CHAPTER VI

CHARACTERIZATION OF PREDOMINANT LACTIC ACID BACTERIA ISOLATED FROM COMMERCIAL HONEY IN MALAYSIA

6.1 Introduction

Lactic acid bacteria (LAB) can be isolated from most fermented foods such as yogurt, fermented meat and sausage, cheese and fermented vegetables. Several LAB strains are characterized by their ability to ferment lactose and improve the digestibility of fermented dairy products (Weinberg *et al.*, 2007) as well as their preservation (Abdelbasset & Djamila, 2008). LABs are also used to improve the taste, texture and viscosity of fermented foods (Soukoulis *et al.*, 2007). LAB has also isolated from honey as reported by several researchers as mentioned earlier chapters.

Identification of LAB may be carried out by antibiotic resistant, phenotypic analyses and molecular methods such as 16S rRNA gene and RAPD analysis (Hertel *et al.*, 1993; Pot *et al.*, 1993; Vandamme *et al.*, 1996; Temmerman *et al.*, 2002) for unknown bacteria.

The molecular identification is based on the similarity with other sequences within the data base. Although it is very useful and simple method for the identification of genus and species of bacteria, it does not allow differentiation of subspecies (Du Plessis *et al.*, 2004; Moreno-Arribas & Polo, 2008). The 16S rRNA gene sequence has been widely used as a molecular method to identify the bacteria especially LAB bacteria. Lim *et al.*, (2009) reported that *Lactobacillus brevis*, *Enterococcus faecium* and *Pediococcus acidilactici* were isolated from children feces and identified using primers: 27f (5 '-AGAGTTTGATCMTGGCTCAAG-3') and1525r (5'-AAGGAGGT-GWTCCARCC-3'). The two universal primers 27F (5_-GCCTTGCCAGCCCGCTC-AGTCGAGTTTATCCTGG-CTCAG-3_) and 338R (5_- GCCTCCCTCGCGCCAT-CAGNNNNNNNNCATGCTGCCTCCCGTAGGAGT-3_) were used for PCR amplification to identify LAB isolates from vagina and the isolates included *L.*

crispatus, *L. iners*, *L. gasseri*, *L. jensenii* and *Streptococcus* as reported by Forney *et al.*, (2010).

The RAPD-PCR technique has been described as a useful technique for both identification and typing of bacteria (Du Plessis & Dicks, 1995; Rodas *et al.*, 2005). The main advantage of the RAPD system deceit in the fact that once a high reproducibility is reached, the method is fast, practical, easy to perform and inexpensive. The RAPD and the other multiplex PCR have been developed for the characterization of different bacterial strains.

Six LAB isolates from honey were previously studied for their antibacterial activity and these isolates are H006-A and H006-C from Libya, H008-D and H008-E from Cameron highlands Malaysia, H009-F from Saudi Arabia and H010-G from New Zealand (Aween *et al.*, 2010). The aim of this study is to identify the LAB isolated from different sources by API CHL50 and 16sRNA, to characterize their antibiotic resistant pattern and to establish the relationship of the LAB isolates by RAPD analysis.

6.2 Materials and Methods

6.2.1 Antibiotic resistant of lactic acid bacteria

Six LAB isolates previously isolated from commercial honey available in Malaysia (Aween *et al.*, 2010) were tested for their resistant against several antibiotics using disk diffusion method (Bauer *et al.*, 1966) with MRS agar. The antibiotics tested were vancomycin (5µm), cephalothin (30µm), nalidixic acid (30 µm), Gentamycin (10 µm), streptomycin (10 µm), tetracycline (30 µm), bacitracine (10 µm), penicillin G (10 µm), chloramphenicol (30 µm) and polymyxin B (300 µm) (Sigma). Growth inhibition zone surrounding the antibiotic disc were measured and the antibiotic resistant patterns were established.

6.2.2 Proteinase detection

The method of Pailin *et al.*, (2001) was followed. Lactic acid bacteria isolates were grown on MRS agar for 48 h at 37 °C, then a loop of LAB was transferred into

skimmed milk agar (skimmed milk and agar prepared separately and after the preparation 25% of skimmed milk was mixed with 75% agar No. 3) as spot and incubated at 37 ºC for 48 h in anaerobic condition followed by incubation at 4 ºC for 48 h. Clear zones around the spots of LAB isolates indicated proteinase activity.

