DOCTORAL (PhD) DISSERTATION THESES

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THE INFLUENCE OF MASTITIS ON MILK COMPOSITION WITH SPECIAL REGARD TO ITS D-AMINO ACID CONTENT

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1. BACKGROUND OF THE RESEARCH, OBJECTIVES

The mastitis of cows producing milk belongs to the so called complicated productive diseases. Keeping the cows in professional conditions, giving up grazing on open pastures, the density of the stock, mechanic milking as well as the disadvantageous effect of high milk production on metabolism all contribute to the fact that micro-organisms multiplying either in the environment or on the mucous membrane of the cow can easily get into the udder where they cause mastitis. Therefore apart from internal and external factors causing to be prone to the disease, various microorganisms also have a crucial role.

The most significant factor in the economic loss is the decrease in the profit from milk trade. The main reason for this is the decline of milk production, but occasionally poor quality, and milk unsuitable for human consumption can be responsible as well.

Even in milk produced in the early degree of subclinical mastitis, certain changes can be recognised, which have a negative influence in terms of both milk procession and milk consumption.

Several publications have reported on the D-amino acid contents of milk and dairy products making it clear that D-amino acid contents were mainly the result of microbial activity and that the technological intervention played here only a minor role. Therefore we supposed that D-amino acids present in traces in the mixed milk drawn from healthy cows are the results of a bacterial infection evolved during subclinical mastitis and that they get into the milk as metabolism products of bacteria.

It can also be interesting for diagnostical purposes how the D-amino acid content changes in accordance with the progress of sublinical mastitis, and whether there is a connection between the microbial species causing the inflammation and the D-amino acid content of the milk.

Main targets of the experiments of these theses can be classified and summarized as follows:

1. Determination of diagnostic value of free amino acid and free D-amino acid content of the cow's milk.

1.1. Determination of the free D-amino acid content of the first two milk flows from healthy cows and the mixed milk of the completely milked udder.

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1.2. Comparison of the free amino acid and free D-amino acid content of milk drawn from healthy and mastitic cows.

2. Examination of separability of bacterium species causing mastitis based on free amino acid and free D-amino acid content.

2.1. The influence of the microbe species on the free amino-acid content of milk.

2.2. The influence of the microbe species on the free D-amino-acid content of milk.

2. MATERIALS AND METHODS

2.1. Milk samples and conditions of the sampling

Milk samples were obtained from mastitic udder from Holstein-Friesian cows of three South-Transdanubian cow farms. All Holstein-Friesian stocks were kept unbound. Genotypes of entities, number and phase of the lactation were not considered as sampling criteria. Feeding was done with TMR (Total Mix Ration).

For the distinction between healthy and mastitic entities and for the identification of the degree of the inflammation, the domestic version of the California-Mastitis-Test (CMT), the Mastitest were applied – keeping the hygienic regulations.

In case of the negative (–) samples, the sample was taken from entities having all the four udder quarter healthy. In positive cases, the entities received a +, ++,+++, ++++ evaluation according to the change of the viscosity of the milk-reagent mixture. Sampling was done in 25 entities by udder quarters.

In order to find out whether the first milk flows rich in bacteria contain D-amino acids, we took the first two milk flows of each udder quarter of 5 cows separately (approx. 10-12 cm^3 for each entity), and after we made sure that the entity was negative according to the Mastitest, we compared the amino acid composition of the first milk flows to that of the milk of the same entities without the first milk flows. The milk samples was cooled immediately after sampling in iced water, then placed them into deep freeze within 2 hours and stored at –25 °C until the preparation for the amino acid analysis.

After a Mastitest was carried out, 200 milk samples taken from udder quarters of the cows involved in the experiment were sent for bacteriological examination. In sterilized plastic pots 10-12 ml milk flows were taken, and the samples were stored deep-frozen (–18 °C) until

they reached the laboratory. The laboratory examinations were done at the National Veterinary Hygienic Station (Budapest).

