

Cell differentiation assisting in evaluating mastitis treatment prognosis

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Abstract

Bovine mastitis is commonly treated with antibiotics, which does not always succeed and therefore, sometimes is unnecessary. Bacteriological cure is the goal of antibiotic therapy and depends on the causing microorganism, the applied therapy, and on animal-related factors. Determining the animal-related part of the probability of bacteriological cure before applying antibiotics might help to reduce unnecessary usage. By now, this is only possible by considering individual cow data including animal-related factors such as age, mastitis history and somatic cell count. Former studies revealed that chronic mastitis lowers the probability of cure and leads to specific characteristics found in the differential cell count. The aim of this study was to develop a flow cytometric cell differentiation tool to determine animal-related factors correlating with a score-based probability of bacteriological cure. Therefore, the proportions of different cell types and their vitality in 874 Dairy Herd Improvement milk samples of 239 cows were determined by flow cytometry. The results were tested for a correlation between data of flow cytometry and the calculated animal-related probability of bacteriological cure of each individual cow by binomial logistic regression analysis. A statistically significant association to the calculated and animal-related probability of bacteriological cure could be shown for highly granulated cells, non-vital cells and macrophages. With this model, 84.4 % of all animals could be allocated to their estimated animal-related probability of bacteriological cure correctly. These findings suggest that flow cytometric cell differentiation might become an innovative tool to estimate animal-related prognosis for bacteriological cure.

Key words: Antibiotics, bacteriological cure, differential cell count, flow cytometry

Introduction

One of the most costly diseases in dairy cows is the inflammatory infection of the udder. In spite of various mastitis control programs its incidence remains high [1]. Farmers need to keep production losses as low as possible and treatment with antibiotics is expected to lead to a cure quickly. However, sometimes antibiotic treatment is ineffective and does not lead to an elimination of bacteria. In these cases, culling or treatment with non-steroidal anti-inflammatory drugs (NSAIDs) should have been the preferred choice of action. In the interest of food safety and consumer acceptance, the usage of antibiotics needs

to be minimized in general, especially by avoiding unnecessary applications [2]. Therefore, it would be beneficial to know if an antibiotic treatment will work successfully before an application. A large variety of factors influencing the probability of bacteriological cure (BC) needs to be considered and often impedes a definite recommendation concerning a mastitic cow [3–5]. These factors can be divided into microbiological factors, therapy related factors and animal-related factors. The latter possess a high diagnostic value and can be obtained quickly and easily for every single cow. Therefore, a supplementary tool to determine the animal-related probability of BC would be very useful. A simple and cost effective method is demanded which can be implied into monthly routine analyses (such as dairy herd improvement [DHI] analyses) and thus help to reduce unnecessary usage of antibiotics.

Measuring the somatic cell count (SCC) is a simple way to monitor udder health on herd level and allows defining status quo and mastitis history on cow level [6]. Although regularly measured SCC can help to distinguish between acute and chronic mastitis, it does not provide direct information about the probability of BC. Different studies revealed that the proportions of individual immune cell types (polymorphonuclear leucocytes [PMNL], macrophages and lymphocytes) change depending on the duration of the disease: Whereas the amount of PMNL is relatively higher during acute infections, the proportion of macrophages increases significantly during later phases and chronic infections [7–9]. Considering that chronic mastitis leads to a lower probability of cure [10–13], the proportions of cell types might also be used as an indicator for the probability of cure.

Immune cells in milk can be differentiated microscopically which is tedious and time-consuming or by flow cytometry which is easier to standardize [14]. Flow cytometry is a relatively quick and low priced analytical method that brings comparable results as microscopy and is recommended to be preferred to microscopy by different authors [8,15,16]. Using a flow cytometer the quantity and vitality of cells can be determined easily by fluorescence staining [14,17]. Besides, different groups of cells can be differentiated by their morphological characteristics (size and granularity) or even more precisely by antibody staining [18]. Differential cell count (DCC) of milk samples taken from healthy as well as mastitic cows at different stages of inflammation lead to very divergent results [19]. They depend on various factors such as method, materials, and the investigator. However, the results within each study were consistent and repeatable

[18–21]. Several authors reported on significant differences between the DCCs of chronic and acute mastitis; those studies describe a great consensus of DCC that gives more detailed information about udder health status and the stage of inflammation than SCC alone [14,16,19,22–24]. Therefore, DCC appears to be a promising tool to investigate variables influencing the animal-related probability of BC but the discrepancies between different studies on DCC need to be overcome.

