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# Evaluation on Antibacterial Activity of *Lactobacillus acidophilus* Strains Isolated from Honey

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**Abstract: Problem statement:** This study reports the isolation of lactic acid bacteria from 13 honey samples produced in Malaysia, Libya and Saudi Arabia and their antibacterial activity against three Gram negative pathogenic bacteria. **Approach:** A modified MRS agar with 0.8% $CaCO_3$ and MRS with 1% glucose was found to facilitate isolation of LAB compared to MRS, tomato juice agar and modified tomato juice agar. 32 isolates were confirmed LAB by catalase test and Gram staining. Six isolates were screened for antibacterial activity and identified as strains of *Lactobacillus acidophilus* 1 by API CH50. **Results:** All the isolates showed very good inhibitory activity against target Gram negative bacteria as indicated by the diameter of inhibition zone: *Salmonella* Typhimurium (23-30 mm), *Escherichia coli* (7-18 mm) and *Enterobacter aerogenes* (10-18 mm) after 24 h incubation at 30°C. Supernatants of *L. acidophilus* 1 strains showed good antibacterial activity against all target bacteria. Heating the supernatants at 90 and 121°C for 1 h enhanced the antibacterial activity against all target bacteria except supernatants H006-A and H010-G against *S. Typhimurium*. Antibacterial activity of supernatants were maintained after pH adjustment to 3, but at pH5 supernatants H006-A, H008-D and H010-G lost the activity against *S. Typhimurium* and *E. coli* within 48 h of incubation while at pH 6 all supernatants lost activity except against *E. aerogenes*. Enzymes treatments of supernatants with RNase II and Proteinase K for 1 h inhibited all target bacteria except supernatants H008-D, H008-E and H006-A which were relatively sensitive to both enzymes against *S. Typhimurium* and *E. coli*. **Conclusion/Recommendations:** In conclusion, honey from different sources contains strains of *L. acidophilus* 1 that produced compounds with good antibacterial activity which may be responsible for the antibacterial properties of honey.

**Key words:** Lactic Acid Bacteria (LAB), Multiple Antibiotic Resistant (MAR), antibacterial activity, demonstrated antimicrobial, antibacterial properties, Intensive Care Units (ICUs)

## INTRODUCTION

Honey is well known for its health benefits and it has been used as traditional medicine for many years (Dobrowolski *et al*., 1991; Bankova, 2005). Honey contains cenamic acid, antioxidant agent and some flavonoids which have been approved for antibacterial applications (Rahman *et al*., 2010). Malika *et al*. (2004) suggested that the activity of honey varies depending on its origin, type of flowers, the region, the nature of bees and the breeding techniques. Zumla and Lulat (1989) reported that honey is very good inhibitor to *Escherichia coli*, *Salmonella* and *Shigella*. Malaysian wild honey known as Tualang honey showed good activity against different pathogens of wound and enteric bacteria and the activity was comparable to that of manuka honey from New Zealand. Tan *et al*. (2009) evaluated the antimicrobial activity of natural honey collected from different aromatic and medicinal plants against antibiotic resistant bacteria isolated from human; all honey samples showed strong activity against *E. coli*, *Staphylococcus aureus*, *Bacillus subtilis* and *Pseudomonas aeruginosa*.

Mundo *et al*. (2004) collected honey samples from different sources and evaluated against food spoilage

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organisms and pathogens namely, *Alcaligenes faecalis, Aspergillus niger, Geotrichum candidum, Penicillium expansum, Lactobacillus Acidophilus*, P. *fluorescens*, B. *cereus*, *E. coli* O157:H7, *Listeria monocytogenes, Salmonella enterica* Ser. Typhimurium, S. *aureus*, S. *aureus* 9144 and B. *stearothermophilus*. The results showed that B. *stearothermophilus* was highly sensitive to honey, *A. faecalis* and *L. acidophilus* were less sensitive and the fungi *A. niger, P. expansum, G. candidum* and *S. aureus* were unaffected to honey. Tumin *et al*. (2005) investigated the antimicrobial activity of five different types of Malaysian honey and observed that three honey samples showed antibacterial activity against *S. typhi, S. aureus, Shigella sonnie, Streptococcus pyogenes* and *E. coli*.

