CHAPTER V

ANTIBACTERIAL ACTIVITY OF LACTIC ACID BACTERIA ISOLATED FROM HONEY AGAINST SELECTED MULTIPLE ANTIBIOTIC RESISTANT (MAR) GRAM-NEGATIVE BACTERIA

5.1 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 & Lulat, (1989) reported that honey is very good inhibitor to *E. 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, S. aureus, B. subtilis* and *Pseudomonas aeruginosa*.

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

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; Tagg *et al.* 1976; Klaenhammer, 1993). Bekele *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. 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.

Salmonella typhimurium and *E. coli* are of the most common type of pathogenic bacteria around the world. *S. typhimurium* causes 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 to 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 (Todar, 2009). 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 (Michael & Abbott, 2006). *E. aerogenes* is known to have drug-resistant characteristics, and the fast development of multidrug resistance strains has become a growingly problem (Neeraja, 2000). Multiresistant strains of *E. aerogenes* have caused outbreaks in intensive care units (ICUs) in France, Belgium, United States and Austria (De Gheldre *et al.* 2001).

Honey is known to have antibacterial activity. Chapter III & IV isolated LAB from honey and was observed to have antibacterial activity against gram positive

phathogenic bacteria. This chapter attempts to evaluate the antibacterial activity of six LAB isolates against multiresistant strains gram negative bacteria.

5.2 Materials and Methods

5.2.1 Antibiotic resistant of target bacteria

S. typhimurium ATCC 13311, *E. coli* ATCC 25922, *E. aerogenes*, *S. marcescens* (ATCC13880) *K. pneumonia* (ATCC 13883) and *S. sonnei* (ATCC 9290) 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 (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).

5.2.2 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.

5.2.3 Determination of antibacterial activity of LAB supernatant using microtiter plates

Cell free supernatant of LAB was obtained from centrifugation (6500 x 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. 100 µl of the supernatant and target bacteria were pipetted into the wells of microtiter plates. 200 µl 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 ELx800 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 x 100.

5.2.4 Effect of heat treatment LAB supernatant on antimicrobial activity

The supernatant of LAB isolates was heat treated at 90 ºC and 121 ºC 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 5.2.3.

5.2.5 Effect of pH adjustment on antimicrobial activity of LAB supernatant

pH of LAB supernatant was adjusted to 3, 5 and 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 5.2.3.

5.2.6 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.

5.3 Results

5.3.1 Antibiotic resistant test of target bacteria

Multiple antibiotic resistant (MAR) patterns of the target bacteria vary with the bacteria and the antibiotic tested (Table 13). MAR index was from 0.4 to 0.5 except *E. aerogenes*. *S. typhimurium* ATCC13311 showed resistance to all the antibiotic especially to bacitracin, cephalothin, penicillin G, vancomycin and strepromycin; *E. coli* ATCC25922 was sensitive to tetracycline, naladixic acid and choloramphenicol but very resistant to bacitracin, penicillin G and vancomycin, and *E. aerogenes* was

sensitive to tetracycline and cholramphinicol but very resistant to nalidixic acid and polymyxin B. *S. sonnei S. marcescens* and *K. pneumoniae* were resistant to bacitracin, cephalothin, pencillin G and vancomycin.

Table 13: Antibacterial activity of selected antibiotics against target pathogenic bacteria measured by diameter of inhibition zone around the discs [a]

Antibiotics	Target bacteria					
	S. Typhimurium	*E. coli*	*E. aerogenes*	*S. marcescens*	*K. pneumoniae*	*S. sonnei*
Bacitracin (10 µm)	0	0	17	0	0	0
Gentamycin (10 µm)	8	10	7	7	11	8
Tetracycline (30 µm)	17	25	22	20	23	18
Naladixic acid (30 µm)	7	21	0	17	25	8
Cephalothin (30 µm)	0	0	12	0	0	0
Polymyxin B (300 µm)	5	6	0	6	5	6
Pencillin G (10 µm)	0	0	16	0	0	0
Vancomycin (5 µm)	0	0	7	0	0	0
Streptomycin (10 µm)	0	15	10	12	15	0
Chloramphenicol (30 µm)	5	20	20	20	20	5
MAR index	0.5	0.4	0.2	0.4	0.4	0.5