The viability of cells has been described as an additional parameter characterizing mastitis by several authors: In one study a significant lower amount of apoptotic cells in milk of healthy cows than in milk of cows suffering from severe mastitis was found [17]. Furthermore, the percentage of viable cells was reported to be influenced by the stage of inflammation; an inflammation in resolution leads to a low proportion of viable PMNL combined with a high SCC [14]. An increasing amount of apoptotic cells during the course of inflammation was also described in recent studies [25,26]. A significant correlation between viability of PMNL and the outcome of clinical mastitis caused by *Escherichia (E.) coli* was described in 2004 [27]. With higher amounts of vital PMNL in the milk pre-infection, the bacterial clearance improved and the recovery occurred earlier. These findings suggest an association between cell viability and the capability of the immune system to induce cure of mastitis.

In case of clinical mastitis, the prognosis for the animal needs to be determined short-dated before antibiotic treatment is applied. Therefore, a method is needed that is simple to repeat and standardize. However, there is no consensus of the percentages of cell types in mastitic milk. This study aims to provide a less specific but more reliable method. The focus is laid on different parameters that can be determined independently from the performing laboratory with a standard method. This study does not try to detect mastitis at early stages. It aims to find additional variables that indicate a low animal-related probability of BC in cases of bovine mastitis quickly and reliably. They might help to decide in cases of clinical mastitis whether antibiotic treatment should be recommended or not.

Material and Methods

Estimating the Animal-related Probability of BC:

Because not every included cow or heifer caught mastitis during the course of the study, their individual cow data were used in order to define the animal-related probability of cure of each individual animal. Animal-related parameters influencing the probability of BC are manifold and discussed in literature broadly [3,4]. For the purpose of

this study the most relevant factors have been integrated in a scoring system shown in table 1. The first parameter that needs to be considered is the number of lactation. A decreased immune defense with increasing lactation number leads to a lower probability of BC [2,28–30]. Secondly, previous clinical mastitis cases have been found to lower the chances of BC [29,30]. The third factor included into the scoring system assembles the previous three SCC of DHI milk samples of the cow: Studies on DHI data and bacteriological findings have shown that exceeding 400,000 cells/mL during the preceding three months lowers the probability of BC significantly (unpublished data of our research group). In order to take previous DHI results into account, the so called individual sum is used. The individual sum is the weighted total of high SCC over 400,000 cells/mL during the last three months. It allows accounting of high SCC relative to distance of time to the present case of clinical mastitis. High SCC in close proximity was accorded a higher number than high SCC that occurred longer ago. The individual sum is calculated as follows: In case of exceeding 400,000 cells/mL during the last month, a summand of 3, during the month before the last month, a summand of 2 and three months ago, a summand of 1 were added. This leads to a total of 0 to 6. For example, a cow showing high SCC over 400,000 cells/mL three months ago (summand 1) and the month before the last month (summand 2), but not during the last month, would have an individual sum of 3. Fourthly, the current SCC has been described to give indication of the probability of BC [2,28,31–33] and was added to the scoring system. The total amount of these 5 scores evaluates the probability of BC of each individual cow. If the total amount is 5 to 11, the probability of BC is called 'favorable', in case of 12 to 15 'poor'.

Animals and Farms:

Holstein-Friesian dairy cows from seven different German dairy farms in Lower Saxony with herd sizes from 26 to 80 lactating cows were included into the study. Cows showing an individual SCC >200.000 cells/mL in the current DHI sample were selected. They were of different ages from 1st to 9th lactation. All the farms provided twice daily milking in milking parlors.

Milk Sampling:

After cows had been selected, composite milk samples were taken monthly during the regular DHI sampling procedures. Composite milk samples were chosen in order to ensure repeatability during routine diagnostics (such as DHI sampling). Thirty mL milk of each cow were sampled into 40 mL polypropylene tubes. The tubes were transported to the microbiology laboratory of the University of Applied Sciences and Arts Hannover (Germany). All samples were kept at 4 °C during

Table 1. Literature based score system evaluating the animal-related probability of bacteriological cure. The total amount of the scores (1.-5.) evaluates the probability of bacteriological cure of each individual cow.

