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Investigation of cefquinome residues in milk after antibiotic dry cow treatment and risk factors for milk leakage in dairy cows

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Meinen Eltern

“The love for all living creatures is the most noble attribute of man.”

(Charles Darwin)

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1 INTRODUCTION

Antibiotic residues in milk should be prevented as they represent a potential risk to human health, a negative effect on milk manufacturing processes and an economic loss for the dairy farmers (Allison, 1985). These facts are known for decades, but they still have not lost actuality and importance for dairy producers, researchers and the general public.

The recording of the Federal Office of Consumer Protection and Food Safety (**BVL**) about the antibiotic dispense quantity displayed, that 6 tons of intramammary (IMM) dry cow antibiotics were used in Germany in 2013 (Wallmann, 2014). Antibiotic dry cow treatment in combination with or without an internal teat sealant is part of the mastitis control program recommended by the National Mastitis Council (NMC, 2006). The IMM treatment with a long-acting antibiotic at dry-off pursues two main goals: the cure of existing intrammamary infections (**IMI**) and the protection against new IMI (Halasa et al., 2009a, 2009b). The National Animal Health Monitoring System (**NAHMS**) collected information about dry-off procedures on US dairy farms in 17 states and reported that 80.3% of operations treated all cows with an IMM dry cow antibiotic (Lombard et al., 2015). Furthermore, a survey of drying-off practices on commercial dairy farms in northern Germany detected a blanket antibiotic dry cow treatment on 79.6% of the farms (Bertulat et al., 2015). These results agree with the recording of the BVL, which reported that in 2013 80% of the German dairy cows were treated with an IMM dry cow antibiotic and in 10% of these cases a fourth-generation cephalosporine was used (Wallmann, 2014).

Cefquinome is a fourth-generation cephalosporine used in dry cow therapy and it offers an antimicrobial activity against gram-positives and gram-negatives as well as a high stability to ß-lactamases (CVMP, 2003). Bradley et al. (2011) investigated the effects of 3 different dry cow treatment protocols (i.e., broad-spectrum antibiotic cefquinome alone, narrow-spectrum antibiotic cloxacillin alone and in combination with a teat sealant) on the cure and the prevention of IMI during the dry period and the occurrence of clinical mastitis during the subsequent lactation. The author reported no difference between the treatment groups concerning the cure of IMI caused by major pathogens. Quarters in the cefquinome group and the cloxacillin combined with teat sealant group were more likely to be free of major pathogens and enterobacterial pathogens post-calving. Furthermore, the cefquinome group was less likely to develop clinical mastitis in the first 100 d of the subsequent lactation than the cloxacillin group.

After antibiotic treatment, milk must be withdrawn for a specific period of time according to the specific product characteristics to avoid antibiotic residues in the food chain. These withdrawal periods for milk are determined utilizing the Time to Safe Concentration method, which calculates the number of milkings after antibiotic treatment, necessary for the residue concentration in milk to fall below the maximum residue limit (**MRL**; CVMP, 2000). To further ensure food safety, the International Dairy Federation recommended the usage of screening tests on farm to detect antibiotic residues in milk (IDF, 2014). Not only the bulk tank milk but milk from individually treated cows should be tested after the recommended withdrawal periods (Jones, 2009). As there is no regulatory requirement for milk from individual cows to be tested for antibiotic residues, the commercially available screening tests, however, were only evaluated by the US Federal Drug Administration (**FDA**) for the usage on raw commingled milk samples (FDA, 1996). We hypothesize that foremilk (i.e., hand collected milk before milking) and stripping samples (i.e., hand collected milk after milking) from individual cows are most commonly used for screening tests, resulting from the easier sampling procedure than for composite milk samples. For an appropriate usage of antibiotic screening tests, knowledge of the relationship between milk fraction and residue concentration in milk samples from individual cows is important. A more than twice as high cephapirin concentration in foremilk than in bucket milk or strippings at the first milking after IMM infusion was reported by Stockler et al. (2009) for a lactating cow antibiotic, but no informations for dry cow antibiotics are available.

Therefore, the objective of my first study was to investigate the residue concentration of an antibiotic dry cow treatment containing cefquinome in two different milk fractions (i.e., foremilk or stripping samples) considering the milk composition (i.e., fat, protein, lactose, urea and somatic cell count) and furthermore, to evaluate the test characteristic of two commercially available screening tests in relationship to the analyzed milk fraction and the milk composition.

Withdrawal periods for dry cow antibiotics depend on the dry period length, thus the residue depletion during the dry-period and post-calving is considered (CVMP, 2006). The optimum dry period length for the cow and dairy producer is still an active field of research. A dry period length of 60 to 65 d was identified to maximize the milk yield in the subsequent lactation (Kuhn et al., 2005). Nevertheless, the NAHMS reported that about 93.0% of US dairy cows in 17 states were dried off approximately 57.1 d before calculated calving date (Lombard et al., 2015) and a survey on commercial dairy farms in northern Germany revealed an averaged dry period length of 7 weeks (Bertulat et al., 2015). Short dry periods of 35 d or less were used

in only 3.7% of the participating dairy farms (Bertulat et al., 2015). Several studies in the last decade have described advantages of short dry periods on milk production (Kuhn et al., 2006), reproductive performance (Shoshani et al., 2014) and energy balance during early lactation (Van Knegsel et al., 2014) without any disadvantages to udder health (Church et al., 2008). Santschi and Lefebvre (2014), however, hypothesized that short dry periods can be a risk factor for antibiotic residues in early lactation when cows calve early. The concrete impact of shortening the residue depletion during the dry period on the residue concentration of dry cow antibiotics after calving has not yet been investigated

Therefore, the objective of our second study was to investigate the residue concentration of a dry cow antibiotic containing cefquinome during the colostrum period and early lactation in quarter foremilk samples after short dry periods.

After dry-off, the occurrence of milk leakage in approximately 30% of cows has been reported (Schukken et al., 1993; Bertulat et al., 2013). Milk leakage is defined as milk dripping or flowing from the teat in the absence of active milking and it is associated with a 4.0 and 6.0 times greater risk for clinical mastitis and new IMI, respectively (Schukken et al., 1993; Waage et al., 1998; Peeler et al., 2000; Waage et al., 2001). Risk factors for milk leakage on the cow level have rarely been investigated. As risk factors for dry cows, high milk yield and high extramammary udder pressure have been reported (Tucker et al., 2009; Bertulat et al., 2013) and for lactating cows short teat canals, teat canal protrusion, high peak milk flow (Klaas et al., 2005) and high IMM pressure (Rovai et al., 2007). The potential influence of the teat canal diameter, physicochemical characteristics of the milk and the application of a dry cow antibiotic on milk leakage has not yet been investigated.

A high number of cows and measurements would have been necessary to investigate plausible risk factors for milk leakage in a field study, due to variance between cows, quarters and teats. Therefore, a laboratory model was developed to determine the risk factors for milk leakage under controlled conditions and thereby reduce the number of study animals. The laboratory model, composed of two viscometers, was used to measure the efflux time (i.e., time interval that the fluid needs to descend from the upper timing mark to the lower timing mark of the viscometers' measuring sphere) and calculate the dynamic viscosity (i.e., internal resistance of a fluid to the application of pressure) of milk samples. Parameters which lead to a decreased efflux time and dynamic viscosity of the milk samples were considered to increase the likelihood of milk leakage in the field.

The objective of the third, in vitro study was to evaluate plausible risk factors for milk leakage in dairy cows before and after dry-off. Specifically, we set out to determine the effects of the teat canal diameter, IMM pressure, milk temperature, milk composition, dry-off and IMM dry cow therapy on efflux time and dynamic viscosity of quarter foremilk samples.

The overall objective of my thesis was to examine the effect of the milk fraction and short dry periods on the residue concentration of cefquinome in milk after calving and thereby improve the application of antibiotic screening tests and verify the validity of established withdrawal periods in the case of short dry periods. Furthermore, plausible risk factors for milk leakage in dairy cows before and after dry-off should be investigated to gain further knowledge about this phenomenon in dairy cows, which is important for the udder health.

2 ADDITIONAL UNPUBLISHED DATA

2.1 Residue concentration of cefquinome considering different milk fractions and performance of two screening tests.

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2.1 Residue Concentration of cefquinome considering different milk fractions and performance of two screening tests

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2.1.1 **Summary**

This Research Communication describes the residue concentration of a dry cow antibiotic and the milk composition in two different milk fractions and furthermore, the effect of milk fraction and milk composition on the test performance of a rapid screening and a microbial inhibitor test. Thirteen dry cows were treated with an intramammary dry cow antibiotic containing 150 mg cefquinome. Quarter foremilk and stripping samples were collected on the first 10 d postpartum. All milk samples were analyzed for milk composition by the local Dairy Herd Improvement Association and were tested for antibiotic residues using the rapid screening test Milchtest BL and the microbial inhibitor test Delvotest BR Brilliant Plates. The residue concentration of cefquinome was determined in foremilk and stripping samples from milkings 1, 2, 3, 5, and 7 after calving using high performance liquid chromatography – tandem mass spectrometry. In our study, the logarithm of cefquinome concentration (logCef) was higher in foremilk than in stripping samples and higher in milk samples with lower lactose content. Furthermore, logCef decreased with the number of milking ($P < 0.001$). The Milchtest BL was more likely to be not evaluable in stripping samples and milk samples with increased protein content. In the Delvotest BR Brilliant Plates milk samples with higher protein content were more likely to have a false positive result. These results indicate that foremilk should be the recommended milk fraction to be tested for residues of cefquinome and that high protein content should be considered as a cause of test failure and false positive results when milk during the first 10 d postpartum is tested for antibiotic residues using screening tests.

Key words: antibiotic residue, screening test, milk fraction, cefquinome

2.1.2 Research Communication

Screening tests should be used regularly on farms to detect antibiotic residues in bulk tank milk as well as in milk from individually treated cows to ensure food safety (Jones, 2009; IDF, 2014b). These screening tests, however, were only evaluated by the US Federal Drug Administration (FDA) for the usage on raw commingled milk samples, as there is no regulatory requirement for milk from individual cows to be tested (FDA, 1996). We hypothesize that foremilk (i.e., hand collected milk before milking) and stripping samples (i.e., hand collected milk after milking) from individual cows are most commonly used for screening tests, because the sampling procedure in the milking parlor is easier than for composite milk samples. For an appropriate use of such screening tests, however, knowledge of the relationship and potential confounding between milk fraction (i.e., foremilk and strippings) and antibiotic concentration in milk samples from individual cows is important.

Stockler et al. (2009) detected more than twice as high cephapirin concentrations in foremilk than in bucket milk or strippings from lactating cows at the first milking after intramammary (IMM) infusion. The authors hypothesized that changes in milk composition among milk fractions might affect the distribution of the antibiotic within the udder (Stockler *et al*., 2009). The highest logarithm of somatic cell count (SCC) and fat content were detected by Vangroenweghe et al. (2002) in residual milk and the highest content of protein in foremilk, cisternal and main milk. We hypothesize that these changes in milk composition between the milk fractions might affect the residue concentration of an antibiotic dry cow treatment and the test characteristic of screening tests.

Therefore, the objective of our study was 1) to investigate the residue concentration of an antibiotic dry cow treatment containing cefquinome in two different milk fractions (i.e., foremilk or strippings) considering the milk composition (i.e., fat, protein, lactose, urea and SCC) and 2) to evaluate the test characteristic of two commercially available screening tests in relationship to the analyzed milk fraction and the milk composition.

2.1.3 Materials and Methods

Data Collection

The study was conducted between November 2014 and May 2015 at the Clinic for Animal Reproduction (Freie Universität Berlin, Berlin, Germany). Thirteen healthy, dry Holstein-Friesian and Holstein-Friesian crossbreed dairy cows were enrolled in the study. Three

quarters of each cow received a single treatment with an IMM dry cow antibiotic (i.e., 150 mg of cefquinome; Virbactan, Virbac Ltd., Carros, France) on d21, 14 and 7 before calculated calving date (i.e., 280 d after artificial insemination), respectively. One quarter of each cow received no treatment. Day of treatment was allocated to each quarter at random. This treatment scheme resulted from the fact, that the study was part of a larger study on antibiotic residues after short dry periods with a quarter-based approach. Therefore, cows were not dried off shortly before calving, but previously dried-off cows were selected and treated again with an IMM dry cow antibiotic following the above-described treatment scheme. All quarters were monitored before and once a day after treatment for signs of clinical mastitis (i.e., firmness, pain, heat, redness, swelling). After calving cows were milked twice daily at 07.00 h and 19.00 h using a portable bucket milking machine.

Milk samples were collected on the first 10 d postpartum during milking times at 07.00 h and 19.00 h (i.e., first to twenty-second milking after calving). Two samples (i.e., 13 mL and 30 mL) each were collected from each quarter before (i.e., foremilk samples) and after milking (i.e., stripping samples), respectively. The foremilk samples were collected after examining the first two streams from each quarter for signs of clinical mastitis (i.e., clots, flakes) as well as subclinical mastitis using the California Mastitis Test. The stripping samples were collected immediately after removing the milking cluster. Antibiotic residues were tested with a lateral flow test Milchtest BL (Packhaus Rockmann GmbH, Sendenhorst, Germany) with a detection limit of 20 µg/kg for cefquinome. Procedures were conducted according to the recommendations of the manufacturer. Residue concentration of cefquinome in foremilk and stripping samples from milkings 1, 2, 3, 5 and 7 (i.e., first, second, third, fifth and seventh milking after calving) were determined using high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) with a limit of quantification of 1 ng/g and a limit of detection of 0.5 ng/g for cefquinome. The 30 mL foremilk and stripping samples were analyzed for milk composition (i.e., fat, protein, lactose, urea, SCC) by trained technicians using the CombiFoss (Foss, Hilleroed, Denmark) at the local DHIA (Landeskontrollverband Berlin-Brandenburg eV., Waldsieversdorf, Germany). Furthermore, all samples were tested for antibiotic residues using the microbial inhibitor test Delvotest BR Brilliant Plates (DSM Food Specialties B.V., Delft, Netherlands) with an incubation time of 2 h 45 min and a detection limit of 100 µg/kg for cefquinome. The test was conducted and evaluated according to IDF 471/2014 (IDF, 2014a).

Statistical Analysis

Data were entered into Excel spreadsheets (version 2010; Microsoft Corp., Redmond, WA) and statistical analyses were performed with IBM SPSS Statistics (version 22.0; IBM Deutschland GmbH, Ehningen, Germany) and Medcalc (version 12.4.0.0, Mariakerke, Belgium). The cefquinome concentration and SCC were transformed to common logarithms to achieve normal distribution (i.e., logCef and logSCC). The effect of milk fraction (i.e., foremilk vs. stripping samples), the number of milking (i.e., first to twenty-second milking after calving), fat (%), protein (%), lactose (%) and urea content (mg/L) and logSCC on logCef was evaluated in a mixed-model ANOVA. The effect of milk fraction on the milk composition was also determined using a mixed-model ANOVA. Mixed models were built according to the model building strategies developed by Dohoo et al. (2009). The random effect of udder quarter within cow was included in all models. The number of milking was considered as repeated factor.

In order to investigate potential associations between the milk fraction, fat, protein, lactose, urea content and logSCC and the test results "not evaluable" and "false positive" of Milchtest BL and "false positive" of Delvotest BR Brilliant Plates binary logistic regressions were used. All values reported are LSM ± SEM. The significance level was set at $P \leq 0.05$ and tendencies were discussed at $P \leq 0.1$.

2.1.4 **Results and Discussion**

Residue Concentration of Cefquinome

No signs of subclinical or clinical mastitis could be detected in any quarter throughout the study period, therefore, 2,027 values of fat, protein and lactose, 2,099 values of SCC and 1,997 values of urea from foremilk and stripping samples could be used in the final analysis. Milk composition differed between foremilk and stripping samples ($P < 0.001$), with higher protein, lactose and urea content in foremilk samples and higher fat content and logSCC in stripping samples (Supplementary Table S1).

One hundred and eighty-four foremilk and 167 stripping samples from milkings 1, 2, 3, 5, and 7 after calving were analyzed for the residue concentration of cefquinome using HPLC-MS/MS. The logarithm of cefquinome concentration (logCef) was affected by the milk fraction ($P = 0.001$), the number of milking ($P < 0.001$) and the lactose content of the milk sample ($P < 0.001$). The logCef was higher in foremilk (1.495 ± 0.080) compared to stripping samples (1.377 ± 0.078; $P = 0.001$). After having retransferred the values of logCef for better

comparability the residue concentration of cefquinome was 31.26 ng/g and 23.82 ng/g in foremilk and stripping samples, respectively. Our results are in agreement with findings from Stockler et al. (2009) for a lactating cow antibiotic containing 200 mg cephapirin sodium. The authors detected 44.2 µg/mL cephapirin in foremilk, 18.5 µg/mL in stripping samples and 15.7 µg/mL in bucket milk at the first milking following the IMM antibiotic treatment. While the cephapirin concentration in the study by Stockler et al. (2009) was 2.39 times higher in foremilk than in stripping samples, in our study the cefquinome concentration in foremilk was only 1.46 times higher. This difference could be explained by the longer exposure time of the drug in the udder, which might lead to a more intense distribution within the udder. The value of logCef decreased with the number of milking ($P < 0.001$) and additionally, an interaction between the milk fraction and the number of milking could be detected ($P = 0.001$; Supplementary Figure S1). This is consistent with residue depletion studies in lactating cows which have detected high concentrations of cefquinome in milk at the first milking after the last administration and concentrations under the maximum residue limit at the 10th milking (CVMP, 1995). The value of logCef was higher in samples with lower lactose content ($P < 0.001$). This was the only effect of milk composition on the cefquinome concentration we could detect in our study and the physiological mechanism remains unclear.

