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ORIGINAL ARTICLE

Antibacterial Activities of Algerian raw Honeys and Isolated *Lactobacillus* against Gram-negative Bacteria

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ABSTRACT

Honey, is used by man since ancient times for many health benefits and nowadays the search for natural antibacterial substances makes its study more and more wide. Recently the discovery of lactic acid bacteria, like the genus Lactobacillus, in raw honey extended its field of study and use. This study aimed to evaluate inhibitory activities, of four (n=4) various Algerian honeys and the effect of Lactobacillus isolated from honey, against Gram-negative bacteria. Lactobacillus was isolated using selective media. Isolates were identified firstly by catalase test, Gram staining and bacterial morphology, and confirmed by MALDI-TOF-MS. Antibacterial activities were tested by agar well diffusion and agar spot assays for honeys and Lactobacillus, respectively. Antibacterial activities of supernatants of Lactobacillus isolates were examined by the agar well diffusion assay. A total of eighteen (n=18) bacteria isolated from honeys were identified and confirmed by MALDI-TOF–MS as Lactobacillus genus, and assumed Lactobacillus pentosus, Lactobacillus plantarum and Lactobacillus paraplantarum species. All tested honey samples showed antibacterial activities with inhibition zone diameters ranging from 13.5 ± 1.5 mm to 23 ± 4.24 mm. Isolates exhibited antimicrobial activity with inhibition zone diameters ranged from 11 ± 1.41 mm to 17.5 ± 0.71 mm. Supernatants from 6 of the 11 isolates of Lactobacillus demonstrated inhibitory activity against all target bacteria. This study reveals the existence of Lactobacillus in Algerian raw Honeys. Honeys and isolated Lactobacillus possess antibacterial properties against Gram-negative bacteria, often responsible of human infections, and can be a favorable substitute to antibiotics.

***Key words**: Algerian raw honeys; Lactobacillus; Antibacterial activity; MALDI-TOF; Botanical origins*

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INTRODUCTION

Honey is a natural sweet substance produced by honey-bees from blossoms nectars or trees and plants exudates. It is used for nutritional, medicinal and industrial purposes, which make it one of the most widely, sought products [1].

The treatment of microbial infections with honey dates back to ancient times. Recently, it has been rediscovered by the medical domain, particularly where conventional modern therapeutic agents are failing [2]. However, the level of antimicrobial activity in honey is highly variable and depends on its geographical source, botanical origin, storage and treatment conditions, and bee factors such as age and health of colonies [3, 4]. Moreover, the antibacterial properties of honey are multiple, mainly related to

the ability to generate hydrogen peroxide by the enzyme glucose oxidase, a naturally low pH and a high osmolarity due to the high sugar content, mainly fructose and glucose [5].

Many researchers have investigated honey antimicrobial properties against a broad range of microorganisms, including aerobes and anaerobes, Gram-positives and Gram-negatives [6, 7, 8, 9, 10]. According to [11], substances such as defensin-1, methylglyoxal, and vegetable inhibitors (lysozymes, flavonoids, aromatic and volatile substances) contribute effectively to its antimicrobial proprieties.

It should be noted that today, the study of lactic acid bacteria (LAB) from different sources is of growing interest, particularly in the scientific community. They are generally recognized and giving them GRAS status "Generally Recognized as Safe", and exploited as probiotics conferring host health benefits [12]. Several LAB strains were isolated from honey [13, 14, 15, 16, 17]. Bacteria, found in honeys, come from honey bees, and possess varied LAB microbiota, essentially *Lactobacillus* and *Bifidobacterium*, acquired by consuming pollen and nectar and through contact with older colony bees [18, 17]. *Lactobacillus* genus is the largest group among lactic acid bacteria group, which includes over 110 listed species[19].

Lactobacilli play an important role in the human and animal gastrointestinal tract. They are characterized by, Gram-positive rods, anaerobic but aerotolerant, non-sporulating and catalase negative. In addition, they represent one of the major groups involved in desirable fermentation and contribute to food preservation [20].

Furthermore the Probiotics such as *Lactobacillus* spp. are reported to exhibit inhibitory activity against common human pathogens [21]. The increase of multidrug-resistant pathogens, due to the overuse of antibiotics in human medicine and its intensive use in the animal industry, requires a second use of new antibacterial agents [22].

