CHAPTER III

ISOLATION AND IDENTIFICATION OF LACTIC ACID BACTERIA FROM HONEY WITH ANTIFUNGAL ACTIVITY AGAINST *CANDIDA* SPECIES

3.1 Introduction

Honey is nectar collected by bees from a wide variety of plants which is concentrated by evaporation of water to form supersaturated solution. Honey is mildly acidic, with a pH between 3.2 and 4.5, the low pH alone is inhibitory to many pathogenic bacteria and fungi (Molan, 1995; Hamouda & Dalia, 2011). Honey has antimicrobial activity against several human pathogens, including *E. coli*, *E. aerogenes*, *S. typhimurium*, *S. aureus* and strains of *Candida* spp. (Irish et al., 2006; Ahmed et al., 2012; Aween et al., 2012b; Zainol et al., 2013). The antifungal and antibacterial potency of honey has been attributed to its high sugar content, strong osmotic effect and naturally low pH can also prevent the microbes from growth (Molan, 1995; Viuda-Martos et al., 2008; Kwakman and Zaat, 2012). Honey has been used for both nutritional and medical purposes, it is one of the oldest traditional medicines considered to be important in the treatment of various diseases including gastrointestinal infection, respiratory ailment, wound infections and other diseases and it has been used effectively as a dressing for wound including surgical wounds, burns and skin ulcers to reduce pain and odor quickly (Mulu et al., 2004; Molan, 2006; Robson et al., 2009; Mandal & Mandal, 2011).

Lactic acid bacteria (LAB) are one of non-pathogenic bacteria that play important role in our everyday life from fermentation, preservation and production of wholesome foods, and vitamins to prevention of certain diseases and cancer due to their antimicrobial activity (Adeniyi & Damsa, 2013).

LAB isolated from different sources are known to produce different antimicrobial compounds that have the ability to inhibit the growth of fungi. Most of the antimicrobial activity was due to organic acid and bacteriocins produced by LAB strains (Fayol-Messaoudi et al., 2005; Valerio et al., 2008; Dalié et al., 2010; Muhialdin & Hassan, 2011). The inhibitory activity of LAB against various *Candida* spp. is in agreement with previous studies. Mohamed et al. (2010) reported that supernatant produced by *L. acidophilus* isolated from yoghurt had antifungal activity against *C. albicans* with zone inhibition 26 mm. Adeniyi and Damsa (2013) reported that the cell free supernatant (CFS) produced by *L. plantarum* showed higher antifungal activity against *C. albicans* ATCC90029. Recently, Chew et al. (2015) 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*.

LABs are well known for their potential ability to prevent diseases in humans (Ljungh & Wadström, 2009). *Candida* species are found everywhere and represent the most common fungal species that harmful to humans (Dixon et al., 2004; Eggimann et al., 2005; Ogunshe et al., 2009; Lingappan et al., 2012). Usually the immune system keeps yeast under control but during sickness or use of antibiotics the fungal can multiply and cause infections such as diaper rash, thrush and vaginitis (Balch & Balch, 2009). The fungus is a single-celled organism present in the genital and intestinal tracts. Candidiasis is a mycotic human infection caused by *Candida* spp. it is one the most common sexually transmissible diseases (Ogunshe et al., 2009).

In the human body, pH can vary from highly acidic in the stomach to mildly acidic in the skin and vagina to neutral in blood stream and alkaline in some part of the gut (Fong et al., 2007). *Candida* species can thrive in most of these sites and tolerant to wide range of environment pH conditions ranged from pH < 2 to > 10. pH adaptation, carbon

metabolism, and interactions with host cells, all of which are critical for the ability of *Candida* spp. to cause disease (Vylkova et al., 2011).

