PhD THESIS

In vitro apiphytotherapy induced changes of the antibiotic resistence profile in mastitic cows

SUMMARY OF Ph.D. THESIS

PhD student **Giupană Radu Mihai**

Scientific coordinators **Prof.univ. dr. Marina Spînu**

ABSTRACT

Diseases in lactating animals have a significant impact on production, with mastitis being one of the most common infectious diseases in dairy cattle worldwide, regardless of the rearing system, type or size. From an economic point of view, mastitis is one of the most damaging diseases, with a significant influence on the quantity and nutritional qualities of milk (OECD/FAO (2019), "OECD-FAO Agricultural Outlook", OECD Agriculture statistics (database), http://dx.doi.org/10.1787/agr-outl-data-en.).

Promoting awareness of the importance of identification and therapeutic elements can lead to a significant increase in production by reducing losses and excessive costs in dairy industry. Currently, the control of mastitis involves the use of antibiotics, thus increasing concern about the overuse of these compounds. Similarly, the possibility of increase in antibiotic resistance leads to a decrease in their therapeutic efficacy and thus to the need for synthesis of new compounds. This process has many unknown sides, the influencial factors being extremely numerous (the infecting species and its intrinsic resistance, the therapeutic schemes used, the pollution of the environment with resistance plasmids, etc.).

The morphological and functional analysis of the bacteriome supports the evaluation of the milk quality and the assessment of the risk faced by the consumers, being defined especially the antibiotic resistance profile. The results serve a dual purpose, first of redefining farm-level therapies in the control of mastitis, and second to prevent the transmission of antibiotic resistance to consumers. The association of results related to the MAR (multiple antibiotic resistance) index with the immunological profile of animals diagnosed with mastitis can direct the therapeutic protocol so as to simultaneously ensure the animal's recovery and increase the quality of milk for consumption (Rindt, 2009a, b, 2011).

Alternative methods for overcoming the difficulties induced by antibiotic resistance have developed on a large scale in recent decades. These include nanotechnologies, phage therapy, plant extracts (including essential oils), various animal protein isolates (IgY) or bacteriocins (Radzikowski et al., 2020). The major advantage of alternative therapies is the lack of resistance in the case these therapeutic compounds are used, in addition to the results obtained *in vitro* and *in vivo* that reveal efficacy by successfully inhibiting the growth / destruction of bacteria from mastitis cows. The intensification of the farming process, particularly complex and aligned with the notion of "more with less" has had unequal effects on the human / animal / environment matrix interface. Currently, in the context of socio-cultural development focused on the concept of "One Health, One Welfare" there is a campaign to raise awareness of the negative impact of industrial intensification in animal exploitation on

the environment (Del Prado et al., 2013) , animal welfare (Koeck et al., 2014), equitable rural development and last but not least, on human health.

The result of such a campaign is beneficial, that of intensifying efforts to design fair and sustainable dairy farming systems and beyond (Jay, 2006).

Mastitis is one of the most common bacterial diseases in most dairy farms, regardless of the rearing system, and is one of the major diseases treatable through the use of antibiotics. The widespread use of antibiotics in the control of mastitis greatly increases the risk of apperance and transfer of antibiotic resistance to consumers. Such a possibility is constantly in the attention of animal and public health authorities, requiring scientifically grounded redefinition of antibiotic therapies taking into account the intersection of animal welfare with social concerns (Ruegg, 2009, 2017; Stevens et al., 2016a and b).

Medicinal plants represent a historically consolidated source of natural therapeutic agents, with increased bioavailability. Plants contain compounds and substances with antibacterial effect and utility as innovative antibiotics, efflux pump inhibitors, anti-virulence components or could even be useful in combination with synthetic drugs (Mushtaq et al., 2018). Bioprospecting is a branch of science that identifies new sources of natural products (microorganisms, plants, fungi, animals) with pharmaceutical utility or applications in biology. Over 80% of current medicinal substances come from natural products, still playing an important role in therapy (Ashforth et al., 2010).

The present research focused on four priority directions, aiming at:

- Etiological investigations, including the definition of antibiotic resistance profile in cattle (cows and buffaloes) with clinical and subclinical mastitis,
- Changes in the immunological profile of animals with clinical and subclinical mastitis in relation to the pathogenic activity of the isolated microbiome,
- Identification of the influencial factors of the antibiotic resistance profile in the case of bacterioma isolated from animals with mastitis,
- Identification of plants with antibacterial potential, increased bioavailability, and evaluation of the *in vitro* effects of these extracts.