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

5.3.2 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 LAB 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 14) (Figure 2). 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, Libya) with

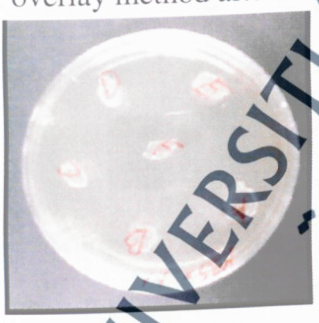
inhibitory zone 30.3±0.60, 30.3±1.41 and 29.3±4.24 mm, respectively. Growth of *E. coli* ATCC2592 and *E. aerogenes* were moderately inhibited by all the isolates; however, these two bacteria were poorly inhibited by H006-C (isolated from Al-Seder honey, Misurata, Libya) with inhibitory zone of 7.5±3.53 and 9.5±1.41mm, respectively.

Table 14: Growth inhibition zone of target bacteria by LAB isolated from honey by dual agar overlay method [a]

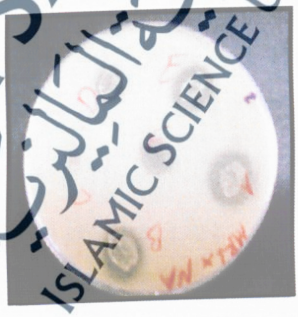
Target bacteria	LAB					
	H006-C	H009-F	H010-G	H008-D	H008-E	H006-A
*S.*Typhimurium	23.2±0.30	30.3±1.41	23.5±0.70	25±2.82	30.3±0.60	29.3±4.24
E. coli	7.5±3.53	14±2.82	18±2.82	12.5±0.70	16.2±0.81	17.5±0.70
E. aerogenes	9.5±1.41	17.5±1.41	16.5±1.41	13.5±2.12	15.5±0.20	12.5±3.53
S. marcescens	7.5±4.24	14.0±0.70	18.0±0.70	12.5±2.82	16.0±0.20	17.5±1.41
K. pneumoniae	19.5±3.53	17.5±2.12	13.5±0.70	21.5±2.12	20±2.82	15.0±0.20
S. sonnei	14.5±7.77	15±5.65	9.0±4.24	19±8.48	22±1.41	14.0±1.41

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

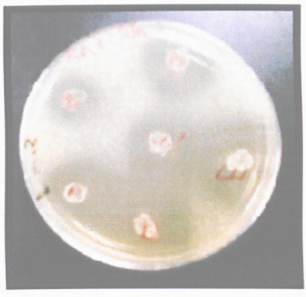
Figure 2: Growth inhibition zone of LAB isolates against pathogenic bacteria by dual agar overlay method after 24 h incubated at 30 °C



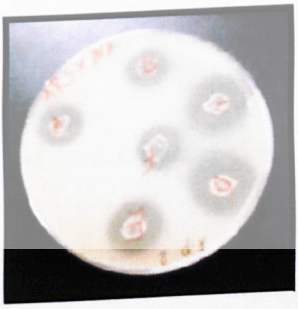
Inhibitory zone of LAB against *S.* Typhimurium



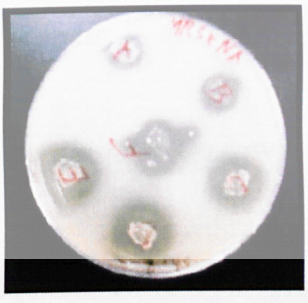
Inhibitory zone of LAB against *E. coli*



Inhibitory zone of LAB against *S. marcescens*



Inhibitory zone of LAB against *K. pneumoniae*



Inhibitory zone of LAB against *Shigella sonnei*

5.3.3 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 15). 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 LAB strains vary with targeted bacteria and the antibacterial compounds produced by these LAB strains.

