Early mastitis detection: Can Lactoferrin evaluation by EU funded project MOLOKO biosensor help?

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Abstract

Mastitis is the most frequent udder disease in dairy cows. It causes huge economic losses, due to less milk production, less milk quality, increasing of drugs usage and, in most severe cases, early culling.

A healthy herd is a fundamental goal not only for animal welfare but also for the environment. Mastitis reduces herd efficiency by the increasing of environmental impact of milk production. In fact, it was calculated that a sick cow produces 6.3 % CO_2 eq./ kg milk more than a healthy one.

To reach efficiency goals, Precision Livestock Farming can give an important contribution. In compliance with that, MOLOKO (Multiplex phOtonic sensor for pLasmonic-based Online detection of contaminants in milK) European funded project aims development of a biosensor to improve milk safety and animal health.

The MOLOKO biosensor working mechanism is based on the optical measurement of an inherent physical property change of the environment in the proximity of the sensing surface.

Lactoferrin was chosen as a mastitis biomarker due to its antimicrobial activities. It is an iron-binding glycoprotein synthesized by neutrophilic polymorphonuclear leukocytes and granular epithelial cells in milk and other exocrine secretions.

A direct immunoassay for the detection of Lactoferrin was developed in buffer and in diluted raw milk using the MOLOKO sensor. The test showed a correct and cross-correlated recognition of Lactoferrin on suitably functionalized channels; promising results were obtained for both buffer (Limit Of Detection ~ 9µg/ml) and diluted raw milk (Limit Of Detection ~40µg/ml) providing information regarding levels of Lactoferrin in only 14 minutes.

A preliminary validation of the sensor for the detection of Lactoferrin was carried out with milk samples collected from two different dairy herds in the north of Italy, respectively 600 milking cows, with a milking parlour and 100 milking cows with automatic milking system.

Samples analysed in the MOLOKO sensor and by certified laboratory were compared for the estimation of lactoferrin concentration. Additionally, known parameters of udder

infections, such as somatic cells count, differential somatic cells count, and finally bacteriologic culture were also determined to better identify true positive.

The preliminary data show that integration of the MOLOKO biosensor on the milking systems could be useful to detect daily lactoferrin fluctuations. Combined with other sensors, it could provide farmers information about inflammatory events.

Keywords: MOLOKO, biosensor, precision livestock farming, mastitis, lactoferrin.

Introduction

Mastitis is the most frequent udder disease in dairy cows. It causes huge economic losses, due to less milk production, less milk quality, increasing of drugs usage and, in most severe cases, early culling (Halasa *et al*., 2011).

Worldwide, was estimated that mastitis costs between 61€ to 97€ per cow with clinical case and between 17€ to 198 € per cow with subclinical one (Hogeveen, 2011).

A healthy herd is a fundamental goal not only for animal welfare but also for the environment. Mastitis reduces herd efficiency by the increasing of environmental impact of milk production. In fact, it was calculated that a sick cow produces 6.3 % CO_2 eq./ kg milk more than a healthy one (Mostert *et al*, 2019).

To reach efficiency goals, Precision Livestock Farming can give an important contribution; indeed, Precision Livestock Farming allows to monitor every animal in real-time using sensors that check their health, welfare, production and reproduction status (Berckmans, 2017).

In compliance with that, MOLOKO (Multiplex phOtonic sensor for pLasmonic-based Online detection of contaminants in milK) European funded project aims development of a biosensor to improve milk safety and animal health.

One of the aspects that MOLOKO biosensor evaluates is mastitis risk; to allow early mastitis detection and improve health, productivity, and welfare of dairy cattle, understanding immune factors and mechanisms involved in the mammary gland defence against infection represents a fundamental task (Chaneton *et al*., 2013).

In the bovine mammary gland, the viable leukocytes offer some degree of cellular protection against microbial invasion through their ability to recognise microorganisms and induce a rapid inflammatory response in an attempt to resolve the intra mammary infection immediately (Alnakip *et al*.,2014).

Lactoferrin (LF) is an iron-binding glycoprotein, found in milk and other exocrine secretions which has antimicrobial/antiviral activities, immunomodulatory activity and antioxidant activity (Wakabayashi *et al*., 2006); LF is synthesized by neutrophilic polymorphonuclear leukocytes that are the first recruited immune cell to sites of infection (Alnakip *et al*., 2014), (Figure 1).

Furthermore, it has been demonstrated that LF concentration changes according to stage of lactation, parity and presence of pathogen, in fact it is significantly higher in cows with subclinical mastitis than healthy ones (Hagiwara *et al*., 2003).

For these reasons Lactoferrin was chosen as a MOLOKO mastitis biomarker.

The aim of this study was to assess MOLOKO biosensor for LF detection in raw cow milk and compare it, as a mastitis biomarker, with a gold standard LF detection.