The first part of the thesis is structured in three chapters, summarizing current literature data on the therapeutic potential and biological effects of some medicinal plants, especially from the Lamiaceae family and their usefulness in animal therapy (Chapter 1), the complex etiology of mastitis in cattle and the pathogenic factors of the different bacterial genera that constitute the pathogenic microbiome in these cases (Chapter 2) and briefly reviews the installation mechanisms and the importance of the antibiotic resistance phenomenon within the “One Health” concept (Chapter 3).

As an introduction to the second part of the thesis which includes the results of personal studies, the aims are presented, followed by ten original published studies, structured in four chapters, focused on investigating the structure and pathogenicity of the microbiome in mastitis in cows (Chapter 4, subchapters 4.1 -4.3), on the

quantification of non-specific immune responses to the isolated microbiome in mastitis cows (Chapter 5, Subchapters 5.1-5.2), characterization of potential factors influencing the mastitis bacteriome and its pathogenicity variations (Chapter 6, Subchapters 6.1-6.2) and on the evaluation of the alternative therapeutic potential of selected plant extracts (chapter 7, subchapters 7.1-7.3). The outcome of the research is presented in the General Conclusions of the thesis.

In subchapter 4.1, the differences in prevalence and etiology of subclinical mastitis in cows and buffaloes from Transylvania, Romania were investigated. The prevalence of clinical mastitis in buffaloes and cows is considered similar by the literature ranging from 8 to 40% and 19.9% to 44.8%, respectively. The percentage is highly variable depending on the region and raising technology. Nevertheless, not much is known about subclinical mastitis agents in buffaloes in Romania. The aim of the study was to comparatively evaluate the prevalence and etiology of mastitis in dairy buffaloes and cows from Transylvania. Additionally, antimicrobial susceptibility profile of microorganisms isolated from milk samples was tested. The research was carried out on a group of buffaloes (n=108) and a group of Romanian Spotted and Red Holstein cows (n= 211). After the physical examination of the mammary gland, R. Mastitest was performed to diagnose subclinical mastitis. Subsequently, a total number of 56 milk samples from the buffaloes, and 28 milk samples from the dairy cows were collected. Classical microbiological methods were used to cultivate the bacteria from the milk samples and the Kirby-Bauer disk diffusion technique was performed to evaluate the sensitivity/resistance to antibiotics commonly used to treat the disease, such as ampicillin, amoxicillin, enrofloxacin, cloxacillin and oxytetracyclin. Antibiotic resistant or highly resistant staphylococci were encountered in almost all milk samples in bovine mastitis (90%) compared to the buffaloes where *Streptococcus spp* was present in the highest percentage (71.42%). The results indicated that frequent and uncontrolled use of antibiotics against mastitis or other current diseases of both buffaloes and dairy cows led to the development of multi- or total resistance to antibiotics. The highest overall sensitivity for the isolated strains was recorded for amoxicillin in both species.

The microbiome, along with other factors, represents one main cause of the bovine mastitis. Antibiotic resistance, a problem human and veterinary medicine confront nowadays, enhances the survival of the bacteria in the host in spite of treatments. Subchapter 4.2. presents the advantages of estimating by automated methods the differences in the bacteriome profile and specifies data related to its antimicrobial behavior in dairy cows, differentiated by the growth system, considered as one of the influencing factors in inducing antibiotic resistance. This study aimed to compare, using automated methods, the antibiotic sensitivity / resistance profile of the

microbiome isolated from dairy cows raised in the respective intensive system extensively.

The research was conducted on two farms, one with intensive the other with extensive raising of dairy cows of Holstein and Maramures Brown breeds. A total of 122 milk samples collected from mastitic animals were processed by classical microbioogical techniques and also WalkAway and Sensititre OptiRead were used to identify the micriobial strains and their antibiotic resistance profile. Isolated cultures were tested by the two systems using Amoxicillin and clavulanic acid, Sulbactam, Ampicillin, Cefazolin, Cephalothin, Clindamycin, Gentamicin, Oxacillin, Penicillin, Rifampin, Tetracyclin, Trimetoprim/sulfa, Vancomycin şi Cefepine. By WalkAway, *S. haemolyticus, S. sciuri, S. werneri*, *S. schleiferi*, *S. auricularis, Micrococcus* spp. were isolated. In the case of *Staphylococcus spp*., the resistance was very high, only some newer generation antibiotics being effective (gentamicine, rifampin) against all of the strains. As opposed to that, the strains are totaly resistant to 33.33% of the antibiotics used. Being betalactamase positive, all are resistant to penicillin, similarly to the data from the literature. The milk samples tested by Sensititre OptiRead contained 48 strains (34 G+, 18 G -). The variety of strains was lesser than on the intensive farm, including: *Staphylococcus aureus*, *Escherichia coli*, *Serratia marcescens*, *Enterobacter aerogenes,* and *Streptococus uberis*. The data indicated the participation of CNS in the etiology of mastitis. Although the mechanisms of some of them are poorly known, the antibiotic resistance profile could support their enhanced pathogenicity. The differences between the microbiome on the two farms were slight, some G- being present on the extensive farms, but a lesser variety of CNS. The antibiotic resistance pattern was similar, suggesting an exchange of resistance genes at different levels.