Honey is a natural, non-toxic and robust antimicrobial. It can advantageously replace an antibacterial agent, in particular against bacteria developing resistance to many antibiotics. In addition, and in recent years, *Lactobacillus* metabolites have captured a substantial interest as natural drugs [23]. However, the possible role of *lactobacillus* in antibacterial activity of honey has received little attention.

The present study aimed to evaluate antimicrobial proprieties of four (n=4) Algerian honeys and Lactobacilli isolated from honey against *Escherichia coli* and *Pseudomonas aeruginosa.*

MATERIALS AND METHODS

Sampling

Between April and August 2015, four (n=4) typical *Apis Melliféra* raw honey samples, of different floral origins, were collected under aseptic conditions, from different Algerian regions, as listed in Table 1 and Figure 1. All honey samples were taken away in sterile bottles and stored at -20 °C, until use. Sample floral origins were identified by pollen analysis according to [24].

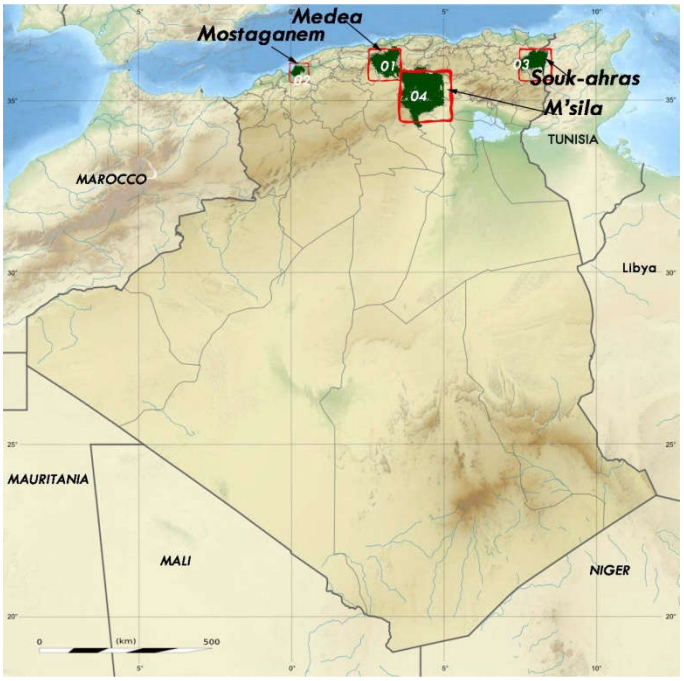

Figure 1. Map of the study area

Culture Method

Ten grams (10 g) of raw honey samples were weighed aseptically into a sterile stomacher bag and mixed with 90 mL of sterile 0.1% peptone saline solution supplemented with Tween 80 (0.9 % w/v NaCl, 0.1 % w/v Tween 80, 0.1 % w/v peptone), during 2 min using a stomacher homogenizer. One milliliter of the homogenate was added to 9 mL of MRS broth and incubated at 30 °C for 24h, followed by appropriate serial dilutions (10^{-1}-10^{-5}) with sterile peptone water (0.1% w/v).

A volume of 100 µl was spread plated on several modified media, namely, MRS agar (de ***Man*, *Rogosa*, *Sharpe***) (Oxoid), MRS agar (with 0.8% $CaCO_3$), MRS (with L-Cysteine 0.1% and fructose 2%) and Rogosa, and then incubated at 37 °C, under anaerobic conditions for 72h. Isolation was undertaken earlier; six days post harvest.

Screening of *Lactobacillus* bacteria

Colonies were initially tested for catalase activity with hydrogen peroxide H_2O_2 (3 %). Catalase-negative colonies were streaked on MRS agar and incubated at 37 °C for 48h. Obtained pure colonies were again tested for catalase activity, followed by Gram stained and bacterial morphology tests. All catalase-negative; Gram-positive and *bacilli* form, isolates were selected and stored in skim milk broth supplemented with glycerol 30 % at –20 °C for further analysis.