Pathogenic fungi cause many symptoms such as constipation, diarrhea, colitis, abdominal pain, itching, sore throat, burning tongue, white spots in the tongue and mouth (Balch & Balch, 2000). Pathogenic fungi represent a serious threat to the lives and health of most the patients. Several *Candida* spp. have become resistance to many antifungal drugs such as amphotericin B, fluconazole, and itraconazole, and there is a need for new antifungal agents (Mishra et al., 2007; Ozcelik et al., 2007). Therefore, the aim of this study was to screen the antifungal activity of LABs isolated from different sources of honey against strains of *Candida* spp. namely, *C. albicans* ATCC 1405, *C. glabrata* ATCC2001, *C. tropicalis* ATCC750, *C. parapsilosis* ATCC22019 and *C. krusei* ATCC6258. Both cells and cell free supernatant (CFS) were used to determine the antifungal activity. LAB which shown good antifungal activity against strains of *Candida* spp. were identify using API CHL50 and 16S rDNA.

3.2 Materials and Methods

3.2.1 Samples of honey

Fifteen honey samples were collected from different sources and were kept at room temperature 28±2 °C before analysis. The samples used in this study were local honey from Malaysia (Madu Tualang, Madu Tani, pure Trigona honey), from Libya (Al-Seder honey, Hanon honey and Zater honey), from Yemen (Al-seder honey and Al-Maray honey), from Saudi Arabia (Al-Shifaa honey) and from NewZealand (Manuka honey). The pH of honey was determined using pH meter (Mettler Toledo).

3.2.2 Isolation of lactic acid bacteria from honey samples

LAB was isolated from honey samples following the method described by Aween et al. (2012b). Approximately 10 g of honey samples were suspended in 90 ml peptone water (0.1% w/v) in stomacher bags and the bags were manually agitated. Then 1 ml was added to 9 ml of MRS broth (Oxoid CM359) incubated at 30 °C for 24 to 48 h until the culture broth became turbid followed by serial dilution with peptone water (0.1% w/v). A 0.1 ml of appropriate dilution was spread plated on several modified media namely, MRS agar (Oxoid) (De Man et al., 1960), MRS agar with 0.8% $CaCO_3$ (Panthavee et al., 2007), MRS agar with 1% glucose, tomato juice agar (Oxoid) with 0.8% $CaCO_3$ and tomato juice agar with 1% glucose. All plates were incubated under anaerobic condition in anaerobic jar at 37 °C for 48 h or until the bacterial colonies were of sufficient size. Colonies were tested for catalase activity with 4% H_2O_2 and catalase negative colonies were streaked on MRS agar containing 0.8% $CaCO_3$ incubated at 37 °C for 48 h to obtain pure colonies. All catalase negative isolates were Gram-stained. All catalase-negative and Gram-positive LAB isolates were maintained in MRS broth with 15% of glycerol and kept at -20 °C for further study.

3.2.3 Preparation of fungi species

The *Candida* species used were obtained from original stock of the microbial collections at the Department of Medical Microbiology, University Putra Malaysia. The *Candida* spp. used in this study were *C. albcans* ATCC14053, *C. parapsilosis* ATCC 22019, *C. tropicalis* ATCC750, *C. krusei* ATCC6258 and *C. glabrata* ATCC2001. All *Candida* spp. were cultured on Sabouraud Dextrose Agar (SDA, Oxoid) incubated at 35 °C for

24 h and 48 h to ensure viability and purity. Fungal strains were maintained on SDA and stored at 4 °C.

3.2.4 Sensitivity of *Candida* spp. to antifungal agents

The *Candida* spp. were tested for their sensitivity to antifungal agents using disc diffusion method as described by Bauer et al. (1966). The antifungal agents used were nystatin (100U), amphotericin B (20µg), fluconazole (100µg), ketoconazole (10µg), itraconazole (50µg), voriconazole and econazole (10µg). All antifungal discs were obtained from SIGMA-Aldrichemie GmbH-Steinheim, Germany. The selection of antifungal agents used in this study was based on the common antifungal agents used in medical practice and health therapy. Multiple antifungal resistant (MAR) index for *Candida* spp. was determined as the number of antifungal agents to which an isolate is resistant/ total number of antifungal tested as described (Subramani & Vignesh, 2012). The 24 h pure cultures of *Candida* spp. cultured in Sabouraud Dextrose Broth (SDB, Oxoid CM147) and were incubated at 37 °C for 24 h. Then, the pathogenic *Candida* cultures were swabbed on SDA plates. The plates followed by drying the agar surface in the laminar flow cabinet at ambient temperature for 15 min. Using a sterilized forceps aseptically the paper discs (6 mm in diameter) of antifungal agents were placed on plates and were incubated at 37 °C for 24 h, aerobically. The diameter of inhibitory zone around each disc was manually measured by ruler and recorded. The experiment was done in replicate.