As shown before, one of the most severe pathologies registered in lactating cows is mastitis, due to the loss of productive capacity of the udder, severe changes in health and the economic impact of the disease, and also the appearance of strains resistant to antibiotic (subchapter 4.3., Increased pathogenicity of antibiotic-resistant *Staphylococcus spp*. strains isolated from mastitis cows).

Milk samples from Holstein dairy cows (n=20) raised intensively, were used. Classical microbiological methods were applied, where broth and agar cultivated strains were identified by API 20 STREP, API STAPH, API 20 E. The *in vitro* antimicrobial susceptibility tests were conducted on total flora and also individual strains, using oxitetracycline, neomycin/bacitracin/ tetracycline, marbofloxacin, ampicillin, amoxicillin/ clavulanic acid, gentamycin, enrofloxacin and tetra-delta. Molecular methods (PCR) were involved in estimating the toxigenic potential of the isolated *Staphylococcus spp.* strains. The results supported the presence of a variable microbiome, involving antibiotic multiresistant bacteria. The lack of the *in vitro* efficacy was noticed in case of several antibiotics commonly used for the mastitis therapy (ampicillin, oxitetracycline). Not only the antimicrobial resistance defined

Staphylococcus spp. isolates, but also the genes enconding toxins, underlying the strong pathological potential.

The intervention of the immune system in conditioning the development and establishing the prognosis of mastitis in cows is of particular importance. In this framework chapter 5, Immunological profile in cows with mastitis, focused on monitoring changes in nonspecific effectors in animals with subclinical mastitis in consecutive years. In subchapter 5.1. Quantification of nonspecific immune system mediators in a population of dairy cows previously diagnosed with subclinical mastitis, the research aimed to monitor changes in non-specific humoral and cell-mediated systemic immunity in animals from Romanian Spotted and Holstein breeds aged 3 to 8 years, diagnosed with subclinical mastitis by the use of precipitation techniques to quantify circulating immune complexes and total immunoglobulins levels from lactoserum.

Furthermore, the neutrophile/lymphocyte ratios were used to estimate the stress levels in the diseased animals and the carbon particle inclusion test to monitor the phagocytosis. The study revealed lower values of circulating immune complexes in whey of mastitic cows (0.002±0.010 optical density units, ODU) when compared with the healthy ones (0.004±0.027 ODU), while the total IgG levels were reversed (0.36±0.13 and 0.29±0.13 ODU, respectively). Stress index (N/L) values and phagocytosis were significantly higher in mastitic animals when compared to the healthy ones (N/L 1.24±0.69 and 0.48±0.15, $p<0.05$ and phagocytosis 1.78±0.21 and 0.44±0.23, $p<0.01$). The results indicated a higher clearance of the immune complexes, higher levels of stress and phagocytosis in cows with subclinical mastitis, suggesting the strong involvement of both non-specific humoral and cell-mediated systemic immunity despite the subclinical course of the disease.

The bacterial etiology of mastitis has an impact on both local immune systems and the system, so the immune status of animals can help predict the outcome of the disease (Giupana et al., 2015, subchapter 5.2). In the following year, similar tests (subchapter 5.2. The changes in non-specific systemic immunity in cattle with subclinical mastitis) The investigations aimed to monitor the changes in non-specific systemic humoral and cell-mediated immunity in cattle of different breeds, previously diagnosed with subclinical mastitis (n=10), when compared to a healthy group (n=9). The cattle originated from private households located in NW Transylvania. Milk and blood samples were collected from each animal, and further tested by the use of precipitation techniques to quantify total immune globulin levels and the carbon particle inclusion test to monitor the phagocytosis from whey and blood, respectively. The study revealed that there were no statistically significant differences between the total immunoglobulin present in the serum of the cattle diagnosed with subclinical

mastitis (0.31±0.079 optical density units, ODU) when compared to the healthy ones (0.26±0.088 ODU). Values for phagocytosis were significantly higher ($p<0.01$) in mastitic (2.24±0.92 ODU) versus healthy (4.33±0.71) animals. Moreover, the higher values obtained for phagocytosis in cows with subclinical mastitis suggested a stronger involvement of non-specific cell-mediated rather than humoral systemic immunity in this category.

