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The role of immune defences in udder health

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The current dairy herd management requires to keep a difficult balance among different management and physiological factors to reduce the frequency of diseases affecting production such as mastitis (de Kruif & Opsomer 2002, Zecconi, 1997). Indeed, it can be demonstrated that achieving a proper animal welfare level is essential to reduce the frequency of diseases, reducing the direct and indirect impairment of immunological defences by external causes (Blaha, 2000).

Udder immune defences include many factors, some of them of blood origin, others synthesized in mammary gland and others specific of the udder (Table 1). The different immune defences have been described in excellent reviews and textbooks (Butler, 1981, Craven and Williams, 1985, Tizard, 2001, Zecconi and Smith, 2003), and the reader is invited to refer to them for further details.

Table 1: Major factors involved in immunity and mediators of inflammation

Type	Factor	Teat / teat secretion	Milk	Blood
Anatomical	Sphincter	X(1)		
	Keratin	X		
	- Basic proteins	X		
	- Fatty acids	X		
Cells	PMN	X	X	X
	Macrophages	X	X	X
	Lymphocytes	X	X	X
Non-specific humoral	Lactoferrin		X	X
	Lysozyme	X	X	X
	Lactoperoxidase		X	X
	Complement		X	X
	Gamma globulins	X	X	X
Specific humoral	Gamma globulins		X (low conc.)	X
Inflammation mediators	Cytokines		X	X
	Acute phase proteins		X	X X
	NAGase	X	X	X
	Nitric oxide		X	X

(1) The mark "X" means that the presence of the specific factor has been demonstrated. The absence of the mark does not necessarily means that it is absent.

The epidemiology of mastitis is classically represented by the interactions among bacteria, cow and environment. The same scheme can be applied to describe the immune response both at animal and at mammary gland level. Indeed, the immune response is the result of the interactions among the animal, the environment and the pathogen (Burvenich, et al., 2003). Among these factors, the cow and its genetics represent an important part of

the immune response. However, the practical impact of genotype on disease risk at udder level is still controversial and it has been covered by recent papers (Burvenich, et al., 2000, Detilleux, et al., 1995). Therefore, we will focus on the role of herd environment and of pathogens on udder immune response, describing some possible ways to modulate udder defences.

The role of bacteria

Pathogens involved in mastitis are generally classified as contagious, environmental and opportunistic bacteria. This classification is based on the main reservoir of the bacteria and on their pathogenic features. The involvement of immune defences in the development of mastitis is documented for *E.coli* acute mastitis. Indeed, its outcome is related both to the release of LPS by *E.coli* invading the udder, but also to the activity of milk PMN (Burvenich, et al., 2003). The immune defences and the mechanisms involved in regulating the different mediators of inflammation are also responsible for the characteristics of mastitis (mild, acute, peracute). The most severe cases are related to the impairment and to the imbalance of the different immune defences, with the release of large quantities of pro-inflammatory mediators (Blum, et al., 2000), without a proper control by immune defences.

The information on the specific role of udder immune defences in mastitis due to other bacteria such as coagulase negative staphylococci (opportunistic) and environmental streptococci are still scarce, whereas an increasing number of researches on the interaction between immune defences and *Staph.aureus* mastitis are available. *Staphylococcus aureus* is the most frequently isolated contagious mammary pathogen in many countries and it causes different pathologies in many species, as well. *Staphylococcus aureus* strains produce several surface-associated and secretory products. The combination of these products influences the pathogenic role of different strains. These differences among strains together with udder immune defences could also affect the infection pattern for *Staph.aureus* intramammary infections (IMI), that is often different from herd to herd (Raimundo, et al., 1999).

Table 2. Values of the different immunological factors assessed in 29 quarter milk samples *Staph.aureus* positive e 41 quarter milk samples *Staph.aureus* negative, collected from cows in five commercial dairy herds (values are expressed as mean ± std)

Immune Factors	*Staphylococcus aureus* positive	*Staphylococcus aureus* negative
PMNs †	0.60 ± 0.16$^{\Psi}$	0.49 ± 0.23$^{\Psi}$
Macrophages †	0.31 ± 0.12	0.38 ± 0.19
Lymphocytes †	0.01 ± 0.02	0.03 ± 0.05
Vitality, %	59.69 ± 19.60$^{\Psi}$	49.79 ± 19.21$^{\Psi}$
Respiratory burst, mV/1000 PMN	7.4 ± 19.57	11.69 ± 14.43
NAGase, pmol/min per ml milk	14.00 ± 8.97$^{\Psi}$	7.66 ± 5.03$^{\Psi}$
Lysozyme, µg/ml	19.71 ± 9.26	23.78 ± 11.95

† Values are expressed as proportions of total cells, mean ± SD

$^{\Psi}$ Difference between positive and negative quarters significant at Student's *t* test ($P<0.05$)

The results of a study summarized in table 2, confirm that *Staph.aureus* intramammary infection is associated with different responses by milk immune factors. Proportion of PMN, viability and NAGase activity were significantly higher in infected quarters than in bacteriologically negative quarters. The same trend could be observed even when results

were analysed by herd, as confirmed by the absence of a statistical difference between herds and by the homogeneity of odds ratios. However, the large variation of mean values among the herds suggests that herd factors (i.e. genetic, management or strain prevalence) could influence the magnitude of the response of the host to the infection.

The role of environment

The term environment includes a number of different factors from housing to bedding, from milking to nutrition. Among these different factors, we would like to focus to one of the best known (nutrition) and to another, much less considered (milking).

