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Evaluation of milk components during whole lactation in healthy quarters

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Several milk components related to immune defences (lysozyme, lactoferrin and γ-globulins, γ-G) and to inflammation (somatic cell counts, SCC; N-acetyl-β-glucosaminidase, NAGase; albumin) were considered. Forty-one quarters and 685 samples of 24 cows were included in the study; among them 534 samples were defined as negative (78·0%), 93 as diseased (13·5%) and 58 (8·5%) as subclinical. The pattern of each milk component in quarters always negative during the follow-up period was evaluated by a mixed model. Statistical analysis showed that days in milk (DIM), age (primiparous, pluriparous), herd and the interaction between herd and days in milk significantly influenced all the markers, with very few exceptions. A subset of samples including the negative quarters before the first outcome of an infection or a subclinical mastitis and the samples from quarters always negative was also selected. The analysis showed that herd, DIM and health status had a significant influence on most markers. Overall, primiparous cows were confirmed to have higher levels of most of the markers than pluriparous cows. The presence of a herd effect on non specific immune defences in fully negative quarters implies that when the mechanisms behind their release are fully elucidated, it might be possible to modulate them. Udder tissues were confirmed as an important source of some immune components, as supported by the inconsistency between SCC mean values and NAGase, lysozyme and lactoferrin values. Overall, quarters with high levels of NAGase, lysozyme and γ-G, exposed to bacteria, did not develop subclinical mastitis. Hence, invading pathogens could induce the development of subclinical IMI when these components and γG are in low concentration.

Keywords: Immunity, mammary gland, milk, innate defences, antimicrobial proteins.

The mammary gland is open to the external environment and therefore exposed to pathogens. Therefore, as in other organs (lung, gut), the role of innate immune defences is fundamental in preventing pathogen invasion (Ganz, 2004). There is increasing interest in innate immune defences, antimicrobial proteins and peptides (APP) both in human and veterinary medicine, even though some of them (*e.g.* lysozyme, lactoferrin) have been known for many years (Skerrett, 2004). Indeed, recent studies show that single APP or their additive and synergic interactions play a major role in preventing airway infections (Singh et al. 2000). Most of these APP are also found in the udder and in milk (Zecconi & Smith, 2003; Rainard & Riollet, 2006) but data on their effective role in preventing intramammary infections are not available.

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We focused our study on several milk components related to immune defences (lysozyme, lactoferrin, γ-globulins) and on inflammation (somatic cell counts, N-acetyl-β-glucosaminidase, albumin).

Lysozyme (LYZ) plays a role in host immune defences by killing ingested bacteria in the phagolysosomes and by the control of colonization through exocytosis of polymorphonuclear leucocyte (PMN) secondary granules (Zecconi & Smith, 2003). In this latter case the killing activity is related to the damage of bacteria cell-walls and to other enzymic means not yet completely investigated (Ganz, 2004). Moreover, LYZ binds to lipopolysaccharide (LPS) and reduces its endotoxic activity (Ohno & Morrison, 1989).

Lactoferrin (LF) is mainly synthesized by glandular epithelial cells and secreted into mucosal fluid (Ward et al. 2005; Rainard & Riollet, 2006). In the presence of a developing infection LF can be rapidly mobilized by PMN

(Harmon & Newbould, 1980) and has well documented antimicrobial properties (Rainard & Riollet, 2006). Among them, antimicrobial activity is probably the first discovered and is based on its affinity for iron, and probably also on its binding to the microbial membrane, dispersing LPS, and thus killing the bacteria. LF can also prevent adhesion of *Escherichia coli* to intestinal cells (Valenti & Antonini, 2005; Ward et al. 2005) and interacts with specific receptors of immune cells (Ward et al. 2005).

Immunoglobulins (γG) are well-known protective immune components for both the udder and the newborn and do not need further description.

N-acetyl-β-glucosaminidase (NAGase) is a glycosidase identified in PMN granules, but also found in macrophages and epithelial cells (Fox et al. 1986). This enzyme is a well-known marker of inflammation (Kitchen et al. 1978; Mattila et al. 1986) but there is no evidence of a specific role in immune defences.

Albumin (ALB) is generally considered a blood component which increases in the presence of udder inflammation (Mattila et al. 1986; Rainard & Riollet, 2006). However, recent studies show that ALB is also synthesized in the udder during inflammation (Shamay et al. 2005) and an anti-oxidant effect has been proposed (Rainard & Riollet, 2006).

