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# L-lactate dehydrogenase and N-acetyl-β-D-glucosaminidase activities in bovine milk as indicators of non-specific mastitis

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Systematic factors affecting the activities of L-lactate dehydrogenase (LDH) and N-acetyl-β-D-glucosaminidase (NAGase) and somatic cell count (SCC), the association between the activities of LDH and NAGase and SCC with respect to udder health status, and the ability of LDH and NAGase to classify cows in udder health categories for early detection of mastitis were studied. A dataset of records from 74 Danish Holstein, 76 Danish Red and 47 Jersey cows on one research farm was used. Cows were grouped into healthy and clinically mastitic. A healthy cow was defined as having no veterinary treatment and SCC<100 000 cells/ml. A clinically infected cow was one receiving veterinary treatment after showing clinical signs of mastitis and SCC >800 000 cells/ml. Breed, month of production, and days in milk significantly influenced ($P<0{\cdot}001$) LDH activity, NAGase activity and SCC in both healthy and clinically mastitic cows. In healthy cows, LDH activity, NAGase activity and SCC started at a high level immediately after calving and decreased to low levels approximately 30–40 d *post partum*. All the three parameters increased due to clinical mastitis. NAGase activity had numerically higher variation in healthy cows than in clinically mastitic cows (CV=56·2% *v.* CV=53·5%). The relationship between LDH activity and SCC was stronger in milk from clinically mastitic than from healthy cows ($r=0{\cdot}76$ *v.* $r=0{\cdot}48$ and $r=0{\cdot}67$ *v.* $r=0{\cdot}44$ for correlation of observed values and residuals, respectively). LDH activity had higher sensitivity than NAGase activity (73–95% *v.* 35–77%) while specificities were in a similar range (92–99%). Further, sensitivities for LDH activity were more robust to changes in the threshold value than those for NAGase activity. Opportunities for automated, in-line real-time mastitis detection are discussed.

**Keywords:** Mastitis, L-lactate dehydrogenase, N-acetyl-β-D-glucosaminidase, somatic cell count.

Traditional indicators for detecting mastitis have some technical drawbacks that affect their efficiency in mastitis detection especially when applied as cow-side methods. For example, bacterial culture and visual inspection using the California Mastitis Test (CMT) do not allow accurate real-time detection (Hillerton, 2000). Further, CMT has limited ability to identify subclinical mastitis (Nielen et al. 1995; De Mol & Ouweltjes, 2001) unless under standardized conditions (Redetzky et al. 2005). Inherent lack of speed in bacteriological culturing and laboratory determination of SCC coupled with high cost per sample limit the use of these methods for early real-time detection (Labohm et al. 1998; Hillerton, 2000). On-farm detection of SCC is also costly (de Haas et al. 2002). In contrast, electrical conductivity is cheap but has poor sensitivity for detecting all types and degrees of mastitis even when used in combination with other data (Nielen et al. 1995; De Mol & Ouweltjes, 2001). Because of such disadvantages, the use of indigenous enzymes has previously been proposed as cow-side indicators of mastitis (Pyörälä & Pyörälä, 1997; Batavani et al. 2003). This has been enhanced by the development in recent years of techniques for automated sampling and measurement of components in milk (Delwiche et al. 2001; Pemberton et al. 2001; Godden et al. 2002) which makes milk a suitable medium for in-line measurements. Two of the indigenous enzymes proposed and used in mastitis diagnosis are N-acetyl-β-D-glucosaminidase (NAGase) (EC: 3.2.1.30) and L-lactate dehydrogenase (LDH) (EC: 1.1.1.27) (Jensen & Knudsen, 1991; Pyörälä & Pyörälä, 1997; Batavani et al. 2003). NAGase is a lysosomal enzyme released from damaged epithelial cells in the mammary gland, as well as from other somatic cells present in milk (Kitchen et al. 1978). Consequently, NAGase activity has been used as an indicator of the degree of udder inflammation in cows

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(Kitchen et al. 1978), ewes (Maisi et al. 1987) and goats (Timms & Schultz, 1985) and has been reported to be a most useful aid in discriminating between minor and major pathogen infections (Berning & Shook, 1992). LDH is an enzyme that is part of the glycolytic pathway, found in the cytoplasm of all cells and tissues in the body. This enzyme is known to consist of five isotypes, designated $LDH_1$ to $LDH_5$ (Larsen, 2005). LDH activity increases with mastitis (Bogin et al. 1977; Harmon, 1994). Although some work on quantification of the association among the activities of LDH; NAGase and their ability to classify udder health status exists (*e.g.* Sommer et al. 1986; Zank & Schlatterer, 1998) studies based on recordings done at every milking over several lactations in naturally occurring mastitis are sparse.

The objectives of the present work were: (1) to describe the systematic factors affecting the activities of LDH, and NAGase and somatic cell count (SCC) in milk; (2) to quantify the association between the activities of LDH, and NAGase and SCC with respect to udder health status; and (3) to determine the ability of LDH and NAGase activities to classify cows into udder health categories.

