Changes in milk L-lactate, lactate dehydrogenase, serum albumin, and IgG during milk ejection and their association with somatic cell count

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Abstract: In both conventional and automatic milking systems (AMS), sensitive and reliable mastitis detection is important for profitable milk production. Mastitis detection parameters must be able to detect mastitis when the somatic cell count (SCC) is only slightly elevated. Owing to the pre-milking teat cleaning process in AMS, sampling cannot take place before the occurrence of alveolar milk ejection and importantly, this can affect the ability of parameters to detect mastitis. The aim of the present study was to examine the effect of alveolar milk ejection on l-lactate, lactate dehydrogenase (LDH), serum albumin (SA) and immunoglobulin G (IgG) compared with SCC, a commonly used indicator of mastitis. In this experiment, milk samples were collected every 20 s from one quarter during a 120-s manual teat stimulation in ten cows. Samples were analysed for SCC, l-lactate, LDH, SA and IgG. Quarters were grouped by low ($<5 \cdot 0$ log10 cells/ml), mid ($5 \cdot 0$–$5 \cdot 7$ log10 cells/ml), and high ($>5 \cdot 7$ log10 cells/ml) SCC using the sample at t=0 s. Neither l-lactate nor LDH could statistically differentiate between low and mid-SCC quarters, but there were a significant difference in levels between the high-SCC quarters and low and mid-SCC quarters. SA could not differentiate between the low and mid-SCC quarters, but the SA levels for the high SCC quarters remained statistically different compared with low and mid-SCC quarters throughout the experiment. IgG could statistically differentiate between low and mid-SCC, although the high-SCC quarters were not statistically different from the mid-SCC quarters after 60 s. In the high-SCC quarters, a decrease was shown in all parameters during milk ejection, after t=60 s. In conclusion, alveolar milk ejection reduces the effectiveness of detection parameters when compared with SCC. With the exception of IgG, the ability of other tested parameters was not satisfactory to differentiate between quarters with low to mid-SCC levels.

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Changes in milk $_L$-lactate, lactate dehydrogenase, serum albumin, and IgG during milk ejection and their association with somatic cell count

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SUMMARY

In both conventional and automatic milking systems (AMS), sensitive and reliable mastitis detection is important for profitable milk production. Mastitis detection parameters must be able to detect mastitis when the somatic cell count (SCC) is only slightly elevated. Due to the pre-milking teat cleaning process in AMS, sampling cannot take place before the occurrence of alveolar milk ejection and importantly, this can affect the ability of parameters to detect mastitis. The aim of the present study was to examine the effect of alveolar milk ejection on $_{L}$-lactate, lactate dehydrogenase (LDH), serum albumin (SA), and immunoglobulin G (IgG) compared to SCC, a commonly used indicator of mastitis. In this experiment, milk samples were collected every 20s from one quarter during a 120s manual teat stimulation in ten cows. Samples were analyzed for SCC, $_{L}$-lactate, LDH, SA, and IgG. Quarters were grouped by low (<5.0 log10 cells/mL), mid (5.0-5.7 log10 cells/mL), and high (>5.7 log10 cells/mL) SCC using the sample at t=0s. Neither $_{L}$-lactate nor LDH could statistically differentiate between low and mid SCC quarters, but there were a significant difference in levels between the high SCC quarters and low and mid SCC quarters. SA could not differentiate between the low and mid SCC quarters, but the SA levels for the high SCC quarters remained statistically different compared to low and mid SCC quarters throughout the experiment. IgG could statistically differentiate between low and mid SCC, although the high SCC quarters were not statistically different from the mid SCC quarters after 60s. In the high SCC quarters, a decrease was shown in all parameters during milk ejection, after t=60s.

In conclusion, alveolar milk ejection reduces the effectiveness of detection parameters when compared to SCC. With the exception of IgG, the ability of other tested parameters was not satisfactory to differentiate between quarters with low to mid SCC levels.