Nutrition, Metabolism & Immunity

The relationship between metabolic diseases and immune status of the dairy cow is often reported as one of the major problems, particularly during periparturient period. It is incontestable that the periparturient period is one of the most important and critical periods in cow life. It has been demonstrated that in this period some impairment of immune defences could be observed (Curtis, et al., 1985, Ingvartsen, et al., 2003, Kehrli and Goff, 1989). This impairment could increase the frequency of reproductive and production diseases (Ingvartsen, et al., 2003), and of clinical mastitis as shown experimentally (Barker, et al., 1998, Curtis, et al., 1985), and in field conditions (Burvenich, et al., 2000). As an example, during periparturient period an important role is played by milk PMN. When they are unable to perform their activity, the mammary gland is at risk of developing mastitis (Burvenich, et al., 2003, Zecconi, et al., 1995). Indeed, a case-control study showed that milk PMN isolated from cases of clinical mastitis had a significant lower respiratory burst (assessed by luminol-enhanced chemiluminescence) in comparison with PMN isolated from homologous control (healthy) quarters (Table 3).

Table 3. Milk PMN respiratory burst (mV) assessed by luminol-enhanced chemiluminescence using different pathogens as challenges (Zecconi, et al., 1995)

	Control		**Cases**	
	Mean	*Std.Dev*	*Mean*	*Std.Dev.*
Str.uberis (mV)	2986	8859	279	1022
Staph.aureus (mV)	3935	7639	148	309
E.coli (mV)	2148	3935	150	238

However, in a recent review (Ingvartsen, et al., 2003), pointed out that even a structured literature selection is inadequate to explain the relationship between production performance and risk of production disease. The Authors suggest that a simple and direct link between metabolic status and immune defences is inadequate to explain why cows are more exposed to disease in presence of altered metabolic status. They focus their interest more on the effects of the “acceleration” in milk production on cow status and therefore on immune defences impairment. If the cow is unable to cope with the accelerated production, an abnormal mobilization of body reserved could be expected, leading to a potential impairment of immunity and therefore to an increase in disease risks.

Milking machine

The importance of milking in the epidemiology of mastitis is well known, but there are few data on the interaction between milking machine and immune defences. Recently it has been shown that teat tissue conditions play an important role on the risk of mastitis. Indeed, as teat tissue conditions deteriorate the risk of mastitis increases (Neijenhuis, et al., 2000, Zecconi, et al., 2004). More recently, it has been shown that milking machine (conventional

or robotic) could influence the immune defences at teat level (Hamann, et al., 2001, Zecconi, et al., 2003,). Table 4 shows the different proportion of PMN and lymphocytes and the NAGase activity in teat secretion, when cows were milked with a conventional milking system (CON) or with a no-pulsation system (NOP), in two different trials. The NOP application did not cause any change in udder health during the Trials 1 and 2 since the SCC level ranged continuously below 50 000 cells/ml. The data suggest that impact of NOP was mainly directed towards an increase in lymphocyte ratios and in increase in NAGase concentrations, andmay indicate the potential of NOP to trigger different biochemical mechanisms that control cell traffic and release of cellular enzymes.

Table 4. Proportion of PMN and Lymphocytes and NAGase activity in teat secretion of cows exposed to conventional or no-pulsation milking (Zecconi, et al., 2003)

		PMN		Lymphocytes		NAGase	
	Trial	*1*	*2*	*1*	*2*	*1*	*2*
Pre-treatment	**CON**	9.0[a]	10.5[a]	1.9[a]	7.9[a]	11.0	22.3[a]
Treatment	**NOP**	11.3[a]	12.1[b]	3.3[b]	13.7[b]	10.4[b]	21.4[a]
Post-treatment	**CON**	12.6[b]	13.6[c]	2.4[a]	18.7[c]	9.2[b]	17.8[b]

Columns with different superscript significantly

Measuring immune defences in field

The direct outcome of this reasoning is the need of biological indicators (biomarkers) that could assess the changes in cow homeostasis, which could affect disease risks. Among the different potential biomarkers, the immune ones are the most suitable (Ingvartsen, et al., 2003). Evaluating cow immune status in field is difficult, for different reasons. Among these reasons, there are the costs and the labour needed for many analytical procedures, the natural variability of some parameters, the disturbance caused to the routine work at farm level and, not last, the absence of reference values to define immune status. In a recent study (Piccinini, et al., 2004b), assessed different immunological indicators in dairy heifers. The data of this study confirm that the first four weeks after calving have a significant influence on non-specific immune defences. The decline in SOD activity during the first two weeks after calving supports the presence of an impairment of oxigen-dependent neutrophil functions, mediated by high concentration of NO (Garboury, et al., 1993). However, if a decrease of immune function is common to all the herds as suggested (Detilleux, et al., 1995), the impairment of some immune functions for both amplitude and length was statistically different across herds.

Immune defences in blood and milk

The level of immune defences at blood level is not necessarily related to what observed at milk level (Mehrzad, 2002, Paape, et al., 2003). Recently, (Piccinini, et al., 2004a), in a field study, showed that milk non-specific humoral defences were higher in the first week after calving, when compared with the samplings taken in the following weeks (Table 5). Two lysosomial enzymes (lysozyme and NAGase) strictly related to SCC, were higher in the first sampling after calving as expected. Unexpectedly, the levels of respiratory burst observed in the first two weeks after calving, were not statistically different from the values observed in samples taken in the following weeks. These data suggest that a significant

reduction in non-specific immune defences cannot be observed during periparturient period at milk level as demonstrated (Mehrzad, et al., 2001) and as observed at blood level (Piccinini, et al., 2004b).

