

FREQUENCY OF SUBCLINICAL MASTITIS AND ITS IMPACT ON THE MAMMARY GLAND AND THE ANTI-BACTERIAL EFFECT OF HONEY ON THE ISOLATED BACTERIUM *STAPHYLOCOCCUS AUREUS*

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ABSTRACT

Mastitis is an infection of the mammary glands in dairy cows. It is characterized by the presence in the milk of inflammatory cells (leukocytes) and possibly bacteria. This inflammation can have clinical consequences with changes in the appearance of the milk and visible inflammation of the udder. However, most often, the disease remains subclinical with no symptoms, the infected cows simply show a rise in somatic cell count (SCC), however, and these consequences are as severe as clinical mastitis, alteration of the composition of the milk and decrease in production. Treatment is mainly based on the use of antibiotics, which is not always effective.

The objective of our study was to see the impact of subclinical mastitis on the structure of the mammary gland. We have performed histological sections of fragments belonging to the mammary gland of dairy cows brought to the slaughterhouse after the drop in milk yield due to subclinical mastitis. Of the 101 samples, 36 showed no change in tissue structure, while 65 of the samples showed significant inflammation. Our second objective was to test the effect of honey directly on the isolated bacterium (*Staphylococcus aureus*). The effect of honey was more active than the antibiotic gentamicin.

Keywords: Dairy cow, Subclinical mastitis, Inflammation, *Staphylococcus aureus*, Antibiogram, honey.

1. INTRODUCTION

Algeria ranks third in the world in importing milk and dairy products due to a production deficit that covers only 40% of the population's needs. [16]. Among the causes of this failure is the decline in milk production due to mastitis problems. Mastitis is an inflammation of the mammary gland that is mainly caused by an infection of bacterial origin. It may also be due to an infection of viral or fungal origin, or may result from physiological changes, trauma or injury [14]. The infection sometimes results in local clinical signs such as the presence of lumps in the milk or a hard, swollen and painful quarter and sometimes even fever, these mastitis are called clinical mastitis, but most often the infection passes unnoticed and mastitis is called subclinical. Prevention against mastitis is a major concern in the rearing of dairy cows. It is among the most expensive diseases of dairy companies. The cost is explained by a decrease in milk production [18], problems of raw material processing [11], premature reform of animals with clinical or chronic mastitis [18], treatment costs (products and veterinary costs) [3] and financial penalties on milk quality as a function of somatic cell count (SCC), not to mention the overload of work for the breeder. Many health problems can also result from the poor quality of the raw material. In fact, the presence of germs in the milk following the milking of infected animals can lead to contamination throughout the dairy chain, causing foodborne illness [6]. In very severe cases, the infection progressed to produce an inflammatory reaction with significant destruction of the secretory tissue.

In bovines, the germs responsible for mastitis are mainly bacterial (*Streptococcus uberis*, *Escherichia coli* and *Staphylococcus aureus*). Breeding mastitis control remains a major health issue. Despite the large number of studies dedicated to this disease, no effective control strategy has been developed. Antibiotherapy remains the most commonly used solution, but its effectiveness is limited.

In our study, we have a dual objective. First, we investigated the relationship between the structure of the bovine mammary gland and the impact of subclinical mastitis by observing histological sections of the breast tissue sampled with mastitis. While our second objective is to try to replace antibiotherapy with an application of honey, we tested the effect of honey on one of the bacteria isolated (*Staphylococcus aureus*.) By diffusion method on agar medium (disk method).

2. MATERIALS AND METHODS

The cows brought to the slaughterhouse had slaughtered certificates for various reasons, but the cows selected for the study were suffering from a decline in milk production due to mastitis. It should be noted that some subjects did not show any clinical signs, the pathological form was subclinical.

Physical and clinical examinations were carried out before the cows were slaughtered. The clinical form of mastitis was detected by the presence of signs of inflammation (redness, heat and pain). Subclinical mastitis was diagnosed by C.M.T. (California mastitis test). 102 mammary gland samples from dairy cows underwent histological sections to reveal potential tissue damage due to mastitis.

These samples taken immediately after slaughter were stored in pots containing formalin (10%) and then transported to the pathology laboratory.

2.1 The stages of the histological study

The mammary glands of dairy cows taken will have to undergo several steps to finish in a fixed blade in order to be able to observe the potential damages of the tissues.