Screening Tests

Three hundred and eighty-three foremilk and 448 stripping samples were tested for residues of cefquinome using the rapid screening test Milchtest BL (Packhaus Rockmann GmbH, Sendenhorst, Germany). Three hundred three test results (i.e., 82.8% of stripping samples, 17.2% of foremilk samples) of the Milchtest BL were not evaluable because no test or control line appeared. Foremilk samples had 38.4 times higher odds to be evaluable in the Milchtest BL than tests of stripping samples (Table 1). High protein content increased and high logSCC tended to increase the odds of non-evaluable results in the Milchtest BL (Table 1). The higher proportion of evaluable foremilk samples might be related to the lower fat content of foremilk compared to stripping samples. Andrew (2000) hypothesized that high milk fat may hinder the milk movement along the gradient in the CITE Snap assay and results in a test failure and false-positive outcomes. In our study the milk samples did not flow far enough through the membrane to reach the test and control line. High fat and protein content cause a higher dynamic viscosity of milk (Alcântara *et al.*, 2012) and we hypothesize that a high viscosity might impede the flow of the milk samples through the membrane of the Milchtest BL resulting in not

evaluable tests. A high logSCC might be a further mechanical obstruction of the milk flow through the test membrane.

Corresponding results of HPLC-MS/MS for residue concentrations of cefquinome were available for 68 test results of the Milchtest BL and they were used as a gold standard. Nineteen test results of the Milchtest BL were false positive (7.6 ± 1.1 ng/g; range: 0.5 to 18.40 ng/g) and no test result was false negative. False positive test results of the Milchtest BL were neither associated with the milk fraction nor milk composition ($P > 0.100$).

Seven hundred and seventy-three foremilk and 775 stripping samples were tested at the local DHIA using the Delvotest BR Brilliant Plates (DSM Food Specialties B.V., Delft, Netherlands). Corresponding HPLC-MS/MS results for residue concentrations of cefquinome were available for 348 test results of the Delvotest BR Brilliant Plates and they were used as a gold standard again. Forty-seven test results of the Delvotest BR Brilliant Plates were false positive (33.8 ± 4.7 ng/g; range: 0.5 to 98.85 ng/g) and six were false negative (180.5 ± 51.2 ng/g; range: 113.03 to 435.11 ng/g). Milk samples with higher protein content were more likely and with a lower urea content tended to be more likely to have a false positive result in the Delvotest BR Brilliant Plates, respectively (Table 2). Natural inhibitors are known to be elevated in colostrum and mastitic milk. They could be the reason for the correlation between increased protein content and false positive results of the Delvotest BR Brilliant Plates in our study, as the natural inhibitors might inhibit the growth of the test organism *Bacillus stearothermophilus.* Kang et al. (2005) collected 73 foremilk samples from lactating cows with clinical mastitis after withdrawal times (i.e., 2 to 5 d) from IMM antibiotic treatment (i.e., beta-lactams, tetracyclines, sulfonamides and aminoglycosides) and tested with the Delvotest SP for antibiotic residues. As 21 from 24 positive results were negative after being heated at 82°C for 5 min, the author concluded that these 21 positive results were caused by natural inhibitors in the milk samples, which were inactivated by the heat treatment. The weak correlation between increased urea content and correct results of the Delvotest BR Brilliant Plates remains unclear and to our knowledge no other study reported a comparable correlation. Milk fraction ($P = 0.799$), fat ($P = 0.887$) and lactose content ($P = 0.110$) as well as logSCC ($P = 0.453$) had no effect on the correctness of the results in the Delvotest BR Brilliant Plates.

Table 1. Association between the evaluability of the Milchtest BL (Packhaus Rockmann GmbH, Sendenhorst, Germany) and milk fraction and milk composition (n = 303 milk samples).

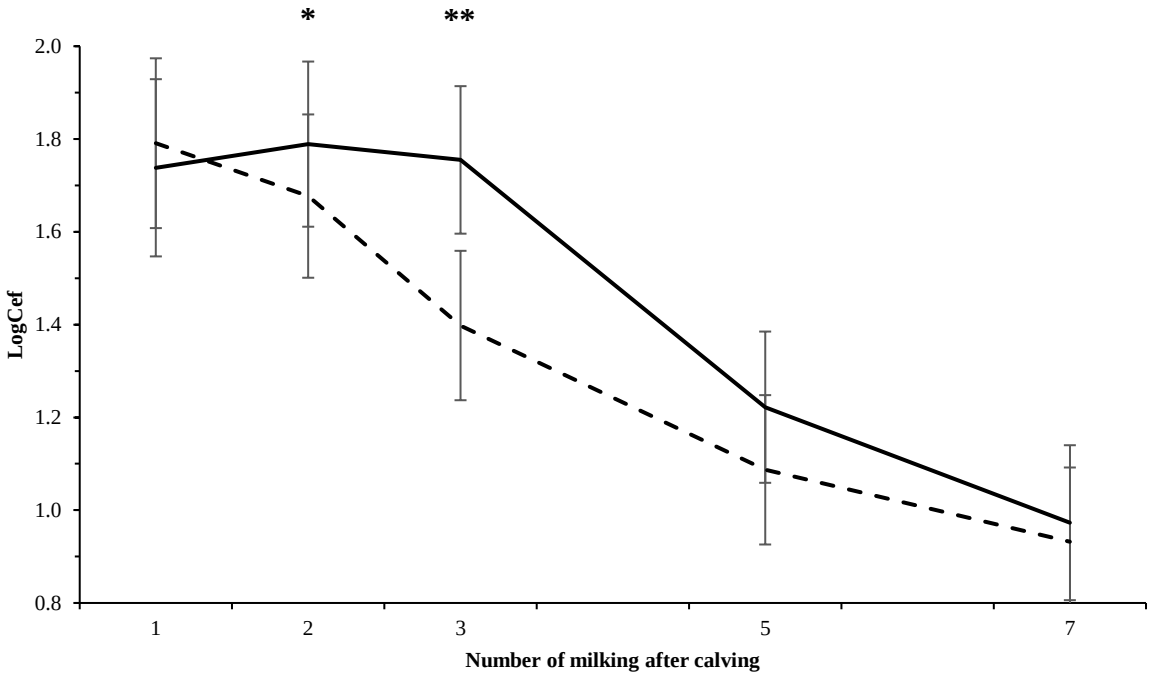
Variable	Estimate	SE	*P*-value	Odds ratio	CI for Odds ratio
Milk fraction					
foremilk	3.647	0.333	< 0.001	38.374	19.973 – 73.728
strippings			reference		
Protein	-0.553	0.068	< 0.001	0.575	0.504 – 0.657
LogSCC	-0.290	0.168	0.084	0.748	0.538 – 1.040

Table 2. Association between false positive results of the Delvotest BR Brilliant Plates (DSM Food Specialties B.V., Delft, Netherlands) and the milk composition (n = 47 milk samples).

Variable	Estimate	SE	*P*-value	Odds ratio	CI for Odds ratio
Protein	-0.212	0.043	< 0.001	0.809	0.744 – 0.880
Urea	0.006	0.003	0.055	1.006	1.000 – 1.012

Supplementary Table S1. Differences in milk composition (mean ± SE) between foremilk and stripping samples (n = 2,027 milk samples for fat, protein and lactose; n = 1,997 milk samples for urea; n = 2,099 milk samples for logSCC)

Milk component	Foremilk samples	Stripping samples	*P*-value
Fat (%)	2.94 ± 0.02	11.62 ± 0.02	< 0.001
Protein (%)	3.84 ± 0.01	3.30 ± 0.01	< 0.001
Lactose (%)	4.57 ± 0.01	4.00 ± 0.01	< 0.001
Urea (mg/L)	132.88 ± 1.20	117.51 ± 1.20	< 0.001
logSCC	5.09 ± 0.01	6.12 ± 0.01	< 0.001



Supplementary Figure S1. Differences in logCef (mean ± SE) between foremilk (solid line) and stripping samples (dashed line) in milking 1, 2, 3, 5 and 7 after calving (n = 351 milk samples). Number of milking marked with * denote $P < 0.05$; number of milking marked with ** denote $P < 0.001$.

2.1.5 **References**

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3 RESEARCH PAPERS

3.1 Residue concentration of cefquinome after intramammary dry cow therapy and short dry periods.

3.2 Viscosity measurements of milk to investigate risk factors for milk leakage in dairy cows before and after dry-off.

For the sake of consistency, small changes to the original formatting style for the journals have been made to both research papers.

3.1 Residue concentration of cefquinome after intramammary dry cow therapy and short dry periods

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3.1.1 **Abstract**

Short dry periods and their effects on milk production, reproductive performance, as well as cow and udder health have been widely studied. A dearth of information is available about the consequences of short dry periods on the residue concentrations of dry cow antibiotics in milk after calving. The objective of our study was to determine the residue concentration of a dry cow antibiotic in milk after short dry periods during the colostrum period and early lactation. Quarters of 19 dry cows were treated with an intramammary (IMM) dry cow antibiotic containing 150 mg of cefquinome on d 21, 14 and 7 before calculated calving date. One quarter of each cow did not receive treatment and served as negative control. After calving, quarter foremilk samples were collected twice daily until 21 d and once daily until 36 d after IMM dry cow treatment (i.e., end of withdrawal period). A total of 588 foremilk samples from odd milking numbers were chosen for the determination of the residue concentration of cefquinome using HPLC – tandem mass spectrometry until the residue concentration fell below the limit of quantification (1 ng/g), which occurred at the latest in milking number 37. The dry period length of the treated quarters was categorized in 3 dry period groups ranging from 1 to 7 d (4.8 ± 2.4), 8 to 14 d (11.5 ± 2.3) and 15 to 26 d (19.5 ± 3.3; ± SEM), in dry period group 1, 2 and 3, respectively. In dry period group 1, the cefquinome concentration increased after calving until the third milking and decreased considerably until the fifth milking. In dry period group 2, the cefquinome concentration peaked at the second milking and decreased considerably until the fifth milking as well. There was no increase in cefquinome after calving in dry period group 3. Up to the 37th milking, the cefquinome concentration was higher in dry period group 1 than in dry period group 2 and 3. On average 31.3 ± 1.2, 19.0 ± 1.1 and 6.7 ± 0.8 milkings and 19.4 ± 0.4, 20.6 ± 0.5 and 24.1 ± 0.7 d after treatment were necessary for the concentration of cefquinome to fall below the maximum residue limit (MRL) in dry period group 1, 2 and 3, respectively. These results indicate that shorter dry periods lead initially to higher cefquinome residues in milk. The residue concentration after experimental short dry periods still falls below the MRL within the recommended withdrawal period for milk of 36 d after IMM dry cow treatment. For the sake of food safety and economics, these short dry periods should not be used in the dry cow management, as they lead up to a maximum of 31.3 ± 1.2 milkings and 19.4 ± 0.4 d after treatment with cefquinome residues above the MRL. Therefore, a considerable number of milkings have to be discarded due to long withdrawal periods after calving.

Key words: dry period, antibiotic residue, cefquinome, withdrawal period

3.1.2 **Introduction**

An effective dry cow management including treatment with an antibiotic or internal teat sealant is an essential part of the international mastitis control program recommended by the National Mastitis Council (NMC, 2006). According to the US National Animal Health Monitoring System, about 93.0% of US dairy cows received an intramammary dry cow antibiotic and averaged a dry period of 57.1 d (Lombard et al., 2015). A survey on commercial dairy farms in northern Germany revealed a blanket dry cow therapy on 79.6% of the farms and an average dry period of 7 wk (Bertulat et al., 2015). Short dry periods of 35 d or less were used in only 3.7% of the participating dairy farms (Bertulat et al., 2015). Short dry periods are considered as a management strategy that could facilitate dry off and the transition period for high-producing cows (Lefebvre and Santschi, 2012).

Recently, several studies investigated the effects of short dry periods on production (Kuhn et al., 2006), reproductive performance (Shoshani et al., 2014), energy balance during early lactation (van Knegsel et al., 2014) and udder health (Church et al., 2008). A dry off 31 to 40 d before calving has been associated with maximized lifetime production after second or later lactation (Kuhn et al., 2006) and improved reproductive performance without any loss in colostrum quality (Shoshani et al., 2014). Furthermore, a dry period length of 30 d resulted in an improved energy balance of dairy cows in the early lactation (van Knegsel et al., 2014) without any untoward effects on udder health (Church et al., 2008).

Santschi and Lefebvre (2014), however, hypothesized that short dry periods can be a risk factor for antibiotic residues in early lactation when cows calve early. To avoid antibiotic residues, withdrawal periods for milk are determined utilizing the time to safe concentration method, which calculates the number of milkings necessary for the residue concentration in milk to fall below the maximum residue limit (CVMP, 2000). For dry cow antibiotics, withdrawal periods for milk depend on the dry period length; thus, the residue depletion during the dry period and postcalving is considered (CVMP, 2006).

The potential effect of shortening the residue depletion during the dry period on the residue concentration of dry cow antibiotics after calving has not yet been investigated. Church et al. (2008) measured antibiotic residues in foremilk samples both from cows with regular dry period length (45 or 60 d) that had been treated with a dry cow antibiotic and from cows with short dry periods (30 d) that had received a lactating cow antibiotic at dry off. Those authors did not find greater risk for antibiotic residues after short dry periods when cows were treated with a lactating cow antibiotic. When dry cow antibiotics are used, however, the risk for

antibiotic residues after short dry periods might be greater, as antibiotics are pharmacologically designed to reach high and prolonged concentrations throughout the udder (Ehinger et al., 2006).

Therefore, the objective of our study was to investigate the residue concentration of a dry cow antibiotic containing 150 mg of cefquinome during the colostrum period and early lactation in quarter foremilk samples after short dry periods.

3.1.3 Materials and Methods

Animals and Eligibility Criteria

The study was conducted between November 2014 and May 2015 at the Clinic for Animal Reproduction (Freie Universität Berlin, Berlin, Germany). To reduce the number of study animals, and in agreement with previous studies that suggested that udder quarters can be considered as independent entities (Schukken et al., 1993; Lindmark-Månsson et al., 2006), we choose a quarter-based approach. Previously dried-off cows (i.e., abrupt dry off with blanket dry cow treatment containing 150mg of cefquinome at least 57 d before calculated calving date) from a commercial dairy farm were selected and treated again with an intramammary (IMM) dry cow antibiotic in an extra-label manner within 21 d before calculated calving (i.e., 280 d after artificial insemination). This approach was favored because it minimized the impact and potential confounding of milk leakage on the residue concentration after calving, as milk leakage occurs most frequently within the first week after dry off (Bertulat et al., 2013). One quarter of each cow was not treated and served as a negative control (i.e., control quarters) to allow a quantification of potential residues from the first dry cow treatment and to determine if antibiotic concentrations could distribute among quarters.

Overall, 19 healthy, multiparous dry Holstein-Friesian and Holstein-Friesian crossbreed dairy cows were enrolled in the study 21 d before calculated calving date and followed until the end of the withdrawal period of the second dry cow treatment (i.e., maximum of 35 d after calving). We only enrolled dry cows with a calculated dry period length of at least 57 d. This allowed a 36-d withdrawal period for the first dry cow treatment plus 21 d between second dry cow treatment and calculated calving. All cows were managed according to the guidelines set by the International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products (Hellmann and Radeloff, 2000). The experimental procedures reported herein were conducted with the approval of the Institutional Animal Care and Use Committee of Freie Universität Berlin. Enrolled cows were in good health (e.g., no mastitis,

metabolic, or infectious disease) and had 4 functional quarters. Cows with clinical mastitis within the past 3 mo or antibiotic treatment (i.e., IMM or systemic) other than dry cow therapy within the past 2 mo before enrolment were excluded from the study.

During their study period, cows were housed in groups of 4 or less in a freestall barn with 6 cubicles equipped with chopped straw-lime-water mixture and smooth concrete floor. For calving, cows were transferred to a calving pen after signs of imminent parturition (i.e., tail raising, vaginal discharge, abdominal contraction) had been identified. The calving pen (3.50 × 3.10 m) was equipped with rubber mats and deep-straw bedding and was separated from the study cows by 2 metal fences. After calving, the cows and calves were separated immediately and cows were transferred back to the study group.

All cows were fed grass silage, haylage and hay ad libitum supplemented with standard dairy concentrate (MLF 18/3 Standard; BKF Belziger Kraftfutter GmbH, Bad Belzig, Germany) according to pregnancy status and milk yield. After calving, cows received a TMR formulated for fresh cows twice a day according to the NRC (2001) guidelines. Main ingredients were corn silage, rape, rapeseed extract, rye, hay, straw and mineral supplements. Fresh water was available ad libitum.

Precalving Study Protocol

An initial examination of cows to be enrolled included a physical examination (i.e., respiration rate, body temperature, heart rate, rumen contraction), a trans-rectal manual pregnancy check, udder inspection (i.e., redness, swelling) and palpation (i.e., firmness, pain and heat), classification of teat end shapes (i.e., round, plate, inverted, pointed; Neijenhuis et al., 2000) and a check for hyperkeratosis (i.e., no ring, smooth or slightly rough ring, rough ring, very rough ring; Mein et al., 2001). The same investigator examined all cows.