## Materials and Methods

### *Experimental animals and milk samples*

Data originated from cows housed at the Danish Cattle Research Centre in Foulum, Denmark. An initial dataset of 76 820 records from 74 Danish Holstein, 76 Danish Red and 47 Jersey cows in parities from one to four recorded in the period between 1 September 2003 and 30 April 2004 was used. Of these, 127 were in first parity, 63 were in second parity and 84 were in parities three and four. All cows were milked with an automatic milking system (three units, on average 2·3 ± 0·83 milkings/cow per day) in which the milk yield was automatically recorded. During each milking, a proportional sample of composite milk (all quarters combined) was collected automatically from each cow in 10-ml tubes. The sampling procedure, at cow level, reflects the prevailing commercial situation where a proportional sample is used in routine mastitis monitoring (Olson & Amick, 1985; IDF, 1997; Rasmussen, 1999). The tubes contained a concentrated solution of Bronopol (2-bromo-2-nitro-1,3 propanediol) to reach 200 ppm (w/v) in the filled tube. The automatic milk sampling system was emptied in the morning and in the afternoon. Milk samples were kept at 4 °C until laboratory analysis, which was done within 24 h of sampling.

### *Milk analysis*

Samples were distributed from the 10-ml tubes to 96-well plates using a Biomek 2000© (Laboratory Automation Workstation, Beckman Coulter) and analysed for LDH and NAGase activity in a spectrophotometer/fluorometer (Fluostar©, BMG Labtechnologies). NAGase activity was determined by fluorometry using kinetic assessment. The assay was as described by Kitchen et al. (1978) and Schüttel (1999). The accuracies (relative bias) were 3·9% and 1·8% for low and high controls, respectively. Intra-assay precisions (CV) were 2·6% and 2·4% for the low and high controls, respectively, and inter-assay precisions were 11·2% and 4·6%, respectively. LDH activity was analysed by a fluorometric, kinetic method as described by Larsen (2005). The accuracies (relative bias) obtained in the present material were 2·7%and 4·9% for low and high controls, respectively. Intra-assay precision (CV) was 8·6% and 3·7% for low and high controls, respectively, and inter-assay precision was 15·8% and 10·4%, respectively. Units used in the assay for both enzymes are $\mu mol\ min^{-1}\ l^{-1}$ SCC was measured at a commercial laboratory (Sønderjysk Kontrolforening, 6500 Vojens, Denmark) using Fossomatic 5000™ automatic equipment (Foss Electric, Hillerød, Denmark).

### *Definition of udder health status*

Cows were subdivided into either healthy or clinically mastitic groups. A healthy cow was defined as having no clinical veterinary treatment. However, in order take care of any misdiagnoses, SCC was used in addition to the absence of veterinary treatments of mastitis. Hence, in the current analysis, a cow was considered healthy when she had no veterinary treatments of mastitis and low SCC (equal to or less than 100 000 cells/ml in composite milk (Hamann, 2001)) from the day of calving up to the point of first rise of SCC above 100 000 cells/ml. Regardless of any drop in SCC after the first rise above 100 000 cells/ml, the part of lactation after the first rise was defined as not healthy and hence was not included in the analysis of healthy data. To distinguish outliers from a genuine rise in SCC, the point of first rise above 100 000 cells/ml was determined as any point when SCC was >100 000 cells/ml in 9 consecutive milkings (approximately 3·9 d). This threshold is in accordance with Laevens et al. (1997), Ma et al. (2000) and Hamann (2005) who indicated that SCC for composite cow milk should not exceed 100 000 cells/ml for an udder with four healthy quarters. Clinical mastitis was identified by the farm staff, confirmed by a veterinarian, and based on clinical signs including udder inflammation or abnormal milk, with or without general clinical signs (International Dairy Federation (IDF), 1997). In addition, to avoid using data that might have been due to misdiagnosis, high SCC of $\geqslant$800 000 cells/ml was used to supplement the classification of mastitic cows. Hence, a cow was only defined as clinically mastitic if it had a veterinary treatment record and also had SCC $\geqslant$ 800 000 cells/ml. The threshold of 800 000 cells/ml was set in oder to ascertain that cows described as clinical mastitis were indeed clinically mastitic. This is in accordance with Hillerton (1999) who pointed out that there exists a grey area when cell count

may be 100 000–400 000 cells/ml and bacteria are not isolated. A veterinarian following the same protocol as consistently as possible performed all veterinary treatments. To be certain of no overlaps between the two groups, any cow with SCC between 100 000 and 800 000 cells/ml was not included in the analysis regardless of veterinary treatment. Data from the first 3 d after calving were excluded. The resulting dataset had 80, 33 and 46 cows in parity classes 1, 2 and 3 in the healthy category; and 37, 18 and 27 cows in parity classes 1, 2 and 3 in the clinically mastitic category. The dataset had detailed information on breed, cow identity, parity, days from calving, date of registration, milking date and disease records. $Log_{10}$ of SCC was used in the analysis. To ascertain that no data sampling error, originating from subdividing the dataset into two using the SCC cut-offs and clinical veterinary treatment, influenced the results, margin of error was calculated for both subgroups at 99% CI.

