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COMPARATIVE RESEARCH ON THE USE OF CLASICAL ANTIBIOTIC AND ALTERNATIVE THERAPIES AGAINST BOVINE MASTITIS

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Abstract

Bovine mastitis is a major problem of dairy animals despite the numerous preventive and therapeutic approaches. Given the increased antibiotic resistance of the involved bacterial strains, this research aimed to evaluate the efficacy 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.

Key words: *cattle, mastitis, antibiotics, honey, propolis.*

INTRODUCTION

Despite numerous preventive and therapeutic approaches, mastitis remains a major problem in dairy animals (Mitchell et al. 1998; Grave et al. 1999). Bovine mastitis, the ascending or descending infection of the mammary gland by various pathogens, leads to considerable economic losses for the dairy industry (Gill et al.2006). Although antibiotics are very useful to treat the infection, they do not directly protect the gland from being damaged (Zhao and Lacasse, 2008)). The impact on public health should be considered, as dairy cows produce milk for consumption (OIE, 2008).

The aim of this study was to evaluate the efficacy of alternative therapy using honey and propolis tincture in comparison with the classical antibiotic therapy.

MATERIALS AND METHODS

The research was carried out on a group of 28 animals of Romanian Spotted and Red Holstein breeds, aged 3 to 11 years, previously diagnosed with clinical mastitis. Samples of mastitic milk from these animals were inoculated in simple broth, incubated for 24 hours at 37ºC temperature. Subsequently, the 24 h cultures were passed to nutrient agar to obtain isolated colonies and smears were stained by Gram method for bacterioscopical recognition. *Staphylococcus spp*. colonies were passed to Chapman agar and they were identified using API Staph 20. Blood agar plates were used to investigate the hemolysis in the isolated colonies.

Susceptibility/resistance to antibiotics was monitored by the Kirby Bauer diffusion method, using standardized antibiotic discs, using 24 h pure cultures. The following antibiotics were used: amoxiclav (AMC), enrofloxacin (ENF), oxytetracycline (OT), ampicillin (AMP), cloxacillin (CX), penicillin (P), trimethoprim - sulfamethoxazole (SXT). The growth inhibition diameters were measured for the sensitive strains, and also the total inhibition or resistant colonies were recorded.

To test the anti-staphylococcal effects of honeydew and polyfloral honey, the respective samples, collected in sterile bottles and stored at (23-25˚C) in a dark place before testing, were used undiluted. Al the samples originated from Transylvania and were characterized by HPLC prior to use.

The minimal inhibitory concentration (MIC) and minimum bactericidal concentrations (MBC) were monitored for the honey samples. In order to achive that, serial microdilution method was used in the broth after the protocol described by Carson et al. (1995).

Initially, 8 v/v successive dilutions of the products to be tested were made in Mueller Hinton broth: D1 (4%), D2 (2%), D3 (1%), D4 (0.5%), D5 (0.25%), D6 (0.125%), D7 (0,0625%) şi D8 (0,031%, followed by addition of an equal volume of the bacterial suspension (0.5 on the McFarland scale) and the 96-well plates were incubated for 24 hours at 37 °C. The turbidity in the liquid medium was observed, considering MIC the lowest concentration which prevented visible growth of germs, the broth remaining clear. The MBC was determined by sub-culturing 10 µl of the test dilutions from MIC wells on fresh Mueller-Hinton agar plates, which were further incubated for 24 h at 37°C. The MIC index (MBC/MIC) was calculated for each honey type and standard control drug to determine whether a type of honey is bactericidal (MBC/MIC <4) or bacteriostatic (MBC/MIC >4). Similarly, the values of MIC index higher than 4 and less than 32 were considered as bacteriostatic (Pavithra et al., 2010).

To investigate the effects of honey and propolis in cultures inseminated on solid agar, the diffusion method in wells was applied (modified Kirby-Bauer test). After insemination of bacteria on the surface of the agar, 3.5 mm wells were performed under sterile conditions and the honey samples to be tested were placed in these wells. The reading of the results was similar to that of the clasical Kirby Bauer method.

Amoxicillin with clavulanic acid (AMC) and enrofloxacin (ENF) served as controls for antibacterial activity of honeys in this experiment.

Statistical interpretation of the results was performed by use of Microsoft Excel software.

