
SUMMARY OF PhD THESIS

The impact of mastitis, pathogens and oxidative stress on nutritional and functional components of goat milk

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INTRODUCTION

Goat milk ranks fourth in terms of milk production worldwide after cow, buffalo and sheep milk. Goat breeding is of great economic importance especially in regions where the climatic conditions are not favorable for cattle breeding (AMIGO and FONTECHA, 2011). According to Eurostat, in 2022 Romania occupies the third place in terms of goat population in the European Union, after Greece and Spain. The demand for goat milk products is increasing, as they are preferred precisely for their nutritional benefits, which include higher digestibility and hypoallergenic properties compared to cow's milk (MILLER and LU, 2019). Although the overall composition of goat milk is influenced by numerous noninfectious factors, the impact of infectious inflammatory processes is by far the most important. Thus, inflammation of the mammary gland causes numerous changes in milk composition (LEITNER et al., 2004).

Since the scientific literature is abundant in studies that are of particular interest to cow's milk, the present thesis complements the information regarding the impact of the inflammatory processes of the mammary gland on the nutritional components of goat's milk, an increasingly popular food product at national level, but also worldwide. Furthermore, the research presented throughout this thesis involves the evaluation of subclinical mastitis in goats, a vast and complex subject that still has much to offer to researchers. The inclusion in the study of native Carpathian goats aimed to bring this breed under the spotlight, as well as to update data regarding the composition of milk, both in clinically healthy animals and in females affected by subclinical mastitis, hoping that the information provided will be useful to the scientific community and to the development of the dairy goat industry in Romania.

WORKING HYPOTHESIS AND RESEARCH OBJECTIVES

Mastitis results in changes in the number of somatic cells, as well as increased activity of inflammatory enzymes. Depending on the severity of injuries, there may be changes in the milk physico-chemical and biochemical parameters, as well as excess formation of reactive oxygen species, which lead to the subsequent induction of oxidative stress, with an increased level of oxidation of some important constituents, such as proteins or fatty acids.

The working hypothesis of this thesis was to demonstrate the presence of changes in the physical and biochemical parameters of goat's milk associated with the installation of subclinical inflammatory processes. Therefore, we aimed to update the scientific knowledge regarding goat milk using certain investigation methods and working protocols.

In order to confirm this hypothesis, the following **general objective** was formulated:

- ✓ Establishing correlations between the severity of mammary infection, the type of pathogens, the state of inflammation induced by subclinical mastitis, and the profile of biochemical parameters, as well as the oxidative and nitrosative stress markers in goat milk.

In order to achieve the general objective, this thesis was structured in four original studies, based on a series of **specific objectives**:

- ✓ Analysis of microflora and hygienic quality of raw goat milk collected from tanks in farms located in Alba, Sălaj and Cluj County, Romania.
 - Collection of bulk milk samples from 18 farms in Transylvania;
 - Characterization of milk microflora, aiming to identify bacterial genera and species and to establish the hygienic quality of analyzed milk by determining the total number of germs (TNG).
- ✓ Identification and characterization of the bacterial microflora of raw goat milk collected from clinically healthy animals and performing the antibiotic sensitivity testing of isolated bacterial strains
 - Collection of individual goat milk samples from clinically healthy animals (without signs of mastitis) from a private farm in Mureș county, Transylvania;
 - Evaluation of milk microflora by isolating and identifying bacterial species, using two identification methods: the Vitek® 2 Compact system and 16S rRNA sequencing;
 - Determining the sensitivity to antibiotics of bacteria in order to assess the presence of antibiotic resistance;
- ✓ Analysis of the somatic cell count (SCC), assessment of milk sediment and determination of milk general composition collected from Carpathian goats.
 - Determination of SCC from the previously collected goat milk and cytological evaluation of the milk sediment, aiming at the differentiation and characterization of the cell population, as well as evaluating the influence of microorganisms on milk somatic cells;
 - Analysis of the general physico-chemical parameters of goat's milk;
 - Observing correlations regarding the presence of pathogens and SCC and also evaluating the influence of pathogens on milk general composition.
- ✓ Establishing correlations between the profile of goat milk microflora and its biochemical parameters
 - Evaluation of certain inflammatory enzymatic markers, represented by milk lactate dehydrogenase and β -glucuronidase;
 - Assessment of oxidative stress indices represented by catalase, glutathione-peroxidase, lipid peroxidation index, total antioxidant status, as well as the evaluation of DNA oxidative degradation by measuring 8-hydroxydeoxyguanosine;

- Assessing the presence of nitric oxide as a marker of nitrosative stress;
- Evaluation of goat milk protein profile, using on chip-electrophoresis (microfluidic system).

