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Identification and Antibacterial Activity of *Lactobacillus* Isolated from Different Raw Honey in Mostaganem Province, Algeria

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ABSTRACT

Background: The increase of multidrug-resistant pathogens causes increased concerns about the efficacy of conventional treatment, which requires the search for new alternatives to antibiotics. The present study aimed to evaluate the antibacterial a ctivity of lactobacilli isolated from eight (n=8) typical *Apis mellifera* honey produced in Mostaganem region against four pathogenic bacteria responsible of bovine mastitis.

Methods: Lacbacilli strains, isolated from different media were firstly presumptively identified by catalase test, Gram staining and cells morphology, than confirmed by partial 16S rRNA sequence analysis. Antibacterial activity of isolates was determined by the overlay agar method. Antibiotic susceptibility of mastitis pathogens was tested by disk diffusion technique.

Result: Two species of lactobacilli were isolated and identified as *Lactobacillus plantarum* and *Lactobacillus pentosus*. Antibiotic sensitivity test demonstrated that mastitis pathogens showed the resistance to antibiotics tested. All the isolates showed antimicrobial activity with inhibition zone diameters ranged from 5±0.02 to 21±2.12 mm. The results revealed that honey can be considered as a source of Lactobacillus with antibacterial activities which can be used as alternative treatment of bovine mastitis.

Key words: Antibacterial activity, Genotypic identification, Honeys, Lactobacilli.

INTRODUCTION

Lactic acid bacteria (LAB) are one of the most microorganisms group studied by researchers. They are fluently used in food and pharmaceutic industries. These bacteria are widely present in nature and can be isolated from different sources such as fermented foods or gastrointestinal tract of humain and animals (Lashani *et al*. 2020). *Lactobacillus* is the largest genus within the group of lactic acid bacteria with more than 237 species (Zare *et al*. 2018). Lactobacilli are characterized by, Gram-positive rods, anaerobic but aerotolerant, non-sporulating and catalase negative. They are generally recognized as healthy, of 3 GRAS 3 (Generally Recognized As Safe) status and play an important role in the fermentation and preservation of foods. In addition, due to their capacity of bioactive compounds production, such as organic acid (lactic acid, acetate acid and formic acid), hydrogen peroxide (H_2O_2), ethanol, enzymes, benzoate, antimicrobial peptides (AMPs), free fatty acid and volatile compounds some *Lactobacillus* species can be developed as natural drugs and novel antimicrobial agent (Niode *et al*. 2019). On the other, some strains of lactobacilli have beneficial effects on human health and are used as a “probiotic”.

Honey is a natural food produced by honey-bees (*Apis mellifera*) from blossoms nectars or honeydew. Due to their nutritional, energetic and therapeutic proprieties this product is very popular by consumers (Fabazadi *et al*. 2020). Recently, some researchers have been interested to lactic acid bacteria from honey and mainly *Lactobacillus* genus. Different species of lactobacilli were detected in honeys such as *Lb kunkeei, Lb plantarum, Lb pentosus, Lb paracasei, Lb casei, Lb brevis, Lb rhamnosus, Lb fermentum* (Hasali *et al*. 2015; Mounia *et al*. 2018; Lashani *et al*. 2020). The community of LAB found in honey is related to digestive tract of honeybees, nectar, pollen, propolis and flowers (Silva *et al*. 2017).

Currently, the conventional treatment of infections is based on antibiotic therapy. However, the increase of multidrug-resistant pathogens, due to the overuse of antibiotics in human medicine and its intensive use in the animal industry, causes increased concerns about the efficacy of this treatment, which requires the search for new alternatives to antibiotics (Abdul Hafeez *et al*. 2019; Geeta *et al*. 2021; Aazami *et al*. 2016)

In the present study we identified the species of *Lactobacillus* detected in honey samples from Mostaganem region in Northwest Algeria by the repetitive element palindromic-polymerase chain reaction (REP-PCR) and we also, evaluated their antimicrobial activity against some pathogenic bacteria responsible of bovine mastitis.