Table 5. Summary of analysis of variance with general linear model for repeated measurements for the blood immune parameters considered (Piccinini, et al., 2004b)

Parameter	Within-subjects factors		Between-subjects factor
	Sampling	Sampling x Herd	Herd
NAGase	0.001	n.s. [1]	n.s.
Haptoglobin	n.s.	n.s.	n.s.
Lysozyme	n.s	n.s.	n.s.
SOD	0.009	0.008	n.s.
Nitric Oxide	0.001	0.001	0.001
Respiratory Burst	0.001	0.039	n.s.
Total proteins	0.001	n.s.	0.032
Albumin	0.001	n.s.	n.s.
Beta-globulins	0.001	n.s.	0.040
Gamma-globulins	0.001	n.s.	n.s.
Albumin/globulin ratio	0.001	0.022	n.s.

[1] Not significant (P>0.05)

As for blood, the non-specific immune response at milk level showed to be significantly influenced by the interaction between sampling and herd. The different pattern at herd level was confirmed by the prevalence of IMI observed in the herds considered, however this could not be related to a significant decrease in immune defences. Indeed, milk PMN from healthy cows showed very low respiratory burst levels, while PMN from infected quarters showed significantly higher values (Figure 1). This could be explained by the presence of activated PMN as hypotyzed by Mehrzad, et al., (2002). These studies suggest that the evaluation of immune defences at blood level should be applied carefully to estimate the level of udder immune defences. If it is true that an impairment of cow immune defences (measured in blood) could be related to an increased risk of *E.coli* acute case, this does not translate directly to an increased risk for subclinical mastitis.

How to improve udder immune defences

Enhancing the mammary gland immune defences is still one of the most studied topics in the veterinary field. In the last 50 years, many efforts have been spent trying to develop vaccines for the different mammary gland pathogens. The mammary gland immune defences can be potentially stimulated either with vaccines or with immunomodulators (Figure 2). Vaccine studies started long before the availability of antibiotics and are still undertaken in many countries. However, great interest has been directed to enhancing non-specific immune defences, by biological response modifiers, which are either chemical or biological substances. Within this general definition, biological response modifiers (BRM) can be distinguished into substances that induce immune reactions (paraimmunization) and substances that substitute primitive immune reactions (cytokines) (Sordillo, et al., 1997).