RESULTS AND DISCUSSIONS

Due to increasing resistance to antibiotics in both human and veterinary medicine, standard treatments no longer work and there is an increased risk of spreading of infections caused by ubiquitous bacteria in the so-called post antibiotic era (WHO, 2014). There is a growing need for alternative antimicrobial strategies, thus, different types of natural products (plant extracts, honey, propolis, etc) find their place in therapy. Most honeys show antimicrobial activity that hinders the growth of microbes due to the enzymatic production of hydrogen peroxide or high osmolarity (Mandal and Mandal, 2011), while propolis had been known for its antibacterial properties against a range of commonly

encountered cocci and Gram-positive rods, but only limited efficacy against Gram-negative bacilli (Grange and Davey, 1990).
The results of HPLC performed on honey samples indicated that the polyphenol content (mg GAE/100g honey) was of 101.45 ± 4.48 for the polyfloral honey and higher, of 126.73 ± 19.16, for the honeydew honey. The total content of flavonoids (mgCE /100g honey) was the highest for honeydew honey (16.73±074), while two floral honey types contained smaller amounts (16.11±2.45 and 10.50 ± 1, 15 respectively). These compounds are known for their antibacterial activity for centuries (Cushnie and Lamb, 2005), confirming the potential of the honeys used in the experiment to act against bacteria.
As a result of microbiological examination of the mastitic milk, nine different bacterial genera have been isolated and identified (Fig. 1). The most frequently isolated was *Staphylococcus spp,* followed by *E. coli, Streptococcus spp., Corynebacterium spp., Pseudomonas spp., Fusobacterium necrophorum, Klebsiella, Listeria spp.* and *Pasteurella spp.*

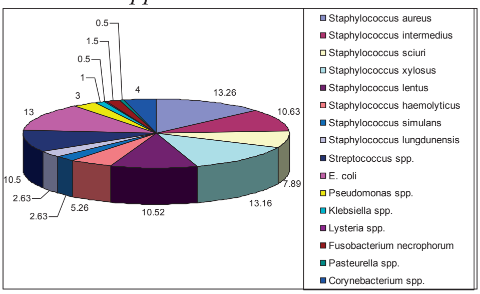

Fig 1. The percentual distribution of bacterial flora in milk sampled during clinical mastitis in cows

Of the Gram positive bacteria, *Staphylococcus xylosus* dominated the microflora, followed by *Staphylococcus aureus*, and other staphylococci and streptococci. Gram negative bacteria, although present, were in a much lower proportion (between 0.5 and 3%), except *E. coli*, present in much higher percentages than other Gram negative rods. API STAPH test and software helped identify the staphylococci species (Figure 2). The most prevalent *S. xylosus*, was more and more frequently cited as inducing mastitis in dairy cows (Bochniarz et al., 2014, Vanderhaeghen et al., 2015).

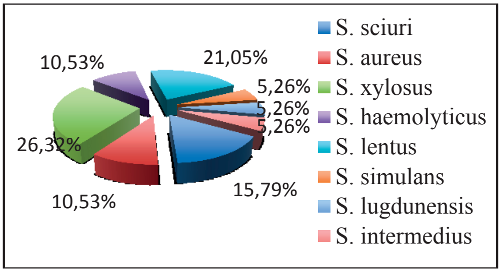

Fig 2. Distribution of *Staphylococcus* species in the mastitic milk

The results of sensitivity/resistance tests performed on the isolated staphylococci were presented in Table 1 and Fig. 3.

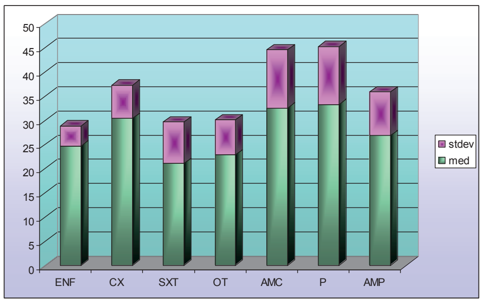

Fig. 3. Average inhibition diameters ranked by antibiotic and tested *Staphylococcus* strains

Table 1. Kirby Bauer test parameters for the tested *Staphylococcus* strains

Category	ENF	CX	SXT	OT	AMC	P	AMP
Sensitive strains	12 (42,85%)	24 (85,71%)	18 (64,28%)	23 (82,14%)	21 (75%)	19 (67,85%)	19 (67,85%)
Strains with resistant colonies RC	7	2	3	1	5	7	8
Resistant strains	9	2	7	4	2	2	1
Average inhibition diameter	24,73± 4,08	30,38± 6,92	21,06± 8,58	22,83± 7,26	32,57± 12,02	33,28± 11,94	26,83± 9,06

An increased resistance to antibiotics is observed, either as total resistance or the presence of resistant colonies to all tested antibiotics. However, *Staphylococcus aureus* still remains the most harmful udder pathogen, since the disease responds poorly to antimicrobial treatment and often remains chronic (Taponen et al., 2003; Wilson et al., 1999). A potential cause is linked to the growth of these bacteria in biofilms (Dunne, 2002; Vuong and Otto, 2002).

MIC and MBC values obtained for honey, honeydew honey and propolis against staphylococcal strains were interconnected (Table 2).These values suggest that honeydew honey, propolis and honey have similar bacteriostatic and bactericidal effects using when simila concentrations were being used.