### *Statistical analysis*

*Systematic factors affecting the activities of LDH and NAGase and SCC.* Effects of systematic factors on LDH, NAGase activity and SCC were analysed using a univariate mixed model. Fixed factors included in the model were breed, parity, days in milk and month of production. Cows in any parity higher than three were combined with those in parity three. Milk yield at the individual milking was included as a covariable to account for differences in milk yield. Analysis was done using restricted maximum likelihood (REML) methodology implemented by the MIXED procedure of SAS version 8.2 (SAS Inst. Inc., 2001). The following statistical model was applied:

$$y_{ijklmn} = \mu + B_i + P_j + M_k + DIM_l + C_m(P)_j + \beta(MY)_{ijklmn} + \varepsilon_{ijklmn}$$

where, $y_{ijklmn}$ = observed value of the milk parameters LDH activity, NAGase activity and SCC within cow; $\mu$ = overall mean; $B_i$ = fixed effect of breed with {i = Danish Holstein, Danish Red, and Jersey}; $P_j$ = fixed effect of parity with {j = 1, 2, and 3 and above}; $M_k$ = fixed effect of month of production with {k = Sep, Oct, Nov, Dec, Jan, Feb, Mar, Apr}; $DIM_l$ = fixed effect of days in milk with {l = 1, 2, 3, …, 336}; $C_m(P)_j$ = random effect of cow m within parity j {m = 1, 2, 3, …, 127 or 63 or 84 for parities one, two and three and above, respectively} with $C_m(P)_j$ being $N(0, \sigma_c^2)$, $\beta(MY)_{ijklmn}$ = regression effect of milk yield at every milking (n) with the regression coefficient, $\beta$. $\varepsilon_{ijklmn}$ = random residual with $N(0, \sigma_e^2)$. Least square means for DIM were used to generate profiles for SCC and the activities of LDH and NAGase. For the clinically mastitic cows, the same statistical model was used. To generate profiles of SCC and the activities of LDH and NAGase around a case of clinical mastitis, days relative to registered clinical mastitis were included in the above model for clinically mastitic cows. The analysis and hence the plot for the profiles of SCC and the activities of LDH and NAGase for clinically mastitic cows were limited to −18 to 9 d around a registered clinical mastitis. The day of registered clinical mastitis was indicated as d = 0 within a given clinical mastitis period.

*Associations among the activities of LDH and NAGase and SCC.* Relationships among LDH, NAGase activities and SCC were quantified in two steps: firstly, through correlation of absolute values; and secondly, through correlation of LDH, NAGase activities and SCC after correcting for systematic effects influencing the variation of each one of the parameters. In the first step, Pearson correlation was used to determine the relationships among the observed values of LDH, NAGase activities and SCC. The intention of this step was to establish the type and direction of any relationship that exists among LDH, NAGase activities and SCC. The second step involved correlation of residues for LDH, NAGase activity and SCC after correcting for systematic effects as described in the previous section under statistical analysis. This was done to ensure that the calculated correlation was the direct correlation among the three parameters in question and not a reflection of the influence from the systematic factors.

*Ability of LDH and NAGase to classify cows in udder health categories.* The ability of LDH and NAGase activity to reflect udder health status was expressed as sensitivity and specificity. Cases where either LDH or NAGase activities predicted a mastitis that coincided with a defined clinical mastitis (defined from SCC values plus veterinary treatment) were characterized as true positives (TP) and cases where LDH and NAGase failed to predict a defined clinical mastitis were considered false negatives (FN). True negatives (TN) represented occasions where no mastitis was predicted and the cows were healthy. Cases where healthy cows were classified as infected based on either LDH or NAGase measurements were defined as false positives (FP). Sensitivity is the percentage of infected cows that were classified as infected $((TP/(TP+FN)) \times 100)$ and specificity is the percentage of uninfected cows that were correctly classified as healthy $((TN/(FP+TN)) \times 100)$. Threshold values for the determination of sensitivity and specificity were determined from percentiles of 60–90% of LDH and NAGase activity of the clinically mastitic subgroup. Threshold values were 4·3, 5·7,6·0 and 6·5 $\mu mol\ min^{-1}\ l^{-1}$for LDH activity, 77·3, 92·8, 105·1 and 117·4 $\mu mol\ min^{-1}\ l^{-1}$ for NAGase activity.

## Results

### *Factors affecting the activities of LDH and NAGase*

Descriptive statistics for LDH, NAGase and SCC in composite milk samples at cow level for healthy and clinical

**Table 1.** Distribution of L-lactate dehydrogenase (LDH), N-acetyl-β-D-glucosaminidase (NAGase) activities (μmol $min^{-1}$ $l^{-1}$ and somatic cell count ($Log_{10}$SCC) (means, SD and CV) in composite milk for healthy and clinically mastitic cows

| | Healthy | | | | Clinically Mastitic | | | |
|---|---|---|---|---|---|---|---|---|
| Variable | *n* | Mean | SD | CV% | *n* | Mean | SD | CV% |
| LDH | 11 110 | 2·52 | 1·24 | 49·2 | 26 660 | 4·07 | 2·37 | 58·2 |
| NAGase | 11 799 | 39·05 | 21·93 | 56·2 | 28 508 | 69·42 | 37·17 | 53·5 |
| $Log_{10}$SCC | 24 563 | 4·65 | 0·22 | 4·73 | 62 630 | 5·44 | 0·62 | 11·4 |

