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Research Article

Antifungal Activity of Lactic Acid Bacteria Strains Isolated from Natural Honey against Pathogenic *Candida* Species

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Abstract The role of lactic acid bacteria (LAB) in honey as antifungal activity has received little attention and their mechanism of inhibitory of fungi is not fully understood. In this study, LAB were isolated from honey samples from Malaysia, Libya, Saudi Arabia, and Yemen. Twenty-five isolates were confirmed LAB by catalase test and Gram staining, and were screened for antifungal activity. Four LAB showed inhibitory activity against *Candida* spp. using the dual agar overlay method. And they were identified as *Lactobacillus plantarum* HS isolated from Al-Seder honey, *Lactobacillus curvatus* HH isolated from Al-Hanon honey, *Pediococcus acidilactici* HC isolated from Tualang honey and *Pediococcus pentosaceus* HM isolated from Al-Maray honey by the 16S rDNA sequence. The growth of *Candida glabrata* ATCC 2001 was strongly inhibited (>15.0 mm) and (10~15 mm) by the isolates of *L. curvatus* HH and *P. pentosaceus* HM, respectively. The antifungal activity of the crude supernatant (cell free supernatant, CFS) was evaluated using well diffusion method. The CFS showed high antifungal activity against *Candida* spp. especially The CFS of *L. curvatus* HH was significantly ($p < 0.05$) inhibited growth of *C. glabrata* ATCC 2001, *C. parapsilosis* ATCC 2201, and *C. tropicalis* ATCC 750 with inhibitory zone 22.0, 15.6, and 14.7 mm, respectively. While CFS of *P. pentosaceus* HM was significantly ($p < 0.05$) effective against *C. krusei*, *C. glabrata*, and *C. albicans* with inhibition zone 17.2, 16.0, and 13.3 mm, respectively. The results indicated that LAB isolated from honey produced compounds which can be used to inhibit the growth of the pathogenic *Candida* species.

Keywords Antifungal activity, Honey, Lactic acid bacteria, Pathogenic *Candida* species

A supersaturated four key sugars solution of fructose, glucose, sucrose and maltose, honey has other elements for example gluconic acid, protein enzymes, amino acid, minerals, vitamins, and water [1]. Slightly acidic with a pH between 3.2 and 4.5, honey's low pH prevents the growth of various pathogenic bacteria and fungi [2]. Honey's antimicrobial activity is affected by how microbial groups including bacteria (Gram-variable pleomorphic bacteria, *Bacillus* spp., Enterobacteriaceae, and lactic acid bacteria [LAB]), molds (primarily aspergilli and penicillia) and yeasts interact with each other [3]. Honey's inherent elements might influence microorganisms' growth and ability to survive via bacteriostatic or bactericidal actions [4]. Comparable to fermented food and silage situation that is both rich in sugar and acid, LAB are honey's major microorganism [5], honey bee (*Apis mellifera*) foragers [6], bees' stomach, flowers, plants, flowers, and fruits [7]. LAB secluded from diverse bases is identified in yielding different antimicrobial compounds that are able to prevent fungi growth. Generally, some species inside LAB might yield bioactive compounds for instance organic acid, hydrogen peroxide, diacetyl, bacteriocins, antimicrobial peptides, and antibiotics [8-10].

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Pathogenic *Candida* types are the commonest reason for hospital-acquired contagions particularly, among cancer and transplant patients who used immunosuppressive therapy. *Candida* species are behind various forms infecting humans for example urinary tract infection, vulvovaginal candidiasis, and nosocomial pneumonias [11]. Several types of *Candida* spp. are resistant to numerous non-natural antifungal drugs for example amphotericin B, fluconazole and itraconazole. These three antifungal agents are normally part of candidemia and invasive candidiasis treatment [11, 12]. Several cases correlated to long term suppressive therapy using antifungal agents was reported to be futile and poisonous to the host [13, 14]. Many patients experiencing fluconazole caused clinical failure will have *Candida* species separates to *in vitro* resisting antifungal [12]. Honey is natural, non-poisonous and robust antimicrobial can be a favorable substitute to antifungal agent. Samplings from honey antifungal activities are different based on their botanical source and geographical origin [15]. Earlier studies emphasized that honey from Algeria [16], Nigeria [17], and Turkey [18], exhibited antifungal activity, however, the studies did not examine honey's LAB role as an antifungal agent. Hence, this study's purpose is determining the LAB isolated antifungal activity from honey from different sources for example Malaysia, Libya, Yemen, Saudi Arabic, and New Zealand against *Candida* spp. strains specifically, *C. albicans* ATCC1405, *C. glabrata* ATCC2001, *C. tropicalis* ATCC750, *C. parapsilosis* ATCC22019 and *C. krusei* ATCC6258.