Key words: mastitis indicator, $_{L}$-lactate, LDH, SA, IgG, alveolar milk ejection

Early detection of mastitis is essential to allow for separation of milk from infected quarters, to improve the cure rate of mastitis treatment, and to minimize economic losses (Milner *et al*., 1997). In conventional milking systems, clinical mastitis cases can be detected by the milker via milk clots in foremilk, signs of udder inflammation, and increased somatic cell count (SCC). Cows with subclinical mastitis can exhibit an elevated SCC, but may not be detected by the milker due to lack of clinical symptoms mentioned above. Nonetheless, both types of mastitis can be detected by the California Mastitis Test (Schalm & Noorlander, 1957). In conventional systems, milkers can also obtain strict foremilk samples (i.e. before alveolar milk ejection), but in automatic milking systems (AMS) this is not possible due to teat cleaning and cup placement. The effects of alveolar milk ejection are particularly important in evaluating mastitis indicators in AMS, because udder health is controlled without interference by the milker. Parameters must be reliable enough to detect not only clinical mastitis where there is a substantial increase in SCC, but also subclinical mastitis when SCC is slightly elevated. It is, therefore, important to test parameters against varying SCC levels to examine if these detection parameters can be useful in AMS. Currently, automatic measurement of electrical conductivity, milk color, milk temperature, milk yield, or a multi-variate approach of several parameters are often used for monitoring udder health in AMS (Hovinen & Pyörälä, 2010). However, the use of some of these parameters including electrical conductivity and milk color are insufficient, (Biggadike *et al*., 2002, Hovinen *et al*., 2006) and various other novel parameters have been tested.

The concentration of a number of milk constituents differs between milk fractions. In particular, milk composition in dairy cows differs between cisternal and alveolar milk (Ontsouka *et al*., 2003; Bruckmaier *et al*., 2004). The cisternal milk is immediately available for milk removal, whereas the alveolar milk fraction is only available in response to the release of oxytocin, i.e. after the occurrence of alveolar milk ejection (Bruckmaier & Blum, 1998). The time between the first contact with the udder and the start of milk ejection is

reported to be 50–100s as a function of the degree of udder filling (Bruckmaier & Hilger, 2001). The decline of physicochemical mastitis indicators such as electrical conductivity and milk electrolytes during alveolar milk ejection, concomitantly with a reduced difference of these parameters and loss of statistical significance between groups has been shown before (Bruckmaier *et al*., 2004). This is also true for SCC to a lesser extent (Sarikaya & Bruckmaier, 2006) likely because an elevation of SCC follows more of an exponential than a linear curve. Therefore, an elevation of SCC still remains visible even if diluted with milk that has low cell content.

Only a few reports are available on the potential mastitis indicator $_{L}$-lactate (hereafter referred to as lactate) in the milk of dairy cows. Lactate is the conjugate base of lactic acid and its concentration in raw milk is approximately 0.1 mmol/L (Morr *et al*., 1957). Davis *et al*. (2004) found an increase in milk lactate concentration in the foremilk of dairy cows during mastitis and concluded that milk lactate can serve as an indicator of clinical and subclinical mastitis.

Lactate dehydrogenase (LDH) has repeatedly been discussed as another indicator of mastitis (Chagunda *et al*., 2006; Hiss *et al*., 2007). The origin of LDH in milk is often attributed to leukocytes (Kato *et al*., 1989) and mammary epithelial cells (Bogin *et al*., 1977). During mastitis, the blood milk barrier is compromised and becomes leaky. This allows for paracellular transport of blood components into the milk and vice versa. Symons & Wright (1974) discussed that LDH originates from the blood and is an indicator of increasing permeability of the blood milk barrier. This hypothesis was confirmed in our recent study (Lehmann *et al*., 2013).