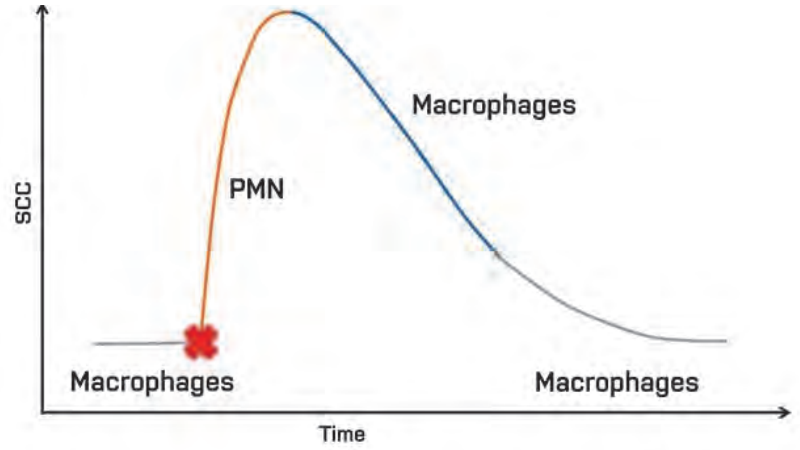

Figure 1. Udder immunologic response after infection (Alnakip et al., 2014).

Material and methods

MOLOKO is a European Horizon 2020 funded project, comprising 12 partners from across Europe, with the aim to optimise production and safety throughout the milk supply chain through the development of a fast and cheap biosensor; its working mechanism is based on the optical measurement of an inherent physical property change of the environment in the proximity of the sensing surface (Figure 2).

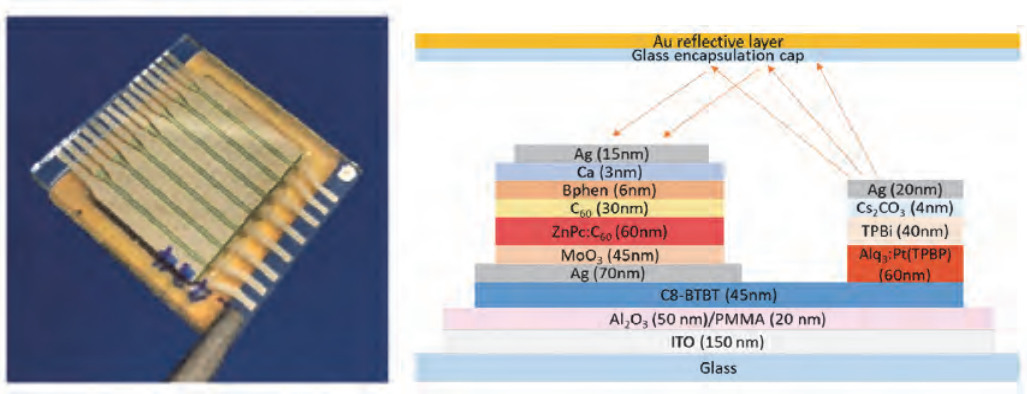

Figure 2. Bottom side of the photonic module. Prosa et al. (2021), Organic Light-Emitting transistor in a Smart-Integrated system for Plasmonic-Based sensing.

MOLOKO partners have decided to research both potential contaminants in milk (e.g. antibiotics, mycotoxins, bacterial toxins), and at the same time create a diagnostic tool useful for the reduction of antibiotics prescriptions, responding to the concerns of WHO and European Commission regarding overuse of antibiotics and subsequent drug resistance.

A direct immunoassay for the detection of Lactoferrin was developed in buffer and in diluted raw milk using the MOLOKO sensor.

The test showed a correct and cross-correlated recognition of Lactoferrin on suitably functionalized channels; promising results were obtained for both buffer (Limit Of Detection ~ 9μg/ml) and diluted raw milk (Limit Of Detection ~40μg/ml) providing information regarding levels of Lactoferrin in only 14 minutes.

Individual milk samples were collected from 2 dairy herds in the North of Italy, from January 2022 to March 2022; these herds were very different: they had respectively 600 milking cows, with a milking parlour and 100 milking cows with automatic milking system.

Cows were selected randomly according to all factors can influence LF concentration, such as stage of lactation, daily milk production, SCC, and parity; all this information, at the beginning of sampling were recorded.

Milk samples were collected in 2 refrigerated aliquots, first one was used for MOLOKO biosensor analysis and the second one was delivered to certified laboratory.

Before analysis with MOLOKO biosensor, samples preparation was needed; milk samples were diluted 1:100 and were filtered twice, first one with 0.45 μm filter and second one with 0.2 μm filter.

ELISA test was performed as a gold standard by certified laboratory in the north of Italy; kit used was “5091LFER- EuroProxima Lactoferrin: lactoferrin in various matrix”.

After analysis the colour intensity of the samples were read automatically and the concentration of lactoferrin were calculated.

Additionally, known parameters of udder infections, such as somatic cells count, differential somatic cells count, and finally bacteriologic culture were also determined by certified laboratory and other partner of MOLOKO Project.

