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Original article

Farming system affecting the *in vitro* activity of a *Hippophae rhamnoides* L. extract on phagocytosis in mastitic cows

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Abstract

Innate cell-mediated defense mechanisms include neutrophils as important factors during phagocytosis and killing of the bacteria. Their presence is considered to be crucial in controlling the *in situ* infection, especially in high yielding cows. The research was carried out to evaluate the impact of clinical mastitis recorded under different farming technologies on the non-specific cell-mediated responses to *Hippophae rhamnoides* L. alcoholic extract and its potential therapeutic use.

Randomly selected dairy cows showing clinical signs of mastitis were sampled for blood on two farms which were different in terms of raising technologies. Causative agents were identified from the milk by Sensititre Opti Read from Mueller Hinton agar plates. Carbon particle inclusion test was performed using an alcoholic Sea buckthorn extract to treat phagocytic cells. Phagocytic activity index was calculated as the difference between the natural logarithms of the optical densities of the phagocytosis divided by time (45 and 15 min, respectively). The statistical significance of the differences between the groups was interpreted by Student t-test.

The results indicated that *Streptococcus uberis* represented the dominant bacterium on one farm while *Staphylococcus aureus* / *E. coli* were present in mastitic milk on the other. The spontaneous phagocytosis was increased in mastitic cows from the tie stall farm, while the response to the *Hippophae* extract significantly (p<0.0004) increased with 132.86% on the free range compared to the tie stall farm. The Sea buckthorn activity was stimulating in healthy cows on the free range farm, but negatively influenced by tie stall raising.

The effects of the *H. hamnoides* alcoholic extract on the phagocytosis were conditioned not only by its composition but also by external factor complex, including the raising technology used on the farm.

Key words: *Hippophae rhamnoides* L., clinical mastitis, phagocytosis, microbiome, dairy cows

1. Introduction

Mastitis represents the most important and expensive pathology in dairy cows. It is characterized by inflammation of mammary gland in response to injury, for the purpose of counter acting the aggressors and to enhance healing and restore the physiological function. In the dairy cow, mastitisis constantly caused by microorganisms; usually bacteria, that invade the udder, multiply in the milk-producing tissues, and produce toxins that are the immediate cause of injury (Saifudeen *et al*., 2017). The primary phagocytic cells of the bovine mammary gland, polymorphonuclear neutrophil leukocytes (PMN), and macrophages, comprise the first line of defence against invading pathogens. In the healthy mammary gland, macrophages dominate and act as sentinels to invading mastitis-causing pathogens. Once invaders are detected, macrophages, and possibly mammary epithelial cells, release chemo-attractants that direct migration of PMN into the area. In the mammary gland, protection is only effective, if rapid influx of PMN from the circulation and subsequent phagocytosis and killing of bacteria occur (Paape *et al*., 2002).

Since economic benefit is the main driving force in dairy cow farming, the improvement of raising technologies was sought lately. Still, technologies which allow only restricted movement (*i.e.*, tie stall) to the animals raise serious welfare and implicit disease resistance concerns (Popescu *et al*., 2015).

Considerable international pressure is applied to limit antimicrobial therapy in animals; this is aimed at lowering the risk of transmission of resistant bacteria and/or antibiotic residues to humans through animal by-products. The consequences of such policies in the field are: (i) the development of new therapeutic strategies and (ii) the development of new cow-side diagnostic tests to allow targeted treatment (Gruet *et. al*., 2001). In spite of numerous antibiotics on

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the market, current treatments are lacking success and cure rates are low. New strategies have recently been investigated. These include particular immune modulators such as lysostaphin or cytokines, and novel formulations (*e.g*., liposomes, microparticles or nanoparticles) that allow uptake of the active component by phagocytes and, thus prolong an enhanced antibacterial activity (Gruet *et al*., 2001).