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MATERIALS AND METHODS

Sampling

During the honey harvesting season starting, between May and August 2019, a total of eight (n=8) raw honey samples were collected, under aseptic conditions, from different localities of Mostaganem region situated in north west of Algeria. All honey samples, were directly obtained from beekeepers and then were stored in sterile bottles at -20°C. Floral origins of these samples were provided by the beekeepers' in relation to the locations where the beehives were situated (Table 1).

Isolation of *Lactobacillus*

Approximately ten grams (10 g) of fresh honey samples were aseptically weighed into a sterile stomacher bag and mixed with 90 mL of sterile 0.1% (w/v) peptone water for 2 min using a stomacher homogenizer. Then, one milliliter (1 ml) of the resulting solution was added to 9 ml of MRS broth and incubated at 30°C for 48 h, followed by serial dilutions with sterile peptone water (0.1% w/v). A volume of 0.1 ml was spread on several specific modified media, namely MRS agar (de Man, Rogosa, Sharpe), MRS agar with (0.8% $CaCO_3$), MRS agar with (0.1% L-cysteine and 2% fructose) and Rogosa agar. These specific seeded isolation media were incubated at 37°C, under anaerobic conditions for 72 h (Feizabadi *et al.* 2020; Homrani *et al.* 2019).

Biochemical screening of *Lactobacillus*

Pure colonies grown on microbiological agar medium were tested biochemically for catalase activity by adding a drop of 3% hydrogen peroxide (H_2O_2). Only catalase-negative colonies (not releasing gas bubbles in the presence of H_2O_2) were retained for Gram staining and cell morphology tests by light microscopy. All catalase-negative, Gram-positive and non spore-forming bacteria were selected and stored in MRS broth supplemented with 15% glycerol and stored at -20°C (Mahmoud Elzeini *et al.* 2020).

Molecular identification

Bacterial identification by the repetitive element palindromic-polymerase chain reaction (REP-PCR) was done as follows:

DNA extraction

The total bacterial DNA of selected isolates and reference strains used was checked and extracted using the method developed by BIO-RAD laboratories and adopted by (Cholet, 2006; Gevers *et al*. 2001; Koenraad *et al.* 2008; Dahou *et al.* 2021).

From a well individual colony, a 1 ml suspension is prepared in 1 ml of sterile distilled water, then centrifuging for 10 min at 3000 rpm. The bacterial pellet is stored at -20°C for 1 hour, the pellet obtained is washed with 1 ml of TES buffer with its resuspensed in 300 µl of STET buffer (1 litter= 80 g Sucrose, 50 ml Triton, 18.6 g EDTA, 6.05 g Tris). An addition of 40µl of an SDS solution (sodium dodecyl sulphate solution) at 20% of a TE buffer composed of 10 Mm Tris-HCL and 1 mM EDTA, pH=8.0, the extraction of the lysate was carried out with an equal distributed between phenol, chloroform and iso-amyl alcohol, then phase separation is carried by centrifugation for 20 min at 3000 rpm. After mixing of the aqueous phase with 70 µl of 5 M NaCl and 1 ml of isopropanol, by the following the DNA precipitate is collected by centrifugation at 3000 rpm for 20 min. Concentration and purity of DNA extract was determined spectrophotometrically by weigh 260 nm.

Table 1: Locality, floral origin and harvesting season of studied honeys.

Sample code	Locality	Floral origin	Harvesting season
S01	Mesra	Polyfloral	Spring
S02	Sidi Fellag	Citrus	Spring
S03	Sidi Fellag	Citrus	Spring
S04	Hchachta	Wild carrot	Summer
S05	Hadjadj	Wild carrot	Summer
S06	Hchachta	Wild carrot	Summer
S07	Sirat	Polyfloral	Summer
S08	Negumaria	Polyforal	Summer

DNA amplification by REP-PCR

The amplification of the DNA fragments obtained from the extraction was performed on a cycler from BIORAD (BIORAD, USA) by the use of specific primers wich will amplify the DNA fragment encoding the 16 S region.