6.2.3 Phenotypic identification of isolates

Six isolates were identified by API 50 CHL (API system, BioMérieux, France). The isolates were tested for catalase and Gram stain. Overnight cultures of isolates were grown on MRS plates (Oxoid) at 37 ºC for 24 and 48 h. The pure colonies were suspended in API 50 CHL medium (API system, BioMérieux, France). The suspension was transferred into each of the 50 wells of the API 50 CH strips. All wells were overlaid with sterile mineral oil to make it anaerobic. Strips were incubated at 37 °C as recommended by the manufacturer. Changes in color from wells were noticed after 24 and 48 h. The results were analyzed with API WEB (BioMérieux).

6.2.4 Genotypic characterization

Genomic DNA was prepared from the six strains of LAB.

6.2.4.1 DNA extraction

Total genomic DNA was extracted from an overnight culture in 20 ml MRS broth at 30 ºC using Master Pure™ Gram positive DNA Purification Kit (USA). One ml of overnight culture was centrifuged 10,500 rpm for 10 min at 25 °C (Eppendorf centrifuge 5804 R) and the pellet was collected. To the pallet 150 µl of TE buffer was added and incubated at 37 ºC overnight. 1 µl of proteinase K (50 µg/µl, Sigma) was mixed to 150 µl of Gram positive lysis solution and then added to TE buffered mixture and mixed thoroughly. The sample was incubated at 65-70 ºC for 15 min and vortexed every 5 min, followed by placing in ice for 5 min. 175 µl of MPC protein precipitation reagent were added to each sample, vortexed and centrifuged at 13000 rpm for 10 min at 4 °C (Eppendorf centrifuge 5804 R). The supernatants were transferred to new tubes and the pellets were discarded. 1 µl of RNase II (5 µg/µl) was added to each sample and mixed thoroughly. The samples were incubated at 37 ºC for 30 min; 500 µl of isopropanol was added to the supernatant, centrifuged at 4ºC

for 10 min at 13000 rpm (Eppendorf centrifuge 5804 R). Isopropanol was removed using a eppendorf pipette without dislodging the DNA pellet. The pellets were rinsed with 200 µl ethanol 70% and centrifuged at 5000 rpm for 2 min at room temperature. The ethanol was removed carefully and the DNA was resuspended with 35 µl of dionised water and kept at -20 ºC for further study.

6.2.4.2 PCR of lactic acid bacteria

Purified DNA of each sample was processed to the PCR using Fail Safe™ Pre Mix Kit Epicentre® (an Illumina® company) Two sets of oligonucleotide primers were used, namely 16S forward: (5-AGAGTTTGATCCTGGCTC-3) and 16S reverse: (5-CGGGAACGTATTCAC-CG-3) (Magnusson *et al.*, 2003), and 27f (5'-AGAGTTTGATCMTGGCTCAAG-3') and 1525r (5'-AAGGAGGTGWTCCARCC-3') (Lim *et al.*, 2009). Primers were synthesized at 1st Base, Malaysia. The settings of PCR were as follows: initial at 95 °C for 2 min, denaturation at 92 °C for 45 s, annealing at 54 °C for 1 min and extension at 72 °C for 1 min, with 35 cycles for each steps.

From each amplification mixture 2 ul were subjected to electrophoresis in 1.5% (w/v) agarose gels in 0.5 x TEA buffer for 45 min and 110 V. 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 were determined by 1st Base, Malaysia and sequences were compared with databases (Gen- Bank).

6.2.4.3 Randomly amplified polymorphic DNA polymerase chain reaction (RAPD-PCR)

The RAPD-PCR procedure was carried out as described by Williams *et al.* (1990). A total of eight random primers were screened for detectable amplification. The primers used in this study were P51, P53, P55, P56, P65, P66, P69 and P70 (Table 23). The PCR was set up as follows: initial denaturation step at 94°C for 5 min, denaturation at 94°C for 1 min, followed by annealing 56°C for 1 min and extension

at 72°C for 1 min. The PCR was run for 45 cycles at each step and amplification products were analyzed by electrophoresis as previously described in 1.5. Two standard molecular weight markers were used from 100 to 100 bp and from 250 to 10,000 bp to evaluate the weight of the fragments. Fragments and the standard markers were separated in 1.5% agarose gel (w/v) in 0.5 x TEA buffer. The gel was stained in ethidium bromide and the separated fragments were visualized and photographed with gel documentation system (BIO RAD). DendroUPGMA was use for dendrograms construction utility.