3.2.5 Initial screening for antifungal activity against *Candida* species

Selected LAB isolates were screened for antifungal activity against *Candida* spp. using dual overlay method as described by Magnusson and Schnürer (2001). Initially, LAB was spot inoculated on MRS agar plates. The plates were incubated 30 °C for 24 h under anaerobic conditions. These plates were overlaid with a layer of 15 ml of SDA (0.75% soft SD agar) containing 10^4 CFU/ml of overnight culture of *Candida* was poured over the plates. The plates were aerobically incubated at 30 °C for 24 h, and zone of growth inhibition of *Candida* by LAB isolate was measured, and this test was done in replicate.

3.2.6 Preparation of supernatant

The LAB isolates were grown in MRS broth at 30 °C for 24 h. The cell free supernatant (CFS) was prepared by centrifuging the broth at 11500 rpm at 4°C for 10 min. (Mini Spin, Eppendorf, AG 22331, Hamburg). Then, the supernatant of each isolate was filtrated using sterile filtered (0.45 µm-pore-size filter, Millipore) (Ogunbanwo, 2005) and used for analysis.

3.2.7 Antifungal activity of LAB supernatants using agar well diffusion method

The LAB isolates that showed strong antifungal activity using dual overlay spot method was further evaluated using well method as described by Magnusson and Schnürer (2001). A 24 h culture of *Candida* spp. (10^4 cell/ml) was mixed with SDA, and poured into the plates. After the agar solidified, wells were made using cork borer of size 6 mm, then cover the base of the well with agar to avoid leaking. Varying amounts of CFS were added to each well and the plates were incubated at 30 °C for 24 h. Growth

inhibition zone around the wells was measured after diminution the well size. The experiment was done in replicate and the means with standard deviations were calculated.

3.2.8 Phenotypic identification of LAB isolates by API 50 CHL Kit

Four LABs that showed strong antifungal activity were further phenotypically identified using API 50 CHL Kit assay following the method described by the manufacturer. Overnight cultures of the LAB isolates grown on MRS plates (Oxoid) at 37 ºC for 24 h anaerobically. The pure colonies were suspended in API 50 CHL medium (API system, Biomerieux, France). The suspension was transferred into each of the 50 wells of the API 50 CHL strips. All wells were overlaid with sterile mineral oil to make it anaerobic. The strips were incubated at 37 ºC and then monitored for change in colour after 24 and 48 h. The change in colour was represented by positive sign while no change in colour was represented negative sign. The results were analysed with API WEB (Bio-Merieux).

3.2.9 Genotypic identification of LAB using 16S rDNA

Chromosomal DNA of the four strains of LAB was isolated using the Wizard® Genomic Gram-positive DNA purification kit (USA). PCR amplification using primers were expected to amplify at approximately 1400 bp of 16S rRNA gene and sequence of the amplified DNA was done by 1st Base, Malaysia.

3.2.9.1 DNA extraction of LAB Isolates

Bacterial DNA was extracted from overnight culture grown in 20 ml of MRS broth incubated at 30 °C, using Master Pure™ Gram positive DNA purification kit (USA).