There are five steps to achieve a histological cut:

a- The fastening

This first step begins directly after removing the organ (mammary gland), its role is the conservation of the different tissues of the organ, it prevents cellular autolysis, also prevents bacterial putrefaction and allows the histological technique and subsequent colorations. We used 35% formalin.

b- The inclusion

The principle of inclusion is to have a rigid consistency tissue, in order to achieve very fine cuts to allow the passage of light through the fabric, in order to have a good microscopic observation. The paraffin embedding includes the infiltration and coating the tissues to be examined with paraffin. We proceed before this coating of two mandatory stages. The first step is dehydration where we will spend the tissues in growing degree of alcohol baths (70 °, 80 °, 90 °, 95 °, 99 °, and finally 100 °), the interest of dehydration is to remove the fixative. In the second step, we proceed to toluene bath which is a solvent miscible with paraffin to replace the alcohol. Finally the fabric is placed in the molten paraffin, heat will accelerate the evaporation of the solvent, then placed in small molds, at room temperature, it will harden, we turn out to obtain fractions of paraffin tissue.

Using a microtome cuts is performed. The instrument moves the block on a knife. The cuts are about 5 microns. All tranches will form a tape in which we find the tissue sample cups. One makes a spreading section on glass slides, heated on a hot plate the paraffin will stick to the blade.

c- The colorations

The most commonly used stain is hematoxylin / eosin / saffron (HES). Haematein is a basic substance, which stains nuclei purple therefore stained nucleic acids. Eosin is an acidic substance, which stains pink cytoplasm, it is the protein staining. Saffron color the yellow collagen fibers. Prior to staining, there is a dewaxing, one passes the blades in toluene baths. Then this is rehydration: the alcohol is mixed with water and toluene, we pass the blades in alcohol baths of decreasing degree (100 ° to 70 °).

d- The mounting

The slides were dehydrated through baths in toluene and then glue glass coverslips over with synthetic resins to preserve the preparations. These slides can be stored for several years [5].

➤ The second stage of our studies and the isolation of the bacteria involved and the direct effect of honey on the bacterial proliferation of one of the bacteria identified (*Staphylococcus aureus*).

2.2. Antibiogram by diffusion on agar medium (disk method)

2.2.1. Preparation of discs

The antibiotics are usually tested in the form of discs 6 mm in diameter. To reproduce the same conditions, we used Watman No. 3 cuts into discs (6 mm). The latter must have a regular contour to give a zone of inhibition that is easy to measure. The discs once cut have been sterilized [7-8]. Each disc is impregnated with 100 µl of honey. The strain tested is that detected "streptococcus aureus". To keep it alive, several transplants per week are carried out. The media used are selective media, which depend on the seed tested [7-8].

2.2.2. Realization of the antibiogram

The Müller-Hinton agar is cast in a petri dish over a thickness of 4 mm, the agar plates are then pre-dried. The inoculum is prepared from an 18-hour culture of isolation medium as follows: the well-isolated colonies are scraped with the aid of the platinum loop and then discharged into 10 ml of sterile physiological water at 0, 9%.

The bacterial suspension is then homogenized. Its opacity must be equivalent to 0.5 Mc Ferland or to an OD of 0.08 to 0.10 to 625 nm. The inoculum can be adjusted by adding culture if it is too weak, or physiological water if it is too strong. [7-8]

Seeding should be done within 15 minutes of inoculum preparation using a sterile swab. The latter is soaked in the bacterial suspension and then drained by pressing it firmly on the inner wall of the tube, in order to discharge it to the maximum. After inoculation by rubbing the swab over the whole of the agar surface (friction is made from top to bottom, in tight grooves this operation is repeated twice, rotating the box by 60 ° each time) the discs are applied. Finally, the cans leave for 15 minutes at room temperature (on the bench). Then incubated for 18 h at 35 ° C. After incubation, the diameters of the zones of inhibition are measured accurately by means of a caliper or ruler. [7-8]

3. RESULTS AND DISCUSSION

3.1. Descriptive characteristics of cows with mastitis

Our samples were dairy cows (101 cows) with mastitis for slaughter. We divided these cows according to their age, the number of gestation, their breed and the type of milking. We observed that the highest number of mastitis was recorded in cows aged 2 to 6 years (Figure 1). Mastitis is also higher in gestures compared to multi-gesture, the greater the number of gestation, and the number of mastitis decreases (Figure 2). We have also noticed that the breeds most susceptible to mastitis are Black Pie and the cross breed (race imported with the local breed) (Figure 3). We also compared between manual and mechanical milking, then the manual and more mastitis-prone than the other (Figure 4).