*n*, number of records

**Table 2.** Estimates (least square means) and SEM for the effects of breed, parity and month of production on lactate dehydrogenase (LDH), N-acetyl-β-D-glucosaminidase (NAGase) activities (μmol $min^{-1}$ $l^{-1}$) and somatic cell count ($log_{10}$SCC) for healthy cows

| | | LDH | | NAGase | | SCC | |
|---|---|---|---|---|---|---|---|
| | | lsmean | SEM | lsmean | SEM | lsmean | SEM |
| Breed[a,b] | Danish Holstein | 2·62 | 0·03 | 38·5 | 1·78 | 4·73 | 0·02 |
| | Danish Red | 2·26 | 0·03 | 34·2 | 1·80 | 4·74 | 0·02 |
| | Jersey | 2·88 | 0·12 | 53·0 | 2·22 | 4·67 | 0·03 |
| Parity | 1 | 2·54 | 0·08 | 39·6 | 1·51 | 4·71 | 0·02 |
| | 2 | 2·62 | 0·12 | 45·1 | 2·32 | 4·72 | 0·03 |
| | 3 and more | 2·59 | 0·11 | 41·2 | 2·08 | 4·71 | 0·02 |
| Month of production[a,b,c] | Sep | 2·09 | 0·08 | 42·9 | 1·55 | 4·68 | 0·02 |
| | Oct | 1·97 | 0·08 | 40·5 | 1·49 | 4·67 | 0·02 |
| | Nov | 2·03 | 0·08 | 41·1 | 1·41 | 4·71 | 0·02 |
| | Dec | 2·47 | 0·08 | 41·9 | 1·39 | 4·71 | 0·02 |
| | Jan | 3·86 | 0·07 | 42·4 | 1·27 | 4·72 | 0·01 |
| | Feb | 2·91 | 0·06 | 45·8 | 1·22 | 4·74 | 0·01 |
| | Mar | 2·53 | 0·06 | 43·1 | 1·18 | 4·74 | 0·01 |
| | Apr | 2·83 | 0·06 | 37·6 | 1·19 | 4·73 | 0·01 |

[a] Factor had significant effect on LDH ($P<0·001$)
[b] Factor had significant effect on NAGase ($P<0·001$)
[c] Factor had significant effect on SCC ($P<0·001$)

mastitis cases are presented in Table 1. Margins of error at a 99% CI for the two subgroups were in the same range (0·09 for healthy cows and 0·14 for clinically mastitic cows) indicating no data sampling error originating from subdividing the dataset using the SCC cut-offs and clinical veterinary treatment. Healthy cows had lower averages for all parameters tested, LDH and NAGase activities and SCC, than clinically mastitic cows. Variation in LDH activity was numerically lower in healthy cows than in clinically mastitic cows (CV, 49·2% *v.* 58·2%). NAGase activity had slighlty higher variation in healthy cows than in clinically mastitic cows (CV, 56·2% *v.* 53·5%,). For SCC, variation in healthy cows was lower in healthy cows than in clinically mastitic cows (CV, 4·7% *v.* 11·4%).

Effects of systematic factors on LDH activity, NAGase activity and SCC in healthy cows are presented in Table 2. Jersey cows had a 38% and 55% ($P<0·001$) higher NAGase activity than Danish Holsteins and Danish Reds, respectively. In contrast, the same Jersey cows had no significantly different SCC to Danish Holsteins and Danish Reds. The activities of LDH, NAGase and the values of SCC were virtually the same in the three parity groups in healthy cows. Month of production also had a significant effect ($P<0·001$) on LDH activity, NAGase activity and SCC. However, no specific trend could be noted in the three studied parameters during the study period, which was between September 2003 and April 2004.

In clinically mastitic cows (Table 3) Danish Holsteins had 22%and 28% higher ($P<0·05$) LDH activity than Danish Reds and Jerseys, respectively. Parity affected LDH activity, NAGase activity and SCC significantly. Similarly to healthy cows, clinically mastitic cows had the highest LDH activity, NAGase activity and SCC in parity three. From parity one to parity three, LDH activity increased by 51% while NAGase activity increased by 38%. All three parameters were significantly affected ($P<0·001$) by month of production. However, no specific trend could be observed due to month of production.

### *Profiles of indicators of mastitis*

Profiles of NAGase, LDH and SCC from three typical cows are presented in Figs 1, 2 and 3. In all three cases SCC,

**Table 3.** Estimates (least square means) and SEM for the effects of breed, parity, and month of production on lactate dehydrogenase N-acetyl-β-D-glucosaminidase (NAGase) activities (μmol $min^{-1}$ $l^{-1}$) and somatic cell count ($log_{10}$SCC) for clinically mastitic cows

| | | LDH | | NAGase | | SCC | |
|---|---|---|---|---|---|---|---|
| | | lsmean | SEM | lsmean | SEM | lsmean | SEM |
| Breed[e,f] | Danish Holstein | 4·75 | 0·26 | 79·3 | 5·15 | 5·51 | 0·06 |
| | Danish Red | 3·88 | 0·26 | 60·9 | 5·19 | 5·45 | 0·06 |
| | Jersey | 3·71 | 0·25 | 78·9 | 6·69 | 5·27 | 0·08 |
| Parity[a,g,d] | 1 | 3·20 | 0·26 | 59·6 | 4·80 | 5·21 | 0·06 |
| | 2 | 4·30 | 0·34 | 77·3 | 6·59 | 5·47 | 0·08 |
| | 3 and more | 4·84 | 0·30 | 82·2 | 5·81 | 5·55 | 0·07 |
| Month of production[a,b,c] | Sep | 4·07 | 0·25 | 90·4 | 4·25 | 5·45 | 0·06 |
| | Oct | 3·35 | 0·28 | 79·8 | 4·47 | 5·41 | 0·06 |
| | Nov | 2·92 | 0·27 | 72·8 | 4·47 | 5·37 | 0·06 |
| | Dec | 3·46 | 0·35 | 69·7 | 5·29 | 5·39 | 0·07 |
| | Jan | 5·95 | 0·27 | 69·4 | 4·65 | 5·45 | 0·06 |
| | Feb | 5·42 | 0·30 | 64·4 | 4·89 | 5·62 | 0·07 |
| | Mar | 3·96 | 0·31 | 71·4 | 5·20 | 5·47 | 0·07 |
| | Apr | 3·80 | 0·52 | 54·2 | 7·49 | 5·10 | 0·11 |