One ml of overnight culture was centrifuged at 13,000 rpm at 25 °C for 2 min (Eppendorf centrifuge 5804 R). The supernatant was discarded and the pellet was collected. Next, the pellet was suspended in 480 µl (50mM EDTA) then 120 µl of Lysozyme were added to each suspended pellet. The suspensions were incubated at 37 °C for 30 min, then centrifuged at 13,000 rpm for 2 min and the supernatant was removed. A 600 µl of Nuclei Lysis solution were added after that incubated at 80 °C for 5 min, then kept at room temperature 28±2 °C. after that 1 µl of RNase solution were added to each sample and mixed thoroughly, then incubated at 37 °C for 30 min, followed by addition of 200 µl of protein precipitation solution finally, vortex to suspend the cell pellet then incubated in ice for 5 min, and centrifuged at 13,000 rpm for 3 min (Eppendorf centrifuge 5804 R). The supernatants were transferred to new tubes containing 600 µl of isopropanol then centrifuged at 4°C for 10 min at 13,000 rpm and decant the supernatant. Pipet was used to remove the isopropanol without dislodging the DNA pellet. Then, the pellets were rinsed with 600 µl of 70% ethanol, centrifuged at 13,000 rpm for 2 min (Eppendorf centrifuge 5804 R), and the ethanol was aspirated and the pellets were air-dried. Then 100 µl of Rehydration solution were added and incubated for one hour at 65 °C. The DNA of LAB isolates were kept at -20 for further study.

3.2.9.2 Genotypic identification of LAB isolates

Purified DNA of each sample was processed to the PCR using Fail SafeTM Pre Mix Kit Epicentre® (an Illumina® company).The oligonucleotide primer used were 16S forward: (5-AGAGTTTGATCCTGGCTC-3) and 16S reverse: (5-CGGGAACGTATTCAC-CG-3) (Magnusson et al., 2003). Primer was synthesized at 1st Base, Malaysia. A standard PCR reaction mixture containing 10 ng *L. plantarum*

genomic DNA instead of sample was used as positive control and the negative control contained water instead of sample. The settings of PCR were as follows: initial at 95 °C for 2 min, 92 °C for 45 s, 54 °C for 1 min and 72 °C for 1 min, 35 cycles for each steps. A 5 ul of each amplification mixture was subjected to electrophoresis in 1.5% agarose gel (1.5 g agarose powder with 100 ml in 1 x TEA buffer) for 45 min and 90 volts. DNA molecular mass marker (250 to 10000 bp) molecular ladders from 1st Base, Malaysia was used as standard. After electrophoresis the gels were stained in ethidium bromide and after washing the gels were visualized and photographed with UV transilluminator (BIORAD). The partial 16S rDNA sequences (approximately 1400 bp) were determined by 1st Base, Malaysia and sequences were compared with databases (Gen- Bank).

3.2.10 Statistical analysis

Data for growth inhibition zone of *Candida* spp. were presented as mean ± standard deviation and were analysed by two-way analysis variance (ANOVA) using general liner model (GLM) procedure of SAS, and Tukey's test was applied for significant means at $P< 0.05$ to evaluate the significant differences between groups.

3.3 Results

3.3.1 Characterization of LAB isolated from honey samples

Among the media evaluated, media with added 0.8% $CaCO_3$ showed good growth of LAB at dilution 10^4 to 10^5. Al-Hanon honey and Tualang honey seem to contain high LAB counts compared to other honey samples (Table 5). The pH of honey samples varies from 3.5 to 5.7. Tualang honey showing lowest pH value of 3.5 and Al-Sedar

honey, Libya showed pH 5.7. A total of twenty-five isolates that showed clear zones on MRS agar with 0.8% $CaCO_3$ (Figure 1) and catalase negative were Gram stained and results showed that the LAB isolates were 52% were rod-shaped and 48% were coccus-shaped (Table 6).

TABLE 5: Effect of media on the isolation of LAB from honey samples

Sample code	Source	pH of sample	Media	Dilution LAB detected
HS	Al-Seder honey, Libya	5.7	MRS+$CaCO_3$	10^4
			MRS+Glucose	10^3
HH	Al-Hanon honey, Libya	4.4	MRS+$CaCO_3$	10^2
			MRS+Glucose	10^1
HM	Al-Maray honey, Yemen	4.1	TJA+$CaCO_3$	10^1
			MRS+$CaCO_3$	10^2
HC	Tualang honey, Malaysia	3.5	MRS+$CaCO_3$	10^5

Notes: MRS = de Man, Rogosa and Sharpe. TJA = Tomato Juice Agar.