Recently, several strains of bacteria were isolated from honey and demonstrated antimicrobial activity against both gram negative and positive pathogenic and spoilage bacteria (Aween *et al*., 2010); Ibarguren *et al*., 2010; Lee *et al*., 2008). Bahiru *et al*. (2006) reported that honey contains yeasts, low amount of spores and lactic acid bacteria. In addition, the heterolactics were of higher count than the homolactics. Olofsson and Vasquez (2008) isolated novel Lactic Acid Bacteria (LAB) in the genera *Lactobacillus* and *Bifidobacterium* from honeybee stomach and the same isolates were also detected in honey. Hosny *et al*. (2009) determined the microbial quality of three different types of honey from Egypt and reported that honey contains bacteria of the genera *Lactobacillus, Streptococcus, Micrococcus* and *Bacillus* namely, B. *butyricum*, B. *subtilis, Enterococcus faecium, L. acidophilus, L. casei, L. plantarum, Lactococcus lacits, Lact. Cremoris* and *Micrococcus luteus*; one of the samples was contaminated with yeasts and molds. Lactic Acid Bacteria (LAB) are known for their antimicrobial activity specially lactobacilli (Klaenhammer, 1993). Most of the antimicrobial activity is due to organic acids and bacteriocins produced by certain LAB strains. Bacteriocins are proteins or protein complex which has inhibition activity against gram positive and negative bacteria (Tagg *et al*., 1976).

*Salmonella Typhimurium* and *E. coli* are of the most common type of pathogenic bacteria around the world. *S. Typhimurium* causes typhoid fever in human and gastroenteritis in human and other mammals. This bacterium usually enters the body through the mouth by contaminated food or water, penetrates the intestinal wall and multiplies in lymphoid tissue. The bacteria could enter bloodstream within 24-72 h causing blood poisoning (McCormick *et al*., 1995; Everest *et al*., 1999). *E. coli* is a type of bacteria that can contaminate food such as beef, fruits and vegetables. This bacterium is found inside human intestines, where it helps the body breaks down and digests the food. Unfortunately, some types of *E. coli* can get from the intestines into the blood and could cause serious infection such as *E. coli* O157:H7. *Enterobacter aerogenes* causes disease in humans and can be transferred in hospitals or from environment. Elderly, infants and people who are in the terminal situation of other disease or immunosuppressed are candidates for its infections (Janda and Abbott, 2006). *E. aerogenes* is known to have drug-resistant and characteristicsand the fast development of multidrug resistance strains has become a growingly problem (Sankaran, 2000). Multiresistant strains of *E. aerogenes* have caused outbreaks in Intensive Care Units (ICUs) in France, Belgium, United States and Austria (Gheldre *et al*., 2001).

Honey is known to have antibacterial activity and LAB has been isolated from honey samples. However, information on the possible role of LAB in honey is lacking. Therefore, this study attempts to evaluate the antibacterial activity of LAB isolated from commercially available honey in Malaysia against multiresistant strains Gram negative bacteria *S. Typhimurium* ATCC 13311, *E. coli* ATCC 25922 and *E. aerogenes*.

## MATERIALS AND METHODS

**Honey samples:** A total of 13 honey samples were collected from different sources in Malaysia. Samples were kept at room temperature before analysis. The samples used in this study were local honey from Cameron Highland (Pure Natural honey and Wild honey), from Melaka (Madu bunga gelam, Pure honey 2, Pure honey 3 and Propolis honey), from Seremban (Madu Tualang and Madu lebah Neron), from Libya (Al-Seder honey and Spring honey), from Saudi Arabia (Al-Shifaa honey) and from New Zealand (Monuka honey). pH of honey was determined using pH meter (METTLER TOLEDO).

**Isolation of lactic acid bacteria from commercial honey samples:** 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 10 $mL^{-1}$ of MRS broth incubated at 30°C for 24-48 h followed by serial dilution with peptone water (0.1% w/v). 0.1 $mL^{-1}$ was spread plated on several modified media namely, MRS agar (Deman *et al*., 1960), MRS agar with 0.8% $CaCO_3$ (Winched *et al*., 2007), MRS agar with 1% glucose, tomato juice agar 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. Pure colonies were again tested for catalase activity and Gram stained. All catalase negative and Gram positive colonies were maintained in MRS broth with 15% of glycerol and kept at-20°C for further study.

**Antibiotic resistant target bacteria** *S. Typhimurium* ATCC 13311, *E. coli* ATCC 25922 and *E. aerogenes* were tested for their resistant to antibiotics using disc diffusion method as described by Bauer *et al*. (1966). The antibiotics used were vancomycin (5µm), cephalothin (30 µm), 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).

**Antimicrobial activity of LAB isolates using dual agar overlay method:** Antimicrobial activity of six LAB isolates was determined against target bacteria using dual agar overlay method. LAB was inoculated in spot on MRS agar plates and grown at 30°C for 24 h in anaerobic jars. The plates were overlaid with 15 ml of nutrient agar containing the target bacteria $10^6$ cells per ml. After 24 h of aerobic incubation at 30°C the diameter of inhibition zone was measured. The tests were done in duplicate and the mean was taken.

