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Noninferiority trial investigating the efficacy of a nonantibiotic intramammary therapy in the treatment of mild-to-moderate clinical mastitis in dairy cows with longer lasting udder diseases

M. Ziesch[1] | N. Wente[1] | Y. Zhang[1] | W. Zaremba[2] | S. Engl[2] | V. Krömker[1]

[1]Department of Bioprocess Engineering - Microbiology, Faculty II, University of Applied Sciences and Arts Hannover, Hannover, Germany

[2]Veyx-Pharma GmbH, Schwarzenborn, Germany

Correspondence
Marco Ziesch, Department of Bioprocess Engineering – Microbiology, Faculty II, University of Applied Sciences and Arts Hannover, Hannover, Germany.
Email: marcoziesch@online.de

A nonblinded, positively controlled, noninferiority trial was conducted to evaluate the efficacy of an alternative, nonantibiotic therapy with Masti Veyxym® to reduce ineffective antibiotic usage in the treatment of nonsevere clinical mastitis (CM) in cows with longer lasting udder diseases. The solely intramammary treatment with Masti Veyxym® (three applications, 12 hr apart) and the combined treatment with Masti Veyxym® and antibiotics as usual on the farm according to label of the respective product were compared with the reference treatment of solely antibiotic therapy. The matched field study was conducted on eight free-stall dairy farms located in Eastern Germany. Cases of mild-to-moderate CM in cows with longer lasting high somatic cell counts in preceding dairy herd improvement test days and with previous CM cases in current lactation were randomly allocated to one of the three treatment groups. A foremilk sample of the affected quarter was taken before treatment and again approximately 14 days and 21 days after the end of therapy for cyto-bacteriological examination. Primary outcomes were clinical cure (CC) and no CM recurrence within 60 days after the end of treatment (no R60). Bacteriological cure (BC) and quarter somatic cell count (QSCC) cure were chosen as secondary outcomes although low probabilities of BC and QSCC cure for selected cows were expected. The study resulted in the following findings: the pathogens mostly cultured from pretreatment samples were *Streptococcus uberis*, followed by *Staphylococcus aureus* and coagulase-negative staphylococci. There were no significant differences between the two test treatments in comparison with the reference treatment regarding all outcome variables. The sole therapy with Masti Veyxym® resulted in a numerically lower likelihood of BC without significant differences to the reference treatment. The combined therapy group showed a numerically higher nonrecurrence rate than the two other treatment groups and noninferiority compared to the reference treatment was proven. Having regard to the selection criteria of cows in this study, the findings indicated that sole treatment with Masti Veyxym® in nonsevere CM cases may constitute an alternative therapy to reduce antibiotics. However, noninferiority evaluations were mostly inconclusive. Further investigations with a larger sample size are required to confirm the results and to make a clear statement on noninferiority.

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1 | INTRODUCTION

Clinical mastitis (CM) is still a common and costly disease on dairy farms all over the world (IDF (International Dairy Federation), 2005; Hogeveen, Huijps, & Lam, 2011). The treatment method of choice to combat CM is antibiotic therapy, which was confirmed by a recently conducted multiherd study on Dutch dairy farms. Santman-Berends, Lam, Keurentjes, and van Schaik (2015) showed that in 72% of CM cases, farmers decided to use antibiotic treatment. Other investigations conducted in 51 large dairy herds in Wisconsin, USA, reported that 95.4% of cows suffering from CM were treated with antibiotics (Oliveira & Ruegg, 2014). This results in high usage of antibiotics, which is currently publicly discussed due to the problem of residuals and potential development of pathogen resistances. Cows suffering from CM should be supplied with evidence-based treatment to ensure prudent use of antibiotics (Mansion-de Vries, Hoedemaker, & Krömker, 2015; Ruegg, 2010) because it is impossible to prevent all CM cases. Trevisi et al. (2014) mentioned that antibiotic treatments for chronic disease cases do not lead to increased animal health and are not reasonable with regard to cost/benefit analysis. The aim of antibiotic therapy must be considered to determine the benefit of antibiotics for such chronic disease cases. Antibiotics are only able to combat micro-organisms. Therefore, the success is assessed by bacteriological cure (BC) and is defined as the elimination of the mastitis-causing pathogen from the infected udder quarter (Krömker, Paduch, Klocke, Friedrich, & Zinke, 2010; Schukken et al., 2013; Swinkels, Krömker, & Lam, 2014; Ziesch & Krömker, 2016). Many studies dealt with the influence of cow-related factors on the BC rate of CM cases treated with antibiotics. The investigations showed a decreasing probability of BC with rising amount of previous CM cases in the same lactation (Pinzón-Sánchez & Ruegg, 2011; Ziesch & Krömker, 2016) and high cow somatic cell counts (CSCC) prior to CM (Bradley & Green, 2009; Pinzón-Sánchez & Ruegg, 2011; Sol, Sampimon, Barkema, & Schukken, 2000; Swinkels, Cox, Schukken, & Lam, 2013; Ziesch & Krömker, 2016). Consequently, lowering the likelihood of BC results in declining efficacy and usefulness of antibiotic treatment. Cows with longer lasting udder diseases are characterized by recurrent CM cases separated by periods without clinical signs and/or constantly elevated CSCC defined as subclinical mastitis (Grieger, Zoche-Golob, Paduch, Hoedemaker, & Krömker, 2014; GVA [German Veterinary Association], 2012). With the help of the aforementioned cow-related factors, CM history in the current lactation and persistent elevation in CSCC, we are able to determine cows expecting a low probability of BC due to antibiotic treatment. If possible such cows should be removed from the herd (Krömker & Friedrich, 2011) or in the case of CM be treated symptomatically to avoid useless application of antibiotics (Degen, Paduch, Hoedemaker, & Krömker, 2015). From a farmer's point of view, such cows, especially the high-yielding animals, are still profitable as long as they show no clinical signs and the milk is saleable. In the case of CM, a low likelihood of BC is expected from a scientific point of view. Maybe an equal clinical cure (CC) rate and recurrence rate during the further course of lactation in comparison with an antibiotic treatment could possibly convince farmers to use an alternative therapy. The medicinal product Masti Veyxym® (Veyx-Pharma GmbH, Schwarzenborn, Germany), an already licensed udder injector containing ointment for intramammary application with proteolytic enzymes and without antibiotics, could be a useful treatment option. In vitro investigations showed an inhibiting activity of containing proteolytic enzymes against udder pathogens (Krüger, Hien, Zaremba, & Penka, 1999). Zander (1997) conducted a clinical study and reported a reduction in CSCC due to sole treatment with proteolytic enzymes of cows with bacteriologically negative subclinical mastitis. Furthermore, he showed a higher cure rate for combined treatment with antibiotics and proteolytic enzymes than solely antibiotic therapy in cows with chronic mastitis and subclinical mastitis with pathogen detection. In addition to the advantage that the drug does not contain antibiotics, a reduced withdrawal period of 1 day for milk and zero days for meat is required resulting in a decrease in discarded milk and an increase in revenue. In this study, we compared the efficacy of the test product Masti Veyxym® to antibiotic treatment as reference therapy. Also, a test group of combined antibiotic and Masti Veyxym® treatment, similar to the investigations of Zander (1997), was included and examined in comparison with solely antibiotic treatment. The aim of the study was to evaluate noninferiority of the test products against the reference product for treatment of mild-to-moderate CM in cows with longer lasting udder diseases.