In an attempt to define the influencing factors of the antibiotic resistance profile in the case of bacteriome isolated from mastitis animals, experiments were performed that included research on the influence of bacterial habitat pH on antibiotic resistance (subchapter 6.1. Antibiotic resistance profile of the mastitic bacteriome in dairy cows could be influenced by changes in pH) as well as the evaluation of the influence of the solvent used in the extraction of active principles from plants in the conditioning of antibiotic resistance (6.2. Extract nature influences the effects of *Melissa officinalis* L. on the milk microbiome in cows with subclinical mastitis).

In subchapter 6.1., the research was conducted on Romanian Spotted dairy cows raised under semi-intensive farming conditions, aged three to five years, showing clinical or subclinical mastitis diagnosed by California mastitis test and somatic cell counts. The milk samples were colected before the morning milking in sterile containers and processed by classical microbiological methods: cultivation on classic and selective media, performing sensitivity testing by Kirby-Bauer disc diffusion method to ampicillin, enrofloxacin, gentamicin, oxytetracycline, ciprofloxacin, amoxicillin clavulanic acid and commercial product Tetra delta® containing novobiocin, neomycin, penicillin procaine, dihydrostreptomycin and prednisolone. To estimate the potential changes in antibiotic sensitivity induced by changes in the environmental pH, the Kirby- Bauer method was repeated on isolated bacteria after their pre-treatment with acid (4-6.5) or alcaline (7.8-8.2) pH.

The dominant bacteria (*Enterococcus* - 45% and *Staphylococcus* - 55%), were sensitive to ciprofloxacine and partially or totally resistant to other antibiotics. Both alcaline (8.0) and acid (5.0) pH proved to be useful in increasing the sensitivity to ciprofloxacine for all isolates. The behaviour of bacteria in presence of antibiotics was highly variabble after pre-treatment with with other pH values, ranging from low sensitivity to total resistance. We concluded that changing the pH could be of help in treating mastitis in dairy cows, while associated with the appropriate antibiotics.

In another study (subchapter 6.2), the influence of the extraction solvent on the antimicrobial effectiveness of *Melissa officinalis* L. was assessed on subclinical mastitis microbiome. Milk and blood samples were harvested from dairy cows at the peak of the lactation. N/L ratios from the blood (Panoptic stain) were calculated as indicators of the degree of stress/immune suppression in subclinical disease. The microbiome components were identified by use of classical bacteriological methods and cultivated against *M. officinalis* alcoholic extract and essential oil (Kirby-Bauer method). Minitab 16.0 was used for the statistical interpretation of the data while the significance of the differences between the groups was interpreted by Student's t test. N/L ratios indicated significantly increased stress ($p < 0.01$) in subclinically affected animals (1.24 ± 0.69)

when compared to healthy ones (0.56 ± 0.12). In bacteria from mastitic milk, *M. officinalis* alcoholic extract was less effective than the essential oil (inhibition zone of 11.3 ± 3.6 mm versus 12.3 ± 4.3 mm) but comparable to amoxicillin, amoxicillin/clavulanic acid and higher than cefoperazone (total resistance). Similarly, in healthy milk isolates the effects were more pronounced for the essential oil than for the tincture (18.67 ± 7.0 mm versus 13.0 ± 8.2 mm) and higher than in cefoperazone (15.67 ± 3.2 mm). The results suggested that the therapeutic use of *M. officinalis* essential oil rather than the alcoholic extract could prove to be efficient against mastitic milk bacteriome, depending on the bacteria genus more than on the extract type.

As bovine mastitis is a disease with an economic impact on lactating animals worldwide, most investigations have focused on defining epidemiological indicators and also control measures. As part of control programs, the most popular therapeutic measures in clinical and subclinical mastitis in lactating cattle is antibiotic treatment, which is less and less effective under the pressure of increasing antibiotic resistance of inducing bacteria. In Chapter 7, Evaluation of the alternative therapeutic potential of selected plant extracts, the results obtained by investigating the activity of plant extracts on bacteria isolated from cows with subclinical mastitis are evaluated, estimating the bioavailability of active components compared to antibiotics with maximum use in these infections.

