All Sciences Proceedings

1st International Conference on Scientific and Innovative Studies

April 18-20, 2023 : Konya, Turkey

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The anti-biofilms effects of Thymus *algeriensis* on isolated strains of *Bacillus cereus*

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Abstract **–**. The ability of some microorganisms to form biofilms has been increasing in recent years. Indeed, biofilms are defined as a way of life that allows bacteria to survive and resist in hostile environments. Therefore, this form of survival represents a major problem for different food industries, including the dairy industry. The objective of this study is the isolation of Bacillus cereus strains from raw cow's milk and the study of their characterization, their ability to form biofilms, as well as the search for an inhibitory effect of the essential oil of 'Thymus algeriensis' on these formed biofilms. For this purpose, samples of cow's milk are collected from the region of Abu El Hassan (Chlef) and submitted to microbiological analyses. The preliminary identification of the isolated bacteria allowed the selection of 06 strains of Bacillus cereus. These strains in question have shown great potential for the formation of biofilm in the wells of a micro plate. The formation of biofilm in the micro plate was analyzed first by a simple observation with the naked eye of the wells after coloration with purple crystal, then using an ELISA device where the absorbance of the populations at 620 nm could be measured. Regarding the in vivo effect of the Thymus algeriensis essential oil, a complete inhibition of the biofilm formed was obtained after 24 hours of contact.

Keywords –Biofilm, Raw Cow's Milk, Bacillus Cereus, Essential Oil, Thymus Algeriensis

I. INTRODUCTION

Algeria is the largest consumer of milk and dairy products in the Maghreb, with nearly three billion litters per year (Kirat, 2007).The dairy industry is one of the main food industries that market a wide range of different dairy products including cow's milk. The Essential oils have a very important spectrum of action, since they inhibit the bacteria growth. Their antimicrobial activity is mainly dependent on their chemical composition and particularly on the nature of their major volatile compounds. They act by preventing the bacteria multiplication, their sporulation and their toxins synthesis (Oussalah et *al.*, 2007). The objectives set by this study are:

Research of *Bacillus cereus* strains in raw cow's milk.

- Characterize *Bacillus cereus* strains on their ability to form a biofilm.
- Look for an inhibitory effect of the essential oil *'Thymus algeriensis*' on the biofilm formed.

II. MATERIALS AND METHOD

This work was carried out at the biotechnology research laboratory of the Hassiba Ben Bouali University of Chlef **(Ouled Fares)**, during a period that extends from the beginning of March until June 2021.Its objective is the isolation and characterization of *Bacillus cereus* strains isolated from raw cow's milk, the study of their ability to

form a biofilm and also the search for an inhibitory effect of *Thymus algeriensis* essential oil on the biofilm formed. A bacteriological examination may be validly interpreted only if it is carried out on a sample properly taken, in a sterile container, according to a precise procedure avoiding accidental contamination, properly transported and stored under satisfactory conditions. (Pasteur Diagnosis, 1987) The collection is carried out by use of traditional methods, in a sterile bottle with a screw cap. The first jets are eliminated and the vial is filled to 2/3 of its capacity (Guiraud, 2003).

Study of biofilm formation in micro plate
There are different methods for detecting the production of a biofilm. These include the Tissue (TCP) culture plate, the Tube (TM) method, and the Red-Congo Agar method (CRA) and also by the titration micro plate (Gordon et *al.*, 1985). In the present work, biofilm formation was carried out in the wells of sterile polystyrene titration micro plates. A bacterial suspension is prepared in physiological water, adjusted to a DO (optical density) of 0.08-0.1, and is subsequently introduced into the wells of the micro plate (50uL), added to 150uL of the TSB. The micro plate is incubated for 48 hours at 37 ° C. The biofilms formed on the surface of the wells undergo the following treatments:
- The plates are first emptied with the micropipette.
-The wells are washed three times with a sterile PBS buffer in order to eliminate free (plank tonic) bacteria. Let dry 10 to 15 min.
-The biofilms formed by the adhesion of organisms are colored with Purple Crystal for 15 min, (Purple Crystal is a small molecule that diffuses through bacterial membranes and penetrates inside bacteria to complex to negatively charged molecules).
-The excess dye is then rinsed by a thorough wash with sterile distilled water and the plates are left at room temperature for drying. Before measuring the optical DO using a 590 nm ELISA reader, the wells are filled with a dissolving solution consisting of Methanol (Stepanovic et *al*., 2000).