2 | MATERIALS AND METHODS

2.1 | Study design

This study was conducted in accordance with the guidelines on good clinical practice (GCP; EMEA (The European Agency for the Evaluation of Medicinal Products), 2000) and designed as a noninferiority study to compare two test treatment groups with one reference treatment group by a previously defined margin of noninferiority (**Δ**; Piaggio, Elbourne, Altman, Pocock, & Evans, 2009; O`Connor et al., 2010; Schukken et al., 2013). The null hypothesis implied that the test product is inferior to the reference product, and the alternative hypothesis implied that the test product is not inferior to the reference product regarding the defined margin (−Δ; Piaggio et al., 2009; Schukken et al., 2013):

H_0: $[P_{outcome}(\text{test}) - P_{outcome}(\text{reference})] \leq -\Delta$

H_A: $[P_{outcome}(\text{test}) - P_{outcome}(\text{reference})] > -\Delta$

Whereby, $P_{outcome}$ is the probability of outcome variables for the test and reference product. To establish noninferiority of a test product to a reference product, the null hypothesis (H_0) must be discarded to accept the alternative hypothesis (H_A). The evaluations of possible study results that were described by Schukken et al. (2013) also apply for this study.

2.2 | Sample size determination

Based on investigations of Schukken et al. (2011), the margin of noninferiority (Δ) was determined as 0.15 for this study. Also, other investigators recommended and applied this value for noninferiority margin in CM studies (Deluyker, Chester, & van Oye, 1999;

Schukken & Deluyker, 1995; Schukken et al., 2013). The confidence interval (CI; 95%) approach was used to calculate required sample size based on the clinical cure rate and CM recurrence rate. In this model, treatments are assumed to achieve similar cure and recurrence rates and we want to assure on the 95% level that the difference is not higher than 15% regarding the margin of noninferiority and the null effect.

If there is truly no difference in clinical cure rates between the reference and test treatment, then 37 CM cases per group are required so as to be 90% sure that the upper limit of a one-sided 95% CI rules out a difference in favour of the reference group of more than 15%.

If there is truly no difference in recurrence rates between the reference and test treatment, then 50 CM cases per group are required so as to be 80% sure that the upper limit of a one-sided 95% CI rules out a difference in favour of the reference group of more than 15%.

Using the estimation of the recurrences due to the higher required sample size, we calculated that if a further 10%–15% of CM cases drop out of the study postadmission, around 60 cases are needed per treatment group. Therefore, a total of 180 cows with CM have to be included.

2.3 | Inclusion criteria for farms and cows

Commercial dairy free-stall farms with interest in and possibilities for performing the study were eligible for inclusion. Study farms have to participate in the German Dairy Herd Improvement (DHI) programme, which records cow data, CSCC, milk yield and milk ingredients on a monthly basis.

Every cow must be registered with a unique ear tag to clearly identify every animal, as stipulated in Germany. Only cows that have had at least three consecutively high CSCC (> 400,000 somatic cells/ml) in the three previous months and/or at least two CM cases in the current lactation directly before the occurrence of the CM were admitted for inclusion in the study. Lactating Holstein-Friesian dairy cows of all parities with CM signs in one or more quarters were eligible for inclusion. Suitable cows showed a period of normal milk secretion without signs of inflammation on the udder quarters until start of CM. The subsequent clinical score was used to characterize and determine CM at occurrence. A quarter was classified as affected by a mild CM if there were only changes in the appearance of the milk (i.e., flaky sediments, watery appearance, discolouration). A moderate CM showed additionally clinical signs of mastitis in the quarter (i.e., swelling, heat, pain, redness) with or without changes in milk secretion as previously described. A CM was classified as severe when a cow suffered from general clinical signs of disease (i.e., fever (rectal temperature >39.5°C), dehydration, anorexia, depression) with or without deviations of the milk and/or the udder quarter (Pinzón-Sánchez & Ruegg, 2011; Swinkels et al., 2014). Cows were excluded from the study if they showed significant udder, teat or teat orifice lesions, suffered from severe CM cases, had been treated with other products in addition to the mastitis treatment or had concurrent diseases at the time of CM.

2.4 | Treatment and randomisation

Treatment was applied by instructed farm staff. Three different treatment regimens were investigated in the study: group 1) AB, antibiotic treatment as usual on the farm according to label of the respective product; group 2) ABMV, antibiotic treatment as usual on the farm according to label of the respective product combined with Masti Veyxym® (Veyx-Pharma GmbH, Schwarzenborn, Germany) comprising three treatments of 10-g disposable syringe containing 120 mg α-tocopherol acetate, 58.83 mg retinol palmitate, 2,400 FIP-U chymotrypsin, 240 FIP-U trypsin and 6 FIP-U papain per syringe at an interval of 12 hr; and group 3) MV, solely Masti Veyxym® comprising three treatments at an interval of 12 hr. All affected quarters of cows in the AB and ABMV treatment groups received intramammary antibiotic therapy, and if desired additionally, a systemic antibiotic therapy was allowed.

For treatment allocation, cows were grouped by lactation number (1, >1). In every lactation number group, cows fulfilling the inclusion criteria were randomly allocated to a treatment group based on a randomization list and therapy applied following strict asepsis by trained farm personnel. Every farm had its own randomization list which was structured in the following way, the first affected cow meeting the inclusion criteria was assigned to the treatment group AB, the second cow was allocated to the treatment group ABMV, the third cow received solely Masti Veyxym®, and the fourth cow commenced again with the treatment group AB and so on. Cows with CM in more than one quarter were also eligible for inclusion in the study, and all affected quarters received the same therapy.

