month interaction. There was an effect of days in milk ($P < 0.001$), but no significance on the interaction was identified between treatment and DIM.

Table 3: Generalized linear mixed models (logistic regression) for quarter-level intramammary infection (IMI) dynamics during the dry period based on treatment group.

	Adjusted risk* (%)	ß	SE	Risk difference (%)	95% CI of risk difference† (%)	*P*-value
IMI at DO						
ORB[1]	31.79	Referent				
SO[2]	30.08	0.08	0.11	-1.71	-5.68, 2.26	0.46
IMI at PC						
ORB	35.46	Referent				
SO	32.89	0.11	0.11	-2.57	-6.64, 1.50	0.31
Cure IMI						
ORB	95.89	Referent				
SO	96.72	-0.23	0.40	0.83	-1.96, 3.62	0.56
New IMI						
ORB	33.11	Referent				
SO	31.51	0.07	0.12	-1.60	-5.62, 2.42	0.54

* Risk estimates using estimated marginal means.

† Risk difference and confidence intervals were derived from generalized linear mixed models using the log odds scale and converted into risk ratio using the formula:

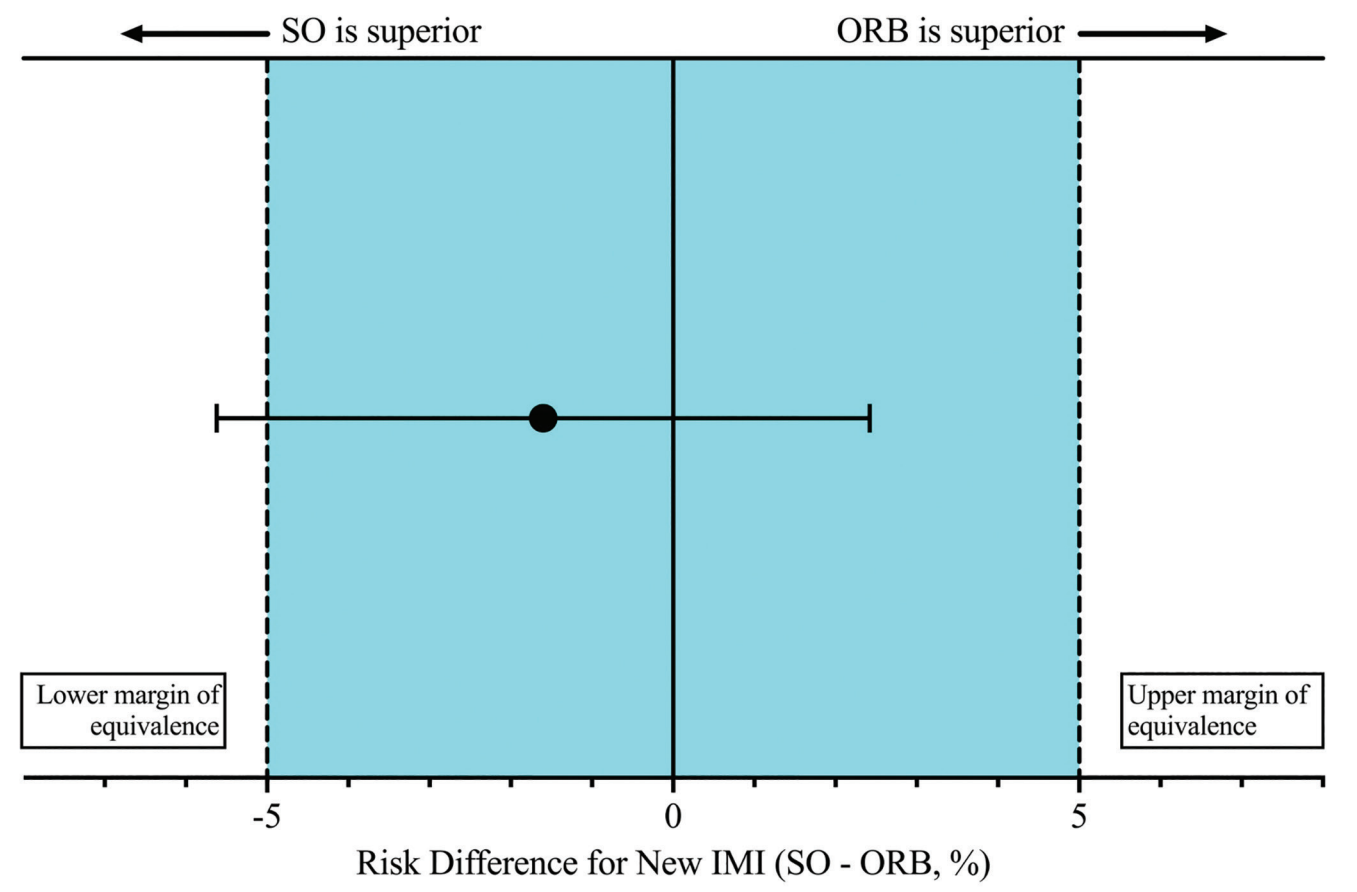
$$\text{Risk difference} = \frac{\textit{Odds ratio}}{1 - \textit{Rc} + (\textit{Rc} \times \textit{Odds ratio})}$$