STRUCTURE OF THE DOCTORAL THESIS

The PhD thesis entitled "The impact of mastitis, pathogens and oxidative stress on the nutritional and functional components of goat milk" is composed according to the editing rules, of two parts: Part I - Current state of knowledge and Part II - Personal contribution, comprising a total of 153 pages, 49 figures, 21 tables and 185 bibliographic references.

PART I - Current state of knowledge

This first part comprises 4 chapters summarizing up-to-date information on goat milk and mastitis in this species.

Chapter I is entitled "Goat milk composition" and presents in a detailed manner aspects related to the physico-chemical and biochemical constituents of goat milk.

Chapter II, entitled "Mastitis in goats", comprises two sub-chapters, "General aspects" and "The etiology of bacterial mastitis".

Chapter III, entitled "Mastitis-induced changes in the main biochemical parameters in goat milk" includes 6 subchapters: "Proteins", "Glucids", "Lipids", "Enzymes", "Electrical conductivity" and "Somatic cell count".

Chapter IV is named "Oxidative stress" and it is organized into 2 subchapters: "General" and "Mastitis-induced changes on oxidative stress markers in goat milk" which bring together relevant information regarding the presence of oxidative stress during inflammatory processes of the mammary gland.

PART II - Personal contribution

Part II is comprised of 8 chapters that present the working hypothesis and objectives of the work, the materials and methods used, the results obtained for each individual study, general conclusions, recommendations, as well as original aspects of the thesis.

Chapter V is entitled "Working hypothesis and research objectives" and presents the working hypothesis, the general objective, as well as the specific objectives of this paper.

Chapter VI is named "Materials and methods - general aspects", being structured in 4 subchapters which describe the organization of the investigations, the biological material used, the investigations that have been carried out in each study and the statistical analysis methods.

Chapter VII is entitled "Study regarding bulk goat milk microflora from Transylvanian farms", being made up of 5 subchapters. The study focuses on the

investigation of raw goat milk from the tanks of 18 farms, with the aim of describing the bacterial microflora, as well as to evaluate the hygienic quality of milk.

Chapter VIII is called "Evaluation of raw goat milk microflora and antibiotic susceptibility testing" and includes 5 subchapters in which are described the objectives of the study, materials and methods used, results and partial conclusions. This chapter aims to identify bacterial species from goat milk, Carpathian breed, collected from a private farm and to evaluate the presence of antibiotic resistance by performing antibiograms using the diffusimetric method.

Chapter IX is entitled "Evaluation of physico-chemical and hygienic parameters of goat milk" and presents aspects related to the number of somatic cells, milk cytology and the main physico-chemical parameters, as well as the influence of pathogens on the milk composition.

Chapter X, named "Evaluation of biochemical parameters, oxidative and nitrosative stress markers of goat milk" is structured in 5 subchapters and presents data related to the impact of microorganisms on inflammatory enzymes' activity and oxidative stress in goat milk. Moreover, the milk protein profile is also investigated, focusing on the changes that occur during subclinical mastitis, as well as presenting various correlations between the analyzed parameters.

Chapter XI, "General conclusions and recommendations", includes the main conclusions drawn from the 4 original studies, as well as a number of recommendations.

Chapter XII is entitled "Originality and innovative contributions of the thesis" and includes the main original elements of the present thesis related to the investigation of the impact of subclinical mastitis on the nutritional and functional components of goat milk.