To achieve antibiotic concentrations representative for short dry periods, 3 quarters of each cow received a single treatment with an IMM dry cow antibiotic (i.e., 150 mg of cefquinome; Virbactan, Virbac Ltd., Carros, France) on a quarter individual basis on d 21, 14 and 7 before calculated calving date, respectively (i.e., treated quarters). This antibiotic treatment scheme falls into the category of extra-label drug usage, as all 4 quarters of a cow should be treated at the same time. It is a result of the quarter-based approach of our study with the above-mentioned advantages of reducing the number of study animals and minimizing the effect of milk leakage on the residue concentration after calving. The day of treatment was allocated to each quarter at random. A randomization list was generated with Microsoft Excel (Microsoft Office 2010, Microsoft, Redmond, WA). All cows were treated by the same investigator

and at the same time (i.e., 12.45 h). Intramammary dry cow antibiotic was administered according to the summary of product characteristics provided by the manufacturer (http://www.vmd.defra.gov.uk/productinformationdatabase/SPC_Documents/SPC_118464.DOC). In brief, teat ends were scrubbed with tissues soaked in 70% alcohol, the antibiotic suspension was infused into the quarter and massaged from the teat tip to the teat base to facilitate dispersion. The recommended withdrawal periods for the used dry cow antibiotic, when administered according to label instructions, are 1 d after calving and 36 d after treatment when the dry period is more and less than 35 d, respectively (CVMP, 2006). The established MRL for cefquinome in milk is 20 ng/g (CVMP, 2003). After treatment, all quarters were monitored once a day at 07.00 h for signs of clinical mastitis (i.e., firmness, pain, heat, redness, swelling) and twice a day for milk leakage (i.e., 07.00 h, 13.00 h; Table 1). Milk leakage was recorded on the udder quarter level and defined as milk dripping or flowing from the teat.

Postcalving Study Protocol

After calving cows were milked twice daily at 07.00 h and 19.00 h using a portable bucket milking machine (i.e., GEA milking bucket with pulsator Vacupuls Constant; GEA Westfalia Separator AG, Oelde, Germany). The milking bucket was connected via vacuum line with a RPS Vacuum Pump (800 L/min) with a 3-phase motor equipped with a typical milking cluster (Classic 300 milking cluster; GEA Westfalia Separator AG, Oelde, Germany). Premilking procedures included wiping each teat wet and dry with an individual paper towel and discarding the first 3 streams of milk utilizing a premilking cup. Furthermore, foremilk from each quarter was checked daily at 07.00 h for signs of clinical mastitis (i.e., clots, flakes) and subclinical mastitis using the California Mastitis Test (eimü Cell-Check 3S; Ferdinand Eimermacher GmbH & Co. KG, Nordwalde, Germany). Udder quarters were monitored once a day at 07.00 h for signs of clinical mastitis (i.e., firmness, pain, heat, redness, swelling) and thrice a day for milk leakage (i.e., 07.00 h, 13.00 h, 19.00 h; Table 1). All cows with signs of clinical mastitis or systemic disease other than subclinical ketosis and all cows that received any antibiotic treatment beyond the IMM dry cow treatment were retrospectively withdrawn from the study.

Sample Collection and Analysis

Two foremilk samples (i.e., 13 mL and 30 mL) from each quarter were collected before the morning milkings (i.e., 07.00 h) from d 1 until 36 after IMM antibiotic treatment (i.e., end of withdrawal period) and before the evening milkings (i.e., 19.00 h) from d 1 until 21 after

IMM antibiotic treatment (Table 1). The foremilk samples were collected immediately after the premilking procedures.

The 30-mL foremilk samples were stored at 6 °C and transported within a maximum of 4 d to the local DHIA (Landeskontrollverband Berlin-Brandenburg eV., Waldsieversdorf, Germany) for milk composition analysis (i.e., fat, protein, lactose, urea, SCC). Fat, protein and lactose were measured by mid-infrared spectroscopy according to IDF 141:2013 (IDF, 2013), and urea was determined by continuous flow analysis according to the guideline 1.13 of the German Association for Performance and Quality testing (DLQ, 2013). Somatic cell count was determined by flow cytometry according to IDF 148-2:2006 (IDF, 2006).

The 13-mL foremilk samples were stored at 6 °C for a maximum of 12 h. Afterwards samples were vortexed for 5 s (2400 min^{-1}), transferred to 5-mL cryotubes (Cryovial, Simport, Bernard-Pilon, Canada) and frozen at -80 °C until analysis. The residue concentration of cefquinome was analyzed by HPLC-MS/MS with a limit of detection (LOD) of 0.2 ng/g and an interassay coefficient between 8.5% (spiked quality control of 15 ng/g of milk) and 3.8% (spiked quality control of 45 ng/g of milk). For the quantification of cefquinome, 2 g of calibrators, controls, or bovine raw milk samples were mixed with 1 ml internal standard solution in water. After protein precipitation and centrifugation (10 min at 3800 x g at 4 °C), the supernatant fluid was transferred into a polypropylene tube and evaporated. Dried extracts were reconstituted to a volume of 1 ml and used for solid phase extraction. Extract fluid (10 µl) was injected onto the analytic column (Gemini-NX C18 110A 150 x 3.0 mm; Phenomenex, Aschaffenburg, Germany). A 3200 QTRAP triple quadrupole mass spectrometer with an ESI ion source from Sciex (Darmstadt, Germany) was used as detector. Initially, the residue concentration of cefquinome was measured from all quarters from milking 1 (i.e., first milking after calving) and from treated quarters from milkings 2, 3, 5 and 7 (i.e., second, third, fifth and seventh milking after calving). Further foremilk samples were individually selected for analysis based on measured residue values of previous milkings. Overall, foremilk samples from odd milking numbers up to maximum of the 37th milking were chosen for the analysis. When the residue concentration of a quarter fell below the limit of quantification, we assumed that this concentration was maintained constantly until milking 37. On d 36 after IMM antibiotic treatment, additional foremilk samples from all quarters were taken from all cows for a bacteriological examination.

Statistical Analysis

Data were entered into Excel spreadsheets (version 2010; Microsoft Corp., Redmond, WA) and statistical analyses were performed with IBM SPSS Statistics (version 22.0; IBM Deutschland GmbH, Ehningen, Germany). Treated quarters were allocated to 1 of 3 groups based on the dry period length relative to the second dry cow treatment (i.e., dry period group 1: 1 to 7 d, dry period group 2: 8 to 14 d, dry period group 3: > 14 d). Effects of various parameters on the cefquinome concentration of the control quarter in the first milking (i.e., cefquinome concentration of the last treated quarter, the parallel quarter and all treated quarters; length of the dry period of the parallel quarter, the last treated quarter, length of the dry period after the first antibiotic dry cow treatment) and the treated quarters in the first milking and up to the 37th milking (i.e., lactation number, dry period length, dry period group, type of quarter (front or rear quarter), milk composition (fat, protein, lactose, SCC and urea), milk yield during a milking) were evaluated using mixed model ANOVA. Mixed models were built according to the model building strategies developed by Dohoo et al. (2009). The number of milkings was considered as repeated factor and quarter within cow was included as random effect. The number of milkings and the days after treatment until the concentration of cefquinome fell below the value for the maximum residue limit (MRL; i.e., 20 ng/g) in the different dry period groups were analyzed with a Kaplan-Meier analysis. Kaplan-Meier survival curves were produced using IBM SPSS Statistics. All values reported are least square means ± standard error of the means. The significance level was set at $P \leq 0.05$ and tendencies were discussed at $P \leq 0.1$.

3.1.4 **Results**

Fourteen cows were used for the final analysis. Five out of 19 cows had to be retrospectively withdrawn from the study due to mastitis (n = 1), ketosis (n = 1), antibiotic treatment (n = 1), and premature calving (n = 1). One cow died during the study period by rupture of the uterine artery. The 14 cows were 62 ± 21 mo old, in the second to sixth lactation and their 305-d milk yield of the previous lactation averaged 10,079 ± 1,267 kg. Two cows calved before the dry cow treatment on d 7 before the calculated calving date, and as a result these cows had 2 treated and 2 control quarters. A total of 56 quarters were used in the final analysis. The distribution of quarters was homogeneous between control quarters (n = 16) and treated quarters of dry period group 1 (n = 11), 2 (n = 14) and 3 (n = 15; $P = 0.195$). The averaged dry period length of the control quarters was 78.1 ± 23.4 d, with 4.8 ± 2.4 d (range =

1 - 7 d), 11.5 ± 2.3 d (range = 8 - 14 d) and 19.5 ± 3.3 d (range = 15 - 26 d), for group 1, 2 and 3, respectively. Only 1 cow showed milk leakage before calving, therefore milk leakage was not considered in our analysis. A total of 2,392 foremilk samples were collected during the study period and 588 samples (i.e., 16 foremilk samples from control quarters and 206, 210 and 156 from dry period group 1, 2 and 3, respectively) were analyzed for the concentration of cefquinome using HPLC-MS/MS. The bacteriological examination at the end of the study period was negative for all cows.

Control Quarters

The average cefquinome concentration of the control quarters in the first milking was 2.91 ± 1.47 ng/g (range: 0.5 – 5.57 ng/g). It was not affected by the cefquinome concentration of the parallel quarter ($P = 0.794$), all treated quarters ($P = 0.715$), the length of the dry period of the parallel quarter ($P = 0.229$), of the last treated quarter ($P = 0.322$), or the length of the dry period after the first antibiotic dry cow treatment ($P = 0.267$). There tended to be an effect of the concentration of the last treated quarter ($P = 0.097$), with higher concentrations in the control quarters when the concentration of the last treated quarter was also higher.

Treated Quarters

We found an exponential relationship between the number of days between treatment and calving and the initial cefquinome concentration. The correlation coefficient between the logarithm of cefquinome and days between treatment and calving was -0.849 ($P < 0.001$; Figure 1). The average cefquinome concentration in the first milking was higher in dry period group 1 (1830.71 ± 355.47 ng/g; $P = 0.002$) and 2 (1155.50 ± 251.94 ng/g; $P = 0.027$) compared with the dry period group 3 (77.59 ± 65.45 ng/g). Differences of the cefquinome concentration between dry period group 1 and 2 could not be detected ($P = 0.201$) in the first milking alone, but differences between the 2 dry period groups could be detected up to the 37th milking, which are described below. The concentration of cefquinome was furthermore related to lactose content, with higher concentrations when the lactose content was higher as well ($P = 0.048$). The cefquinome concentration of the treated quarters in the first milking was not affected by the number of lactation ($P = 0.856$), the type of quarter ($P = 0.441$), fat ($P = 0.626$), protein ($P = 0.161$), urea content ($P = 0.682$), SCC ($P = 0.841$), or milk yield during the first milking ($P = 0.567$).

The excretion of cefquinome of the treated quarters up to the 37th milking was affected by the number of milking ($P < 0.001$) and the dry period group ($P < 0.001$), as well as by an interaction between the number of milking and the dry period group ($P < 0.001$). The average

cefquinome concentration was higher in dry period group 1 (581.40 ± 90.71 ng/g) than in dry period group 2 (115.41 ± 15.38 ng/g; $P = 0.001$) and 3 (21.28 ± 6.00 ng/g; $P < 0.001$). Cefquinome values measured in dry period group 1 remained higher than in dry period group 2 and 3 until the fifth milking ($P < 0.05$). No statistical difference could be detected between the cefquinome concentration of dry period group 2 and 3 for any of the milkings ($P = 0.471$). Interestingly, in dry period group 1, the cefquinome concentration increased after calving until the third milking ($P = 0.027$), decreased considerably until the fifth milking ($P = 0.001$) and slowly decreased afterward. In dry period group 2, cefquinome concentration peaked at the second milking ($P < 0.05$), retained the same level as in milking 1 in milking 3 ($P = 0.441$), decreased considerably until the fifth milking ($P < 0.001$), and slowly decreased afterward. In dry period group 3, cefquinome concentration did not differ between the first and second milking ($P = 0.994$) but decreased between milking 2 and 3 ($P = 0.069$); afterward it slowly decreased until the end of the measuring period (Figure 2).

The cefquinome concentration after calving was additionally affected by the milk yield with higher concentrations when the milk yield during a milking was lower ($P = 0.006$). The number of milking and protein ($r = 0.833$) and lactose content ($r = 0.746$) were highly correlated; as a result, only the number of milking was used in the analysis. The number of lactation ($P = 0.510$), type of quarter ($P = 0.135$), fat ($P = 0.901$) and urea content ($P = 0.709$) as well as SCC ($P = 0.241$) had no effect on the cefquinome concentration of the treated quarters.

MRL

The first survival curve shows the time from the first milking after calving to the first milking with a cefquinome concentration below the MRL dependent on the dry period group (Figure 3). On average 31.3 ± 1.2, 19.0 ± 1.1, and 6.7 ± 0.8 milkings were necessary for the concentration of cefquinome to fall below the MRL in dry period group 1, 2 and 3, respectively ($P < 0.001$). The number of days needed after treatment until the cefquinome concentration fell below the MRL differed between the dry period groups as well. The second survival curve shows the time from the day of treatment to the first day after treatment with a cefquinome concentration under the MRL depending on the dry period group (Figure 4). In dry period group 3, 24.1 ± 0.7 d were necessary, whereas in dry period group 2 and 1 only 20.6 ± 0.5 ($P = 0.003$) and 19.4 ± 0.4 d ($P < 0.001$) were needed, respectively. A maximum of 22, 23 and 28 d after treatment in dry period group 1, 2 and 3, respectively, were needed for the cefquinome concentration to fall under the MRL. These results indicate that in all dry period groups,

cefquinome concentration went below the MRL before the regular withdrawal period of 36 d after IMM dry cow treatment ended and they show a safety margin of at least 1 wk.

3.1.5 **Discussion**

Control Quarters

In our study, 1 quarter of each cow was not treated a second time with an IMM dry cow antibiotic to serve as a negative control quarter. Minor cefquinome residues (range = 0.5 – 5.59 ng/g), albeit far below the MRL (i.e., 20 ng/g), could be detected in the foremilk samples of the control quarters of the first milking. Interestingly, we found no association between the concentration of the control quarters and the dry period length after the first dry cow treatment, but a weak association with the last treated quarters. These results challenge the assumed independence of the udder quarters in our study design, as they indicate a certain diffusion of cefquinome between the quarters and not a persistence of residues from the first dry cow treatment. The assumption of independent udder quarters was based on previous observations. Schukken et al. (1993) performed a pilot study on 10 cows and treated 1 udder half (left or right) of each cow with a dry cow antibiotic containing procaine penicillin (300,000 IU), nafcillin (100 mg) and dihydrostreptomycin (100 mg). Those authors tested milk samples from the untreated quarters during the first 10 d of the dry period with the Delvotest SP assay (i.e., limit of detection for penicillin 2 ng/mL, nafcillin 5 ng/mL and dihydrostreptomycin 300 - 500 ng/mL; DSM Food Specialties B.V., Delft, the Netherlands) and reported that none of the untreated quarters showed any evidence that concentrations of the dry cow antibiotic crossed from treated to untreated quarters. Likewise, the milk composition in udder quarter milk samples were considered independently by Lindmark-Månsson et al. (2006).

In contrast to these studies, Barkema et al. (1997) detected a strong interdependence between high SCC and IMI of quarters within a cow and the authors recommended statistical correction of the interdependence of quarters in studies on subclinical mastitis. A most recent work (Li et al., 2014) published after our study started, measured cefquinome concentrations in milk samples from treated and untreated parallel quarters after 1 and 3 consecutive IMM administrations of 75 mg of cefquinome in lactating cows. Those authors detected cefquinome concentrations from 5 to 80 ng/g at 6 h after treatment in untreated quarters. This could be observed in 4 out of 6 cows, and those authors explained the occurrence with diffusion or distribution of cefquinome between the quarters (Li et al., 2014). These and our findings, however, are in contrast to the pharmacokinetic characteristics of cefquinome. In general, the

ability of cefquinome to distribute through the udder and diffuse through lipophilic membranes is low, as it is a weak organic acid with a low fat solubility. Furthermore, it has an acid dissociation constant (i.e., pKa) of 2.51 and 2.91, which means that in milk with a pH of 6.4 to 7.0 a higher proportion of molecules is ionized (CVMP, 1995; Gehring and Smith, 2006). These pharmacokinetic characteristics of cefquinome would support our quarter-based approach; however, further research is necessary to elucidate the origin of the cefquinome residues in the control quarters and to investigate if the assumption of independent quarters is in fact valid. In previous studies, minor residue concentrations in control quarters might not have been detected due to a higher LOD of the used measurements methods. For comparison, the Committee for Veterinary Medicinal Products validated a reversed-phase HPLC for the determination of cefquinome in milk with a LOD of 10 ng/g and a LOQ of 15 ng/g (CVMP, 1995). Li et al. (2014) used an HPLC-MS/MS with a LOD of 2 ng/g and a LOQ of 5 ng/g for the determination of the cefquinome concentration, whereas the HPLC-MS/MS in our study had a LOD of 0.2 ng/g and a LOQ of 1 ng/g. These high sensitivities might have allowed the detection of minor cefquinome residues in the control quarters that might not have been detected before.

Overall, the residues detected in our control quarters averaged 0.28% of the antibiotic concentration measured in all quarters. Considering the proportion of cefquinome in the treated and control quarter, the measured cefquinome concentrations of the treated quarters might be slightly underestimated. Even if the detected numerical association between the cefquinome concentration of the last treated quarter and the control quarter would be based on a certain diffusion between the quarters, the effect of this diffusion on the concentrations of the treated quarters would be negligible.

Treated Quarters

We measured cefquinome concentrations in foremilk samples after a single, extra-label IMM treatment with a dry cow antibiotic followed by experimental short dry periods from 1 d to a maximum of 26 d. These dry period lengths could occur when cows with short dry periods of 20 to 40 d calve early. With our study design, which includes a second dry cow treatment of previously dried-off cows, we could minimize the losses of antibiotic residues by milk leakage before calving and thus confounding of results, as IMM administered cefquinome is mainly excreted with the milk (CVMP, 1995).

