

Original article

## Extract nature influences the effects of *Melissa officinalis* L. on the milk microbiome in cows with subclinical mastitis

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### Abstract

Mastitis in dairy cows represents one of the most economically impacting pathology, the management costs being augmented by difficulties in diagnosing the subclinical form of the disease. The biased and non-discriminatory use of antibiotics led to their lesser efficacy, therefore therapeutic alternatives are looked for. In this study, 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 ( $1.24 \pm 0.69$ ) when compared to healthy ones ( $0.56 \pm 0.12$ ). In bacteria from mastitic milk, *M. officinalis* alcoholic extract was less effective than the essential oil (inhibition zone of  $11.3 \pm 3.6$  mm versus  $12.3 \pm 4.3$  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 ( $18.67 \pm 7.0$  mm versus  $13.0 \pm 8.2$  mm) and higher than in cefoperazone ( $15.67 \pm 3.2$  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.

**Key words:** Dairy cows, subclinical mastitis, *Melissa officinalis* L., microbiome, therapy

### 1. Introduction

Considered to be a multifactorial disease, mastitis exerts a high economic impact due to increased production costs, health related costs and control and preventive measures to be implemented (Kvapilík *et al.*, 2015). Difficulties to diagnose subclinical forms of the disease, further complicate the control of the disease (Asfaw and Negash, 2017). *Staphylococcus* spp. stands for the most prevalent etiological agent of this complex infection in dairy cows, but also expresses an important zoonotic potential, thus posing a health risk for contacts and consumers. Furthermore, the etiology of the disease could be complicated by the presence of environmental and also gut origin microflora, acquired by the immediate contact of

infected animals with their habitat (floors, bedding, fodder, caretakers, *etc.*) (Machado *et al.*, 2008). The interaction is reciprocal, the animals infected with multiple microbial strains shedding those to the environment, further perpetuating the disease to naive incoming dairy animals and increasing its prevalence.

The management of this pathology is mainly based on the extensive antibiotic use (Pieterse and Todorov, 2010), which has triggered the development of high levels of antimicrobial resistance (Motlagh *et al.*, 2013). Lesser efficacy of antibiotics used to treat both clinical and subclinical mastitis due to their irrational and extensive involvement in therapeutic schemes, led to numerous researches on finding efficient alternatives, such as probiotics, phytobiotics and plant essential oils (De and Mukherjee, 2013).

Millenia of phytomedical practice with positive results, high physical and biological availability, low costs, lack of side effects in most of them, regional and local traditions were some of the reasons that supported the development of scientific research in the area of medicinal plant uses in both human and veterinary medicine (Biradar, 2015). According to WHO, almost 80% of the

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world's population, located mainly in developing countries rely on affordable medicinal plants as "first solution" therapeutic means (Nayanabhirama, 2016), but a continuously increasing percent of drug companies and physicians are producing and, respectively, prescribing treatments based on medicinal plants and their components (Biradar, 2015).

The antimicrobial properties of essential oils were looked upon in many studies (Babu *et al.*, 2015; Ncube *et al.*, 2008; Pozzo *et al.*, 2012). Recent review of the literature (Shakeri *et al.*, 2016) indicated *M. officinalis* as having numerous biological influences, of which anti-inflammatory (Pushpangadan *et al.*, 2015), antitumor, antioxidant (Miraj *et al.*, 2017; Pereira *et al.*, 2014), hypoglycemic, hypocholesterolemic (Zarei *et al.*, 2014), antimicrobial and antiprotozoal (Nayanabhirama, 2016; Nasri and Salem, 2012), antiproliferative and spasmolytic properties (Weidner *et al.*, 2015).