RESEARCH RESULTS

Chapter VII - Study regarding bulk goat milk microflora from Transylvanian farms

Bacteriological examination

All 36 bulk milk samples were positive for bacterial growth, with a high prevalence of Gram-positive bacteria. The highest prevalence was registered by the genus *Staphylococcus* (25%), followed by *Escherichia* (15%), *Micrococcus* (14%), *Raoultella* (2%), *Trueperella* (2%) and *Enterococcus* (1%). The following bacterial species were identified (Vitek® 2 and API® Staph): *S. xylosus*, *S. epidermidis*, *S. hominis*, *Kocuria varians*, *Kocuria rosea*, *Aerococcus viridans*, *Streptococcus uberis*, *B. licheniformis*, *E. durans*, *E. faecium* and *Micrococcus luteus*. The following Gram negative species were identified: *E.coli*, *K. oxytoca*, *K. pneumoniae*, *Hafnia alvei*, *Raoultella planticola*, *Aeromonas sobria* (fig. 1).

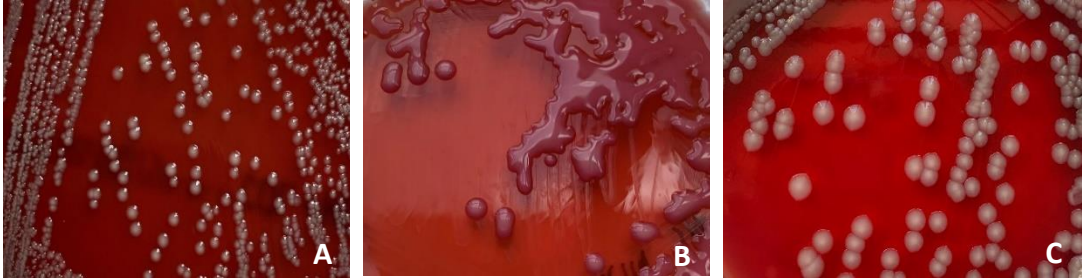


Fig. 1 Colony morphology: A - *E. coli* (blood agar), B - *Klebsiella* spp. (MacConkey), C - *Aeromonas* spp. (blood agar)

Total number of germs

The samples collected from farms in Alba county recorded the highest values, however no sample from this study exceeded the norms provided by Regulation (EC) no 853/2004 of the European Parliament regarding the specific hygienic rules for raw milk from species other than cow's, being therefore suitable for human consumption.

Chapter VIII - Evaluation of raw goat milk microflora and antibiotic susceptibility testing

Bacteriological examination. Molecular analysis.

A high percentage of positive samples was highlighted (86.84%), with an increased prevalence of the genus *Staphylococcus* (32 isolates), *Enterococcus* (24 isolates) and *Bacillus* (18 isolates), the most frequently isolated species being *E.durans*, *B. licheniformis* and *S. aureus*. Other identified bacterial genera were *Macrococcus*, *Aerococcus* and *Streptococcus*, but with a lower prevalence.

The present study reports for the first time the isolation of *Staphylococcus petrasii* ssp. *jettensis* from raw goat milk. The only Gram negative bacteria identified were *Moraxella osloensis* and *Aeromonas hydrophila*, suggesting the low involvement of this category in the etiology of subclinical mastitis in goats.

Antibiotic susceptibility testing

Regarding general antibiotic sensitivity, in the present study an increased efficiency of florenicol (86.9%) was observed, followed by neomycin (72.62%) and gentamicin (69.05%), a high percentage of resistance being recorded in the case of bacitracin (100% of isolates), cefquinome (91.67%), amoxicillin with clavulanic acid (61.9%) and oxytetracycline (59.52%).

38.10%, of the tested microorganisms were resistant to agents belonging to four antimicrobial classes, and the highest resistance, to one agent from seven classes, was observed for 2.38% of the tested strains, demonstrating the presence of multidrug resistant isolates in milk goat.

Chapter IX - Evaluation of physico-chemical and hygienic parameters of goat milk

The analysis of the somatic cell count (SCC) highlighted significant differences between the analyzed categories, as well as the influence of different mycoorganisms on this parameter. Thus, the lowest number of somatic cells was recorded in the case of microbiologically negative milk, and the highest average value was found in the case of *S. aureus* infection, demonstrating the evolution of a subclinical inflammatory process (Table 1).