Table 15: Growth percentage of target bacteria with LAB supernatant in microtiter plate incubated at 30 °C for 48 h [a]

Target bacteria	Time (h)	LAB						
		H006-C	H009-F	H010-G	H008-D	H008-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	75.40
	48	0.90	8.48	6.15	1.38	NG	NG	155.32
E. aerogenes	24	14.75	17.55	NG	10.78	5.55	6.58	68.69
	48	9.43	21.99	NG	12.35	0.17	12.82	77.00
S. marcescens	24	15.25	21.27	0.72	9.67	12.79	3.81	48.24
	48	8.98	141.10	NG	10.64	6.40	8.40	90.32
K. pneumoniae	24	8.52	1.06	3.49	11.17	5.17	3.09	80.52
	48	8.77	123.96	3.49	10.15	3.20	11.62	146.08
S. sonnei	24	7.01	0.50	0.60	12.48	5.92	7.27	44.87
	48	3.85	72.17	NG	3.74	NG	9.94	76.06

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

5.3.4 Effect of heat treatment on antimicrobial activity of LAB supernatant

Antibacterial activity of LAB supernatants were further enhanced by heating at 90 °C and 121 °C for 1 h compared to control (Table 16 & 17). 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.*

Table 16: 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]

Target bacteria	Time (h)	LAB						
		H006-C	H009-F	H010-G	H008-D	H008-E	H006-A	Control
*S.*Typhimurium	24	3.36	4.61	1.10	8.85	6.33	3.46	191.09
	48	12.86	14.85	19.79	17.70	9.69	12.60	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
S. marcescens	24	0.30	1.18	NG	6.02	6.77	1.60	202.67
	48	NG	NG	NG	100.15	3.01	1.06	366.55
K. pneumoniae	24	0.22	1.37	NG	6.34	6.76	NG	175.08
	48	0.37	NG	NG	1.79	1.81	NG	288.61
S. sonnei	24	NG	0.86	NG	2.91	1.60	1.87	361.12
	48	NG	NG	NG	0.81	NG	3.34	569.21

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

Table 17: 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]

Target bacteria	Time (h)	LAB						
		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	2.10	2.35	196.61
	48	NG	NG	NG	5.25	NG	0.11	345.48
E aerogenes	24	NG	0.30	NG	0.51	NG	1.37	227.99
	48	NG	NG	NG	NG	NG	1.43	286.48
S. marcescens	24	1.82	0.35	NG	1.90	3.17	2.49	217.22
	48	NG	NG	1.02	NG	1.56	0.55	389.16
K. pneumoniae	24	0.44	0.35	NG	2.63	2.81	0.26	193.05
	48	NG	NG	NG	0.87	0.09	NG	221.98
S. sonnei	24	NG	0.46	NG	0.97	0.97	1.63	367.95
	48	NG	NG	NG	NG	NG	1.01	566.81

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

5.3.5 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* 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 18). 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 19), and similarly percent growth of *S.* Typhimurium ATCC13311 increased in supernatants H010-G, H008-D and H006-A at similar pH. However, at pH 6 all LAB supernatants lost their antibacterial activity against *S.*

Typhimurium ATCC13311 and *E. coli* ATCC25922 (data not shown) except *E. aerogenes* (Table 20).

Table 18: Growth percentage of gram negative bacteria with LAB supernatant pH 3 in microtiter plate incubated at 30 °C for 48 h [a]

Target bacteria	Time (h)	LAB						
		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	3.58	124.77
E. aerogenes	24	NG	NG	NG	NG	NG	1.24	175.78
	48	NG	NG	NG	NG	NG	0.62	323.09
S. marcescens	24	2.22	1.63	1.89	3.52	2.81	5.97	107.41
	48	2.69	2.15	1.74	5.17	3.49	4.57	160.63
K. pneumoniae	24	1.48	2.01	0.85	0.85	2.47	1.00	49.34
	48	2.26	2.17	NG	2.01	2.06	1.59	91.38
S. sonnei	24	2.24	1.00	1.15	1.80	0.88	0.5	340.37
	48	2.56	2.59	0.46	1.72	1.61	0.83	493.33

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

Table 19: Growth percentage of gram negative bacteria with LAB supernatant pH 5 in microtiter plate incubated at 30 °C for 48 h [a]

Target bacteria	Time (h)	LAB						
		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	608.8
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
S. marcescens	24	NG	NG	NG	211.01	NG	241.81	102.07
	48	102.06	NG	176.71	302.77	NG	280.60	154.63
K. pneumoniae	24	NG	NG	NG	NG	NG	167.48	86.63
	48	100.19	NG	145.15	239.37	NG	235.61	280.61
S. sonnei	24	18.19	NG	NG	6.31	NG	3.32	314.66
	48	17.85	NG	18.35	6.95	NG	5.69	487.21