Agarose gel electrophoresis

Visualization of the REP-PCR amplicons was performed by electrophoresis on 1.2% agarose electrophoresis was achieved using a fluorescent dye ethidium bromide (BET).

Comparison of DNA sequences obtained

The acquisitions obtained by the REP-PCR are compared with reference species *Lactobacillus plantarum* subsp. plantarum ATCC 14917 and *Lactobacillus pentosus* ATCC 8041 and with those from the Gene Bank data base using the program blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi) from NCBI.

Pathogenic bacterial strains

Four pathogenic strains (n=4) responsible for bovine subclinical mastitis which are *Staphylococcus xylosus*, *Staphylococcus simulans*, *Klebsiella pneumonea* and *Enterobacter* spp, provided by the Laboratory of Sciences and Technics of Animal Production, University of Mostaganem were used as indicator strains for assessment of antimicrobial activity. These strains were identified by standard biochemical test (Bio-Merieux, France), API Staph and API 20E.

Antibiotic susceptibility test of bacterial strains

Antibiotic susceptibility, for the four pathogen strains, was performed using the disc diffusion method, according to the national committee of clinical laboratory standards (NCCLS) (CLSI. 2018). Broth cultures, of each pathogen strains were diluted in saline solution to about 10^7 CFU/ml (0.5 Units of McFarland turbidity standard). Subsequently, 100 µL of these

growing cultures was spread on Mueller-Hinton (MH) agar. The antibiotic discs were then, placed on the surface of plates. After incubation at 37°C for 24 h, the diameters of the zone of inhibition around the discs were measured in millimeter using a ruler. The tested antibiotics and their concentrations were as follows: Amoxicillin + Acid clavulanic 20+10 µg, Penicillin 6 µg, Oxacillin 1 µg, Bacitracine 130 µg, Ampicillin 10 µg, Streptomycin 300 µg, Trimetoprim + Sulfametoxazol 1.25+23.75 µg.

Antibacterial activity of *Lactobacillus* isolated from honey

The overlay method, described by Fleming *et al*. (1975) was used to evaluate the antibacterial potential of Lactobacillus isolates on selected pathogenic bacteria. Fresh culture of *Lactobacillus* realized in MRS broth was inoculated in spot on MRS agar plates and grown at 30°C for 18 h under anaerobic conditions. The plates were then, overlaid with 8 mL of MRS soft agar (0.7% agar) seeded by 100 µl of indicator strain cultivated overnight. After 24 h of aerobic incubation at 30°C the diameter of inhibition zone was measured in millimeters (mm).

RESULTS AND DISCUSSION

Isolation and screening of *Lactobacillus*

After pre-enrichment in MRS broth followed by plating in four microbiological media a total of fifty seven (n=57) from one hundred and forty four (n=144) isolates were identified as presumed of *Lactobacillus* based on their catalase negative, gram positive and bacilli form (Table 2). The results found proved the presence of lactobacilli in raw honey produced in Mostaganem region. Majority of lactobacilli were isolated from MRS medium followed by MRS supplemented by $CaCO_3$ and MRS supplemented by fructose and cysteine. The origin of lactobacilli detected in honey was honey stomacher of honey bees and the variation of lactobacilli number depending on honey bee health and nectar source (Olofsson *et al.* 2016).