Table 23: List of RAPD-PCR primers

Primer code	Primer sequence	References
P51	5′-CAG GCC CTT C-3′	1st Base, Malaysia
P53	5′-AGT CAG CCA C-3′	1st Base, Malaysia
P55	5′-AGG GGT CTT G-3′	Ashmaig *et al.*, (2009)
P56	5′-GGT CCC TGA C-3′	1st Base, Malaysia
P65	5′-TTC CGA ACC C-3′	1st Base, Malaysia
P66	5′-AGC CAG CGA A-3′	1st Base, Malaysia
P69	5′-CAA ACG TCG G-3′	Ashmaig *et al.*, (2009)
P70	5′-GTT GCG ATC C-3′	Ashmaig *et al.*, (2009)

6.3 Results

6.3.1 Antibiotic resistant test of LAB isolates

All LAB isolates were resistant to nalidixic acid and streptomycin. Isolate H006-A was resistant to penicillin G, and isolate H010-G showed resistant to gentamycin and polymyxin B. All LAB isolates were sensitive to vancomycin, cephalothin, tetracycline, bacitracine and chloramphenicol. MAR index was 0.2 to 0.4. (Table 24).

Table 24: Antibiotics resistant test result against lactic acid bacteria[a]

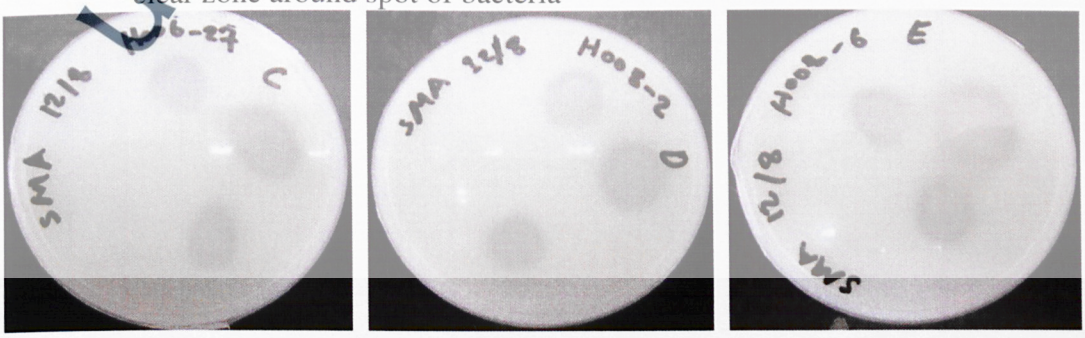
Antibiotics	LAB					
	H006-A	H006-C	H008-D	H008-E	H009-F	H010-G
Vancomycin	S	S	S	S	S	S
Cephalothin	S	S	S	S	S	S
Nalidixic acid	R	R	R	R	R	R
Gentamycin	S	S	S	S	S	R
Streptomycin	R	R	R	R	R	R
Tetracycline	S	S	S	S	S	S
Bacitracine	S	S	S	S	S	S
Penicillin G	R	S	S	S	S	S
Chloramphenicol	S	S	S	S	S	S
Polymyxin B	S	S	S	S	S	R
MAR index	0.3	0.2	0.2	0.2	0.2	0.4

[a]S: Sensitive (> 5 mm diameter) and R: resistant (No inhibitory zone).

6.3.2 Proteinase activity of LAB isolates

Three of the LAB isolates namely H006-C from Libyan honey, H008-D and H008-E from Malaysian honey produced proteinase enzyme as indicated by clear zone around the bacteria spot on milk agar plates (Figure 3). Proteinase activity was not detected in isolates H006-A from Libyan, H009-F from Saudi Arabian and H010-G from New Zealand honey.

Figure 3: Proteinase activity of lactic acid bacteria on skim milk agar as indicated by clear zone around spot of bacteria



6.3.3 Phenotypic identification of LAB using API 50CHL Kit

There was variation in the utilization of carbohydrates sources of the API CHL 50 systems by all the isolates (Table 25). It was observed that all the isolates fermented galactose but not sorbitol except H006-A. *N*-Acetyl glucosamine was utilized by all isolates except H008-E. Amygdaline was utilized by H006-C and H009-F but not others. Lactose was fermented by H006-C, H008-D and H009-F. H009-F did not utilize trehalose. H006-C, H008-E and H010G ferment inulin. H006-A and H010-G did not utilize D-raffinose. H008-D did not ferment D-gentibiose. Therefore, the isolates were identified as *Lactobacillus acidophilus* of which H006-C, H009-F and H010-G were *Lactobacillus acidophilus* 1 with very good percentage of similarity (above 90%) isolated from honey samples produced in Libya, Arab Saudi and New Zealand, respectively. Isolates H006-A, H008-D and H008-E were from honey produced in Malaysia, and identified as *Lactobacillus acidophilus* 1 strains of poor percentage similarity (less than 60 %) (Tables 25 and 26).