Medicinal plants representing an important tool of mankind to combat infection and diseases since the dawn of civilization. These natural products waned or disappeared from the medical prescription with the advent of modern medicine (Mukherjee *et al*., 2010; Manoharachary and Nagaraj, 2016; Rajeshwari *et al*., 2013; Udupa, 2016). Sea-buckthorn (*H. rhamnoides*), a wild shrub of family Elaeagnaceae, is being used in different parts of the world for its properties (Gupta *et al*., 2006; Mishra *et al*., 2008). It is a thorny nitrogen-fixing deciduous shrub of cold arid region native to Europe and Asia. It has been used in traditional Chinese medicine since the Tang Dynasty, going back more than 1000 years (Suryakumar and Gupta, 2011). Still, its efficacy in influencing the immune system has not been explored in depth. The detailed molecular mechanisms of their effects remain mostly undetermined (Mishra *et al*., 2008). Monitoring the complex relationship between leukocyte subpopulations and the microbiome involved in pathological episodes can provide viable therapeutic solutions to the paradigm defined (Leitner *et al*., 2015).

Within this frame work, the study aimed to evaluate the impact of clinical mastitis on non-specific cell-mediated responses to *H. rhamnoides* alcoholic extract and its potential therapeutic use.

2. Material and Methods

2.1 Animals and biological samples

The research was carried out on two farms: a tie stall and on a free range farm on cows showing clinical mastitis (n=10) and on healthy cows (n=20). The presence of edema, induration of the mammary gland and increase of its temperature, macroscopic changes in the milk aspect and quality were considered to define the clinical mastitis group and milk samples were collected prior to antibiotic treatment.

Milk and blood were taken simultaneously from each animal in sterile conditions and processed at the Faculty of Veterinary Medicine (Cluj-Napoca, Romania) in maximum 4 h after sampling for detecting the milk bacteriome and establishing the blood phagocytic activity. The blood sampling was carried out within the frame of the National Disease Surveillance Program, and further processed for the diagnosis of bovine leucosis and brucellosis by the accredited state laboratory.

The first few drops of milk were discarded and subsequently the samples were collected into sterile recipients. Blood was sampled on heparine (50 IU/ml) and subjected to carbon particle inclusion test.

2.2 *Hyppophae rhamnoides* L. extract

The alcoholic extract of *H. rhamnoides* was a commercial product for human use (Plant extract, Romania), obtained according to the procedures described in the German Pharmacopoeia. For the aim of this project, the extract was used solely *in vitro*.

2.3 Bacteriological examination

Subsequent to a microscopic examination of the milk sediment stained by Gram method, the samples were cultivated on simple broth/agar and also Mac Conkey agar plates. Then, isolated bacteria were identified by use of Sensititre Opti Read device (Thermo Fisher Scientific, USA).

2.4 Carbonparticle inclusion test

Phagocytic cells engulf *in vitro* inert particles such as carbon as their ability to protect the organism from intruders. The measurement of this activity suggests their defensive capacity against real, such as microbial assault. Aliquots of 1.5 ml of heparinised blood (0.5 ml /variant) were mixed each with 2 microliters of an Indian ink supernatant, obtained by centrifugation at 6000 rpm for 40 min (Hettich centrifuge, Germany). Three variants were performed: control for spontaneous phagocytosis (no additives, except Indian ink), alcohol treated control and plant extract treated blood. 15 microliters of the mixture were transferred to 2 ml of saline immediately and after 15 and 45 min. of incubation at 37°C. All the final tubes containing the saline and aliquots of different *in vitro* experimental variants were centrifuged at 1800 rpm and the supernatants were subjected to spectrophotometrical reading (λ=535 nm, d=1 cm) (Crisan *et al*., 2012). The results were expressed as the 'ln' of the slope of phagocytosis divided by time (first and second incubation intervals).