where Rc = adjusted risk of the reference group (Orbeseal).

1 ORB = Orbeseal (Zoetis, Parsippany, NJ)

2 SO = ShutOut (Merck & Co., Inc., Rahway, NJ)

Figure 3: Demonstration of equivalence by determining new intramammary risk difference (•) and 95% confidence interval of risk difference (⊢⊣) of two internal teat sealants.[1] The a priori margin of equivalence (Δ) for risk difference was ±5%.



[1] ORB = Orbeseal (Zoetis, Parsippany, NJ) and SO = ShutOut (Merck & Co., Inc., Rahway, NJ)

Log_e SCC [log_e (cells/1,000) per mL)] for each treatment group by DIM in lactation for the first 120 days is illustrated in Figure 4b and Table 4. Least square means test day log_e SCC was 2.09 for the ORB group versus 2.08 for the SO group. A repeated measures model was utilized to evaluate this data with the fixed effects of treatment and DIM by level included and no additional covariates. Based on this model, no difference was identified between treatment groups (log_e SCC difference = -0.01, 95% CI -0.24, 0.22), but there was an effect of DIM ($P <$ 0.001), with no significance determined on the interaction between treatment and DIM.

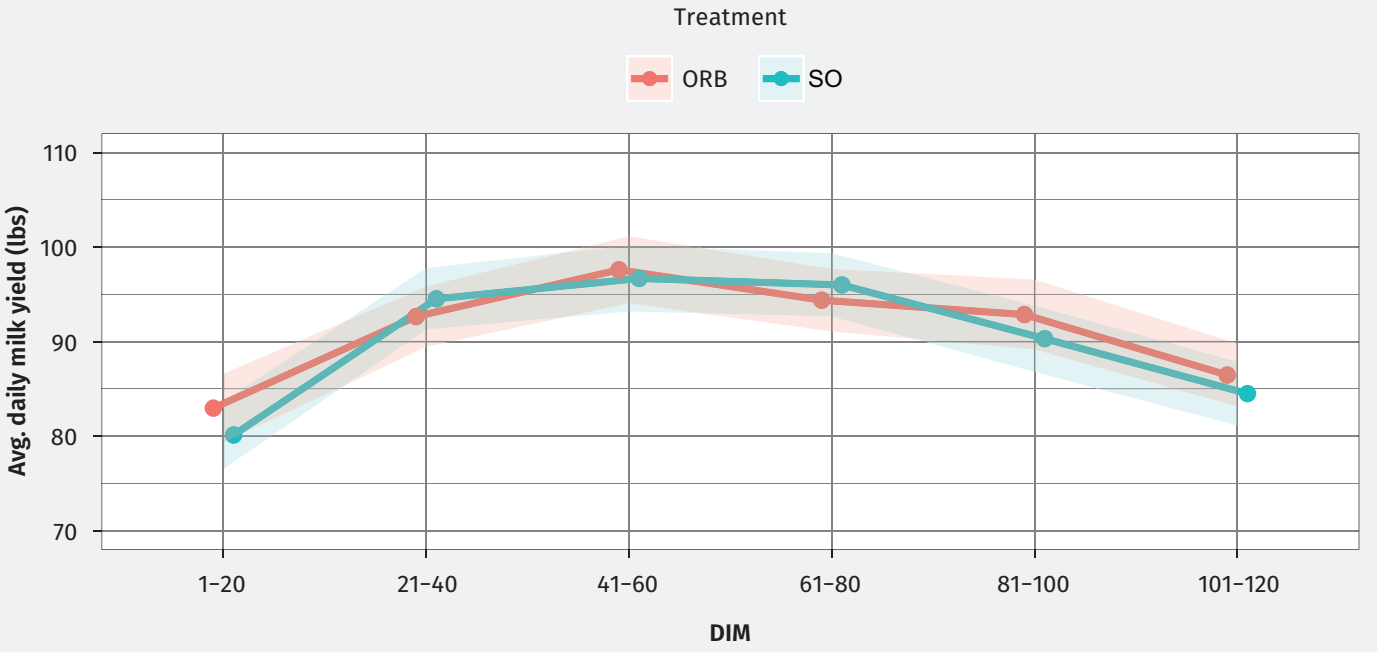
Cox proportional hazards regression models were utilized to examine time to event for mastitis, culling, and death within the first 120 DIM. Outputs from this analysis are shown in Figure 5 and Table 5. Among the entire study population, no differences were detected for the risk of clinical mastitis, culling or death between the treatment groups. The incidence of clinical mastitis within the first 120 DIM was 11.7%, with no difference detected between treatment groups. At least 1 mastitis event was noted for 12.84% of ORB cows versus 11.72% of SO cows affected (hazard ratio [HR] of SO compared to OBR [referent] = 0.98 [95% CI of HR 0.79, 10.5]). Risk of death within the first 120 DIM was also similar between groups with an overall crude death loss of 2.47% of ORB cows affected, while 2.74% of SO cows were affected (HR of SO compared to OBR [referent] = 1.13 [95% CI of HR 0.83, 1.54]). Finally, cull risk was similar between treatment groups with 10.37% of ORB cows affected versus 7.73% of SO cows (HR of SO compared to OBR [referent] = 0.79 [95% CI of HR 0.57, 1.08]) (Table 5).

