COMPARATIVE EFFICIENCY OF AN ANTIBIOTHERAPY AND A HONEY-BASED ALTERNATIVE TREATMENT OF ACUTE SHEEP STAPHYLOCOCCAL MASTITIS IN ALGERIA

Mostefa Ghallal[1,2], Hamza Khaled[1,2], Mohamed Besbaci[1,2], Mohammed Ramdani[3], Abdallah Bouyoucef[1,2]

[1]Institute of Veterinary Sciences, University Blida 1, Blida, Algeria. [2]Laboratory of Animal Reproduction Biotechnologies (LBRA), University of Blida 1, Blida, Algeria. [3]Department of Zoology and Animal Ecology, Scientific Institute, University Mohammed V of Rabat, Morocco. ghallalmostefa2021@gmail.com; khaledhamz@yahoo.fr; besbaci@gmail.com; ramdanimed@gmail.com; abdallahbouyoucef@gmail.com

Abstract: This investigation aims to assess the efficiency of honey/starch in treatment of clinical mastitis in ewes and to compare it with a conventional treatment. Acute mastitis was induced in 60 healthy ewes by inoculating a reference strain of Staphylococcus aureus. Ewes were divided randomly into three groups and received one of the following treatments; Group 1 (n=20): honey/starch, Group 2 (n=20): amoxicillin/clavulanic acid, Group 3 "control" (n=20) which received no treatment. The assessment of treatment efficiency was realized based on clinical assessment: disappearance of mastitis clinical signs in the first 7 days, bacteriological assessment: negative bacteriological culture in the first 7 days, cellular assessment: negative California mastitis test in the 4 first weeks, zootechnical assessment: normal milk yield in the first 7 days. Udders treated with antibiotic had a complete clinical healing of 62.5% on day 4 and 100% on day 6 and day 7, a bacteriological healing of 100% on day 1 and until day 7, a cellular healing of 100% on week 3 and week 4, and a zootechnical healing of 77.5% on day 4 and 100% on day 6 and day 7. Udders treated with honey/starch had a complete clinical healing of 65% on day 3 and 95% on day 5 and until day 7, a bacteriological healing of 95% on day 1 and until day 7, no cellular healing on week 4, and a zootechnical healing of 75% on day 3 and 95% on day 5 and until day 7. The control group had no healing. By reconsidering the adverse effects of antibiotics, honey could be an interesting alternative to antibiotics for treating acute mastitis in sheep.

Keywords: mastitis, ewe, *Staphylococcus aureus*, antibiotic, honey.

INTRODUCTION

Mastitis is defined as a mammary gland inflammation affecting all lactating animals (De Vliegher et al., 2012). Clinical and subclinical mastitis cause severe transitory inflammatory signs due to traumatic, pathological, and bacteriological changes in mammary glands that can lead to transient or permanent blocking of milk ducts (Monsang et al., 2014).

Mastitis is known to seriously impact the general health and the animals' well-being causing significant animal welfare concerns impacting consumer and public perspectives as well as contributing to economic losses for farmers (Alekish et al., 2017; Dhakal et al., 2016).

Antibiotic therapy is the primary tool for mastitis control in dairy herds, and antibacterial agents have been the mainstay of mastitis treatment and prevention programs for decades. However, antibiotics are not always effective against mastitis

pathogens. Variable success rate for curing infections with antibiotics and misuse or overuse of antimicrobial drugs has led to the emergence of resistant bacteria (Gomes and Henriques, 2015). Consequently, the non-responsiveness of animals to antibiotics therapy and the transfer of microbial resistance to human food chain have a lot of human health consequences (De Jong et al., 2018). The infectious multidrug-resistant bacteria have become a major health problem and limited the option for effective treatment in human and animal population worldwide (Gheldof et al., 2002). Moreover, the risk of emerging antimicrobial resistance against mastitis pathogens has been reported in many studies, particularly for drugs with highly therapeutic value in human medicine (Benhanifia et al., 2019). Although the new generation of advanced antibiotics was produced by pharmacological industries, numbers of drug-resistant bacteria have been increasing. In addition, the adverse effects of antibiotics harm vital organs such as liver, kidneys, pancreas and spleen (Hadjazi et al., 2015). All these limitations presented by antibiotics and the current and rising issue associated with multidrug-resistant bacteria, which is an urgent global health threat, have led to the urgency of finding new and innovative alternative treatments as effective strategies to control mastitis. In the past few decades, many researches have been focused on characterizing the antibacterial effects of different natural substances for the treatment of various animal diseases including mastitis (Benhanifia et al., 2019).

Honey is a natural remedy which has been used as a medication since ancient time, which is known as "traditional medicine" in various cultures. It has a valuable role in folk medicine worldwide for thousands of years (Irish et al., 2011). It has been reported that honey has an inhibitory effect to around 60 species of bacteria including aerobes, anaerobes, Gram-positives, and Gram-negatives pathogens (Olaitan et al., 2007). The potent *in vitro* activity of honey against antibiotic-resistant bacteria (Lehtopolku et al., 2010; Patel and Chauhan, 2017) and its successful application in treatment of many infections is a promising research topic as it has been documented in previous works (Al-Waili et al., 2012; Lehtopolku et al., 2010; Elmenoufy, 2012). Recently, there is a renewed interest in the study of honey as a natural product for therapeutic purposes (Al-Waili et al., 2012). In addition, there has been a renewed interest in honey composition and biological properties such as antimicrobial, antioxidant, anti-inflammatory, immunomodulatory and antitumor (Fernandez-Cabezudo et al., 2013; Samarghandian et al., 2017; Cianciosi et al., 2018; McLoone et al., 2016; Bourabah et al., 2014).

In the present study, we evaluated the *in vivo* efficiency of a honey/starch-based treatment, compared to an antibiotic therapy of acute mastitis in sheep caused by *S. aureus*.

MaterialS and Methods

Ethical approval

This present study was performed in accordance with Algerian laws and regulations in force on animal welfare and according to the Guidelines of the Ethics and Animal Welfare Committee of the Veterinary Sciences Institute of Blida, Algeria.

Animals and study area

Experimental study was conducted in a dairy farm in the province of Tiaret (35° 23' 17' N, 1° 19' 22' E), north-western of Algeria. A total of 60 Rumbi lactating ewes

were used. They gave birth since about one month and were aged between one and two years. They did not present any signs of clinical or subclinical mastitis at the time of the experiment.

Physical examination and clinical signs

For the detection of a clinical mastitis, all ewes were subjected to a physical examination and an assessment of milk quality in a dark plate was carried out; clinical data were then recorded. They presented no signs of clinical mastitis, such as temperature ≤ 39 °C, no udder inflammatory signs and visibly normal milk. The quantity of milk yield was also assessed and measured for each ewe.

California Mastitis Test (CMT)

California mastitis test was performed for subclinical mastitis detection; it was carried out according to the Clinical and Laboratory Standard Institute (CLSI, 2020), the National Mastitis Council (NMC) guidelines (NMC, 2004), and Zeedan et al. (2014) (Zeedan et al., 2014).