Parameter	Score		
	1	2	3
1. Number of lactation	1	2	3
2. Number of previous cases of clinical mastitis during current lactation	0	1	>1
3. Number of previous cases of clinical mastitis during previous lactation	0	1	>1
4. Individual sum 400 ¹	0-1	2-3	4-6
5. Current SCC ² (x 1,000 cells/mL)	<400	400-1,000	>1,000

¹ Individual sum is calculated as follows: In case of exceeding 400,000 cells/mL during the last month, a summand of 3, during the month before the last month, a summand of 2 and three months ago, a summand of 1 were added. This leads to a total of 0 to 6.

² SCC = somatic cell count

all procedures and analyzed within 18 hours.

DCC – Isolation of Milk Cells:

After the SCC was determined using a flow cytometer, all samples showing a SCC exceeding 200,000 cells/mL were used for further procedures. Each sample was divided into two parts of 10 mL each and filled into two tubes (tube A and B) and then was centrifuged for 15 min at 200 x g and 4°C. Cream layers and supernatant were discarded and residual cream was removed with a swab. Cell pellets were resuspended with 10 mL of PBS and after a second centrifugation for 15 min at 200 x g and 4°C, the supernatant was discarded again.

DCC – Staining of Milk Cells and Flow Cytometry Analysis:

The cell pellet in tube A was resuspended with 10 mL PBS, and 2 mL were transferred into a 3-mL tube. Acridin orange (0,4 ng/mL final concentration) and propidium iodide (2 µg/mL final concentration) were added. The flow cytometer was equipped with a blue solid state laser and a red diode laser with excitation wavelengths of 488 and 638 nm, respectively. After a live gate was set on green fluorescing particles (acridin orange-positive) in order to disregard non-cellular events, 20,000 events were acquired. By additionally gating on events showing high side scatter (SSC), highly granulated cells could be identified (figure 1). Gating on events showing high forward scatter (FSC) revealed the proportion of comparatively large cells (figure 2). Gating on propidium iodide-positive events revealed the number of non-vital cells (figure 3).

The cell pellet in tube B was resuspended with 10 mL of PBS and centrifuged for 5 min at 250 x g and 4°C. After removing the supernatant carefully, the cell pellet was resuspended with PBS and transferred into a tube for flow cytometry. The suspension was stained with propidium iodide (2 µg/mL final concentration) and 10,000 cells were measured by setting a live gate on FSC in order to disregard non-cellular events. A gate in the FSC versus SSC dot plot on macrophages was determined in studies before by labelling cells with antibodies (CD14 and CD11b) (data not published) (figure 4) and served in this study as a gate on macrophages (figure 5A). Setting a gate on propidium iodide-positive events the proportion of non-vital macrophages was identified (figure 5B).

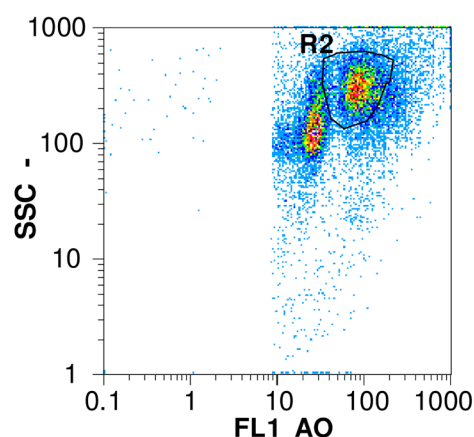


Figure 1. Dot plot of the flow cytometric analysis of milk leucocytes: Fluorescence channel for acridine orange (FL1 AO) versus side scatter (SSC) showing the granularity of green fluorescing particles. A population of highly granulated cells (R2) shows high SSC intensity.

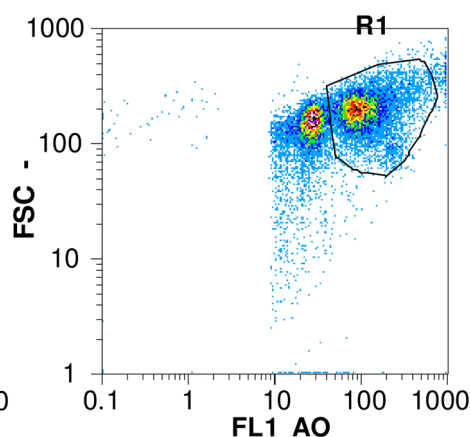


Figure 2. Dot plot of the flow cytometric analysis of milk leucocytes: Fluorescence channel for acridine orange (FL1 AO) versus forward scatter (FSC) showing the size of green fluorescing particles. A population of relatively large cells (R1) shows high FSC intensity.