Discussion

This randomized, positive controlled, equivalence study demonstrated that SO had a NIMI risk difference of -1.60% for the development of new intramammary infections during the dry period in dairy cattle compared to ORB. As a result, we determined that SO was equivalent to ORB at prevention of NIMI during the dry period. It should be noted that the lower end of the 95% confidence interval for risk difference being lower than the lower margin of equivalence in Figure 3 does not demonstrate that SO was superior to ORB as superiority tests were not conducted.

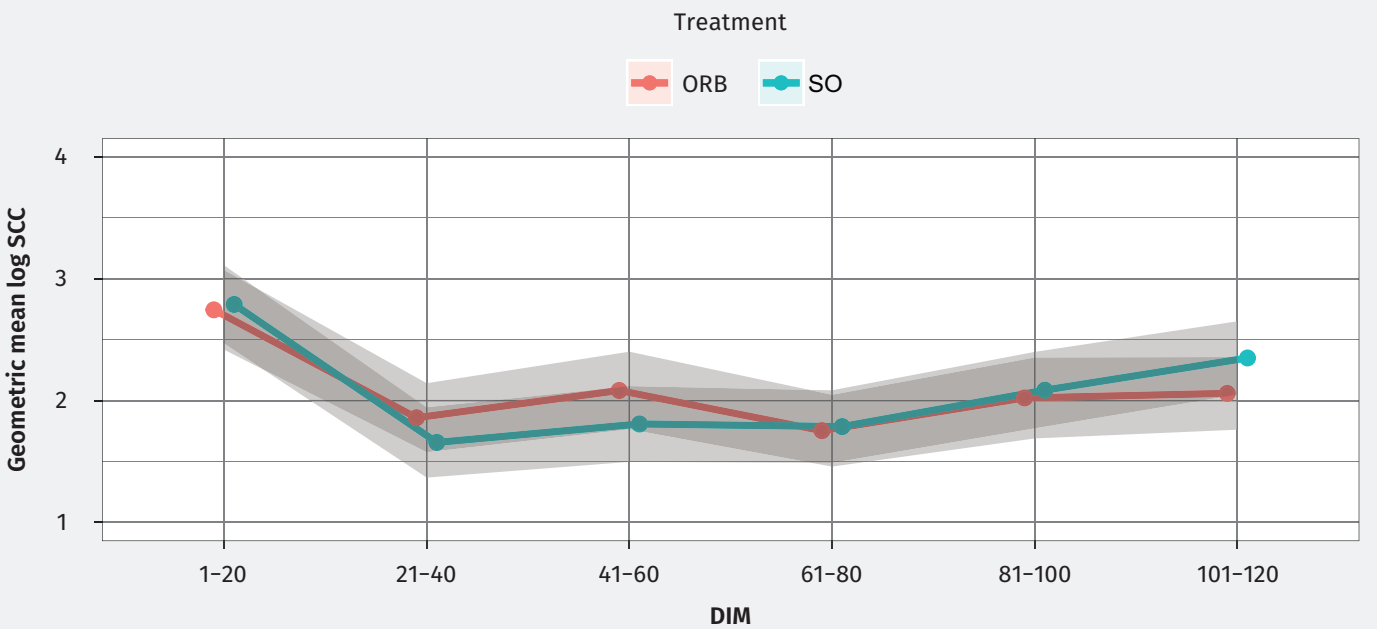
The crude prevalence of IMI at dry-off was 32.8%, which was nearly identical to data from Godden et al.[6] and the 34.7% presented by Johnson et al.[10], but higher than several other studies[8,11,15] which presented crude prevalence rates of 25.4%, 22% and 19.2%, respectively. Non-aureus staphylococci (Group A) were the most prevalent group of pathogens identified in dry-off cultures infecting 13.6% of quarters sampled, followed by other Gram-positive pathogens (Group C) excluding streptococci and *Streptococcus*-like species. These findings are also consistent with the studies mentioned previously. The present study demonstrated a prevalence of coliform bacteria and