Milk sampling for bacteriological examination

A bacteriological examination was also performed for detection of subclinical or incubating mastitis. Milk samples were collected from each ewe using procedures recommended by the NMC (NMC, 2004) and according to the standard procedures of International Dairy Federation (IDF, 2011). Bacterial isolation from milk samples was carried out following aseptic procedures as described by the NMC (NMC, 2004). Milk samples were cultivated on various culture media including nutrient agar, Columbia blood agar and nutrient broth. All plates were then incubated aerobically at a temperature of 37 °C during 24 h.

Inoculum preparation and mastitis induction

To induce mastitis, a reference strain of *Staphylococcus aureus* ATCC 33862 was used. The inoculum was obtained by taking five colonies from the 24 hour-old culture grown on specific medium Chapman agar. The colonies were suspended in 5 ml of sterile saline solution (0.9% NaCl), and the mixture was shaken for 15 seconds. The density was adjusted to the turbidity of a 0.5 Standard McFarland. Once the bacterial suspension is prepared, it was placed in an ice box and transported to the farm and used within 2 h. Teats were disinfected with disposable disinfectant towels and 0.5 ml of the bacterial suspension was inoculated in each udder using sterile micropipettes.

Mastitis confirmation

Physical examination, milk quantity assessment, CMT, and bacteriological examination were realized 24 h and 48 h after bacterium inoculation to diagnose a mastitis. For the bacteriological procedures, milk samples were cultivated on selective medium Chapman agar and then incubated aerobically at 37 °C for 24 h. The plates were examined at 24-48 h. Colonies of *S. aureus* were identified by cultural characteristics, based on colony size, morphology, appearance and pigmentation. After the purification of bacterium, the selected colonies were subjected to the Gram staining, biochemical tests (catalase reaction and coagulase test) and finally the Analytical Profile Index (API-Staph 20 Kit), which was used according to the manufacturer's instructions and

according to methods described by Taponen et al. (2006) (Taponen et al., 2006) and López-malo et al. (2005) (López-Malo et al., 2005).

Mastitis treatment

Ewes were divided randomly into three main groups; group 1 (n=20): received 3 injections of a half of an intramammary preparation Amoxicillin/clavulanic acid (200 mg/50 mg; injector of 3 g) intended for cattle, i.e., 1.5 g in each udder half at 12 h intervals according to the manufacturer's instructions, group 2 (n=20): received an amount of 5 ml of a natural preparation (75% fennel honey+25% cornstarch solution) in each teat at 12 h intervals for 3 consecutive days, and group 3 (n=20) received no treatment (group "control"). Note that all ewes were milked twice a day at 6:00 a.m. and 6:00 p.m. All treatments were administered aseptically and atraumatically in affected udders by intramammary route through the teat canal immediately after full milking: the antibiotic preparation of an intramammary injector (intramammary tube) intended for udders was directly infused and the natural preparation was introduced using a syringe and an intramammary probe which was cleaned and disinfected in an antiseptic solution between each application and another and from a teat to another. Before administering the treatments, the udders were carefully washed with soapy water and then completely dried with a towel. The orifice and the entire teat end were disinfected using a single-use disinfectant wipe soaked in alcohol. After inserting the treatments into the teat, the udders were massaged from bottom to top for well infusing medication throughout the surrounding tissue; in the meantime, teats were pinched preventing treatments from flowing back out.

Honey samples

Pure honey fennel used in our investigation was obtained from a local beekeeper in the region of Tiaret (35° 23' 17' N, 1° 19' 22' E), in the north-west of Algeria. The honey sample was stored in a sterile black bottle (dark place) and kept in refrigerator at 4 °C until use.

Antibiotic susceptibility testing

The antibiotic assay was performed using disk containing the antibiotic combination Amoxicillin (20 µg)/clavulanic acid (10 µm) against the reference strain of *S. aureus* ATCC 33862. The bacterial suspension (standard inoculum) was inoculated into Muller Hinton Agar (MHA) according to KIRBY-BAUER. Diameter of inhibition zone was interpreted according to the interpretive standards of the National Committee of Clinical Laboratory Standards (NCCLS, 1997).

Determination of antibacterial activity of alternative formula

Antibacterial activity i*n vitro* of alternative formula was tested against the reference strain of *S. aureus*. The evaluation of this activity was performed by determining of minimal inhibitory concentration (MIC) using the diffusion method on MHA according to the method of Hegazi (2011) (Hegazi, 2011) and to the National Committee of Clinical Laboratory Standards (NCCLS, 1997). Graded concentrations of alternative formula from 5 to 10% (volume/volume; v/v) were incorporated into MHA (Tan et al., 2009; Mullai and Menon, 2007) and the final volume of formula and culture medium in each plate was adjusted to a total of 5 ml. The MIC value was done according

to the method described by Clinical and Laboratory Standard Institute (CLSI, 2006). It was defined as the lowest concentration of formula that completely inhibited the visible bacterial growth after 24 h of incubation. To valid our result, a positive control was realized.

Assessment of treatment efficiency

All ewes were kept under observation after treatment administration for any eventual immediate adverse effects and were monitored for all the duration of treatment. Nevertheless, the main aim of keeping the ewes under surveillance is to assess the efficiency of the two treatments implemented.

The assessment of treatment efficiency was based on four healing criteria:

Clinical assessment: Ewes were subjected to a daily clinical examination for the first 7 days and in a month later, to follow the evolution of mastitis clinical signs: inflammatory signs (heat, redness, swelling and pain), pus and lumps, changes in consistency and color milk, and abscesses;

Bacteriological assessment: Milk samples were taken daily during the first 7 days and subjected to a usual bacteriological examination;

Cellular assessment: A CMT was carried out every week for a month after treatment starting;

Zootechnical assessment: Milk production was measured daily for the first 7 days and compared to that recorded before mastitis induction.

Fate of untreated ewes (group "control"):

On the 7^{th} day, we began a massive treatment of all the "control" ewes with antibiotic therapy and corticosteroid therapy by local and general route. Once the ewes were cured, respecting the withdrawal period for prescribed medication, they were reformed and were destined for slaughter.

Statistical analysis

Statistical calculations were performed by R software (version 3.2.3; R basis; Vienna; Austria) using statistical packages "Survival". The recovery follow-up was established from an analysis of survival with or without event by the Kaplan-Meier method. The qualitative variables comparison was done by the Log-Rank test. A comparison between the two-by-two groups was realized by the Bonferroni adjustment method. A comparison between variables was considered significant when the p value was less than 0.05.

Results

Clinical assessment

Udders treated by Amoxicillin/clavulanic acid: The first 3 days after the treatment beginning, no udder has completely healed. On day 4, mastitis clinical signs disappeared completely in 62.5% of udders. From day 6, clinical signs have not been observed in 100% of udders (Table 1.a). The same result was recorded 1 month later.

Table 1.a.

Healing rates of udders treated with Amoxicillin/clavulanic acid.