2.5 | Flow of events for a cow in the study

Every month, a list containing eligible cows from every farm was prepared by the first author (MZ) based on the monthly DHI results and the farm records of cow CM history. Farm staff was instructed and trained to perform mastitis identification, clinical data collection, sampling, treatment and to fill in treatment protocols in accordance with the study procedure. A cow with a mild or moderate CM case in one or more quarters was identified by the milkers and checked for suitability of inclusion in the study using the list of eligible cows. When a cow fulfilled the inclusion criteria, the cow was allocated to one of the three treatment groups in accordance with the aforementioned randomization list. Before aseptic application of treatment, a foremilk sample of the affected quarter was taken by trained milkers respecting the guidelines of aseptic milk sampling (GVA [German Veterinary Association], 2009). Treatment was performed according to the label of the respective product. In the combined therapy group (ABMV), first the antibiotic and immediately afterwards the Masti Veyxym® were injected. At every milking period, the clinical score of the affected quarter was assessed by the milkers until 7 days after the end of treatment. When clinical signs deteriorated, the farmer was allowed to treat the cow with an additional or different treatment and the case was documented as treatment failure. CM cases where the

clinical signs disappeared until day 7 after the end of treatment and without classification as treatment failure were assessed as clinically cured. These cured quarters were observed from day 8 to day 60 after the end of treatment for recurrent CM cases, and in the case of recurrence, a quarter foremilk sample was collected. Furthermore, instructed farm staff took quarter foremilk samples at day 14 (±2) and day 21 (±2) after the end of treatment of all clinically cured quarters. Milk samples were stored in the on-farm refrigerators and picked up once a week by the first author. During these regular farm visits, the first author exchanged information with the herd personnel to resolve inaccuracies and ensure data quality. Any deviations from the study protocol were noted and investigated for eligibility to include in the study. Commonly used cow-level data including lactation number, affected quarter location, milk yield, CSCC of the three most recent DHI recordings prior to CM, days in milk (DIM) at CM occurrence and concurrent diseases and treatments for a period of 30 days after enrolment were recorded.

2.6 | Blinding

It was not possible to blind either the study personnel or the farmers/herdspersons to product administration by virtue of the differences in treatment regimens. The personnel at the laboratory culturing for mastitis pathogens was unaware of the treatment given to the quarter being sampled.

2.7 | Laboratory procedure

All milk samples were collected aseptically and were stored below 8°C until analysis. Ly20, containing boric acid as preserving agent, was used in test tubes (GVA [German Veterinary Association], 2009). The samples were sent to the microbiological laboratory at the University of Applied Sciences and Arts Hannover (Germany). Microbiological examinations were performed in accordance with the guidelines of the German Veterinary Association (GVA [German Veterinary Association], 2009), which are based on National Mastitis Council recommendations (NMC (National Mastitis Council), 1999); 10 µl of each milk sample was plated on a quadrant of an aesculin blood agar plate (Oxoid, Germany) and incubated at least for 48 hr at 37°C under aerobic conditions. By the assessment of Gram staining, morphology of the colonies and cells, hemolysis patterns, aesculin hydrolysis and activity of catalase (3% H_2O_2; Merck, Germany), an initial evaluation of the grown colonies was performed. Subsequently, several biochemical tests were performed to determine the growing micro-organisms. The clumping factor test (DiaMondiaL Staph Plus Kit, Sekisui Virotech, Germany) instead of the coagulase test was used to differentiate presumptive *Staphylococcus* (*S.*) *aureus* from coagulase-negative staphylococci (CNS). Different aesculin-negative streptococci were distinguished by the serological tests for Lancefield Group B (*Streptococcus (Sc.) agalactiae*), C (*Sc. dysgalactiae*) and G (DiaMondiaL Streptococcal Extraction Kit Sekisui Virotech, Germany). To differentiate between *Sc. uberis* and *Enterococcus* spp., the modified Rambach agar according to Watts, Salmon, and Yancey (1993) was used. Gram-positive, beta-haemolytic and catalase-negative irregular rods with a V-or Y-shaped configuration were identified as *Trueperella (T.) pyogenes*. Coryneform bacteria form small colonies on aesculin blood agar. They are gram-positive and catalase-positive. Both *T. pyogenes* and coryneform bacteria are asporogen. *Bacillus* spp. form large colonies on aesculin blood agar. *Bacillus* spp. are gram-positive, catalase-positive rods and can form endospores. Coliform bacteria are gram-negative, catalase-negative and cytochrome oxidase-negative (Bactident oxidase, Merck, Germany) rod-shaped bacteria, which can metabolize glucose fermentatively (OF basal medium with addition of D (+)-glucose monohydrate, Merck, Germany). On Chromocult Coliform Agar (Merck, Germany), *Escherichia* (*E.*) *coli* forms blue colonies under aerobic incubation at 37°C for 24 hr, and other coliforms form pink-red colonies. *Klebsiella* spp. are immobile during the performance of the OF test. Pseudomonads were identified as gram-negative, catalase-positive and cytochrome oxidase-positive rod-shaped bacteria that break down glucose oxidatively. Yeasts, moulds and *Prototheca* spp. were differentiated microscopically after subculturing on YGC agar (Merck, Germany). Environment-associated, mastitis-causing micro-organisms (*Sc. uberis*, *E. coli*, CNS, *Klebsiella* spp., coliform bacteria, yeasts, *Pseudomonas* spp. and *Prototheca* spp.) were recorded as a microbiologically positive result if ≥5 cfu/0.01 ml were cultured to reduce bias due to contamination. If two pathogens were cultured, the case was included in the study and both micro-organisms were documented. A milk sample was considered as contaminated when more than two pathogens were identified, except in cases where also *S. aureus*, *Sc. agalactiae*, *Sc. dysgalactiae* and *T. pyogenes* were cultured. Then, only the growth of these pathogens was recorded, and the cases were classified as contaminated if the samples contained more than two of these pathogens. The Somascope Smart (Delta Instruments, The Netherlands) was used to determine the quarter SCC (QSCC) by flow cytometry.

2.8 | Outcome variables

Primary outcomes were clinical cure (CC) and no CM recurrence within 60 days after the end of treatment (no R60). Secondary outcomes were bacteriological cure (BC) and quarter somatic cell count (QSCC) cure. clinical cure (CC) was defined as the absence of clinical signs in milk, this means without flaky sediments, watery appearance or discolouration and on udder quarter, this means without swelling, heat, redness or pain at days 5–7 after the end of treatment. Clinical mastitis (CM) cases of cows which received additional or different treatment due to deterioration of clinical score within the 7 days after the end of initial therapy or were removed from the herd due to udder disease were assessed as failure of CC.

Quarters with clinically cured cases were observed for the time frame of day 8 to day 60 after the end of treatment and defined as recurrent quarters when one or more CM cases were detected. A quarter showed no R60 if it was free of CM within the observed time frame.

Bacteriological cure was defined as the absence of the pathogen-cultured pretreatment in both post-treatment samples at days 14 and 21. If a bacterial species other than the pathogen-cultured pretreatment was isolated in the post-treatment samples, the case was still defined as bacteriologically cured. In case one post-treatment sample was contaminated, the outcome of the other post-treatment sample was used to determine the BC. If two pathogens were isolated in the pretreatment sample, the case was enrolled as mixed infection and applied as bacteriologically cured if neither of the two pathogens were cultured in both of the post-treatment samples. When a clinically cured quarter suffered from a CM recurrence within day 8 to day 21 after the end of treatment, available post-treatment samples and the recurrence sample were used to determine BC.

QSCC cure was defined as a QSCC being <200,000 cells/ml in both post-treatment samples at days 14 and 21. In case one post-treatment sample was missing, the QSCC of the other post-treatment sample was used to determine the outcome. When a clinically cured quarter suffered from a CM recurrence within day 8 to day 21 after the end of treatment, the CM case was assessed as failure of QSCC cure. Quarters with CM cases experiencing no CC were also included in the analysis as failure of BC and QSCC cure to take the principle of "intention-to-treat" into account (O`Connor et al., 2010; Schukken et al., 2013).