Figure 4a: Least square means of DHIA test data for average daily milk yield (lb) during the first 120 days in milk (DIM) following dry treatment including an internal teat sealant.[1] Colored shaded areas represent 95% confidence intervals for each treatment. To account for inconsistencies in time between tests for cows and across farms, 6 time levels were created, which were forced into the model as a fixed effect.



[1] ORB = Orbeseal (Zoetis, Parsippany, NJ) and SO = ShutOut (Merck & Co., Inc., Rahway, NJ)

Figure 4b: Geometric means of DHIA test data for $\log_e$ SCC during the first 120 days in milk (DIM) following dry treatment including an internal teat sealant.[1] Colored shaded areas represent 95% confidence intervals for each treatment. To account for inconsistencies in time between tests for cows and across farms, 6 time levels were created, which were forced into the model as a fixed effect.



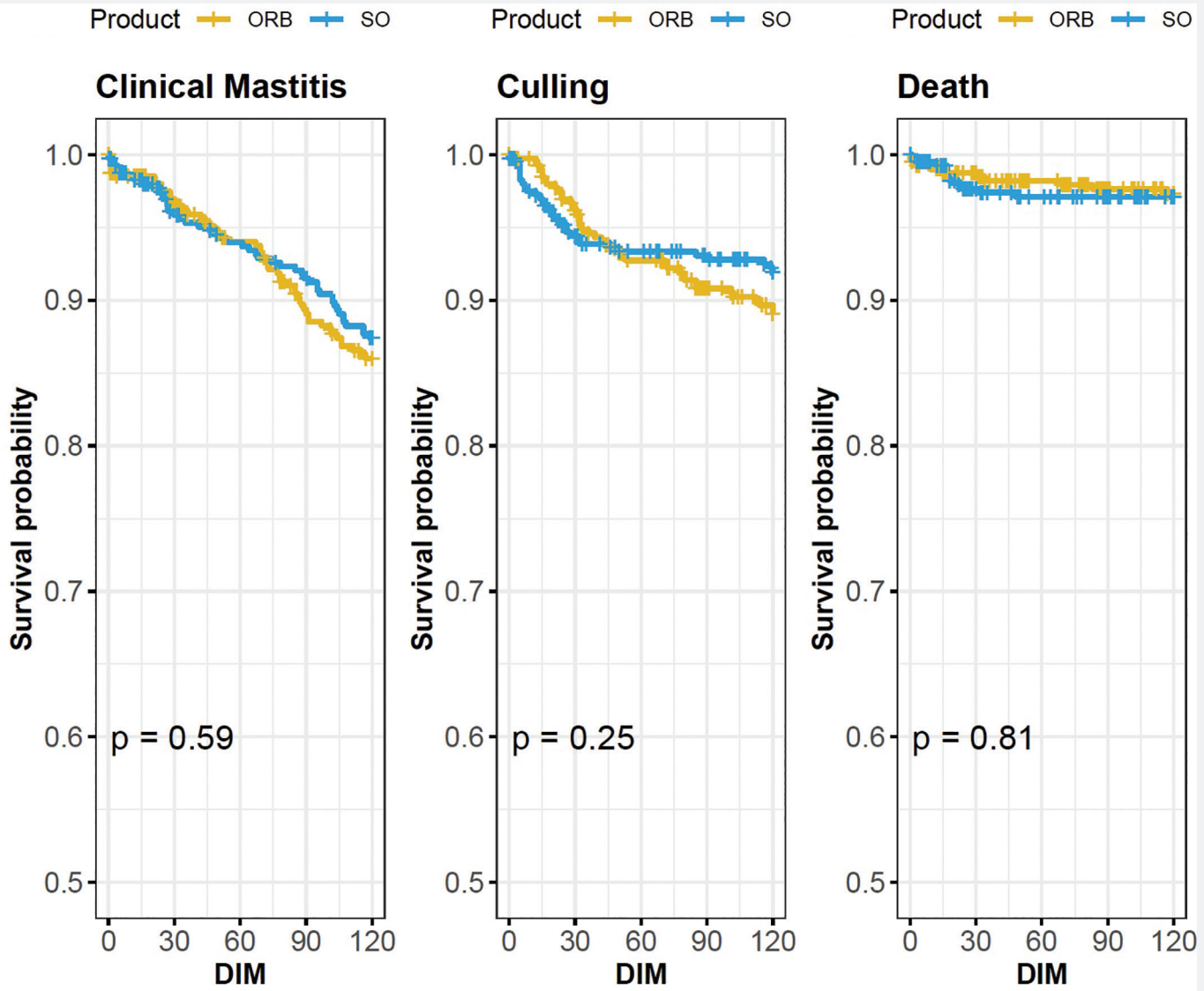
[1] ORB = Orbeseal (Zoetis, Parsippany, NJ) and SO = ShutOut (Merck & Co., Inc., Rahway, NJ)