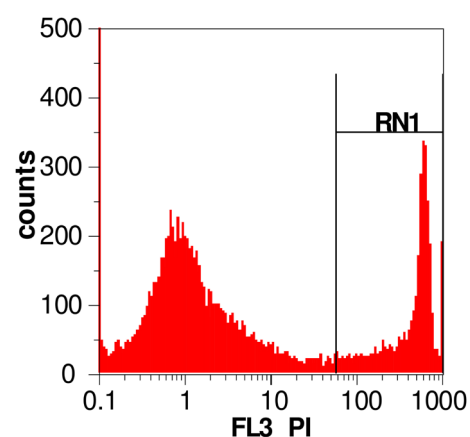


Figure 3. Histogram of the flow cytometric analysis of milk leucocytes demonstrating propidium iodide positivity (FL3 PI). Non-vital cells (RN1) show high propidium iodide intensities.

Statistical Analysis:

Data were collected and analyzed using Excel 2010 (Microsoft Corporation, Redmond, USA) and SPSS (SPSS 22.0, Chicago, USA). A monthly observation of a cow was recognized as the statistical unit. Relevant covariates influencing the probability of BC were identified using generalized linear mixed models. The relationships between the normally distributed proportions of large, granulated, non-vital cells, macrophages and non-vital macrophages, and probability of BC (poor/favorable) were tested using Student's T-test. As a second step, variables that were associated with the outcome variables at $P < 0.10$ were included in binary logistic regressions with favorable (1) and poor (0) probability of BC as the binary outcome. A random cow within a herd effect was included in the model. A forward stepwise process was used for final model selection, applying a P value < 0.05 for inclusion. For the final regression model, the linear predictor was given by

$$\text{Logit}(\text{animal-related probability of BC}) = \text{granulated cells} + \text{non-vital cells} + \text{macrophages} + \text{herd (random)} + e$$

Likelihood-ratio tests were used for significance test to include predictors. Statistical significance was assumed at $P = 0.05$. No predictors had to be excluded from the model to avoid multicollinearity due to strong correlation with each other ($r > 0.70$). Goodness of fit of models was assessed by the Hosmer-Lemeshow goodness of fit statistics [34]. The predictive power of a model was measured by a rescaled pseudo R^2 with the maximum of 1 [35]. Odds ratios (OR) with 95 % confidence intervals (95 % CI) were calculated.

Results

A number of 874 milk samples of 239 cows were analyzed by flow cytometry and proportions of large, granulated, non-vital cells and macrophages and dead macrophages were determined. The results of descriptive statistics are presented in table 2. 728 milk samples were allocated to cows with a favorable probability of BC and 146 samples were taken from cows with a poor probability of BC. On average, milk of cows assigned to have a poor probability of BC contained