2.9 | Statistical analysis

The data were collected and analysed using Excel, Office 2010 (Microsoft Corporation) and SPSS (IBM SPSS 23.0.0.0, Armonk, USA). The statistical unit was the CM case of an udder quarter. For every CM case, CC or no CC, R60 or no R60, BC or no BC and QSCC cure or no QSCC cure (encoded as 1 or 0, respectively) were determined according to the aforementioned definitions, constituting the binary dichotomous-dependent variables. Outcomes were analysed using generalized linear mixed models including lactation number, DIM and pathogen-cultured pretreatment as important covariates. As clustering was present in the design (i.e., gland within cow and cow within herd), the analysis was corrected using random effects, but had no relevant influence. The treatment group was the main variable of interest. Statistical significance was assumed at $\alpha = 0.05$.

The linear predictor was calculated as

Logit (outcome) = intercept + treatment + lactation number + DIM + pathogen + herd * cow * gland (random).

CC, no R60, BC or QSCC are the outcomes and lactation number is the lactation number of the included cow grouped as 1, 2 and over 2. DIM is days in milk of the cow at CM occurrence grouped as 0–100, 101–200 and over 200. Pathogen-cultured pretreatment were grouped into *Enterobacteriaceae*, streptococci, staphylococci, other pathogens, contaminated samples and no growth.

For CC, no R60 and BC, the model was used to calculate least-square means of the various treatment groups. Thereby, the differences between treatments were estimated. Confidence intervals of the therapy differences were calculated utilising the least-square means and the standard deviation (Schukken et al., 2013).

3 | RESULTS

3.1 | Descriptive results

The time frame for data collection ranged from September 2014 to September 2015. The study was conducted on eight free-stall dairy farms located in Eastern Germany. All farms were conventional and commercially oriented with a herd size between approximately 140 and 800 lactating Holstein-Friesian dairy cows. The milk production ranged from 8,000 and 9,700 kg/cow/year with bulk milk SCC (BMSCC) between 181,000 and 382,000 cells/ml. All farms were equipped with modern milking systems and used common hygiene management methods. Milkers wore gloves during milking, used one tissue per cow to clean the teats before milking and utilized teat disinfection after milking. All herds were milked twice a day, except on one farm where the high-yielding and fresh cow group was milked three times a day. A rotary milking parlour was installed on five farms, three farms milked with a herringbone parlour and no automatic milking system was present. All cows were fed with total mixed rations.

In total, 174 CM cases were enrolled in the study and no adverse events of treatment were observed. The median of lactation number for all CM cases amounted to 2 (minimum 1; maximum 6), of CSCC last DHI before CM onset 594,000 cells/ml (minimum 16,000 cells/ml; maximum 9,694,000 cells/ml) and of milk yield last DHI before CM occurrence 30.15 kg (minimum 10.3 kg; maximum 58.4 kg). In 89 cases the front quarters and in 85 cases the rear quarters suffered from CM. The severity of clinical signs at CM case occurrence was classified as mild in 106 cases and as moderate in 68 cases. A proportion of 26.4% of the CM cases arose in cows being 0–100 days in milk (DIM), 40.3% being 101–200 DIM and 33.3% being over 200 DIM, respectively. Solely, antibiotic treatment (AB) was applied in 59 CM cases, 63 quarters received combined treatment (ABMV) and solely, Masti Veyxym® (MV) was used in 52 CM cases. Consequently, 122 quarters were treated with antibiotics, the used ingredients of which are shown in Table 1. Thereof, 74.6% of quarters suffering from CM

TABLE 1 Number and percentage of the used antibiotic ingredients for clinical mastitis (CM) treatment in the study

Antibiotic ingredients	Number of treated CM cases (%)
Cefquinome	57/122 (46.7)
Ampicillin, Cloxacillin	25/122 (20.5)
Cefoperazone	20/122 (16.4)
Cefalexin, Kanamycin	5/122 (4.1)
Procaine benzylpenicillin	4/122 (3.3)
Lincomycin, Neomycin	3/122 (2.5)
Cefalexin	1/122 (0.8)
Mixed ingredients	7/122 (5.7)

received solely intramammary antibiotic therapy, and in 25.4% of CM cases, combined intramammary and systemic antibiotic treatment was applied.

Foremilk samples of the affected quarters at CM occurrence were available in 169 cases, the remaining five samples forgotten to be taken by the milkers. The results of bacteriological culture are presented in Table 2. The pathogen mostly cultured from the pretreatment sample was *Sc. uberis* (14.2%), followed by *S. aureus* (11.8%), and CNS (9.5%), respectively. No micro-organisms were cultured in 28 cases (16.6%), 26 quarters showed mixed infections (15.4%) and four samples were contaminated (2.3%). In 53.8% of the mixed infections, *Sc. uberis* was one of the cultured pathogens, and in 30.8%, *S. aureus* was one of the isolated micro-organisms.

3.2 | Homogeneity of treatment groups

No significant differences between treatment groups in DIM and pathogen-cultured pretreatment were found ($p > .2$). Treatment was allocated at herd level and at the lactation number level grouped in lactation number 1 and >1. For good measure, herd as random effect, DIM, lactation number and pathogen-cultured pretreatment were included in the generalized linear mixed models to take these factors into account.

TABLE 2 Bacteriological culture results of the 169 clinical mastitis (CM) pretreatment samples

Micro-organism	Number	%
Enterobacteriaceae	8	
Coliforms (other than *Escherichia coli* and *Klebsiella* spp.)	5	3.0
Escherichia coli	3	1.8
Streptococci	35	
Streptococcus uberis	24	14.2
Streptococcus dysgalactiae	8	4.7
Other streptococci	3	1.8
Staphylococci	36	
Staphylococcus aureus	20	11.8
Coagulase-negative staphylococci (CNS)	16	9.5
Other pathogens	32	
Coryneforms	12	7.1
Pseudomonas spp.	7	4.1
Prototheca spp.	5	3.0
Enterococci	4	2.3
Trueperella pyogenes	3	1.8
Yeasts	1	0.6
No growth	28	16.6
Mixed infections	26	15.4
Contaminated	4	2.3
Total	169	100

3.3 | Clinical cure

The overall CC rate was 62.6% (109/174). The probability of CC in the AB group was 62.7% (37/59), in the ABMV group 63.5% (40/63) and in the MV group 61.5% (32/52), respectively.

Results of the generalized linear mixed model showed least-square means of 63.8% for the AB group, 62.2% for the ABMV group and 58.3% for the MV group. However, no significant differences in CC of the reference treatment AB to the test treatment MV ($p = .61$) and to the test treatment ABMV ($p = .875$) were found (Table 3). Cows suffering from CM within 101–200 DIM showed a significantly lower probability of CC than cows affected with CM over 200 DIM ($p = .023$). The point estimate of the calculated differences in CC from the logistic regression and the associated 95% CI is shown in Figure 1. Noninferiority is inconclusive for both test treatments in comparison with the reference treatment.

