Effect of dietary zinc upon in vitro bacterial adherence to ovine mammary epithelium

IM SAIANDA1, CMV BETTENCOURT2, MC QUEIROGA3, G FERREIRA-DIAS1, CL VILELA1*

1 Centro de Investigação Interdisciplinar em Samidade Animal (CIIASA)/Faculdade de Medicina Veterinária, Lisboa, PORTUGAL
2 DRAAL - Centro de Experimentação do Baixo Alentejo - Herdade da Abóbada, Serpa, PORTUGAL
3 Departamento de Sanidade Animal e Vegetal, Universidade de Évora, PORTUGAL
* Corresponding author: E-mail: clvilela@fmv.utf.pt

SUMMARY
Mastitis remains one of the main causes of ruminant disease in milk-producing farms. Frequently, the causative microorganisms are coagulase-negative *Staphylococcus* that are normal inhabitants of the udder skin, but that may cause infection when natural defences are compromised. Amongst these, preservation of the integrity of mammary epithelium plays an essential role, as penetration of microorganisms within the mammary gland and subsequent epithelial colonisation are important steps to the establishment of intramammary infections. Zinc is one of the trace elements that have been related to reduction of new intramammary infections in cows. The present work aimed at studying the effect of zinc on bacterial adhesion to ovine mammary epithelium and the influence of the trace element’s form (mineral or proteinate) in the diet. Three groups of 24 adult ewes were used: one group with diet supplementation with 60ppm zinc oxide, another group fed with 30ppm zinc oxide and 30ppm of zinc proteinate, and a negative control group. Diet supplementation was kept for 3 months, after which 2 animals of each group were euthanised. Bacterial adhesion assays were performed with a field strain of *Staphylococcus xylosus*, the most frequently isolated species. Three different techniques were used: suspended epithelial cells; epithelial cells previously fixed to slides and epithelial tissue from the mammary cistern. The results of these 3 techniques were similar. Bacterial adhesion was significantly reduced in sheep receiving zinc supplementation in the diet. Furthermore, zinc proteinate associated to mineral zinc resulted in a significant reduction in bacterial adherence, when compared to zinc oxide alone. These results suggest that supplementation of sheep’s diet with zinc proteinate associated to zinc oxide may contribute to the preservation of epithelial integrity and therefore reduce the risk of intramammary infections.

Keywords: Ovine mastitis, zinc, bacterial adherence, *Staphylococcus xylosus*.

RéSUMÉ

Mots-clés: Mammite ovine, zinc, adhérence bactérienne, *Staphylococcus xylosus*.

Introduction
Whenever bacteria meet a mucosal surface, close proximity between the microorganism and the host cells must be established in order to initiate successful colonization. In the mammary gland, adherence of both Gram positive and Gram negative bacteria to epithelial cells of the teat and lactiferous sinuses has been related to the prevalence and pathogenesis of mastitis [1,6,9]. Bacterial adherence to epithelial cells represents a way of ensuring bacterial persistence within the mammary gland, by avoiding elimination by host’s cell renewal mechanisms as well as by the flushing effect of milking.

Experiments performed with dairy cattle have shown that zinc contributes to cell integrity and to the maintenance of mammary gland defences, acting upon the keratinization process and reducing the incidence of new infections [8] This reduction has been related to the form in which zinc is incorporated in the diet [16]. It has been suggested that zinc supplied as proteinate is more efficient than mineral zinc, accelerating the keratinization process [5].

The present study aimed at assessing in vitro the influence of diet supplementation with zinc upon *Staphylococcus xylosus* adherence to ovine mammary gland epithelium.

Materials and Methods

ANIMALS

The assay was carried out in a farm with animals from different breeds: Lacaune, Serra da Estrela Branca and Preta, Merino Branco and Preto, Merino Espanhol and Merino Alemão.

Three groups, of 24 adult ewes each, were fed, during 3 months, with different diets: group BZ - fed with 50% zinc proteinate (30ppm) and 50% zinc oxide (30ppm); group ZO - supplemented with zinc oxide (60ppm) and a negative control group, with no diet supplementation - CO.

The assays were carried out in two Merino Branco ewes from each group, sacrificed at the end of the assay.

BACTERIA

A field strain of *Staphylococcus xylosus*, isolated from a subclinical case of mastitis from the same flock was used. The bacterial strain, frozen at 20°C, was cultured onto blood agar plates and incubated overnight at 37°C. From this subculture a bacterial suspension in 10ml phosphate saline buffer, pH 7.4 (PBS) was prepared. Viable bacteria counts were performed [15] and the concentration of bacterial suspension correlated with its optical density at 610nm wavelength, in order to obtain bacterial suspensions of 10^6 colony forming units (CFU)/ml.