During the periparturient period, some impairment of immune defences can be observed (Kehrli et al. 1989; Mallard et al. 1998). However, the amplitude of this impairment is influenced by various factors such as age of the cow (Mehrzad et al. 2002), stage of lactation (Mehrzad et al. 2001), energy balance (Dosogne et al. 1999; Stabel et al. 2003) and herd (Piccinini et al. 2004; Piccinini et al. 2005). Moreover, recent field studies (Piccinini et al. 2004; Piccinini et al. 2005) showed that innate immune defences, such as LF and milk γG, have higher mean values in the first week after calving than in susequent samplings. However, effects of such variations on the risk of intramammary infections are still unclear.

To address this latter aspect, the present work aimed to evaluate the pattern of some milk components related to innate immune defences and to inflammation during lactation in relation to quarter health status.

Materials & Methods

Herds, cows and samples

Cows were selected in four commercial dairy herds with automatic milking systems (AMS). These systems allow milking of each quarter separately; hence the influences of the health status of different quarters should be reduced. Herd characteristics are described in Table 1. In each herd 15 cows were randomly selected and followed for the whole lactation. Quarter milk samples (QMS) were collected in the morning at least 6 h after the previous milking, by an aseptic procedure at day 7±1, and then every 20±4 d until 300 days in milk (DIM). Among the 240 available quarters (60 cows), only those both bacteriologically negative and with SCC <100 000 cells/ml for the first two samplings were included in the study.

Table 1. Herd characteristics

Herd	Breed	Lactating cows	AMS brand	Number
A	Italian Holstein	120	DeLaval	2
B	Italian Holstein	105	Lely	2
C	Italian Holstein	185	Lely	3
D	Italian Holstein	100	DeLaval	2

Milk bacteriological analysis

At the laboratory, an aliquot (0·01 ml) of each QMS was spread on blood agar plates. Colonies were isolated and identified by methods according to National Mastitis Council (NMC, 1999). Somatic cells were counted on a Bentley Somacount 150 (Bentley Instruments, Chaska MN, USA).

Definitions

QMS status was defined following the scheme described by Pyorala (2003) with slight modifications. Samples with SCC ⩾200 000 cells/ml (either bacteriologically positive or negative) were defined as subclinical; bacteriologically negative samples with SCC <100 000 cells/ml were defined as negative; when the sample had SCC in the range 100 000–200 000 cells/ml (either bacteriologically positive or negative) it was defined as diseased.

Whey preparation

Whey was obtained from milk by centrifugation at 60 000 ***g*** at 4 °C for 30 min, then aliquoted in 1500 µl tubes and immediately frozen at −80 °C until enzyme analyses. These analyses were performed in a single session for each enzyme, at the end of the follow-up period.

Biochemical assays

LZ was assessed in duplicate by a fluorescence-based procedure (EnzChek Lysozyme Kit, Invitrogen, Carlsbad CA, USA). The method is based on the lysis of *Micrococcus lysodeycticus* labelled with fluorescine to such a degree that fluorescence is quenched. LZ activity is measured by changes of fluorescence on a microplate fluorimeter at 355 exc and 460 em (Ascent, Thermo Labsystem, FL), against a standard curve obtained for each test with a range of 8–500 units. One unit of LZ is defined as the quantity of enzyme that produces a decrease in turbidity of 0·0001 OD units per min at 450 nm measured at pH 7·0 (25 °C) using 0·3 mg/ml.

NAGase was assessed in duplicate by the procedure described by Kitchen et al. (1978), and expressed as units (pmol of 4-methylubelliferon released per min at 25 °C

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Table 2. Frequency of infections and subclinical intramammary infection (IMI) in primiparous and pluriparous cows by days in milk (DIM)

	Diseased infections		Subclinical IMI	
DIM	Primiparous	Pluriparous	Primiparous	Pluriparous
0–30	0	0	0	0
31–60	8·70	14·71	0	0
61–90	2·44	7·89	0	2·63
91–120	5·13	6·45	0	12·90
121–150	11·36	16·67	2·27	13·89
151–180	15·15	27·59	9·09	20·69
181–210	21·74	25·00	8·70	18·75
211–240	25·00	14·29	16·67	20·00
241–270	23·33	21·43	3·35	16·67
271–300	25·00	17·50	6·25	17·50