FIGURE 1: Growth of LAB on MRS agar with 0.8% $CaCO_3$ showing clear zones around the colonies

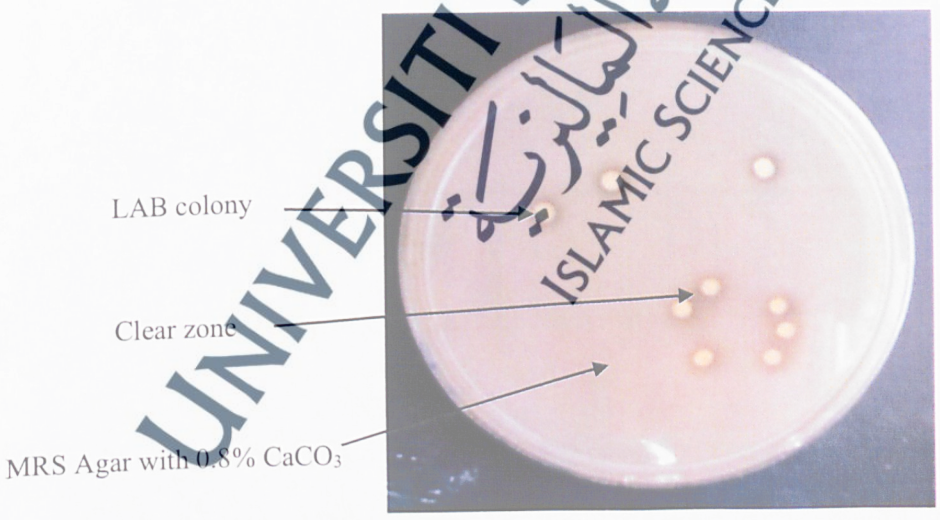


TABLE 6: Characteristics of lactic acid bacteria isolated from honey samples

Samples codes	Catalase test	Gram stain	Clear zone on modified MRS	Shape
H012	_	+	+	Rod, chain
HA1	_	+	+	Rod, single
HA2	_	+	+	Cocci
HA4	_	+	+	Rod, chain
HH4	_	+	+	Rod, single
HH9	_	+	+	Rod, single
HM	_	+	+	Short rod
HM8	_	+	+	Short rod
HM10	_	+	+	Short rod
HN4	_	+	+	Short rod
HN5	_	+	+	Short rod
HN6	_	+	+	Cocci
HP06	_	+	+	Rod, chain
HP08	_	+	+	Cocci
HS	_	+	+	Short rod
HT2	_	+	+	Rod, single
HT6	_	+	+	Cocci
HUS4	_	+	+	Cocci
HUS7	_	+	+	Cocci
HUS9	_	+	+	Cocci
HZ1	_	+	+	Cocci
HZ3	_	+	+	Cocci
HZ7	_	+	+	Cocci

Notes: Growth (+); No growth (-)

3.3.2 Sensitivity of *Candida* spp. to Antifungal Agents

The sensitivity of *Candida* spp. to antifungal agents varied with species and antifungal agents evaluated, the diameter of inhibition zone varies between 0 and 22 mm. All the *Candida* spp. was sensitive to voriconazole (10 µg), but resistant to ketoconazole (10µg)

and itraconazole (50 µg). It was observed that *C. albicans* ATCC 14053 was sensitive to nystatin (100U), amphotericin B (20µg) and fluconazole (100µg) while *C. tropicalis* ATCC 750, *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were resistant to nystatin, amphotericin B and fluconazole. It was also observed that *C. glabrata* ATCC 2001 was sensitive to nystatin but very resistant to amphotericin B, fluconazole and ketoconazole. The MAR index was from 33% to 83%. The highest MAR index percentage (83%) was noted for *C. parapsilosis* ATCC22019 compared to 33% showed by *C. albicans*ATCC14053 and *C. glabrata* (33%) and, *C. tropicalis*ATCC750 and *C. krusei* ATCC6258 (66%) (Table 7).