2.2. Microbial analysis

During the laboratory processing procedure 0.01 ml milk was placed on a Columbia agar *(Merck KgaA, Darmstadt, Germany)* containing 5% sheep blood. After incubing the nutrient media at 37 °C in aerobic atmosphere, the cultures were assessed in 24 and 48 hrs. Cultures containing at least 5 bacterium colonies with the same morphology were considered as suspicious in mastitis pathogenic respect. The evolved pathogenic bacterium species, classified on the basis of the colony morphology , Gram dyeing, catalase and oxidase test, were identified on species level, using the appropriate panels of the ATB bacterium identification system *(bioMérieux s.a., Marc-l'Etoile, France)* based on examination of the biochemical properties.

2.3. Chemical analysis

The amount of free amino acids and free D-amino acids was measured after the precipitation of proteins with trichloroacetic acid. Before the analysis diastereoisomers were produced from D-and L-enantiomers with OPA (o-phthaldialdehyde) and TATG (1-thio-β-D-glucose-tetraacetate) in case of free D-amino acids, with OPA and 2-mercaptoethanol in case of free amino acids. The derivatization and the analysis were carried out with a LaChrom type MERCK-Hitachi high performance liquid chromatograph (HPLC) and the data conversion utility for D-700 HPLC system manager.

2.4. Statistical analysis

Data analysis was carried out with the use of SPSS for Windows 10.0 (1999) statistical program.

In order to decide whether there is a statistically verifiable difference between free D-amino acid concentration of the first milk flows and the mixed milk, respectively, independent samples „t" test was carried out.

The difference between free amino acid and free D-amino acid content of the healthy and the mastitic milk samples was examined using one-way analysis of variance. For the comparison of the mean values of the examined variables Student-Newman-Keuls test was used at a 5% confidence level.

Discriminant analysis was employed in order to find out based upon what variables (independent variables: free amino acid, free D-amino acid) the groups the mostly differ from each other (Dependent variables: degrees of the MastiTest , bacterial species), that is, whether the belonging to the group can be predicted based on a chosen group of the indipendent variables.

3. RESULTS AND DISCUSSION

3.1. Free D-amino acid content of the first two milk flows and that of the mixed milk withouth the first milk flows from healthy cows

During our measurements we established that bacteria rich first two milk flows contain significantly more D-Asp, D-Glu and D-Ala than the mixed milk without the first milk flows. Besides the above, we were able to identify even D-allo-isoleucine in the first milk flows which we could not find even in traces in the mixed milk.

3.2. Free amino acid and free D-amino acid content of milk milked from healthy and mastitic cows

In higher degree of mastitis the quantity of the free amino acids increases considerably. Milk rated + contains twice, milk rated ++ three and a half times and the milk rated +++ and ++++ five to five and a half times more free amino acid than the normal milk (ie negative as per the mastitest). Compared to the values of normal milk, the most spectacular increase is seen for isoleucine, leucine, alanine, aspartic acid, proline and glutamic acid.

In case of the rest of the amino acids the increase is not substantial due to the relatively small absolute amounts. Carried out the discriminant analysis it was established that the examined groups (degrees of the Mastitest) was well separated based on the absolute amount of free amino acids, and each group could be in 100% categorized on the basis of the given variable.

The two, three and four cross rating milk samples resemble more and more the free amino acid composition of colostrum. This tendency was already noted in the case of all other milk components. Because of the minor absolute quantities, in the case of the other amino acids the increase is negligible.

As regards the free D-amino acid content (in mg/100 cm^3) of the milk of healthy and unhealthy cows as rated by the mastitest, we found that even the milk samples rated as negative by the mastitest do contain free D-aspartic acid, D-glutamic acid and D-alanine. However, the quantities present are almost negligible as compared to the quantities occurring in the milk samples corresponding to various mastitest ratings. In the + rated samples besides D-Asp, D-Glu and D-Ala one also finds D-valine, D-allo-isoleucine, D-leucine and D-lysine. In the case of ++, +++ and ++++ rated samples we were able to identify two further D-amino acids, namely D-serine and D-proline. Not even traces could be identified of the D-enantiomers of other amino acids that are building blocks of proteins.