We were able to demonstrate that the dry period length affected the residue concentration of cefquinome in foremilk samples after calving. In the first milking, the cefquinome concentration was significantly higher in dry period groups 1 and 2 than in dry

period group 3. A residue depletion study (CVMP, 2006) in milk from dairy cows after an IMM treatment with 150 mg cefquinome, followed by short dry periods varying from 16 to 36 d, found that the dry period had a significant effect on the residues at the first milking due to the wide range of dry periods and the substantial depletion of residues from the udder quarters during the dry period. The depletion of cefquinome during the dry period is followed by a more rapid depletion after calving (CVMP, 2006), which could be confirmed by our results. Up to the fifth milking, the cefquinome concentration in dry period group 1 was significantly higher than in dry period group 2 and 3. The more rapid, exponential depletion after calving seemed to compensate for the effect of the dry period group on the cefquinome concentration in our study. The difference between dry period group 2 and 3 in the first milking was not detectable in the second ANOVA, and no difference could be detected between the 2 dry periods groups in any later milking. In cistern milk samples from healthy quarters, cefquinome concentrations could be detected until the ninth milking from lactating cows after 3 consecutive IMM treatments with 75 mg cefquinome (Zonca et al., 2011). Li et al. (2014) reported maximum levels of 598 ± 323 µg/mL (i.e., 598,000 ± 323,000 ng/g) cefquinome in milk at 2 h after a single IMM administration of 75 mg cefquinome; those authors reported a mono-exponential decline to low levels of 0.01 ± 0.005 µg/mL (i.e., 10 ± 5 ng/g) at 72 h after the administration. In contrast to these 2 studies investigating a lactating cow antibiotic with 75 mg cefquinome and showing a continuous decline of the residue concentration, our study showed that in dry period group 1 and 2 the concentration of cefquinome increased until the third milking and second milking, respectively. Afterward, the cefquinome concentration decreased until the fifth milking. We hypothesize, that some characteristics of the colostrum might hinder the release of cefquinome from the dry cow formulation, but might speed up after the first milking and thus lead to the higher concentrations in the second and third milking. This effect might be weaker when the dry cow antibiotic remains longer in the dry udder, which could explain the smaller increase of the cefquinome concentration in dry period group 2 and no increase in dry period group 3, respectively. The considerable decrease of the cefquinome concentration between the third and fifth milking in dry period group 1 might be related to the transition from colostrum to milk accompanied by an increasing milk yield and the exponential depletion of the cefquinome concentration.

We did not detect an effect of the milk composition on the cefquinome concentration after calving, except for the lactose content in the first milking. The cefquinome concentration in the first milking was higher when the lactose content was also higher. The physiological background of this effect remains unclear and, to our knowledge no other study reported a

similar effect. Due to the high correlation between protein and lactose content and the number of milkings, protein and lactose were excluded from further analysis and a potential effect on the cefquinome concentration up to the 37th milking could not be investigated in our study. As mentioned above, cefquinome has a low fat solubility (CVMP, 1995), thus an effect of the fat content of the milk samples on the cefquinome concentration could be presumed. This hypothesis, however, could not be confirmed in our study, as the fat content had no effect on the cefquinome concentration in the first and up to the 37th milking.

We found a negative relationship between the cefquinome concentration and the milk yield. This is in contrast to previous reports, where a correlation between milk production and drug elimination of cefquinome or cefoperazone after IMM treatment in lactating cows could not be detected (Cagnardi et al., 2010; Zonca et al., 2011). Similar to our results, a correlation between milk yield and drug clearance after IMM administration of the lincosamide pirlimycin (Whittem, 1999) and various antibiotics against mastitis were described (Mercer et al., 1970). Low-producing cows (i.e., < 9 kg/d) have been reported to eliminate antibiotics more slowly from the milk; however, the milk yield only partially explained the variations in milk-out rates, as several high-producing cows (i.e., > 23 kg/d) also eliminated antibiotic residues slowly (Mercer et al., 1970). As these results are inconsistent, and might be drug dependent (Gehring and Smith, 2006) and confounded through less sensitive methodologies, further research is necessary in this field.

MRL

The mean number of milkings and days after treatment until the cefquinome concentration fell below the MRL differed significantly between the 3 dry period groups. The mean number of milkings was higher when the dry period length was shorter. Dry period groups 1, 2 and 3 led to an average of 31, 19 and 7 milkings with a cefquinome concentrations above the MRL. That shows a potential risk for food safety in the field if the recommended withdrawal periods are disregarded. In general, milk from individual cows with short dry periods should not be sold for human consumption before the withdrawal period is finished; furthermore, milk should be tested with an antibiotic screening test before selling for human consumption to ensure food safety. The total number of days necessary after treatment until the cefquinome concentration reached the MRL averaged 19, 21 and 24 d in dry period groups 1, 2 and 3, respectively; therefore, it was lower in cows with shorter dry periods. This is a further proof of the more rapid depletion of cefquinome after calving compared to the dry period. The cefquinome concentration in our study fell below the MRL in all quarters within the

recommended withdrawal period for milk of 36 d after IMM dry cow treatment, but the extra label use of the dry cow antibiotic in our study has to be considered. Short dry periods, as investigated in our study, should not be used in the dry cow management, as they lead to a considerable number of milkings that have to be discarded due to long withdrawal periods after calving, which displays an immense economically disadvantage in the field.

Nevertheless, further research is necessary to investigate the relationship between short dry periods and residue concentrations in milk for other dry cow antibiotics, as different pharmacokinetics and formulations can affect the residue depletion during the dry period and after calving. Therefore, the effect of short dry periods on the residue concentration after calving and the days after treatment needed for the residue concentration to fall below the MRL has to be evaluated for each dry cow formulation separately.

3.1.6 **Conclusions**

The results of this study indicate that shorter dry periods lead initially to higher cefquinome residues in milk. The residue concentration after experimental short dry periods still falls below the MRL within the recommended withdrawal period for milk of 36 d after IMM dry cow treatment. For the sake of food safety and economics, these short dry periods should not be used in the dry cow management, as they lead up to a maximum of 31.3 ± 1.2 milkings and 19.4 ± 0.4 d after treatment with cefquinome residues above the MRL; likewise, a considerable number of milkings have to be discarded due to long withdrawal periods after calving. These results cannot be transferred to other IMM dry cow antibiotics; therefore, further research on the effect of short dry periods on residue concentrations is needed. Furthermore, the validity of the independence of quarters should be investigated in order to decide, if a quarter-based approach is an appropriate study design for future pharmacokinetic and residue studies.

3.1.7 **Acknowledgments**

We gratefully thank the staff of the Clinic of Animal Reproduction (Freie Universität Berlin, Berlin, Germany) for their support during the practical part of the study. Furthermore, we thank the staff of the DHIA (Landekontrollverband Berlin-Brandenburg e. V., Waldsieversdorf, Germany) for their superb cooperation. This study was funded in part by the Federal Office of Consumer Protection and Food Safety (BVL, Braunschweig, Germany).

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Table 1. Study protocol summarizing pre- and postcalving examinations.

Day relative to calving	Time	Milk leakage	Udder examination	CMT	Milk samples
21d[1] – 1d a.p.[2]	7.00h	×	×		
	13.00h	×			
0d p.p.[3] – 21d[4]	7.00h	×	×	×	×
	13.00h	×			
	19.00h	×			×
22d[4] – 36d[4]	7.00h	×	×	×	×
	13.00h	×			
	19.00h	×			

[1]Days before calculated calving date.

[2]a.p. = antepartum.

[3]p.p. = postpartum.

[4]Days after last IMM antibiotic treatment.

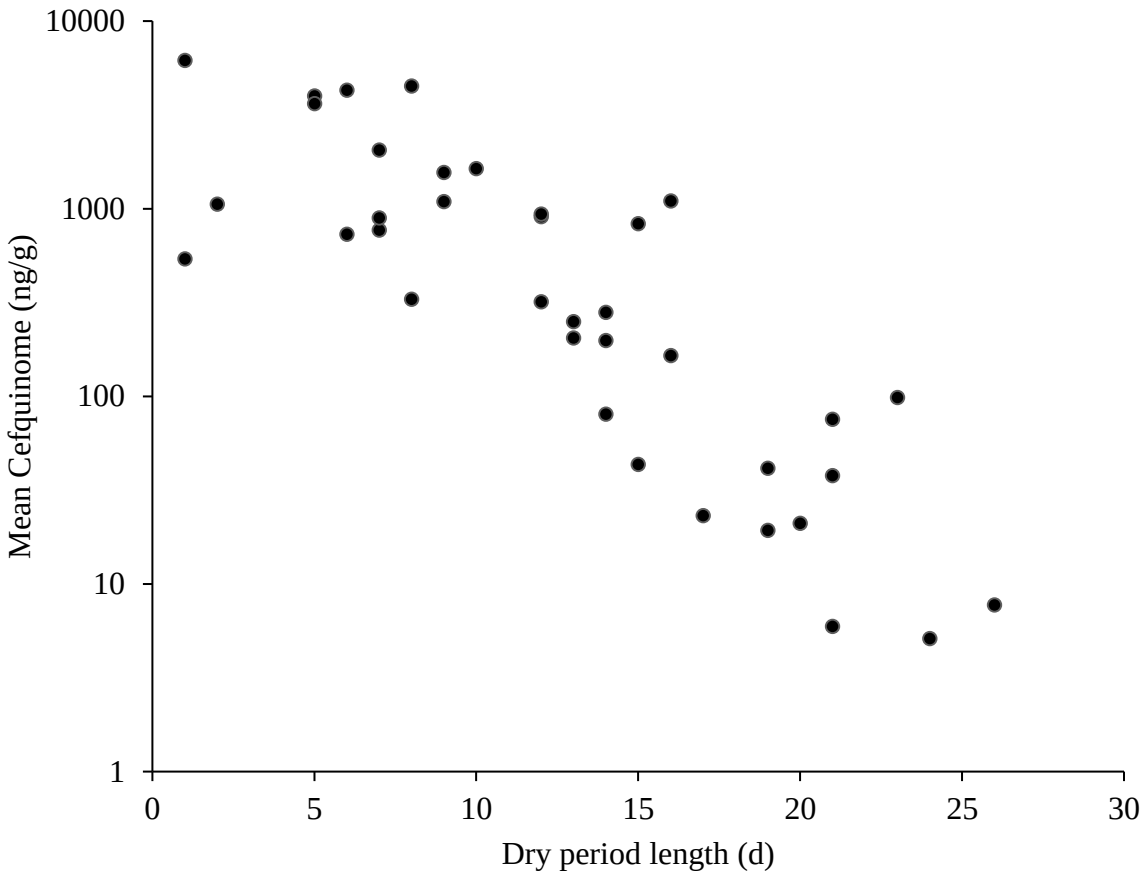

Figure 1. Relationship between initial cefquinome concentration in the first milking after calving and the dry period length. Mean concentration of cefquinome on logarithmic scale.

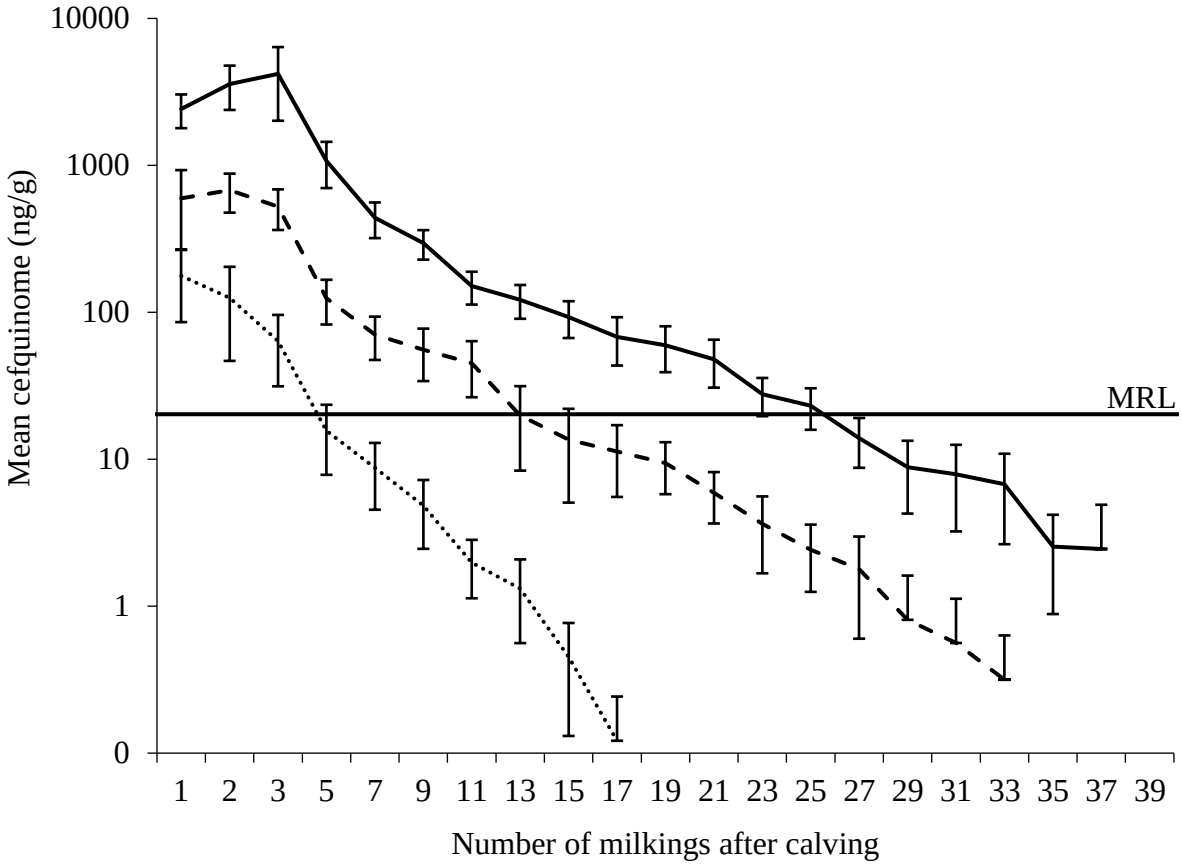

Figure 2. Mean concentration of cefquinome on logarithmic scale during the whole study period in dry period group 1 (1 to 7 d; solid line), group 2 (7 to 14 d; dashed line), and group 3 (> 14 d; dotted line) plotted with maximum residue limit (MRL; 20 ng/g). Error bars show SEM.

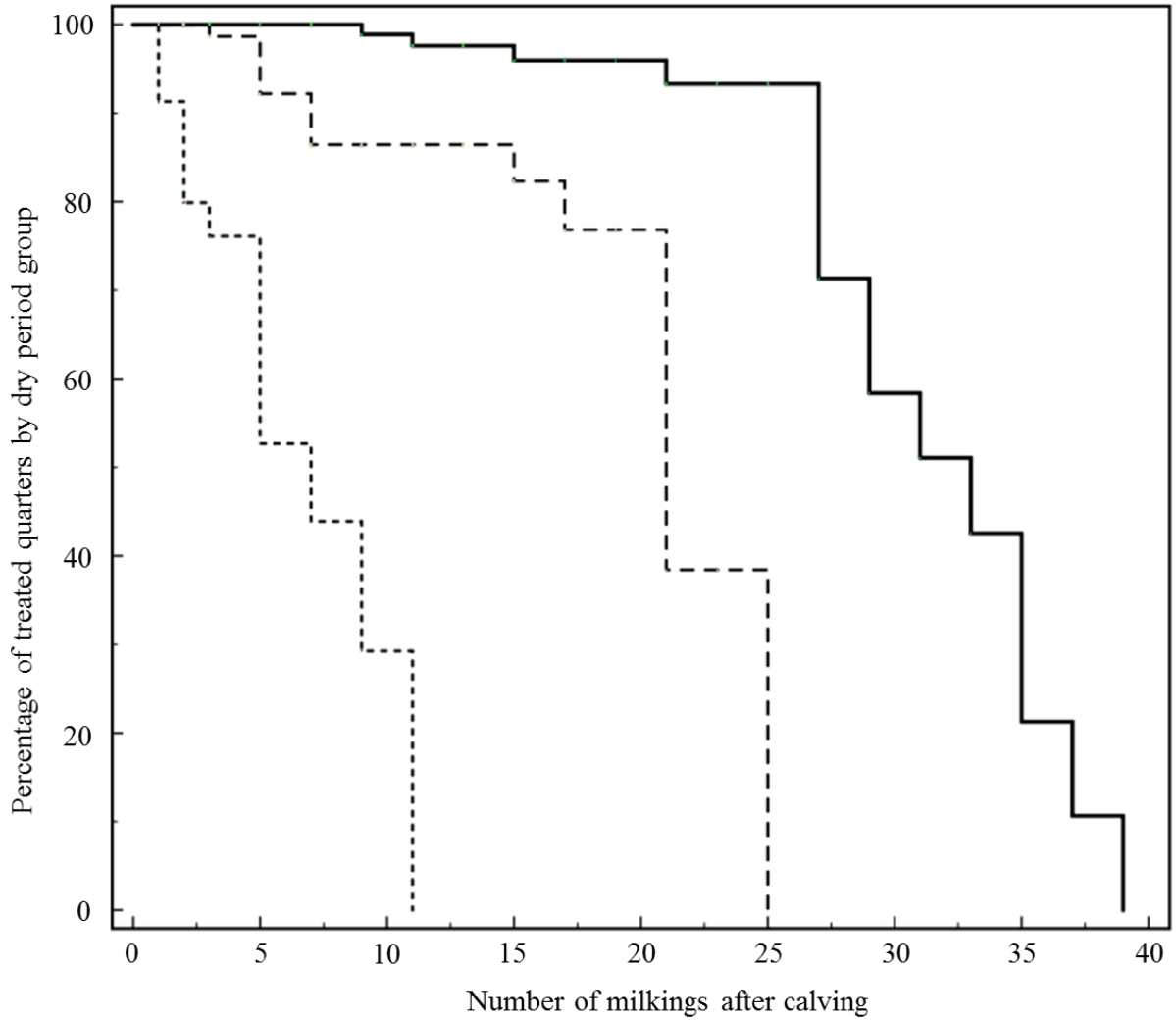

Figure 3. Kaplan-Meier curve from the first milking after calving to the first milking with a cefquinome concentration under the maximum residue limit (MRL) in dry period group 1 (1 to 7 d; solid line), group 2 (7 to 14 d; long dashed line) and group 3 (> 14 d; short dashed line).