[a]Factor had significant effect on LDH ($P<0·001$)
[b]Factor had significant effect on NAGase ($P<0·001$)
[c]Factor had significant effect on SCC ($P<0·001$)
[d]Factor had significant effect on SCC ($P<0·005$)
[e]Factor had significant effect on LDH ($P<0·05$)
[f]Factor had significant effect on NAGase ($P<0·05$)
[g]Factor had significant effect on NAGase ($P<0·01$)

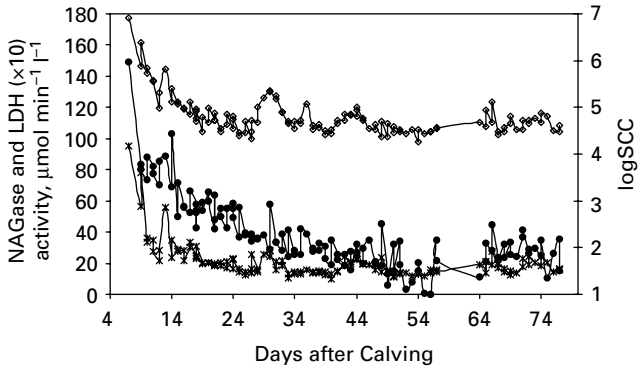


**Fig. 1.** An example of profiles for Lactate dehydrogenase (LDH) (solid line with asterisk and scaled up 10 times), N-acetyl-β-D-glucosaminidase (NAGase) activities (μmol $min^{-1}$ $l^{-1}$) (solid line with solid dots) and $log_{10}$SCC (solid line with open diamonds) for a cow that had no record of clinical mastitis.

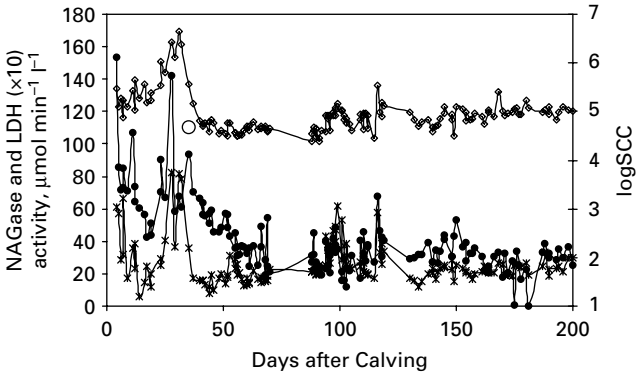


**Fig. 2.** An example of profiles for Lactate dehydrogenase (LDH) (solid line with asterisk and scaled up 10 times), N-acetyl-β-D-glucosaminidase (NAGase) activities (μmol $min^{-1}$ $l^{-1}$) (solid line with solid dots) and $log_{10}$SCC (solid line with open diamonds) of a cow that had a registered clinical mastitis record on day 35 after calving (open circle).

LDH and NAGase activities were elevated at calving but decreased rapidly until about day 30–40 of lactation. The profiles demonstrate the corresponding increase and fall in LDH and NAGase activity relative to any increase and fall in SCC. For example, the cow presented in Fig. 1, which had lactation records that went up to 77 d after calving, had an elevation in SCC, LDH and NAGase at calving which decreased drastically within the first 2 d after calving and then gradually decreased until about 26 d after calving. On day 27 after calving, there was another elevation in all three parameters, which subsided about day 34 after calving. Between days 42 and 50 after calving there was another elevation of all three parameters. However, for this particular elevation, SCC subsided about 2 d earlier than the LDH and NAGase activity. The rest of the lactation is followed by more or less constant values of all three parameters. This particular cow had no records of clinical mastitis.

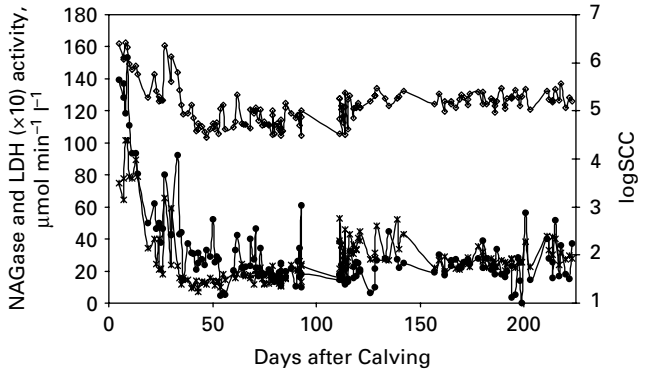


**Fig. 3.** An example of profiles for Lactate dehydrogenase (LDH) (solid line with asterisk and scaled up 10 times), N-acetyl-β-D-glucosaminidase (NAGase) activities (μmol $min^{-1}$ $l^{-1}$) (solid line with solid dots) and $log_{10}$SCC (solid line with open diamonds) for a cow that had no record of clinical mastitis.