Table 3. Results of mixed model statistical analysis for the different milk components considered in 14 quarters that were healthy for the whole follow-up period. Results are displayed as *P*-values with $\alpha = 0{\cdot}05$

	Fixed effects and interactions			
Markers	Herd	Age	DIM†	Herd × DIM
SCC	0·0245	<0·0001	0·0054	0·0005
NAGase	0·0018	<0·0001	<0·0001	<0·0001
Lysozyme	0·0004	<0·0001	<0·0001	<0·0001
Albumin	<0·0001	<0·0001	<0·0001	0·0337
Lactoferrin	NS‡	<0·0001	<0·0001	0·0291
γ-Globulin	<0·0433	<0·0001	<0·0001	NS

† Days in milk
‡ Not significant

catalysed by 1 μl of milk) on a microplate fluorimeter at 355 exc and 460 em (Ascent, Thermo Labsystem, FL).

Whey proteins were assessed by agarose gel electrophoresis with Hydragel 30 (Sebia, F), a kit intended for separation of serum proteins on automated multiparametric agarose gel electrophoresis system (Hydrasis, Sebia, F). Gels were analysed by a densitometer and dedicated software (Phoresis, Sebia, F). Protein standards (ALB, α- and β-globulins, LF and γ-G) were added as a reference for the densitometer analysis.

Statistical analysis

To assess the pattern of the different milk components, data were analysed using the MIXED procedure of SAS 9.1 (SAS Institute, USA). The model included herd, sampling, parity, DIM and the interaction between herd and DIM, and random effects accounting for variance between cows. Effects of repeated samples were accounted for by choosing the appropriate covariance structure by Akaike's information criteria. A significance level of $\alpha = 0{\cdot}05$ was chosen for all the statistical analyses.

Results

Distribution of intramammary infections

A total of 685 samples from 41 quarters of 13 primiparous cows and 11 pluriparous cows were included in the study. Based on the definitions described above, 534 samples were defined as negative (78·0%), 93 as diseased (13·5%) and 58 (8·5%) as subclinical. The distribution of aetiological agents showed that coagulase-negative staphylococci were isolated from 66·1% of bacteriologically positive quarters, environmental streptococci from 27·5% of quarters, Gram-negative and *Corynebacterium bovis* from 3·2% of quarters each. The frequency of infected and subclinical quarters (Table 2) showed a clear increase as lactation proceeded, with a peak for diseased quarters after 181–210 DIM in primiparous cows and at 211–240 DIM for pluriparous cows. The highest frequency of subclinical samples was observed at 211–240 DIM in both group of cows.

When data were evaluated for the whole follow-up period, 14 quarters (34·1%) were always negative, 19 (46·3%) had at least one sample with subclinical intramammary infection (IMI) and 8 (19·6%) had one or more diseased samples.

Pattern of milk components in quarters negative for the whole lactation

The pattern of each milk component in quarters always negative during the follow-up period was evaluated by a mixed model which included the proper assessment of repeated measurements, and the analysis of variance for the final model is reported in Table 3. The analysis showed that DIM, age (primiparous, pluriparous), herd and the interaction between herd and DIM had a significant influence on all the markers, with very few exceptions.

Mean marker values (±SE) during lactation in negative quarters are reported in Figs 1 and 2. SCC (Fig. 1, top) were in the range 3–4·5 $\log_{10}$/ml (1000–31 600 cells/ml) during the whole follow-up period. As lactation proceeded, the dispersion of values increased, with no clear trend. The pattern of NAGase (Fig. 1, middle) showed the highest values in the first 20 DIM, then the values declined as DIM proceeded to reach a herd level around 20 units/ml after 160 DIM. For LZ (Fig. 1, bottom) the pattern was completely different from SCC and NAGase. Indeed, the values were lower in the first 140 DIM, then an increasing trend was observed in the following part of lactation. ALB proportion (Fig. 2, top) showed relatively steady values, after the peak in the first 20 DIM. LF proportion showed the highest mean values in the first 20 DIM (Fig. 2, middle), then decreased to a level of about 4% for the following part of lactation. Also mean γG proportions (Fig. 2, bottom) had the highest mean values at 20 DIM, followed by a decrease between 180 and 240 DIM, then they increased to reach the initial levels.