MAMMARY GLAND EPITHELIUM

Two Merino Branco ewes from each group were sacrificed at the end of the assay. Mammary glands, refrigerated immediately after slaughter and processed within 4 hours after sacrifice, were aseptically dissected, from teat canal to the cistern and washed with Hanks Balanced Salt Solution (HBSS) to eliminate any secretion still present. Epithelial cells were obtained by gently brushing mammary gland cistern with a toothbrush, as described by [18]. Cell viability was at least 90% as assessed by trypan blue staining, and cell concentration was adjusted to obtain 10^6 cells/ml in PBS. Two samples of cistern epithelium with approximately 5mm^3 were also aseptically collected from each mammary gland, and washed with PBS.

ADHERENCE ASSAYS

Three methods for evaluating *S. xylosus* adherence to mammary epithelium were used:

Adherence to epithelial cells in suspension: The experimental protocol was based on the methods described by Wanasinghe [20] and Olmested and Norcross [13]. Volumes of 0,5ml of cell suspension (10^6 cells/ml) and 0,5ml of bacterial suspension (10^8 CFU/ml) were incubated for 1 hour at 4°C, shaking every 10 minutes. Cells were then washed 3 times in PBS, to eliminate unattached bacteria. Smears in glass slides were prepared in duplicate and stained by the Gram’s method. As negative controls, cell suspensions were subjected to the same protocol, but incubated with sterile PBS instead of bacterial suspension.

Bacterial adherence was evaluated following two parameters: quantification of the number of cells with adherent bacteria, and counting the average number of bacteria adherent to each cell. A total of 100 cells per ewe, randomly selected, was counted.

Adherence to epithelial cells previously fixed to slides: This protocol was based in an adaptation of the method described by Moreira et al. [4]. Epithelial cells were prepared as previously described. Volumes of 0.1ml of cell suspension were deposed on microscope slides by centrifugation (Cytospin 2, Shandon, Wolf Laboratories, UK). The slides, prepared in duplicate, were subjected to mild fixation with 1% paraformaldehyde, for 15 minutes at 4°C. Slides were then incubated during 1 hour at 4°C with 20ml of bacterial suspension, washed 10 times with PBS/Tween 20 (0,05% v/v) to eliminate unattached bacteria and fixed with methanol during 15 minutes at room temperature and Gram stained.

Bacterial adherence was evaluated following two parameters: quantification of the number of cells with adherent bacteria, and counting the average number of bacteria adherent to each cell. A total of 200 cells per ewe, randomly selected, was counted.

Adherence to epithelial tissue from the mammary cistern: The experimental protocol was based in the adaptation of the method described by Ferreira-Dias et al. [7]. Two samples of epithelial tissue from the mammary gland cistern were used. One sample was incubated with 0.5ml of bacterial suspension, 1 hour at 4°C, shaken every 10 minutes. The other sample (as a negative control) was incubated under similar conditions in 0.5ml sterile PBS. After incubation, the samples were rinsed 3 times by immersion in PBS for 10 minutes, fixed in 3% glutaraldehyde in PBS for 3 hours, dehydrated in a serial ethanol baths for 10 minutes and xylol for 1 hour. Samples were then embedded in paraffin, cut in 4 m sections and placed onto glass slides. The slides were deparaffined, rehydrated, Gram stained, dehydrated again with absolute ethanol and xylol and mounted with entellan.

A total of 1000 cells per ewe (500 cells in each slide) were randomly counted as well as the number of bacteria attached to the apical pole of each cell.

Results

The three methods used showed that *Staphylococcus xylosus* adhered to mammary epithelial cells. The adherence assay performed with the epithelial cell suspension incubated with *S. xylosus* showed bacteria attached to all the exposed cell surfaces as well as to clumps and cell debris (figure 1). There were no visible bacteria in the slides except for the ones associated with cells or cell debris. When epithelial cells were fixed onto glass slides and then incubated with the bacterial suspension, bacteria were shown to adhere to all cell surfaces, as well as to cell clumps and debris (figure 2). When epithelial tissue from the mammary cistern was used to evaluate bacterial adherence, some cells showed one or more adherent bacteria; nevertheless, the majority of cells did not show any attached bacteria (figure 3).
None of the negative control slides showed cells with adhering bacteria.

Adherence results, presented in figures 4 and 5, were obtained by random counting of 200 cells per diet group for suspended epithelial cells, 400 cells per diet group for fixed epithelial cells and 2000 cells per diet group for mammary cistern epithelial tissue.

In all assays, Group BZ has shown the smallest number of cells with adherent bacteria, with 142 cells in suspension, 30 fixed cells and 8 cells in the mammary tissue showing adherent bacteria. This group also showed the smallest number of attached bacteria per cell. The average count of bacteria per cell was 2.08 for the cells in suspension, 0.11 for the fixed cells and 0.01 for cells in the mammary tissue. For group ZO, intermediate values for both parameters were observed. The number of cells with adherent bacteria was 162, 70 and 20, respectively for the assay with cells in suspension, fixed cells onto a slide and cells in the mammary tissue. Regarding the average number of bacteria attached to a cell, the results were 30 for the suspended cells, 0.25 for the fixed cells and 0.02 for the cells in the mammary tissue. The control group (CO) showed high statistical significance (p=0.042) when mammary cistern epithelial was used.