MALDI-TOF MS identification of isolates

Selected isolates were identified by MALDI-TOF MS (Matrix Assisted Laser Desorption Ionization/Time Of Flight, Mass Spectrometry), using Vitek MS system (Biomerieux, la Balme, France). The procedure was performed according to manufacturer's instructions. Briefly, fresh part of the cultivated colony on blood agar was smeared on Vitek MS-DS target slide, and immediately covered with 1µL of matrix solution. Bacterial suspension spectra were performed using Vitek-MS mass spectrometer and microbial identification results were done by MYLA software (Biomerieux, France). The Vitek MS identifications (i.e., species level, genus level, or no result) were based on confidence level. Whenever the software provides identification with only one choice, an acceptable confidence value may range from 60 to 99.99%. Two or more results with the same genus but multiple species were considered acceptable for genus identification only.

Pathogenic Bacterial strains

The pathogenic bacterial strains, *Escherichia coli* ATCC 25922 *and Pseudomonas aeruginosa* ATCC 27853, used as indicator Bacteria, were provided by the regional veterinary laboratory of Mostaganem.

Antimicrobial activity of honey

The agar well diffusion assay was used to determine the antibacterial activity of the raw honeys according to [25]. To do this, a sterile cotton swab was dipped into a fresh culture suspension of indicator strains. The turbidity of the bacterial culture was adjusted to 0.5 Mc-Farland to obtain the final concentration (1.5×10^8 cfu / mL) of each test microorganism.

The cotton swab which was soaked in fresh culture slurry of indicator strains was then streaked uniformly over the entire Muller-Hinton agar surface, rotating the plate at about 60 ° each time. After inoculation, wells of about 5 mm in diameter were hollowed and filled with 100 µl of raw honeys. After incubated at 37 °C for 24 h, the plates were examined for any zones of inhibition, and the diameter of these zones were measured in millimeters (mm).

Antibacterial activity of *Lactobacillus* isolated from honey

The agar spot assay described by [26] was used to determine the antibacterial activity of Lactobacillus isolates. Overnight cultures, of Lactobacilli isolates realized in MRS broth, were deposited on the surface of MRS agar plates and incubated at 37 ° C for 18 h under anaerobic conditions to allow the colonies to grow. Aliquots (100 µl) of indicator strain cultivated overnight were inoculated into 8 ml of MRS soft agar (1% agar) and poured onto the plate on which the LAB isolates were grown. After incubation at 30 ° C for 24 hours under aerobic conditions, the antimicrobial activity of *Lactobacillus* and the susceptibility of the pathogenic indicator strain were evaluated by checking for inhibition zones around the spots and measuring there diameters in mm.

Antibacterial activity of *Lactobacillus* supernatant

The agar Well diffusion assay was performed to determine the antibacterial activity of Cell Free Supernatants (CFS) of *Lactobacillus* according to [25]. After incubation at 30 °C during 18h in MRS broth, CFS of *Lactobacillus* was obtained by centrifugation at 6000 rpm, during 20 mn at 4 °C and filtration. Wells were hollowed, into the MRS agar plates containing indicator strain, and filled with 100 µl of CFS. The plates were incubated at 37 °C for 18 h and then subsequently examined for inhibition zones.

RESULTS AND DISCUSSION

More than 100 colonies developed on four selective Media were picked up and evaluated on the basis of their staining properties, catalase reactions and bacterial morphology. The rod- shaped gram-positive and catalase-negative single colonies that could belong to the *Lactobacillus* genus were selected.

In our study, a total of 18 presumptive *Lactobacillus* were isolated from four various Algerian raw honey samples by combination of pre-enrichment in MRS broth followed by plating in three microbiological media: MRS-$CaCo_3$; MRS 2% fructose and 0.1% L- cysteine; and Rogosa medium (Table 2). Among isolates, eight (44%) were detected in *brassica* honey from Souk-ahras of which five isolated on Rogosa Medium; two on MRS (2 % fructose and 0.1% L-cyctein), one on MRS $CaCO_3$. In addition, eight (44 %) were detected in *Eucalyptus* honey from M'sila of which four isolated on MRS $CaCO_3$ and four on Rogosa. One *Lactobacilli* was detected and isolated on MRS (2 % fructose + 0.1 % cystein) medium in each of two *polyfloral* honeys from Mostaganem (6 %) and Medea (6 %). None *Lactobacillus* growth was detected on MRS agar medium.