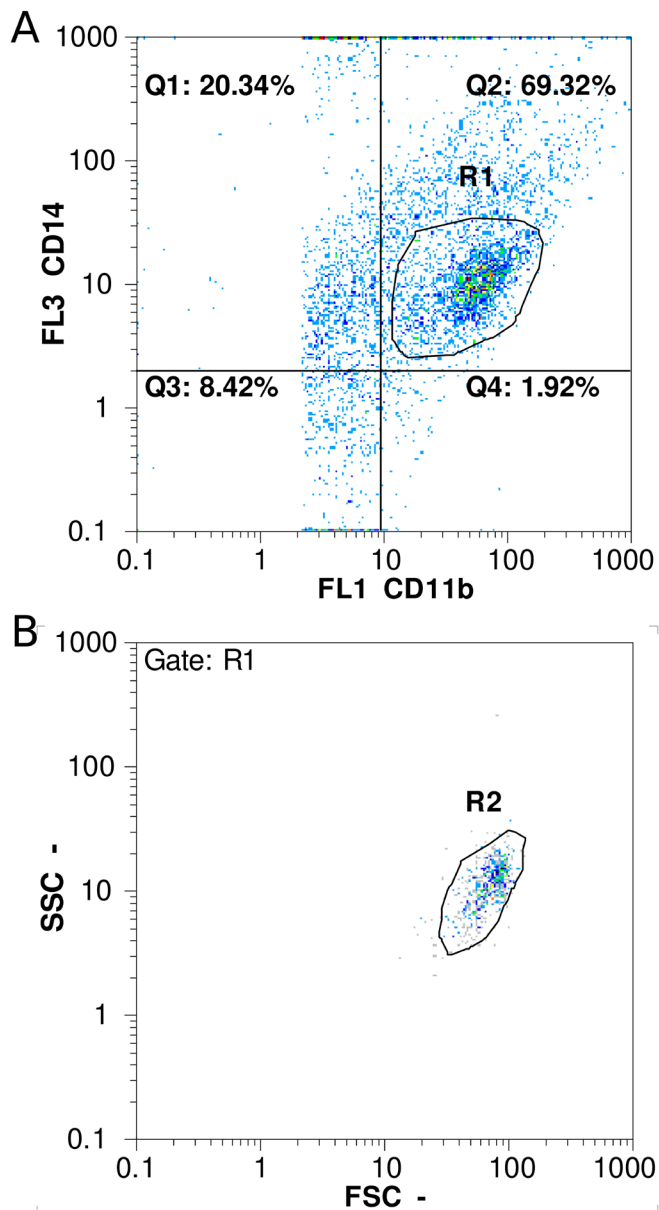


Figure 4. Flow cytometric analysis of milk macrophages: The FL1 CD11b versus FL3 CD14 plot (a) shows the results of the fluorescence analysis of antibody labelled leukocytes. Dot plot of forward scatter (FSC) versus side scatter (SSC) (b) only shows events from Region 1 (R1) and provides a gate on macrophages that does not require antibody labelling (R2).

52.2% large cells, 30.0% granulated cells, 26.0% non-vital cells, 7.0% macrophages and 28.2% non-vital macrophages. In milk of cows with a favorable probability of BC on average 50.1% large cells, 24.5% granulated cells, 21.8% non-vital cells, 4.9% macrophages and 27.5% non-vital macrophages were identified.

The rescaled R^2 of the final model was 0.289. The goodness of fit statistic did not give any reason to doubt the validity of the model ($P < 0.05$). Significant risk factors for a poor probability of BC identified by the final logistic regression model were non-vital cells, macrophages and granulated cells (table 3). With increasing proportions of granulated, non-vital cells and macrophages, the animal-related probability of BC decreases (figure 6).

Animals tended to belong to the group with a poor animal-related probability of BC if their milk contained more granulated cells, non-vital cells and macrophages. With the final model containing these

three variables, 84.4% of all animals could be allocated to their score-based animal-related probability of BC. Cows with more than 25.5% granulated cells in the milk can be identified to have a poor probability of BC with a sensitivity and specificity of 61.2% and 60.8%, respectively (figure 7). Using a threshold of 22% of non-vital cells, cows can be identified to have low chances of being cured with a sensitivity and specificity of 57.8% and 58.2%, respectively (figure 8). Concerning the macrophages, cows with milk with more than 4.5% macrophages can be classified to have a poor probability of BC with a sensitivity and specificity of 61.9% and 61.7%, respectively (figure 9).

Discussion

For the sake of sustainable milk production it would be of great advantage to know about the animal-related probability of BC without knowing the relevant pathogen before antibiotics are ap-