	Inflammatory signs	Pus and lumps	changes in milk consistency and color	Abscesses	Clinical healing	Milk yield (zootechnical healing)	Bacteriological culture (bacteriological healing)	CMT (cellular healing)
Day 1	00 %	00 %	00 %	00 %	00 %	00 %	100 %	/
Day 2	00 %	00 %	00 %	00 %	00 %	00 %	100 %	/
Day 3	75 %	80 %	82.5 %	00 %	00 %	00 %	100 %	/
Day 4	85 %	97.5 %	90 %	62.5 %	62.5 %	77.5 %	100 %	/
Day 5	100 %	100 %	100 %	90 %	90 %	87.5 %	100 %	/
Day 6	100 %	100 %	100 %	100 %	100 %	100 %	100 %	/
Day 7	100 %	100 %	100 %	100 %	100 %	100 %	100 %	00 %
Week 2	/	/	/	/	/	/	/	00 %
Week 3	/	/	/	/	/	/	/	100 %
Week 4	/	/	/	/	/	/	/	100 %

Table 1.b: Morbidity rates of udders treated with Amoxicillin/clavulanic acid.

	Inflammatory signs	Pus and lumps	changes in milk consistency and color	Abscesses	Clinical morbidity	Milk yield (zootechnical morbidity)	Bacteriological culture (bacteriological morbidity)	CMT (cellular morbidity)
Day 1	100 %	100 %	100 %	100 %	100 %	100 %	00 %	/
Day 2	100 %	100 %	100 %	100 %	100 %	100 %	00 %	/
Day 3	25 %	20 %	17.5 %	100 %	100 %	100 %	00 %	/
Day 4	15 %	2.5 %	10 %	37.5 %	37.5 %	22.5 %	00 %	/
Day 5	00 %	00 %	00 %	10 %	10 %	12.5 %	00 %	/
Day 6	00 %	00 %	00 %	00 %	00 %	00 %	00 %	/

Day 7	00 %	00 %	00 %	00 %	00 %	00 %	00 %	100 %
Week 2	/	/	/	/	/	/	/	100 %
Week 3	/	/	/	/	/	/	/	00 %
Week 4	/	/	/	/	/	/	/	00 %

Udders treated by honey/starch: On day 1 and day 2 after the treatment beginning, no udder has completely healed, the milk was liquefied in consistency and brown in color. On day 3, mastitis clinical signs disappeared completely in 65% of udders. From day 5, i.e., 48 hours after stopping treatment, clinical signs disappeared completely in 95% of udders and the milk returned into its normal consistency and color. A failure rate of 5% was therefore reported, in which the unhealed udders retained their abscesses with the presence of pus and lumps on day 7 (Table 2.a). The same result was recorded 1 month later.

Table 2.a: Healing rates of udders treated with the honey/starch.

	Inflammatory signs	**Pus and lumps**	**changes in milk consistency and color**	**Abscesses**	**Clinical healing**	**Milk yield (zootechnical healing)**	**Bacteriological culture (bacteriological healing)**	**CMT (cellular healing)**
Day 1	00 %	00 %	00 %	00 %	00 %	00 %	95 %	/
Day 2	70 %	75 %	77.5 %	00 %	00 %	00 %	95 %	/
Day 3	80 %	85 %	90 %	65 %	65 %	75 %	95 %	/
Day 4	100 %	95 %	100 %	75 %	75 %	82.5 %	95 %	/
Day 5	100 %	95 %	100 %	95 %	95 %	95 %	95 %	/
Day 6	100 %	95 %	100 %	95 %	95 %	95 %	95 %	/
Day 7	100 %	95 %	100 %	95 %	95 %	95 %	95 %	00 %
Week 2	/	/	/	/	/	/	/	00 %
Week 3	/	/	/	/	/	/	/	00 %
Week 4	/	/	/	/	/	/	/	00 %

Table 2.b: Morbidity rates of udders treated with the honey/starch.

	Inflammatory signs	Pus and lumps	changes in milk consistency and color	Abscesses	Clinical morbidity	Milk yield (zootechnical morbidity)	Bacteriological culture (bacteriological morbidity)	CMT (cellular morbidity)
Day 1	100 %	100 %	100 %	100 %	100 %	100 %	5 %	/
Day 2	30 %	25 %	22.5 %	100 %	100 %	100 %	5 %	/
Day 3	20 %	15 %	10 %	35 %	35 %	25 %	5 %	/
Day 4	00 %	5 %	00 %	25 %	25 %	17.5 %	5 %	/
Day 5	00 %	5 %	00 %	5 %	5 %	5 %	5 %	/
Day 6	00 %	5 %	00 %	5 %	5 %	5 %	5 %	/
Day 7	00 %	5 %	00 %	5 %	5 %	5 %	5 %	100 %
Week 2	/	/	/	/	/	/	/	100 %
Week 3	/	/	/	/	/	/	/	100 %
Week 4	/	/	/	/	/	/	/	100 %

Udders "control": On day 7 after mastitis induction, inflammation signs were found to have disappeared in 15% of untreated udders, but no udder had a complete clinical healing (0% of clinical healing) (Table 3.a).

Table 3.a: Healing rates of untreated udders "control" after mastitis induction.

	Inflammatory signs	Pus and lumps	changes in milk consistency and color	Abscesses	Clinical healing	Milk yield (zootechnical healing)	Bacteriological culture (bacteriological healing)	CMT (cellular healing)
Day 1	00 %	00 %	00 %	00 %	00 %	00 %	00 %	/
Day 2	00 %	00 %	00 %	00 %	00 %	00 %	00 %	/
Day 3	00 %	00 %	00 %	00 %	00 %	00 %	00 %	/
Day 4	00 %	00 %	00 %	00 %	00 %	00 %	00 %	/
Day 5	00 %	00 %	00 %	00 %	00 %	00 %	00 %	/
Day 6	10 %	00 %	00 %	00 %	00 %	00 %	00 %	/

Day 7	15 %	00 %	00 %	00 %	00 %	00 %	00 %	00 %
Week 2	/	/	/	/	/	/	/	00 %
Week 3	/	/	/	/	/	/	/	00 %
Week 4	/	/	/	/	/	/	/	00 %

Table 3.b: Morbidity rates of untreated udders "control" after mastitis induction.

	Inflammatory signs	**Pus and lumps**	**changes in milk consistency and color**	**Abscesses**	**Clinical morbidity**	**Milk yield (zootechnical morbidity)**	**Bacteriological culture (bacteriological morbidity)**	**CMT (cellular morbidity)**
Day 1	100 %	100 %	100 %	100 %	100 %	100 %	100 %	/
Day 2	100 %	100 %	100 %	100 %	100 %	100 %	100 %	/
Day 3	100 %	100 %	100 %	100 %	100 %	100 %	100 %	/
Day 4	100 %	100 %	100 %	100 %	100 %	100 %	100 %	/
Day 5	100 %	100 %	100 %	100 %	100 %	100 %	100 %	/
Day 6	90 %	100 %	100 %	100 %	100 %	100 %	100 %	/
Day 7	85 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %
Week 2	/	/	/	/	/	/	/	100 %
Week 3	/	/	/	/	/	/	/	100 %
Week 4	/	/	/	/	/	/	/	100 %

Table 4.a: Kaplan Meier estimates of median time to recovery with 95% confidence intervals (CI) for each parameter in the three groups of ewes with mastitis.