Table 25: Carbohydrates fermentation by lactic acid bacteria isolates using API 50 CHL identification systems

Carbon sources	LAB					
	H006-A	H006-C	H008-D	H008-E	H009-F	H010-G
(1) Glycerol	-	-	-	-	-	-
(2) Erythritol	-	-	-	-	-	-
(3) D-Arabinose	-	-	-	-	-	-
(4) L-Arabinose	-	-	-	-	-	-
(5) Ribose	-	-	-	-	-	-
(6) D-Xylose	-	-	-	-	-	-
(7) L-Xylose	-	-	-	-	-	-
(8) Adonitol	-	-	-	-	-	-
(9) β-Methyl-xyloside	-	-	-	-	-	-
(10) Galactose	-	+	+	+	+	+
(11) D-Glucose	+	+	+	+	+	+
(12) D-Fructose	+	+	+	+	+	+
(13) D-Mannose	+	+	+	+	+	+
(14) L-Sorbose	-	-	-	-	-	-
(15) Rhamnose	-	-	-	-	-	-
(16) Dulcitol	-	-	-	-	-	-
(17) Inositol	-	-	-	-	-	-
(18) Mannitol	-	-	-	-	-	-
(19) Sorbitol	+	-	-	-	-	-
(20)α-Methyl-D-mannoside	-	-	-	-	-	-
(21) α-Methyl-D-Glucoside	-	-	-	-	-	-
(22) *N*-Acetyl glucosamine	+	+	+	+	-	+
(23) Amygdaline	-	+	-	-	+	-

Antibiotics	LAB					
	H006-A	H006-C	H008-D	H008-E	H009-F	H010-G
(24) Arbutine	+	+	+	+	+	+
(25) Esculine	+	+	+	+	+	+
(26) Salicine	+	+	+	+	+	+
(27) Cellobiose	+	+	+	+	+	+
(28) Maltose	+	+	+	+	+	+
(29) Lactose	-	+	+	-	+	-
(30) Melibiose	-	-	-	-	-	-
(31) Saccharose	+	+	+	+	+	+
(32) Trehalose	+	+	+	+	-	+
(33) Inulin	-	+	-	+	-	+
(34) Melezitose	-	-	-	-	-	-
(35) D-Raffinose	-	+	+	+	-	+
(36) Amidon	-	-	-	-	-	-
(37) Glycogène	-	-	-	-	-	-
(38) Xylitol	-	-	-	-	-	-
(39) β-Gentiobiose	+	+	+	+	+	+
(40) D-Turanose	-	-	-	-	-	-
(41) D-Lyxose	-	-	-	-	-	-
(42) D-Tagatose	-	-	-	-	-	-
(43) D-Fucose	-	-	-	-	-	-
(44) L-Fucose	-		-	-	-	-
(45) D-Arabitol	-	-	-	-	-	-
(46) L-Arabitol	-	-	-	-	-	-
(47) Gluconate	-	-	-	-	-	-
(48) 2 Ceto-gluconate	-	-	-	-	-	-
(49) 5 Ceto-gluconate	-	-	-	-	-	-

Table 26: Identification of isolates using API 50 CHL Kits and API web

Sample code	Percentage of similarity	Identification (ID)
H006-A	53.2	*Lactobacillus acidophilus 1*
H006-C	97.2	*Lactobacillus acidophilus 1*
H008-D	56.7	*Lactobacillus acidophilus 1*
H008-E	56.7	*Lactobacillus acidophilus 1*
H009-F	99.5	*Lactobacillus acidophilus 1*
H010-G	99.5	*Lactobacillus acidophilus 1*

6.3.4 Molecular characterization for LAB isolates

6.3.4.1 PCR of lactic acid bacteria

The results of PCR using 16S forward: (5-AGAGTTTGATCCTGGCTC-3) and 16S reverse: (5-CGGGAACGTATTCAC-CG-3) (Magnusson *et al.*, 2003) resulted in clear bands of all isolates (Figure 4) with approximate molecular weight 1400 bp. BLASTING analysis sequence in the NCBI showed that the isolates were uncultured bacteria (Figure 4). It was observed that 27f primer (5 '-AGA-GTTTGATCMTGGCTCAAG-3') and 1525r primer (5'-AAGGAGGTGWT-CCARCC-3') could produce observable bands for H006-A and H006-C but not for other isolates (Figure 5).