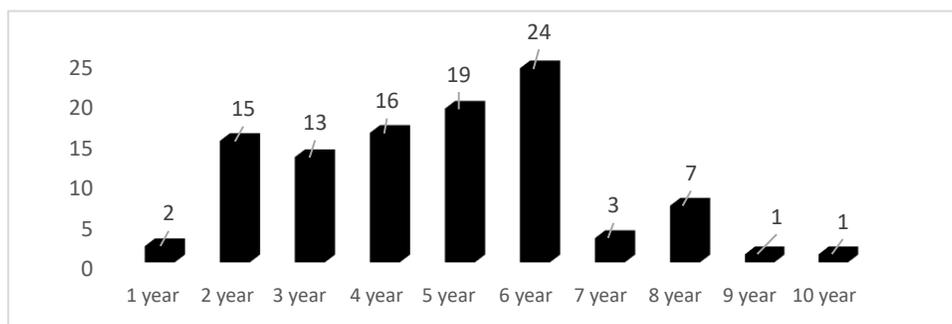


Figure 1: Distribution of affected cows by age.

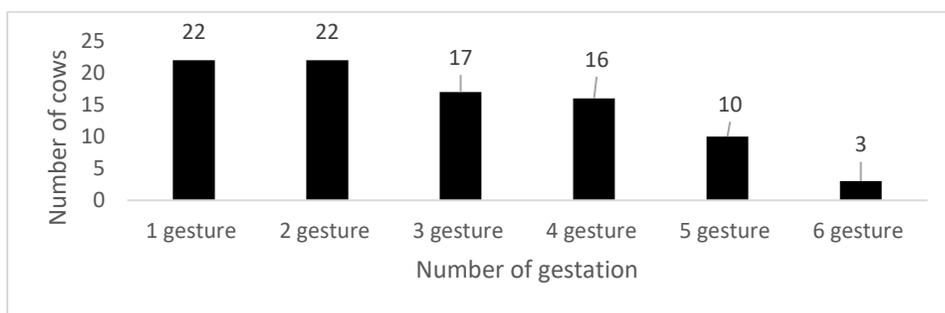


Figure 2: Breakdown of affected cows by gestation number.

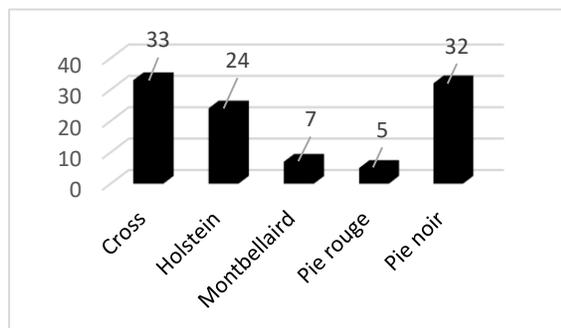


Figure 3: Breeding of cows with mastitis by breed.

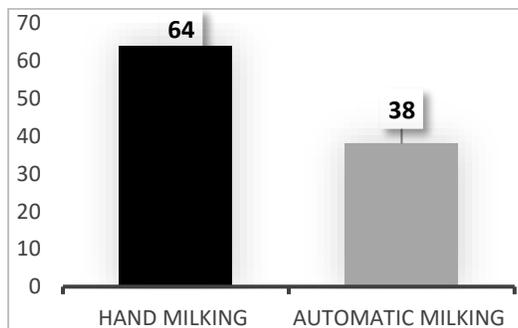


Figure 4: Distribution of cows with mastitis according to the milking method.

3.2. Histological characteristics

Histological analyzes showed that among our 101 cows with mastitis, 36 cows (36.36%) had structurally normal tissues, active alveoli with large acines and cells with normal contours (Fig 5). Other samples showed images of an udder at the end of activity, showing basal and cubic cells (Fig. 6).

However, 65 cows (65.65%) showed different inflammatory lesions, which manifested by the disappearance of alveolar lumen, fibrosis, complete destruction of the parenchyma and invasion of blood cells (Figure 7, 8, 9).