Table 1

SCC results based on microbiological category

Category	SCC ($\times 10^3$ cel./mL)	Log ₁₀ NCS	Significant difference ($p \leq 0.05$)
N	236.4 \pm 64.1	5.36 \pm 0.11	SNA, B, SA
SNA	710.52 \pm 458.02	5.76 \pm 0.27	N, E, SA
E	251.75 \pm 112.7	5.36 \pm 0.17	SNA, B, SA, A
B	709.83 \pm 385.91	5.79 \pm 0.22	N, E, SA
SA	4377.83 \pm 1426.65	6.62 \pm 0.12	N, SNA, E, B, A
A	871.90 \pm 1478.12	5.64 \pm 0.54	E, SA

N - microbiologically negative samples, SNA - non-aureus staphylococci samples, E - enterococci, B - *Bacillus* genus, SA - *S. aureus*, A - other pathogens

Regarding milk cytology, milk under N category recorded values of cell subpopulations that fall within the reference ranges of the species, with a high percentage of neutrophils and macrophages, followed by lymphocytes and epithelial cells. Numerous cytoplasmic particles and other cellular debris commonly found in goat's milk were also highlighted.

When analyzing the milk categories using the statistical test of multiple comparisons, a high percentage of neutrophils was observed in the microbiologically positive milk samples, with a marked increase in the case of *S. aureus* infection (Table 2). Similarly, a decrease in the percentage of lymphocytes and macrophages was found for SNA, E, B, SA and A categories.

Table 2

Milk cells percentage for each sample category

Category	N %	B %	E %	L %	M %	CE %
N	42.50 \pm 1.17	0.08 \pm 0.04	0.8 \pm 0.42	18.10 \pm 1.52	29 \pm 1.24	9.5 \pm 0.70
SNA	53.91 \pm 6.02	0.06 \pm 0.04	1.91 \pm 1.50	16.29 \pm 3.96	19.82 \pm 6.10	8.45 \pm 1.28
E	49.91 \pm 5.43	0.05 \pm 0.05	1.82 \pm 1.64	17.91 \pm 2.25	21.82 \pm 5.41	8.08 \pm 1.08
B	51.44 \pm 3.97	0.03 \pm 0.05	1.77 \pm 1.35	16.61 \pm 2.68	21.60 \pm 3.58	8.55 \pm 1.14
SA	71.00 \pm 1.09	0.01 \pm 0.04	3.16 \pm 0.98	9.83 \pm 1.32	8.68 \pm 1.64	7.16 \pm 0.98
A	48.22 \pm 3.86	0.04 \pm 0.05	1.55 \pm 1.13	17.11 \pm 4.13	24.43 \pm 2.83	8.66 \pm 1.11

N-neutrophils, B-basophils, E-eosinophils, L-lymphocytes, M-macrophages, CE-epithelial cells

The analysis of the general physico-chemical parameters of milk (total proteins, lipids, lactose, non-fat dry matter, density, pH) did not reveal statistical differences between categories, suggesting that subclinical mastitis in goats does not significantly influence the general physico-chemical composition of milk. The only parameter that

showed changes was the electrical conductivity, higher in the case of milk samples within E and SNA category compared to N, demonstrating the increased influx of electrolytes in milk during inflammatory processes.

Chapter X - Evaluation of biochemical parameters, oxidative and nitrosative stress markers of goat milk

Inflammatory enzymatic indices

Mastitic goat milk showed a much more pronounced inflammatory enzyme activity (lactate dehydrogenase and β -glucuronidase) ($p \leq 0.05$) compared to healthy milk, activity that was associated with the pathogenicity of isolated bacteria, aspects positively correlated with SCC (fig. 2).

Oxidative and nitrosative stress markers

The antioxidant enzymes, namely catalase (CAT) and glutathione-peroxidase (GPx), registered a significantly increased activity ($p \leq 0.05$) in goat milk positive for microorganisms, having the highest level in *S. aureus* infection. The total antioxidant status (TAS) was lower in case of subclinical mastitis, indicating that the antioxidant capacity of goat milk can be significantly influenced by both the presence of bacterial agents and their pathogenicity (fig. 3).