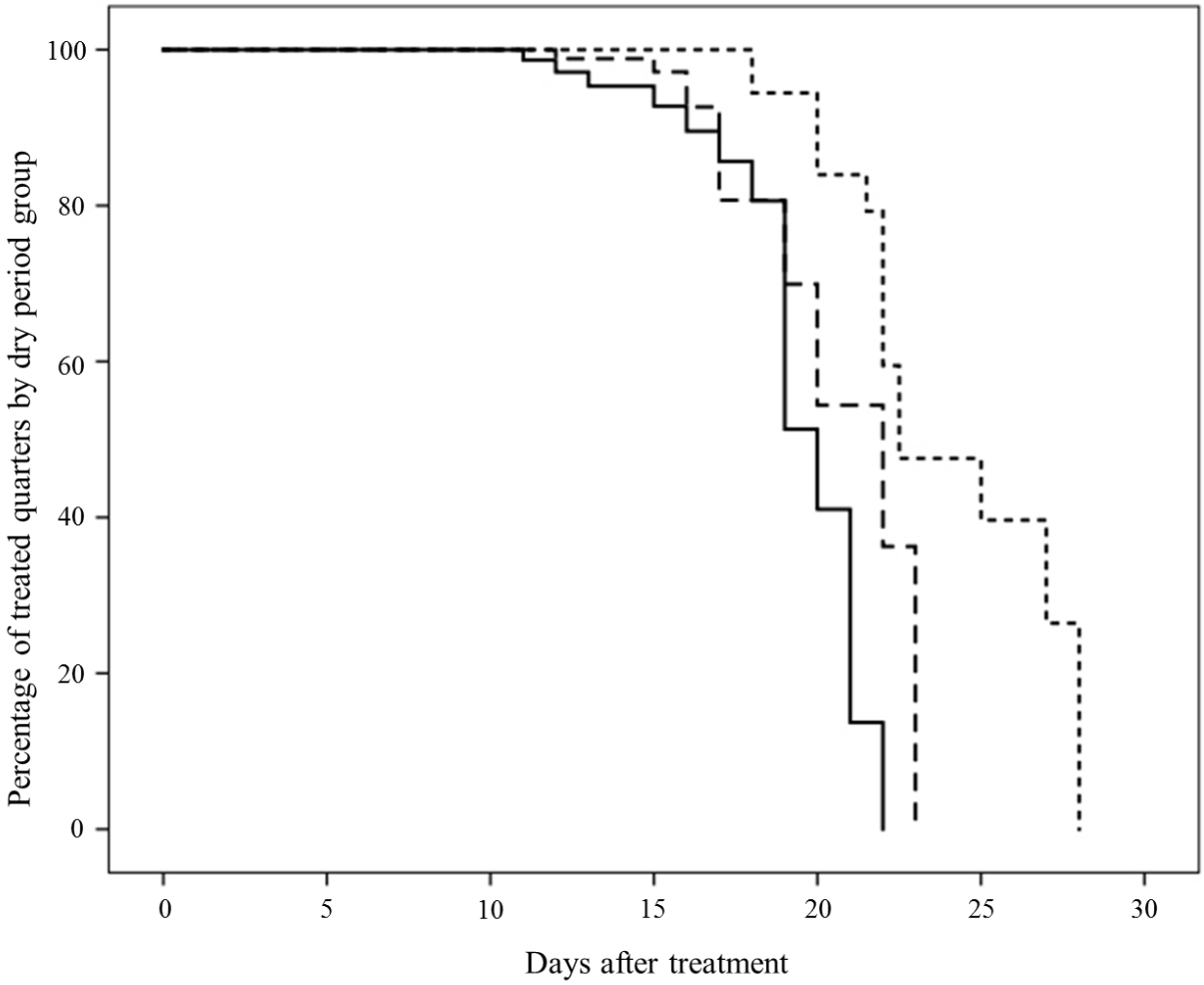

Figure 4. Kaplan-Meier curve from the day of treatment to the first day after treatment with a cefquinome concentration under the maximum residue limit (MRL) in dry period group 1 (1 to 7 d; solid line), group 2 (7 to 14 d; long dashed line) and group 3 (> 14 d; short dashed line).

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3.2 Viscosity measurements of milk to investigate risk factors for milk leakage in dairy cows

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3.2.1 **Abstract**

Milk leakage is a prevalent phenomenon in dairy cows and it is known to negatively affect udder health, as it increases the risk for clinical mastitis and new intramammary (IMM) infections. The determination of risk factors for milk leakage might be the first step towards the development of potential prevention measures. Therefore, the objective of this in vitro study was to evaluate the effect of the teat canal diameter, IMM pressure, milk temperature, milk composition, dry-off and antibiotic dry cow therapy on the occurrence of milk leakage in dairy cows. Two Ubbelohde viscometers (type no. 50100 and 50110 according to DIN 51 562 Part 1; SI Analytics GmbH, Mainz, Germany) were used to mimic the teat canal diameter, IMM pressure and milk temperature by suitable choice of the capillary diameter, hydrostatic pressure and milk sample temperature. Nineteen quarter foremilk samples each were collected on the day of dry-off and 2d after dry-off in cows dried off with and without antibiotic dry-cow therapy, respectively. All milk samples were analysed for milk composition (protein, fat, lactose, somatic cell count (SCC)) by the local Dairy Herd Improvement Association. The viscometers were used to measure the efflux time of all milk samples and afterwards the dynamic viscosity was calculated on basis of the Hagen–Poiseuille equation. Parameters that lead to shorter efflux times and smaller dynamic viscosities were considered as potential risk factors for milk leakage in vivo, because the milk could flow faster and more easily. In our study, the efflux time was shorter at wider capillary diameter, higher hydrostatic pressure, higher milk sample temperature and lower concentrations of fat and protein ($P < 0.001$). The dynamic viscosity was determined to be smaller at higher milk sample temperature and lower concentrations of fat and protein ($P < 0.001$). These results indicate that wider teat canal diameter, higher IMM pressure, higher milk temperature and lower concentrations of fat and protein might be risk factors for milk leakage in dairy cows.

Key words: milk leakage, dairy cow, dynamic viscosity, dry-off

3.2.2 **Introduction**

Milk leakage is defined as milk dripping or flowing from the teat in the absence of active milking. In dairy cows it frequently occurs shortly before milking [1], when cows were recumbent between milkings [2] and during the first days after dry-off [3]. While there are several studies describing milk leakage in lactating cows, milk leakage before or after dry-off has rarely been investigated. Klaas et al. [1] reported that overall 5.3% of lactating cows showed milk leakage while entering the milking parlor. After dry-off, however, approximately 30% of cows had milk leakage [3, 4]. Milk leakage should be considered a serious condition, because it is associated with a 4.0 and 6.0 times greater risk for clinical mastitis and new intramammary (IMM) infections, respectively [4-7]. Especially, an association with clinical mastitis caused by Escherichia coli has been reported [8].

There are few studies that have assessed the risk factors for milk leakage on cow level. Reported external risk factors are short teat canals, teat canal protrusion, high peak milk flow and high IMM pressure [1, 9] for lactating cows and high milk yield and high extramammary udder pressure for dry cows [3, 10]. Rovai et al. [9] detected that the reason for the high IMM pressure in lactating cows is a large amount of cisternal milk. Persson Waller et al. [2] concluded that the high IMM pressure can be caused by an activated milk ejection and that milk leakage occurs when the IMM pressure overcomes the closing mechanism of the teat canal. The teat canal is known to be a barrier for preventing milk leakage and defending the udder against new IMM infections [11]. Several studies investigated the influence of teat canal length and diameter on udder health and associated shorter and wider teat canals with an increased risk for mastitis [12-14], but an association with milk leakage was not evaluated.

Rovai et al. [9] discovered decreased concentrations of lactose in milk leaking from teats compared to foremilk collected after udder preparation. Based on that finding, we hypothesized that parameters of the milk composition might be potential internal risk factors for milk leakage. Changes in milk composition are caused by several factors, such as nutrition [15, 16], season [17-20], milking omission [15, 21] or mammary involution after dry-off [22]. The relationship between milk composition changes and milk leakage and the influence of antibiotic dry cow therapy on milk leakage after dry-off, however, have not yet been investigated.

In order to investigate risk factors for milk leakage in a field study, a high number of cows and measurements would be necessary due to variance between cows, quarters and teats. For this purpose, a measuring method evaluating risk factors for milk leakage under controlled conditions would be advantageous in order to reduce the use of study animals [23]. Therefore,

a laboratory model composed of two capillary viscometers was developed to determine risk factors for milk leakage under various conditions. Capillary viscometers are used to measure the efflux time of fluids (time interval that the fluid needs to descend from the upper timing mark to the lower timing mark of the viscometers' measuring sphere) and calculate their dynamic viscosity (internal resistance of a fluid to the application of pressure) [24]. Fluids with short efflux time and low dynamic viscosity flow fast and easy. In food engineering, milk composition and viscosity of milk are determined as important factors for manufacturing processes of dairy products [20, 24]. Alcântara et al. [24] reported increased dynamic viscosity values for milk as contents of fat, protein and lactose increased and decreased values as temperature increased. Physical properties such as the viscosity of milk might be useful factors to optimize milk processing [20] and they might also be plausible factors to investigate risk factors for milk leakage. We hypothesized that milk with decreased efflux time and decreased dynamic viscosity has a higher likelihood to leak from the teat.

Overall, the objective of this in vitro study was to evaluate plausible risk factors for milk leakage in dairy cows before and after dry-off. Specifically, we set out to determine the effects of 1) teat canal diameter, IMM pressure and milk temperature, 2) milk composition, 3) dry-off, and 4) IMM dry cow therapy on efflux time and dynamic viscosity of quarter foremilk samples.

3.2.3 **Materials and Methods**

Laboratory Model

Two calibrated Ubbelohde viscometers (type no. 50100 and 50110 according to DIN 51 562 Part 1; SI Analytics GmbH, Mainz, Germany) were used in this in vitro study. Capillary diameters (0.36 and 0.63 mm, referred to as capillary No. 0 and I) of the viscometers were chosen based on teat canal diameters reported in the literature [12, 14, 25, 26]. The viscometers were placed in a tempered water bath, for heating the milk sample to a certain temperature (26, 32 and 38°C) and for keeping this temperature constant during the measurements. Temperatures were chosen based on previously reported teat [27] and milk temperatures [28]. In order to simulate varying udder pressures values, different hydrostatic pressures (60, 80 and 100 mbar) were applied on the capillary tubes of the viscometers. Pressure values were chosen based on previously reported IMM pressure values [29, 30] and were adapted to actual milk yields at dry-off (15 to 20 kg). The two viscometers were used to measure the efflux time and calculate the dynamic viscosity of quarter foremilk samples before and 2 d after dry-off, with and without antibiotic dry-cow therapy.

Sample Collection

Six multiparous Holstein-Friesian crossbreed dairy cows from the Clinic of Animal Reproduction (Freie Universität Berlin, Berlin, Germany) were enrolled in this study. The six cows were in the first to sixth lactation and their 305d milk yield of the previous lactation averaged 8,019 ± 1,449 kg. All cows were in good health, had 4 functional quarters and were milked twice daily. All cows were fed grass silage, haylage and hay ad libitum supplemented with standard dairy concentrate according to their milk yield (MLF 18/3 Standard; BKF Belziger Kraftfutter GmbH, Bad Belzig, Germany). Fresh water was available ad libitum.

Quarter foremilk samples were collected in two trials conducted between September and November 2015. During the first trial, three hundred and fifty mL foremilk samples were collected from each quarter on the day of dry-off (d0) and 2 d after dry-off (d2), because this is the day with the highest likelihood of the occurrence of milk leakage after dry-off [3]. All samples were collected immediately after examining foremilk from each quarter for signs of clinical mastitis (clots, flakes) utilizing a pre-milking cup. Quarters with signs of clinical mastitis were withdrawn from the study. Thirty mL of the 350 mL quarter foremilk samples were send to the local DHIA (Landeskontrollverband Berlin-Brandenburg e. V., Waldsieversdorf, Germany) for milk composition analysis (fat, protein, lactose, SCC). All samples were analysed using the CombiFoss (Foss, Hilleroed, Denmark). After sample collection (samples d0) and milking on d0, each quarter was treated with an IMM dry cow antibiotic (150 mg of cefquinome; Virbactan, Virbac Ltd, Carros, France). Antibiotic dry cow therapy (AB) was administered according to the summary of product characteristics provided by the manufacturer. After antibiotic treatment on d0 cows were not milked for 48h for simulating dry-off until sample collection on d2 (samples d2 AB).

During the second trial the procedures were repeated as described above using the same cows, however, cows were not treated with an IMM dry cow therapy on d0. Foremilk samples were again collected from each quarter 2d after dry-off (samples d2). The efflux time of all samples (samples d0, samples d2 AB and samples d2) was measured the same day as samples were collected using the two viscometers.

Viscosity Measurements

Seventy mL of each milk sample were transferred into the viscometers and tempered to the starting temperature (26 °C). The time interval (efflux time) that the milk sample needed to descend from the upper timing mark to the lower timing mark of the measuring sphere was measured manually with a stopwatch. Each combination of the 2 capillary diameters, 3 pressure

values and 3 temperature values was measured with 3 repetitions for every milk sample. The dynamic viscosity η (mPa s) was calculated on the basis of the Hagen–Poiseuille equation: $\eta = K \times \Delta p \times t$. The viscometer constant K was determined individually for both viscometers by measurements with distilled water at 38 °C. Delta p (mPa) is the sum of the pressure difference between the capillary ends and the additional hydrostatic pressure applied on the capillary tubes, and t (s) is the mean efflux time for each capillary-temperature-pressure combination. The mean and SD of the efflux time and the dynamic viscosity for each capillary-pressure-temperature combination were calculated.

Statistical Analysis

Data were entered into Excel spreadsheets (version 2010; Microsoft Corp., Redmond, WA) and statistical analyses were performed with IBM SPSS Statistics for Windows software (version 22.0; IBM Deutschland GmbH, Ehningen, Germany). The association and differences between efflux times and dynamic viscosities of capillary 0 and I, were investigated using Pearson`s correlation and paired *t*-test. Further analyses of efflux time and dynamic viscosity were carried out with two mixed model ANOVAs. Models were built according to the model building strategies developed by Dohoo et al. [31]. The effect of capillary diameter, hydrostatic pressure, milk sample temperature, fat, protein and lactose content, SCC, dry-off and their potential interactions on efflux time were tested in the first mixed model ANOVA. The effect of capillary diameter, hydrostatic pressure, milk sample temperature, fat, protein and lactose content, SCC, dry-off, antibiotic dry cow therapy and their potential interactions on dynamic viscosity were tested in the second mixed model ANOVA. The random effect of cows was included in both models and days were considered as the repeated measure. All values reported are LSM ± SEM. The significance level was set at $P < 0.05$.

3.2.4 **Results**

Twenty-four quarters of six cows were enrolled in the study. Five quarters had to be excluded from final analysis due to deviations from the study protocol (e.g., clinical mastitis) and these quarters were treated with an IMM antibiotic for lactating cows. A total of 57 foremilk samples from 19 quarters (19 samples d0, 19 samples d2 and 19 samples d2 AB) were collected and analysed for milk composition. Three thousand and seventy-eight efflux time measurements were carried out, and 1026 dynamic viscosity values were calculated. Both decreased efflux time and decreased dynamic viscosity of the quarter foremilk samples were considered as a measure for a higher likelihood of occurrence of milk leakage, because the milk

could flow and potentially leak faster and more easily. Therefore, parameters which lead to decreased efflux time and decreased dynamic viscosity were considered as potential risk factors for milk leakage in the field.

Capillary Diameter, Hydrostatic Pressure and Milk Sample Temperature

Efflux time and dynamic viscosity differed ($P < 0.001$) between capillary 0 and capillary I, respectively, but showed a high correlation ($r = 0.996$; $r = 0.989$, $P < 0.001$). The efflux time decreased whereas the dynamic viscosity increased with wider capillary diameter ($P < 0.001$; Table 1).

An overall effect of the hydrostatic pressure and milk sample temperature ($P < 0.001$) on the efflux time and dynamic viscosity could be evaluated. The efflux time decreased as the hydrostatic pressure and milk sample temperature increased ($P < 0.001$; Table 1). The dynamic viscosity, however, just slightly increased as the hydrostatic pressure increased (Fig. 1a), but decreased as the milk sample temperature increased ($P < 0.001$; Fig. 1b). In addition to the effect of the capillary diameter, the hydrostatic pressure and milk sample temperature on the efflux time and dynamic viscosity, an interaction between the capillary diameter and hydrostatic pressure and furthermore between the capillary diameter and milk sample temperature could be demonstrated ($P < 0.001$). A slightly greater decrease of efflux time by increasing hydrostatic pressure was detected in capillary 0 and a greater and consistent increase of dynamic viscosity was seen in capillary I. A greater decrease in efflux time and dynamic viscosity by increasing milk sample temperature was seen in capillary 0.