Parameters	Groups	n	Event (Recovery)	Median (time to recovery)	0.95LCL	0.95UCL	P value
Bacteriological healing	Control	40	0	NA	NA	NA	< 0,0001
	Antibiotic	40	40	1	NA	NA	
	Honey	40	38	1	1	1	
Zootechnical healing	Control	40	0	NA	NA	NA	< 0,0001
	Antibiotic	40	40	4	4	4	
	Honey	40	38	3	3	3	
Clinical healing	Control	40	0	NA	NA	NA	< 0,0001
	Antibiotic	40	40	4	4	5	
	Honey	40	38	3	3	4	

Table 4.b: Pairwise comparisons using Log-Rank test (P value adjustment method: Bonferroni).

Parameters	Groups	Control	Honey
Bacteriological healing	Honey	p < 0,0001	-
	Antibiotic	p < 0,0001	p = 0,46
Zootechnical healing	Honey	p < 0,0001	-
	Antibiotic	p < 0,0001	p = 0.00035
Clinical healing	Honey	p < 0,0001	-
	Antibiotic	p < 0,0001	p = 0.01

Bacteriological assessment

Udders treated by Amoxicillin/clavulanic acid: On day 1 and until day 7 after the treatment beginning 100% of milk samples had a negative bacteriological culture (Table 1.a).

Udders treated by honey/starch: On day 1 and up to day 7 after the treatment beginning, 95% of milk samples were all negative. It was therefore found that 5% of udders which did not clinically heal had a positive bacteriological culture even on day 7 (Table 2.a).

Udders "control": On day 7 after mastitis induction, all udders had a positive bacteriological culture (0% of bacteriological healing) (Table 3.a).

Cellular assessment

Udders treated by Amoxicillin/clavulanic acid: All milk samples of week 1 and week 2 after the treatment beginning reacted positively to CMT. Then for the 2 weeks later, 100% of udders gave a negative result (Table 1.a).

Udders treated by honey/starch: For the 4 weeks after the treatment beginning, 100% of udders had a positive CMT (Table 2.a).

Udders "control": For the 4 weeks after mastitis induction, all udders had a positive CMT (0% of cellular healing) (Table 3.a).

Zootechnical assessment

Udders treated by Amoxicillin/clavulanic acid: Until day 3 after the treatment beginning, all udders had a decreased milk secretion. From day 4, normal milk production has been resumed in 77.5% of udders. From day 6, 100% of udders recovered a normal milk production equivalent to that appreciated before the mastitis induction (Table 1.a).

Udders treated by honey/starch: On day 1 and day 2 after the treatment beginning, all udders had an approximately normal quantity of milk secretion, thus equivalent to a reduced quantity (bias of the honey's osmotic effect). From day 3, a milk production increase was observed in 75% of udders, thus equivalent to a normal quantity (bias of the honey's osmotic effect). From day 5, i.e., 48 hours after stopping treatment, 95% of udders resumed their normal milk production. It was therefore recorded that 5% of udders that did not clinically heal had a decreased milk production on day 7 (Table 2.a).

Udders "control": 7 days after mastitis induction, untreated udders did not restore their initial milk production (0% of zootechnical healing) (Table 3.a).

Adverse effects

All ewes tolerated the intramammary infusion of the alternative formula very well. No local or systemic effects were observed.

Statistical study

A high significant difference ($p<0.0001$) was observed between the three groups of animals: antibiotic, honey/starch, control (Table 4.a), between the antibiotic group and the control group, and between the honey/starch group and the control group (Table 4.b); regarding clinical, zootechnical and bacteriological healing.

There was a significant difference between the antibiotic group and the honey/starch group in clinical healing ($p=0.01$) and zootechnical healing ($p=0.00035$),

but no significant difference (p=0,46) was found between these two groups in bacteriological healing (Table 4.b).

DISCUSSION

The antibiotic treatment was 100% effective (clinical, zootechnical, bacteriological and cellular healing).

In the present work, *S. aureus* showed a susceptibility to amoxicillin/clavulanic acid. A similar susceptibility of *S. aureus* to β-lactams was reported in ewes, cows (Pengov and Ceru, 2003) and does mastitis (Bourabah et al., 2014). Alekish et al. (2017) have treated 5 ewes suffering from subclinical mastitis with 3 ml of Amoxicillin, one injection every 12 hours for 3 successive days and they have observed, 24 hours and 48 hours after treatment stopping, in 100% of the treated ewes a cellular healing and a milk quality improvement, indicating efficient therapeutic effects against ovine mastitis and a possible improvement in health udder (Alekish et al., 2017). On the other hand, the study of Monsallier (2000) has demonstrated a 7-day healing rate of acute bovine mastitis caused by *S. aureus*, treated with the amoxicillin/clavulanic acid combination in 86% of cases (Monsallier, 2000). Variation in results could be due to the difference in a lot of parameters: the therapeutic protocol (dose, concentration, duration and use of Amoxicillin as monotherapy or in combination with other active ingredients), the animal species affected by mastitis (ovine or bovine), the number of experimental animals, the mastitis type (acute or subclinical), the species and strain of bacteria implicated with mastitis.

Bacteriological healing was fast, 24 hours after the treatment beginning. This is reasonable because of the early onset of treatment, the speed and the potency of its bactericidal effect (Monsallier, 2000). Furthermore, the antibiogram carried out also demonstrated a high sensitivity of *S. aureus* to the antibiotic treatment used with inhibition zone diameter of 24 mm. Monsallier (2000) has shown, in a study carried out on 339 strains of *S. aureus*, a sensitivity rate to Amoxicillin/clavulanic acid of 100% (Monsallier, 2000). The antibiotic treatment administered, which is probably massive, could also be responsible for this result. Indeed, the commercialized antibiotic preparations for intramammary treatment possessing a marketing authorization in ewes are currently very rare, even non-existent; we can therefore only use products intended for cows (Bergonier et al., 2003).

The udders treated with the antibiotic had late cellular healing, i.e., the 3rd week after the treatment beginning, and the 2nd week after clinical, zootechnical and bacteriological healing.

In fact, to achieve the CMT, a time limit must be respected, and the CMT must be repeated, because the cells decrease less quickly after clinical healing (Monsallier, 2000).

The alternative treatment was effective in 95% of the treated udders (clinical, zootechnical, bacteriological and cellular healing).