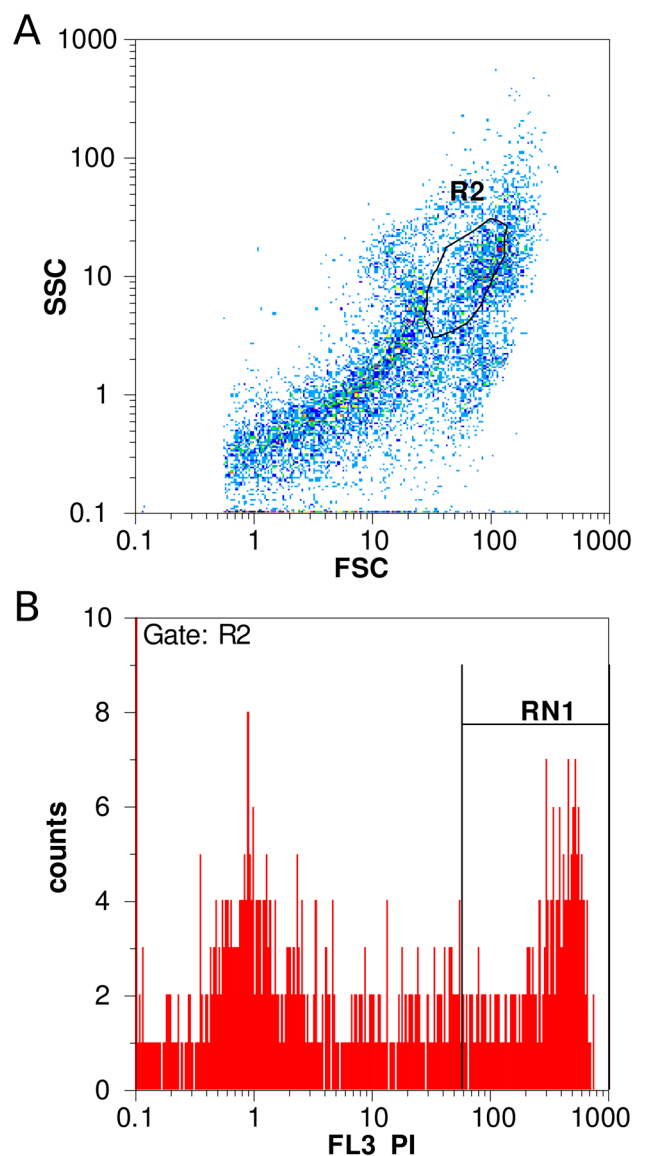


Figure 5. Flow cytometric analysis of milk macrophages: Forward scatter (FSC) versus side scatter (SSC) showing size and granularity of leukocytes (A). The gate of antibody-labelled macrophages (R2) shows the region where macrophages are to be expected. A Histogram histogram (B) demonstrates propidium iodide (FL3 PI) positivity of events in R2. Non-vital cells (RN1) show high propidium iodide intensities.

Table 2. Mean proportions of cells with certain characteristics in milk of cows with favorable and poor animal-related probabilities of bacteriological cure in 874 composite milk samples.

Variable (mean ± SE ¹)	Animal-related probability of bacteriological cure	
	Favorable (n = 728)	Poor (n = 146)
Large cells	50.1 ± 15.6	52.2 ± 16.8
Granulated cells	24.5 ± 12.5 ^a	30.0 ± 13.6 ^b
Non-vital cells	21.8 ± 10.2 ^c	26.0 ± 12.3 ^d
MAK ²	4.9 ± 4.7 ^c	7.0 ± 4.8 ^d
Non-vital MAK ³	27.5 ± 13.1	28.2 ± 12.4

^{a-b} Means within a row with different superscripts differ (P < 0.05)

^{c-d} Means within a row with different superscripts differ (P < 0.01)

¹ SE = standard error

² MAK = macrophages

³ % of all MAK

plied. There is evidence that immune cells in milk not only provide information about presence or absence of a disease but also about state, process and prognosis of mastitis [14,16,19,22–24]. However, to our knowledge, no applicable method has been described that is able to define the animal-related probability of BC. The aim of this study was to find a parameter that is simple to determine and implicates a correlation to the animal-related part of BC probability.

As reference, a simple literature based score system was designed. It includes the most important parameters weighted accordingly to their influence on the animal-related probability of BC. Age, mastitis history, current SCC and SCC history were taken into account. The score of each cow was determined in order to compare the animal-related probability of BC to the results of cell differentiation. DHI milk samples were analyzed for large and granulated cells, macrophages and their vitality. The results were compared to a literature-based score system estimating the probability of BC of each cow. It was found that the amount of granulated, non-vital cells and macrophages increases with a decreasing probability of BC. The results support assumptions acquired from literature.

Macrophages are predominantly present in late infection because they are involved in the resolution process of intramammary infections [25]. Chronic mastitis usually results from an inability of the immune system in defeating the infection successfully and the healing process is impaired. Therefore, the percentage of macrophages increases in long lasting or even chronic infections [7–9]. Given the fact that longer lasting infections and chronic mastitis lower the probability of BC significantly [10–13], an association between the percentage of macrophages and the probability of BC was suspected. The results of this study show that in milk of cows with a low animal-related probability of BC the proportions of macrophages were higher than in the milk of cows with higher probabilities of BC. However, in this stu-

dy only milk samples with SCC > 200,000 cells/mL were examined. Some studies have shown that macrophages are predominant in the milk of healthy udders as well [18]. This leads to the assumption that the findings of this study are not applicable to low SCC milk. Nevertheless, the probability of BC only needs to be determined if the SCC of a cow indicates mastitis by exceeding 200,000 cells/mL [36] and there is no indication to apply this method to milk of healthy cows. The intention of this study was to identify animals with poor probabilities of BC under all cows that are chronically diseased and show long term high SCC in order to discourage application of antimicrobials.