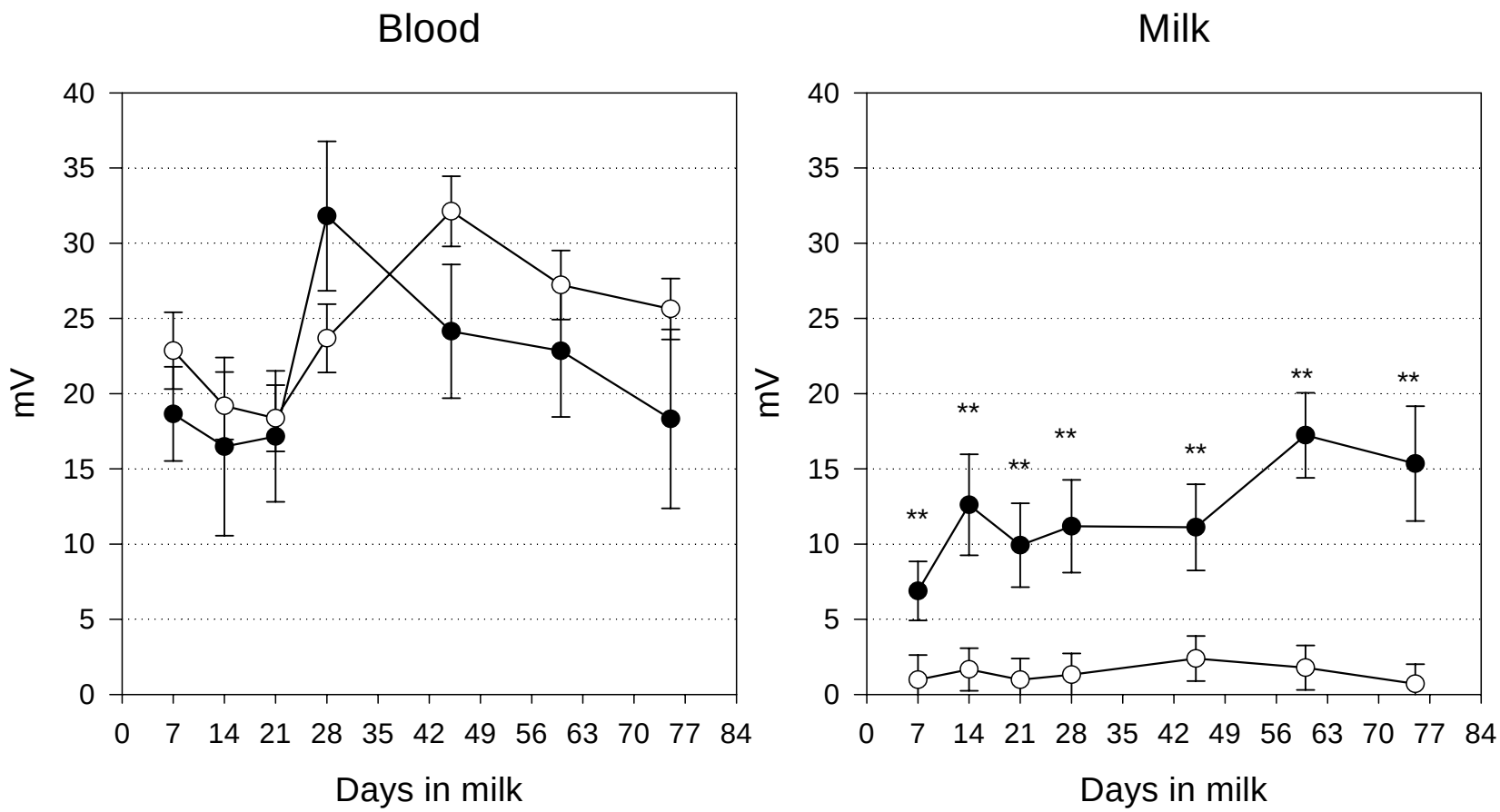


Figure 1: Distribution of the mean± SE of respiratory burst in blood and milk, by mammary gland health status (●; IMI - ○; healthy) during the follow-up period. Stars on sampling means a statistical significant difference among blood and milk samples (*: P<0.10; **: P<0.05) (Piccinini, et al., 2004a)

Biological response modifiers

There are several studies on the application of cytokines to modulate the immune response of the mammary gland. The treatment with rbIL-2 improved clearance of bacteria from the mammary gland; however, the mechanism through which this occurred is a matter of speculation. IL-2 also may activate phagocytic cells already in the udder, but further research is necessary before conclusions are drawn (Quiroga, et al., 1993). Therefore, rbIL-2 has been proposed as an adjuvant for antibiotic treatment of intramammary infections (Daley et. al. 1991, 1992) However, some of the studies showed the application of IL-2 could have same indesiderable side effects such as an increased rate of abortions (Erskine, et al., 1998). Studies on Granulocyte/Macrophage colony stimulating factor (GM-CSF) (Kehrli, et al., 1991a;1991b);.Nickerson, (1991) demonstrated the potential immunomodulating effect of rBG-CSF during the peripartum period. The treatment induced neutrophilia, an increase in $CD5^+$, $CD4^+$ and $CD8^+$ $CD5^+$ T lymphocytes, IgM, B-lymphocytes and monocytes. These effects resulted in an improvement of PMN immune functions such as Fc receptor-mediated phagocytosis, cytotoxic activity and chemiluminescence. However, there is no evidence of studies in field conditions. Even if papers on the application of cytokines for mastitis prevention are now less frequent than in nineties, still research groups are working on them and new developments can be expected.

The applications para-immunity inducers introduced a different approach to the modulation of mammary gland immune response even if these substances have been developed to control respiratory diseases. The application of this BRM in the periparturient period showed interesting results (Zecconi, et al., 1999), Indeed the number of *Staph. aureus* IMI after calving was significantly reduced in the BRM-treated group of cows and heifers. Quarters of cows in the BRM-treated group had significantly fewer new IMI for the first 21 days. At 28 days, *Staph. aureus* IMI had the highest prevalence within the follow-up period, because of the increase in bacteria shedding. Even at this time, the number of new IMI in the BRM-treated group was significantly lower than that in the placebo-group.

Vaccines

There are a series of different vaccines commercially or experimentally available against different mastitis pathogens. Some of them have been developed many years ago, other have been introduced recently, such as *E.coli* J5 vaccine. Actually, only *E.coli* vaccine is currently sold worldwide to prevent severe clinical *E.coli* mastitis. Whereas, two major groups of vaccines are in development, the one directed against environmental Streptococci and the other directed against *Staph.aureus*.

Kitt and Leigh, (1997) showed that *Str. uberis* is auxotrophic for 10 -13 amino acids, 8 of them were commonly required by all strains and that the growth of *Str. uberis* would be facilitated by the ability to hydrolyze host proteins (Leigh, 1993). This ability depends on the availability of activators of the cellular biochemistry leading to hydroxylation of host proteins. Plasminogen activator (PauA) is currently the only molecule to be assigned a putative role in this process. The PauA is produced by most of strains isolated from clinical cases of bovine mastitis; and it appears to be the major bovine plasminogen activator produced by *Str. uberis* (Lincoln and Leigh, 1997). A subunit vaccine based on the plasminogen activator, PauA, showed to be cross- protective in experimental challenge with values in the range 37.5-62.5%, but its efficacy in field trial is yet to be shown.