TABLE 7: Susceptibility of *Candida* spp. to antifungal drugs measured by diameter of inhibition (mm) zone around the discs*

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

* Diameter of inhibition zone around the discs (mm). Diameter of paper discs = 6 mm

3.3.3 Sensitivity of *Candida* spp. to LAB isolate

Screening for antifungal activity of all twenty-five LAB isolates were determined against five *Candida* spp. observed that the antifungal activity of LAB was effected by the *Candida* spp. Two LAB isolates HM and HH showed good inhibitory activity (> 15.0 mm) against *C. glabrata* with inhibition zone >15 and 10-15 mm, respectively, while four isolates (HS, HH, HC and HM) showed good activity (6-10 mm) against *C. albicans* and *C. tropicalis* (Figure 2 and Table 8). However, the antifungal activity of

LAB against *C. albicans, C. glabrata, C. krusei, C. parapsilosis* and *C. tropicalis* was reduced to a lesser extent (diameter of inhibition zone < 6mm) by other LAB isolates as shown in Table 8. Similarly, LAB (HM10, USH7, HN5 and HH4) inhibited *C. albicans* and *C. glabrata*. On the other hand, three isolates (HT2, HM8, and H012) failed to inhibit the growth of all *Candida* spp.

FIGURE 2: Plates showing growth inhibition of *Candida* spp. by overlay method incubated at 30°C for 24 h

TABLE 8: Antifungal activity of LAB isolates against *Candida* species after 24 h incubation at 30 °C determined by dual agar overly method*

	Inhibitory activity				
LAB isolates	*C. albicans*	*C. glabrata*	*C. krusei*	*C. parapsilosis*	*C. tropicalis*
HS	++	+	+	+	++
HH	++	+++	++	+	++
HC	++	+	+	+	++
HM	++	++++	+	+	++
HA1	-	-	-	+	-
HZ1	+	-	-	-	+
HZ3	+	-	-	+	-
HZ7	-	-	-	+	-
HH4	+	+	-	+	+
HH9	-	-	+	+	+
HT2	-	-	-	-	-
HT6	-	-	-	+	+
HUS4	-	-	-	+	+
HUS7	+	+	-	-	-
HUS9	+	-	+	-	-
HM8	-	-	-	-	-
HM10	+	+	-	-	-
H012	-	-	-	-	-
HP06	-	-	+	+	+
HP08	-	-	-	+	+
HN4	-	-	-	-	+
HN5	+	+	-	-	+
HN6	-	-	-	+	-
HA2	-	-	-	+	+
HA4	-	-	+	+	+

*Inhibitory activity of selected lactic acid bacteria isolates against *Candida* spp. after 24h 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.

3.3.4 Antifungal activity of LAB supernatant against *Candida* spp. by well diffusion method

Four LAB isolates namely, HS, HC, HH and HM were selected to further evaluate the antifungal activity of the CFS against five strains of pathogenic *Candida* spp. using the agar well diffusion method. It was observed that all the CFS inhibited the growth of the pathogenic *Candida* spp. with the inhibition diameter between 10.0 mm to 17.2 mm and, exceptionally greatest inhibition of 22.0 mm was shown by HH against *C. glabrata* ATCC2001 (Table 9). The inhibitory activity of CFS HH was significantly higher ($P< 0.05$) against *C. glabrata* ATCC2001 then inhibitory activity against *C. parapsilosis* ATCC2201 and *C. tropicalis* ATCC750 with growth inhibitory zone 22.0, 15.6 and 14.7 mm, respectively. While, CFS of HM was significantly different ($P< 0.05$) against *C. krusei*, *C. glabrata* and *C. albicans* with inhibition zone 17.2, 16.0 and 13.3 mm, respectively. Furthermore, CFS of HS showed significantly less inhibition ($P< 0.05$) against *C. albicans* and *C. krusei* with inhibition zone 15.3 and 13.1 mm, respectively. The CFS of HH was significantly higher ($P< 0.05$) against *C. glabrata* compared to the other *Candida* spp.