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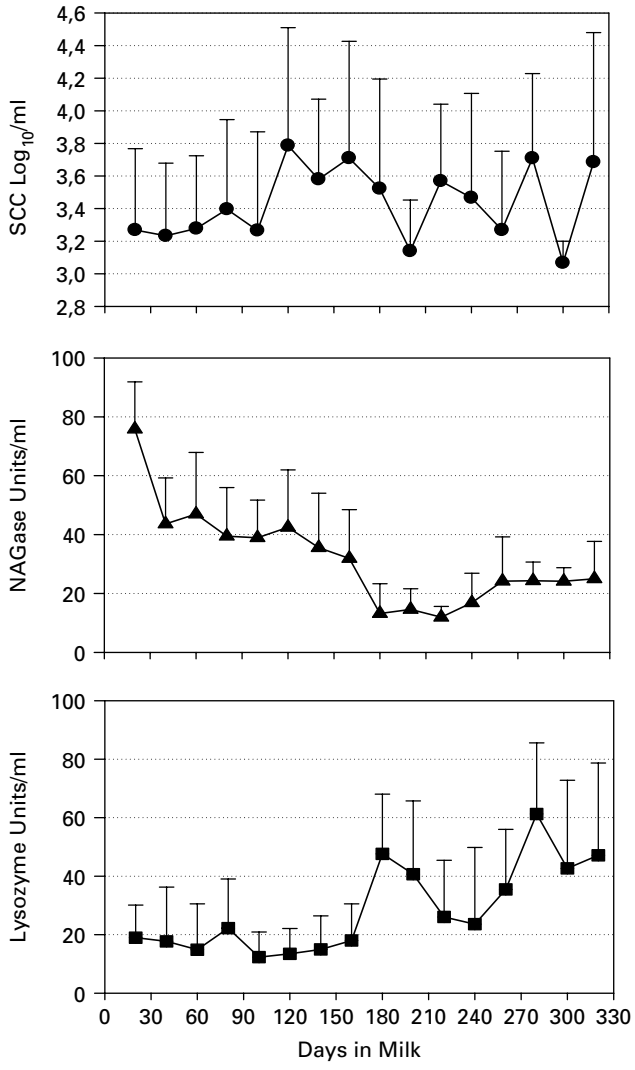

Fig. 1. Mean values (±SE) observed for SCC (●), NAGase (▲) and lysozyme (■) in always-negative quarters during lactation.

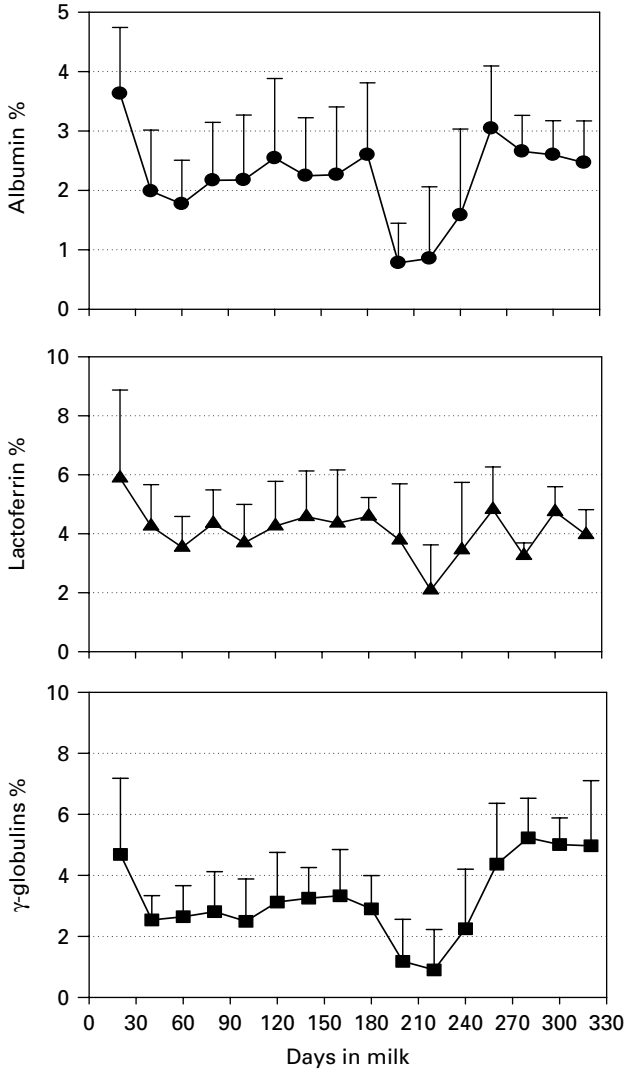

Fig. 2. Mean NAGase values (±SE) observed for albumin (●), lactoferrin (▲) and γ-globulins (■) in always-negative quarters during lactation.