Extraction of essential oil *T.algeriensis*
This plant was harvested in the region of Oued Chorfa, Wilaya of Ain Defla, during the month of March 2021, the parts used are the leaves and flowering tops. Their identification was carried out by Dr. Mohamed Chorfa professor at the University of Khemis Meliana., Algeria.

Results
Biochemical characteristics of isolates
Growth on Mosel medium
The search for *B.cereus* was highlighted by promoting the germination of spores following a heat treatment carried out on all samples. Preliminary identification made it possible to select 06 strains of *B.cereus*, which will then be seeded in a selective Mosel environment. Biochemical tests performed on isolated strains show the presence of catalase. Generally, this is the case for the majority of bacteria in members of the *Bacillaceae* family **(Oren, 2002).** Table 03.*B.cereus* bacteria are easily distinct from other *Bacillus* by their inability to ferment Mannitol and their lecithinase production **(Fritze, 2004; Logan and Berkely, 1984)**.thus; the cultures in our study were identified as B.cereus.

Evaluation of biofilm Formation
06 strains of *B.cereus* isolated from a sample were tested to demonstrate their potential for biofilm formation on a micro plate of 96 wells. The method used made it possible to quantify the rate of biofilm formation for all isolated strains. The adherent biofilm layer in each well is considered positive because a visible film is seen bordering the bottom of the wells and the surface of the micro plate. The formation of biofilm in the micro plate is analyzed first by a simple observation with the naked eye of the wells after a staining with purple crystal, then using an ELISA device where the absorbance of the populations at 620 nm could be measured**. (Racha et *al*., 2012).** The results obtained after the measurement of the absorbance by the ELISA

apparatus for all strains are introduced in the following table:

Table 01: Results obtained after the measurement of the absorbance by the ELISA apparatus.

	Repetition 01	Repetition 02	Repetition 03	Medium ± standard deviation
Control (-)	0.03	0.02	0.03	**0.02±0.05**
S1	0.18	0.17	0.16	**0.17±0.008**
S2	0.17	0.17	0.18	**0.17±0.004**
S3	0.28	0.32	0.61	**0.40±0.14**
S4	0.14	0.22	0.23	**0.19±0.04**
S5	0.09	0.11	0.11	**0.10±0.009**
S6	0.11	0.13	0.20	**0.14±0.03**

In order to interpret and evaluate the formation of the biofilm in relation to the introduced control (-), a calculation of the mean was made for the control (-) and for all strains of *B.cereus*. The values obtained were converted to histogram on Excel and the standard deviations were converted to error bars as shown in Figure 01

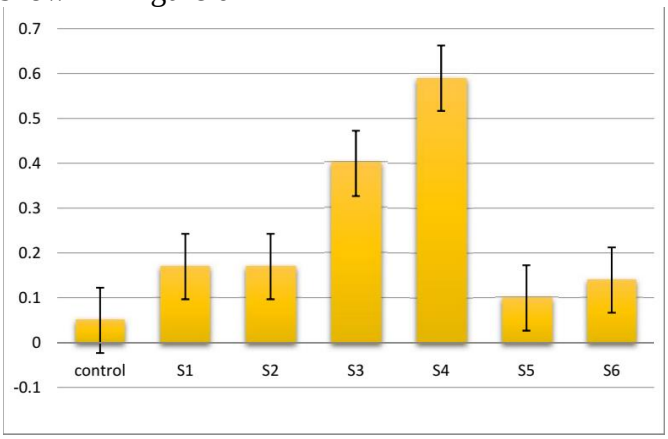

Figure 01: Evaluation of biofilm formation of isolated strains after absorbance measurement by the ELISA apparatus.