TABLE 9: Growth inhibition zone of *Candida* species by CFS using agar well diffusion method after 24 h incubation at 37°C*

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

*Diameter of growth inhibitory zone was measured in (mm) after 24 h, size the wells was 6 mm.
The results are expressed as mean± standard deviations of values obtained from triplicate experiments [a-i] Mean±SD. Means with different superscripts are differ significantly ($P < 0.05$).

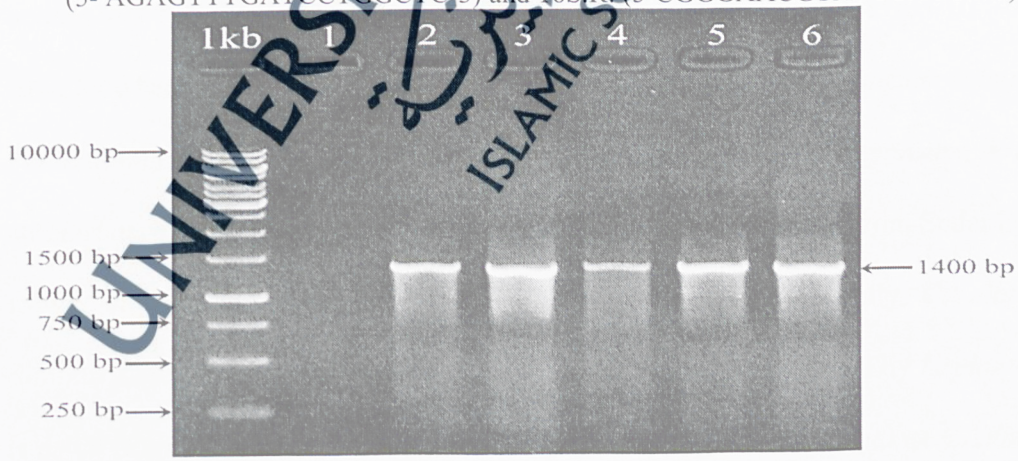
3.3.5 Identification of LAB using API 50 CHL assay and 16S rDNA

The identification of four LAB isolated from honey samples that showed antifungal activity against five strains of pathogenic *Candida* spp. is presented in Table 10. The results from API 50CHL kit identified the isolate HS from Al-Sedar honey as *Lactobacillus plantarum*2, and other three isolates HH from Al-Hanon honey, HC from Tualang honey and HM from Al-Maray as *Lactobacillus curvatus*. However, the results from 16S rDNA sequence were slightly different (Figure 3): HS was identified as *L. plantarum*, HH as *L. curvatus*, HC as *Pediococcus acidilactici* and HM as *P. pentosaceus* (Appendix A, B, C and D).

TABLE: 10 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-Sedar honey, Libya	HS	*L. plantarum*2	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%	*P. acidilactici*	99.0%
Al-Maray honey, Yemen	HM	*L. curvatus*	97.4%	*P. pentosaceus*	99.0%

FIGURE 3: DNA bands of LABs on the 1.5 % agarose gel using primers 16S.S: (5- AGAGTTTGATCCTGGCTC-3) and 16S.R: (5-CGGGAACGTATTCACCG-3).



DNA ladder 1: Negative control (no DNA), 2: HM, 3: HS, 4: HC, 5: HH and 6: Positive control (*L. plantarum* JCM 1149).