Breast tissue lesions reduce the number and activity of epithelial cells, and consequently, contribute to decreased milk production. These mammary lesions are generally induced by apoptosis or by significant cell necrosis. These tissue lesions can be caused by bacteria that are able to invade and multiply in mammary epithelial cells of the cow before causing cell death. Some bacteria secrete toxins that destroy cell membranes and damage the milk-producing tissues. As a defense there is the migration of immune cells into the mammary gland and the collapse of the blood-blood barrier, so neutrophilic polynuclear cells release proteolytic enzymes and cause intense damage to the mammary tissues. When the infection persists and the canals are blocked, there is an increase in milk in the alveoli, the pressure increases, thus the secretory cells lose their ability to synthesize and the cells to atrophy.

Among the bacteria that caused this mastitis, we isolated *Staphylococcus aureus*. According to several studies, Trinidad et al. [19], Mehrzad et al [12] the mammary glands infected with *Staphylococcus aureus*, showing an increase in intercellular connective tissue and a reduction of epithelial cells and alveolar light. These same studies also reported that proteases in mastitis milk eliminate casein, gelatin, collagen, hemoglobin, mammary gland membrane proteins and lactoferrin, indicating that mastitis milk proteases have a wide range of activities.

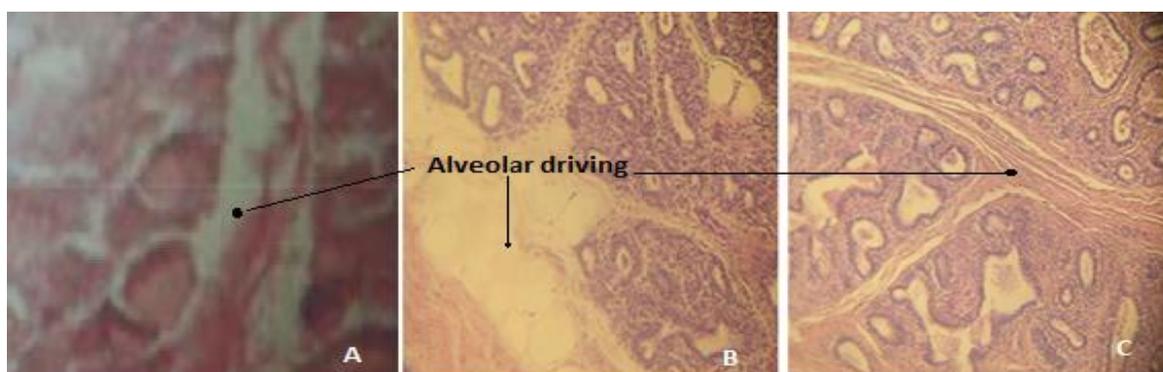


Figure 5: Breast acini in lactating udder activity (A: Grx100, B & C: Grx40).

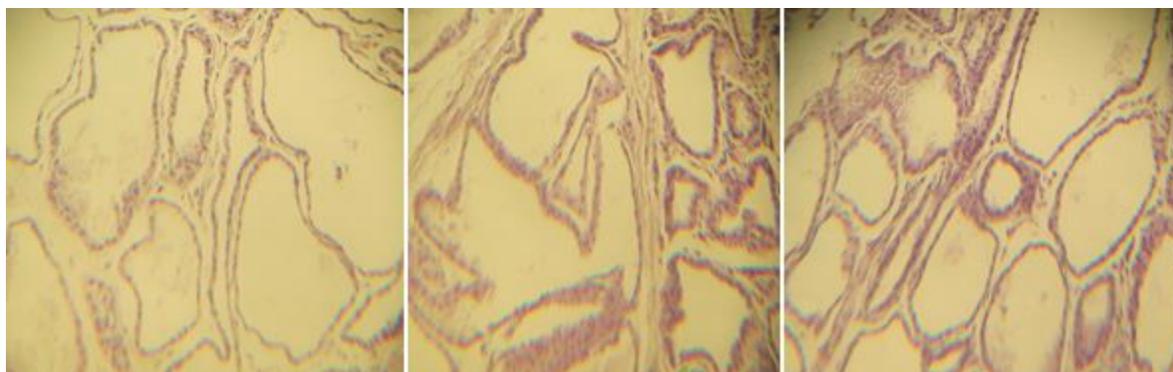


Figure 6: udders at the end of activity, cells in cubic form, distended cells, acini with broad light (Gr x 40).