In vivo, our result was not in agreement with those found by other authors who treated subclinical bovine mastitis with honey. Nahed et al. (2011) have injected 10 ml of 10% fennel honey in 10 cows once a day for 3 days successively, and 10 ml of 10% fennel honey in 15 cows day after day for 3 successive doses with intramuscular

administration of an antihistamine. They had 100% cell healing on the 3rd and 10th day, but, based on other healing criteria (milk cytology and milk production), they concluded that the 2nd protocol could be used to treat subclinical bovine mastitis (Nahed et al., 2011). Benhanifia et al. (2019) have treated 3 cows with *S. aureus* subclinical mastitis, by injecting 5 ml of pure multifloral honey (undiluted) once a day for 2 consecutive days. They found that 66.6% of the cows are cured on the 7th day after the treatment beginning and 100% of the cows on the 21st day (Benhanifia et al., 2019). Abdel-hafeez et al. (2005) have demonstrated a significant decrease in the bacteria total number and a very significant increase in milk production, following intramammary infusion with fennel honey (Abdel-Hafeez et al., 2005). These highly disparate results could be explained by variation in several factors, namely the therapeutic protocol (dose, concentration, duration and use of honey as monotherapy or in combination with other substances), botanical origin of the honey, the species and strain of bacteria implicated in mastitis, the mastitis type (acute or subclinical), the animal species affected by mastitis (ewes or cows) and the number of experimental animals.

Bacteriological healing was early, i.e., 24 hours after the treatment beginning, the precocity of treatment installation and the very rapid diffusion of honey due to its anti-inflammatory effect (Al-Waili, 2003) could explain this spectacular result. The alternative treatment used could also be massive and of ample duration. Standardized therapeutic protocols for our alternative formula were almost absent. Some researchers have proposed therapeutic protocols using honey as monotherapy for the treatment of subclinical mastitis in cows: Nahed et al. (2011) have injected 10 ml once a day for 3 consecutive days or day by day for 3 successive doses (Nahed et al., 2011); while Benhanifia et al. (2019) have injected 5 ml for 2 consecutive days (Benhanifia et al., 2019).

In the current investigation, we noticed that the presently tested natural preparation (honey/starch) was effective against *S. aureus* with a MIC of 7% (*v/v*) as it completely inhibits its growth. This result was in agreement with other authors (Benhanifia et al., 2019; Boukraa et al., 2008; Hegazi, 2011; Abdalhamed et al., 2018). This clearly indicates that the honey tested was of high antimicrobial quality. The antiseptic and antibacterial properties of honey have been known since immemorial time (Mullai and Menon, 2007). It is documented and proven that *S. aureus* is one of the most sensitive species to the antimicrobial activity of honey and in all bacterial strains studied (Jenkins and Cooper, 2012; Sayed et al., 2009). Applying honey to wounds infected with *S. aureus* and other *Staphylococci* results in their asepsis within a few days and the wounds heal promptly (Mullai and Menon, 2007). Many authors studied the *in vivo* antibacterial activity of manuka honey and found that *S. aureus* was well inhibited (Kwakman et al., 2008; Hassanein et al., 2010). Honey has potent *in vitro* activity against bacteria (Lehtopolku et al., 2010) and its successful application in the treatment of certain infections is confirmed by reports of numerous researchers (Al-Waili et al., 2012; Lehtopolku et al., 2010; Elmenoufy, 2012). Egyptian honey has some effect on microorganisms isolated from clinical sheep mastitis at different concentrations, of which *S. aureus* was the most affected microbe (Abdalhamed et al., 2018). On the other hand, an effective additive and synergistic action *in vitro* of starch and honey against *S. aureus* (Boukraa and Amara, 2008) and fennel honey and propolis has already been revealed (Aamer et al., 2015).

Many reports literature revealed that honeys originating from different geographical locations (countries and regions) showed wide variability in their antimicrobial activity. This could be attributed to the raw materials utilized by bees in preparing their valuable honey (Hegazi, 2011). The antibacterial activity of Algeria honey, in combination with starch, is more significant than that of other honeys, tested as monotherapy, which showed higher MIC: Egyptian honey 12.5% (Abdel-Hafeez et al., 2005), fennel honey 13.3% (Aamer et al., 2015), fennel honey 33.33% (Basson and Grobler, 2008), as well as honeys of different botanical and geographical origins (countries and regions) 8 to 50% or more (Hegazi, 2011). Bourabah et al. (2014) have found that Algerian honey (collected in Tiaret) was effective against different pathogens isolated from goat's subclinical mastitis at a MIC varying from 11 to 14%. It showed higher activity against *S. aureus* (13.25±0.95%) (Bourabah et al., 2014). In contrast, the antibacterial activity of Algeria honey, in combination with starch, remains less efficient than that of other varieties of honey, used as monotherapy, which showed lower MIC: honeys of different botanical and geographical origins (countries and regions) 3 to 6% or even less (Hegazi, 2011), Malay honey 5% (Ruiz et al., 2013), honey with antibacterial peroxidase activity 5% and manuka honey of United Kingdom 6% (Ruiz et al., 2013) and Ethiopian honey 6.25% (Moussa et al., 2012).

The large spectrum of the antibacterial activity of honey is multi-factorial in nature, it may be in relation to the presence of hydrogen peroxide (Almasaudi et al., 2017) and the osmotic effect of honey (high osmolarity) (Descottes, 2009; Pascual-Maté et al., 2018). Its high sugar contents (80%) create a high osmotic pressure which is unfavorable to bacterial growth and proliferation. The acidity of honey is also incriminated. Honey has acidic pH ranging from 4.31 to 6.02, which plays a role in microbial inhibition (Pascual-Maté et al., 2018). In addition, the antibacterial action of honey may also be due to the propolis which contains flavonoids (Hegazi et al., 2017) and the gluconic acid which originates from the dissolution of sugar by honey's glucose-oxidase (Almasaudi et al., 2017). Egyptian honey has inhibitory effect which may be due to its complex composition including hydrogen peroxide and bee-derived enzyme glucose oxidase which has a bactericidal effect. It may be also attributed to natural honey extract which is rich in contents of active compounds such as alkaloids and flavones (Abdalhamed et al., 2018). This result is in consistent with Nelson et al. (2007) (Nelson et al., 2007) and Saad et al. (2013) (Saad et al., 2013). The antibacterial effect of New Zealand honey is very strong although it does not contain hydrogen peroxide or glucose-oxidase. A recent study reported that the compound of honey responsible for higher anti-bacterial activity is methylglyoxal which is effective against both Gram-positive and Gram-negative bacteria with inhibitory effect ranging from 85.7 to 100% (Lu et al., 2013). In addition, the components of honey have more than 181 constituents (Pascual-Maté et al., 2018).

No udders treated with honey/starch had cell healing even a month later after the treatment beginning.

California mastitis tests performed for the udders treated with the alternative treatment were positive the 4th week after the treatment start, i.e., 3 weeks after clinical, zootechnical and bacteriological recovery. These reactions cannot be taken into account, because the CMT gives extremely false results by excess. Our results are consistent with those of Nahed et al. (2011), all cows when examined for CMT before mastitis treatment with honey, scored results ranging from traces (T) up to positive reactions (++ve), while

after treatment, scored extremely positive reactions (+++ve). They are considered false positive reactions since the honey infusion is accompanied by a significant increase in lymphocytes percentage (Nahed et al., 2011). The same results were recorded by Abdel-hafeez et al. (2005) and persisted for three months post intramammary honey infusion (Abdel-Hafeez et al., 2005).