Table 4: Final linear mixed models on test-day milk yield and SCC during the first 120 days in milk examining the effect of internl teat sealant at dry off.

Item	LS Means	SE	95% CI	Difference	95% CI of difference
Milk yield (lb/day)					
ORB[1]	91.15	1.00	89.19, 93.11	Referent	
SO[2]	90.35	1.00	88.39, 92.32	-0.80	-3.55, 1.95
Log_e SCC [log_e (cells/1,000) per mL)]					
ORB	2.09	0.08	1.92, 2.25	Referent	
SO	2.08	0.08	1.92, 2.24	-0.01	-0.24, 0.22

[1] ORB = Orbeseal (Zoetis, Parsippany, NJ)
[2] SO = ShutOut (Merck & Co., Inc., Rahway, NJ)

Figure 5: Kaplan-Meier survival curves for time to development of clinical mastitis, culling or death after calving following treatment with an internal teat sealant.[1]



[1] ORB = Orbeseal (Zoetis, Parsippany, NJ) and SO = ShutOut (Merck & Co., Inc., Rahway, NJ)

Table 5: Cox proportional hazard models results for development of clinical mastitis, culling or death by 120 days in milk examining the effect of internal sealant at dry off.

	Crude 120 d risk (%)	Hazard ratio	Robust SE	95% CI for hazard ratio
Clinical mastitis				
ORB[1]	12.84	Referent		
SO[2]	11.72	0.91	0.07	0.79, 1.05
Culling				
ORB	10.37	Referent		
SO	7.73	0.79	0.17	0.57, 1.08
Death				
ORB	2.47	Referent		
SO	2.74	1.13	0.16	0.83, 1.54

[1] ORB = Orbeseal (Zoetis, Parsippany, NJ)
[2] SO = ShutOut (Merck & Co., Inc., Rahway, NJ)

Staphylococcus aureus each infecting 0.4% and 0.3% of quarters, respectively. This is consistent with expected margins based on previously published data.[11]

The quarter-level prevalence of NIMI over the dry period was 30.5%, which was substantially higher than previous studies including Rowe et al. at 11.0%,[11] Arruda et al. at 14.7%,[15] and slightly higher than Rowe et al.[8] and Johnson et al.[10] which both presented a crude NIMI prevalence of 23.0%. The overall crude CIMI risk during the dry period was 95.9%, which is similar to published literature,[10,11] which both evaluated internal teat sealants. The use of ITS was not expected to impact cure risk nor was it an objective of this study to determine the effect of ITS on cure risk. The main reason for reporting CIMI risk was to provide comparability between ours and previous work. However, in vitro studies have shown inhibition of bacterial growth when in contact with bismuth subnitrate.[16] The previous work by Johnson et al. has shown that cloxacillin is an efficacious dry cow antimicrobial product.[10] It is hypothesized that the high cure risk was likely due to the prevalence of Gram-positive pathogens identified at DO (87.3%, 796 of 950 isolates), which are generally susceptible to narrow spectrum beta-lactam intramammary antimicrobials such as cloxacillin.[17]