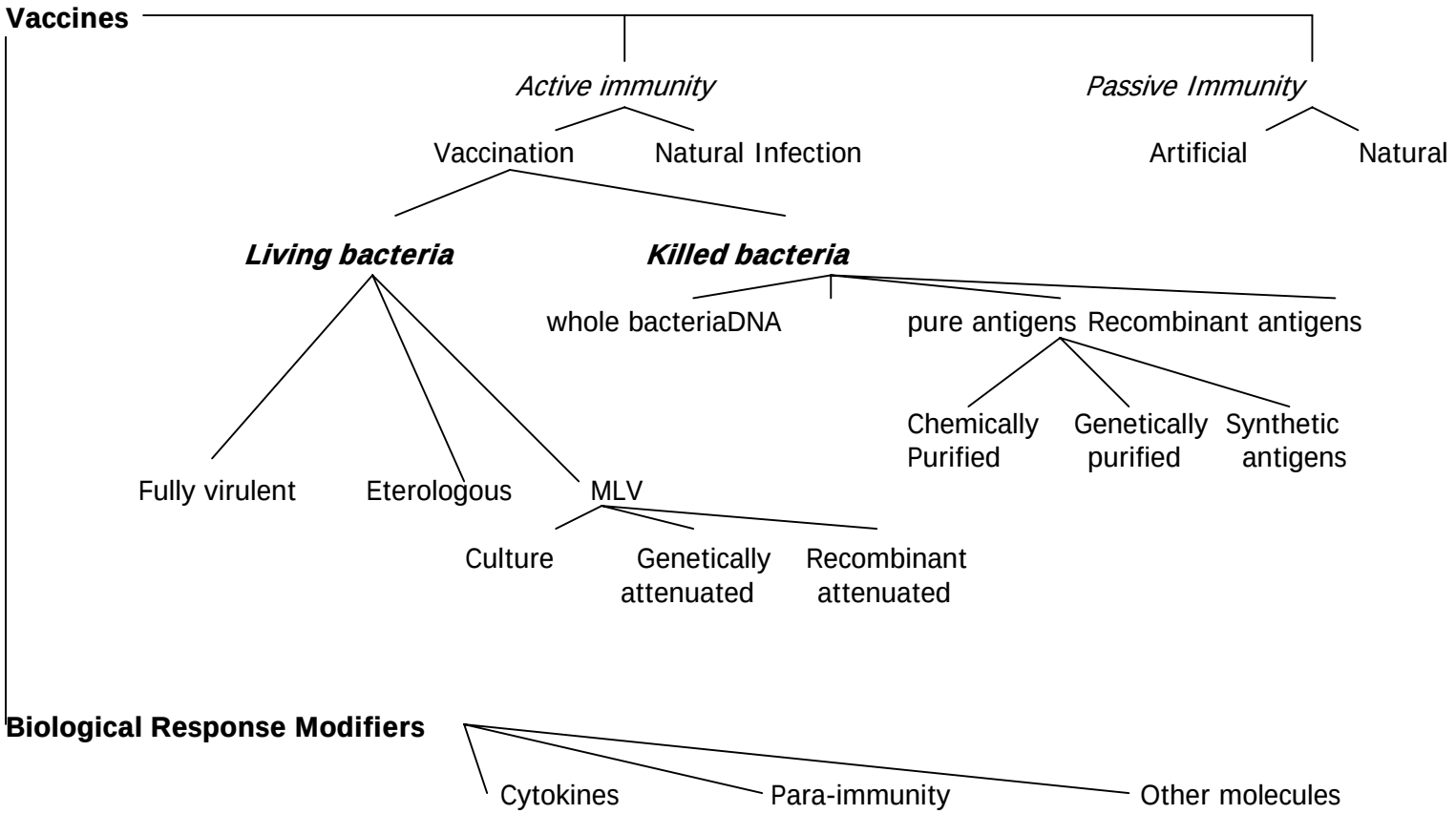


Figure 2: Scheme of the possible methods to improve immune defences (From (Tizard, 2001) and (Zecconi and Smith, 2003))

Very recently, (Potter, 2002) reported new information on a different approach to the vaccination against *Str.uberis* and *Str.dysgalactiae*. The vaccine was formulated after a detailed analysis of the possible factors contributing to the pathogenic activity of *Str.uberis*. Two putative molecules were identified: glyceraldehydes-3-phospate dehydrogenase (GapC) and a multiple FC-receptor protein (Mig) (Song, et al., 2001). In a subsequent trail vaccine with GapC from *Str.uberis*, GapC from *Str.dysgalactiae* and CAMP molecule from *Str.uberis* were compared. GapC and CAMP from *Str.uberis* showed the lowest level of SCC, suggesting that these two recombinant molecules could be the best candidates to be included in a vaccine.