Molecular identification

A total of eleven (n=11) isolates were selected and performed by partial analysis of 16S rRNA sequence using the NCBI blast program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to the research for 16S rRNA sequence similarities. Results confirmed that all isolates belonging to the *Lactobacillus genus*, three isolates were identified as *Lactobacillus pentosus* (lb38, lb174, lb176) and eight as *Lactobacillus plantarum* (lb75, lb108, lb133, lb87, lb21, lb234, lb109, lb175) with 100% genetic sequence similarities. The results from this study are in agreement with previous studies of (Homrani *et al.* 2019) who also, detected the species of *Lactobacillus plantarum* and *Lactobacillus pentosus* in Algerian honeys. Feizabadi *et al*. (2020) proved that the predominance of *Lactobacillus* species is mainly by *Lactobacillus plantarum*, followed by *Lactobacillus pentosus* in *Apis mellifera* honeys produced in Iran. Other species of *Lactobacillus* were isolated from honeys such as *Lb acidophilus, Lb brevis, Lb fermentum, Lb paracasei, Lb kunkeei* (Aween *et al*. 2012; Hasali *et al*. 2015; Bulgasem *et al*. 2016; Olofsson *et al*. 2016; Lashani *et al*. 2020).

Table 2: Isolation of *Lactobacillus* from Mostaganem honeys.

Honey	Catalase	Gram	Number of *lactobacillus*	Medium
S01	-	+	3	MRS
S01	-	+	2	MRS- fructose+cysteine
S01	-	+	3	MRS-$CaCO_3$
S01	-	+	2	Roggosa
S02	-	+	3	MRS
S02	-	+	3	MRS- fructose+cysteine
S02	-	+	5	MRS-$CaCO_3$
S03	-	+	5	MRS
S03	-	+	1	MRS-$CaCO_3$
S04	-	+	2	MRS
S04	-	+	2	MRS-fructose+cysteine
S05	-	+	3	MRS
S05	-	+	6	MRS-fructose+cysteine
S06	-	+	1	MRS
S07	-	+	2	MRS
S07	-	+	1	MRS-fructose+cysteine
S07	-	+	1	MRS-$CaCO_3$
S07	-	+	1	Roggosa
S08	-	+	5	MRS
S08	-	+	3	MRS-fructose+cysteine
S08	-	+	3	MRS-$CaCO_3$

(+): Positive (-): Negative.

Antibiotic susceptibility test

Sensitivity of bacteria isolated from bovine subclinical mastitis to antibiotics are presented in Table 3. We noticed that *Klebsiella pneumonia* strain showed a resistance to all antibiotics tested. *Enterobacter* spp strain was susceptible only to ampiciline. *Staphylococcus simulans* and *Staphylococcus xylosus* showed resistance to penicillin, streptomycin and oxacillin. The same results was also reported by Singh *et al*. (2016) who observed that the bacteria isolated from bovine mastitis resists to cefotaxime, ampicillin-sulbactam, cefixime and ceftriaxone. Bolte *et al.* (2020) reported that some mastitis pathogens on German dairy farms have developed resistance to frequently used antibiotics.

Antibacterial activity of *Lactobacillus* isolated from honey

The eleven (n=11) *Lactobacillus* produce different zones of inhibition by the double agar overlay method and the results are presented in Table 4. The diameter of the inhibition zones ranged from 5 to 21.5 mm (*Klebsiella pneumonae*), from 8 to 19.5 mm (*Enterobacter* spp), from 6 to 14.5 mm (*Staphylococcus simulans*) and from 5 to 12.5 mm (*Staphylococcus xylosus*).

The highest zone of inhibition was observed by lb176 (21.5±2.12 mm) against *Klebsiella pneumonae* and lb133 (19±1.41 mm) against *Enterobacter* spp while the minimum antibacterial effect was observed in lb87 against *Staphylococcus xylosus* (5±0.02 mm) and *Staphylococcus simulans* (6±0.01 mm). Piccart *et al.* (2016) reported that 13 lactic acid bacteria (*Lactobacillus* and *Bifidobacterium*) isolated from honey and honey bees havehigh and promising *in vitro* antibacterial activity against bovine mastitis pathogens. Olofsson *et al.* (2016) tested lactic acid bacteria symbionts in honey bees against severe pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa* and vancomycin-resistant *Enterococcus* (VRE). LAB from honey bees digestive tract produce broad-spectrum antibacterial compounds such as *Serratia marcescens, Eschericia coli, methicillin-resistant Staphylococcus aureus, Klebsiella aerogenes, Salmonella typhi, Pseudomonas* spp, *Klebsiella* spp, *Proteus* spp and *Bacillus subtilis* (Niode *et al.* 2019). Geetha *et al.* (2015) demonstrated the efficacy of purified antimicrobial peptides of LAB isolated from partially decayed of food against pathogenic bacterial strains of bovine mastitis.