2.5 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 Discussion

The complexity, heterogeneity and multilevel structure and functioning of the immune system stand for the conditioning of the multifaceted, not always pattern-like relationships with microbial aggressors (Kurakin, 2009). Thus, increased number of phagocytes would lead, logically, to enhanced protection. Further, increased phagocytosis, as proven by practical experiments, is not always triggered by high numbers of neutrophils (Leitner *et al*., 2015). Dairy cows' raising systems are well-known to induce various stress levels and disease is representing and addition to this stress. Thus, we aimed to investigate how the raising system inducing mastitis to various extents would condition the phagocytosis in sick cows and if and how can the physiological activity be restored by a *H. rhamnoides* extract.

The microbiological results indicated that *Streptococus uberis* represented the dominant bacteria on the free range farm while more numerous bacteria, including *Staphylococcus aureus, E. coli, Serratia marcescens* and *Enterobacter aerogenes* were isolated on the tie stall farm.

Healthy milk samples contained potentially pathogenic bacteria on both farms, with higher numbers on the tie stall farm (*Staphylococcus xylosus*, *Streptococus uberis*, *Staphylococcus warneri*, all known udder pathogens). Similarly, *E. coli* and *Citrobacter freundii* were isolated from the healthy milk on the free range farm. Although, the investigation of antimicrobial resistance was not a purpose of this study, the obtained data are in harmony with those of Wilhelm *et al*. (2009) who concluded that antimicrobial load and resistance was lower in organic dairy production.

The phagocytosis was significantly (p<0.05) increased in the free range healthy cows by the *H. rhamnoides* alcoholic extract when compared to the mastitic cows and also both categories on the tie stall farm. This might stands for the higher responsiveness of the

phagocytes due to lesser stress levels induces by a lesser microflora and also the raising system, ensuring more freedom and a better welfare. Similarly, a statistically non-significantly increased value was recorded subsequent to the comparison of the spontaneous phagocytic response in tie stall mastitic versus free range mastitic cows.

The response of the *Hippophae* extract was significantly (p<0.0004) higher (132.86%) on the tie stall farm in mastitic cows when compared to the healthy ones (0.74 versus 1.63 units). Still, on the tie stall farm, the Sea buckthorn activity was inhibiting when compared to the non-stimulated and alcohol controls.

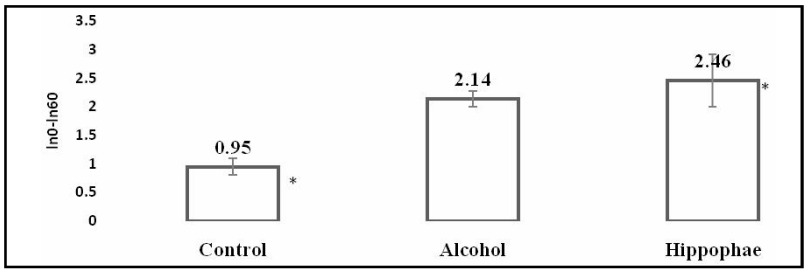

Figure 1: Phagocytosis in free range healthy cows was significantly (p<0.05, *) increased by *H. rhamnoides* alcoholic extract treatment.

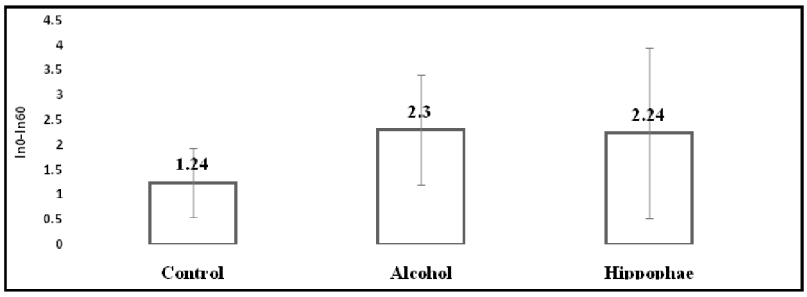

Figure 2: *H. rhamnoides* alcoholic did not augment the phagocytic response in free range mastitic cows, as compared to alcohol control.