**Determination of antibacterial activity of LAB supernatant using microtiter plates:** Cell free supernatant of LAB was obtained from centrifugation (6500× g for 15 min) and filtration of overnight MRS broth inoculated with LAB isolates incubated at 30°C 24 h anaerobically. Nutrient broth was prepared and mixed with the target bacteria $10^4$ cell $mL^{-1}$. 100 $\mu L^{-1}$ of the supernatant and target bacteria were pipetted into the wells of microtiter plates. 200 $\mu L^{-1}$ of target bacteria in nutrient broth was used as positive control. All microtiter plates were incubated at 30°C for 24 and 48 h. Bacterial growth was monitored using Optical Density (OD) 560 nm using BioTek EL×800 ELISA reader. The analysis was carried out in duplicates and the mean was taken. The percentage growth of target bacteria was measured using the equation: OD 560 nm of MRS broth with bacteria or supernatant after incubation 24 and 48 h-OD560 nm of MRS broth with the bacteria at time 0h / OD560 nm of MRS broth with the bacteria at time 0h ×100.

**Effect of heat treatment LAB supernatant on antimicrobial activity:** The supernatant of LAB isolates was heat treated at 90 and 121°C for 1 h and tested against the target bacteria using microtiter plate as described above. All plates were incubated at 30°C for 48 h and bacterial growth was monitored at 24 h interval. Percent growth of target bacteria was calculated as described in 1.5.

**Effect of pH adjustment on antimicrobial activity of LAB supernatant:** pH of LAB supernatant was adjusted to 3-6 using drops of HCl (0.1 N) and NaOH (0.2 N) and pH determined by pH meter (METTLER TOLEDO) then tested against the target bacteria. The microtiter plates were incubated at 30°C for 48 h and bacterial growth was monitored at 24 h interval. Percent growth of target bacteria was calculated as described in 1.5.

**Effect of enzymes on antimicrobial activity of LAB supernatant:** The supernatants were treated with Proteinase K and RNase II separately. 1µl of each enzyme was inoculated to 1 ml of supernatant and left for 1 h at room temperature. After that, the supernatant was tested against target bacteria in microtiter plates followed by incubation at 30°C for 48 h and bacterial growth was monitored at 24 h interval as described above.

**Phenotypic identification of LAB isolates:** Phenotypic identification of the six LAB isolates evaluated for their antimicrobial activity was carried out. Overnight cultures of the isolates were grown on MRS plates (Oxoid) at 37°C for 24 h anaerobically. 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 of wells were noticed after 24 and 48 h. The results were analyzed with API WEB (BioMérieux).

## RESULTS

**Isolation of lactic acid bacteria using different media:** Isolation of LAB form honey was possible by including a pre-enrichment in MRS broth followed by plating in selective media. A total of 32 isolates were isolated from the honey samples and were catalase

negative and Gram positive. Highest number of LAB was isolated from MRS agar with 0.8% $CaCO_3$ (Table 1). LAB present in Al-Sedar honey from Libya, pure honey from Malaysiaand Al-Shifaa honey from Saudi Arabia were isolated from MRS with 1% glucose, while LAB from Madu Bunga Gelam, Malaysia was isolated on tomato juice agar with 0.8% $CaCO_3$. MRS agar and tomato juice agar with 1% glucose did not support growth of LAB in all honey samples. pH of honey seems to affect the population of LAB; high number of LAB ($>10^5$ $mL^{-1}$) was isolated from Al-Seder honey (pH 5.6), followed by Alshifa honey ($> 10^4$ $mL^{-1}$, pH 3.66) and Monuka honey ($> 10^4$ $mL^{-1}$, pH 4.38). It was observed that Malaysia honey contained low number of LAB (>10 $mL^{-1}$) and lower pH. It seems that pH of honey seems to influence number of LAB present. Six of these LAB isolates were selected for antibacterial study against Multiple Antibiotic Resistant (MAR) Gram negative bacteria.

**Antibiotic resistant test of target bacteria:** Multiple Antibiotic Resistant (MAR) patterns of the target bacteria vary with the bacteria and the antibiotic tested (Table 2). *S. Typhimurium* ATCC13311 showed resistance to all the antibiotic s especially to bactracin, cephalothin, penicillin G, vancomycin and strepromycin; *E. coli* ATCC25922 was sensitive to tetracycline, naladixic acid and choloramphinicol but very resistant to bactotracin, penicillin G and vancomycin and E. aerogenes was sensitive to tetracycline and cholramphinicol but very resistant to nalidixic acid and polymyxin B.

**Antimicrobial activity of lactic acid bacteria isolates against target bacteria by dual agar overlay method:** Growth of all the MAR gram negative target bacteria was inhibited by all six *L. acidophilus* strains isolated from different honey samples, especially *S. Typhimurium* ATCC13311 with growth inhibitory zone between 23.2±0.30 to 30.3±1.41 mm (Table 3). Growth of this bacteria was easily inhibited by H008-E (isolated from pure honey, Cameron Highlands, Malaysia), H009-F (isolated from Al-Shifaa honey, Saudi Arabia and H006-A (isolated from Al-Seder honey, Misurata honey, Libya) with inhibitory zone 30.3±0.60, 30.3±1.41 and 29.3±4.24 mm, respectively. Growth of *E. coli* ATCC2592 and *E. aerogenes* were moderately inhibited by all the isolates; these two bacteria were poorly inhibited by H006-C (isolated from Al-Seder honey, Misurata, Libya) with inhibitory zone of 7.5±3.53 and 9.5±1.41mm, respectively.