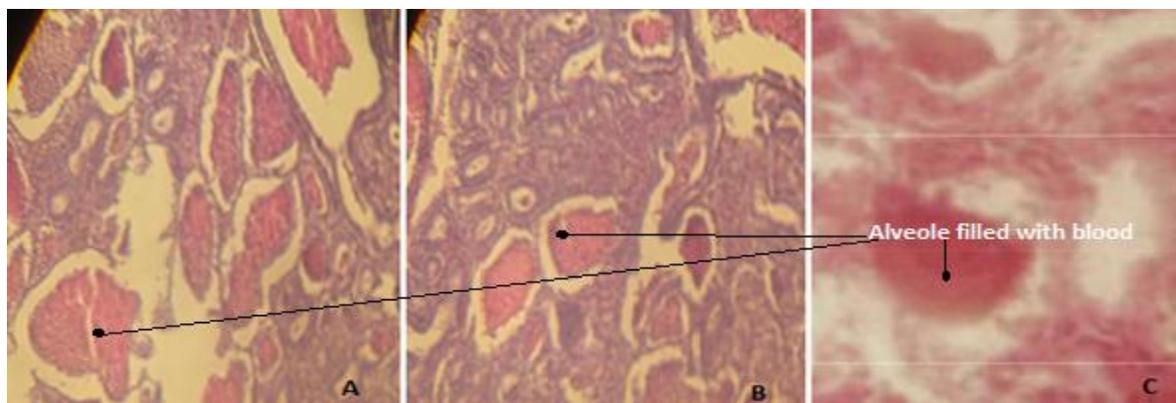


Figure 7: Intense inflammatory reaction, polynuclear invasion, blood-filled alveoli (A & B: Grx40, see: Grx100).

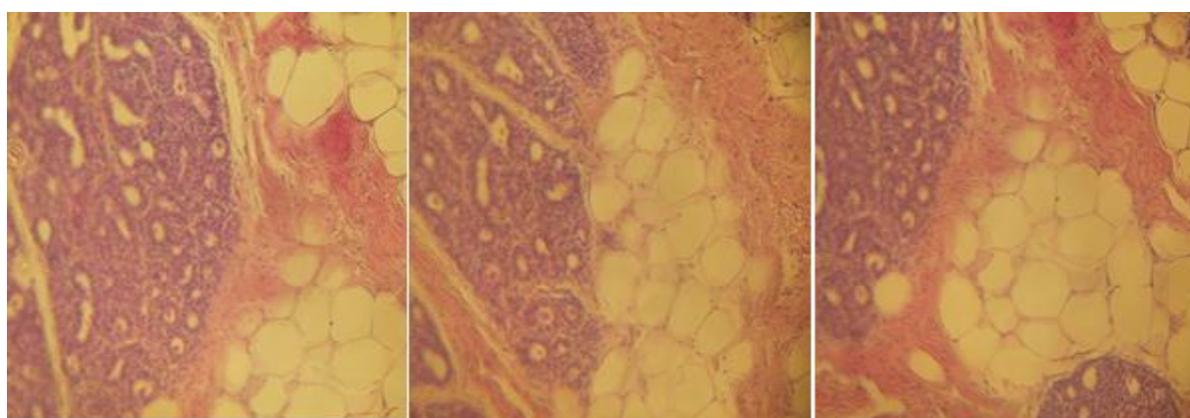


Figure 8: Invasion of blood into alveolar, congestion, udder inter-lactation (Gr x 40).