Figure 4: The DNA bands of LABs on the 1.5 % agarose gel using primers 16S.S: (5-AGAGTTTGATCCTGGCTC-3) and 16S.R.: (5-CGGGAACGTATTCACCG-3), Lane 1:. H006-A, 2. H006-C, 3.H008-D, 4. H008-E, 5.H009-F and 6. H010-G.

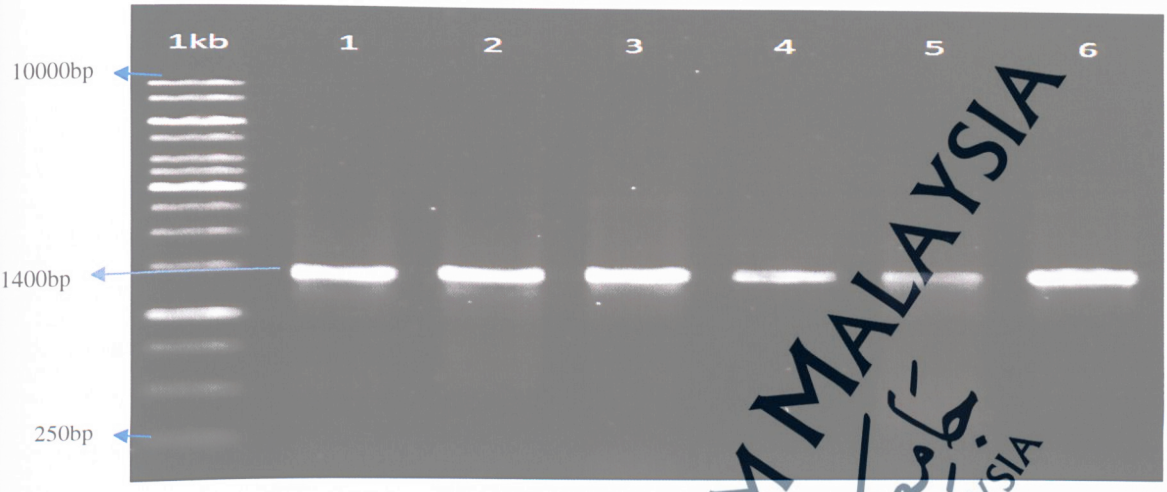


Figure 5: The DNA bands of LABs on the agarose gel, 1. H006-A, 2. H006-C, 3.H008-D, 4. H008-E, 5.H009-F and 6. H010-G. The primers: 2′ AGAGTTTGATCMTGGCTCAA-G-3') and1525r AAGGAGGTGWT-CCARCC-3')

6.3.4.2 RAPD-PCR analysis

The results of strain typing of six LAB isolates by RAPD fingerprinting using eight primers are shown in Figure 6 & 7. *Lactobacillus acidophilus* (M), *Lactobacillus paracasei* (X), *Lactobacillus pentoses* (Y) and *Pseudococcus pentoses* (Z) were used as indicator strains. Variations in number of bands, fragment size and intensity were

observed by primers P-66 5´-AGC CAG CGA A-3´, P-53 5´-AGT CAG CCA C-3´ and P-70 5´-GTT GCG ATC C-3´ (Table 27 & Figures 6 & 7).

Primer P-53 5´-AGT CAG CCA C-3´ discriminated the indicator *P. pentoses* from other isolates in which H009-F and H006-C were grouped together as one cluster while H010-G was grouped together with *L. paracasei* (Figure 8). However, Primer P-70 5´-GTT GCG ATC C-3´ discriminated 2 major groups, isolate H006-A from Libyan honey and was grouped together with indicator strain of *P. pentoses*. The second cluster consists of 2 main groups in which isolates H008-D and H008-E were in one group while H010-G and H009-L were in another group (Figure 9). Primer P-66 5'-AGCCAGCGAA-3' showed two clusters, one cluster consisted of 7 strains (H006-A, H008-E, H006-C, H008-D, H010-G, H001-H and H009-F) and another cluster of 7 strains (H005-I, H010-J, *L. acidophilus*, *L. paracasei*, *L. pentoses* and *P. pentoses*). Isolate H006-A was grouped together with H008-E at Pearson coefficient 10.89% as well as H010-G with H001-H at Pearson coefficient 40.83%. Indicator strain of *L. acidophilus* from milk was in the other cluster different from LAB isolates from honey (Figure 10). Thus, Primer P-66 (5'-AGCCAGCGAA-3') is suitable to give distinct patterns which were highly reproducible for all the isolates indicating there were genetic differences among the LAB used.