Statistical analysis showed a significant influence, among the others, of age. Therefore, we compared the mean values observed for the different markers in primiparous and pluriparous cows (Table 4). Primiparous cows showed a significantly lower level of LYZ, while NAGase, ALB and LF were significantly higher than in pluriparous cows.

Comparison of marker patterns showed a drop in mean values between 180 and 240 DIM for ALB, LF, γG and NAGase, while an increase was observed for LYZ. This outcome coincided with the peaks observed for both diseased and subclinical quarters (Table 2).

Table 4. Mean values (±SE) for the considered markers assessed in 14 quarters that were healthy for the whole follow-up period in primiparous and pluriparous cows

Marker	Units	Primiparous	Pluriparous
SCC	Log_{10}/ml	$3{\cdot}45 \pm 0{\cdot}59^{a}$†	$3{\cdot}43 \pm 0{\cdot}58^{a}$
NAGase	Units	$33{\cdot}88 \pm 1{\cdot}86^{a}$	$25{\cdot}70 \pm 2{\cdot}04^{b}$
Lysozyme	Units	$11{\cdot}86 \pm 7{\cdot}66^{a}$	$26{\cdot}95 \pm 22{\cdot}83^{b}$
Albumin	%	$5{\cdot}43 \pm 1{\cdot}36^{a}$	$3{\cdot}88 \pm 1{\cdot}58^{b}$
Lactoferrin	%	$3{\cdot}29 \pm 1{\cdot}23^{a}$	$2{\cdot}03 \pm 1{\cdot}08^{b}$
γ-Globulin	%	$3{\cdot}37 \pm 1{\cdot}51^{a}$	$3{\cdot}09 \pm 1{\cdot}82^{a}$

†Means in cells in the same row with a different superscript letter are statistically different ($\alpha = 0{\cdot}05$)

Milk markers prior to the development of an intramammary infection

To assess whether marker values showed changes prior to the development of an IMI, we selected a subset of samples including quarters always negative until the first outcome of a diseased or subclinical samples and those always negative. This subset was classified as

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Table 5. Results of mixed model statistical analysis for the different milk components considered in healthy quarters. Group has been coded as negative ($n=14$) when quarters were healthy for the whole follow-up period, latent ($n=19$) when it came from a quarter with one or more samples defined as diseased and subclinical ($n=8$) when it came from a quarter with one or more samples with subclinical IMI. Results are displayed as *P*-values with $\alpha=0{\cdot}05$

	Fixed effects and interactions					
Markers	Herd	DIM†	Group	Herd × DIM	Group × DIM	Group × Herd
SCC	NS‡	0·0149	<0·0001	0·0048	NS	0·0029
NAGase	<0·0001	<0·0001	<0·0001	0·0027	NS	NS
Lysozyme	<0·0001	<0·0001	<0·0001	0·0201	0·0479	NS
Albumin	<0·0001	0·0137	<0·0001	0·0200	0·0193	NS
Lactoferrin	<0·0001	0·0231	<0·0001	NS	NS	NS
γ-Globulin	<0·0001	<0·0001	<0·0001	0·0258	0·0070	NS

† Days in milk
‡ Not significant

Table 6. Mean values (± SD) for the considered markers assessed in healthy quarters healthy by health status up to 200 days in milk. Group has been coded as negative ($n=14$) when quarters were healthy for the whole follow-up period, latent ($n=19$) when it came from a quarter with one or more samples defined as diseased and subclinical ($n=8$) when it came from a quarter with one or more samples with subclinical intramammary infection