The antibacterial effect of *Lacobacillus plantarum* and *Lactobacillus pentosus* isolated from Algerian honeys

Table 3: Antibiotic susceptibility of bacterial isolates from bovine sub clinical mastitis.

	References of antibiotics tested		Pathogenic bacteria tested	
	Klebsiella pneumonae	*Enterobacter* spp	*Staphylococcus simulans*	*Staphylococcus xylosus*
BCT	R	R	S	S
SXT	S	S	S	S
P	R	R	R	R
AMC	R	S	S	S
S	R	R	R	R
AMP	R	R	S	S
OX	R	R	R	R

BCT: Bacitracine; SXT: Trimetoprim+Sulfame toxazol; P: Penicillin; AMC: Amoxicillin acid clavulanic; S: Streptomycin; AMP; Ampicillin OX: Oxacillin. S: Sensible; R: Resistant according to algerian network for monitoring bacterial resistance to antibiotics, standardization of antibiogram at the national level (human and veterinary medicine) document edited with the collaboration of WHO, 6th edition 2011.

Table 4: Antibacterial activity of lactobacilli against bacterial isolates of bovine mastitis.

Bacillus strains	Diameter of inhibition zone (mm)			
	Klebsiella pneumonae	*Enterobacter* spp	*Staphylococcus simulans*	*Staphylococcus xylosus*
Lb75	13±1.41	15±1.41	11.5±2.12	11±0.01
Lb108	10.5±0.71	13±1.41	6.5±2.12	9.5±0.71
Lb133	13.5±0.71	19±1.41	10±0.71	10±1.41
Lb87	5±0.05	8±2.83	6±0.01	5±0.02
Lb21	18.5±2.12	18±2.83	12±2.83	14±1.41
Lb38	8±1.41	14±2.83	11±1.41	10.5±0.71
Lb234	11.5±0.71	8.5±0.71	14.5±0.71	11.5±2.12
Lb109	5.5±0.71	13±1.41	6.5±2.12	9.5±0.71
Lb174	10±0.05	15±1.41	13.5±2.12	10.5±0.71
Lb176	21.5±2.12	19.5±2.12	8±2.83	11±1.41
Lb175	14±1.41	13.5±0.71	13.5±0.71	12.5±0.71

Values are means three replicates±standard deviation.

against gram negative bacteria is showed by Homrani *et al*. (2019). Lashani *et al*. (2018) showed that Iranian honey can be a source for different *Lactobacillus* such as *Lb plantarum* and *Lb paracasei* and their inhibitory effects against pathogen bacteria as *Staphylaococcus aureus*. Ugras *et al*. (2017) found that Lb Kunkeei isolated from the stomach of bee inhibits *Escherichia coli* ATCC 35218, Listeria monocytogenes ATCC 7644, Yersinia pseudotuberculosis ATCC 911, *Pseudomonas aeruginosa* ATCC 27853.

CONCLUSION

This preliminary experimental study revealed the antibacterial potential of two different *Lactobacillus* species, isolated from honeys produced in Mostaganem region (*Lactobacillus plantarum* and *Lactobacillus pentosus*) against the agents of bovine subclinical mastitis. The conventional treatment by antibiotic therapy has showed insufficiencies against the pathogenic agents of the bovine mastitis and in this frame, the lactobacilli strains isolated could be promising alternative treatments of the bovine mastitis. In this perspective, it will be necessary to confirm the antibacterial effect of the combination of honey and lactobacilli by the development of experimental curative tests *in vivo*.

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