3.4 Discussion

The presence of LAB in honey was reported by several researchers (Ruiz-Argueso & Rodriguez-Navarro, 1975; Bahiru et al., 2006; Hosny et al., 2009; Forsgren et al., 2010). Aween et al. (2012a) isolated LAB from honey and identified as strains *L. acidophilus* and demonstrated that they have antibacterial activities against Gram-positive and Gram-negative bacteria. In this study LAB was detected in 10 of 15 honey samples with variable antifungal activity against *Candida* spp. Four of the LAB that showed good antifungal activity against *Candida* spp. were identified as *L. plantarum* HS, *P. acidilactici* HC, *L. curvatus* HH and *P. pentosaceus* HM. Atanassova et al. (2003) reported that *L. paracasei* subsp. *paracasei* M3 had antifungal activity against *C. albicans*, *C. pseudointermedia* and *C. blankii*. Similarly, Jin et al. (2007) also observed that strains of *Pendiococcus* spp. had strong antifungal activity against *C. albicans* ATCC10231 and *C. parapsilosis* ATCC22019 but moderate activity against *C. tropicalis* ATCC 13803 and *C. kefir* ATCC46764. Ogunshe et al. (2011) reported that *L. acidophilus* and *L. plantarum* from vaginal isolates had antifungal activity against strains of pathogenic *Candida* spp. Cizeikiene et al. (2013) also found that *P. acidilactici* KTU05-7, *P. pentosaceusjo* KTU05-8, KTU05-9 and KTU05-10 isolated from food had inhibitory activity against *C. parapsilosis* as well as *Fusarium culmorum*, *Penicillium chrysogenum*, *Aspergillus fumigatus*, *A. versicolor*, *P. expansum*, *A. niger* and *Debaryomyces hansenii*. In this study *L. plantarum* HS isolated from Sedar honey, Libya showed antifungal activity against *Candida* spp. especially *C. albicans*. Similarly, Adeniyi and Damsa (2013) reported that the CFS produced by *L. plantarum* isolated from fresh salad vegetables had higher antifungal activity against *C. albicans* ATCC 90029 with inhibition zone 25 mm.

Oluwafemi and Adetunji (2011) reported that *L. plantarum* isolated from Oqi showed inhibitory activity against *C. albicans*. In contrast *L. plantarum* was generally reported by several researchers effective against different fungi. Laref and Guessas (2013) reported that five strains of *L. plantarum* isolated from silage, camel milk and carrot had antifungal activity against *Aspergillus* spp., *F. roseum, Trichoderma* spp., *Penicilium* spp. and *Stemphylium* spp. Muhialdin and Hassan (2011) observed that *L. pentosus* G004, *L. fermentum* Te007 and *P. pentosaceus* Te010 isolated from Malaysian fermented foods and fruits had strong antifungal activity against *A. oryzae*.
This study observed that LAB isolated from honey samples had good antifungal activity against *Candida* spp. as evaluated by the dual agar overlay method. 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. It was also observed that CFS of LAB showed good inhibitory activity against *Candida* spp. when evaluated by the well diffusion method. The highest antifungal activity was obtained with CFS of *L. curvatus* HH that showed significantly higher ($P< 0.05$) antifungal activity against *C. glabrata* ATCC2001 with inhibition zone 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 voriconazole and fluconazole. Voriconazole was highly active against all *Candida* spp. while fluconazole was moderately effective against *C. albicans*. This is in agreement with Al-Abeid et al. (2004) who reported that non-albicans spp. showed higher resistance rates against fluconazole than *C. albicans*.

Our findings observed that both the LAB cells and their CFS isolated from honey samples could inhibit the growth of *Candida* spp. similar results were obtained by

Monthon (2005) 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. (2009) who reported that *L. rhamnosus* and *L. paracasei* isolated from human stool had antifungal activity against *C. albicans* ATCC10291, while Kariptas et al. (2010) 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. (2015) 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. (2007) 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. (2012) also report on *L. paracasei* inhibits the growth of *C. albicans* BCC6120 using dual agar overly method.

LAB produced organic acids, hydrogen peroxide, diacetyl and bacteriocins among others that have both antibacterial and antifungal activity. The antifungal activity against *Asperogillus fumigatus* of *L. casei*, *L. lactis* subsp. *lactis* and *L. pentosus* was from the peptides (Kim, 2005). The current study showed that bacteria cells and their CFS have antifungal activity against *Candida* spp. It is difficult to explain the mechanism of antifungal action of the CFS against *Candida* spp. due to the complex interactions between different compounds present. This may suggest that accumulation of soluble compounds in the CFS of LAB is responsible for the growth inhibitory activity of *Candida* spp.

3.5 Conclusion

The results obtained in this study indicated that LAB isolated from honey have antifungal activity that can be used to inhibit the 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.