The nature of the solvent influences the extent of biological effects of the plant extract due to the presence of various extractable components with affinity towards the solvent used. Thus, mixtures of methanol/water and ethanol/water proved to be more efficient in extracting a higher amount of active principles from *Moringa oleifera*, *Azadirachta indica*, *Terminalia arjuna*, *Acacia nilotica*, *Eugenia jambolana*, *Aloe barbadensis* than pure alcohols also improving the antioxidant activity of the extracts (Sultana *et al.*, 2009). A comparison for antioxidant content of red fruit (grapes, black and red currant) with the same alcohols led to the conclusion that antioxidant activity was much higher than in water extracts of the same fruits (Lapornik *et al.*, 2005). Another investigation carried out to compare six types of solvents (distilled water, methanol, acetone, chloroform, ethyl acetate and hexane) for their efficacy in extracting biologically active compounds from *Datura metel* (devil's trumpet) showed a high variation based on the solvent, indicating the methanol as best option to enhance antioxidant activity (Dhawan and Gupta, 2017).

We hypothesized that the antimicrobial efficacy of extracts from *M. officinalis*, a plant from Lamiaceae family known for its strong antibacterial effectiveness, will be influenced by the extraction solvent and the microbiome component species isolated from bovine mastitis. Thus, in this study, we explored the antibacterial effect of essential oil and alcoholic extract of *M. officinalis* involving the bacterium from subclinical mastitis.

## 2. Materials and Methods

### 2.1 Biological samples

Milk samples were collected from a 250-headed herd within the commune, where 64 clinically healthy cows at different stages of lactation, responded positive to the California Mastitis Test. Other 40 healthy samples were considered negative controls.

Samples were collected prior to antibiotic treatment. Subsequent to recording milk clotting degree, the comparison with the standard for mastitis diagnosis was performed as indicated by Nichols (2009).

Animals were sampled for blood during the compulsory routine testing for seroconversion within the official surveillance program for bovine enzootic leucosis and brucellosis. Milk samples were simultaneously collected from all selected animals, transported to the laboratory and processed in maximum 4 h after sampling.

### 2.2 Plant extracts and propolis

Essential oil and alcoholic extracts of *M. officinalis* for human use were obtained from Plant extract, SRL Romania and produced according to the German Homeopathic Pharmacopeia. Professor Dr. M. Tamas, Department of Pharmaceutical Botany, University of Medicine and Pharmacy, Cluj-Napoca, Romania carried out the identification of the plant. Aqueous and alcoholic extracts of propolis, standardised by HPLC, obtained from the Apiculture Department of the University of Agricultural Sciences and Veterinary Medicine, known for their antibacterial activity were used as natural controls to evaluate the *M. officinalis* antibacterial activity.

Some preliminary experiments (unpublished data with the author) were carried out to define the dosage of both plant and propolis extracts, which would allow reproducible results.

### 2.3 Methods

#### 2.3.1 Establishment of the stress index

Neutrophile/lymphocytes (N/L) ratios (Davis *et al.*, 2008) from the blood were calculated as stress indicators (Panoptic stain) to estimate the degree of stress induced by the subclinical disease.

#### 2.3.2 Classical bacteriological tests

Milk samples were transferred to simple broth, incubated for 24 h at 37°C, then further passed to glucose agar and incubated for another 24 h under the same conditions. Chapman and McConkey culture media were used for selective isolation of bacteria, while Api 20E and ApiStaph special galleries were implied for the identification of the isolated species.

To evaluate the antibacterial effect of two different types of extracts with different solvents, bacteria were cultivated against *M. officinalis* alcoholic extract and essential oil in the Kirby-Bauer well diffusion method. Similarly, aqueous and alcoholic propolis extracts were used as active antibacterial complexes (Rindt *et al.*, 2009). Common antibiotics used in mastitis therapy were also tested in parallel, for comparison, including amoxicilline/clavulanic acid (AMC), amoxicilline (AX), chloramphenicol (C), cefoperazone (CFP), ciprofloxacin (CIP), oxytetracycline (T).

#### 2.3.3 Statistical interpretation of the results

The statistical significance of the differences was interpreted by Student's t test in the Excel program.