On the 1st day after the alternative treatment start, the milk was liquefied and brown in color (honey color) and did not return to its initial qualities until 48 hours after the treatment stopping.

It could be attributed to the osmotic effect exerted locally by honey in the udder, which is probably the cause of the liquefaction and the brown color of the milk. In reality, its osmolarity (ability to extract water from living cells), resulting from its high sugar content, promotes exudation by generating an outward flow of blood and lymphatic fluids (Descottes, 2009).

On the 1st and 2nd day after the alternative treatment starting, all the udders had a milk secretion of approximately normal quantity, it was therefore equivalent to a reduced quantity (bias of the honey osmotic effect is incriminated). From the 3rd day, a slight increase in milk production was observed in 75% of the udders, equivaling approximately a normal quantity (always due to the bias of the honey osmotic effect). From the 5th day, i.e., 48 hours after treatment stopping, 95% of the udders resumed their normal milk production.

This could also be explained by the honey osmotic effect. The exudation it generates in the udder is believed to be the cause of the milk production increase (Descottes, 2009). Such an increase in milk production following honey intramammary application has been reported by Nahed et al. (2011) (Nahed et al., 2011).

There was a failure rate of the alternative treatment of 5%, whose udders still had abscesses, pus and lumps, as well as decreased milk production and a positive bacteriological culture on the 7th day.

S. aureus is a pathogen agent that persists in mammary glands, teat canals, and teat lesions of infected animals (Peton and Le Loir, 2014). In addition, it has virulence factors, which can aid in escape to host immune defenses, resist antibiotic treatment, and increase the infections severity (Scali et al., 2015). This bacterium has the particularity of encysting in udder tissue and forming fibrosis, biofilms and micro-abscesses where it escapes, which is the cause of chronic mastitis (Fitzgerald, 2012). This situation allows *S. aureus* to easily evade all means of defense, whether natural or therapeutic, and it is sometimes very difficult to be eliminated after lactation treatment (Thatcher et al., 2014). Biofilm-associated bacteria show an innate resistance to antibiotics and disinfectants by host defense mechanisms, which is considered as a potential contributor to the poor response of chronic *S. aureus* to antibacterial therapy (Melchior et al., 2006). With the presence of abscesses in unhealed udders, it is reasonable to assume that honey activity was limited by the same factors that limit the usefulness of antibiotics in these cases (Thatcher et al., 2014).

All ewes tolerated the intramammary infusion of the alternative formula very well. Negative local or systemic effects were not observed.

This result has also been reported by other authors in 100% of ewes (Alekish et al., 2017) and 84% of cows (Nahed et al., 2011) of the study.

Finally, our study shows a healing rate of 100% and 95% of udders treated with Amoxicillin/clavulanic acid and honey/starch, respectively. Experimentally, the

conventional treatment overcomes acute staphylococcal mastitis and wins over the alternative formula, so it has the upper hand. But statistically, there was no significant difference (p=0,46) between the efficiency of these two treatments in bacteriological healing.

Antibiotic treatment has drawbacks: on the one hand its high cost, and on the other hand, the milk must be discarded and cannot be marketed before all traces of antibiotics have disappeared, because the presence of these chemical therapeutic residues could promote the development of antibiotic resistant bacterial strains (Nahed et al., 2011).

From the results discussed in this document, the alternative treatment was safe, efficient and had a strong antibacterial effect, it gave good results which were generally satisfactory. Indeed, it has shown a clinical and zootechnical healing faster, earlier, and therefore more satisfactory than that of conventional treatment. The clearest economic benefit of the alternative intramammary infusion is its low cost and its almost perfect safety, since honey has very few adverse effects. The milk from animals treated with honey during mastitis is not contaminated with unwanted residues like antibiotics, so there is no withdrawal period, knowing that the most obvious economic merit of the intramammary honey infusion is that there is no rejection of milk. In addition, its recurrent use does not generate any microbial resistance. Honey is harmless to tissues and it is interesting to note that there is no interaction between honey and drugs (Nahed et al., 2011; Benhanifia et al., 2019).

This aspect undeniably favors a wider therapeutical use of honey which, with its desirable advantages, represents a leading therapeutic possibility and therefore appears to be an interesting alternative to antibiotic therapy for treating ovine mastitis.

CONCLUSION

Our results proved that the alternative formula had an obvious strong antibacterial effect and indicated the efficiency of honey/starch mixture as one of the new and promising alternative therapeutic formulas against *S. aureus* clinical mastitis in ewes.

However, future extensive studies are needed to elucidate the therapeutic properties of honey, the active ingredients responsible for the honey antimicrobial effect, the susceptibility of other pathogen microorganisms implicated in clinical or even subclinical sheep or cattle mastitis, the biodynamic of honey (distribution, dissemination) in the mammary gland, and an appropriate intramammary infusion of honey for mastitis prevention and therapy.

Finally, honey is harmless to tissues without undesirable residues in milk and its repeated use does not produce any microbial resistance. From this perspective it seems relevant to conclude that honey could be a good choice for clinical mastitis treatment, and it represents one of the rare alternatives to fight against antibiotic resistant bacteria which emerge more and more over time.

Acknowledgments

The authors highly acknowledge the beekeeper who supplied honey sample for the survey. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