MATERIALS AND METHODS

Honey samples. The collection of fifteen samples of honey from various sources was stored at room temperature prior to analysis. This study's samples were honey locally attained from Malaysia (Madu Tualang, Madu Tani, pure Trigona honey) Libya (Al-Seder honey, Al-Hanon honey, and Al-Zater honey), Yemen (Al-Seder honey and Al-Maray honey), Saudi Arabic (Al-Shifaa honey), and New Zealand (Manuka honey). A pH meter (METTLER TOLEDO, Greifensee, Switzerland) was used in determining the diverse honey's pH range.

Isolation of LAB from honey samples. Isolation of LAB from honey samples succeeding the technique defined by Bulgasem *et al.* [19]. The suspension of roughly 10 g of honey samples in 90 mL peptone water (0.1% w/v) in sterile stomacher bags and the bags' agitation were manually done. Then, the addition of 1 mL into 9 mL of de Man, Rogosa & Sharpe (MRS) broth (Oxoid CM359) and the incubation was at 30°C for 24 to 48 hr followed by diluting serially with peptone water (0.1% w/v). Subsequently, 0.1 mL was spread plated on a number of adapted media specifically, MRS agar (Oxoid) [20], MRS agar with 0.8% $CaCO_3$ [21], MRS agar with 1% glucose, tomato juice agar with 0.8% $CaCO_3$, and tomato juice agar with 1% glucose. The incubation of plates was under anaerobic situation in anaerobic jar at 37°C for 48 hr or until the bacterial colonies grown sufficiently in size. The testing of colonies for catalase activity with 4% H_2O_2 and the streaking of catalase negative colonies on MRS agar that contained 0.8% $CaCO_3$ was kept warm at 37°C for 48 hr to attain pure colonies. The isolates' validation for Gram staining and culture purity was inspected using morphology and microscopic. All negative catalase and gram-positive LAB isolates were preserved in MRS broth with 15% of glycerol and set aside at −20°C for more inspection.

Culturing of *Candida* spp. The *Candida* strains in this study were attained from microbial collections original stock cultures of Department of Medical Microbiology, University Putra Malaysia. All *Candida* spp. including *C. albcans* ATCC14053, *C. parapsilosis* ATCC22019, *C. tropicalis* ATCC750, *C. krusei* ATCC 6258, and *C. glabrata* ATCC2001 strains which were cultured on Sabouraud dextrose agar (SDA, Oxoid) at 35°C for 24 and 48 hr to confirm feasibility and clarity. Fungal strains were kept on SDA at 4°C until further use.

Cell free supernatant preparation. The isolates were injected into MRS broth and keep warm at 30°C for 24 hr. The preparation of cell free supernatant (CFS) was by centrifuging the broth at 11,500 rpm for 10 min at 4°C. (Mini Spin, AG 22331; Eppendorf, Hamburg, Germany). Each filtration of isolate supernatant used sterile filter (0.45 μm-pore-size filter; Millipore, Darmstadt, Hesse, Germany) [22], and this filtrate was utilized for analysis.