The higher percentage of granulated cells in the milk of cows with lower chances of BC can be explained by the fact that macrophages contain high numbers of embedded particles after they have phagocytized bacteria or apoptotic cells [37]. This results in an increase of granulated cells when the percentage of macrophages increases. Next to macrophages, PMNL also show high granularity, as they perform phagocytosis as well [15]. Therefore, an increase of highly granulated cells could also be the consequence of an increase of PMNL. In early inflammation, when bacteria have entered the udder, the first reaction is an increase of PMNL percentage [38], but their presence does not necessarily indicate a low probability of BC. But a persistent accumulation of PMNL is also associated to chronic mastitis [39]. Thus, the combination of higher percentages of PMNL and macrophages together might be a sign of a mastitis that is less likely to be cured. At the same time, high amounts of bacteria trigger an accumulation of PMNL and a long lasting infection leads to high amounts of macrophages in the milk. This kind of mastitis is characterized by a low probability of BC [2,12]. An increase of PMNL and macrophage percentages would explain the increase of granulated cells in milk of cows with low animal-related probabilities of BC.

The impact of cell viability has been investigated in several studies,

Table 3. Final logistic regression model for the animal-related probability of bacteriological cure including non-vital cells, macrophages and granulated cells.

Variable	Coefficient		OR ¹	95% CI ²	P-value
	X	SE ³			
Non-vital cells	-0.026	0.009	0.974	0.957-0.991	0.003
MAK	-0.065	0.021	0.937	0.899-0.976	0.002
Granulated cells	-0.018	0.008	0.982	0.966-0.998	0.028

¹ OR = odds ratio

³ SE = standard error

² CI = confidence interval

⁴ MAK = macrophages

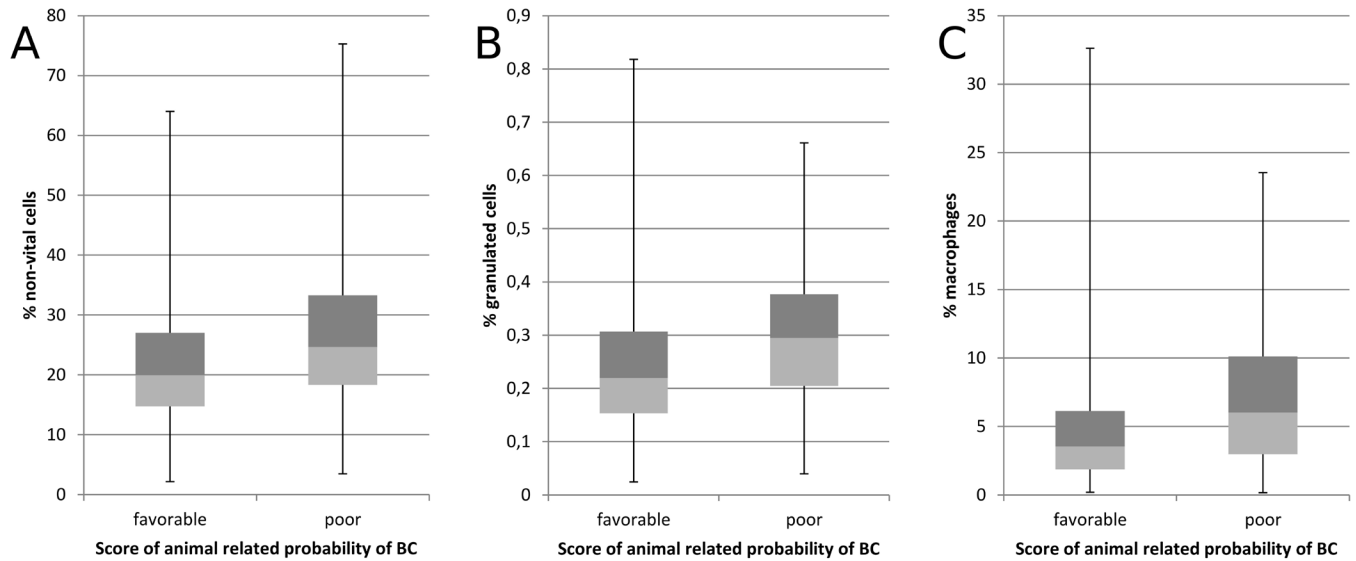


Figure 6. Boxplots of the proportions of non-vital (A), granulated cells (B) and macrophages (C) in the milk of cows with favorable and poor animal-related probabilities of bacteriological cure (BC) (bottom box: 1st quartile; top box: 3rd quartile; central line: median; bottom/top of whiskers: minimum/maximum).