		Status		
Marker	Units	Healthy	Diseased	Subclinical
SCC	Log_{10}/ml	$3{\cdot}44 \pm 0{\cdot}69^{a}$†	$3{\cdot}66 \pm 0{\cdot}64^{b}$	$3{\cdot}68 \pm 0{\cdot}63^{b}$
NAGase	Units	$32{\cdot}55 \pm 1{\cdot}94^{a}$	$39{\cdot}81 \pm 1{\cdot}62^{b}$	$33{\cdot}88 \pm 1{\cdot}82^{a}$
Lysozyme	Units	$19{\cdot}18 \pm 9{\cdot}66^{a}$	$30{\cdot}69 \pm 12{\cdot}80^{b}$	$25{\cdot}90 \pm 12{\cdot}04^{c}$
Albumin	%	$4{\cdot}23 \pm 1{\cdot}76^{a}$	$3{\cdot}91 \pm 1{\cdot}64^{a,b}$	$3{\cdot}85 \pm 1{\cdot}46^{b}$
Lactoferrin	%	$2{\cdot}21 \pm 1{\cdot}25^{a}$	$1{\cdot}74 \pm 1{\cdot}22^{b}$	$2{\cdot}28 \pm 1{\cdot}58^{a}$
γ-Globulin	%	$2{\cdot}91 \pm 1{\cdot}70^{a}$	$2{\cdot}38 \pm 1{\cdot}60^{a,b}$	$1{\cdot}88 \pm 1{\cdot}73^{b}$

† Means in cells in the same row with a common superscript letter are statistically different ($\alpha=0{\cdot}05$)

follows: samples from quarters always negative as healthy (HEA); samples from quarters which would have shown only diseased outcome as latent (LAT) and quarters which would develop subclinical infection as subclinical (SUB). The variable describing these different subgroups was defined as group. This subset included only samples up to 200 DIM, because afterwards the sequences of negative samples in group LAT and SUB were changed.

Table 5 reports the analysis of variance obtained by the final mixed model applied to compare marker values in these three subgroups. The analysis showed that herd, DIM and group had always a significant influence on all markers, out of herd for SCC. The interaction between group and herd was significant only for SCC, the interaction between group and DIM only for LYZ, ALB and γG, while herd × DIM was always significant, but not for LF.

Comparison of marker values by group showed interesting results (Table 6). Indeed, we found statistically similar levels of NAGase and LF in HEA and SUB groups. LZ and SCC levels were significantly lower in the HEA group when compared with LAT and SUB, while ALB and γG was significantly higher. Also unexpected were the significantly higher levels observed in SUB group for NAGase and LYZ and the significantly lower level of LF, when compared with the other two groups.

Discussion

Non-specific antimicrobial factors are particularly important in the udder defence system. In bovine milk several factors have been identified for their antimicrobial or modulating activity or both. Even though some of them (*e.g.* LZ, LF) have long been known, a better understanding of the potential role of milk components in udder immune defences has been obtained only recently (Cross & Gill, 2000). Indeed, an increasing number of studies evaluate the role and elucidate the mechanism of action of proteins such as LF and LZ as components of non-specific host defences (Singh et al. 2000; Ganz, 2004; Sarikaya et al. 2006).

Most of the milk components considered in this study have been proposed as diagnostic tools for detecting mastitis (Pyorala, 2003). We tried a different approach, focusing on the pattern of these components during lactation in relation to quarter health status and to their levels

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before the development of IMI. These components were selected because of the feasibility of their measurement under field conditions and their biological characteristics.

To investigate the role of non-specific udder defences under field conditions, two major problems should be solved: the bias introduced by machine milking; and the influence of undetected intramammary infections. The introduction of AMS allows milking cows with separate units for each teat, thus reducing the physiological and inflammatory interactions among quarters due to conventional milking machines. Moreover, the inclusion of only healthy quarters (<100 000 SCC/ml and bacteriologically negative) defined on the basis of at least two samples at the beginning of lactation, reduces the probability that the concentration of milk components could be influenced by concurrent undetected intramammary infections (Bansal et al. 2005).