Sensitivity of *Candida* spp. to antifungal agents. *Candida* spp. was examined to see how sensitive it is to antifungal agents via disc diffusion technique as defined by Bauer *et al.* [23]. The antifungal utilized were nystatin (100 U), amphotericine B (20 μg), fluconazole (100 μg), ketoconazole (10 μg), itraconazole (50 μg), voriconazole (10 μg), and econazole (10 μg). The antifungal disks were attained from SIGMA-Aldrich Chemie GmbH (Steinheim, Germany). Antifungal agents selected to be utilized in this study was established on the common antifungal utilized in medical practice and health therapy. *Candida* spp.'s multiple antifungal resistant (MAR) index was decided when the number of antifungal agents which is resistant to an isolate/whole quantity of antifungal tried as defined by Subramani and Vignesh [24]. *Candida* spp.'s 24-hr pure cultures cultured in Sabouraud dextrose broth (Oxoid CM147) and kept warm at 37°C for 24 hr. The pathogenic *Candida* cultures were later swabbed on SDA (Oxoid) plates. The plates' agar surface were dried in the laminar flow cabinet at ambient temperature for 15 min. With a sterilized forceps, the antifungal agents' paper discs (6 mm in diameter) were aseptically placed on plates and the incubation was at 37°C for 24 hr aerobically. The inhibitory zone's diameter measurement surrounding each disc was taken using a

ruler and documented. The experiment was carried out two times and the mean and standard deviation was noted.

Antifungal activity of LAB isolates using dual agar overlay method. Four LAB isolates antifungal activities was decided against *Candida* spp. using the overlay technique as defined by Magnusson and Schnürer [25]. LAB was injected in spot on MRS agar plates. The incubation of the plates was at 30°C for 24 hr under anaerobic situations. These plates were covered with a layer of 15 mL of SDA (0.75% soft SD agar) that contained 10^4 CFU/mL of overnight *Candida* culture poured over the plates. The plates were kept warm aerobically at 30°C for 24 hr, and the zone inhibiting *Candida* growth located above respective LAB culture was measured. LAB inhibition tests against *Candida* spp. was carried out in duplication.

Antifungal activity of LAB supernatants using agar well diffusion method. The LAB isolates exhibited strong antifungal activity via dual overlay spot technique was additionally assessed by the well technique defined by Magnusson and Schnürer [25]. *Candida* spp. (10^4 cells/mL) cultured 24 hr was diversified with SDA, and dispensed into the plates. After the solidification of agar, cork borer were used to make 6-mm wells. It then covered the well's base with agar to evade leaks. The addition of CFS invariable quantities of LAB to each well and incubating the plates aerobically at 30°C for 24 hr. The measurement of growth inhibition zone surrounding the wells was taken after well size decreases. The experiment was carried out in duplicates and the means with calculations of standard deviations.

Identification of LAB isolates by API 50 CHL and 16S rDNA gene sequences. The four LAB isolates showing antifungal activity were recognized with API 50 CHL kit assay resulting from the technique used as described by the manufacturer [26]. The LAB isolates identified were further established by 16S rDNA gene sequences. The LAB chromosomal DNA's four strains were taken out using the Wizard Genomic gram-positive DNA purification kit (Promega, Madison, WI, USA). The sample's purified DNA was processed to the polymerase chain reaction (PCR) by utilising the Fail Safe Pre Mix Kit Epicentre (an Illumina company, San Diego, CA, USA). One thousand four hundred base pair fragment of 16S rDNA gene was amplified using PCR with primers based on the preserved 16S forward (5′-AGAGTTTGATCCTGGCTC-3′) and 16S reverse (5′-CGGGAACGTATTCAC-CG-3′) regions [27]. The PCR protocols were as follows: denaturation at 95°C for 2 min, followed by 35 denaturation cycles at 92°C for 45 sec, hardening at 54°C for 1 min and elongating at 72°C for 1 min, and final extension at 72°C for 5 min. Five microliters of each amplified mixture were exposed to electrophoresis in 1.5% (1.5 g agarose powder with 100 mL in 1× TEA buffer) for 45 min at 90 V. Amplified products were examined more by the First BASE Laboratories Sdn Bhd., Malaysia. The aligned nucleotide sequence was later exposed to the Nucleotide-nucleotide Basic Local Alignment Search Tool (BLASTN) software ver. 2.2.14 to be compared to other accessible isolates in the GenBank Database of the National Centre for Biotechnology Information (NCBI) to be confirmed. The multiple sequence alignment was done using Multiple Sequence Comparison by Log-Expectation (MUSCLE) [28], and phylogenetic tree was constructed by the neighbor-joining method [29]. *Bacillus cereus* ATCC14579 used as an out-group organism with 1,000 bootstrap replicates. The nucleotide sequences for the 16S rDNA described in this study were deposited with the GenBank database of Bethesda, MD, USA under accession Nos. KX346613~KX346616 for the strains HS, HC, HH, and HM.