Subchapter 7.1. Identification of plant extracts as potential alternative therapeutic means for dairy cows diagnosed with subclinical mastitis, describes investigations related to the definition of the immunostimulating therapeutic potential of plant extracts such as *Calendula officinalis, Echinacea angustifolia, E. purpurea, Hippophae rhamnoides, Urtica dioica, Allium sativum, Mentha piperita* which grow on most Romanian pastures.

The research was carried out on a group of 27 dairy cows, divided in two groups, healthy (n=10) and diagnosed with subclinical mastitis (n=17). Blood samples collected by puncturing the jugular vein were diluted with RPMI 1640 (1:4) and dispensed in 96-well plates. Duplicates of alcoholic vegetal extract treated variants (1.5 μl/well) were compared to cultures treated with PHA and LPS standard mitogens (1.0 μl/well). Glucose concentrations were measured by means of an orto-toluidine colorimetric test and stimulation indices (SI%) were calculated. The spontaneous SI was lower in the subclinical mastitis group (48.03±10.23) than in the healthy animals' group (53.44±9.34). There was no increase of SI in mastitic cows under the influence of plant extracts, that acted inhibiting (SI% from 17.54±13.97 to 39.90±7.89), when compared with the spontaneous (SI% 48.03±10.23) and alcohol induced (SI% 40.06±8.57) ones and the effects obtained in healthy animals (SI% garlic extract 56.71±12.91, SI% sage 58.26±12.31, SI% mint 54.60±15.97). The results indicated that the plant extracts used according to the described protocol failed to restore the *in vitro* cell-mediated immune

response, suggesting the continuation of the research to establish the adequate dosage for these extracts.

Thymus marschallianus Willd. is a Lamiaceae species spread in a large variety of habitats worldwide. The aim of the research presented in Chapter 7.2 (Phytochemical profile and antibacterial potential of extracts obtained from *Thymus marschallianus* Willd) was the analysis of two different samples of this species, one obtained from spontaneous flora and one from culture. The total polyphenols, flavonoids, and phenolic acid contents were spectrophotometrically determined. Qualitative and quantitative analysis of polyphenols was performed by an *HPLC-DAD-ESI (+)-MS* method. For the antibacterial assay, the well-diffusion and the broth microdilution methods were used. Analysis of polyphenols revealed for both samples the presence of flavonoids like luteolin, quercetin, apigenin and their derivatives, but also of rosmarinic acid and methyl-rosmarinate. Differences regarding the amount of these compounds were emphasized. Significantly larger amounts of flavonoids were found for the sample harvested in the spontaneous flora, while for the rosmarinic acid, larger amounts were found for the cultured sample. Both samples displayed promising antibacterial activity, particularly towards Gram positive organisms. *T. marschallianus* represents, therefore, a rich source of polyphenolic compounds that prove its promising potential as a medicinal species.

The last subchapter of the study (7.3. Comparative research on the use of clasical antibiotic and alternative therapies against bovine mastitis) was dedicated to the comparative evaluation of the effects of alternative therapies (apiproducts) and common antibiotics on the mastitis microbiome. Given the increased antibiotic resistance of the bacterial strains involved, this research aimed to evaluate the effectiveness of alternative therapy with honey and propolis in treating mastitis in cattle.

The research was carried out on a group of 28 animals, aged 3 to 11 years, of Romanian Spotted and Red Holstein breeds. The investigations aimed the isolation and identification of bacteria involved in cases of clinical mastitis in cows, evaluation of their sensitivity/resistance to commonly used antibiotics, as well as the assessment of honey and propolis efficacy on bacteria isolated from mastitis cases of intensively managed cows. Main methods used were classical cultivation and Kirby-Bauer disk diffusion susceptibility test. Antibiotic resistant or highly resistant staphylococci were encountered in almost all milk samples. The comparative study regarding the use of various propolis tincture concentrations showed maximum efficacy for the 20% concentration, with decreasing effects for larger concentrations, which denied the hypothesis according to which increased concentrations produce increased effect. The efficacy of honey products depended upon concentration and bacterial strain, individualized tratment schemes being absolutely necessary.The results indicated that frequent and uncontrolled use of antibiotics against mastitis led to the development of multi- or total resistance to antibiotics, thus honey and propolis represented valuable therapeutical alternatives, especially in case of Staphylococcus. The obtained results are

encouraging, mainly for the clinical use of propolis in therapy alone or in combination with antibiotics, after standardization of the method through in vivo studies and finding a method for diminishing the irritative effects of the propolis tincture.

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