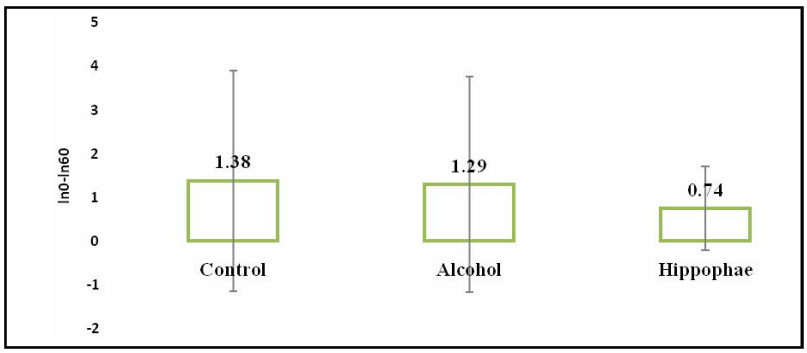

Figure 3: In tie stall healthy cows, the active principles of *Hippophae* acted inhibiting, below the level of both spontaneous and alcohol control induced phagocytosis.

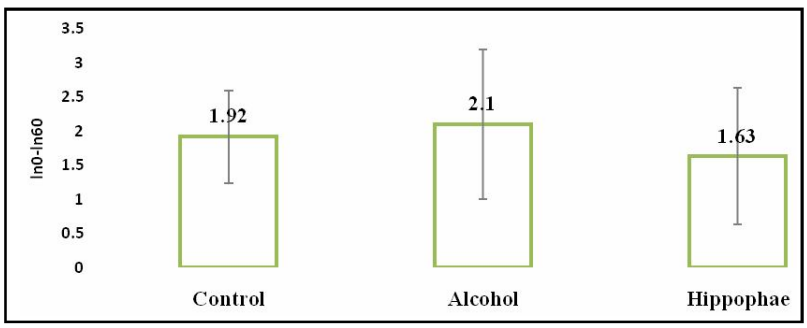

Figure 4: Decreased phagocytosis in the presence of *Hippophae* extract in mastitic cows from the tie stall farm.

Regulation of PMNs migration from the peripheral circulation to the diseased udder is of great importance with regards to the further development of fundamental methods for treatment of mastitis. Appropriate elimination of pathogen requires both the effectiveness of the drug and optimum functioning of the hosts' immune system. This is especially important for animals with an impaired immune function during peri-parturient period (Hoeben *et al.* 1997). Neutrophils are the dominant cell type found in the mammary gland during inflammation and play vital role in udder defence mechanism (Sordillo *et al*., 1997).

Antioxidant effects and phogocyte activation are described for vegetal extracts containing polysaccharides (Das *et al*., 2017; Gonda *et al*., 1992, 1992a), also in case of *H. rhamnoides*. Geetha *et al.* (2005) proved that the extract alone stimulated IL-2 and g-IFN production in the absence of Con A and also inhibited chromium induced decline in IL-2 and g-IFN production, but did not alter IL-4 production suggesting that Sea buckthorn has significant immunomodulatory activity and specifically activates the cell-mediated immune response.

In this study, the *H. rhamnoides* alcoholic extract exerted a differentiated effect on the phagocytes from healthy and mastitic animals raised under different technologies, suggesting that the extract could act as a phagocytosis enhancer in healthy and to some extent mastitic animals raised in the free range system, but was not active where the stress levels were higher, such as the tie stall farm.

4. Conclusion

Phagocytosis was significantly enhanced on the free range farm, in spite of lesser bacteria found, probably due to the lesser stress exerted on the immune system and a better response to the active principles in the alcoholic Sea buckthorn extract**.** On the tie stall farm, the mastitic cows had a more active phagocytosis as a protective measure against various bacteria**.** The effects of the plant extract on the phagocytosis were conditioned not only by its composition but also by external complex factors, such as disease, and also the type of farming technology applied.

Conflict of interest

We declare that we have no conflict of interest.

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