Statistical analysis. All data were presented as mean ± standard deviation and were analysed by two-way analysis variance using general liner model procedure of SAS (SAS Institute Inc., Cary, NC, USA), and Tukey's test was applied for significant means at $p < 0.05$ to evaluate the significant differences between groups.

RESULTS

Screening of antifungal LAB. Isolation of LAB from honey was performed by using modified media. Among the different medium evaluated, medium with added 0.8% $CaCO_3$ showed good growth of LAB at dilution 10^4 to 10^5. The higher the dilution of LAB were detected, the higher number of LAB population correspond to the samples. In

Table 1. Characterization of LAB that showed high antifungal activity against *Candida* species

Sample code	Source	Shape	pH of sample	Media	Dilution LAB detected
HS	Al-Seder honey, Libya	Short rod	5.7	MRS + $Caco_3$	10^{-4}
				MRS + Glucose	10^{-3}
HH	Al-Hanon honey, Libya	Cocci	4.4	MRS + $Caco_3$	10^{-5}
				MRS + Glucose	10^{-3}
HC	Tualang honey, Malaysia	Cocci	3.5	MRS + $Caco_3$	10^{-5}
HM	Al-Maray honey, Yemen	Short rod	4.1	TJA + $Caco_3$	10^{-1}
				MRS + $Caco_3$	10^{-2}

LAB, lactic acid bacteria; MRS, de Man, Rogosa & Sharpe; HS, *Lactobacillus plantarum*; HH, *L. curvatus*; HC, *Pediococcus acidilactici*; HM, *P. pentosaceus*.

this study, Tualang honey contained high numbers of LAB. The pH of honey samples varies from 3.5 to 5.7 with Tualang honey showing the lowest pH value of 3.5 (Table 1). A total of twenty-five isolates that showed clear zones on MRS agar with 0.8% $CaCO_3$ and catalase negative were gram-positive stained and results showed that the LAB isolates were 52% rod-shaped and 48% coccus-shaped. Four of these LAB isolates namely, HS, HC, HH, and HM were selected for antifungal study against five strains of pathogenic *Candida* spp.

Identification of LAB isolates by API 50 CHL and 16S rDNA. The identification of four LAB isolates from honey samples that showed antifungal activity against five strains of pathogenic *Candida* spp. is presented in Table 2. The results from API 50 CHL kit identified the isolate HS from Al-Seder honey, as *Lactobacillus plantarum*2, and other three isolates are HH from Al-Hanon honey, HC from Tualang honey, and HM from Al-Maray as *L. curvatus*. The results from 16S rDNA gene sequence identified HS as *L. plantarum*, HH as *L. curvatus*, HC as *Pediococcus acidilactici*, and HM as *P. pentosaceus*. Phenotypic and 16S rDNA molecular characterization of these isolates showed high degree of similarity of *Lactobacillus* and *Pediococcus* which belong to LAB. In this study, the sequencing of 16S rDNA gene of all isolates was confirmed by phylogenetic analysis. The phylogenetic tree was constructed by the neighbour-joining method as shown in Fig. 1, the isolate HS was the most closely related to the species *L. plantarum* 645320660 and isolate HH was the most closely related to the species *L. curvatus* 343201711 (Fig. 1) with similarities 99% and 96% in the 16S rDNA gene sequences, respectively. While isolate HC was the most closely related to the species *Pediococcus acidilactici* 343201331, supporting the 100% value from bootstrap analysis of the phylogenetic tree (Fig. 1) with 99% similarity in their 16S rDNA gene sequences. The isolate HM was closely related to the species *Pediococcus pentosaceus* 343201332 with 99% similarity in the 16S rDNA gene sequences.