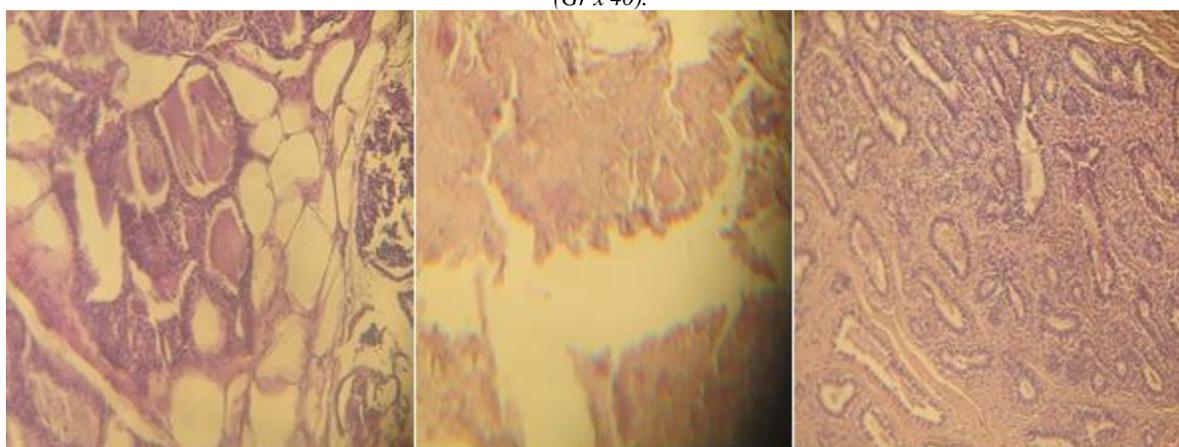


Figure 9: loss of structure, alveolar destruction and extensive alveolar-lobular erasure (Grx40).

3.3. Evaluation of the antibacterial power of honey

In this section, we will present the results obtained from the effect of honey on the growth of *Staphylococcus aureus*. We tested a series of antibiotic, we chose the one where the bacteria to show the most sensitivity to then compare it to the antibacterial effect of honey.

3.3.1. Antibiotics containing antibiotic discs

Table 1: Antibiogram of Staphylococcus aureus

Antibiotics	CN	AMC	SXT	GEN
<i>Inhibition diameter (cm)</i>	0	0	0	1.6



Figure 10: Antibiogram of Staphylococcus aureus.

3.3.2. Antibiograms containing honey discs

Table 2: Effect of honey on Staphylococcus aureus

The disks of honey	1	2	3	4	5
<i>Inhibition diameter (cm)</i>	2.2	2.5	2.5	2.5	2.2



Figure 11: Effect of honey on Staphylococcus aureus.

4. CONCLUSION

Mammary infections can range from subclinical-chronic mastitis to gangrenous mastitis [10], causing significant economic losses and public health risks. The factors of the host intervene in the outcome of the infection (genetic background, general health status, animal age, number of lactations, etc.) [17].

Staphylococcus aureus is one of the most isolated bacteria in the case of mastitis in cattle. Unfortunately, apart from antibiotic therapy, there is no effective solution to deal with this pathology both in preventive and curative.

The results of our study showed that in a herd of 101 cows with mastitis the most important number was in cows between the age ranges of 2 to 6 years. We also noted that mastitis was more severe in cows with only one to two gestations. Concerning the breeds, then the most concerned of the mastitis were the crossed breeds (resulting from a coupling of a local breed and an imported breed) and the race imported Black Pie. We also noticed that manual milking was more concerned with mastitis than mechanical milking.

The observation of the histological sections showed that among the 101 cows with mastitis, 36 cows had tissue structures that showed no abnormalities, alveoli in activity with large acini, cells with normal contours and among them, there were samples whose tissue structures represent the basal and cubic cells of an udder at the end of the activity.

However, 65 cows showed different inflammatory lesions, which manifested by the disappearance of alveolar light, fibrosis, complete destruction of the parenchyma and invasion of blood cells, leading the mammary cell, either to programmed suicide (apoptosis) or to death due to the poor living conditions of the cell (necrosis). These two modes of mortality of the cell are caused by bacteria, either by their enzymes or by their secret toxins.

Staphylococcus aureus have the ability to colonize host tissues by adhering to abiotic surfaces (e.g. milking equipment) or directly to eukaryotic cells and to components of the extracellular matrix. The bacteria can adhere to the host epithelium or endothelium via its affinity to fibronectin (Fn), fibrinogen (Fg), elastin, collagen and Von Willebrand factor [9].

The penetration of *Staphylococcus aureus* into mammary epithelial cells involves a zipper mechanism. a process of interaction between bacteria and cellular proteins leading to the formation of pseudopoda invaginating the adhered bacteria [1-2-4-13]. Since the use of antibiotics is limited in all livestock systems due to the risk of the emergence and spread of resistant strains and the direct consequences in public health, there is a real need for alternative means of control and non-antibiotic in the context of sustainable agriculture (Ecoantibio Action Plan 2012-2017) [15].

Thought to traditional therapy like honey is a way to fight infections caused by these resistant bacteria. We tested the effect of honey on *Staphylococcus aureus*. We performed antibiograms containing antibiotic discs as well as antibiograms containing discs of honey and we compared the diameter of the inhibition zones. Then we noticed that the honey to give very good results with diameters of inhibition of 2.5cm compared to the antibiotic Gentamicin where the zone of inhibition was that of 1.6 cm. Whereas it is considered as the most reliable to *Staphylococcus aureus*.

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