The presumptive *Lactobacillus* was identified by MALDI-TOF MS using the VITEK MS system. The results confirmed that all isolates were classified as *Lactobacillus* genera*,* but uncertainty species identification between *Lb. pentosus*, *Lb. plantarum* and *Lb. paraplantarum*, with 99.9 % confidence value (Table 3).

The bacteria presented in raw honey can not grow or reproduce and lose viability over time. Pre-enrichment in MRS broth for 24 hours allows regenerating the *Lactobacillus* present in the honey and promotes their growth.

The isolation of *Lactobacillus* from honey by classical media, such as MRS, commonly used for most (fermented) food products, is difficult to achieve because of the high concentration of sugars thus limiting the detection of these bacteria. The use of supplemented MRS with $CaCO_3$, fructose and L-cysteine, and Rogosa medium permits the detection and isolation of lactobacillus. The high number of *lactobacillus* isolated during our experiments from Rogosa medium, demonstrate its ability, to isolate the *lactobacilli* from the honeys.

MALDI-TOF- MS based microbial identification relies on the generation of an organism-specific mass spectrum or "protein footprint" examined against a reference database to provide identification of the organism [27]. Our Results of MALDI-TOF identification was not able to differentiate spectra from *Lb. plantarum*, *Lb. pentosus*, and *Lb. paraplantarum*, indicating the need for additional testing or the use of another method, like the sequencing of the rRNA 16S gene, for definitive differentiation.

[28] reported the presence of *Lb.* brevis in melipionine honey, while [29] detected *Lb. acidophilus* in Malysian, Libyan and Saudi honey. In previous studies, [30] detected *Lb. plantarum,. Lb. Cuvatus*, *Pediococcus acidilactici* and *Pediococcus pentos**aceus*** in 10 of the 15 honey samples. To our knowledge, information about lactic acid bacteria from Algerian honeys was not reported.

The different types of *Lactobacillus* species, detected in honey, may come from honey stomacher of honeybees and vary depending on nectar source, honeybee health and the presence of other microorganisms [31].

Pseudomonas aeruginosa and *Escherichia coli* are amongst some of the main bacteria with multidrug resistance and are included in the category of community and hospital acquired pathogens [32]. The inefficacy of existing medical treatments has necessitated the search for novel natural effective alternative to tackle this problem.

The *in vitro* antimicrobial activity of four various Algerian honeys on Gram-negative strains (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853) were tested and compared. The results expresses as the size (mm) of the inhibition halo are presented in Figure 2. Data obtained, demonstrated that all varieties of honeys exhibits an antimicrobial effect. They produced zones of inhibition at the different diameters ranged from 13.5 ±1.5 mm to 21.25 ± 0.25 mm for *E. coli* and from 13.75 ± 0.35 mm to 23 ± 4.24 mm for *P. aeruginosa*. the highest inhibitory activities against *E. coli* were shown by *brassica* honey (M'sila), followed by *polyfloral* honey (Mostaganem), *polyfloral* honey (Medea) and *Eucalyptus* honey (Souk-ahrass). While, Polyfloral honey (Medea) showed the highest inhibitory activity against *P.* aeruginosa, followed by *polyflora* honey (Mostaganem), *Brassica* honey (M'sila) and *Eucalyptus* honey (Souk-ahrass). The differences between honey samples in terms of antibacterial activity can be attributed to the natural variations in floral sources of nectar and the different locations, the same results have been described by [33].