## 3. Results and Discussions

Plants and their extracts were well-known for their antimicrobial activity in humans for many years. Scientific research in this field expanded rapidly in the last few decades and is continuously increasing (Reynolds, 1996; Lis-Balchin and Deans, 1997; Saranraj and Sivasakthi, 2014). The traditional ethno-veterinary medicinal practices are being followed by the local communities to treat a number of human and animal diseases especially in developing countries due to low costs.

In the post antibiotic era, the use of antibiotics is banned for probiotic use in a number of countries because of risk posed to human health (Jamane *et al.*, 2007). Due to the non-discriminatory use of antimicrobial, their efficacy is decreasing lately, especially in farm animals, therefore, alternative therapies were sought.

Experiments indicated the efficacy of plant extracts on bacteria, *i.e.*, *Staphylococcus aureus*, isolated from cows with mastitis, comparable to that of antibiotics and encouraged their use, as for example, the use of a hydroalcoholic extract of common guava (*Psidium guajava* L) (da Silva Menezes *et al.*, 2016). Further trying to clarify the influence of different plant compounds on the pharmacokinetics of antibiotics, the researchers found that quercetine, an antiallergic and immune stimulating compound (Kasturi *et al.*, 2016) enhanced the activity of enrofloxacin.

In this study, the investigations were carried out to evaluate the effects of two extracts from *M. officinalis* on the bacteria isolated

from mastitic milk, in order to provide a viable alternative to antimicrobial therapy. A total of 20 bacterial strains were identified, of which ten were isolated from healthy milk : *Staphylococcus spp.* (*S. xylosus* 3 strains, *S. sciuri* 6 strains, *S. lentus* one strain) and other ten strains from mastitic milk, belonging to several genera and species : *Staphylococcus spp.* (*S. xylosus* and *S. lentus*, two and three strains, respectively), *Shigella spp.* (one strain), *Enterobacter spp.* (2 strains of *E. cloacae*) and *Escherichia vulneris*, one strain of *Acinetobacter baumani* and one of *Chryseomonas luteola* (Figure 1). The results indicated a relatively broad spectrum of both Gram-positive and Gram-negative bacteria.

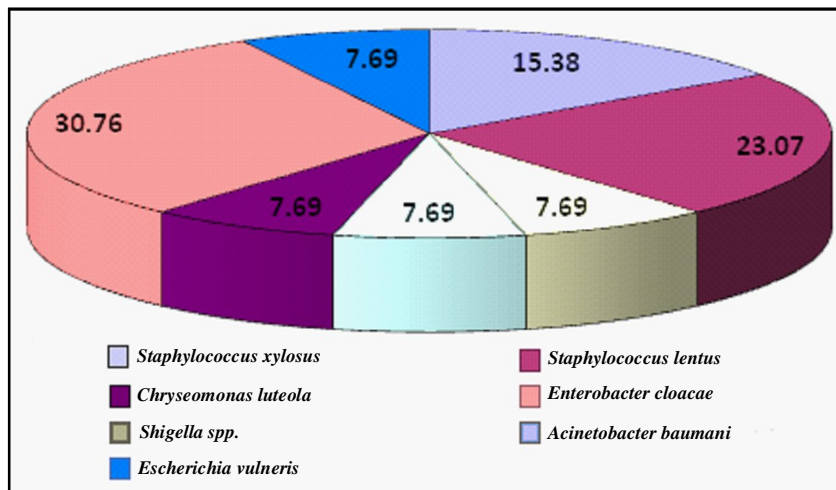
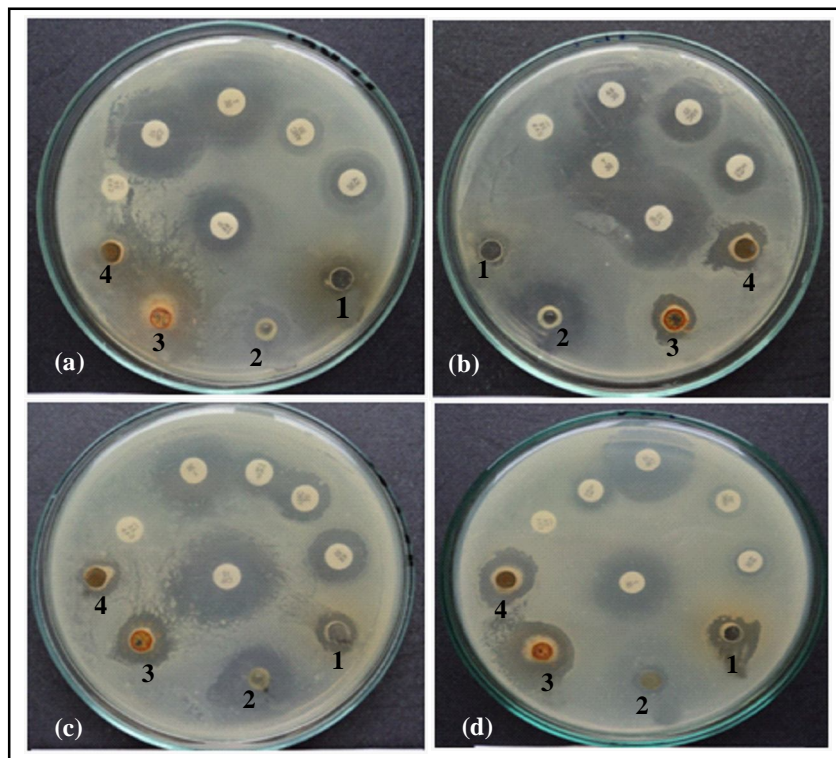