3.4 | No recurrence 60 days

Only quarters with clinically cured cases of cows, which were still in milk 60 days after the end of treatment, were included in this analysis (Pinzón-Sánchez & Ruegg, 2011). Of the 109 clinically cured quarters, 23 cases were excluded because the cow had been dried-off (13 cases) or sold (ten cases) within the considered time frame. Consequently, 86 CM cases were included in the analysis. The overall no R60 rate was 58.1% (50/86). The probability of achieving no CM recurrence within 60 days after the end of treatment in the AB group was 53.6% (15/28), in the ABMV group 65.7% (23/35) and in the MV group 52.2% (12/23), respectively.

Results of the generalized linear mixed model showed numerically different least-square means of 66.8% for the AB group, 88.2% for the ABMV group and 55.6% for the MV group. However, no significant differences in no R60 of the reference treatment AB to the test treatment MV ($p = .556$) and to the test treatment ABMV ($p = .087$) were found (Table 4). Cows suffering from CM in the first lactation ($p = .026$) and in the second lactation ($p = .009$) showed a significantly higher probability of no R60 than cows affected with CM in the third or higher lactation. Furthermore, animals contracting CM over 200 DIM had a significantly higher likelihood of no R60 compared to cows suffering from CM within 0–100 DIM ($p = .009$) and cows affected with CM within 101–200 DIM ($p = .043$). CM cases caused by staphylococci showed a significantly lower probability of no R60 than CM cases where no pathogen was cultured pretreatment ($p = .048$). The point estimate of the calculated differences in no R60 from the logistic regression and the associated 95% CI is shown in Figure 1. Noninferiority is inconclusive for the MV treatment in comparison with the AB treatment. The ABMV therapy is noninferior to the AB therapy.

3.5 | Bacteriological cure

Bacteriological cure was determined for 129 CM cases. The remaining 45 cases were excluded because of growth-negative (28 cases), missing (five cases) or contaminated (four cases) pretreatment samples and

TABLE 3 Final mixed logistic regression model results for the primary outcome variable clinical cure. Three different treatment regimens were investigated: MV, solely Masti Veyxym®, comprising three treatments at an interval of 12 hr; ABMV, antibiotic treatment as usual on the farm according to label of the respective product combined with Masti Veyxym®, comprising three treatments at an interval of 12 hr; AB, antibiotic treatment as usual on the farm according to label of the respective product

	Coefficient				
Variable	***X***	***SE***	**OR**	**95% CI**	***p*-value**[a]
Intercept	−0.399	0.594	0.671	0.206–2.189	.504
Treatment					
MV	0.231	0.451	1.260	0.513–3.093	.610
ABMV	0.069	0.440	1.072	0.446–2.574	.875
AB (reference)	0				
Lactation number of the cow at the day of clinical mastitis occurrence					
1	0.256	0.448	1.291	0.529–3.151	.570
2	0.182	0.404	1.199	0.537–2.680	.654
>2 (reference)	0				
Days in milk at the day of clinical mastitis occurrence					
0–100	−0.433	0.460	0.649	0.260–1.620	.349
101–200	−0.959	0.414	0.383	0.168–0.873	**.023**
>200 (reference)	0				
Pathogen cultured from the pretreatment milk sample					
Enterobacteriaceae	0.813	0.917	2.254	0.363–13.984	.378
Streptococci	0.714	0.559	2.043	0.672–6.209	.205
Staphylococci	0.082	0.580	1.086	0.342–3.441	.888
Other pathogens	−0.178	0.540	0.837	0.286–2.454	.743
Contaminated sample	−0.530	1.304	0.589	0.044–7.882	.686
No growth (reference)	0				

[a]Significance set at $p < .05$. Bold value indicates significant value.

missing post-treatment samples (eight cases). The overall BC rate was 34.9% (45/129). The probability of BC in the AB group was 37.2% (16/43), in the ABMV group 39.1% (18/46) and in the MV group 27.5% (11/40), respectively.

Results of the generalized linear mixed model showed the least-square means of 38.2% for the AB group, 31.6% for the ABMV group and 20.9% for the MV group. However, no significant differences in BC of the reference treatment AB to the test treatment MV ($p = .151$) and to the test treatment ABMV ($p = .570$) were found (Table 5). Cows suffering from CM within 101–200 DIM showed a significantly lower probability of BC than cows affected with CM over 200 DIM ($p = .002$). The point estimate of the calculated differences in BC from the logistic regression and the associated 95% CI is shown in Figure 1. Noninferiority is inconclusive for both test treatments in comparison with the reference treatment.

3.6 | Quarter somatic cell count cure

Overall twelve CM cases were excluded from this analysis due to missing post-treatment samples (seven cases) or the cow was dried-off (three cases) or slaughtered (one case) or suffered from a teat injury (one case) before at least the first post-treatment sample was collected. The pretreatment CSCC and the course of QSCC for the examined treatment groups are shown in Table 6. The overall QSCC cure was 9.88% (16/162). Including the important covariates of the aforementioned generalized linear mixed model, there are no significant differences between the investigated treatment groups for the outcome variable QSCC cure ($p = .159$; data not shown).

4 | DISCUSSION

The main aim of this study was to evaluate the efficacy of a nonantibiotic intramammary treatment with Masti Veyxym® (MV) in comparison with a reference therapy with antibiotics (AB) of nonsevere CM in cows with longer lasting udder diseases. Furthermore, the influence of Masti Veyxym® on the efficacy of antibiotic treatment for such CM cases was investigated by inclusion of a combined treatment group (ABMV).

Regarding CC as one primary outcome, descriptive results showed small differences in cure rates ranging from 62.7% for the reference treatment (AB) to 63.5% for ABMV and 61.5% for the nonantibiotic treatment. This is in accordance with further investigations, which reported a probability of CC of approximately 60% for CM cases treated with antibiotics, respecting different definitions of CC (Schukken et al., 2013; Swinkels et al., 2014). The small variations in CC rates were