whereas most of them dealt with PMNL which are the predominant cell type in early clinical mastitis [38]. It has been shown, that the amount of non-vital PMNL is associated with the course of infection (a peak occurs 48 h post-infection) [40,41] and lactation stage (significantly higher percentages of non-vital PMNL in early lactation) [20,42]. Non-vital macrophages have been found to increase after ingesting necrotic PMNL and to indicate the resolution of inflammation [25]. Little is known about the impact for the outcome of mastitis in cases of high percentages of non-vital cells, but in case of apoptotic PMNL, it has been suggested, that an adequate immune response is impeded [20,27]. Two possible reasons could be the following. Firstly, the phagocytic capacity of non-vital cells in the milk is decreased and secondly, they might compete with microorganisms for phagocytosis by macrophages [20]. One study found that a higher viability of PMNL before infection leads to a more successful removal of bacteria and a faster recovery of clinical *E. coli* mastitis [27]. An association with the animal-related probability of BC has also been proven in the present study. The higher the percentage of non-vital cells, the less likely it is for affected cows to cure.

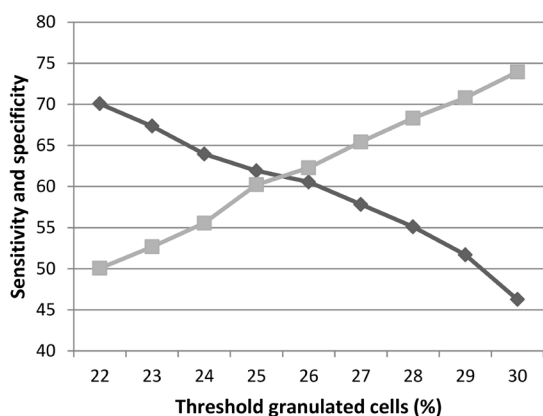


Figure 7. Sensitivity (dark grey) and specificity (light grey) for evaluating the animal-related probability of bacteriological cure at different thresholds of granulated cells.

Conclusion

The results of this study prove that animal-related probability of cure is associated with results from differential cell count analyses. An as-

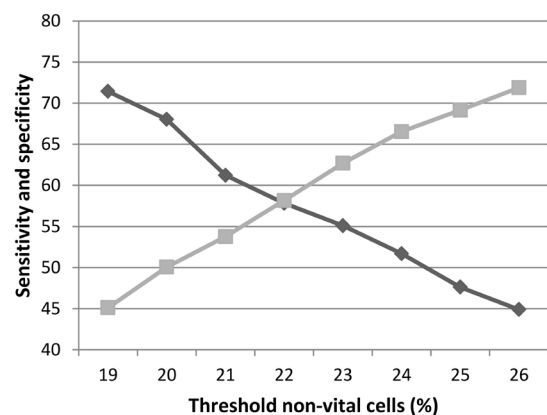


Figure 8. Sensitivity (dark grey) and specificity (light grey) for evaluating the animal-related probability of bacteriological cure at different thresholds of non-vital cells.

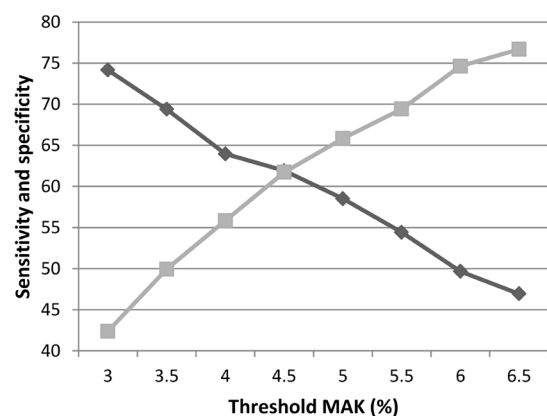


Figure 9. Sensitivity (dark grey) and specificity (light grey) for evaluating the animal-related probability of bacteriological cure at different thresholds of macrophages (MAK).

sociation between the percentages of granulated, non-vital cells and macrophages and the probability of BC could be shown. However, it needs to be validated, if these cells provide variables that are supplementary to animal-related factors such as age, mastitis history and SCC for a model providing solid prognosis for the outcome of BC. The association to the real probability of BC needs to be proven by additional studies implementing culture analyses. This method included in routine diagnostics such as DHI could prevent unnecessary usage of antibiotics and thereby improve sustainability and efficiency of milk production.

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Conflict of interest

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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