The free D-amino acid content of milk milked from healthy and sick cows expressed in percentage of the total free amino acids. The calculation was made by dividing the quantity of total amino acids as determined by HPLC with the quantity of free amino acids as determined by ion exchange chromatography (IEC) and the result was multiplied with one hundred. It can be said from the data featured that the relative concentration of the free D-amino acids is the lowest in the case of the negative and + rated mastitest subjects. In the case of most amino acids as the mastitest rating increases so does the relative quantity of D-amino acids. Exceptions are D-leucine and D-lysine where we found no difference between the samples originating from ++, +++ and ++++ rated subjects.

3.3. Microbial analysis of the milk samples

In the mastitic milk samples eight bacterial species were identified during the microbial analysis. Chemical analysis was carried out only in case of monocontaminated samples. The studied bacterial species typically mastitis pathogenic in Hungary are as follows:

Streptococcus dysgalactiae, Streptococcus uberis, Escherichia coli, Staphylococcus aureus, Pasteurella multocida, Corynebacterium bovis, Arcanobacter pyogenes, Pseudomonas aeruginosa.

3.4. The influence of the microbe species on the free D-amino acid content of milk

Enantiomer pairs of aspartic acid, glutamic acid and alanine were measured in milk samples derived from mastitic udders with Mastitest degree of +++ and ++++ where mastitis was caused by the above eight bacterial species. Since in peptidoglycans of cell walls of bacteria and in metabolism products of bacteria these three amino acids are present in highest concentration therefore their quantities can be evidenced without any doubt. Free D-amino acid and free amino acid contents of samples with +++ and ++++ do not differ significantly, therefore they are suitable for examination of amino acid contents jointly.

We concluded that D-aspartic acid ratio (D/D+L*100) of bacteria-free, Mastitest-negative milk sample significantly differ from those of milk samples containing bacterial species. Out of the microbe species D-aspartic acid contents of the species *Staphylococcus aureus* were significantly higher than those of other species. The species *Streptococcus dysgalactiae, Streptococcus uberis, Corynebacerium bovis, Pseudomonas aeruginosa, Arcanobacter pyogenes* and *Pasteurella multocida* do not differ from each other based on D-aspartic acid contents, however. Based on percentage of D-aspartic acid therefore only the Mastitest-negative, bacteria-free milk sample and the species *Staphylococcus aureus*, respectively, can be identified. Thus, it can be concluded that D-aspartic acid is not suitable for the identification of the pathogen microbes.

Examining average values and absolute amount of D-glutamic acid of the species groups it can be established that this amino acid is suitable for the identification of the microbe species because between the groups the difference in quantity of the amino acid in question is significant.

That means that on the basis of examination of glutamic acid enantiomers identification of the pathogen microbe species would be possible.

Based on examination of the average values of D-alanine it can be established that the species *Escherichia coli, Staphylococcus aureus* and *Pseudomonas aeruginosa* cannot be identified on the basis of this amino acid, D-alanine contents of the other species show significant differences, however.

3.5. The influence of the microbe species on the free amino acid content of milk

Examination of free amino acids was carried out from the same samples from which the enantiomers were determined.

On the basis of the absolute amount of the D-amino acids it can be established that the Mastitest-negative milk sample significantly differed from all of the other samples, except the Phe content. Between the species there was significant difference in case of some amino acid. Milk from mastitic udder with mastitis caused by *Escherichia coli* contains significantly more Val than all of the other milk samples.

Tyr content of milk infected by *Streptococcus uberis and Escherichia coli* do not significantly differ from each other, but they significantly do more from mastitic milk samples with mastitis caused by *Staphylococcus aureus* and *Arcanobacter pyogenes.* In the latter cases of two bacteria species we concluded that Tyr contents of milk significantly increased compared to Mastitest-negative milk sample and the other sample.

Considering sum of the amino acid there was significantly lower compared to all of the bacteria species. In case of inflammation caused by *Corynebacterium bovis* and *Streptococcus dysgalactiae* sum of the amino acid content of milk do not differ from each other, but they significantly do lower from the other samples. The same is true in