REFERENCES

1. Aamer, A. A., Abdul-Hafeez, M. M., Sayed, S. M. (2015). Minimum inhibitory and bactericidal concentrations (MIC et MBC) of honey and bee propolis against multidrug resistant (MDR) *Staphylococcus sp.* isolated from bovine clinical mastitis. Global Journal of Science Frontier Research, 15(2), 20-28.
2. Abdalhamed, A. M., Zeedan, G. S. G., Abou Zeina, H. A. A. (2018). Isolation and identification of bacteria causing mastitis in small ruminants and their susceptibility to antibiotics, honey, essential oils, and plant extracts. Veterinary World, 11(3), 355-362.
3. Abdel-Hafeez, M. M., Ali, M. M., Abdel-Rahman, M. F., Nahed-Wahba, M. (2005). Antibacterial activity of honey for treatment of subclinical bovine mastitis: intramammary infusion as a tool to manage non responding antibiotic cases. Proceedings of the 8th Scientific Congress of Egyptian Society for Cattle Disease, 11(13), 147-151.
4. Alekish, M. O., Ismail, Z. B., Awawdeh, M. S., Shatnawi, S. (2017). Effects of intramammary infusion of sage (*Salvia officinalis*) essential oil on milk somatic cell count, milk composition parameters and selected hematology and serum biochemical parameters in Awassi sheep with subclinical mastitis. Veterinary World, 10(8), 895-900.
5. Almasaudi, S. B., Al-Nahari, A. A. M., Abd El-Ghany, E. S. M., Barbour, E., Al Muhayawi, S. M., Al-Jaouni, S., Azhar, E., Qari, M., Qari, Y. A., Harakeh, S. (2017). Antimicrobial effect of different types of honey on *S. aureus*. Saudi J. Biol. Sci., 24(6), 1255-1261.
6. Al-Waili, N., Al-Ghamdi, A., Ansari, M. J., Al-Attalm, Y., Salom, K. (2012). Synergistic effects of honey and propolis toward drug multi-resistant and *Candida albicans* isolates in single and polymicrobial cultures. Int. J. Med. Sci., 9(9), 793-800.
7. Al-Waili, N. S. (2003). Identification of nitric oxide metabolites in various honeys, effects of honey on plasma and urinary nitrite/nitrate concentration. J. Med. Food, 6(4), 359-364.
8. Basson, N. J., Grobler, S. R. (2008). Antimicrobial activity of two South African honeys produced from indigenous *Leucospermum cordifolium* and *Erica species* on selected micro-organisms. BMC Complement. Altern. Med., 8(41).
9. Benhanifia, M., Ayad, A., Mohamed, W. (2020). Intramammary honey preparation for treatment of subclinical bovine mastitis: a preliminary study. Org. Agr., 10(1), 1-8.
10. Bergonier, D., De Crémoux, R., Rupp, R., Lagriffoul, G., Berthelot, X. (2003). Mastitis of dairy small ruminants. Vet. Res., 34(5), 689-716.
11. Boukraa, L., Amara, K. (2008). Synergistic effect of starch on the antibacterial activity of honey. J. Med. Food, 11(1), 195-198.
12. Boukraa, L., Niar, A., Benbarek, H., Benhanifia, M. (2008). Additive action of royal jelly and honey against *S. aureus*. J. Med. Food, 11(1), 190-192.
13. Bourabah, A., Ayad, A., Hammoudi, S. M., Boukraa, L., Benbarek, H. (2014). Antimicrobial activity of Algerian honey on subclinical mastitis pathogens isolated from goat's milk. Veterinary World, 7(4), 248-252.
14. Cianciosi, D., Forbes-Hernández, T. Y., Afrin, S., Gasparrini, M., Reboredo-Rodriguez, P., Manna, P. P., Zhang, J., Bravo Lamas, L., Martínez Flórez, S., Agudo Toyos, P., Quiles, J. L., Giampieri, F., Battino, M. (2018). Phenolic compounds in honey and their associated health benefits: a review. Molecules, 23(9), 2309-2322.
15. CLSI (Clinical and Laboratory Standards Institute) (2006). Methods for broth dilution susceptibility testing of bacteria isolated form aquatic animals, Approved guideline. CLSI document M49-A, Wayne, PA, USA.
16. CLSI (Clinical and Laboratory Standards Institute) (2011). Performance standards for antimicrobial susceptibility testing, Twenty-first informational supplement. CLSI document M100-S21, Wayne, PA, USA.
17. De Jong, A., Garch, F. E., Simjee, S., Moyaert, H., Rose, M., Youala, M., Siegwart, E., VetPath Study Group (2018). Monitoring of antimicrobial susceptibility of udder pathogens

recovered from cases of clinical mastitis in dairy cows across Europe: VetPath results. Vet. Microbiol., 213, 73-81.
18. De Vliegher, S., Fox, L. K., Piepers, S., McDougall, S., Barkema, H. W. (2012). Invited review: mastitis in dairy heifers: nature of the disease, potential impact, prevention, and control. J. Dairy Sci., 95(3), 1025-1040.
19. Descottes, B. (2009). Cicatrisation par le miel, l'expérience de 25 années. Phytother., 7(2), 112-116.
20. Dhakal, K., Tiezzi, F., Clay, J. S., Maltecca, C. (2016). Causal relationships between clinical mastitis events, milk yields and lactation persistency in US Holsteins. Livest. Sci., 189, 8-16.
21. Elmenoufy, G. A. M. (2012). Bee honey dose-dependently ameliorates lead acetate-mediated hepatorenal toxicity in rats. Life Sci. J., 9(4), 780-788.
22. Fernandez-Cabezudo, M. J., El-Kharrag, R., Torab, F., Bashir, G., George, J. A., El-Taji, H., Al-Ramadi, B. K. (2013). Intravenous administration of manuka honey inhibits tumor growth and improves host survival when used in combination with chemotherapy in a melanoma mouse model. PLoS One, 8(2), e55993.
23. Fitzgerald, J. R. (2012). Livestock-associated *Staphylococcus aureus*: origin, evolution and public health threat. Trends Microbiol., 20(4), 192-198.
24. Gheldof, N., Wang, X. H., Engeseth, N. J. (2002). Identification and quantification of antioxidant components of honeys from various floral sources. J. Agric. Food Chem., 50(21), 5870-5877.
25. Gomes, F., Henriques, M. (2015). Control of bovine mastitis: old and recent therapeutic approaches. Curr. Microbiol., 72(4), 377–382.
26. Hadjazi, D., Larbi, D. K., Reffas, F. Z. I., Benine, M. L., Abbouni, B. (2015). Antibacterial activity of the essential oils of *Nigella sativa L.* against pathogens bacteria. Glob. J. Biotechnol. Biochem. Res., 10(2), 100-105.
27. Hassanein, S. M., Gebreel, H. M., Hassan, A. A. (2010). Honey compared with some antibiotics against bacteria isolated from burn wound infections of patients in Ain Shams university hospital. J. American Sci., 6(10), 301-320.
28. Hegazi, A. G. (2011). Antimicrobial activity of different Egyptian honeys as comparison of Saudi Arabia honey. Res. J. Microbiol., 6(5), 488-495.
29. Hegazi, A. G., Al Guthami, F. M., Al Gethami, A. F. M., AbdAllah, F. M., Saleh, A. A., Fouad, E. A. (2017). Potential antibacterial activity of some Saudi Arabia honey. Veterinary World, 10(2), 233-237.
30. IDF (International Dairy Federation) (1999). Suggested interpretation of mastitis terminology. Bulletin of IDF, 338, Brussels, Belgium, 3-20.
31. Irish, J., Blair, S., Carter, D. A. (2011). The antibacterial activity of honey derived from Australian flora. PLoS One, 6(3), e18229.
32. Jenkins, R., Cooper, R. (2012). Improving antibiotic activity against wound pathogens with manuka honey *in vitro*. PLoS One, 7(9), e45600.
33. Kwakman, P. H. S., Johannes, P. C., Vanden Akker, A. G., Aslami, H., Binnekade, J. M., Leonie De Boer, L., Boszhard, L., Paulus, F., Middelhoej, P., Te Velde, A. A., Vandenbroucke-Grauls, C. M. J. E., Schultz, M. J., Zaat, S. A. J. (2008). Medical-grade honey kills antibiotic resistant bacteria *in vitro* and eradicates skin colonization. Clin. Infect. Dis., 46(11), 1677-1682.
34. Lehtopolku, M., Nakari, U. M., Kotilainen, P., Huovinen, P., Siitonen, A., Hakanen, A. J. (2010). Antimicrobial susceptibilities of multidrug-resistant *Campylobacter jejuni* and *C. coli* strains: *in vitro* activities of 20 antimicrobial agents. Antimicrob. Agents Chemother., 54(3), 1232-1236.
35. López-Malo, A., Vigil, E., Palou, M. E., Parish, P. M. (2005). Methods for activity assay and evaluation of results. *In* P. M. Davidson, N. J. Sofos, & A. L. Branen (Eds.),