Table 2. MIC, MBC and bactericidal index against *S. aureus* isolated from cows with mastitis

***S. aureus* strains**	**Honeydew honey**		**MBC/ MIC index**	**Polifloral honey**		**MBC / MIC index**	**Propo lis**		**MBC / MIC index**
N=10	**MIC**	**MBC**		**MIC**	**MBC**		**MIC**	**MBC**	
Mean	3%	3%	100%	2%	2%	100%	1%	1%	100%
Stdev	0.010	0.010		0.005	0.005		0.006	0.006	

As indicated in Table 2, the isolated *Staphylococcus* strains were sensitive to both honey and propolis while the resistance to AMC was high. The importance of the results obtained for the bee products is supported by the broad use of AMC, regarded as a broad spectrum antibiotic, in therapy of human and animal infections.

To investigate the influence of the source of origin on the antibacterial efficacy, the effects of different concentrations and types of honey, in diverse combinations and alone and the effects of various types of propolis were also compared. According to the results, the highest efficiency was present in propolis extract PP1. At the same time it was noticed that at a concentration as low as 1%, the antibacterial effect is low- similar values for both PP1 and PP2 being noticed. Inhibition zones at concentrations of 20, and 40% were non significantly different (table 3).

Table 3. An estimate of the efficacy of different types and concentrations of propolis against *Staphylococcus* spp. isolates

Dilution/ sample	1%				20%				40%			
Propilis type	PS1	PS2	PP1	PP2	PS1	PS2	PP1	PP2	PS1	PS2	PP1	PP2
Mean	8.69	8.00	10.08	8.78	11.73	11.40	14.46	11.23	11.93	11.67	14.08	11.08
Stdev	1.65	1.83	1.71	1.72	2.91	1.90	3.23	1.79	2.76	1.73	3.15	1.78
Resistant strains	2	5	1	3	0	2	0	0	0	1	1	0
Sensitive strains (%)	13 (81,25)	10 (62,5)	13 (81,25)	9 (56,25)	15 (93,75)	10 (62,5)	13 (81,25)	13 (81.25)	15 (93,75)	9 (56,25)	13 (81,25)	12 (75)
RC	1	1	2	4	1	4	3	3	1	6	2	4

Table 4. Average inhibition areas in the Kirby Bauer test using propolis against staphylococcal strains

Staphylococcus spp.	**propolis 1%**	**propolis 20%**	**propolis 40%**	**Alcool**
S. sciuri	9.76	15.05	14.75	0
S. aureus	7.62	11.37	11.12	0
S. xylosus	4.75	11.8	11.48	0.4
S. lentus	5.27	8.56	7.89	1
S. haemolyticus	8.87	11.62	11.75	0
S. lugdunensis	7.5	8.75	8.5	0
S. simulans	7.33	8.5	8.75	0
S. intermedius	7.75	12	12.75	0
Mean	**7.36**	**10.96**	**10.87**	**0.18**
Stdev	1.67	2.26	2.35	0.36

Results from table 3 show that the activity depends on the concentration of propolis and the tested strain.. Thus, the most active propolis was PP1, which, at all concentrations detected the highest number of sensitive strains (n= 13 out of a total of 16). RC were observed in all cultures, while the total resistance to propolis was not recorded for PS1, PP1şi PP2 at concentrations of 20% and 40%. The efficacy of propolis was comparable to that of the oxytetracycline and cloxacillin in terms of the total number of inhibited strains.

Monitoring the effects of the propolis against various species of staphylococci (Table 4) it was observed that. the propolis tincture inhibited the most *Staphylococcus sciuri*, followed by *Staphylococcus intermedius* .

Because of its popularity in folk medicine, propolis has become the subject of intense pharmacological and chemical studies for the last 30 years. Numerous studies have proven its versatile pharmacological activities: antibacterial, antifungal, antiviral, anti-inflamatory, hepatoprotective, antioxidant, antitumoral (Banskota et al., 2001a, b; Rindt et al., 2009)

A significant number of papers dealing with propolis chemistry were also published and researchers began to understand that its chemical composition was highly variable and depended on the local flora at the site of collection (Marcucci,1995 and Bankova et al., 2000). Although the biological activity of bee glue and especially its activity against microorganisms was always present, in samples from different geographic and climatic zones this activity was the result of completely different chemical composition (Kujumgiev et al., 1999). As a result, recently almost every publication on propolis biological activity includes some kind of chemical characterization of the bee glue used (Bankova, 2005). However, to be formally accepted in therapy, propolis require chemical standardization that can guarantee the quality, safety and efficacy of the product (Rindt et al., 2009a; Rindt et al., 2009b).

The obtained data indicated a higher number of resistant strains or strains with RC to the antibiotics than towards propolis and honey. Meanwhile, there was a directly dose-dependent activity of the propolis up to 20% which decreased for higher doses against the majority of isolated staphylococcal strains.

CONCLUSIONS

The high number of antibiotic resistant bacterial species isolated from dairy cows with clinical mastitis, stress the importance of alternative therapies to be used on the tested farms. Honey and propolis both represented valuable therapeutical alternatives, showing increased activity against the numerous *Staphylococcu*s spp. strains isolated from clinical cases of bovine mastitis. The obtained results are encouraging, mainly for the clinical use of propolis in therapy, after composition studies and aleviation of its irritative effects *in vivo*.

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