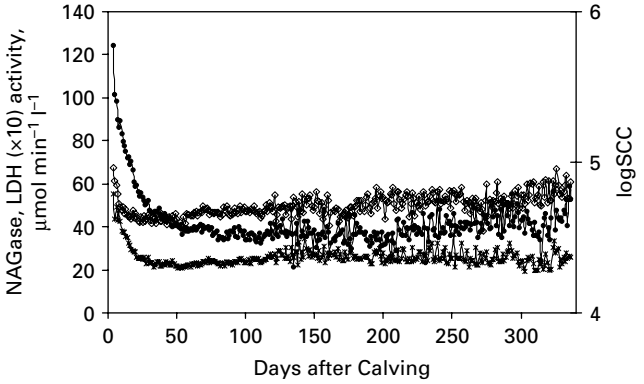


**Fig. 4.** Profiles for least squares means of Lactate dehydrogenase (LDH × 10) (solid line with asterisk), N-acetyl-β-D-glucosaminidase (NAGase) (solid line with solid dots) activity (μmol $min^{-1}$ $l^{-1}$) and $log_{10}$SCC (solid line with open diamonds) for healthy cows. All three variables were elevated at calving and declined drastically as the lactation progressed. SCC was observed to increase again with time. The plot was limited to 210 d after calving.

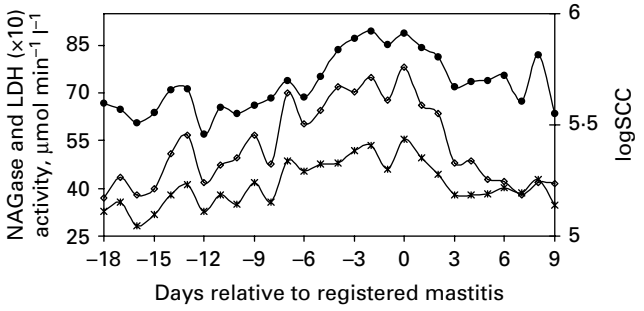


**Fig. 5.** Profiles for least squares means of Lactate dehydrogenase (LDH × 10) (solid line with asterisk), N-acetyl-β-D-glucosaminidase (NAGase) (solid line with solid dots) activity (μmol $min^{-1}$ $l^{-1}$) and $log_{10}$SCC (solid line with open diamonds) around clinical mastitis. All three variables were elevated prior to the mastitis record (day 0) and declined after treatment. The plot was limited to 18 d before and 9 d after the registered clinical mastitis.

In Fig. 2, the general picture of the profiles for all three parameters is similar to that presented in Fig. 1. However, the cow presented in Fig. 2 had a clinical mastitis record on day 35 after calving. Starting from about day 24 after calving, there was a marked elevation in all three parameters, which subsided at about day 40 after calving. Between days 96 and 104 after calving there was another noticeable elevation. The cow shown in Fig. 3 had milk records up to day 223 after calving, but no record of clinical mastitis. The figure shows the same drastic decrease in LDH and NAGase activities and SCC after calving. From day 5 after calving when the first set of records was available, LDH and NAGase activity and SCC gradually decreased until about day 35 after calving when the values for the three parameters stabilized and remained more or less constant. The average trend of SCC, NAGase and LDH with respect to DIM is shown in Fig. 4. SCC had a nadir at 30 d after calving while LDH and NAGase activities reached stable levels after the initial decline at about days 34 and 40 after calving, respectively. After the nadir, SCC gradually increased for the rest of the lactation. LDH and NAGase activities, however, remained almost constant after 50 and 60 d after calving, respectively.

Profiles for the period before and after a clinical mastitic record are presented in Fig. 5. LDH activity, NAGase activity and SCC increased before registered cases of clinical mastitis and decreased thereafter. The profiles showed that the curves for LDH and NAGase activity and SCC displayed consistent rising trend a couple of days before a registered clinical mastitis. In the period between day 8 before a registered mastitis and the day of the registered mastitis, LDH, NAGase activities and SCC increased by 56%, 30% and 8%, respectively (Fig. 5). After the registered mastitis case, which by definition includes mastitis treatment, all three parameters, LDH, NAGase activities and SCC decreased. Within 3 d after the registered mastitis case (treatment) there was a decrease of 32%, 19% and 7%, for LDH, NAGase activities and SCC, respectively. Changes occurring around registered mastitis cases in individual cows, however, varied substantially between cows. For example, the cow presented in Fig. 2 had dramatic increases of LDH, NAGase activities and SCC before the registered mastitis. Relative to the lowest values (Fig. 2), the highest values in the period of 8 d before the registered mastitis were 2·2, 2·1and 1·2 times higher. The decrease of LDH, NAGase activity and SCC was similarly dramatic. Within 3 d after a registered mastitis case, LDH activity reduced by 55%, NAGase reduced by 29% while SCC reduced by 14%.