Sensitivity of *Candida* spp. to antifungal agents. The sensitivity of *Candida* spp. to antifungal agents varied with species and antifungal agents evaluated with the diameter of inhibition zone varied between 0 and 22 mm. All the *Candida* spp. were sensitive to voriconazole (10 μg), but *C. tropicalis* ATCC750 and *C. parapsilosis* ATCC22019 were resistant to ketoconazole (10 μg) and *C. glabrata* ATCC2001 and *C. tropicalis* ATCC750 were resistant itraconazole

Table 2. Similarity index of LAB isolated from honey samples as determined by API 50CHL and 16S rDNA

Sources	Code	API CHL 50	Similarity (%)	16S rDNA	Similarity (%)
Al-Seder honey, Libya	HS	*Lactobacillus plantarum2*	99.4	*L. plantarum*	99.0
Al-Hanon honey, Libya	HH	*L. curvatus*	99.4	*L. curvatus*	96.0
Tualang honey, Malaysia	HC	*L. curvatus*	99.4	*Pediococcus acidilactici*	99.0
Al-Maray honey, Yemen	HM	*L. curvatus*	97.4	*Pediococcus pentosaceus*	99.0

LAB, lactic acid bacteria.

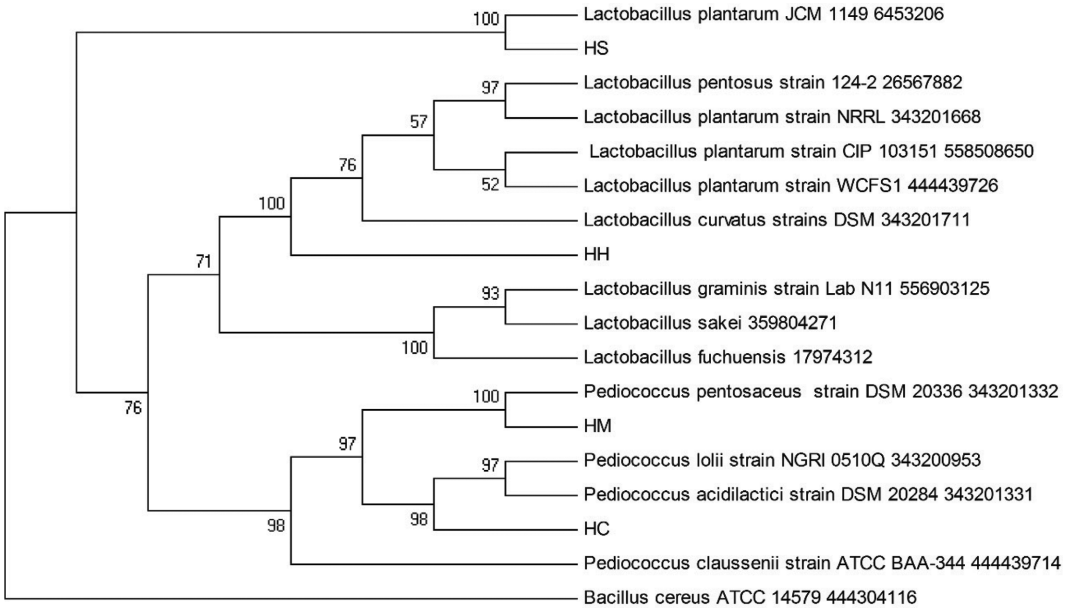


Fig. 1. Phylogenetic tree of partial 16S rDNA sequences of isolated strains and sequences of identified bacteria in the nucleotide database of GenBank. The bar indicates 1% sequence divergence.

Table 3. Susceptibility of *Candida* spp. to antifungal drugs measured by diameter of inhibition (mm) zone around the discs[a]

Antifungal agents	*Candida* spp.				
	C. albicans	*C. glabrata*	*C. tropicalis*	*C. parapsilosis*	*C. krusei*
Nystatin (100 U)	10	11	4	0	0
Amphotericine B (20 μg)	12	0	3	0	2
Fluconazole (100 μg)	13	6	5	3	6
Ketoconazole (10 μg)	4	5	0	0	3
Itraconazole (50 μg)	4	0	0	3	0
Voriconazole (10 μg)	13	16	18	20	22
MAR index %	33	33	66	83	66

MAR, multiple antifungal resistant.
[a]Diameter of inhibition zone around the discs (mm). Diameter of paper discs = 6 mm.