**Growth inhibition of pathogenic bacteria in the microtiter plate:** Addition of LAB supernatants to MAR target bacteria generally resulted in reduced growth compared to control (Table 4). Supernatant H010-G completely inhibited *E. aerogenes* and reduced growth of *S. Typhimurium* ATCC13311, while *E. coli* ATCC25922 was inhibited by supernatant H008-E and H006-A after 48 h incubation. However, supernatants H006-C, H009-F and H008-E were not effective in preventing the growth of S.Typhimurium ATCC13311. The results demonstrate that the antibacterial activity of *L. acidophilus* strains vary with targeted bacteria and the antibacterial compounds produced by these LAB strains.

**Effect of heat treatment on antimicrobial activity of LAB supernatant:** Antibacterial activity of LAB supernatants were further enhanced by heating at 90 and 121°C for 1 h compared to control (Table 5 and 6). And However, when comparison is made among the LAB supernatants, heating seems to result in increase percentage growth of *S. Typhimurium* ATCC13311 for all the supernatants. In contrast, both heat treatments enhanced the antibacterial activity of LAB supernatants against *E. coli* ATCC25922 and *E. aerogenes*.

**pH sensitivity of LAB supernatant:** Adjusting LAB supernatants to pH 3 resulted in good inhibitory activity against all target Gram negative bacteria especially *E. aerogenes* (Table 7) within 48 h incubation compared to control. It was noteworthy that growth of *S. Typhimurium* ATCC13311 was inhibited when all the supernatants were reduced to pH 3, which was not observed in unadjusted supernatants (Table 4). Both *S. Typhimurium* ATCC13311 and *E. aerogenes* were inhibited at pH 5. In contrast, an increase in percent growth of *E. coli* ATCC25922 was observed in all supernatants except H009-F and H008-E at pH 5 (Table 8) and similarly percent growth of *S. Typhimurium* ATCC13311 increased in supernatants H010-G, H008-D and H006-A at this pH. All LAB supernatants lost their antibacterial activity against *S. Typhimurium* ATCC13311 and *E. coli* ATCC25922 (data not shown) except *E. aerogenes* (Table 9) at pH 6.

**Enzymes sensitivity of LAB supernatant:** Both proteinase K and RNase II treated LAB supernatants showed growth inhibitory activity against all the target bacteria compared to control (Table 10 and 11). While proteinase K treated LAB supernatant showed antibacterial activity against all target bacteria, all RNase II treated LAB supernatants allowed growth of *S. Typhimurium* ATCC13311 after 48 h incubation (Table 11).

Table 1: Isolation of lactic acid bacteria from honey samples using different media incubated at 37°C for 48 h[a]

| Sample code | Source | Honey sample | pH of honey | Media | Gram stain | Catalase | Dilution LAB detected |
|---|---|---|---|---|---|---|---|
| H001 | Melaka, Malaysia | Madu Bunga gelam | 3.57 | MRS agar | | | NG |
| | | | | MRS+ $CaCO_3$ | + | - | 101 |
| | | | | MRS+ Glucose | | | NG |
| | | | | TJA+ $CaCO_3$ | + | - | 101 |
| | | | | TJA+ Glucose | | | NG |
| H005 | Melaka, Malaysia | Wild Honey | 3.41 | MRS agar | | | NG |
| | | | | MRS+ $CaCO_3$ | + | - | 101 |
| | | | | MRS+ Glucose | | | NG |
| | | | | TJA+ $CaCO_3$ | | | NG |
| | | | | TJA+ Glucose | | | NG |
| H006 | Misurata , Libya | Al-seder honey | 5.06 | MRS agar | | | NG |
| | | | | MRS+ $CaCO_3$ MIRS+ Glucose | + | - | 105 |
| | | | | TJA+ $CaCO_3$ | + | - | 103 |
| | | | | TJA+ Glucose | | | NG |
| | | | | | | | NG |
| H008 | Cameron Highlands, Malaysia | Pure honey | 3.61 | MRS agar | | | NG |
| | | | | MRS+ $CaCO_3$ | + | - | 103 |
| | | | | MRS + Glucose | + | - | 103 |
| | | | | TJA + $CaCO_3$ | | | NG |
| | | | | TJA + Glucose | | | NG |
| H009 | Saudi Arabia | Al-Shifaa | 3.66 | MRS agar | | | NG |
| | | | | MRS+ $CaCO_3$ | + | - | 105 |
| | | | | MRS + Glucose | | | NG |
| | | | | TJA+ $CaCO_3$ | | | NG |
| | | | | TJA+ Glucose | | | NG |
| H010 | New Zealand | Monuka honey | 4.38 | MRS agar | | | NG |
| | | | | MRS+ $CaCO_3$ | + | - | 105 |
| | | | | MRS + Gulcose | | | NG |
| | | | | TJA+ $CaCO_3$ | | | NG |
| | | | | TJA+ Glucose | | | NG |