Results and discussion

Preliminary analyses were made both in buffer and milk; after this analysis, we could observe a clear signal of 0.014% in the specific channel for the detection of lactoferrin in buffer (Figure 3) which indicated that the OPM was responding well to the detection of lactoferrin.

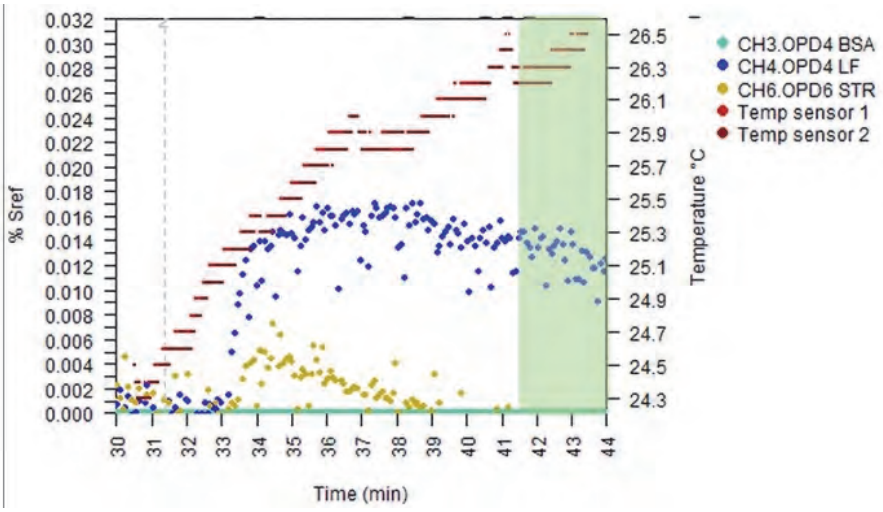

Figure 3. Sensorgram of the interaction of Lactoferrin at 100 μg/ml in buffer.

In the sensorgram for the milk samples diluted 100 times (Figure 4) and after dissociation (green region), we did not observe an increasement in the signal respect to the baseline. That indicates that the concentration of Lactoferrin in those samples is below the limit of detection of this OPM (approximately 0.001% which would correspond to 10 μg/ml of Lactoferrin in buffer and 1000 μg/ml in milk sample).

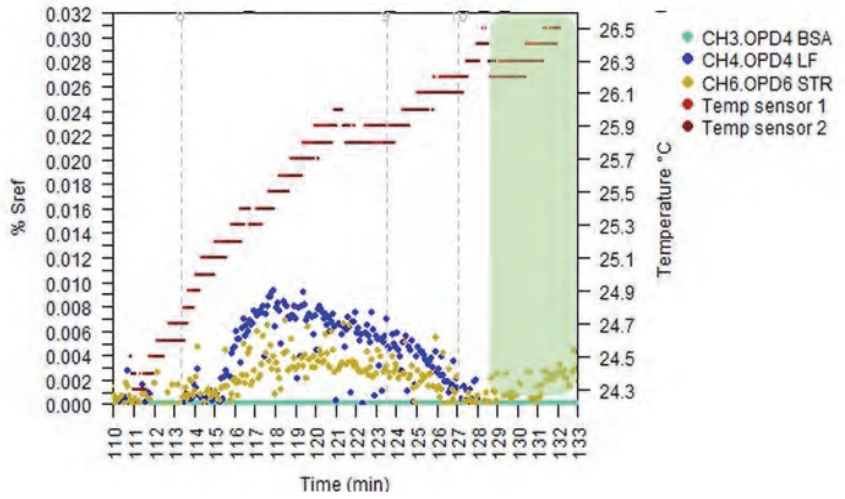

Figure 4. Sensorgram of the interaction of milk sample diluted 100 times.

However, we can say that qualitatively the concentration of Lactoferrin in milk samples was lower than 1000 μg/ml, according to ELISA test the level was 361 μg/ml.

Kutila T. (2004) have reviewed sundry studies and have reported main results about milk LF concentration in healthy and mastitic cows:

- Lactating cow: 20-35 μg /ml.
- Cow with subclinical mastitis: 20-120 μg /ml.
- Cow with clinical mastitis: 20-230 μg /ml.

With data collected from the sampling, further assessment will help in confirming the given threshold especially due to LF influence by parities and different stage of lactation (Hagiwara *et al*., 2003), also bacterial presence will be assessed.

Conclusion

The preliminary data showed that LF is correctly recognized by MOLOKO biosensor; for this reason, its integration on the milking systems could be useful to detect daily lactoferrin fluctuations. Combined with other sensors, it could provide farmers information about inflammatory events, furthermore, another fundamental goal it could be define a more accurate LF thresholds to distinguish clinical mastitis from subclinical ones and effectiveness at drying off.

However, more study is needed to define a sample preparation that is consistent with lactoferrin level in raw milk.

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