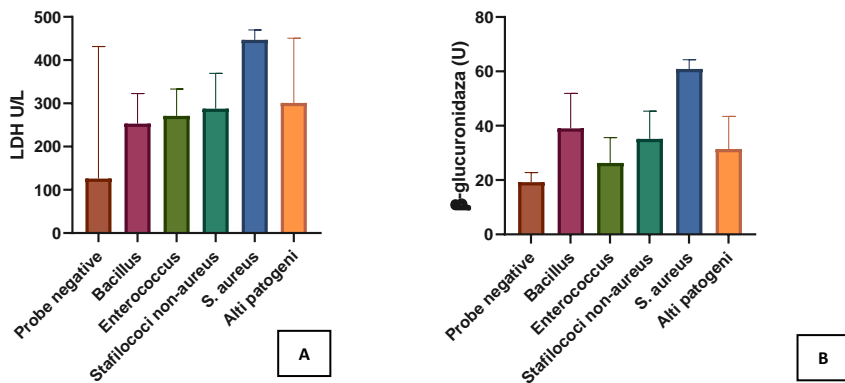


Fig. 2 Changes in milk LDH (A) and β -glucuronidase (B) according to bacteriological status

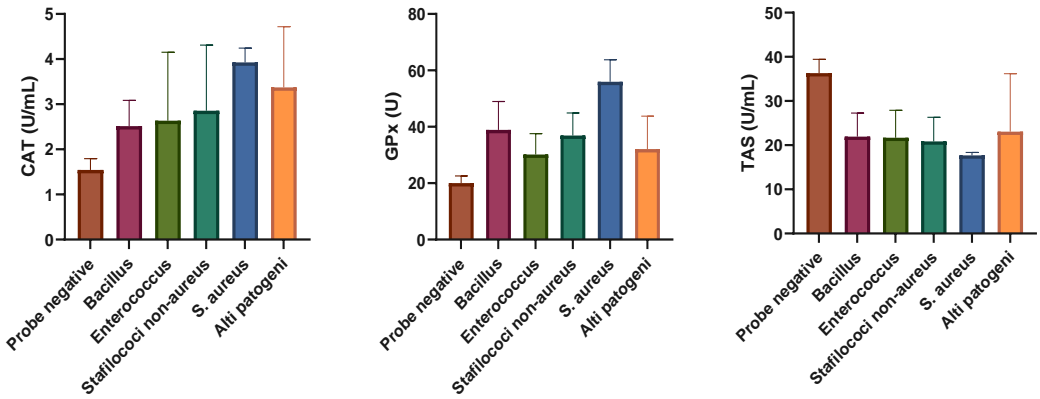


Fig. 3 CAT, GPx and TAS in goat milk

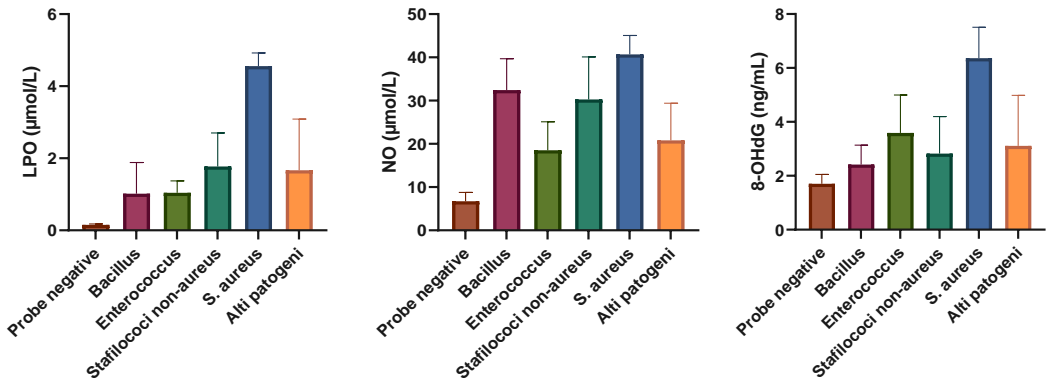


Fig. 4 LPO, NO and 8-OHdG in goat milk

Microbiologically positive milk samples are associated with significantly higher mean values ($p \leq 0.05$) of lipid peroxides (LPO), nitric oxide (NO) and hydroxydeoxyguanosine (8-OHdG), suggesting the presence of oxidative stress and the consecutive oxidation of biomolecules in the context of subclinical inflammation (fig. 4).

Goat milk protein profile

Regarding milk soluble proteins, the following major proteins were identified: α -lactalbumin (α -LA) and β -lactoglobulin (β -LG), as well as minor proteins: immunoglobulins (Ig), serum albumin (SA) and lactoferrin (Lf). The identified caseins were represented by α s-casein (α s-CN), β -casein (β -CN) and κ -casein (κ -CN).