[a] G: glucose (1.0 %), $CaCO_3$: (0.8 %), NG = no growth

Table 2: Antibacterial activity of selected antibiotics against target pathogenic bacteria measured by diameter of inhibition zone around the discs [a]

| | Target bacteria | | |
|---|---|---|---|
| Antibiontics | S. Typhimur ATCC1331 | *E. coil* ATCC2592 | *E. aerogenes* |
| Bacitracin | 0 | 0 | 17 |
| Gentamycin | 8 | 10 | 7 |
| Tetracycline | 17 | 25 | 22 |
| Naladixic acid | 7 | 21 | 0 |
| Cephalorthin | 0 | 0 | 12 |
| Polymyxin B | 5 | 6 | 0 |
| Pencillin G | 0 | 0 | 16 |
| Vancomycin | 0 | 0 | 7 |
| Streptomycin | 0 | 15 | 10 |
| Choloramphinicol | 5 | 20 | 20 |

[a] Diameter of inhibition zone around the discs (mm)

Table 3: Growth inhibition zone of target bacteria by LAB isolated from honey by dual agar overlay method[a]

| | LAB | | | | | |
|---|---|---|---|---|---|---|
| Target bacteria | H006-C | H009-F | H010-G | H008-D | H008-E | H006-A |
| *S. Typhimurium* | 23.2±0.30 | 30.3±1.41 | 23.5±0.70 | 25±2.82 | 30.3±0.60 | 29.3±4.24 |
| *E. coli* | 7.5±3.53 | 14±2.82 | 18±2.82 | 12.5±0.70 | 16.2±0.81 | 17.5±0.70 |
| *E. aerogenes* | 9.5±1.41 | 17.5±1.41 | 16.5±1.41 | 13.5±2.12 | 15.5±0.20 | 12.5±3.53 |

[a]Diameter of growth inhibitory zone was measured in mm after 24 h incubation at 30°C

Table 4: Growth percentage of target bacteria with LAB supernatant in microtiter plate incubated at 30°C for 48 h [a]

| | | LAB | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Target bacteria | Time (h) | H006-C | H009-F | H010-G | H008-D | H00S-E | H006-A | Control |
| *S. Typhimurium* | 24 | 15.25 | 21.27 | 0.72 | 9.67 | 12.79 | 3.81 | 48.24 |
| | 48 | 16.19 | 24.27 | 1.27 | 9.95 | 21.30 | 9.92 | 101.68 |
| *E. coli* | 24 | 4.76 | 3.85 | 4.10 | 4.35 | 2.12 | 2.21 | 75.00 |
| | 48 | 0.90 | 8.48 | 6.15 | 1.38 | NG | NG | 155.32 |
| *E. aerogenes* | 24 | 14.75 | 17.55 | NG | 1078.00 | 5.55 | 6.58 | 68.69 |
| | 48 | 9.43 | 21.99 | NG | 12.35 | 0.17 | 12.82 | 77.00 |

[a] Growth was measured as OD at 560 nm, NG: No growth

Table 5: Growth percentage of gram negative bacteria with LAB supernatant after heat treatment at 90°C in microtiter plate incubated at 30°C for 48 h[a]

| | | LAB | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Target bacteria | Time (h) | H006-C | H009-F | H010-G | H008-D | H00S-E | H006-A | Control |
| *S. Typhimurium* | 24 | 3.36 | 4.61 | 1.10 | 8.85 | 6.33 | 3.46 | 191.09 |
| | 48 | 12.48 | 14.85 | 19.79 | 17.70 | 9.69 | 12.6 | 257.48 |
| *E. coli* | 24 | NG | 5.30 | NG | 6.86 | 6.06 | NG | 195.87 |
| | 48 | NG | 0.08 | NG | 2.87 | 3.26 | 2.36 | 339.14 |
| *E. aerogenes* | 24 | NG | 4.85 | NG | 4.50 | 2.34 | NG | 239.07 |
| | 48 | NG | 18.41 | NG | 5.20 | NG | 1.99 | 304.64 |