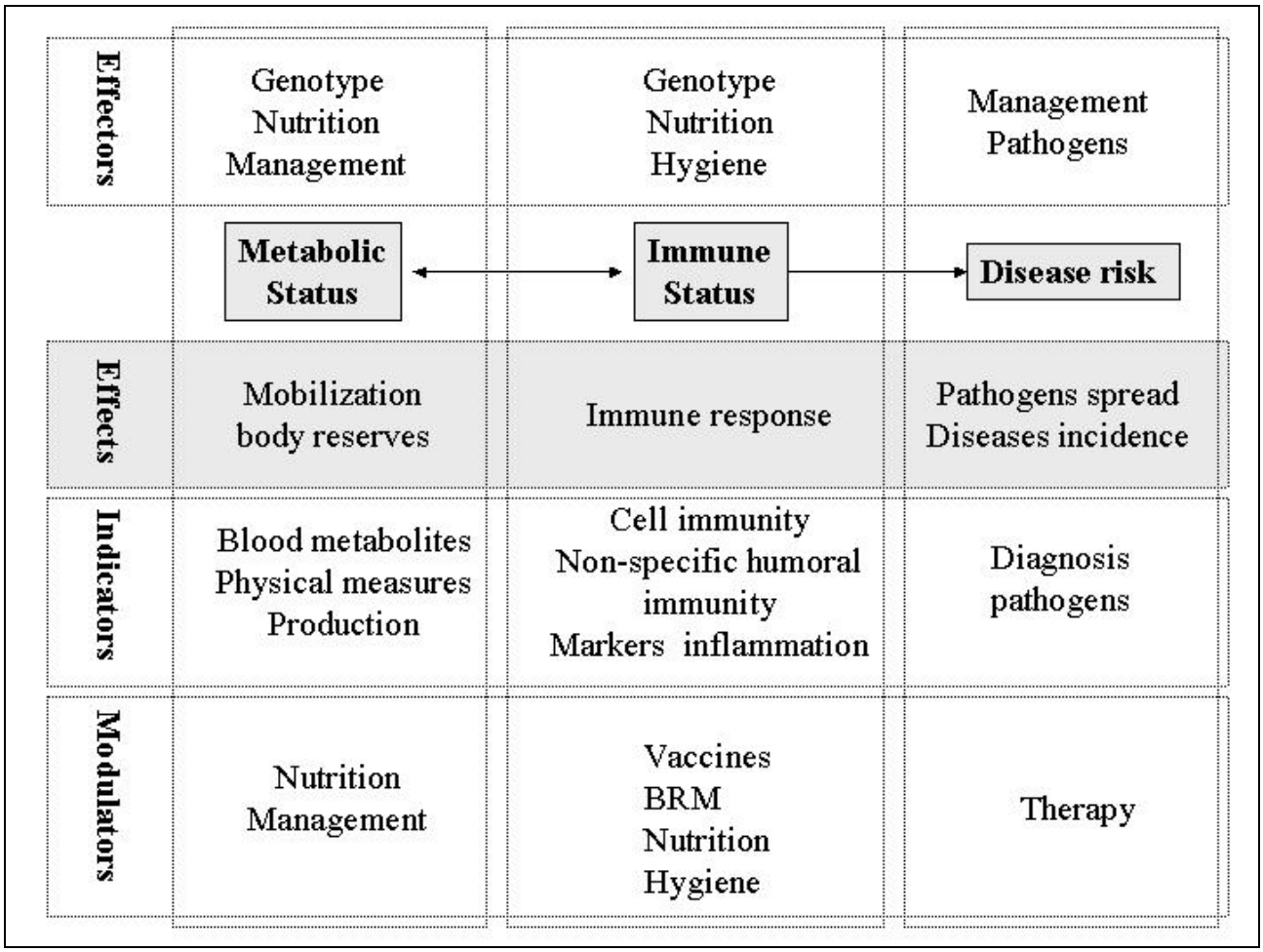
As it happened for Streptococci, the application of antibiotics reduced for a while the interest in developing staphylococcal vaccine both in human and veterinary medicine. However, the rapid appearance of resistance and the low cure rate generally achieved with even new antimicrobial molecules, supported further research on *Staph.aureus* vaccine development. Most of humans and cows have antibody against *Staph. aureus* antigens, but they are not protective. The different possible targets for an immune response are reviewed by Foster and Hook, (1998) and summarized in the following table 6. In general, there are two different approaches to the development of *Staph.aureus* vaccines, one using capsular polysaccharide and the other targeting extracellular matrix binding proteins or adhesins. Vaccines in the first group have been already produced and the efficacy of some of them has been investigated, but the results in field are inconsistent. The other group of vaccines is still in the development stages, even if it is probably the most promising group in both human and veterinary medicine.

Table 6: Potential antigens to be used for vaccination against *Staph.aureus* IMI (Foster and Hook, 1998) modified)

Antigen	Characteristics
Capsules	Virulence factor produced by some strains which form diffuse colonies in serum soft agar (macrocapsule). 90% of clinical human strains showed a microcapsule. Macrocapsule increases virulence in laboratory animals, while microcapsule does not.
Adhesins	Adhesion is one of the most important pathogenic factors for the development of IMI. *Staph.aureus* have different adhesins and their expression could justify the observed different pathogenic characteristics of the strains.
Surface proteins	There are several cell wall bound proteins on *Staph.aureus* surface: protein A, fibronectin-binding proteins, clumping factor. Protein A showed to inhibit phagocytosis and is expressed in a variable amount in *Staph.aureus* strains isolated from mastitis cases. Fibronectin-binding proteins have been identified and biochemically characterized, even if their importance in the pathogenesis of infection is still controversial. Clumping factors is a well-know factor, different from coagulase that allows the formation of small clamps in the presence of plasma.
Toxins	Toxins are probably the best-known pathogenic factors and the most used antigens in vaccine development. The use of these antigens allowed decreasing the severity and the frequency of clinical mastitis in ewes and cows.

Conclusions

The importance of udder immune defences, will increases as the consumers increase their demand for safe, wholesome dairy products. However, udder immune defences can not be considered by themselves, but they should be evaluated in an holistic way as suggested by the following scheme, inspired by the work of Ingvartsen, et al., (2003), and completed with the aspect related to diseases and modulators.



In conclusion, the most important points to consider, to improve dairy cow health through the improvement of immune defences, are:

1. immune response is the result of the interaction of environment, pathogen and animal, at herd level, therefore any herd has its own characteristics;
2. a change of cow homeostasis doesn't cause immediately an impairment of immune defenses, particularly when mammary gland is involved; the pathogens involved must be identified prior to implement prevention measures;
3. to prevent impairment of immune defences we should focus not only on general herd status, but we target specific groups at risk (heifers, periparturient cows, dry-cows....);
4. cow metabolic and immunological status should be assessed objectively by selecting proper biomarkers and measuring them in specific groups at risk;
5. pathogens characteristics cannot be ignored, being involved in the development of the immune (inflammatory) response.