Antimicrobials in Food (pp. 659-680). 3rd edition, Boca Raton, FL, USA: Taylor and Francis Group.
36. Lu, J., Dee, A., Turnbull, C. L., Rosendale, D., Hedderley, D., Stephens, J., Gannabathula, S. G. S., Schlothauer, R. C., Whitchurch, C. B., Harry, E. J. (2013). The effect of New Zealand Kanuka, Manuka and clover honeys on bacterial growth dynamics and cellular morphology varies according to the species. PLoS One, 8(2), e55898.
37. McLoone, P., Warnock, M., Fyfe, L. (2016). Honey: an immunomodulatory agent for disorders of the skin. Food Agric. Immunol., 27(3), 338-349.
38. Melchior, M. B., Vaarkamp, H., Fink-Gremmels, J. (2006). Biofilms: a role in recurrent mastitis infections?. Vet. J., 171(3), 398-407.
39. Monsallier, D. (2000). Comparative evaluation of the antibacterial efficacy of honey *in vitro* and antiplaque efficacy in a 4-day plaque regrowth model *in vivo*: preliminary results. J. Periodontol., 83(11), 16-21.
40. Monsang, S. W., Pal, S. K., Kumar, M., Roy, J., Sharma, C. S., Norjit, S. M. N. (2014). Bilateral mastectomy for successful management of chronic suppurative mastitis in a black Bengal doe (*Capra hircus*). J. Anim. Health Prod., 2(2), 28-30.
41. Moussa, A., Noureddine, D., Hamoudi, S. M., Saada, A., Bourabeh, A., Houari, H. (2012). Additive potential of ginger starch on antifungal potency of honey against *Candida albicans*. Asian Pac. J. Trop. Biomed., 2(4), 253-255.
42. Mullai, V., Menon, T. (2007). Bactericidal activity of different types of honey against clinical and environment isolates of *Pseudomonas aeruginosa*. J. Altern. Complement. Med., 13(4), 439-441.
43. Nahed, M. W., Neveen, A. E. N., Sayed, S. M., Abdellah, M. R., Abdel-Hafeez, M. M., Aamer, A. A. (2011). Intramammary honey infusion: a new trend in the management of bovine subclinical mastitis. Journal of Animal and Veterinary Advances, 10(20), 2740-2744.
44. NCCLS (National Committee of Clinical Laboratory Standards) (1997). Performance standards for antimicrobial disk susceptibility tests, Approved standards. NCCLS document M2-A6, Wayne, PA, USA.
45. Nelson, C. A., Reginald, A. O., Okoro, N., Janet, K. (2006). Antibacterial activity of *Allium cepa* (Onions) and *Zingiber officinale* (Ginger) on *Staphylococcus aureus* and *Pseudomonas aeruginosa* isolated from high vaginal swab. Int. J. Trop. Med., 3(2), 1-5.
46. NMC (National Mastitis Council) (2004). Microbiological procedures for the diagnosis of bovine udder infection and determining milk quality. 3rd edition, Arlington.
47. Olaitan, P. B., Adeleke, E. O., Lyabo, O. O. (2007). Honey: a reservoir for microorganisms and an inhibitory agent for microbes. Afr. Health Sci., 7(3), 159-165.
48. Pascual-Maté, A., Osés, S. M., Fernández-Muiño, M. A., Sancho, M. T. (2018). Methods of analysis of honey. J. Apicult. Res., 57(1), 38-74.
49. Patel, A., Chauhan, B. P. (2017). Antimicrobial effect of honey on MRSA isolated from pus samples. Int. J. Drug Res. Technol., 6(2), 58-63.
50. Pengov, A., Ceru, S. (2003). Antimicrobial drug susceptibility of *Staphylococcus aureus* strains isolated from bovine and ovine mammary glands. J. Dairy Sci., 86(10), 3157-3163.
51. Peton, V., Le Loir, Y. (2014). *S. aureus* in veterinary medicine. Infect. Genet. Evol., 21, 602–615.
52. Ruiz, L., Porto, A. V., Al-Habsi, N., Vera, S., San Andrés, M. P., Jauregi, P. (2013). Antioxidant, antibacterial and ACE-inhibitory activity of four monofloral honeys in relation to their chemical composition. Food Funct., 4(11), 1617-1624.
53. Saad, B., AL Masaudi, M. O., Bureikan, A. (2013). Antimicrobial activity of garlic juice (*Allium sativum*), honey, and garlic-honey mixture on some sensitive and multi-resistant microorganisms. J. Biol. Life Sci., 10(4), 2429-2435.
54. Samarghandian, S., Farkhondeh, T., Samini, F. (2017). Honey and health: a review of recent clinical research. Pharm. Res., 9(2), 121-127.

55. Sayed, S. M., Abou El-Ella, G. A., Wahba, N. M., El Nisr, N. A., Raddad, K., Abd El Rahman, M. F., Abd El Hafeez, M. M., Aamer, A. A. (2009). Immune defense of rats immunized with fennel honey, propolis, and bee venom against induced staphylococcal infection. J. Med. Food, 12(3), 569-575.
56. Scali, F., Camussone, C., Calvinho, L. F., Cipolla, M., Zecconi, A. (2015). Which are important targets in development of *S. aureus* mastitis vaccine?. Res. Vet. Sci., 100, 88-99.
57. Tan, H. T., Rahman, R. A., Gan, S. H., Halim, A. S., Sulaiman, S. A., Kirnpal-Kaur, B. (2009). The antibacterial properties of Malaysian Tualang honey against wound and enteric microorganisms in comparison to Manuka honey. BMC Complement. Altern. Med., 9(1), 1-8.
58. Taponen, S., Simojoki, H., Haveri, M., Larsen, H. D., Pyorala, S. (2006). Clinical characteristics and persistence of bovine mastitis caused by different species of coagulase-negative *Staphylococci* identified with API or AFLP. Vet. Microbial., 115(1-3), 199-207.
59. Thatcher, A., Paz Martin, N., Petrovski, K. R. (2014). A study of subclinical mastitis in two herds, one managed organically, the other conventionally, and the effect of different management strategies. Organic Agriculture, 4(4), 1-5.
60. Zeedan, G. S. G., Abdalhamed, A. M., Ottai, M. E., Abdelshafy, S., Abdeen, E. (2014). Antimicrobial, antiviral activity and GC-MS analysis of essential oil extracted from *Achillea fragrantissima* plant growing in Sinai Peninsula, Egypt. J. Microb. Biochem. Technol., S, 8(6).