The study of [34] conducted on four Algerian honeys from different sites, the Annaba region revealed that the honey from the Seraidi and Chetaibi site showed a strong antimicrobial activity compared to those of Berrehal and El -Bouni, against *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli and Pseudomonas aeruginosa.* [35] showed that Algerian honey, diluted at concentrations ranging from 11 to 14%, completely inhibits the growth of bacteria isolated from subclinical mammary goat milk (*Streptococcus* D,

Corynebacterium spp., *Enterobacter* spp., *E. coli*, *Bacillus* spp). [36] Compared between 32 honeys from the Algerian Sahara from 8 different botanical origins revealed that the antibacterial effect of the *Fabaceae sp* honey was more effective against *Bacillus subtilis*, *Clostridium perfringens*, *Escherichia coli* and *Staphylococcus aureus*. *E. coli* was the most sensitive species with an inhibition zone of 10.1 ± 4.7 mm.
In recent years, many researchers have investigated the antibacterial properties of Manuka honeys against various microorganisms [37, 38, 39, 40, 41, 42]. Other factors, not studied, could also be responsible of the variations observed, such as osmotic pressure, pH, water activity and the effect of hydrogen peroxide [43]. Flavonoids, protein compounds and the high sugar content have also been reported to play a role in antimicrobial activity [44].
A total of 11 selected bacterial cultures of *Lactobacillus* isolated from four Algerian honeys were evaluated for their inhibitory activity. For this we used the agar spot method and the results are presented in Figure 3. All bacterial cultures showed an inhibitory action against target Gram-negative bacteria. The diameter of the inhibition zones varied between 12.5 ± 0.71 - 17.5 ± 0.71 mm (*E. coli*) and 11 ± 1.41 - 15 ± 0.82 mm (*P. aeruginosa*). Two isolates of *Lactobacillus*, lb47 of *polyfloral* honey (Mostaganem) and lb44 of *Eucalyptus* honey (Souk-ahras) show greater inhibition against *E. coli.* However, Lb38 of *eucalyptus* honey (Souk-ahrass) and lb67 of *brassica* honey (M'sila) show greater inhibition against *P. aeruginosa*. Minimum activities were observed against *E. coli and P. aeruginosa* in lb38 and lb 65, respectively.
The cell free supernatant (CFS) of the *Lactobacillus* was also tested against Gram-negative bacteria using well diffusion agar method and the results were summarized in Figure 4. Cell-free supernatants from 6 of the 11 isolates of *Lactobacillus* (lb92, lb47, lb03, lb38, lb12, lb67) demonstrated inhibitory activity against *E. coli* and *P. aeruginoss*. Average diameters of inhibition zones ranged from 6±1.73 to 11±1.41 mm**.** (*E. coli*) and from 5.5± 0.71 to 11±1.41 mm (*P. aeruginosa*).
Lactic acid bacteria including *lactobacillus* can produce antimicrobial agents that exert strong antagonistic activity against many gram positive and negative microorganisms [45].
Metabolites such as organic acids (lactic and acetic acid), hydrogen peroxide, ethanol, diacetyl, acetaldehyde, acetoïn, carbon dioxide, reuterin, reutericycline and bacteriocins are examples of antimicrobial agents produced by LAB microorganisms.
Organic acid metabolites produced by lactic acid bacteria result in reduced pH and increase hydrogen production [46]. [47] reported that *Lb. plantarum* and *Lb. paracasei* isolated from Iranian honeys had wonderful inhibitory effects against *S. aureus*. [28] found that strains of *lactobacilli* isolated from meliponin honey from Heterotrigona itama showed significant antibacterial activity against *S. epidermidis*, *P. aeruginosa* and *L. monocytogenes* and could be applied in the food and pharmaceutical industries. [48] tested 13 isolated lactic acid bacteria (*Lactobacillus* and *Bifidobacterium* species) from honey and honey bees against bovine mastitis and observed that the synergism between lactic acid bacteria and honey was able to inhibit growth of bacteria that cause for inflammatory mastitis; even those that were resistant to other antibiotics. [49] demonstrated that Lb. acidophilus supernatants isolated from different sources of honeys were effective against Gram-negative pathogenic bacteria. They suggest the possible role of lactic acid bacteria in improving the antibacterial activity of honey.

Table 1. Region, Locality, Floral origin, Harvesting season, and Geographic location of Algerian honeys

Samples n°	Region	Locality	Floral Origin	Harvesting Season	Geographic location
01	Medea	Moudjbar	*Polyfloral*	Summer	34°28'0" N et 3°25'0" E
02	Mostaganem	Sirat	*Polyfloral*	Spring	35° 46′ 48″ N, 0° 11′ 31″ E
03	Souk-Ahras	Ouled-Driss	*Eucalyptus*	Summer	36°21'0" N et 8°1'0" E
04	M´Sila	Bou-Saâda	*Brassica*	Summer	35° 13′ 09″ N, 4° 10′ 54″ E

Table 2. Isolation of *lactobacillus* from Algerian honeys using different Media.