Table 4. Growth inhibition zone of *Candida* species by LAB isolated from honey by dual agar overlay method after 24 hr incubation at 30°C[a]

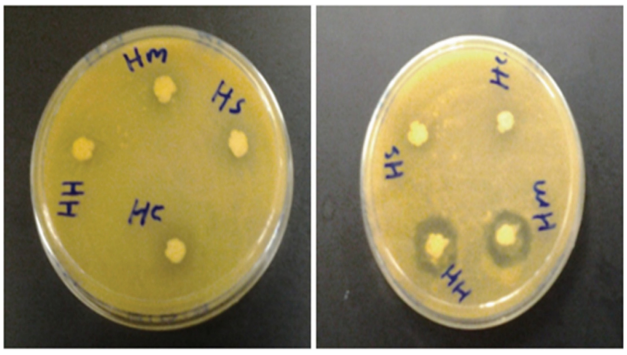
LAB isolates	Inhibitory activity				
	C. albicans	*C. glabrata*	*C. krusei*	*C. parapsilosis*	*C. tropicalis*
HS	++	+	+	+	++
HH	++	+++	++	+	++
HC	++	+	+	+	++
HM	++	++++	+	+	++

LAB, lactic acid bacteria; HS, *Lactobacillus plantarum*; HH, *L. curvatus*; HC, *Pediococcus acidilactici*; HM, *P. pentosaceus.*
[a]Inhibitory activity of selected lactic acid bacteria isolates against *Candida* spp. after 24-hr incubation at 30°C by dual agar overlay method. The inhibition was measured using the following scales: (–), no inhibition; (+), inhibition zone of less than 6 mm; (++), inhibition zone of 6~10 mm; (+++), inhibition zone of 10~15 mm; (++++), inhibition zone of more than 15 mm.

(50 μg). It was observed that *C. albicans* ATCC14053 was sensitive to nystatin (100 U), amphotericine B (20 μg), and fluconazole (100 μg) while *C. tropicalis* ATCC750, *C. parapsilosis* ATCC22019, and C. *krusei* ATCC6258 were resistant to nystatin, amphotericine B and fluconazole. It was also observed that *C. glabrata* was sensitive to nystatin but very resistant to amphotericine B and itraconazole. The MAR index was between 33 and 83. The highest MAR index percentage (83%) was noted for *C. parapsilosis* compared to 33% exhibited by both *C. albicans* and *C. glabrata*, and 66% showed by both *C. tropicalis* and *C. krusei* (Table 3).

Antifungal activity of LAB isolates against *Candida* spp. by the dual agar overlay method. Growth of all *Candida* spp. were inhibited by all four LAB isolated from different honey samples, by the dual agar overlay method, especially the growth of *C. glabrata* ATCC2001, where this yeast was strongly inhibited by *P. pentosaceus* and *L. curvatus* isolated from Al-Maray honey, Yemen (Sample HM) and Al-Hanon honey, Libya (Sample HH), respectively. While growth of *C. albicans* ATCC14053 and *C. tropicalis* ATCC750 were moderately inhibited by all LAB isolates with inhibitory zone (6~10 mm) (Table 4, Fig. 2).

Antifungal activity of LAB supernatant against *Candida* spp. by well diffusion method. Four LAB isolates (HS, HC, HH, and HM) were selected for further



Candida albicans *Candida glabrata*

Fig. 2. Growth inhibition of the isolates against *Candida* spp. by overlay method after 24 hr incubation at 30°C. HS, *Lactobacillus plantarum*; HH, *L. curvatus*; HC, *Pediococcus acidilactici*; HM, *P. pentosaceus*.

evaluation on the antifungal activity of CFS against five strains of pathogenic *Candida* spp. using the agar well diffusion method. It was observed that all the CFS of LAB had significantly ($p < 0.05$) inhibited the growth of the pathogenic *Candida* spp. with the inhibition diameter between 10.0 and 17.2 mm while the greatest inhibition of 22.0 mm was exceptionally exhibited by HH against *C. glabrata* ATCC2001 (Table 5). The CFS of *L. curvatus* in the sample HH had significantly ($p < 0.05$) inhibited the growth of *C. glabrata* ATCC2001, *C. parapsilosis* ATCC2201,