Two other components that can be used as indicators of blood milk barrier permeability and mastitis are serum albumin (SA) and immunoglobulin G (IgG). SA is a ubiquitous blood constituent that increases in milk when the blood milk barrier is compromised (Stelwagen *et al*., 1994). During an infection, IgG2 is the most important immunoglobulin in bovine milk

and it plays an important role in udder defense against mastitis (Burton and Erksine, 2003). During an immune response to mastitis, there is an increase in IgG in the milk due to passive transfer through the leaky blood milk barrier.

The objective of the present study was to evaluate milk lactate, LDH activity, SA, and IgG concentrations compared to low, mid, and high SCC levels. Special emphasis was put on the influence of alveolar milk ejection on these parameters for the possible application in AMS, since teat cleaning in AMS induces milk ejection before foremilk can be sampled (Dzidic *et al*., 2004*a*; Dzidic *et al*., 2004*b*).

MATERIALS AND METHODS

Animals and milking management

Ten Holstein cows with a daily milk yield of 32.3 ± 1.9 kg were used in this experiment. They were kept in free stall housing and were milked in a milking parlor at 05.00 and 16.00. Cows were 65-199 days in milk and in their first to sixth lactation.

Experimental design

Repeated milk sampling was carried out during pre-stimulation in ten cows. The manual stimulation time was 120s and included all teats. Milk samples (~6 mL) were collected from the start until the end of stimulation from one single quarter at 20s intervals, while the other quarters were continuously stimulated. Therefore, seven consecutive milk samples were taken. The udder was not touched before the start of sampling and stimulation. The procedure was repeated at four milkings per cow and every quarter was sampled only once. Four quarters were excluded from the experiment, thus 36 quarters were used.

Sample analysis

Milk SCC was determined immediately after sampling by using a DeLaval cell counter, and

125 samples were frozen at -20°C for further analyses. Lactate concentrations were measured
126 enzymatically in raw milk using a prototype of Lact-Sens-PoC (Foerster-Technik GmbH,
127 Gerwigstrasse 25, D-78234, Engen, Germany). The coefficient of variation of this method
128 was 6.2% and the minimum detectable concentration was 0.01 mmol/L. To validate the
129 measuring technique, lactate values in milk, measured by Lact-Sens-PoC, were compared
130 with lactate values measured in milk serum (milk serum was isolated by a two-step
131 centrifugation procedure at 4,000 x *g* for 15 min and at14,000 x *g* for 30 min) using the test
132 kit Lactate PAP (bioMérieux, Marcy l`Etoile, F-69280, France) and an automated analyzer
133 (COBAS MIRA, Roche Diagnostics, Grenzacherstrasse 124, CH-4070, Basel, Switzerland).
134 LDH activity was measured in milk serum (prepared as described above) using the test kit
135 LDH IFCC (Axon Lab AG, Täferstrasse 15, CH-5405, Baden, Switzerland) and an automated
136 analyzer (COBAS MIRA, Roche Diagnostics, Grenzacherstrasse 124, CH-4070, Basel,
137 Switzerland) according to manufacturer's instructions. Minimum detectable activity was 5
138 U/L.
139 Serum albumin and total IgG were measured in milk serum by ELISA using a commercial kit
140 (Bethyl Laboratories, Montgomery, TX, USA) according to the manufacturer's instructions.
141 A slight modification of the protocol was used for IgG as samples were blocked in 5% fish
142 skin gelatin (Sigma-Aldrich, St. Louis, MO, USA) diluted in double distilled water. Samples
143 were diluted in wash buffer (50mM Tris, 0.14M NaCl, 0.05% Tween 20, adjusted to pH 8.0)
144 to ensure the samples were in range of the standards. Absorbance measurements were read on
145 the Synergy Mx plate reader (Bio Tec Instruments, Winooski, VT, USA). The inter and intra
146 assay coefficients of variation were ~4 and ~5% for SA and ~3 and ~8% for IgG. The
147 minimum detectable concentration was 6.25 ng/ml for SA and 9.375 ng/ml for IgG.
148
149 *Statistical analysis*
150 Results are presented as means±SEM. Somatic cell count is presented and statistically