[a] Growth was measured as OD at 560 nm, NG: No growth

Table 6: Growth percentage of gram negative bacteria with LAB supernatant after heat treatment at 121°C in microtiter plate incubated at 30°C for 48 h [a]

| | | LAB | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Target bacteria | Time (h) | H006-C | H009-F | H010-G | H008-D | H008-E | H006-A | Control |
| *S. Typhimurium* | 24 | 1.25 | 1.05 | NG | 3.59 | 2.85 | 2.21 | 201.36 |
| | 48 | 4.87 | 8.66 | 9.47 | NG | 3.64 | 8.41 | 284.43 |
| *E. coli* | 24 | 1.13 | 1.21 | 0.44 | 2.57 | 2.1 | 2.35 | 196.61 |
| | 48 | NG | NG | NG | 5.25 | NG | 0.11 | 345.48 |
| *E. aerogenes* | 24 | NG | 0.3 | NG | 0.51 | NG | 1.37 | 227.99 |
| | 48 | NG | NG | NG | NG | NG | 1.43 | 286.48 |

[a] Growth was measured as OD at 560 nm, NG: No growth

Table 7: Growth percentage of gram negative bacteria with LAB supernatant pH 3 in microtiter plate incubated at 30°C for 48 h [a]

| | | LAB | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Target bacteria | Time (h) | H006-C | H009-F | H010-G | H008-D | H008-E | H006-A | Control |
| *S. Typhimurium* | 24 | NG | NG | 0.67 | 0.31 | NG | 2.62 | 612.50 |
| | 48 | 0.21 | 0.70 | 0.75 | 1.03 | NG | 2.52 | 686.63 |
| *E. coli* | 24 | NG | 2.81 | 1.87 | 3.68 | 1.15 | 3.53 | 93.24 |
| | 48 | 1.95 | 3.25 | 2.09 | 3.48 | 1.94 | 3.53 | 124.77 |
| *E. aerogenes* | 24 | NG | NG | NG | NG | NG | 1.24 | 175.78 |
| | 48 | NG | NG | NG | NG | NG | 0.62 | 323.09 |

[a] Growth was measured as OD at 560 nm, NG: No growt

Table 8: Growth percentage of gram negative bacteria with LAB supernatant pH 5 in microtiter plate incubated at 30°C for 48 h [a]

| | | LAB | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Target bacteria | Time (h) | H006-C | H009-F | H010-G | H008-D | H008-E | H006-A | Control |
| *S. Typhimurium* | 24 | NG | NG | NG | NG | NG | 107.18 | 539.72 |
| | 48 | 1.51 | NG | 81.03 | 357.52 | NG | 463.51 | 608.80 |
| *E. coli* | 24 | 24.11 | NG | 3.90 | 203.93 | 5.05 | 212.51 | 88.63 |
| | 48 | 97.04 | NG | 204.98 | 223.97 | NG | 229.47 | 131.27 |
| *E. aerogenes* | 24 | NG | NG | NG | NG | NG | NG | 100.46 |
| | 48 | NG | NG | NG | NG | NG | 1.01 | 316.24 |

[a] Growth was measured as OD at 560 nm, NG: No growth

Table 9: Growth percentage of gram negative bacteria with LAB supernatant pH 6 in microtiter plate incubated at 30°C for 48 h [a]

| | | LAB | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Target bacteria | Time (h) | H006-C | H009-F | H010-G | H008-D | H008-E | H006-A | Control |
| *E. aerogenes* | 24 | 15.42 | 13.37 | 16.27 | 10.44 | 14.48 | 27.6 | 356.52 |
| | 48 | 20.50 | 19.28 | 14.7 | 22.48 | 18.18 | 51.06 | 645.65 |

[a] Growth was measured as OD at 560 nm, NG: No growth

Table 10: Growth percentage of gram negative bacteria with LAB supernatant after treatment with Proteinase K in microtiter plate incubated at 30°C for 48 h [a]

| | | LAB | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Target bacteria | Time (h) | H006-C | H009-F | H010-G | H008-D | H008-E | H006-A | Control |
| *S. Typhimurium* | 24 | NG | NG | NG | NG | 0.86 | NG | 195.86 |
| | 48 | NG | NG | NG | NG | NG | NG | 178.41 |
| *E. coli* | 24 | 2.61 | 3.86 | NG | 7.70 | 7.19 | 3.65 | 129.97 |
| | 48 | NG | NG | NG | NG | NG | NG | 175.21 |
| *E. aerogenes* | 24 | NG | 0.84 | NG | NG | NG | NG | 163.33 |
| | 48 | NG | NG | NG | NG | NG | NG | 376.40 |

[a] Growth was measured as OD at 560 nm, NG: No growth

Table 11: Growth percentage of gram negative bacteria with LAB supernatant after treatment with RNase II in microtiter plate incubated at 30 °C for 48 h [a]

| | | LAB | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Target bacteria | Time (h) | H006-C | H009-F | H010-G | H008-D | H008-E | H006-A | Control |
| *S. Typhimurium* | 24 | 4.02 | 2.22 | 0.12 | 6.99 | 6.87 | 1.27 | 328.95 |
| | 48 | 2.09 | NG | 0.38 | 3.77 | 1.30 | 0.06 | 371.30 |
| *E. coli* | 24 | 3.76 | 3.62 | NG | 9.93 | 9.13 | 3.79 | 177.36 |
| | 48 | NG | NG | NG | 3.50 | 2.89 | 2.32 | 257.50 |
| *E. aerogenes* | 24 | NG | NG | NG | NG | NG | NG | 163.33 |
| | 48 | NG | NG | NG | NG | NG | NG | 376.40 |