Table 5. Inhibition zone of *Candida* species by CFS using agar well diffusion method after 24-hr incubation at 37°C[a]

Candida species	LAB			
	HH	HM	HC	HS
C. albicans	13.5 ± 0.25f	13.3 ± 0.20f	11.4 ± 0.21h	15.3 ± 0.20d
C. krusei	12.4 ± 0.22g	17.2 ± 0.15b	12.0 ± 0.05g	13.1 ± 0.15f
C. glabrata	22.0 ± 0.05a	16.0 ± 0.10c	10.0 ± 0.05i	11.0 ± 0.15h
C. tropicalis	14.7 ± 0.10e	12.2 ± 0.17g	11.3 ± 0.10h	10.0 ± 0.05i
C. parapsilosis	15.6 ± 0.06c	10.0 ± 0.00i	12.4 ± 0.08g	10.2 ± 0.05i

The results are expressed as mean ± standard deviations of values obtained from triplicate experiments. Means with different letters were significantly different ($p < 0.05$).

CFS, cell free supernatant; LAB, lactic acid bacteria; HH, *Lactobacillus curvatus*; HM, *Pediococcus pentosaceus*; HC, *P. acidilactici*; HS, *L. plantarum*.

[a]Diameter of growth inhibitory zone was measured in millimeter after 24 hr, size the wells was 6 mm.

and *C. tropicalis* ATCC750 with inhibitory zones 22.0, 15.6, and 14.7 mm, respectively. In addition, CFS of *P. pentosaceus* in the sample HM was significantly ($p < 0.05$) effective against *C. krusei*, *C. glabrata*, and *C. albicans* evident by inhibition zones of 17.2, 16.0, and 13.3 mm, respectively. Subsequently, CFS of *L. plantarum* in the sample HS had significantly ($p < 0.05$) inhibited the growth of *C. albicans* and *C. krusei* with inhibition zone 15.3 and 13.1 mm, respectively.

DISCUSSION

The presence of LAB in honey was reported by several researchers [30-33]. Aween *et al.* [34] isolated LAB from honey and the isolates were identified as strains of *L. acidophilus* that have antibacterial activities against gram-positive and gram-negative bacteria. In this study, LAB was detected in 10 from the 15 honey samples with variable antifungal activity against *Candida* spp. Four of the LAB that displayed good antifungal activities against *Candida* spp. were identified as *L. plantarum* HS, *P. acidilactici* HC, *L. curvatus* HH, and *P. pentosaceus* HM. Atanassova *et al.* [35], reported that *L. paracasei* subsp. paracasei M3 had antifungal activity against *C. albicans*, *C. pseudointermedia*, and *C. blankii*. Similarly, Jin *et al.* [36] also observed that strains of *Pendiococcus* sp. had strong antifungal activity against *C. albicans* ATCC10231 and *C. parapsilosis* ATCC22019 but moderate activity against *C. tropicalis* ATCC13803 and *C. kefir* ATCC46764. Ogunshe *et al.* [37] reported that *L. acidophilus* and *L. plantarum* from vaginal had antifungal activity against strains of pathogenic *Candida* spp. Cizeikiene *et al.* [38] also found that *Pediococcus acidilactici* KTU05-7, *Pediococcus pentosaceus* KTU05-8, KTU05-9 and KTU05-10 isolated from food had inhibitory activity against *C. parapsilosis* including *Fusarium culmorum*, *Penicillium chrysogenum*, *Aspergillus fumigatus*, *Aspergillus versicolor*, *Penicillium expansum*, *Aspergillus niger*, and *Debaryomyces hansenii*. In this study the *L. plantarum* isolated from Seder honey, Libya showed antifungal activity against *Candida* spp. especially *C. albicans*. Similarly, Adeniyi and Iveren [39] reported that the CFS produced by *L. plantarum* isolated from fresh salad vegetables had higher antifungal activity against *C. albicans* ATCC90029 with inhibition zone (25 mm). Oluwafemi and Adetunji [40] reported that *L. plantarum* isolated from Oqi had inhibitory activity against *C. albicans*. Similarly, the antifungal activity of *L. plantarum* were generally reported by several researchers against different fungi. Laref and Guessas [41] reported that five strains of *L. plantarum* isolated from silage, camel milk, and carrot had antifungal activity against *Aspergillus* spp., *Fusarium roseum*, *Trichoderma* spp., *Penicilium* spp., and *Stemphylium* spp. In this study, the highest zone of inhibition (> 15 mm and 10~15 mm) was recorded by *P. pentosaceus* HM and *L. curvatus* HH, respectively against *C. glabrata* ATCC2001. The strong antifungal activity by *P. pentosaceus* from foods was also supported by Muhialdin *et al.* [42] where they observed that *L. pentosus* G004, *L. fermentum* Te007, and *P. pentosaceus* Te010 isolated from Malaysian fermented foods and fruits had strong antifungal activity against *Aspergillus oryzae*. Their study also observed that LAB isolated from honey samples had good antifungal activity against *Candida* spp. as evaluated by the dual agar overlay method. In addition, it was also established that CFS of LAB show good inhibitory activity when evaluated by the well diffusion method. In this study, the highest antifungal activity was obtained with CFS of *L. curvatus* HH that showed significant ($p < 0.05$) antifungal activity against *C. glabrata* ATCC2001 with inhibition zone of 22.0 mm.