evaluated at a logarithmic scale (log_{10}) to ensure normal distribution. Results were tested for significance ($P<0.05$) using the repeated measures analysis of the MIXED procedure of SAS (SAS Institute Inc., 2002-2008, Release 9.2 Cary, NC, USA). For this experiment, quarters were classified based on the SCC of the first sample obtained at t=0s. Three groups were used: SCC < 5.0 log_{10} cells/ml (SCC I), 5.0 – 5.7 log_{10} cells/ml (SCC II) and > 5.7 log_{10} cells/ml (SCC III). The model included the SCC group and the time of sampling as fixed effects and the animal was considered as a repeated factor. Lactate, LDH, IgG, and SA were the dependent variables.

RESULTS

SCC

Results are presented as means±SEM. In the first sample (t=0s), mean SCC was 4.57 ± 0.07, 5.50 ± 0.05, and 6.08 ± 0.09 log_{10} cells/mL in SCC I (n=14 samples), SCC II (n=12 samples), and SCC III (n=10 samples), respectively. SCC decreased within 120s and was significantly lower at 80s, 100s, and 120s than at the start of sampling (t=0s) in SCC II quarters and at 100s and 120s in SCC III quarters. The SCC group differences between SCC I, SCC II, and SCC III quarters remained significant throughout the entire 120s sampling period (figure 1a).

Lactate

From 0 to 60s and from 0 to 80s, the lactate concentration in SCC III quarters was significantly higher than in SCC II and SCC I quarters, respectively. Lactate concentrations did not differ significantly between SCC I and SCC II quarters at any time-point. A decrease of lactate occurred between 40 and 100s of sampling in both the SCC II and SCC III quarters. In SCC III and SCC II quarters, lactate was significantly lower at t=120s than at t=0s. The difference of lactate concentrations between SCC III and SCC I quarters were no longer significant from t=100s, i.e., lactate concentrations were similar in SCC III, SCC II and SCC I

quarters 100s after the start of sampling (figure 1b).

LDH

Lactate dehydrogenase activity in SCC III quarters was significantly higher than in SCC II and SCC I quarters from 0 to 80s and from 0 to 100s, respectively. LDH activity did not differ significantly between SCC I and SCC II quarters during the entire sampling period. Between 60 and 100s a decrease of LDH activity in SCC II and SCC III quarters was detected. In SCC II quarters, LDH activity was significantly lower at 100 and 120s than at t=0s. The difference of LDH activity between SCC III and SCC I quarters was no longer significant from t=120s, i.e. 120s after the start of sampling LDH activity was similar in SCC III, SCC II and SCC I quarters (figure 1c).

Serum Albumin

Serum albumin concentration was significantly higher in the SCC III quarters than in the SCC I and SCC II quarters throughout the entire sampling period. The SA concentration was higher in the SCC II quarters compared to the SCC I quarters only at 20s and 40s. In SCC III quarters, SA concentration was significantly lower at 120s than t=0s. There were no significant decreases in SA concentration in the SCC I and SCC II quarters (figure 1d).

IgG

Total IgG concentration was significantly higher in SCC II quarters than SCC I quarters throughout the entire sampling. SCC III quarters were significantly higher than SCC I throughout the experiment, but only significantly higher than SCC II quarters from t=0s to t=60s. There was a significant decrease in IgG concentration at t=120s compared to t=0s in SCC III quarters. There were no significant decreases in IgG concentration in both the SCC I and SCC II quarters throughout the experiment (figure 1e).