Milk Composition, Dry-off and Antibiotic Dry Cow Therapy

There were considerable changes in the milk composition between samples d0 and samples d2. The protein content increased from 3.80 ± 0.39 in samples d0 to 4.44 ± 0.39% in samples d2 ($P < 0.001$), SCC increased from 84,153 ± 22,671 to 151,153 ± 22,671 SCC/mL ($P < 0.001$) and the lactose content decreased from 4.57 ± 0.14 to 4.13 ± 0.14%, respectively ($P < 0.001$). No difference in the fat content could be evaluated between samples d0 and samples d2 (1.83 ± 0.29 and 1.87 ± 0.29%, $P = 0.340$). Changes in the milk composition between samples d2 AB and samples d2 could be detected as well. In these samples, the fat content decreased from 2.50 ± 0.38 in samples d2 AB to 1.88 ± 0.38% in samples d2 ($P < 0.001$), SCC decreased from 467,819 ± 52,240 to 132,345 ± 52,240 SCC/mL ($P < 0.001$), and the lactose content increased from 3.63 ± 0.15 to 4.13 ± 0.15%, respectively ($P < 0.001$). No difference in the protein content could be observed between samples d2 AB and samples d2 (4.44 ± 0.42 and 4.45 ± 0.42%, $P = 0.296$).

Changes in the milk composition affected the efflux time and dynamic viscosity. The efflux time and dynamic viscosity increased as the fat and protein content increased ($P < 0.001$). The lactose and SCC content, however, had no effect on the efflux time ($P = 0.495$ and $P = 0.423$, respectively). An effect of the interaction between the lactose content and antibiotic dry cow therapy ($P < 0.001$) and between SCC and antibiotic dry cow therapy ($P = 0.025$) on the dynamic viscosity could be evaluated. In samples d2 AB, the dynamic viscosity increased with increasing lactose content and in samples d2, the dynamic viscosity decreased with increasing lactose content. Furthermore, in samples d2 AB, the dynamic viscosity decreased with increasing SCC, however, in samples d2 the dynamic viscosity was not affected by SCC.

An overall effect of dry-off on the efflux time and dynamic viscosity could be evaluated. In samples collected after dry-off, the efflux times and dynamic viscosities were higher than in samples before dry-off ($P < 0.001$), respectively. Antibiotic dry cow therapy had no effect on the efflux time ($P = 0.485$). The interaction between antibiotic dry cow therapy and lactose content and between antibiotic dry cow therapy and SCC on the dynamic viscosity is described above.

3.2.5 **Discussion**

Increasing milk yield resulting in higher udder pressure before and after dry-off might aggravate the occurrence of milk leakage in dairy cows. Factors such as missed or incomplete milking in automatic milking systems [2] and an abrupt dry-off [32] without reduction of feed allowance during the dry-off period [10] can increase the prevalence of milk leakage. Considering milk leakage as a risk factor for clinical mastitis, the investigation of its aetiology is important for the development of potential prevention methods. To our knowledge, this is the first study evaluating risk factors for milk leakage in lactating cows and 2 d after dry-off with and without antibiotic dry-cow therapy in an in vitro study utilizing a laboratory model. Our results demonstrate that a wider capillary diameter, higher hydrostatic pressure, higher milk sample temperature and lower concentration of fat and protein are associated with a decreased efflux time of quarter foremilk samples. Furthermore, we detected an association between higher milk sample temperature, lower concentration of fat and protein and a decreased dynamic viscosity of quarter foremilk samples. As we considered a decreased efflux time and decreased dynamic viscosity as a measure for higher likelihood of the occurrence of milk leakage in the field, we identified a wider teat canal diameter, higher IMM pressure, higher

milk temperature and lower concentrations of fat and protein as potential risk factors for milk leakage.

Capillary Diameter

The efflux time decreased with a wider capillary diameter, which is well expectable based on the Hagen–Poiseuille equation:

$$\frac{dV}{dt} = \frac{\pi \times r^4 \times \Delta p}{8 \times \eta \times l}.$$

The equation states that the volumetric flow rate (d*V*/d*t*) of a fluid through a capillary depends on the fourth power of the capillary radius (*r*), which is half the capillary diameter. Consequently, the efflux time (*t*) decreases with wider radius and diameter, respectively. Transferred to milk leakage, these findings indicate that cows with wider teat canal diameter would be more likely to show milk leakage than cows with narrow teat canal diameter. Nevertheless, the influence of the teat canal diameter on the occurrence of milk leakage has not been investigated in a field study, yet. Shorter and wider teat canals were associated by several authors with an increased risk for mastitis [12-14], as was milk leakage [4-7]. A higher association between milk leakage and mastitis caused by Escherichia coli than by Staphylococcus aureus has been detected [8]. As milk leakage is a sign for an open teat canal, the udder might be more susceptible for IMM infections caused by environmental pathogens. An association between teat canal configuration, milk leakage and mastitis has not yet been investigated.

In our study, dynamic viscosities calculated from the measured efflux times using the Hagen–Poiseuille equation differed between the two capillaries but were highly correlated. We speculate that uncertainties in the manual measurements or in the determination of the viscometer constant, *K* might be the potential reason for discrepancy between the two capillaries.

Hydrostatic Pressure

The efflux time of the quarter foremilk samples was affected by hydrostatic pressure and decreased as the hydrostatic pressure increased. Transferred to the teat, this finding indicates that higher IMM pressure might facilitate milk leakage in the field. This conclusion confirms earlier results of Bertulat et al. [3], who demonstrated that the occurrence of milk leakage after dry-off was significantly associated with udder pressure and that cows with a high udder pressure were more likely to show milk leakage than cows with low pressure values. A similar relationship between greater IMM pressure and milk leakage was demonstrated by

Rovai et al. [9] in lactating cows. These authors hypothesized that the elevated IMM pressure caused by a large amount of cisternal milk is the trigger for milk leakage. Along with the efflux time, the dynamic viscosity of the quarter foremilk samples was also affected by hydrostatic pressure. The dynamic viscosity increased slightly as the hydrostatic pressure increased (Fig. 1a). Transferred to the teat, those findings indicate that high IMM pressure leads to faster but not easier flowing or leaking of milk.

Milk Sample Temperature

Viscosity measurements were carried out at three different temperatures, aiming to represent milk temperatures in the teat cistern at different ambient temperatures. Direct data on milk temperature in the teat cistern are not available, but indirect estimates have been reported. We speculate that temperatures of milk in the teat cistern were lower than in the udder cistern and more affected by ambient temperatures, as it is physiological for acra. Therefore, we have chosen 26, 32 and 38°C as experimental temperatures, thereby representing cisternal milk temperatures during a cool, moderate and hot period.

In our study an overall effect of temperature on the efflux time and dynamic viscosity of the quarter foremilk samples could be evaluated. The efflux time and dynamic viscosity decreased as the temperature increased (Fig. 1b), similar to results from previous studies analysing dynamic viscosities of whole or raw milk samples [24, 33, 34]. In our study, the plot of the milk viscosity against inverse temperature is linear (Fig. 1c), substantiating Arrhenius-type thermal activation of the milk flow. For all our samples, an energy of activation of 19 ± 1 kJ/mol could be determined from this type of data evaluation, in quantitative agreement to earlier findings from Alcântara et al. [24]. Those results indicate that the likelihood of occurrence of milk leakage might be higher when the ambient temperature, and with that, the teat and cisternal milk temperatures increase. This observation warrants further research on teat and cisternal milk temperatures depending on the ambient temperatures, and with that, seasonality of milk leakage in the field.

Milk Composition, Dry-off and Antibiotic Dry Cow Therapy

In response to simulated dry-off, the milk protein content and SCC increased, whereas the lactose content decreased. These changes in the milk composition can be attributed to changes in the mammary epithelial tissue permeability in response to milking omission. Alveolar distension due to milk accumulation leads to impaired tight junctions and an efflux of milk lactose and influx of blood proteins [21]. In response to antibiotic dry cow therapy, the milk fat content and SCC increased, whereas the lactose content decreased. The physiological

background of these antibiotic dry cow therapy related changes in the milk composition remains unclear. The increased fat content might be related to the paraffin content of the dry cow antibiotic.

An overall effect of the fat and protein content on the efflux time and dynamic viscosity could be demonstrated. The efflux time and dynamic viscosity increased as the fat and protein content increased. A similar relationship between the dynamic viscosity and fat and protein content was reported by several authors [24, 33, 34]. McCarthy [34] reported that lactose and whey proteins have a relatively small influence on the dynamic viscosity of whole milk and the fat and casein content had a major and the greatest influence, respectively. Transferred to the teat, those findings indicate that a decrease of the fat and protein content might facilitate milk leakage due to faster and more easily flowing of the milk. Furthermore, it might be hypothesized that breeds with a naturally higher fat and protein content (e.g., Jersey cows) are less likely to show milk leakage. Again, further studies are warranted to elucidate this association.

In the current study, the lactose content and SCC had no effect on the efflux time. An interaction between the lactose content and antibiotic dry cow therapy and between SCC and antibiotic dry cow therapy on the dynamic viscosity, however, could be detected. Similar to the results of Alcântara et al. [24] the dynamic viscosity decreased with decreasing lactose content in samples with antibiotic dry cow therapy. In samples without antibiotic dry cow therapy, however, dynamic viscosity increased with decreasing lactose content. Rovai et al. [9] detected lower concentrations of lactose in milk leaking from teats of lactating cows, which let us hypothesize that low lactose content might facilitate milk leakage. Our results contradict this hypothesis, because the lactose content was not found to have any effect on the efflux time and decreasing lactose content of samples without antibiotic dry cow therapy let to an increased dynamic viscosity. To our knowledge, there are no studies that report an effect of SCC on dynamic viscosity of raw milk. In the current study, increased SCC resulted in a decreased dynamic viscosity in samples with antibiotic dry cow therapy. But in samples without antibiotic dry cow therapy the dynamic viscosity was not affected by SCC.

The efflux time and dynamic viscosity increased after dry-off, which is in agreement with the effects of dry-off related changes in milk composition. Those findings, however, are in contrast to the prevalence of milk leakage, which is with approximately 30% [3, 4] 6 times higher in cows the first days after dry-off than in lactating cows with 5.3% [1]. Transferred to the teat, dry-off and dry-off related changes in milk composition might not be a risk factor for milk leakage and not the reason for the higher prevalence of milk leakage after dry-off.

Antibiotic dry cow therapy does not affect the efflux time but does affect the dynamic viscosity in interaction with lactose content and SCC, respectively. Transferred to the teat, antibiotic dry cow therapy in interaction with decreased lactose content and increased SCC might be a risk factor for milk leakage on the milk level. To our knowledge, there are no other studies investigating the effect of antibiotic dry cow therapy on the likelihood of the occurrence of milk leakage.

3.2.6 **Conclusions**

To our knowledge our laboratory model is the first in vitro approach for a bovine teat. We are aware that the anatomical and physiological traits of a living teat and teat canal which are influenced by physiological changes such as blood flow, tissue tonus or contractions of smooth muscles could not be perfectly simulated by the constant diameter of a cylindrical shaped capillary in a viscometer. The laboratory model allowed us to reduce our study animals and to perform less invasive procedures on them. It might be useful in pilot studies, to provide indications for parameters which should be studied more extensively or it might be a good basis for further, improved models. The results of this in vitro study indicate that various factors influence the efflux time and dynamic viscosity of milk, which might influence the occurrence of milk leakage in the field. Considering decreased efflux time and decreased dynamic viscosity as a measure for higher likelihood of the occurrence of milk leakage, we confirmed wider teat canal diameter, higher IMM pressure, higher milk temperature and lower concentrations of fat and protein as plausible risk factors for milk leakage in dairy cows. Future research studies should investigate the influence of a teat sealant on the occurrence of milk leakage, the potential seasonality of milk leakage regarding the milk temperature and the potential lower prevalence for milk leakage in breeds with a naturally higher milk fat and protein content, which could be a useful information for future breeding programs.

3.2.7 **Acknowledgments**

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Table 1. Differences in efflux time and dynamic viscosity in quarter foremilk samples on the day of dry-off (d0) and 2 d after dry-off (d2) with and without antibiotic dry cow therapy.

	Efflux time (s)			Dynamic viscosity (mPa s)		
Variable	LSM	SEM	*P*-value	LSM	SEM	*P*-value
Dry-off						
d0	102.95	4.80	< 0.001	1.93	0.02	< 0.001
d2	108.61	3.40		2.03	0.01	
Antibiotic						
with	-	-	0.485	2.04	0.17	< 0.001
without	-	-		1.98	0.12	
Temperature						
26 °C	122.58	5.40	< 0.001	2.27	0.01	< 0.001
32 °C	105.75	4.69		1.98	0.01	
38 °C	91.84	4.11		1.74	0.01	
Pressure						
60 mbar	132.17	5.70	< 0.001	1.97	0.02	< 0.001
80 mbar	103.25	4.47		2.00	0.02	
100 mbar	84.76	3.70		2.02	0.02	
Capillary						
0 (0.36 mm)	189.67	1.92	< 0.001	1.93	0.01	< 0.001
1 (0.63 mm)	23.78	0.55		2.03	0.01	
Antibiotic × Lactose			-			< 0.001
Antibiotic × SCC			-			0.025
Capillary × Temperature			< 0.001			< 0.001
Capillary × Pressure			< 0.001			< 0.001

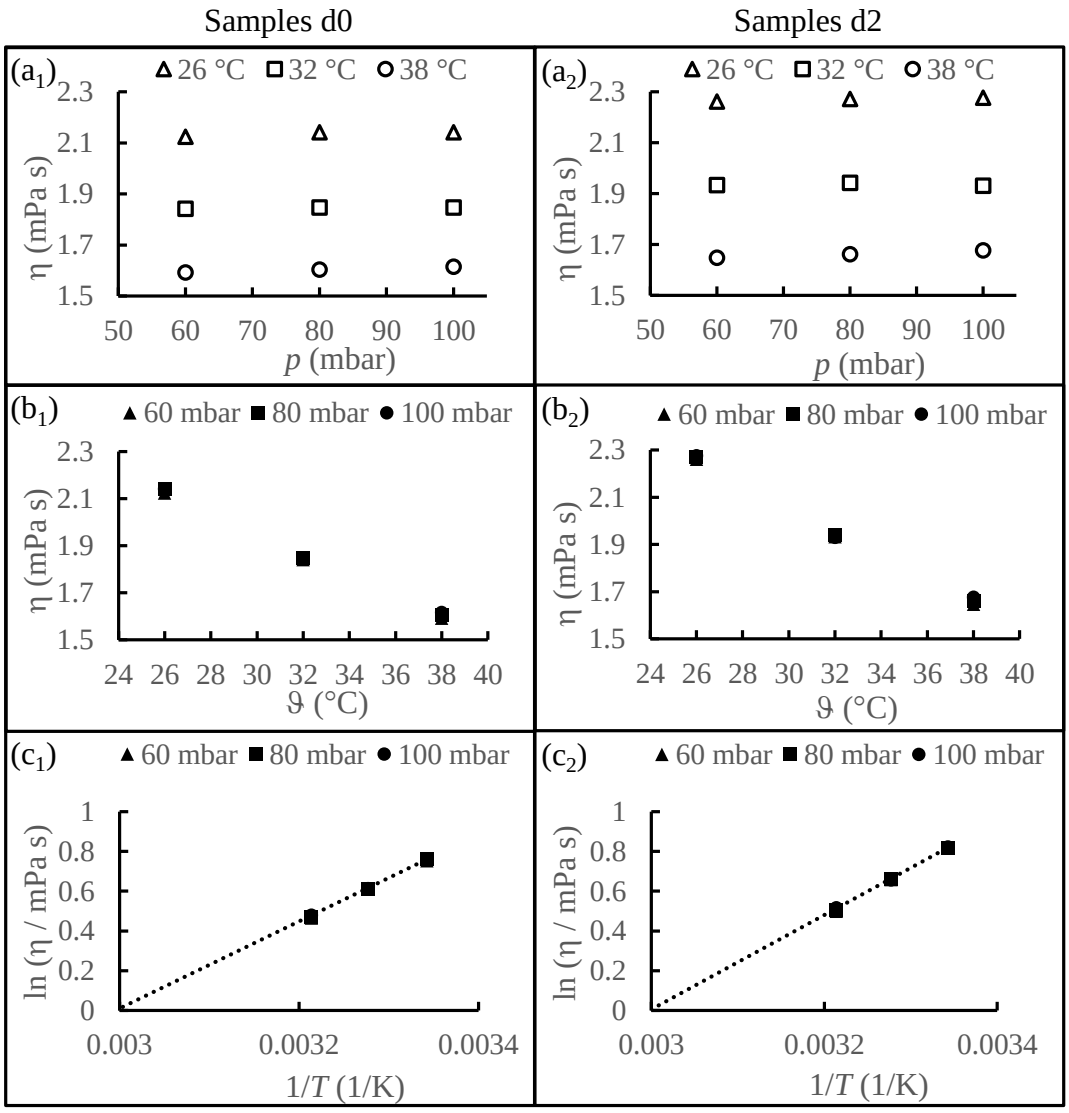

Figure 1. Average dynamic viscosities, η, of samples d0 (left column, panel label index "1") and samples d2 (right column, panel label index "2") as a function of (a) hydrostatic pressure, *p*, and (b) sample Celsius temperature, ϑ. (c) Replotting the data in a form of ln η vs. the inverse Kelvin temperature, 1/*T*, yields a linear Arrhenius plot that allows the energy of activation on flow to be determined from the slope of the linear fit (dotted line). All error bars are smaller than the data symbols.