Fourteen quarters maintained their healthy status for the whole follow-up period (330 DIM), and represent an interesting subset to benchmark the pattern of different milk components during lactation. Statistical analysis of this subset showed that factors such as age and herd, in addition to DIM, significantly influenced the levels of the markers considered. The importance of age confirmed previous studies on blood and milk non-specific immune defences (Mehrzad et al. 2002; Piccinini et al. 2004, 2005). However, in this study the levels of NAGase and LF are significantly higher in primiparous than in pluriparous cows, and this result is partially in disagreement with analogous studies on cellular functions (Mehrzad et al. 2002). One possible explanation for these differences is herd influence. Indeed, there are significant differences among herds for all the parameters considered, out of LF, confirming the results of analogous studies on heifers (Piccinini et al. 2004, 2005). In our opinion, the presence of a herd effect on non-specific immune defences has two important implications: the possibility of modulating the mechanisms behind the release of non-specific immune components, when they are fully elucidated; and that experimental studies on small groups of animals or single herds could not fully reflect the conditions we find under field conditions.

The pattern of the different milk markers also showed interesting features. Indeed, SCC were <10 000 cells/ml during the whole follow-up period. However, NAGase, generally considered correlated to SCC, showed a different pattern with initial high levels that declined during the remainder of lactation. In contrast, LYZ showed lower levels at the beginning of lactation, increasing after 160 DIM. Two other important components of non-specific immune defences, LF and γG, showed high levels at the beginning of lactation as expected, decreasing after 20 DIM, as expected in the absence of IMI. However, while LF levels remained low, γG increased after 240 DIM to reach levels equal to or higher than the initial ones. These results suggest the presence of tissue factors contributing to the release of some immune components. Indeed, inconsistency between SCC mean values and NAGase, LYZ and LF suggests that SCC cannot be the major source of these components, in the absence of an inflammatory reaction (Bruckmaier, 2005; Sarikaya et al. 2006). The increase of γG in the last part of lactation suggests that it could play a larger role in preventing IMI than generally hypothesized (Zecconi & Smith, 2003).

The peculiar changes observed for ALB, LF, γG, NAGase and LYZ between 180 and 240 DIM can be only matter for speculation. The presence of an increase of IMI during the same period of time suggests that quarters challenged by invading pathogens could deplete immune components from healthy ones (Bansal et al. 2005). The opposite behaviour of LYZ compared with the other markers could be explained by the different secretion mechanism for antimicrobial proteins (Schmitz et al. 2004).

Another unexpected observation was the presence of significantly higher levels of NAGase and LYZ in LAT quarters in comparison with both HEA and SUB, while it was the opposite for LF. Based on these results we can draw the following scenario: healthy quarters with high levels of γG, exposed to bacteria, secreted NAGase and LYZ and consequently did not develop a subclinical mastitis because these components, and probably other APP, could control the invading pathogens. Hence, invading pathogens could induce the development of subclinical IMI when these components are insufficient and γG are low. Further research is needed to understand the underlying mechanism, and to identify possible ways to increase these innate immune defences.

The role of γG and LYZ seems to be more important in the last part of lactation. The presence of significantly lower levels of LF in LAT quarters suggests that this protein has a minor role in preventing intramammary infections, but this does not exclude a role in the subsequent inflammatory process (Schmitz et al. 2004; Bruckmaier, 2005).

Overall, the results of this study suggest that when SCC are low, their role in preventing intramammary infections is probably less important than other innate immune components produced by udder tissues (Bruckmaier, 2005; Sarikaya et al. 2006). Therefore, low NAGase and LYZ levels, in the absence of an established inflammation, could represent a marker indicating reduced reactivity of udder tissue innate immune defences and a higher risk of developing udder infections.

Among the different components of the innate immune defences, LF does not seem to play a major role during lactation. The importance of this component during drying-off is well known (Smith & Oliver, 1981; Oliver & Smith, 1982) and our results suggest that its activity could be important in the first month after calving, but not afterwards. On the other hand, γG was significantly higher in HEA quarters and after 240 DIM, suggesting a preventive role probably through increasing the efficiency of milk PMN functions (Paape et al. 2002). This hypothesis is partially supported also by the observed increase of LYZ in the last part of lactation.

Conclusions

The present results confirmed that in absence of established inflammation, some milk components related to innate immune defences are poorly related to milk leucocyte counts. Therefore, udder tissue could be an important source of APP and healthy primiparous cows have values not inferior to pluriparous cows. The definition of reference values could be useful in monitoring udder health status. Indeed, a panel of markers including SCC, LYZ, NAGase and γG could be suggested as indicators to identify cows with potentially impaired tissue-related immune defences during the first 2 months of lactation.

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