Figure 1: Percentages of isolated bacterial strains in mastitic milk.



(a) *Enterobacter cloacae*, (b) *Acinetobacter baumani/calcoaceticus*, (c) *Staphylococcus xylosus* and (d) *Shigella spp.*

Figure 2: Inhibition areas against bacteria isolated from mastitic milk. Numbers indicate the *M. officinalis* (1, 2) and propolis (3, 4) extract activity.

Traditional uses of *M. officinalis* have been recorded mostly in European countries, Mediterranean region and Middle East countries. The most common therapeutic properties of lemon balm are its sedative, antioxidant, antispasmodic, carminative, antibacterial, antiviral and anti-inflammatory cases (Burt, 2004).

Steam distillation or chemical extraction are both methods used for the obtainment of essential oils. The lemon grass essential oil, acquired by any of the methods, contains mainly citronellal, citral (33-39%) and a small amount of geranial (2%) (Bahtiyarca Bagdat and Cosge, 2006) with pronounced antibacterial effect. The alcoholic extract of the plant contained mainly rosmarinic acid, with a marked cytotoxic effect on glioblastoma cells (Ramanauskiene *et al.*, 2016).

In mastitic microbial strains, *Melissa* tincture was less effective than the essential oil ( $11.3 \pm 3.6$  mm versus  $12.3 \pm 4.3$  mm), but comparable to amoxicillin, amoxicillin/clavulanic acid and higher than cefoperazone (total resistance) but much smaller inhibition area than that of ciprofloxacin which encountered a value of 30 mm (Figure 2).

Ciprofloxacin is the most commonly used antimicrobial fluoroquinolone, both in humans and veterinary practice. In healthy milk strains, the effects were more pronounced for the essential oil than for the tincture ( $13.0 \pm 8.2$  mm versus  $18.67 \pm 7.0$  mm) and higher than in cefoperazone ( $15.67 \pm 3.2$  mm). This could be the result of the differentiated activity of rosmarinic acid, lesser on bacteria than on cancer cells.