The protein profile revealed higher concentrations of lactoferrin, serum albumin and immunoglobulins in mastitic milk. Among caseins, only β -CN showed variations between the analyzed categories, with lower average values in the case of milk from positive for staphylococci, suggesting that this fraction is more susceptible to milk's own

proteases, as well as bacterial ones. α -CN and κ -CN did not show significant variations between sample categories.

Subclinical mastitis does not significantly influence the level of β -LG and α -LA in goat milk.

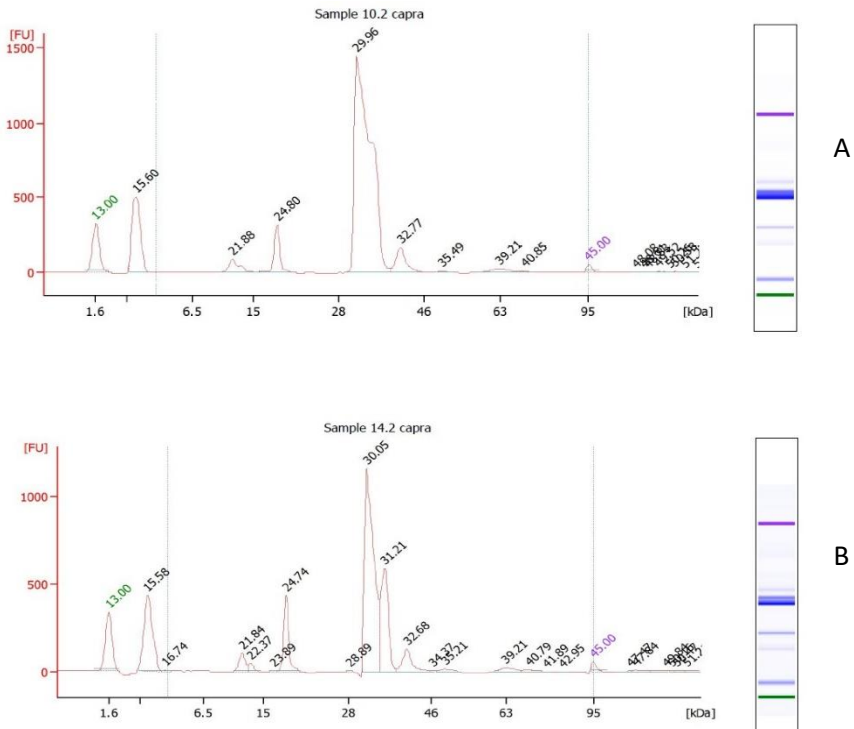


Fig. 5 A - Electropherogram of a negative milk sample, B - milk sample - *S. aureus*

In conclusion, the present thesis demonstrated the influence of mammary inflammatory processes on milk constituents, processes that result in the installation of oxidative stress and the oxidation of biomolecules. All these changes are much more significant following the infection with microorganisms with increased pathogenicity, such as *S. aureus*.

RECOMMENDATIONS

Taking into account the data presented throughout this thesis, we strongly recommend carrying out further investigations on both healthy and mastitic milk from goats belonging to more diverse populations, of different breeds, physiological and environmental conditions, this paper opening new perspectives in the field of small ruminant mastitis. Therefore, we recommend the following:

- ✓ Carrying out microbiological examinations of milk and antibiotic sensitivity testing in case of bacterial mastitis confirmation, in order to avoid therapy failure and the occurrence of antibiotic resistance phenomenon.
- ✓ Using molecular biology methods to identify bacterial species with greater accuracy.
- ✓ Carrying out complementary investigations of milk as alternative tools in the diagnosis of mastitis.
- ✓ Evaluation of inflammatory enzymes and oxidative stress markers in milk as a predictive method of an intramammary infection, especially in the case of major pathogens.
- ✓ Updating current knowledge with studies carried out on a larger population, which would allow a detailed assessment of the impact of each bacterial species on milk components and oxidative and nitrosative stress indices.
- ✓ Continuing research in the field of mastitis in goats and developing new and reliable diagnostic techniques for this condition.

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