[a] Growth was measured as OD at 560 nm, NG: No growth

Table 12: Phenotypic 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 |

Similarly, growth of *E. coli* ATCC25922 was not inhibited by RNase II treated LAB supernatant H008-D, H008-E and H006-A after 48 h incubation. E. aerogenes was inhibited by all RNase II treated LAB supernatants. This observation suggests that there exist protein- like compounds in these LAB supernatants that have antibacterial activity against *S. Typhimurium* ATCC13311 and *E. coli* ATCC25922 but not against *E. aerogenes*.

**Phenotypic identification of LAB isolates using API 50CHL Kit:** Six randomly selected LAB were identified as *Lactobacillus Acidophilus* 1 from API 50CH test kits and API web. Isolates H006-C, H009-F and H010-G were identified *Lactobacillus Acidophilus* 1 with very good percentage of similarity (above 90%) and were isolated from honey produced in Libya, Saudi Arabia and New Zealand, respectively. Isolates H006-A, H008-D and H008-E were from honey produced in Malaysia and the *L. acidophilus* 1 detected present were poor percentage of similarity (less than 60%) (Table 12).

## DISCUSSION

Honey contains high sugar concentration that normally limits the detection of LAB by normal media such as MRS and tomato juice agar. Modification of media as carried out in this study allows the isolation of LAB from honey which was otherwise difficult. The pre-enrichment in MRS broth overnight allows LAB in honey to resuscitate, together with the addition of 0.8% $CaCO_3$ or 1% glucose to MRS further permits detection and isolation of LAB from honey samples. The high numbers of LAB isolated from MRS media with added 0.8% $CaCO_3$ further support the suitability of this media for the isolation of LAB from food samples as reported by (Panthavee *et al*., 2007).

Earlier reports detected the presence of lactic acid bacteria in raw honey (Forsgren *et al*., 2009; Ruiz-Argueso and Rodriguez-Navarro, 1975; Bahiru *et al*.,

2006; Hosny *et al*., 2009). Additionally, many studies reported the isolation of LAB from stomach of bee, flowers and plants. Recently, Endo *et al*. (2009) isolated lactic acid bacteria from flowers and fruits and identified them as *Lactobacillus kunkeei, Fructobacillus pseudoficulneus* and *Fructobacillus fructosus*. Isolates of lactic acid bacteria, belonging to *Lactobacillus, Lactococcus* and Leuconostoc genera were also isolated from flowers (Tavaria *et al*., 2002), from plant surfaces and plant associated products suggesting that LAB which present in honey may come from plant sources and the bees.

However, the identification of the LAB isolates has not been reported (Lee *et al*., 2008). The LAB isolates from honey in this study were identified *L. acidophilus* 1. The detection of *L. acidophilus* 1 in all the honey samples from different sources reflects the ubiquitous nature of this bacteria and its ability to survive in products with high sugar content and low water activity such as honey.

The presence of multiple resistance pathogenic bacteria has led to the investigation of natural effective alternatives to known antibiotics. Lactic acid bacteria are well known producer of antimicrobial compounds especially bacteriocins which have high antimicrobial activity (Jay, 1982; Klaenhammer, 1993; Piard and Desmazeaud, 1991). All LABs isolated from different honey samples possess very good antimicrobial activity against target Gram negative bacteria with inhibitory zone greater between 7.5±3.53 to 30.3±0.60 mm diameter (Table 3 and Fig 1). The highest activity was obtained from H00-F from Saudi Arabia against *S. Typhimurium* and *E. aerogenes* and H010-G from New Zealand against *E. coli*. Earlier reports showed that *L. acidophilus* isolated from human intestine have antimicrobial activity against a wide range of Gram-negative and Gram-positive pathogens *in vitro* and in vivo (Chauviere *et al*., 1992; Coconnier *et al*., 1993; Coconnier *et al*., 1998).

In contrast, Oh *et al*. (2000) observed that bacteriocin from *L. acidophilus* 30SC obtain from dairy microbiology laboratory did not inhibit the growth of rang of Gram negative bacteria including *K. pneumoiae*, *E. coli* and *S. Typhimurium*. Recently, Gharaei-Fathabad and Eslamifar (2011) reported that strain of Lactobacillus paraplantarum isolated from tea leaves has antimicrobial activity against *E. coli* and *S. Typhimurium*.