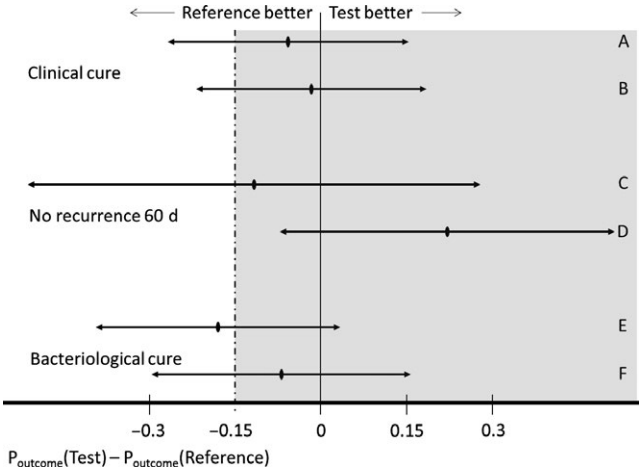

FIGURE 1 Main results of this noninferiority trial. Black point presents point estimate of difference in outcome variables between the test treatments (MV; ABMV) and the reference treatment (AB) with the associated 95% CI indicated by the arrowheads. Dark field represents area of noninferiority. Clinical cure = difference in CC between test treatments MV (A) and ABMV (B) in comparison with AB group, the CI spans both 0 and noninferiority margin (−Δ), noninferiority is inconclusive and there are no significant differences between the two treatments. No recurrence 60 days = difference in no R60 between test treatments MV (C) and ABMV (D) in comparison with AB group. C: The CI spans both 0 and −Δ, noninferiority is inconclusive and there are no significant differences between the two treatments. D: The CI spans 0 but not −Δ, noninferiority is proven and there are no significant differences between the two treatments. Bacteriological cure (BC) = difference in BC between test treatments MV (E) and ABMV (F) in comparison with AB group, the CI spans both 0 and −Δ, noninferiority is inconclusive and there are no significant differences between the two treatments

confirmed by statistical analysis, showing no significant differences for the two test treatments in comparison with the reference treatment (Table 3). Noninferiority was inconclusive, because the CI spans both the noninferiority margin (−Δ) and 0 (Figure 1). An inconclusive result could possibly occur due to a wide range of the CI. A method to reduce this range is to increase sample size. The noninferiority margin also influences the outcome and was chosen according to previous CM trials (Deluyker et al., 1999; Schukken & Deluyker, 1995; Schukken et al., 2013). Sample size was calculated to give the study sufficient power and to show a difference between test and reference therapy if there was a real difference of at least 15% according to Schukken et al. (2013). The nonantibiotic treatment showed a numerically almost identical CC rate and no significant differences to the reference treatment; noninferiority was inconclusive due to the lack of power.

The other primary outcome variable was no R60. The probability of achieving no CM recurrence within 60 days after the end of treatment was almost numerically identical for AB (53.6%) and MV (52.2%). Statistical analysis showed no significant differences between these two treatments. Noninferiority was inconclusive because the CI showed a very wide range and spans −Δ and 0. Recurrences were observed only for clinically cured cases, and some cows dropped out due to the fact that they were not in milk until 60 days after the end of treatment. Hence, the amount of evaluable cases was low and the CI increased. The no R60 rate of ABMV (65.7%) was numerically better than the rate of the reference product; only a tendency but no significant differences was shown (p = .087; Table 4). The CI of the difference between these two treatments spanned only 0; thus, ABMV is noninferior to the solely antibiotic therapy (Figure 1). In a previous study, Pinzón-Sánchez and Ruegg (2011) reported an overall nonrecurrence rate of approximately 80% within 60 days. That value is much higher than the overall no R60 rate of this study (58.1%), although they used the recurrence definition on cow level expecting actually a lower nonrecurrence rate in contrast to the quarter level used in this study. A reason could be that in this study, only cows with a longer lasting high CSCC and with previous CM cases were included. CM is a disease with recurrent character (Schukken, Bar, Hertl, & Gröhn, 2010), and Cha et al. (2016) showed that a cow with two CM cases in current lactation had a higher risk of contracting a third case. Therefore, the low overall no R60 rate could be an indication that the used eligibility criteria are able to select cows with a higher recurrence rate as previously intended.

Bacteriological cure was investigated as secondary outcome because a poor probability of BC for included CM cases was expected. This study resulted in much lower BC rates for CM cases treated with antibiotics (AB group 37.2%; ABMV group 39.1%) in comparison with other studies, which showed a BC rate of approximately 70% (Schukken et al., 2013; Swinkels et al., 2014). The high differences in BC rates support the selection criteria used in this study to choose cows suffering from CM with a low likelihood of BC. Nevertheless, a tendency for the efficacy of antibiotic treatment against mastitis pathogens was shown. The probability of BC in the nonantibiotic treatment group (MV) was 27.5% and therefore numerically lower than the BC rate in the reference treatment group. However, there were no significant differences between MV and AB group (p = .151). Noninferiority was inconclusive because CI spans both −Δ and 0. This may be due to the fact that the real difference not being higher than the observed 15%. Between the ABMV and AB group were small numerical differences without significant associations and noninferiority was inconclusive.

The evaluations of noninferiority resulted mostly in inconclusive findings. A larger sample size in all treatment groups is required to confirm the detected results of the study and to make a clear statement on noninferiority.

The main interests of the farmers are disappearance of clinical signs, a low recurrence rate and a short time of discarding milk (Ruegg, 2010). With respect to the primary outcomes, Masti Veyxym® seems to show similar results in comparison with the reference group treated with antibiotics. Furthermore, advantageous properties of Masti Veyxym® are the short withdrawal period for milk of 1 day, which decreases time of discarding milk, and that it contains no antibiotics, resulting in a reduced risk of residues and improved safety.

Another interesting outcome variable for farmers assessing a successful treatment is the course of the CSCC, because it is used as a measure of milk quality. In this study, cows with persistent high CSCC were chosen and a low probability of BC was expected and confirmed.

TABLE 4 Final mixed logistic regression model results for the primary outcome variable no recurrence 60 days. Three different treatment regimens were investigated: MV, solely Masti Veyxym®, comprising three treatments at an interval of 12 hr; ABMV, antibiotic treatment as usual on the farm according to label of the respective product combined with Masti Veyxym®, comprising three treatments at an interval of 12 hr; AB, antibiotic treatment as usual on the farm according to label of the respective product

	Coefficient				
Variable	***X***	***SE***	**OR**	**95% CI**	***p*-value[a]**
Intercept	1.759	1.323	5.805	0.405–83.149	.190
Treatment					
MV	−0.475	0.804	0.622	0.125–3.086	.556
ABMV	1.306	0.753	3.693	0.822–16.590	.087
AB (reference)	0				
Lactation number of the cow at the day of clinical mastitis occurrence					
1	1.843	0.809	6.315	1.260–31.665	**.026**
2	2.137	0.791	8.476	1.752–41.006	**.009**
>2 (reference)	0				
Days in milk at the day of clinical mastitis occurrence					
0–100	−2.815	1.050	0.060	0.007–0.486	**.009**
101–200	−1.939	0.941	0.144	0.022–0.940	**.043**
>200 (reference)	0				
Pathogen cultured from the pretreatment milk sample					
Enterobacteriaceae	−1.002	1.744	0.367	0.011–11.887	.567
Streptococci	0.628	0.980	1.873	0.265–13.221	.524
Staphylococci	−2.028	1.008	0.132	0.018–0.982	**.048**
Other pathogens	−0.510	0.884	0.601	0.103–3.501	.566
Contaminated sample	−1.888	1.767	0.151	0.004–5.128	.289
No growth (reference)	0				

[a]Significance set at $p < .05$. Bold value indicates significant value.