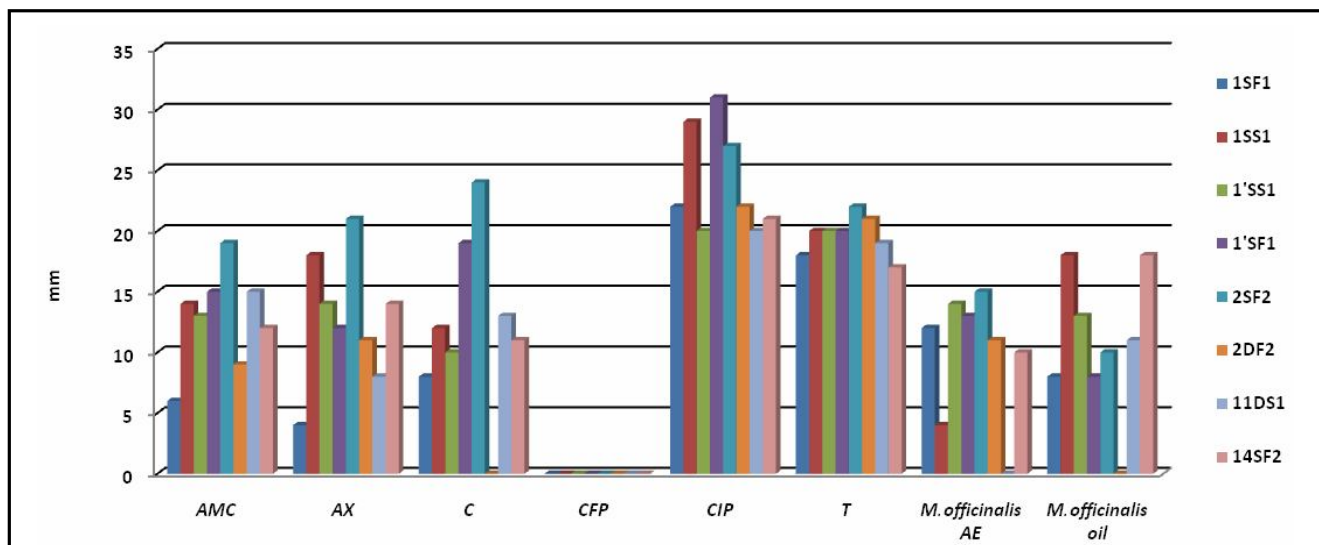
To overcome the problem of antibiotics resistance, effective and safer alternatives to the current antimicrobial drugs need to be

urgently assessed. The use of plant extracts like *M. officinalis* in control of bovine mastitis caused by *Staphylococcus spp.* is a promising solution that could contribute to the reduction of the occurrence of dairy food industry contaminations, providing considerable benefits to agro-industries on the formulation of high-quality and safety dairy products (Martins *et al.*, 2014).

In this study, the total or partial resistance (resistant colonies) of the isolated zoonotic or non-zoonotic strains to several antibiotics is worrying, suggesting the lack of controlled use of antibiotics in the field. Under these circumstances, the extracts obtained from *M. officinalis* and tested for their antibacterial activity proved to be efficient. The solubility of active substances from the plant also defined its activity, the essential oil proving to be more active than the alcoholic extract, therefore in further *in vivo* therapeutic attempts, the essential oils should be considered.

#### 4. Conclusion

The use of botanical preparation dates back from the beginning of human civilization, effectively used in a wide variety of health conditions. The World Health Organization (WHO) states that 70% of the plants derived medicines have a modern indication that correlates with their traditional, cultural and sometimes ancient uses (Wynn, 2001). The present study showed that *M. officinalis* tincture and essential oil proved to be quite efficient against bacteria from mastitic milk, depending more on the strain than on the solvent type, also suggesting a therapeutic alternative to antibiotic treatment.



**Figure 3:** The effects of two extractive preparations of *M. officinalis*, essential oil and alcoholic extract, on bacteria isolated from mastitic milk, compared to antibiotic efficacy.

#### Conflict of interest

We declare that we have no conflict of interest.

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