Figure 6: The RAPD-PCR products obtained from 14 LAB isolated from honey using primer P-70 5´-GTT GCG ATC C-3´

Figure 7: The RAPD-PCR products obtained from 14 LAB isolated from honey using primer P-66 5'-AGCCAGCGAA-3'

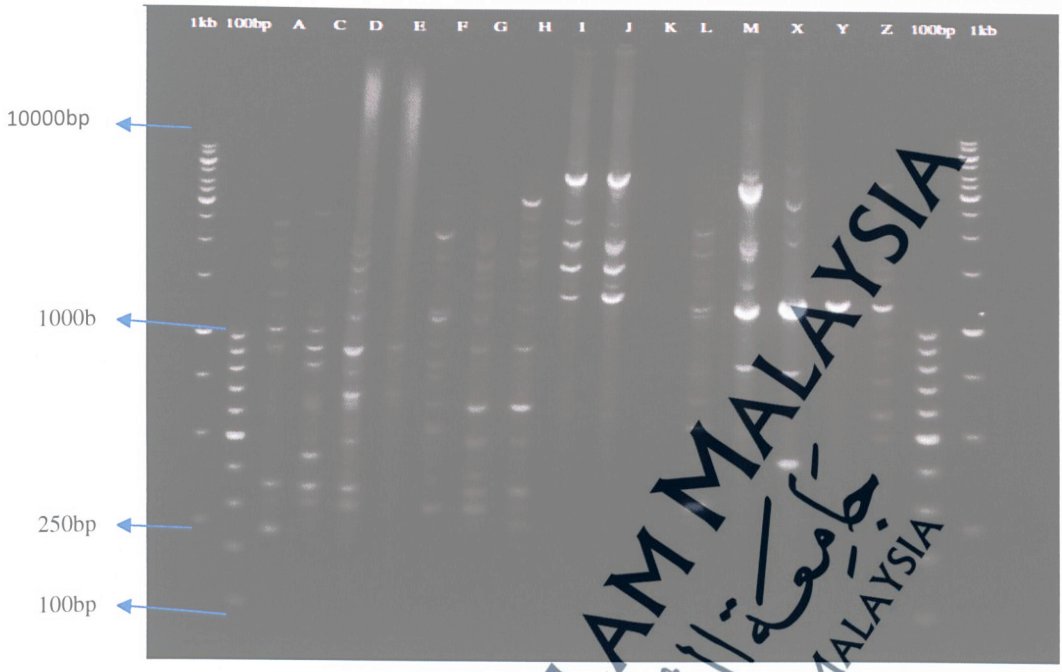


Table 27: Number of produced bands for all LAB samples using primer 5'-AGCCAGCGAA-3'

Sample code	Name of isolate	No. bands using P-53	No. bands using P-70	No. bands using P-66
A	H006-A	0	1	8
C	H006-C	1	0	9
D	H008-D	0	5	11
E	H008-E	1	5	3
F	H009-F	3	2	11
G	H010-G	2	3	12
H	H001-H	1	2	15
I	H005-I	2	4	5
J	H010-J	2	1	5
L	H009-L	1	4	9
M	*Lactobacillus acidophilus*	2	5	6
X	*Lactobacillus paracasei*	2	4	7
Y	*Lactobacillus pentoses*	0	1	1
Z	*Pseudococcus pentoses*	2	1	17

Figure 8: The dendogram of RAPD-PCR products of LAB isolates using primer P-53 5′-AGT CAG CCA C-3′, M: *Lactobacillus acidophilus*, X: *Lactobacillus paracasei,* Y: *Lactobacillus pentoses* and Z: *Pseudococcus pentoses.*

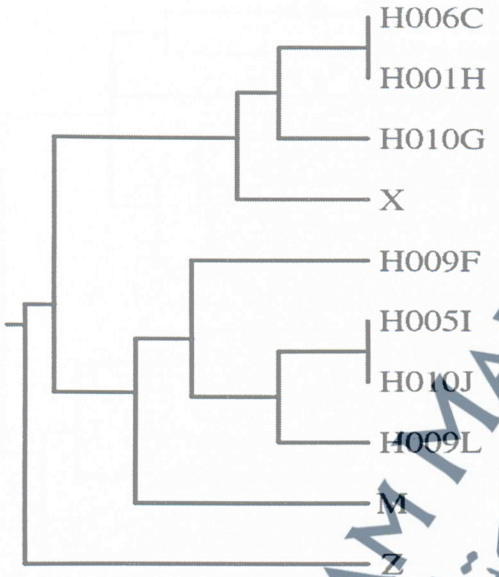


Figure 9: The dandogram of RAPD-PCR products of LAB isolates using primer P-70 5′-GTT GCG ATC C-3′, M: *Lactobacillus acidophilus*, X: *Lactobacillus paracasei,* Y: *Lactobacillus pentoses* and Z: *Pseudococcus pentoses.*