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

Table 20: Growth percentage of gram negative bacteria with LAB supernatant pH 6 in microtiter plate incubated at 30 °C for 48 h [a]

Target bacteria	Time (h)	LAB						
		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

5.3.6 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 (Tables 21 & 22). While proteinase K treated LAB supernatant showed antibacterial activity against all target bacteria, all RNase II treated LAB supernatants, however, allowed growth of *S.* Typhimurium ATCC13311 after 48 h incubation (Table 22). 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. In contrast *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.*

Table 21: 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]

Target bacteria	Time (h)	LAB						
		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
S. marcescens	24	3	NG	0.38	10	10.33	4.89	128.58
	48	NG	NG	NG	0.47	0.86	2.752	160.51
K. pneumoniae	24	NG	NG	66.96	NG	NG	NG	219.05
	48	1.91	2.25	NG	5.12	5.68	2.19	145.24
S. sonnei	24	NG	NG	NG	NG	NG	NG	198.77
	48	NG	NG	5.84	NG	NG	NG	241.30

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

Table 22: 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]

Target bacteria	Time (h)	LAB						
		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	0.60	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	257.5
E. aerogenes	24	NG	NG	NG	NG	NG	NG	163.33
	48	NG	NG	NG	NG	NG	NG	376.40
S. marcescens	24	0.38	0.15	NG	8.27	9.60	1.37	134.56
	48	NG	NG	NG	2.45	3.46	0.96	175.18
K. pneumoniae	24	2.21	103.28	NG	8.49	6.58	2.02	165.35
	48	NG	96.71	NG	3.59	NG	0.90	250.40
S. sonnei	24	4.94	NG	3.28	4.60	6.70	2.75	425.29
	48	4.94	NG	2.06	4.11	7.15	4.66	471.22

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

5.4 Discussion

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 & Desmazeaud ,1991). All LABs isolated from different honey samples possess antimicrobial activity against target Gram negative bacteria with inhibitory zone between 7.5±3.53 to 30.3±0.60 mm diameter by dual overlay method (Table 14 & Figure 2). 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*. Recenty, Fathabad & Eslamifar *et al.* (2011) reported that strain of *Lactobacillus paraplantarum* isolated from tea leaves has antimicrobial activity against *E. coli* and *S.* Typhimurium. 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* (Chauvie`re *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 gram negative bacteria including *K. pneumoniae*, *E. coli* and *S.* Typhimurium. However, in this study all the LAB isolates from honey showed inhibitory activity against *K. pneumoniae*.

The cell free supernatants of LAB strains showed antibacterial activity against the target Gram negative bacteria evaluated in which growth was reduced between 45 to 70% compared to control after 24 h of incubation (Table 15). 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 & Okanlawon, 2008).

All the six LAB cell free supernatants were heat stable at 90 and 121 °C for 1 h, and the antibacterial activity of LAB supernatants was enhanced after heat treatments (Tables 16 & 17). Similarly, Coconnier *et al*. (1997) observed that the antimicrobial activity of supernatant of *L. acidophilus* strain LB from human was heat stable at 110 °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. Oliveira *et al*. (2008) reported that *L. acidophilus* 30SC produced antimicrobial compound that was heat stable at 95°C for 20 min, and 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 °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 LAB supernatants was active under acidic conditions between pH 3 to 5 against all target Gram negative bacteria (Tables 18 & 19). Growth of *E. aerogenes* was reduced by all supernatants even at pH 6 compared to control (Table 20). 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. pneumoniae*, *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 7, and 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 to 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* and the activity was stable at pH 4 to 9.

The effect of enzymes RNase and Proteinase K on 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* (Tables 21 & 22), indicating the protein-like compound produced by these strains. Coconnier *et al.* (1997) isolated *L. acidophilus* strain LB from human was relatively sensitive to trypsin, proteinase K, and 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.

5.5 Conclusion

This study demonstrates that strains of LAB in honey, both the cells and the supernatants have good inhibitory properties against MAR gram negative pathogens.

The antibacterial compounds produced by these LAB *L.* 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.