TABLE 5 Final mixed logistic regression model results for the secondary outcome variable bacteriological cure. Three different treatment regimens were investigated: MV, solely Masti Veyxym®, comprising three treatments at an interval of 12 hr; ABMV, antibiotic treatment as usual on the farm according to label of the respective product combined with Masti Veyxym®, comprising three treatments at an interval of 12 hr; AB, antibiotic treatment as usual on the farm according to label of the respective product

	Coefficient				
Variable	***X***	***SE***	**OR**	**95% CI**	***p*-value[a]**
Intercept	0.563	0.544	1.756	0.598–5.153	.302
Treatment					
MV	0.808	0.559	2.243	0.741–6.786	.151
ABMV	0.303	0.531	1.353	0.473–3.871	.570
AB (reference)	0				
Lactation number of the cow at the day of clinical mastitis occurrence					
1	−0.284	0.539	0.753	0.259–2.189	.599
2	0.274	0.464	1.315	0.525–3.297	.556
>2 (reference)	0				
Days in milk at the day of clinical mastitis occurrence					
0–100	0.186	0.604	1.204	0.364–3.985	.759
101–200	−1.577	0.488	0.207	0.079–0.543	**.002**
>200 (reference)	0				
Pathogen cultured from the pretreatment milk sample					
Enterobacteriaceae	0.103	0.887	1.109	0.191–6.424	0.908
Streptococci	0.766	0.510	2.151	0.784–5.905	0.136
Staphylococci	0.747	0.528	2.111	0.742–6.008	0.160
Other pathogens (reference)	0				

[a]Significance set at $p < .05$. Bold value indicates significant value.

TABLE 6 Illustrated are the mean log of the cow somatic cell count (CSCC pretreatment, cells/ml) of the most recent milk recording before the occurrence of the clinical mastitis (CM) case and the course of the mean log of the quarter somatic cell count (QSCC, cells/ml) after treatment of CM cases of cows with longer lasting udder diseases at CM occurrence (QSCC d0) as well as at days 14 (QSCC 14d) and 21 (QSCC 21d) after the end of treatment with the associated standard deviations (±). Three different treatment regimens were investigated: MV, solely Masti Veyxym®, comprising three treatments at an interval of 12 hr; ABMV, antibiotic treatment as usual on the farm according to label of the respective product combined with Masti Veyxym®, comprising three treatments at an interval of 12 hr; AB, antibiotic treatment as usual on the farm according to label of the respective product

	CSCC pretreatment	QSCC d0	QSCC d14	QSCC d21
MV	5.73 ± 0.62	6.80 ± 0.36	6.12 ± 0.85	5.76 ± 0.86
ABMV	5.67 ± 0.68	6.83 ± 0.53	5.80 ± 0.88	5.48 ± 1.04
AB	5.77 ± 0.53	6.79 ± 0.46	6.11 ± 0.86	5.69 ± 0.92

Differences between the treatment groups were not significant ($p > .05$).

The antibiotic treatment can only affect the BC and at best eliminate the causing pathogen. Only in the successful case of BC can be achieved a CC and a noticeable reduction in CSCC (Degen et al., 2015). Overall, only 9.88% of the examined CM cases reached a QSCC cure and no significant differences between the treatment groups were observed. This percentage is lower than results of a recently published study of Swinkels et al. (2014). They showed an overall QSCC cure of 22%, which suggests that a cow with the used selection criteria for this study has got a very low probability to recover a normal SCC in the affected udder quarter. The low QSCC cure rate by a CC rate of approximately 60% indicates that the observed CM cases may convert into a subclinical stage with still elevated QSCC.

Our intention was to reflect the situation in daily practice on dairy farms. That implies no information about the causative pathogen at the time of CM treatment. Therefore, and because power calculations were made on overall therapy level, evaluations of treatment efficacy at a pathogen level gave no reliable results due to lack of power. Moreover, farmers were allowed to use their normal mastitis treatment procedure. That resulted in a wide range of used antibiotics with different durations of treatment and withholding periods. However, there were no indications of the various therapies affecting the study results.

No completely untreated control group was included in our investigation. There are some reasons for this. Clinical mastitis is a disease which is accompanied with pain, suffering and harm for the cow. Therefore, solely for reasons of the animal welfare a treatment is indicated. The participated farms were all geared to economic principles and in the normal production cycle. It was not possible to leave cows with CM untreated without any evidence-based information. Furthermore, they mostly did not differentiate between a first CM case and a chronic mastitis, all CM cases were treated with antibiotics. Finally, prior to the study we were unaware if the chosen selection criteria for cows are really suitable to identify cows with CM expecting a poor probability of BC.

5 | CONCLUSIONS

A randomized, multiherd, noninferiority study was conducted evaluating the efficacy of the test treatments Masti Veyxym® (MV) and combined Masti Veyxym® with antibiotics (ABMV) in comparison with antibiotic treatment (AB; reference) of mild-to-moderate CM in cows with longer lasting udder diseases. The two test treatments showed no significant differences to the reference treatment with respect to the outcome variables such as clinical cure, no CM recurrence within 60 days after the end of therapy, bacteriological cure and quarter somatic cell count cure. The solely nonantibiotic therapy showed a numerically lower probability of bacteriological cure without significant differences to the reference treatment. The combined treatment group (ABMV) resulted in a numerically higher nonrecurrence rate than the two other therapy groups (MV and AB) and noninferiority compared to the reference treatment was proven. The study findings indicate that using solely Masti Veyxym® in treatment of mild-to-moderate CM in cows with longer lasting udder diseases may constitute an alternative therapy to reduce antibiotics. However, to give a reliable noninferiority evaluation, a higher sample size is needed. The selection criteria of cows have to be respected, and it is recommended to remove such animals from the herd if possible.

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CONFLICT OF INTEREST

The authors planned, designed and conducted this study. Veyx-Pharma GmbH supported the investigations. The authors declare no conflict of interest.

REFERENCES

Bradley, A. J., & Green, M. J. (2009). Factors affecting cure when treating bovine clinical mastitis with cephalosporin-based intramammary preparations. *Journal of Dairy Science*, *92*, 1941–1953.

Cha, E., Hertl, J., Schukken, Y., Tauer, L., Welcome, F., & Gröhn, Y. (2016). Evidence of no protection for a recurrent case of pathogen specific clinical mastitis from a previous case. *Journal of Dairy Research*, *83*, 72–80.

Degen, S., Paduch, J.-H., Hoedemaker, M., & Krömker, V. (2015). Factors affecting the probability of bacteriological cure of bovine mastitis. *Tierärztliche Praxis Ausgabe Grosstiere/Nutztiere*, *43*, 222–227.

Deluyker, H. A., Chester, S. T., & van Oye, S. N. (1999). A multilocation clinical trial in lactating dairy cows affected with clinical mastitis to compare the efficacy of treatment with intramammary infusions of a lincomycin/neomycin combination with an ampicillin/cloxacillin combination. *Journal of Veterinary Pharmacology and Therapeutics*, *22*, 274–282.