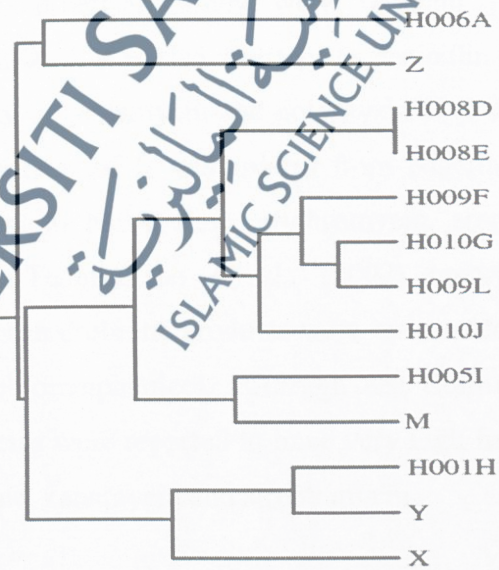
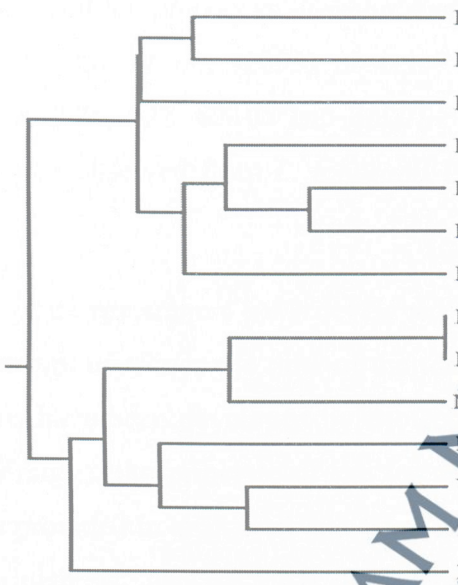


Figure 10: The dendogram of RAPD-PCR products of LAB isolates using primer P-66 5'-AGCCAGCGAA-3', M: *Lactobacillus acidophilus*, X: *Lactobacillus paracasei,* Y: *Lactobacillus pentoses* and Z: *Pseudococcus pentoses.*



6.4 Discussion

LAB isolated from honey were multi-antibiotic resistant which make them unique and different from common LAB associated with fermented food. All the strains of LAB isolated from commercial honey were resistant to nalidixic acid and streptomycin. Isolate H006-A was also resistant to penicillin G, and isolate H010-G showed resistant activity to gentamycin and polymyxin B (Table 24). Olukoya *et al.*, (1993) observed that isolates of *L. acidophilus* from Nigerian fermented foods and beverages were resistant to tetracycline, erythromycin, ampicillin, cloxacillin and penicillin. Similarly, Temmerman *et al.*, (2002) reported that *Lactobacillus acidophilus* from European probiotic products were resistant to tetracycline, penicillin G, erythromycin and chloramphenicol. Curragh and Collins, (1992) reported that several lactobacilli species were reported to have very high frequency of spontaneous mutation to nitrofurazone, kanamycin and streptomycin.

Isolates H006-C from Libya, H008-D and H008-E from Malaysia showed proteinase activity while the other isolates did not. *Lactobacillus acidophilus* isolated from kefir grains showed proteolytic activity (Kabadjova-Hristova *et al.* 2006).

Several researchers reported that the proteinase are extracellular cell bound as shown by *Lactobacillus casei* HN14 isolated from cheese (Kojic *et al.*, 1991), and *Lactobacillus bulgaricus* and *Streptococcus thermophilus* cultures from yogurt (Pailin *et al.*, 2001). Similarly, Fira *et al.*, (2005) observed that the natural strains of *Streptococcus* sp. M104, F86, F22, S2105 and S2007 produced proteinase enzyme. Protease activity was also observed from *L. johnsonii* LT17 and *L. crispatus* LT11 (Taheri *et al.*, 2009).