3.2.8 **References**

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4 DISCUSSION

The overall objective of this thesis was to examine the effect of the milk fraction and short dry periods on the residue concentration of cefquinome in milk after calving and thereby improve the application of antibiotic screening tests and verify the validity of established withdrawal periods. Furthermore, risk factors for milk leakage in dairy cows should be investigated to gain further knowledge about this phenomenon in dairy cows.

The aim of our first study was to investigate the residue concentration of a dry cow antibiotic containing cefquinome in two different milk fractions. The effect of the milk fraction on the concentration of a lactating cow antibiotic containing cephapirin at the first milking after treatment has been reported by Stockler et al. (2009). The author detected similar to our results higher residue concentrations in the foremilk than in the stripping samples and furthermore reported no significant difference between the cephapirin concentrations of bucket milk and stripping samples. We investigated foremilk and stripping samples only, as the focus of our study laid on the consequences of the milk fraction on the performance of antibiotic screening tests. Due to the higher cefquinome concentration in the foremilk samples, we recommend this milk fraction for the use in screening test in respect of food safety.

Another important result for the on-farm usage of screening tests is the fact, that over 80% of the not evaluable test results of the lateral flow test Milchtest BL in our study were stripping samples. Andrew (2000) hypothesized that high fat content may hinder the milk movement in the CITE Snap assay, another lateral flow test. In our study, a high protein content and high logSCC caused not evaluable test results and we hypothesized that these milk components might impede the flow of the milk samples through the membrane of the Milchtest BL due to higher viscosity and mechanical obstruction. The detection of cefquinome residues on MRL-level and no false negative results in our study are advantages of the Milchtest BL compared to the Delvotest BR Brilliant Plates.

The aim of our second study was to investigate the impact of short dry periods on the residue concentration of a dry cow antibiotic containing cefquinome in foremilk samples during the colostrum period and early lactation. Dry periods of 40 d or less have been associated with maximized lifetime production after second or later lactation (Kuhn et al., 2006) and improved reproductive performance without any loss in colostrum quality (Shoshani et al., 2014). Furthermore, dry periods of 30 d resulted in an improved energy balance of dairy cows in the early lactation (Van Knegsel et al., 2014) without any untoward effects on udder health

(Church et al., 2008). However, the aspect of a potential higher risk for antibiotic residues from the dry cow treatment in milk after calving should not be neglected.

In our study with experimental short dry periods from 1 to 26 d, which were categorized into three dry period groups (dry period group 1: 1 to 7 d, dry period group 2: 8 to 14 d, dry period group 3: more than 14 d), shorter dry periods lead initially to higher cefquinome residues after calving. Interestingly, less days after treatment were needed for the residue concentrations to fall below the MRL-value after shorter dry periods. These results agree with the CVMP (2006), which reported, that the depletion of cefquinome during the dry period is followed by a more rapid depletion after calving. The cefquinome concentration in our study fell below the MRL in all quarters within the recommended withdrawal period for milk of 36d after IMM dry cow treatment. Due to long withdrawal periods after calving resulting in a considerable number of milkings that have to be discarded, short dry periods as investigated in our study should not be used in the dry cow management. Further research is necessary, to investigate the relationship between short dry periods and the residue concentration of other dry cow antibiotics with different pharmacokinetic characteristics than cefquinome.

We detected an increase in the cefquinome concentration in dry period group 1 and 2 until the third and second milking, respectively. This fact contrasts with studies on the pharmacokinetic of a lactating cow antibiotic containing cefquinome, which reported a continuous decline of the residue concentration from 2 h to 72 h after treatment and up to a maximum of the 10th milking after treatment, respectively (Zonca et al., 2011; Li et al., 2014). We hypothesized, that characteristics of the colostrum might hinder the release of cefquinome from the dry cow formulation, which might take place more enhanced after the first milking, but the concrete physiological background remains unclear.

Minor cefquinome concentrations in the untreated control quarters and a tendential positive correlation to the cefquinome concentrations of the last treated quarters could be detected in our study. These findings challenge the assumed independence of quarters in our study design. The occurrence of cefquinome residues in untreated quarters of a quarter-based approach has been reported before (Li et al., 2014) and the author explained it with diffusion or distribution of cefquinome through the quarters. In general, the ability of cefquinome to distribute through the udder and diffuse through lipophilic membranes is low, as it is a weak organic acid with a low fat solubility. Our study design included a second dry cow treatment of previously dried-off cows, in order to minimize the losses of residues by milk leakage before calving. Therefore, two potential origins of the cefquinome residues in the control quarters

could be detected: the at least 57 d ago first dry cow treatment or the dry cow treatment of the treated quarters during the study. For the elucidation of the concrete origin of the cefquinome residues in the control quarters further research is needed. Furthermore, the validity of the independence of quarters and the usage of quarter based approaches in pharmacokinetic studies should be investigated.

The aim of our third study was to investigate risk factors for milk leakage in dairy cows before and after dry-off. Specifically, we set out to determine the effects of 1) teat canal diameter, IMM pressure and milk temperature, 2) milk composition, 3) dry-off, and 4) IMM dry cow therapy on efflux time and dynamic viscosity of quarter foremilk samples. In order to reduce the use of study animals (Russell and Burch, 1959), a measuring method to evaluate the risk factors under controlled conditions was developed. To our knowledge our laboratory model is the first in vitro approach for a bovine teat, simulating the teat canal, IMM pressure and milk temperature in two capillary viscometers. However, we are aware that the anatomical and physiological traits of a living teat and teat canal could not be perfectly represented by the constant diameter of a cylindrical shaped capillary in a viscometer, but we see a good basis for further improved in vitro models in this model.

The finding of Bertulat et al. (2013), who demonstrated that milk leakage after dry-off was associated with higher udder pressure could be confirmed in our study, where the efflux time of milk samples decreased as the hydrostatic pressure increased. Further detected potential risk factors for milk leakage in our study, as wider teat canal diameter, higher milk temperature and lower concentrations of fat and protein have not been reported before but might be interesting fields for further research studies with the aim of finding potential prevention methods for milk leakage. The 6 times higher prevalence for milk leakage in cows the first days after dry-off (Schukken et al., 1993; Bertulat et al., 2013) compared to lactating cows (Klaas et al., 2005) could not be explained by dry-off or dry-off related changes in the milk composition in our study, as they both increased the efflux time and dynamic viscosity of the milk samples. The antibiotic dry cow therapy in interaction with decreased lactose content or increased SCC lead to a decreased dynamic viscosity of the milk samples. Further research is necessary to investigate the effect of antibiotic dry cow therapy on the likelihood of the occurrence of milk leakage, where widening the teat canal diameter with the injector of the dry cow antibiotic might be a further interesting aspect.

Overall, the effect of the milk fraction and short dry periods on the cefquinome concentration in milk after calving was investigated and risk factors for milk leakage in dairy

cows before and after dry-off were determined in an in vitro approach. The results of these three studies contribute to the improvement of the application of antibiotic screening tests, to the determination of the optimum dry period length in the sake of food safety and economics and to the udder health of dairy cows, due to the gain of knowledge about the aetiology and potential risk factors for milk leakage.

5 SUMMARY

Investigation of cefquinome residues in milk after antibiotic dry cow treatment and risk factors for milk leakage in dairy cows

The overall objective of my thesis was to examine the effect of the milk fraction and short dry periods on the residue concentration of cefquinome in milk after antibiotic dry cow treatment and to investigate risk factors for milk leakage in dairy cows before and after dry-off.

The knowledge of the relationship between the milk fraction and antibiotic concentration in milk samples from individual cows is important for an appropriate use of antibiotic screening tests on farm. Therefore, the first study investigated the residue concentration of an antibiotic dry cow treatment containing cefquinome considering the milk composition in two different milk fractions (foremilk and strippings) and evaluated the test characteristic of two commercially available screening tests in relationship to the analyzed milk fraction and the milk composition. Thirteen dry dairy cows were enrolled in this study. One quarter each per cow was treated with an intramammary (IMM) dry cow antibiotic containing cefquinome on d 21, 14 and 7 before calculated calving date. Milk samples were collected from each quarter before (foremilk samples) and after milking (stripping samples) twice daily during the first 10 d postpartum. All samples were tested for antibiotic residues with the lateral flow test Milchtest BL and the microbial inhibitor test Delvotest BR Brilliant Plates and were analyzed for milk composition (fat, protein, lactose, urea, somatic cell count (SCC)). The residue concentration of cefquinome in foremilk and stripping samples from the first, second, third, fifth and seventh milking after calving were determined using high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS).

The logarithm of cefquinome concentration (logCef) was higher in foremilk (1.495 ± 0.080) compared to stripping samples (1.377 ± 0.078; $P = 0.001$). The value of logCef decreased with the number of milking ($P < 0.001$) and additionally, an interaction between the milk fraction and the number of milking could be detected ($P = 0.001$).

Foremilk samples had 38.4 times higher odds to be evaluable (test and control line appeare) in the Milchtest BL than tests of stripping samples. High protein content increased and high logarithm of SCC (logSCC) tended to increase the odds of not evaluable results (no test and/or control line appeare) in the Milchtest BL. False positive test results of the Milchtest BL were neither associated with the milk fraction ($P = 0.424$) nor milk composition ($P > 0.100$). Milk samples with higher protein content were more likely and with a lower urea content tended

to be more likely to have a false positive result in the Delvotest BR Brilliant Plates. Milk fraction (P = 0.799), fat (P = 0.887) and lactose content (P = 0.110) as well as logSCC (P = 0.453) had no effect on the correctness of the results in the Delvotest BR Brilliant Plates.

Overall, the results of this study indicate that foremilk should be the recommended milk fraction to be tested for residues of cefquinome and to be used in lateral flow tests to avoid test failure. Furthermore, high protein content should be considered as a cause of test failure and false positive results when milk during the first 10 d postpartum is tested for antibiotic residues using lateral flow or microbial inhibitor tests.

The optimum dry period length is still an active field of research. Several studies in the last years have detected advantages of short dry periods on milk production, reproductive performance and energy balance during early lactation. The impact of short dry periods on the residue concentration of dry cow antibiotics in milk after calving has not yet been investigated. Therefore, the objective of our second study was to investigate the residue concentration of a dry cow antibiotic containing cefquinome during the colostrum period and early lactation in quarter foremilk samples after short dry periods. Overall, 19 dry dairy cows were enrolled in the study. In order to achieve antibiotic concentrations representative for short dry periods three quarters of each cow were treated with an IMM dry cow antibiotic containing 150 mg of cefquinome on d21, 14 and 7 before calculated calving date (treated quarters). One quarter of each cow was not treated (control quarter). Foremilk samples from each quarter were collected twice daily until 21 d and once daily until 36 d after IMM dry cow treatment (end of withdrawal period), respectively. The residue concentration of cefquinome was measured from all quarters from the first milking after calving and from treated quarters from the second, third, fifth and seventh milking after calving. Further foremilk samples from odd milking numbers were individually selected for the analysis until the residue concentration fell below the limit of quantification, which occurred at the latest in milking number 37.

Minor cefquinome residues (range: 0.5 – 5.59 ng/g), albeit far below the maximum residue limit (MRL; 20 ng/g), could be detected in the foremilk samples of the control quarters of the first milking. There tended to be an effect of the concentration of the last treated quarter on the concentration of the control quarter with higher concentrations, when the concentration of the last treated quarter was also higher (P = 0.097). Treated quarters were allocated to 1 out of 3 groups based on the dry period length after the dry cow treatment (dry period group 1: 1 to 7 d, dry period group 2: 8 to 14 d, dry period group 3: more than 14 d). The cefquinome concentration up to the 37th milking was higher in dry period group 1 (581.40 ± 90.71 ng/g)

than in dry period group 2 (115.41 ± 15.38 ng/g; $P = 0.001$) and 3 (21.28 ± 6.00 ng/g; $P < 0.001$), respectively. In dry period group 1, the cefquinome concentration increased after calving until the third milking ($P = 0.027$) and decreased considerably until the fifth milking ($P = 0.001$). In dry period group 2 the cefquinome concentration peaked at the second milking ($P < 0.05$). On average 31.3 ± 1.2, 19.0 ± 1.1 and 6.7 ± 0.8 milkings ($P < 0.001$) and 19.4 ± 0.4, 20.6 ± 0.5 and 24.1 ± 0.7 d ($P < 0.05$) after treatment were necessary for the concentration of cefquinome to fall below the MRL-value in dry period group 1, 2 and 3. The results of this study indicate that shorter dry periods lead initially to higher cefquinome residues in milk after calving, but due to the more rapid depletion of cefquinome after calving fewer days after treatment are necessary for the cefquinome concentration to fall below the MRL. The residue concentration after short dry periods still fell below the MRL within the recommended withdrawal period for milk of 36 d after IMM dry cow treatment. For the sake of food safety and economics, these short dry periods should not be used in the dry cow management, as they lead up to a maximum of 31.3 ± 1.2 milkings and 19.4 ± 0.4 days after treatment with cefquinome residues above the MRL and a considerable number of milkings that have to be discarded, due to long withdrawal periods after calving.

The occurrence of milk leakage has been reported in approximately 30% of cows after dry-off, with the highest likelihood of the occurrence 2 d after dry-off and in approximately 5.3% of lactating cows while entering the milking parlor. Risk factors for milk leakage on cow level have rarely been investigated. We developed a laboratory model to determine risk factors for milk leakage under controlled conditions. The laboratory model, composed of two capillary viscometers, was used to measure the efflux time (time interval that the fluid needs to descend from the upper timing mark to the lower timing mark of the viscometers' measuring sphere) and calculate the dynamic viscosity (internal resistance of a fluid to the application of pressure) of milk samples. Parameters which lead to a decreased efflux time and dynamic viscosity of the milk samples were considered to increase the likelihood of milk leakage in the field. Overall, the objective of the third, in vitro study was to determine the effects of the teat canal diameter, IMM pressure, milk temperature, milk composition, dry-off and IMM dry cow therapy on efflux time and dynamic viscosity of quarter foremilk samples and thereby evaluate plausible risk factors for milk leakage in dairy cows before and after dry-off.

Quarter foremilk samples from 6 dairy cows were collected each on the day of dry-off and 2d after dry-off with and without antibiotic dry cow therapy. Milk composition was analyzed and efflux time and dynamic viscosity of all foremilk samples were measured. The

effect of 2 different capillary diameters, 3 different pressures and 3 different temperatures, milk composition, dry-off and antibiotic dry cow therapy on efflux time and dynamic viscosity was evaluated. The efflux time decreased as the hydrostatic pressure and milk sample temperature increased ($P < 0.001$). The dynamic viscosity slightly increased as the hydrostatic pressure increased, but decreased as the milk sample temperature increased ($P < 0.001$). The efflux time and dynamic viscosity increased as the fat and protein content increased ($P < 0.001$). The lactose content ($P = 0.495$) and SCC ($P = 0.423$) had no effect on the efflux time. In samples collected after dry-off, the efflux time and dynamic viscosity were higher than in samples before dry-off ($P < 0.001$). Antibiotic dry cow therapy had no effect on the efflux time ($P = 0.485$). An effect of the interaction between the lactose content and antibiotic dry cow therapy ($P < 0.001$) and between SCC and antibiotic dry cow therapy ($P = 0.025$) on the dynamic viscosity could be evaluated. In samples drawn 2d after antibiotic dry cow therapy, the dynamic viscosity increased with increasing lactose content and in samples collected without previous antibiotic dry cow therapy, the dynamic viscosity decreased with increasing lactose content. Furthermore, in samples with antibiotic dry cow therapy, the dynamic viscosity decreased with increasing SCC, however, in samples without antibiotic dry cow therapy the dynamic viscosity was not affected by SCC.

The results of this study indicate that various factors influence the efflux time and dynamic viscosity of milk, which might influence the occurrence of milk leakage in the field. Considering decreased efflux time and decreased dynamic viscosity as a measure for higher likelihood of the occurrence of milk leakage, we could report wider teat canal diameter, higher IMM pressure, higher milk temperature and lower concentrations of fat and protein as plausible risk factors for milk leakage in dairy cows.

Regarding the overall hypothesis of my thesis, the results of the three studies indicate the impact of milk fraction and short dry periods on the residue concentration of cefquinome after IMM dry cow therapy during the colostrum period and early lactation. Furthermore, the results show risk factors for milk leakage before and after dry-off. These findings are relevant for the appropriate usage of antibiotic screening tests and the determination of the optimum dry period length in the sake of food safety and economics. The developed laboratory model represents a basis model for other in vitro studies regarding the bovine teat, conducted with the aim of reducing study animals or performing less invasive procedures on them.

6 ZUSAMMENFASSUNG

Untersuchung der Rückstandskonzentration von Cefquinom in der Milch nach dem antibiotischen Trockenstellen und Risikofaktoren für Milchabtropfen bei Milchkühen

Das Ziel dieser Arbeit war es die Auswirkung der Art des Gemelkes und kurzer Trockenstehdauern auf die Rückstandskonzentration von Cefquinom in der Milch nach dem antibiotischen Trockenstellen zu untersuchen und Risikofaktoren für Milchabtropfen bei Milchkühen vor und nach dem Trockenstellen zu ermitteln.

Für eine sachgerechte Anwendung von Hemmstofftests auf milcherzeugenden Betrieben ist die Kenntnis über den Zusammenhang zwischen der Art des untersuchten Gemelkes und der Rückstandskonzentration wichtig. Deshalb wurde in der ersten Studie die Rückstandskonzentration eines cefquinomhaltigen Trockenstellers in zwei verschiedenen Gemelken (Vor- und Nachgemelk) unter Berücksichtigung der Milchzusammensetzung untersucht und das Testergebnis zweier kommerziell erhältlicher Hemmstofftests im Hinblick auf das Gemelk und die Milchzusammensetzung bewertet.