Samples n°	Nomber of *lactobacillus* isolated	*Lactobacilius* code	Gram stain	Catalase test	Cell morphology	Media
01	01	Lb92	+	-	Rods	0.1% Cysteine+2% Fructose
02	01	Lb47	+	-	Rods	0.1% Cysteine+2% Fructose
03	08	Lb3	+	-	Rods	0.1% cysteine+2% Fructose
		Lb35	+	-	Rods	Rogosa
		Lb38	+	-	Rods	Rogosa
		Lb40	+	-	Rods	Rogosa
		Lb44	+	-	Rods	MRS-$CaCO_3$
		Lb49	+	-	Rods	0.1% Cysteine+2% Fructose
		Lb119	+	-	Rods	Rogosa
		37r07	+	-	Rods	Rogosa
04	08	Lb12	+	-	Rods	Rogosa
		Lb29	+	-	Rods	Rogosa
		Lb65	+	-	Rods	MRS-$CaCO_3$
		Lb66	+	-	Rods	MRS-$CaCO_3$
		Lb67	+	-	Rods	Rogosa
		L84	+	-	Rods	Mrs-$CaCO_3$
		Lb116	+	-	Rods	Mrs-$CaCO_3$
		Lb32	+	-	Rods	Rogosa

Table 3. Maldi-Tof identification of isolates

Code LB	*Maldi-tof identification*	confidence value (%)
Lb92	*Pentoses-plantarum-paraplantarum*	99.9
Lb47	*Pentoses-plantarum-paraplantarum*	99.9
Lb3	*Pentoses-plantarum-paraplantarum*	99.9
Lb35	*Pentoses-plantarum-paraplantarum*	99.9
Lb38	*Pentoses-plantarum-paraplantarum*	99.9
Lb40	*Pentoses-plantarum-paraplantarum*	99.9
Lb44	*Pentoses-plantarum-paraplantarum*	99.9
Lb49	*Pentoses-plantarum-paraplantarum*	99.9
Lb119	*Pentoses-plantarum-paraplantarum*	99.9
37r07	*Pentoses-plantarum-paraplantarum*	99.9
Lb12	*Pentoses-plantarum-paraplantarum*	99.9
Lb29	*Pentoses-plantarum-paraplantarum*	99.9
Lb65	*Pentoses-plantarum-paraplantarum*	99.9
Lb66	*Pentoses-plantarum-paraplantarum*	99.9
Lb67	*Pentoses-plantarum-paraplantarum*	99.9
L84	*Pentoses-plantarum-paraplantarum*	99.9
Lb116	*Pentoses-plantarum-paraplantarum*	99.9
Lb32	*Pentoses-plantarum-paraplantarum*	99.9

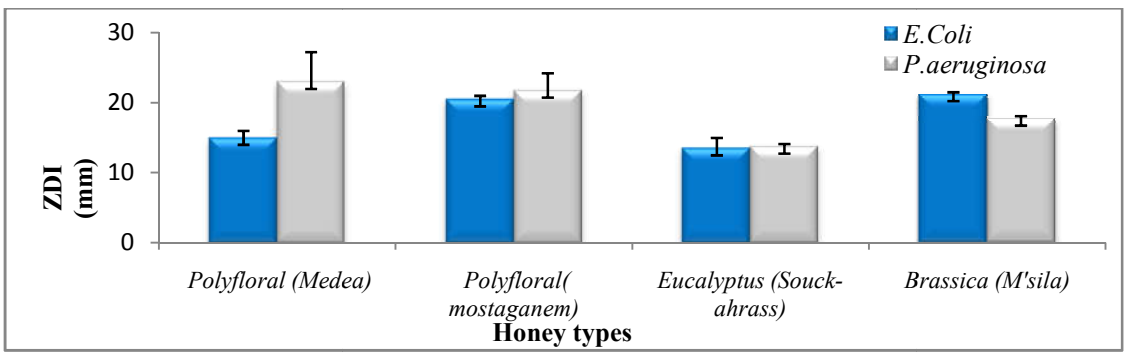

Figure2. Antibacterial activity of different Algerian honeys types against Gram negative bacteria by well agar diffusion; ZDI: Zone diameter of inhibition