To our knowledge, this is the first publication that has looked at the efficacy of SO for prevention of new intramammary infections during the dry period in U.S. dairy cattle. There was one study conducted in Brazil which compared dry period IMI and post-partum infection dynamics in cattle treated with a first-generation cephalosporin (cefalonium[h]) dry cow preparation alone versus a combination of cefalonium and SO. In Brazil, however, SO is sold under a different trade name.[i] This work demonstrated that dairy cattle treated with ITS in combination with an antimicrobial had a NIMI risk of 14% compared with 19% for the dry antimicrobial alone.[18] This result is similar to the results of the efficacy study for ORB,[6] and given the results of the current study, similar reduction of NIMI risk should be expected on U.S. cattle.

The most apparent weakness of this study is the high number of contaminated quarters (15.2%) and quarters that did not have PC samples collected (n = 304). This resulted in less than the target number of quarters of 1,320 per treatment available for quarter-level risk assessments of dry period IMI dynamics. Our a priori estimate of NIMI risk over the dry period to calculate the target number of quarters was 20%, thus a target of 264 quarters acquiring a NIMI per treatment group. We ended up with a crude NIMI risk of 30.6%; therefore, we had 332 and 306 newly infected quarters in the ORB and SO groups, respectively. As a result, we still acquired the target number of quarters to support the risk assessment and determined that the risk difference for NIMI of -1.60% was well within the margins of equivalence (Figure 3, Table 3). This weakness points out one of the major challenges of carrying out research on large commercial dairies. On one hand, dairy producers want to see results on modern farms but on the other hand, time is a priority which pushes the envelope on providing time to do pristine sample collection. The benefit of this approach is that the study demonstrates substantial external validity, which should give veterinarians and dairy producers confidence in the results.

When evaluating cow-level performance, SO had similar levels of clinical mastitis, culling, death, milk production and somatic cell count in the first 120 DIM in the lactation following treatment. This is supported by 95% confidence intervals for mean differences in milk yield and SCC encompassing zero and hazard ratios for clinical mastitis, culling and death encompassing one. This allows producers to weigh factors other than efficacy more heavily in their decision on which internal teat sealant to utilize in their dry cow program.

Given the results of this study, the choice between utilizing SO and ORB becomes one of ease of use given the product delivery systems and price. As the price for pharmaceuticals vary by farm based on volume purchased and other pricing promotions from manufacturers, it is beyond the scope of this manuscript to complete a cost comparison of the two products.

Conclusions

In this study, we tested the hypothesis that SO would be equivalent to ORB in the efficacy of preventing new intramammary infections in the subsequent lactation after use. We conclude that SO is equivalent to ORB when evaluated at a margin of equivalence of ±5% for risk difference of new IMI infections over the dry period. Cattle treated with either product had similar quarter-level risk of cured infections, as well as clinical mastitis, culling and death during the first 120 DIM, as well as had similar milk production and SCC. With the ever-growing economic challenges that dairy producers face, comparing the efficacy of multiple products in preventing new intramammary infections can provide peace of mind for producers while providing a wider array of products and improving competitive pricing within the market.

Endnotes

[a]ShutOut®, Merck & Co., Inc., Rahway, NJ

[b]Orbeseal®, Zoetis, Parsippany, NJ

[c]Orbenin DC®, Merck & Co., Inc., Rahway, NJ

[d]Excel, Microsoft Corporation, Redmond, WA

[e]Bruker Daltonics, Inc., Billerica, MA

[f]Box, Box, Inc., Redwood City, CA

[g]R Statistical Programing Environment, v.4.1.0, R Core Team (2022), Boston, MA

[h]Cepravin®, MSD Animal Health, São Paulo, Brazil

[i]Masti-Seal®, MSD Animal Health, São Paulo, Brazil

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Conflicts of interest

The study investigators (MB, JB, SG, GS, and PG) have no financial interest to declare.

Co-authors TT and BEM are employees of Merck & Co.

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Author contributions

Author roles were: MB was involved in local and multi-state coordination, fieldwork, laboratory work, data management, statistical analysis and manuscript preparation. JB, TT and BEM were involved in study conceptualization and manuscript editing. SMG was involved in study conceptualization, local site coordination and fieldwork in MN, and manuscript editing. GSS was involved in study conceptualization, statistical analysis, and manuscript editing. PJG was involved in study conceptualization, herd recruitment in IA, supervision of fieldwork, data management, statistical analysis and manuscript editing.

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