Dreizehn trockenstehende Milchkühe wurden in die Studie aufgenommen. Jeweils 1 Euterviertel pro Kuh wurde an Tag 21, 14 und 7 vor dem errechneten Abkalbetermin mit einem cefquinomhaltigen Trockensteller behandelt. Während der ersten 10 Tage post partum wurden zweimal täglich vor (Vorgemelk) und nach dem Melken (Nachgemelk) Milchproben von jedem Euterviertel genommen. Alle Milchproben wurden mit dem Lateral Flow Test Milchtest BL und dem mikrobiellen Hemmstofftest Delvotest BR Brilliant Plates auf Antibiotikarückstände untersucht und ihre Milchzusammensetzung (Fett, Protein, Lactose, Harnstoff, somatische Zellzahl (SCC)) wurde bestimmt. Die Rückstandskonzentration von Cefquinom in den Vor- und Nachgemelken von Gemelksnummer 1, 2, 3, 5, und 7 nach der Abkalbung wurden mittels Hochdruckflüssigchromatographie mit Tandem-Massenspektrometrie-Kopplung (HPLC-MS/MS) analysiert.

Der Logarithmus der Konzentration von Cefquinom (LogCef) war im Vorgemelk (1.495 ± 0.080) höher als im Nachgemelk (1.377 ± 0.078; $P = 0.001$). Der LogCef nahm in unserer Studie mit der Gemelksnummer ab ($P < 0.001$) und des Weiteren konnte eine Interaktion zwischen Gemelk und Gemelksnummer nachgewiesen werden ($P = 0.001$).

Vorgemelke hatten eine 38.4-fach höhere Wahrscheinlichkeit auf ein auswertbares Ergebnis (Test- und Kontrolllinie erscheinen) im Milchtest BL als Nachgemelke. Hoher Proteingehalt und hohe logarithmierte SCC (LogSCC) erhöhten tendenziell die

Wahrscheinlichkeit von nicht auswertbaren Ergebnissen (Test- und/oder Kontrolllinie erscheinen nicht) im Milchtest BL. Falsch positive Ergebnisse des Milchtest BL waren weder mit dem Gemelk ($P = 0.424$) noch mit der Milchzusammensetzung ($P > 0.100$) verbunden. Milchproben mit hohem Proteingehalt führten und die mit einem geringeren Harnstoffgehalt führten tendenziell zu falsch positiven Ergebnissen in den Delvotest BR Brilliant Plates. Das Gemelk ($P = 0.799$), Konzentration von Fett ($P = 0.887$) und Laktose ($P = 0.110$), sowie LogSCC ($P = 0.453$) hatten keine Auswirkung auf die Richtigkeit der Testergebnisse der Delvotest BR Brilliant Plates.

Insgesamt verdeutlichen die Ergebnisse dieser Studie, dass Vorgemelke die empfohlenen Gemelke für die Bestimmung der Rückstandskonzentration von Cefquinom sein und in Lateral Flow Tests verwendet werden sollten, um nicht auswertbare Testergebnisse zu vermeiden. Des Weiteren sollte ein hoher Proteingehalt als Grund für nicht auswertbare und falsch positive Ergebnisse während der ersten 10 Tage post partum in Betracht gezogen werden, wenn Lateral Flow Tests oder mikrobielle Hemmstofftests verwendet wurden.

Die Bestimmung der optimale Trockenstehdauer ist weiterhin ein aktuelles Forschungsgebiet. Zahlreiche Studien in den letzten Jahren haben Vorteile von kurzen Trockenstehdauern auf die Milchproduktion, die Reproduktionsfähigkeit und die Energiebilanz während der Frühlaktation dargestellt. Die Auswirkungen von kurzen Trockenstehdauern auf die Rückstandskonzentration von antibiotischen Trockenstellern in der Milch nach dem Abkalben wurde noch nicht untersucht. Deshalb war das Thema unserer zweiten Studie die Rückstandskonzentration eines cefquinomhaltigen Trockenstellers nach kurzer Trockenstehdauer in Vorgemelksproben während der Kolostralperiode und Frühlaktation zu bestimmen.

Insgesamt wurden 19 trockenstehende Kühe in die Studie aufgenommen. Um Rückstandskonzentrationen zu erreichen, die kurze Trockenstehdauern wiederspiegeln, wurden jeweils drei Euterviertel jeder Kuh an Tag 21, 14 und 7 vor dem errechneten Abkalbetermin mit einem cefquinomhaltigen Trockensteller behandelt (behandelte Viertel). Ein Euterviertel jeder Kuh blieb unbehandelt (Kontrollviertel). Vorgemelksproben von jedem Viertel wurden jeweils zweimal täglich bis 21 Tage und einmal täglich bis 36 Tage nach dem antibiotischen Trockenstellen (Ende der Wartezeit des Trockenstellers) genommen. Die Rückstandskonzentration von Cefquinom von allen Vierteln von Gemelksnummer 1 und von allen behandelten Vierteln von Gemelksnummer 2, 3, 5, und 7 nach der Abkalbung wurden bestimmt. Weitere Vorgemelksproben wurden viertelindividuell für die Bestimmung der

Rückstandskonzentration von Cefquinom ausgewählt, bis die Konzentration unter die Quantifizierungsgrenze fiel, was spätestens in Gemelksnummer 37 eintrat.

Geringfügige Rückstandskonzentrationen von Cefquinom (0.5 – 5.59 ng/g) weit unter der zulässigen Rückstandshöchstmenge (MRL; 20 ng/g) konnten in Gemelksnummer 1 in den unbehandelten Kontrollvierteln festgestellt werden. Eine tendenzielle, positive Korrelation zwischen der Konzentration von Cefquinom der letzten behandelten Viertel und der Konzentration von Cefquinom der Kontrollviertel konnte ausgemacht werden. Die behandelten Viertel wurden anhand ihrer Trockenstehdauer in drei Trockenstehgruppen aufgeteilt (Trockenstehgruppe 1: 1 bis 7 d, Trockenstehgruppe 2: 8 bis 14 d, Trockenstehgruppe 3: mehr als 14 d). Die Rückstandskonzentration von Cefquinom bis zur Gemelksnummer 37 war in der Trockenstehgruppe 1 höher als in Trockenstehgruppe 2 und 3. In der Trockenstehgruppe 1 stieg die Rückstandskonzentration von Cefquinom bis zur dritten Gemelksnummer an und fiel danach deutlich bis zur fünften Gemelksnummer ab. In Trockenstehgruppe 2 erreichte die Rückstandskonzentration von Cefquinom ihren Höchstwert in der zweiten Gemelksnummer. Im Durchschnitt waren jeweils 31.3 ± 1.2, 19.0 ± 1.1 und 6.7 ± 0.8 Gemelke ($P < 0.001$) und jeweils 19.4 ± 0.4, 20.6 ± 0.5 und 24.1 ± 0.7 Tage ($P < 0.05$) nach dem antibiotischen Trockenstellen in Trockenstehgruppe 1, 2 und 3 nötig, bis die Rückstandskonzentration von Cefquinom den MRL unterschritt. Die Ergebnisse dieser Studie zeigen, dass kurze Trockenstehdauern zwar anfangs zu höheren Rückstandskonzentrationen in der Milch nach dem Abkalben führen, aber aufgrund der schnelleren Ausscheidung von Cefquinom nach der Abkalbung weniger Tage nach dem antibiotischen Trockenstellen nötig sind, um den MRL zu unterschreiten. Die Rückstandskonzentration fiel auch nach kurzen Trockenstehzeiten innerhalb der empfohlenen Wartezeit von 36 Tagen nach dem antibiotischen Trockenstellen unter den MRL. Im Sinne der Lebensmittelsicherheit und Ökonomie sollten diese kurzen Trockenstehzeiten nicht im regulären Trockenstellmanagement verwendet werden, da sie zu Höchstwerten von 31.3 ± 1.2 Gemelken und 19.4 ± 0.4 Tagen nach dem antibiotischen Trockenstellen führen können, in denen die Konzentration von Cefquinom oberhalb des MRL liegt. Dies führt zu einer beachtlichen Menge an Gemelken, die aufgrund langer Wartezeiten verworfen werden müssen.

Das Auftreten von Milchabtropfen wurde in Studien in circa 30% der Milchkühe am häufigsten an Tag 2 nach dem Trockenstellen und in circa 5.3% der laktierenden Kühe kurz vor dem Melken festgestellt. Risikofaktoren für Milchabtropfen sind bisher nur geringfügig untersucht worden. Wir haben ein Labormodell entwickelt, um Risikofaktoren für

Milchabtropfen unter kontrollierten Bedingungen zu bestimmen. Das Labormodell, bestehend aus zwei Kapillarviskosimetern, wurde verwendet, um die Ausflusszeit (Zeitspanne, die eine Flüssigkeit benötigt um von der oberen Ringmessmarke zur unteren Ringmessmarke des Messgefäßes eines Viskosimeters abzusinken) und die dynamische Viskosität (innerer Widerstand einer Flüssigkeit gegen Druck- oder Schubspannung) von Milchproben zu untersuchen. Parameter, die zu einer geringeren Ausflusszeit und geringerer dynamischen Viskosität führten, wurden mit einer höheren Wahrscheinlichkeit für Milchabtropfen in vivo in Verbindung gesetzt. Das Ziel dieser dritten, in vitro Studie war es, die Auswirkungen des Strichkanaldurchmessers, des intramammären Druckes, der Milchtemperatur, der Milchzusammensetzung, des Trockenstellens und des antibiotischen Trockenstellers auf die Ausflusszeit und die dynamische Viskosität zu bestimmen und damit plausible Risikofaktoren für das Milchabtropfen bei Milchkühen vor und nach dem Trockenstellen herauszufinden.

Viertelvorgemelksproben von 6 Milchkühen wurden jeweils am Tag des Trockenstellens, 2 Tage nach dem Trockenstellen mit und ohne antibiotischen Trockensteller genommen. Die Milchzusammensetzung wurde bestimmt und die Ausflusszeit und dynamische Viskosität aller Vorgemelksproben mit Hilfe des Labormodells gemessen. Die Auswirkungen von 2 verschiedenen Kapillardurchmessern, 3 verschiedenen Drücken und Milchtemperaturen, der Milchzusammensetzung, dem Trockenstellen und dem antibiotischen Trockensteller auf die Ausflusszeit und die dynamische Viskosität wurden bestimmt.

Die Ausflusszeit sank mit steigendem Druck und steigender Temperatur ($P < 0.001$) und die dynamische Viskosität stieg leicht mit zunehmendem Druck und sank ebenfalls mit steigender Temperatur der Milchproben ($P < 0.001$). Sowohl die Ausflusszeit als auch die dynamische Viskosität stiegen mit zunehmender Konzentration von Fett und Protein in den Milchproben ($P < 0.001$). Die Konzentration von Laktose ($P = 0.495$) und die SCC ($P = 0.423$) hatten keine Auswirkungen auf die Ausflusszeit. In Milchproben, die nach dem Trockenstellen genommen worden waren, war die Ausflusszeit und die dynamische Viskosität höher als in den Proben die vor dem Trockenstellen genommen worden waren ($P < 0.001$). Der antibiotische Trockensteller hatte keine Auswirkungen auf die Ausflusszeit der Milchproben ($P = 0.485$). Ein Effekt der Interaktion zwischen der Konzentration von Laktose und dem antibiotischem Trockensteller ($P < 0.001$) sowie zwischen der SCC und dem antibiotischen Trockensteller ($P = 0.025$) auf die dynamische Viskosität konnte festgestellt werden. In Milchproben, die 2 Tage nach dem antibiotischen Trockenstellen genommen wurden, stieg die dynamische Viskosität mit steigender Konzentration von Laktose und in Milchproben, die 2 Tage nach dem

Trockenstellen ohne antibiotischen Trockensteller genommen wurden, fiel die dynamische Viskosität mit steigender Konzentration von Laktose. Des Weiteren fiel die dynamische Viskosität in Milchproben, die 2 Tage nach dem antibiotischen Trockenstellen genommen wurden, mit steigender SCC. In Milchproben, die 2 Tage nach dem Trockenstellen ohne antibiotischen Trockensteller genommen wurden, konnte kein Einfluss der SCC auf die dynamische Viskosität festgestellt werden.

Die Ergebnisse dieser Studie deuten darauf hin, dass verschiedene Faktoren die Ausflusszeit und dynamische Viskosität von Milch beeinflussen, wodurch gleichzeitig das Auftreten von Milchabtropfen in vivo beeinflusst werden könnte. Da wir eine geringere Ausflusszeit und geringere dynamische Viskosität als Maß für eine höhere Wahrscheinlichkeit für Milchabtropfen betrachtet haben, können wir einen weiteren Strichkanal, höheren intramammären Druck, höhere Milchtemperatur und geringeren Fett- und Proteingehalt als Risikofaktoren für Milchabtropfen bei Milchkühen benennen.

Zusammenfassend zeigen die Ergebnisse der von mir im Rahmen meiner Doktorarbeit durchgeführten Studien, den Einfluss des Gemelkes und kurzer Trockenstehzeiten auf die Rückstandskonzentration von Cefquinom während der Kolostralphase und Frühlaktation und stellen Risikofaktoren für Milchabtropfen bei Milchkühen vor und nach dem Trockenstellen dar. Diese Ergebnisse sind sowohl für die sachgerechte Anwendung von Hemmstofftests relevant als auch für die Bestimmung der optimalen Trockenstehzeit im Sinne der Lebensmittelsicherheit und Ökonomie. Das entwickelte Labormodell stellt ein Ausgangsmodell für weitere in vitro Studien dar, die die bovine Zitze betreffen und das Ziel verfolgen, die Anzahl an Studientieren zu reduzieren oder weniger invasive Maßnahmen an ihnen durchzuführen.

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8 PUBLICATIONS

8.1 Research articles

J. Bachmann, S. Bertulat, S. Seiffert, W. Heuwieser. Viscosity measurements of milk to investigate risk factors for milk leakage in dairy cows before and after dry-off. 2017. Milk Science International 70:20-24.

J. Bachmann, C. Helmschrodt, A. Richter, W. Heuwiser, S. Bertulat. Residue concentration of cefquinome after intramammary dry cow therapy and short dry periods. 2018. Journal of Dairy Science 101:1-11.

8.2 Oral presentations

S. Bertulat, J. Bachmann, W. Heuwieser. 2017. Messung von Antibiotikarückständen in Milch-Einflussfaktoren und Verlässlichkeit von Screeningtestsystemen. 42. Leipziger Fortbildungsveranstaltung – Labordiagnostik in der Bestandsbetreuung. Leipzig, 16.06.2017.

S. Bertulat, J. Bachmann, W. Heuwieser. 2017. Lebensmittelsicherheit-Einfluss verkürzter Trockenstehzeiten auf Rückstände antibiotischer Trockensteller in der Milch. 63. Jahreskongress der DVG-DGK, Tagung der DVG-Fachgruppen Deutsche buiatrische Gesellschaft DbG, Fortpflanzung und ihre Störungen und Tropenveterinärmedizin & internationale Tiergesundheit. Berlin, 08.11.-12.11.17. In: 63. Jahreskongress der DVG-DGK, Tagung der DVG-Fachgruppen Deutsche buiatrische Gesellschaft DbG, Fortpflanzung und ihre Störungen und Tropenveterinärmedizin & internationale Tiergesundheit - DVG Vet-Congress (Hrsg.), Gießen: Verlag der DVG Service GmbH, S. 31-34, ISBN 978-3-86345-394-7.

S. Bertulat, J. Bachmann, W. Heuwieser. 2018. Einfluss verkürzter Trockenstehzeiten auf Antibiotikarückstände in der Milch nach Abkalbung. Tagung der AG „Sachverständigenausschuss Subklinische Mastitis“. Berlin, 22.03.2018.

8.3 Poster presentations

Bachmann, J.; Bertulat, S.; Seiffert, S.; Heuwieser, W. 2016. Milk leakage in dairy cows-investigation of risk factors in a teat model. 9. Doktorandensymposium & DRS Präsentationsseminar "Biomedical Sciences" Berlin – 16.09.2016. In: 9. Doktorandensymposium & DRS Präsentationsseminar "Biomedical Sciences": von Doktoranden für Doktoranden: 16. September 2016, Programm & Abstracts – Dahlem Research School (DRS), Freie Universität Berlin (Hrsg.) Berlin: Mensch und Buch Verlag, S. 39 ISBN: 978-3-86387-744-6.

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10 DECLARATION OF INDEPENDENCE

Hiermit erkläre ich, dass ich, Joana Bachmann, alle Studien selbständig durchgeführt und die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Tabelle 1. Eigener Anteil[1] an den Forschungsprojekten der vorliegenden Dissertation

	Studie 1[a]	Studie 2[b]	Studie 3[c]
Studienplanung	+++	+++	+++
Datenerhebung	+++	+++	+++
Datenanalyse	+++	+++	+++
Verfassen des Manuskripts	+++	+++	+++
Editieren des Manuskripts	++	++	++

[1]Legende: +++: > 70%

++: 50−70%

+: < 50%

[a] Residue concentration of cefquinome considering different milk fractions and the performance of two screening tests

[b] Residue concentration of cefquinome after intramammary dry cow therapy and short dry periods

[c] Viscosity measurements of milk to investigate risk factors for milk leakage in dairy cows before and after dry-off

Berlin, den 11.01.2019

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