DISCUSSION

The most widely accepted indicator of mammary inflammation is the SCC (Harmon, 1994), although recommended SCC thresholds are different among authors. According to Hillerton (1999), a SCC threshold of 100,000 cells/mL was used to define mastitis, i.e. a quarter was classified as healthy when the milk SCC was < 100,000 cells/mL, whereas an unhealthy quarter was defined when the milk SCC was > 100,000 cells/mL. In addition, two less rigid definitions of mastitis with SCC thresholds of 200,000 and 500,000 cells/mL have been considered (Smith, 1996; IDF, 1971). For adequate mastitis detection in AMS, mastitis indicators should be highly effective at SCC levels around 100,000 cells/mL. For the purpose of this study, samples were grouped into SCC I (SCC < 5.0 $\log_{10}$ cells/ml), SCC II (SCC 5.0 – 5.7 $\log_{10}$ cells/ml) and SCC III (SCC > 5.7 $\log_{10}$ cells/ml).

In the present study, IgG concentration could successfully differentiate between the SCC I and SCC II quarters and also between SCC II and SCC III until t=60s. The reason that this specific parameter can detect a slightly elevated SCC throughout the 120s sampling period is unknown, although it is important to note that IgG is the only studied parameter with a known immune function. Specifically, IgG2 is the major opsonization protein for neutrophil phagocytosis in the udder. As previously stated, during mastitis the blood milk barrier loses integrity and becomes open allowing IgG to enter the milk (Burton and Erksine, 2003). Since IgG can detect slightly elevated SCC before and after milk ejection which is essential for its use in AMS, it could be suitable for use as a mastitis indicator. More studies of this nature either with manual stimulation or sampling in AMS are needed to examine the use of IgG.

Lactate and LDH activity could not significantly differentiate between low SCC (SCC I) and slightly increased SCC (SCC II). This is indicative that neither lactate nor LDH would be suitable to identify mastitis with a slightly elevated SCC in conventional or AMS systems, even before alveolar milk ejection. Both of these parameters could identify the high SCC quarters (when SCC is ≥ 500,000 cells/ml), until t=100s for lactate and t=120s for LDH. It is

229 possible that these parameters could distinguish a quarter with substantially elevated SCC in
230 AMS.
231 Serum albumin is a ubiquitous blood protein that could be used as an indirect mastitis
232 indicator, although this parameter has not yet been studied in AMS. SA concentration could
233 not be used to distinguish between SCC I and SCC II (with the only statistically significant
234 differences at t=20 and 40s) after milk ejection, although it could differentiate high SCC
235 quarters at all time-points. This again demonstrates that this parameter could be used to detect
236 quarters with high SCC, but not in quarters that are only slightly elevated.
237 Somatic cell count, LDH activity, and concentrations of lactate, SA, and IgG decreased
238 during the sampling period but they did not change before 60s after the start of udder
239 stimulation. In a similar experiment by Bruckmaier *et al*. (2004), a decrease of the electrical
240 conductivity after 60s was shown when different parameters were measured. It is clear that
241 the continuous teat stimulation induced alveolar milk ejection after approximately 60s, and
242 caused a mixture of alveolar and cisternal milk. This fact needs to be considered in case of
243 foremilk sampling for measurements, i.e. foremilk can only be expected to be cisternal milk
244 until about 40s from the first contact with the udder (Bruckmaier & Hilger, 2001).
245 The reason for higher SCC, lactate, SA, IgG and LDH concentrations in cisternal milk
246 compared to alveolar milk is still not known. It is possible that the blood milk barrier is
247 regulated differently in the teat end compared to the rest of the mammary gland. Nickerson &
248 Pankey (1983) suggested that cells are recruited in the Furstenberg's rosette area to serve as
249 protection against invading pathogens. It is also possible that during milk let down, the
250 composition of milk changes while travelling through the milk ducts reducing the
251 concentration of SA, IgG, lactate, and LDH in alveolar milk.
252 In conclusion, IgG could detect a slightly elevated SCC both before and after alveolar milk
253 ejection whereas ability of lactate, LDH, and SA for detecting quarters with only a slightly
254 increased SCC was not satisfactory even in milk samples of the cisternal fraction. LDH

activity, and the concentration of lactate and SA are usable parameters in detecting only quarters with high SCC in strict foremilk samples, however in AMS it seems to be almost impossible to collect milk samples before the occurrence of alveolar milk ejection. More studies need to be conducted to examine the use of these parameters in AMS.