**Table 4.** Correlations among lactate dehydrogenase (LDH), N-acetyl-β-D-glucosaminidase (NAGase) activities and somatic cell count ($log_{10}$SCC) for healthy cows and clinically mastitic cows determined using observed data and residuals after correcting for systematic effects

| | | Udder status | |
|---|---|---|---|
| Correlation | Variables | Healthy | Clinically mastitic |
| Correlation of observed values | LDH *v.* SCC | 0·48 | 0·76 |
| | NAGase *v.* SCC | 0·41 | 0·59 |
| | LDH *v.* NAGase | 0·43 | 0·56 |
| Correlation of residuals after correcting for effects of systematic factors | LDH *v.* SCC | 0·44 | 0·67 |
| | NAGase *v.* SCC | 0·46 | 0·50 |
| | LDH *v.* NAGase | 0·48 | 0·54 |

### *Associations among LDH, NAGase activity and SCC*

Correlation coefficients between LDH activity, NAGase activity and SCC for healthy and clinically infected cows are presented in Table 4. In clinically mastitic cows the correlation coefficient between LDH activity and SCC was higher than in healthy cows ($r$=0·76 *v.* $r$=0·48 for observed values and $r$=0·67 *v.* 0·44 for correlation of residuals). Similarly, the relationship between NAGase activity and SCC was lower in healthy cows than in clinically mastitic cows ($r$=0·41 *v.* 0·59). A slight decrease was observed when the correlation between NAGase activity and SCC was based on residuals ($r$=0·46 *v.* $r$=0·50). The relationship between LDH and NAGase was lower in healthy than in clinically mastitic cows ($r$=0·43 *v.* $r$=0·56). The picture did not change when the correlation was done on residuals after correcting for systematic effects ($r$=0·48 *v.* $r$=0·54).

### *Classification of cows according to health status*

Sensitivities and specificities for the ability of LDH and NAGase activity to distinguish between healthy and clinically mastitic cows using four different thresholds are presented in Table 5. The sensitivity for detecting clinically infected cows using LDH activity and NAGase activity was 73–95%. The equivalent sensitivity for detecting clinically infected cows by NAGase activity was 35–77%. The specificity for correctly classifying healthy cows using LDH activity and NAGase activity was 92–99% and 94–99%, respectively. The change in the threshold value affected the ability of LDH and NAGase activity to classify udder health status differently. For example, an increase in the threshold value from the 70% to the 80% percentile reduced the sensitivity of NAGase and LDH activities by 17·6% and 16·1%, respectively, but increased their specificity by 1·3 and 0·6%, in that order. A further increase in the threshold value to 90% percentiles decreased the sensitivity of NAGase by 12·2% but only changed the LDH sensitivity by 2·4%. The corresponding increase in the specificity was 0·7% and 0·4% for NAGase and LDH, respectively.

## Discussion

### *Effect of mastitis on LDH and NAGase activity*

We found increased activities of LDH and NAGase and higher SCC values in clinically mastitic cows compared with healthy cows. These results are in accordance with previous reports (Bogin et al. 1977; Kato et al. 1989). The increased activities of LDH and NAGase and the higher values of SCC in clinically infected cows compared with healthy cows demonstrate the changes that occur in the inflamed udder as a result of disrupted cells or tissue due to mastitis (Bogin et al. 1976; Kato et al. 1989). Our results, from naturally occurring mastitis, confirm those from induced mastitis (*e.g.* Bogin et al. 1977) where mammary infection causes a cascade of changes including increased enzyme activity in milk. We found that NAGase and LDH activities in milk were responsive indicators of mastitis in cow composite milk samples showing that indigenous enzymes, in particular LDH, offered an important opportunity for early detection of bovine mastitis.

The present results showed that the activities of LDH and NAGase and SCC values of healthy cows were affected by parity, days from calving and month of production. Increased parity was associated with increased LDH and NAGase activities and the level of SCC in the milk. Further, the general profiles of the activities of LDH and NAGase and the values of SCC were elevated at calving, declining until 30–40 d of lactation and gradually increasing again in the case of SCC through lactation. Although high SCC is usually associated with mastitis, elevation of SCC at calving can be a normal physiological phenomenon (Honkanen-Buzalski et al. 1981). According to Honkanen-Buzalski et al. (1981), Wiggans & Shook (1987) and Weller et al. (1992), a typical lactation curve for SCC starts off high shortly after parturition, decreases in the first 50–60 DIM to the lowest point, and increases slowly from then on towards the end of the lactation. The present results showed that these changes and the curvilinear lactation curve are not only typical in SCC but also in the activities of LDH and NAGase although the enzymes seem relatively constant in mid-lactation. This entails that, before any inferences are made on enzyme activity at cow level, it is important to take the effect of parity, days after calving and milk yield into account in order to avoid classifying healthy cows as sick. The increase in the activity of the two enzymes just like the increase in SCC prior to a registered clinical mastitis shows that the enzymes are indicators of mastitis. Around a registered mastitis, there was a general gradual increase in all the three parameters, which was followed by a decrease after veterinary treatment. The decrease in LDH, NAGase activities and SCC after treatment took a shorter