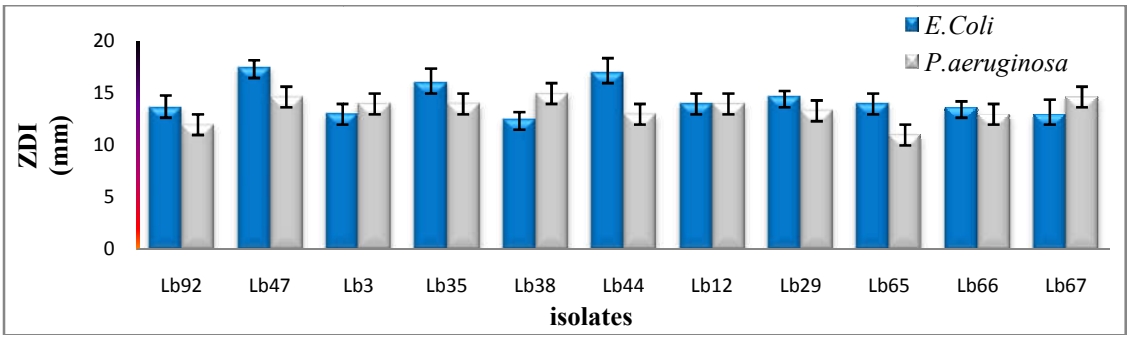

Figure3. Antibacterial activity of *lactobacillus* isolated from different Algerian honey types against Gram negative bacteria by spot agar test; ZDI: Zone diameter of inhibition

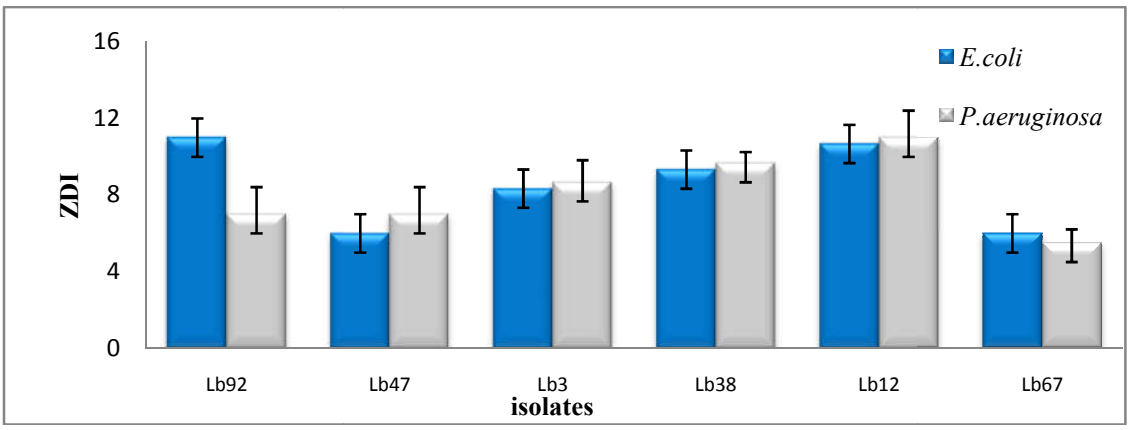

Figure4. Antibacterial Activity of *Lactobacillus* Supernatants against Gram negative bacteria; ZDI: Zone diameter of inhibition

CONCLUSION

Our study, revealed the presence of different species of *Lactobacillus* in different types of Algerian raw honey, this result is all the more interesting as lactobacilli can be used as probiotics. The emergence of increasingly antibiotic-resistant bacteria leads researchers to try to find new strategies to fight them. The honeys described in this study have shown significant inhibitory effects, for this it would be interesting to know the exact composition and quantification of their inhibitory factors. Honey, by all the inhibitors it contains, is described as an excellent inhibitor of dangerous bacteria and if it is associated with lactic bacteria the inhibitory power, against Gram-negative bacteria responsible for infections, will be improved.

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