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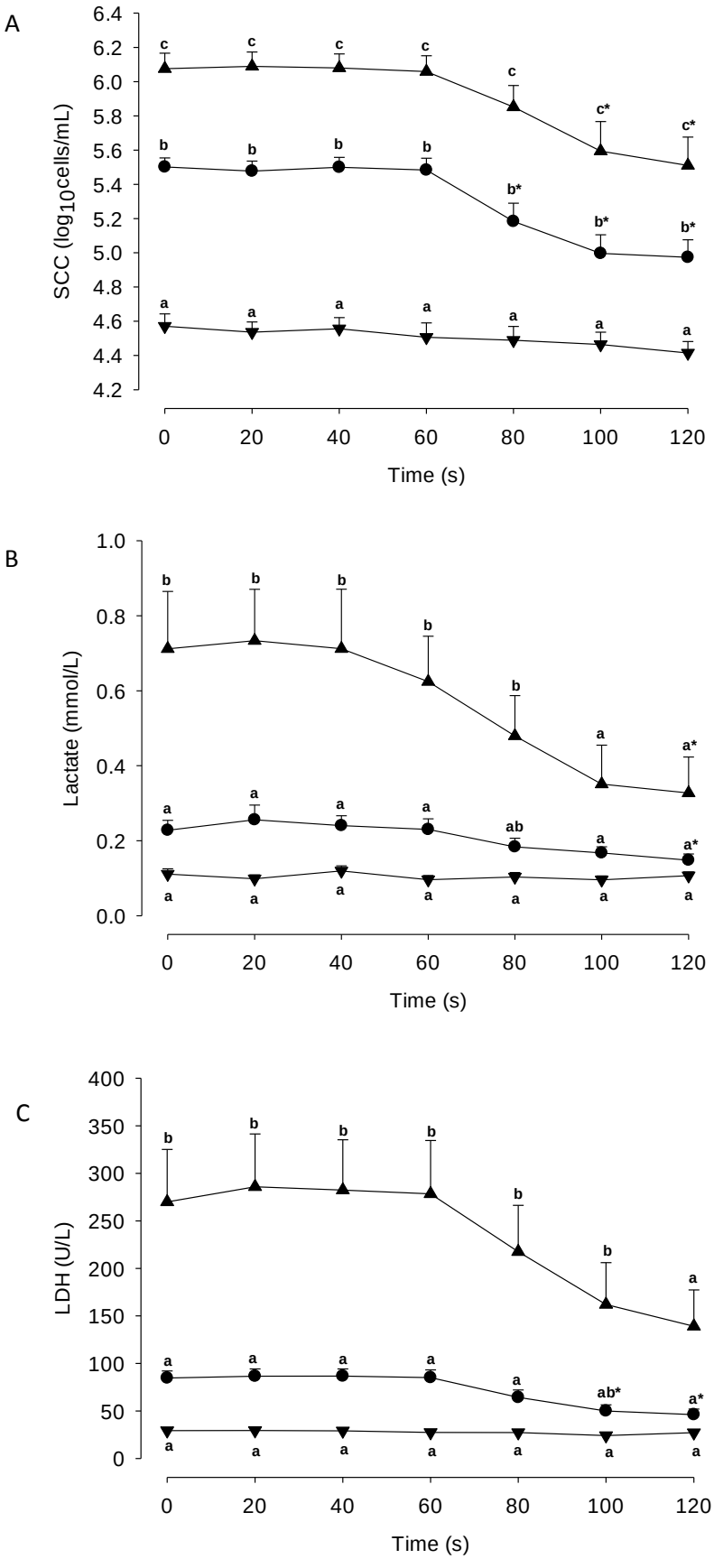
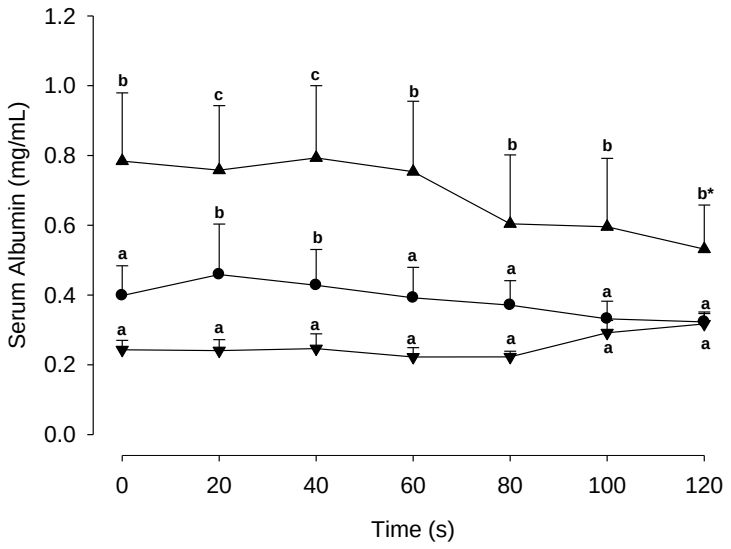
Figure legends