Both the cells and cell free supernatants of *L. acidophilus* 1 strains showed antibacterial activity against the target Gram negative bacteria evaluated in which growth was reduced between 45-70% compared to control after 24h of incubation (Table 4). Supernatant H010-G completely inhibited *E. aerogenes* and reduced growth of *S. Typhimurium* ATCC13311, while *E. coli* ATCC25922 was inhibited by supernatant H008-E and H006-A after 48 h incubation. However, supernatants H006-C, H009-F and H008-E were not effective in preventing the growth of *S. Typhimurium* ATCC13311. In contrast, Coconnier *et al*. (1997) reported that supernatant of *L. acidophilus* strain LB from human showed antimicrobial activity against *S. Typhimurium*, *E. coli* and Enterobacter spp. Bacteriocins produced by *L. acidophilus* U1 isolated from pygmy goat meat showed antimicrobial activity against *E. coli* but did not inhibit the growth of *S. Typhimurium* (Ogunbanwo and Okanlawon, 2008).

All the six LAB cell free supernatants were heat stable at 90 and 121ºC for 1 hand the antibacterial activity of LAB supernatants was enhanced after heat treatments. Similarly, Coconnier *et al*. (1997) observed that the antimicrobial activity of supernatant of *L. acidophilus* strain LB from human was heat stable at 110$^{o}$C for 1 and the activity was increased under acidic condition against *S. Typhimurium*, *E. coli*, *Listeria monocytogenes*, Shigella flexneri, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Enterobacter* spp. Ollveira *et al*. (2008) reported that *L. acidophilus* 30SC produced antimicrobial compound that was heat stable at 95°C for 20 minand 50% of activity was lost after heating at 121°C for 20 min Heating supernatant of L casei GC subgroup A isolated from vacuum packaged beef at 100$^{o}$C for 10 min showed antagonistic activity against reference strains *L. acidophilus*, *L. fermentum and L. plantarum* (Oh *et al*., 2000).

The antibacterial activity of strains of *L. acidophilus* 1 supernatants was active under acidic conditions between pH 3-5 against all target Gram negative bacteria (Table 7 and 8). Growth of *E. aerogenes* was reduced by all supernatants even at pH 6 compared to control. Coconnier *et al*. (1997) observed that the antimicrobial activity of supernatant of *L. acidophilus* strain LB from human was increased under acidic condition against *E. coli*, *K. pneumonia*, *S. Typhimurium*, *Shigella flexneri*, L. *monocytogenes*, *Enterobacter* spp and *Pseudomonas aeruginosa*. Oh *et al*. (2000) studied antimicrobial activity of proteinaceous compound produced by *L. acidophilus* 30SC strain, the bacteriocin was completely stable at pH 6 and 7and 50% of activity lost after adjusting to the various pH values between 3 and 10. Maurad and

Meriem (2008) reported that the antimicrobial activity of L. plantarum isolated from camel milk butter was stable at pH 2-6 but the activity was lost at pH 8 against indicator strain of *Lactococcus lactis* B8. Ollveira *et al*. (2008) isolated *Lactobacillus casei* GC subgroup A from vacuum packaged beef and had antagonistic activity against indicator strains of *L. acidophilus, L. fermentum and L. plantarum;* the activity was stable at pH 4-9.

The effect of enzymes RNase and Proteinase K on *L. acidophilus* 1 supernatants showed variable inhibitory activity against *S. Typhimurium*, *E. coli* and *E. aerogenes*. Supernatants H008-E and H008-D (obtained from pure honey from Cameron Highlands, Malaysia) were slightly sensitive to both enzymes Proteinase K and RNase II when tested against *E. coli* and *S. Typhimurium* (Table 10 and 11), indicating the protein-like compound produced by these *L. acidophilus strain*. Coconnier *et al*. (1997) isolated *L. acidophilus* strain LB from human was relatively sensitive to trypsin, proteinase Kand pronase and showed antimicrobial activity against K. *pneumoniae, Enterobacter* spp., *S. Typhimurium, E.coli, L. monocytogenes, S. flexneri* and P. aeruginosa. However, Maurad and Meriem (2008) reported that antibacterial activity of *L. plantarum* isolated from butter made from camel milk against indicator strain of *Lactococcus lactis* B8 was lost when treated with α-chymotrypsin and proteinase K.

## CONCLUSION

This study demonstrates that strains of *L. acidophilus* 1 are present in honey obtained from different sources and further supports the complexity of *L. acidophilus* strains isolated from food such as honey. The antibacterial activity against Gram negative bacteria was variable depending on source of honey, *L. acidophilus* 1strain and target bacteria evaluated. Among the honey studied Manuka honey from New Zealand and Al-Seder honey contains the highest number of LAB compared to the other honey samples from Malaysia and Saudi Arabia. The antibacterial compounds produced by these *L. acidophilus* 1 strains were stable at low pH (3 and 5) and high temperature (90 and 121°C), an important consideration in the preparation of honey for pharmaceutical, preservation and health care applications. Additionally, this study suggests the possible role of LAB in enhancing the antibacterial activity of honey.

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