References

1. Barker, A. R., F. N. Schrick, M. J. Lewis, H. H. Dowlen, and S. P. Oliver. 1998. Influence of clinical mastitis during early lactation on reproductive performance of Jersey cows. Journal of Dairy Science. 81(5):1285-1290.
2. Blaha, F. 2000. Immune System Response to Stress. Pages 111-121 *in* The Biology of Animal Stress. G. P. Moberg and J. A. Mench, eds. CAB International, Oxford.
3. Blum, J. W., H. Dosogne, D. Hoeben, F. Vangroenweghe, H. M. Hammon, R. M. Bruckmaier, and C. Burvenich. 2000. Tumor necrosis factor-α and nitrite/nitrate responses during acute mastitis induced by *Escherichia coli* infection and endotoxin in dairy cows. Domestic Animal Endocrinology. 19:223-235.
4. Burvenich, C., J. Deteilleux, M. J. Paape, and A. M. Massart Leen. 2000. Physiological and Genetic Factors that Influence the Cows Resistance to Mastitis, Especially During Early Lactation. Int.Symposium on Immunology of Ruminant Mammary Gland, Stresa It 11-14 June 2000:9-20.
5. Burvenich, C., V. Van Merris, J. Mehrzad, A. Diez-Fraile, and L. Duchateau. 2003. Severity of E.coli mastitis is mainly determined by cow-factors. Vet.Res. 34(5):521-564.
6. Butler, J. E. 1981. A concept of humoral immunity among ruminants and an approach to its investigation. Pages 3-56 *in* The ruminant immune system. Vol. 137. J. E. Butler, ed. Plenum Press, New York.
7. Craven, N. and M. R. Williams. 1985. Defences of the bovine mammary gland against infections and prospects for their enhancement. Vet.Immun.Immunopath. 10:71-127.
8. Curtis, C. R., H. N. Erb, C. J. Sniffen, R. D. Smith, and D. S. Kronfeld. 1985. Path analysis of dry period nutrition, postpartum metabolic and reproductive disorders, and mastitis in Holstein cows. Journal Dairy Science. 68(9):2347-2359.
9. Daley, M., P. Coyle, T. Williams, G. Furda, R. Dougherty, and P. Hayes. 1991. Staphylococcus aureus mastitis: pathogenesis and treatment with bovine interleukin-1 beta and interleukin-2. Journal of Dairy Science. 1991. 74(ref):12, 4413-4424.
10. Daley, M., G. Furda, R. Dougherty, P. Coyle, T. Williams, and P. Johnston. 1992. Potentiation of antibiotic therapy for bovine mastitis by recombinant bovine interleukin-2. Journal of Dairy Science. 1992. 75(ref):12, 3330-3338.
11. de Kruif, A. and G. Opsomer. 2002. Integrated dairy herd health management as the basis for prevention. XXII World Buiatric Congress, Hannover D, 18-23 Aug 2002:410-419.
12. Detilleux, J. C., M. E. Kehrli, J. R. Stabel, A. E. Freeman, and D. H. Kelley. 1995. Study of immunological dysfunction in periparurient Holstein cattle selected for high and average milk production. Vet.Immunol.Immunopath. 44:251-267.
13. Erskine, R. J., P. C. Bartlett, S. R. Tavernier, L. H. Fowler, R. D. Walker, J. H. Seguin, and D. Shuster. 1998. Recombinant Bovine Interleukin-2 and Dry Cow Therapy: Efficacy to cure and Prevent Intramammary Infections, Safety, and Effect on Gestation. J. Dairy Sci. 81:107-115.
14. Foster, T. and M. Hook. 1998. Surface protein adhesins of Staphylococcus aureus. Trends Microbiol. 6(12):484-488.
15. Garboury, J., R. C. Woodman, D. N. Granger, P. Reinhardt, and P. Kubes. 1993. Nitric oxide prevents leukocyte adherence: role of superoxide. Am J. Physiol Hearth Circ Physiol. 265:862-867.
16. Hamann, J., V. Bronzo, P. Moroni, A. Casula, and A. Zecconi. 2001. Conventional and positive pressure pulsation effects on bovine teats and on immunological components of different milk fractions. Milchwissenschaft. 56(8):423-427.
17. Ingvartsen, K. L., R. J. Dewhurst, and N. C. Friggens. 2003. On the relationship between lactational performance and health: is it yield or metabolic imbalance that cause production diseases in dairy cattle ? A position paper. Livestock Production Science. 83:277-308.
18. Kehrli, M., J. , J. Cullor, and S. Nickerson. 1991a. Immunobiology of hematopoietic colony-stimulating factors: potential application to disease prevention in the bovine. Journal of Dairy Science. 1991. 74(ref):12, 4399-4412.
19. Kehrli, M. E., Jr. and J. P. Goff. 1989. Periparturient hypocalcemia in cows: effects on peripheral blood neutrophil and lymphocyte function. Journal of Dairy Science. 72(5):1188-1196.
20. Kehrli, M. E., Jr., J. P. Goff, M. G. Stevens, and T. C. Boone. 1991b. Effects of granulocyte colony-stimulating factor administration to periparturient cows on neutrophils and bacterial shedding. Journal of Dairy Science. 74(8):2448-2458.
21. Kitt, A. and J. A. Leigh. 1997. The Auxotrophic Nature of Streptococcus uberis: The Acquisition of Essential Amino Acids from Plasmin Derived Casein Peptides. Advances in Experimental Medicine & Biology. 418:647-650.