III. DISCUSSION

In this study, we were able to isolate strains of B.cereus from raw cow's milk from the Abu El Hassan (Chlef) region. The approach we followed for the identification is as follows: Initially, we ensured that our strains belonged to the genus *Bacillus* (Claus and Berkeley, 1986) by studying certain morphological and biochemical characteristics, namely the study of colony characteristics (shape, appearance and size) on 48-hour cultures in nutrient agar, The results of morphological characterization show that the isolated strains are isolated short or long rods or chained with rounded ends. Generally, this is the case for the majority of bacteria in members of the *Bacillaceae* family (Oren, 2002).According to Bergey's Manual of Systematic biology; the characteristics described above represent the typical traits of species belonging to the genus *Bacillus.* Indeed a large part of these strains are assigned to this genus. To prove that it is indeed *Bacillus cereus*, we used Mosel agar: Obtaining pink colonies indicates the absence of fermentation of Mannitol by *Bacillus cereus. B.cereus* bacteria are easily distinct from other Bacillus by their inability to ferment Mannitol and their production of lecithinase (Fritze, 2004; Logan & Berkely, 1984). The 06 strains of *B. cereus* Isolated were tested to demonstrate their potential for biofilm formation on a micro plate of 96 wells. From the results obtained, it can be seen that all strains isolated in the region show a potential for biofilm formation within the micro plate compared to the control; Strain S3, has the best potential for formation. It is also found that the strain S4 isolated at the Chlef region is the most biofilm-forming strain, S'1 and S'2 are less biofilm formative, so that S'5 has the lowest biofilm formation potential Table 01.This difference reflects on the conditions of breeding and the positioning of the cows in each region. Cows in the mountainous region of Chlef are more accustomed to withstanding harsh environmental conditions. Biofilm formation was observed in *B.cereus* strain ATCC 10987 in a study conducted by Auger et *al*.2006 under hostile conditions. In another study, free iron availability was observed to increase biofilm formation of strain ATCC 14579 (Hayrapetyan et *al.*, 2015).The authors of this study concluded that biofilm formation was highly dependent on incubation time, temperature and medium. The 06 strains of *Bacillus cereus* isolated from raw cow's milk were tested to highlight the inhibition capacity of *Thymus algeriensis* essential oil on Muller Hinton medium by the disc method. The concentration of the essential oil (5ul) inhibited the growth of bacterial strains of the genus *Bacillus cereus* with different diameters which are respectively (86 /45/52/73/32/ and 74 mm). An antimicrobial effect was noted for all strains compared to the control. The antimicrobial properties of *Thymusalgeriensis* essential oil have been known for a long time and have been the subject of much work over the past thirty years. These results were confirmed by the standardization of the aromatogram technique. (Zohary et *al.*, 2004). The antimicrobial activity of essential oils and plant extracts is due to the various chemical agents present in them, in

particular Thymol and carvacrol for some EOs, flavonoides and terpenoids as well as other phenolic compounds or free hydroxyl groups, which are classified as highly active antibiotic compounds (Marjorie, 1999).Thymol may also be involved in inhibiting electron transport processes, intracellular protein transport, phosphorylation steps, and other enzymatic reactions (Burt, 2004; Ultee et *al.*, 1999; Knobloch et *al.*, 1986)

IV. CONCLUSION

The surface microbial contamination is still an important problem in many sectors of the food industry, especially in the dairy sector. The presence of biofilm-forming microbial flora in cow's milk is a alarm major source, because biofilms are known to cause serious economic and sanitary problems. In this study, we have isolated *B. cereus* strains from raw cow's milk from the region of Abu El Hassan (Chlef).The strains are tested for their biofilm forming potential in a microplate. We were also interested in the antibacterial evaluation and the anti-biofilm activity of the *T. algeriensis* essential oil against all strains. The results obtained showed that *B. cereus* strains are a good candidate for biofilm formation, as described by the Purple Crystal technique. Moreover, an inhibitory effect of the *Thymus algeriensis* essential oil is obtained for all the strains tested in the tonicplate and biofilm phase

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