EMEA (The European Agency for the Evaluation of Medicinal Products) (2000). *VICH Topic GL9 (GCP): Guideline on good clinical practices*. London: United Kingdom.

Grieger, A.-S., Zoche-Golob, V., Paduch, J.-H., Hoedemaker, M., & Krömker, V. (2014). Recurrent clinical mastitis in dairy cattle – importance and causes. *Tierärztliche Praxis Ausgabe Grosstiere/Nutztiere*, *42*, 156–162.

GVA [German Veterinary Association] (2009). *Guidelines for aseptic milk sampling and guidelines to isolate and identify mastitis pathogens*, 2nd ed. Germany: Gießen.

GVA [German Veterinary Association] (2012). *Guidelines for combating bovine mastitis as a stock problem*, 5th ed. Germany: Gießen.

Hogeveen, H., Huijps, K., & Lam, T. J. G. M. (2011). Economic aspects of mastitis: New developments. *New Zealand Veterinary Journal*, *59*, 16–23.

IDF (International Dairy Federation) (2005). *Economic consequences of mastitis. Bulletin No 394*. Brussels, Belgium.

Krömker, V., & Friedrich, J. (2011). Recommendations for diagnostic measures regarding mastitis control on herd level. *Der Praktische Tierarzt*, *92*, 516–524.

Krömker, V., Paduch, J.-H., Klocke, D., Friedrich, J., & Zinke, C. (2010). Efficacy of extended intramammary therapy to treat moderate and severe clinical mastitis in lactating dairy cows. *Berliner und Münchener Tierärztliche Wochenschrift*, *123*, 10–15.

Krüger, M., Hien, T. T., Zaremba, W., & Penka, L. (1999). Investigations on the influence of the proteolytic enzymes trypsin, chymotrypsin, and papain on udder pathogenic microorganisms. Part 1: Influence of enzymes on growth behaviour, survival rate, chain formation (streptococci) and morphism (yeasts). *Tierärztliche Praxis Ausgabe Grosstiere/Nutztiere*, *27*, 207–215.

Mansion-de Vries, E. M., Hoedemaker, M., & Krömker, V. (2015). Evidence-based aspects of clinical mastitis treatment. *Tierärztliche Praxis Ausgabe Grosstiere/Nutztiere*, *43*, 287–295.

NMC (National Mastitis Council) (1999). *Laboratory handbook on bovine mastitis*, Revised ed. Madison WI, USA: NMC.

O`Connor, A.M., Sargeant, J.M., Gardner, I.A., Dickson, J.S., Torrence, M.E., Dewey, C.E., … Wills, R. (2010). The REFLECT statement: Methods and processes of creating reporting guidelines for randomized controlled trials for livestock and food safety. *Journal of Veterinary Internal Medicine/American College of Veterinary Internal Medicine*, *24*, 57–64.

Oliveira, L., & Ruegg, P. L. (2014). Treatments of clinical mastitis occurring in cows on 51 large dairy herds in Wisconsin. *Journal of Dairy Science*, *97*, 5426–5436.

Piaggio, G., Elbourne, D. R., Altman, D. G., Pocock, S. J., & Evans, S. J. W. (2009). Reporting of non-inferiority and equivalence randomized trials: An extension of the CONSORT statement. *Journal of the American Medical Association*, *295*, 1152–1160. 1842

Pinzón-Sánchez, C., & Ruegg, P. L. (2011). Risk factors associated with short-term post-treatment outcomes of clinical mastitis. *Journal of Dairy Science*, *94*, 3397–3410.

Ruegg, P. L. (2010). *The application of evidence based veterinary medicine to mastitis therapy*. Santiago, Chile: World Buiatrics Congress.

Santman-Berends, I. M. G. A., Lam, T. J. G. M., Keurentjes, J., & van Schaik, G. (2015). An estimation of the clinical mastitis incidence per 100 cows per year based on routinely collected herd data. *Journal of Dairy Science*, *98*, 6965–6977.

Schukken, Y. H., Bar, D., Hertl, J., & Gröhn, Y. T. (2010). Correlated time to event data: Modeling repeated clinical mastitis data from dairy cattle in New York State. *Preventive Veterinary Medicine*, *97*, 150–156.

Schukken, Y. H., Bennett, G. J., Zurakowski, M. J., Sharkey, H. L., Rauch, B. J., Thomas, M. J., … Zadoks, R. N. (2011). Randomized clinical trial to evaluate the efficacy of a 5-day ceftiofur hydrochloride intramammary treatment on nonsevere gram-negative clinical mastitis. *Journal of Dairy Science*, *94*, 6203–6215.

Schukken, Y. H., & Deluyker, H. A. (1995). Design of field trials for the evaluation of antibacterial products for therapy of bovine clinical mastitis. *Journal of Veterinary Pharmacology and Therapeutics*, *18*, 274–283.

Schukken, Y. H., Zurakowski, M. J., Rauch, B. J., Gross, B., Tikofsky, L. L., & Welcome, F. L. (2013). Noninferiority trial comparing a first-generation cephalosporin with a third-generation cephalosporin in the treatment of nonsevere clinical mastitis in dairy cows. *Journal of Dairy Science*, *96*, 6763–6774.

Sol, J., Sampimon, O. C., Barkema, H. W., & Schukken, Y. H. (2000). Factors associated with cure after therapy of clinical mastitis caused by *Staphylococcus aureus*. *Journal of Dairy Science*, *83*, 278–284.

Swinkels, J. M., Cox, P., Schukken, Y. H., & Lam, T. J. G. M. (2013). Efficacy of extended cefquinome treatment of clinical *Staphylococcus aureus* mastitis. *Journal of Dairy Science*, *96*, 4983–4992.

Swinkels, J. M., Krömker, V., & Lam, T. J. G. M. (2014). Efficacy of standard vs. extended intramammary cefquinome treatment of clinical mastitis in cows with persistent high somatic cell counts. *Journal of Dairy Research*, *81*, 424–433.

Trevisi, E., Zecconi, A., Cogrossi, S., Razzuoli, E., Grossi, P., & Amadori, M. (2014). Strategies for reduced antibiotic usage in dairy cattle farms. *Research in Veterinary Science*, *96*, 229–233.

Watts, J. L., Salmon, S. A., & Yancey, R. J. J. (1993). Use of modified Rambach agar to differentiate *Streptococcus uberis* from other mastitis streptococci. *Journal of Dairy Science*, *76*, 1740–1743.

Zander, H.(1997) *Examinations about the effectiveness of enzymes and enzyme/antibiotic-combinations in the therapy of different kind of bovine mastitis*. Thesis Freie Universität Berlin.

Ziesch, M., & Krömker, V. (2016). Factors influencing bacteriological cure after antibiotic therapy of clinical mastitis. *Milk Science International*, *69*, 7–14.

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