22. Leigh, J. A. 1993. Activation of Bovine Plasminogen by Streptococcus uberis. FEMS Microbiology Letters. 114:67-72.
23. Lincoln, R. A. and J. A. Leigh. 1997. Characterisation of a Novel Plasminogen Activator from Streptococcus uberis. Advances in Experimental Medicine & Biology. 418:643-645.
24. Mehrzad, J. 2002. Respiratory burst activity and viability of bovine blood and milk neutrophils during different stages of lactation and mastitis. Universitet Gent, Merelbeke, B.
25. Mehrzad, J., H. Dosogne, E. Meyer, R. Heyneman, and C. Burvenich. 2001. Respiratory burst activity of blood and milk neutrophils in sairy cows during different stages of lactation. J.Dairy Res. 68:399-415.
26. Mehrzad, J., L. Duchateau, S. Pyorala, and C. Burvenich. 2002. Blood and Milk Chemiluminescence and Viability in Primiparous and Pluriparous Dairy Cows During Late Pregnancy, Around Parturition and Early Lactation. J.Dairy Sci. 85:3268-3276.
27. Neijenhuis, F., H. W. Barkema, H. Hogeveen, and J. P. T. M. Noordhuizen. 2000. Classification and longitudinal examination of callused teat ends in dairy cows. J. Dairy Sci. 83:2795-2804.
28. Nickerson, S. 1991. Effect of cytokines on bovine mammary gland immunity. SAAS Bulletin Biochemistry and Biotechnology. 1991. 4(ref):60-67.
29. Paape, M. J., D. D. Bannerman, X. Zhao, and J. W. Lee. 2003. The bovine neutrophils: structure and function in blood and milk. Vet. Res. 34:597-628.
30. Piccinini, R., E. Binda, M. Belotti, A. Casirani, and A. Zecconi. 2004a. Comparison of blood and milk non-specific immune parameters in heifers after calving. Vet. Res. submitted.
31. Piccinini, R., E. Binda, M. Belotti, G. Casirani, and A. Zecconi. 2004b. Evaluation of non-specific immune status of heifers in field conditions during the periparturient. Vet. Res. in press.
32. Piccinini, R. and A. Zecconi. 2000. Relationship between udder cellular response and Staph.aureus strains characterized by plasmid fingerprinting. International symposium on immunology of ruminant mammary gland, Stresa 11-14 June 2000:352-354.
33. Potter. 2002. Vaccines for streptococcal pathogens have arrived. National Mastitis Council 41st Annual Meeting, Orlando FL 3-6 Feb 2002:81-88.
34. Quiroga, G., L. Sordillo, R. Adkinson, and S. Nickerson. 1993. Cytologic responses of Staphylococcus aureus infected mammary glands of heifers to interferon gamma and interleukin-2 treatment. American Journal of Veterinary Research. 1993. 54(ref):11, 1894-1900.
35. Raimundo, O., M. Deighton, J. Capstick, and N. Gerraty. 1999. Molecular typing of Staphylococcus aureus of bovine origin by polymorphisms of the coagulase gene. Vet Microbiol. 66(4):275-284.
36. Song, X. M., J. Perez-Casal, A. Bolton, and A. A. Potter. 2001. Surface-expressed Mig protein protects Streptoccoccus dysgalactiae against phagocytosis by bovine neutrophils. Infection & Immunity. 69:6030-6037.
37. Sordillo, L., K. Shafer-Weaver, and D. DeRosa. 1997. Immunobiology of the mammary gland. Journal of Dairy Science. 80(ref):8, 1851-1865.
38. Tizard, I. 2001. Veterinary Immunology: an introduction. 6th ed. W.B.Saunders, Philadelphia.
39. Zecconi, A. 1997. Intramammary infections and udder defences : the search for a new equilibrium. Flem.Vet.J. 66:85-91.
40. Zecconi, A., V. Bronzo, A. Casula, C. Luzzago, P. Moroni, R. Piccinini, and G. Spreafico. 1999. Efficacy of a biological response modifier in preventing Staphylococcus aureus intramammary infections after calving. Journal of Dairy Science. 82(10):2101-2107.
41. Zecconi, A., V. Bronzo, J. Hamann, and A. Casula. 1995. Case-control study on defence functions of milk cells in clinically infected bovine quarters. Proceedings of the Third IDF International Mastitis Seminar Tel-Aviv, Israel, 28 May - 1 June 1995. Book 1. National Mastitis Reference Center, Beit-Dagan, Israel: 1995. s-1,28-32. 9 ref.
42. Zecconi, A., P. Moroni, A. Casula, V. Bronzo, and J. Hamann. 2003. Effects of conventional and no pulsation milking systems on bovine teats and on immunological components of different milk fractions. Milchwissenschaft. 58:355-359.
43. Zecconi, A., R. Piccinini, G. Casirani, E. Binda, and L. Migliorati. 2004. Introduction of AMS in Italian dairy herds. Effects on teat tissues, intramammary infection risk, and spread of contagious pathogens. Pages 161-168 *in* Automatic Milking: a better understanding. Vol. 1. A. Meijering, H. Hogeveen, and C. J. A. M. de Koning, eds. Wageningen Academic Publishers, Wageningen.
44. Zecconi, A. and K. L. Smith. 2003. Ruminant Mammary Gland Immunity. FIL-IDF, Bruxelles.