Candida spp. are not easily killed by normal antifungal agents used for health therapy. *Candida* spp. used in this study were resistant to several antifungal agents such as amphotericin B, fluconazole, and itraconazole except for voriconazole and fluconazole. Voriconazole is highly active against all *Candida* spp. while fluconazole is moderately effective against *C. albicans*. This is in agreement with Al-Abeid *et al.* [43] who reported that non-albicans spp. showed higher resistance rates against fluconazole than *C. albicans*. Our findings depicted that both the LAB cells and their CFS isolated from honey samples could inhibit the growth of *Candida* spp. and similar results were obtained by Lertcanawanichakul [44] who reported that the supernatant produced by *Lactococus lactis* showed inhibitory activity against *C. albicans* DMST 5239. The

results from this study are in agreement with previous studies of Verdenelli *et al.* [45] who reported that *L. rhamnosus* and *L. paracasei* isolated from human stool had antifungal activity against *C. albicans* ATCC 10291, while Kariptaş *et al.* [46], observed that *Lactobacillus* isolated from human stool had antifungal activity against *C. albicans* (M29, M36), *C. parapsilosis* (M25, M26), *C. famata* (M28), and *C. guilliermondii* (M38) isolated from blood cultures. Recently, Chew *et al.* [47] reported that the CFS produced by the probiotic *L. rhamnosus* GR-1 and *L. reuteri* RC-14 have high antagonistic activities against five strains of *C. glabrata*. This is consistent with Rönnqvist *et al.* [48] who also reported that CFS produced by *L. fermentum* Ess-1 isolated from human had strong antifungal activity against *C. albicans* and *C. glabrata*. Sungsri *et al.* [49] also report on *L. paracasei* inhibits the growth of *C. albicans* BCC6120 by using dual agar overly method. The antimicrobial properties of CFS is contributed by the metabolites produced by LAB as reported by Olofsson *et al.* [50]. LAB produced organic acids, hydrogen peroxide, diacetyl, and bacteriocins are among others that have both antibacterial and antifungal activity. The current study shows that bacteria cells and their CFS isolated from different geographic location of natural honey samples have antifungal activity against *Candida* spp. It is no definite explanation on the mechanism of antifungal action of the CFS against *Candida* spp. due to the complex interactions between different metabolite compounds present in the CFS. Certain strains of LAB are able to produce natural antifungal compounds that can inhibit the growth of *Candida* spp. This may suggest that accumulation of soluble compounds in the CFS from LAB is responsible for inhibiting the growth of *Candida* spp.

The results obtained in this study indicated that LAB isolated from honey produced bioactive compounds that can be used to inhibit growth of the pathogenic *Candida* spp. namely, *C. albicans* ATCC14053, *C. glabrata* ATCC2001, *C. tropicalis* ATCC750, *C. parapsilosis* ATCC22019, and *C. krusei* ATCC6258, that often cause many human infections. The CFS of these LAB isolates showed greater inhibitory activity than the cells against *Candida* spp.

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