Figure 1 SCC (A), lactate (B), LDH (C), SA (D), and IgG (E) in the different milk fractions, 0 to 120s after the first touch of the udder and start of stimulation; results are presented as means±SEM; data are clustered according to the SCC of the first sample (t=0s), ▼ < 5.0 $\log_{10}$ cells/ml (SCC I), ● 5.0-5.7 $\log_{10}$ cells/ml (SCC II), ▲ > 5.7 $\log_{10}$ cells/ml (SCC III); n=10 cows; four milkings/cow; a,b,c: means with different letters indicate differences between clusters within time points (P<0.05*)*; * indicates a significant (P<0.05) difference compared to the sample t=0 s.

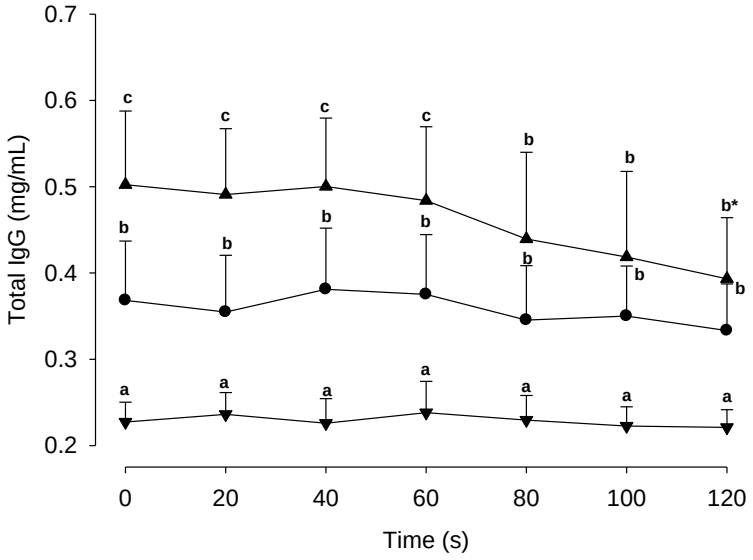
Figure 1


A
SCC (log10cells/mL)
Time (s)
B
Lactate (mmol/L)
Time (s)
C
LDH (U/L)
Time (s)

D


Serum Albumin (mg/mL)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
120
Time (s)

E


Total IgG (mg/mL)
0.7
0.6
0.5
0.4
0.3
0.2
0
20
40
60
80
100
120
Time (s)