**Table 5.** Sensitivities and specificities to separate healthy and clinically infected cows by using lactate dehydrogenase (LDH) and N-acetyl-β-D-glucosaminidase (NAGase) activities within cow and milking determined at different threshold levels. Each row indicates a different threshold level

| *NAGase* | | | *LDH* | | |
|---|---|---|---|---|---|
| Threshold (μmol $min^{-1}$ $l^{-1}$) | Sensitivity | Specificity | Threshold (μmol $min^{-1}$ $l^{-1}$) | Sensitivity | Specificity |
| 77·3 | 77·4 | 94·1 | 4·3 | 95·2 | 92·0 |
| 92·8 | 65·2 | 97·5 | 5·7 | 91·1 | 97·5 |
| 105·1 | 47·6 | 98·8 | 6·0 | 75·0 | 98·1 |
| 117·4 | 35·4 | 99·5 | 6·5 | 72·6 | 98·5 |

time to reach low levels than did the increase to reach the highest level. Although this is the case, there was quite high variation between cows and even between parities within cows as reflected in the standard deviations and the substantial difference between the average profiles and individual cows' profiles. These differences could be due to mastitis cases caused by different pathogens, some veterinary treatment being misplaced, or individual cows' reactions to disease. Quantifying these factors was beyond the scope of the current study. However, the variation as shown in the profiles, calls for data prediction methods that would extract only the useful information that would reflect the onset of mastitis when using LDH, NAGase activity and SCC as indicators.

### *Enzymes as alternatives to SCC*

The present results showed that the association between LDH and SCC is stronger in clinically mastitic cows than in healthy cows. The higher correlation coefficients between LDH activity and SCC in clinically mastitic cows than in healthy cows show that the strength of the relationship between LDH activity and SCC increases with mastitis. Similarly, the correlation between NAGase activity and SCC increased slightly in clinically mastitic compared with healthy cows when the correlation was calculated based on observed values. When the correlation was based on residuals after accounting for systematic factors, the relationship was slightly reduced. Although enzymic and biochemical activity may increase in milk due to mastitis, the relationship between NAGase and SCC only changes slightly. Regardless of udder health status, the relationship between the two enzymes, LDH and NAGase, remained in the same range. This was noted in both the correlation of observed values and the correlation of residuals after correcting for systematic effects. The positive and strong relationship between the two enzymes and SCC at cow level shows that the two enzymes have great potential as alternatives to SCC in identifying the tendency in the process of clinical mastitis. There has been some controversy about the origin of the enzymes in milk. The potential origins of milk NAGase and LDH are plasma, disrupted mammary epithelial cells, and milk somatic cells (Kitchen et al. 1984; Fox et al. 1988). However, other reports (*e.g.* Timms & Schultz, 1985) indicate that, in general, the contribution of NAGase from plasma to milk may not be important. During the dry period, for example, NAGase activity in mammary secretions is significantly higher than in blood plasma (Timms & Schultz, 1985), which suggests that the enzyme present in mammary secretion comes mainly from within the mammary gland. Another point of controversy has been how many of the enzymes originate from milk phagocytes or from damaged epithelial cells. Considerable NAGase activity can also originate from epithelial cells (Kitchen et al. 1978; Kitchen et al. 1984; Fox et al. 1988; Mattila et al. 1988). According to Bogin (1977), the origin of the elevated LDH in milk is the leucocytes while Kato et al. (1989) found that the pattern of LDH isoenzyme distribution of granulocytes and lymphocytes was similar in mastitic milk suggesting that heavy introduction of granulocytes and lymphocytes, at least partly, contributed to the displacement of LDH isoenzymes in mastitic milk. The present results indicate that the association between these two enzymes has a link to somatic cells. However, more investigations are needed on the precise origin of milk constituents in order to improve mastitis diagnosis techniques.

### *Enzymes' ability to classify udder health status*

The present results indicated that LDH activity was better than NAGase activity at classifying clinically mastitic cows as sick. The ability of LDH and NAGase activities to classify healthy cows as healthy was in a similar range. Changing the threshold showed that sensitivity and specificity values of LDH activity were more stable than those for NAGase activity. This indicates that LDH activity is a more stable indicator of clinical mastitis than NAGase activity. This agrees with Bogin et al. (1977) who reported that activity of LDH in milk is a sensitive indicator of mastitis. Stable and high sensitivity and specificity values are the desired characteristics of a disease indicator. This is because, although the numerical changes may not be great, misclassifying udder health status may have considerable practical and economic implications. As Rasmussen et al. (2005) pointed out, correct classification of cows according to their health status not only puts

heavy demands on the detection system, but also requires a high degree of agreement between the detection system and the reference measurement of mastitis. The relatively high sensitivity and specificity obtained using LDH-activity in the current study show that LDH activity has great potential as a predictor of clinical mastitis.

### *Perspectives*

As biosensor assays for enzymes in milk are now becoming available, they provide an opportunity for improved, automated, real-time, in-line mastitis detection. However, the inherent between-milking variation as reflected in the high CV (Table 1), the changes that occur as the lactation progresses (Fig. 4), pose some practical implementation challenges when trying to predict mastitis, as early as possible, based on the the increase prior to a clinical mastitis case (Fig. 5). Any data coming from the mastitis indicator needs to be processed to provide reliable information. As Smith & West (1983) pointed out, the detection and interpretation of abrupt changes in the pattern of time series data are of paramount importance in disease surveillance systems. This entails pre-handling of the data before using it to provide decision support information for mastitis detection. Several data-processing techniques for smoothing time-series data exist. System-state probability methods, such as the multiprocess Kalman filter, which provide probabilities based on whether the change is due to normal evolution, an outlier, slope change or level change, have been proposed for biologically relevant changes (Smith & West, 1983; Korsgaard & Løvendahl, 2002) like the ones discussed in the present study. We are currently working on developing such a system as described in Chagunda et al. (2006).

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