In the last twenty years researchers have developed fast and reliable methods to identify LAB in an attempt to reduce the time of the identification process. Some of the mentioned methods have been developed by Biotech (Gen2 and Gen3) and API center (BioMérieux, France), the principle of the test is based on the carbohydrate fermentation which is provided in each well and takes 24 to 48 h and consider faster than other chemicals methods. The six LAB that were selected were identified as *Lactobacillus acidophilus 1* but with varying similarity percentage (Table 26) by API CHL 50. The variability of the isolates was supported by the differences in antibiotic patterns and proteinase activity as observed in this study. T6SrDNA pattern using universal primer revealed that the isolates were uncultured while 27f could discriminate only two isolates (H006-A and H006-C) and no bands were obtained from other LAB isolates (Figures 4 & 5).

Phenotypic characterization which based on sugar fermentation may not always offer sufficient basis for the reliable identification of LAB as observed by several researchers (Nigatu, 2000; de Angelis *et al.* 2001; Muyana *et al.* 2003; Ashmaig *et al.* 2009). Hosny *et al.*, (2009) isolated lactic acid bacteria from Egyptian honey and identified these isolates using API Kits (BioMérieux, France), Gram staining and catalase activity, and the isolates were identified as *Lactobacillus acidophilus*, *L. casei*, *L. plantarum*, *Lactococcus lacits*, *Lact, cremoris* and *Micrococcus luteus*. Adiguzel & Atasever, (2009) isolated lactic acid bacteria from Turkish fermented sausages (sucuk) and identified the isolates phenotypicaly by morphology, Gram staining, catalase test and API Kits (BioMérieux, France) and genotypicaly using primer BOX A1R (CTA CGG CAAGGC GAC GCT GAC G). These researchers faced contrasted results for identifying LAB isolates between API Kits and BOX-PCR genotypic fingerprinting.

Magnusson *et al.*, (2003) isolated lactic acid bacteria from different environments and identified these isolates as *Lactobacillus coryniformis*. *Lactobacillus plantarum* and *Pediococcus pentosaceus* using primers 16S.S (5P-AGAGTTTGATCCTGGCTC-3P) and 16S.R (5P-CGGGAACGTATTCACCG-3P). Chen *et al.*, (2010) used Primer: 16S rDNA 5′CTGCTGCCTC-CCGTAG-3′ (27F) for PCR amplification following the conditions and methods prescribed and identified *Lactobacillus plantarum*, *Weissella cibaria* and *Lactococcus lactis subsp. lactis* isolated from ripe mulberries. That shows that the use of the 16S rDNA is good for almost lactic acid bacteria species.

PCR-RAPD has been used to differentiate between species (Nigatu, 2000; Tamang *et al.* 2005; Ashmaig *et al.* 2009; Sathishkumar *et al.* 2010). Of the 8 primers used, 3 of the primers showed bands for some isolates while primer P-66 was able to produce bands for all the isolates and discriminate the isolates into clusters, different of each other. RAPD-PCR with primer (P-66 5'-AGCCAGCGAA-3') distinguished *L. acidophilus* from honey and from milk, indicating that the isolate from honey is different from isolates present in milk. Tamang *et al.*, (2005) identified lactic acid bacteria isolated from fermented vegetable products and identified them using RAPD-PCR using primer M13 (5V-GAG GGT GGC GGT TCT-3V). The major representatives of the LAB involved in these fermentations were identified as *L. brevis*, *L. plantarum*, *Pediococcus pentosaceus*, *Pediococcus acidilactici* and *Leuconostoc fallax*. Ashmaig *et al.*, (2009) isolated LAB from fermented camel's milk and identified the isolates using API 50 CHL and RAPD-PCR analysis using primers: 5′-GTT GCG ATC C-3′, 5′-CAA ACG TCG G-3′ and 5′-AGG GGT CTT G-3′.

Phenotypically identification of LAB isolates showed that isolate H009-F is similar to isolate H010-G although they were from different origins while H008-E is similar to H008-D and from the same origin, but genetically showed that these isolates are different. Isolates H006-C, H008-E, H008-D and H009-F showed similar resistant activity against antibiotic tested, while H006-A and H010-G were different.

This study indicates that LAB isolates from different origin could belong to the same gen, but with different species. The bacteria *L. acidophilus* is complex group of LAB, *Lactobacillus acidophilus* isolates were different from each other and from indicator strains used.

5.5 Conclusion

This study observed that honey contains different strains of LAB. Identification of the LAB strains using phenotypic method identified the isolates as *L. acidophilus*. However, antibiotic resistance pattern, proteolytic activity and RAPD analysis indicated the variability of the isolates depending on the origin of honey. Therefore, more work should be done in developing methods to characterize and identify LAB strains isolated from different honey sources.