**Discussion**

Mastitis remains one of the major health problems for ruminants, including sheep, due to the failures and costs of therapy and associated economical losses in subclinical forms of the disease. The prevention of new intramammary infections is a key aspect of mastitis control that may be achieved through diet manipulation. Trace elements, like selenium and vitamin E, are known to participate in non-specific defences of the organism [14]. Zinc has also been related to the defence mechanisms present at epithelial level, namely in the mammary gland [10, 16]. Zinc deficiency may result in weakened resistance of the epithelial tissue to infection [17].
Bacterial adherence to mammary epithelium has been shown to occur with several mastitis pathogens, including coagulase-negative Staphylococci [2]. In the present study, a field isolate of Staphylococcus xylosus was selected to perform the adherence studies to ovine mammary epithelial cells, based on preliminary bacterial prevalence studies which showed that S. xylosus was the most common mastitis pathogen isolated in the farm (data not published).

The degree of bacterial adherence to epithelial cells has been evaluated by three methods described in the literature that differ in the exposure surface of epithelial cells. When adherence to dispersed epithelial cells, in suspension and previously fixed onto slides, was studied, cellular apical pole and basal membrane were exposed to the S. xylosus in the bacterial suspension. In the adherence assays performed with samples of epithelial tissue obtained from the mammary gland cistern was studied, only the apical pole of epithelial cells was exposed to the bacteria.

When dispersed epithelial cells were used, both the number of cells with adherent bacteria and the number of bacteria attached to each cell were higher, as compared with results obtained when mammary cistern epithelial tissue was used. In other in vitro research, in which adherence to and internalisation of Streptococcus uberis into bovine mammary epithelial cells was studied, it was suggested that extra-cellular matrix proteins may induce or up-regulate proteins involved in adherence/internalisation [3]. The difference observed in our study may be caused by non-specific attachment of bacteria to cellular components, such as basal membrane, when cellular suspensions were used. The assays performed with mammary cistern epithelial tissue are more likely to reflect specific adherence, as bacteria were only detected at the cellular apical pole. It has been shown that adherence to mammary epithelium by other mastitis pathogens involves interaction between bacterial surface proteins and host cell receptors [1,6,9]. ALMEIDA and OLIVER [2] have shown that S. xylosus adherence to and internalisation into cultured bovine mammary epithelium required the intervention of protein kinase C (PKC) and tyrosine kinase (TPK) pathways, as host cell cytoskeleton polymerisation and protein kinase (PK) phosphorylation were required for internalisation. Significant differences in the reduction of bacterial adherence to epithelial cells between the groups fed with zinc proteinate and control group were detected, independently of the method used in the assay. Group BZ has shown a percentage of reduction in adherence attributable to the diet from 66.6 and 77.5%, depending on the method used. When the control group and the group fed with mineral zinc were compared, reduction of bacterial adherence due to the diet had low significance, with values ranging from 25.9 to 43.3%. Taken together, these results suggest that dietary zinc may have a protective action upon the udder and that the association of zinc proteinate and zinc oxide is more efficient in reducing bacterial adherence than mineral zinc.

Commercial diets have adequate levels of zinc, although only small doses of this mineral are available to the animal. This may be due to interaction of elements like calcium, cadmium, cooper, iron, selenium, magnesium, as well as histidine and the level of protein, known to interfere in zinc’s metabolism [12]. Intestinal absorption of zinc seems to be a carrier mediated process, which probably involves interaction with zinc in a chelate form [12]. When this trace element is supplied in the proteinate form it is more easily absorbed and its action is more efficient than when it is fed in the form of zinc oxide [18]. The use of zinc proteinate allows a reduction in the doses of zinc incorporated in the diet as well as in the quantity excreted by the animal, and so contributing to reduce pollution with zinc oxide [11]. An association of proteinate and mineral zinc has been proven to be the most equilibrate and efficient way to supply zinc to the animal [18].

In conclusion, the results of this study suggest that zinc supplementation of sheep diet may contribute to enhancing mammary gland resistance to infection, by reducing the degree of bacterial adherence to epithelium. Furthermore, the association of zinc proteinate to mineral zinc has shown better in vitro results than supplementation with zinc oxide alone. Future work must be carried out, in order to confirm these in vitro results and to analyse the cost/benefit relation of this combination. The inclusion of proteinate in the diet may also contribute to the environmental preservation by reducing the level of this trace element excreted by the animals.

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References


