CHAPTER IV

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

4.1 Introduction

The continuous use of antibiotics as antimicrobial chemotherapy for more than fifty years ago has been a leading reason for the increase of average life expectancy. However, disease-causing microbes that have become resistant to antibiotic drug therapy have become an increasing public health problem world-wide (Hsueh *et al.* 2005). To encounter such bacterial resistance to antibiotics, natural health remedies and supplements have been studied extensively in finding potential antimicrobial properties as alternative therapeutic agents (Mandal *et al.* 2010). Honey has been used since ancient times as food and become an effective natural remedy for certain ailments, such as, treatments of some respiratory diseases and for the healing of skin wounds (Basualdo *et al.* 2007). The therapeutic property of honey has received wide recognition from the medical field (Zumla & Lulat, 1989).

It is believed that naturally occurring lactic acid bacteria from different sources are known to produce different compounds that have the ability to inhibit the growth of both bacteria and fungi (Muhialdin *et al.* 2011a & b). Antimicrobial activity of both heated (100 °C for 10 min) and pH adjusted (4 to 9) cell free supernatant of lactic acid bacteria isolated from vacuum packaged beef was found to inhibit the growth of *L. acidophilus* indicating LAB are bacteriocin producers (Oliveira *et al.* 2008).

Staphylococcus aureus is a commensal bacterium that colonizes, in addition to the nose, the pharynx, axillae, vagina, and skin surfaces (Lowy, 1998). It can survive on disciplined animals, such as dogs, cats, and horses. This bacterium may stay alive for hours to weeks, some cases for months on dry environmental surfaces depending on the strain susceptibility to environmental conditions (Cimolai, 2008). *S. aureus* also

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can infect infants where it can cause a severe disease- staphylococcal scalded skin syndrome (Curran & Al-Salihi, 1980). *S. epidermis* could develop biofilm on intravenous catheters and on medical prostheses (Hedin, 1993), and can survive on plastic plates (Otto, 2009). The contaminated medical devices by this microorganism may easily infect patients in the hospital, especially dialysis patients. Bacteria that adhere to different devises and form biofilms are more resistant to known antibiotic, where it could eventually cause infections in the implemented devises in the body (Philip & William, 2001). *B. subtilis* is not a human pathogen, but it could contaminate several foods and may results in food poisoning but in rare cases. It produces proteolytic enzyme subtilisin. *B. subtilis* spores are very difficult to be killed by heating during cooking and it may spoil bread dough (Ryan and Ray, 2004). People who suffer from infection due to the contamination by these Gram-positive bacteria were commonly treated by antibiotics by medical practitioners. Therefore, these target pathogenic bacteria may easily become multiple antibiotic resistant (MAR).

Malaysian local honey has been reported to have antibacterial effect (Aljadi & Yusoff, 2003; Tan *et al.* 2009). Gelam honey and coconut honey contained non-peroxide factors, such as phenolic acids and these honey were shown to inhibit the growth of *E. coli* and *S. aureus* (Aljadi & Yusoff, 2003). Similarly, Tualang honey has been shown to inhibit the multidrug resistant *Acinetobacter baumanni* and *Stenotrophomonas maltophilia* (Tan *et al.* 2009). However, lack of study has been investigated in detail on the potential antibacterial activity from honey marketed in Malaysia against *S. aureus*, *S. epidermis* and *B. subtilis*. Therefore, this chapter reports both the cells and their supernatants were evaluated for their antimicrobial activities against selected multi-antibiotic resistant (MAR) Gram-positive bacteria. Information obtained from this study may provide an insight to the possible contribution of the naturally present LAB in honey for their antimicrobial activities and possibly the medical benefits of honey.

4.2 Materials and methods

4.2.1 Antibiotic resistant of selected target bacteria

Staphylococcus aureus ATCC25923, *Staphylococcus epidermis* ATCC12228 and *Bacillus subtilis* ATCC21332 were used as target bacteria and tested for their

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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) (Sigma). The selection of antibiotics used in this study was based on the common antibiotics used in medical practice and health therapy.

4.2.2 Antimicrobial activity of LAB isolates using dual agar overlay method

Antimicrobial activity of six LAB isolates was determined against target bacteria using the 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 with 10^6 CFU/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 of diameter of inhibitory zones was taken.

4.2.3 Determination of antibacterial activity of LAB supernatant using microtiter plates

Cell free supernatant 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 (Oxoid) was prepared and mixed with the target bacteria containing 10^4 CFU/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 without the addition of supernatant was used as positive control. All microtiter plates were incubated at 30 °C for 24, 48, and 72 h. Bacterial growth was monitored at OD_{560} of MRS broth using BioTek ELx800 ELISA reader. The analysis was carried out in duplicates and the mean of optical density was taken. The percentage growth of target bacteria was calculated using the formula established by Muhialdin *et al*., 2011a: [OD 560 nm with bacteria/supernatant after incubation – OD560 nm of MRS broth with the bacteria at time 0h] / [OD560 nm of MRS broth with the bacteria at time 0h] x 100.

4.2.4 Effect of heat treatment of LAB supernatant on antimicrobial activity

Cell free supernatants were 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 72 h and bacterial growth was monitored at 24 h interval. Percentage growth of target bacteria was calculated as described in section 4.2.3.

4.2.5 Effect of enzymes on antimicrobial activity of LAB supernatant

The cell free supernatants were treated with Proteinase K and RNase II separately. 1 μl 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 72 h and bacterial growth was monitored at 24 h interval. Percentage growth of target bacteria was calculated as described in section 4.2.3.

4.2.6 Effect of pH adjustment on antimicrobial activity of LAB supernatant

pH of cell free supernatants were adjusted to 3, 5 and 6 using drops of HCl (0.1 N) and NaOH (0.2 N), then tested against the target bacteria. The microtiter plates were incubated at 30 °C for 72 h and bacterial growth was monitored at 24 h interval. Percentage growth of target bacteria was calculated as described in section 4.2.3.

4.3 Results

4.3.1 Antibiotic resistant test of target bacteria

The target bacteria showed high resistance to several antibiotics tested. The diameter of inhibition zone varies between 8 to 25 mm, MAR index was from 0.4 except *B. subtilis* (Table 4). *S. aureus* and *S. epidermis* were resistant to tetracycline, naladixic acid, polymyxin B and chloramphenicol. The three target bacteria were not inhibited by cephalothin while *S. epidermis* was resistant to gentamycin and bacitracin.

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Table 4: The antibacterial activities of selected antibiotics against target bacteria[a]

Antibiotics	Target bacteria		
	S. aureus ATCC25923	*S. epidermis* ATCC12228	*B. subtilis* ATCC21332
Bacitracin (10 µm)	19	5	25
Gentamycin (10 µm)	14	2	10
Tetracycline (30 µm)	0	0	15
Naladixic acid (30 µm)	0	0	13
Cephalothin (30µm)	5	5	4
Polymyxin B (300 µm)	0	0	15
Penicillin G (10 µm)	20	10	25
Vancomycin (5µm)	25	21	15
Streptomycin (10 µm)	10	8	7
Chloramphenicol (30 µm)	0	0	11
MAR index	0.4	0.4	0

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

4.3.2 Antimicrobial activity of lactic acid bacteria isolates against target bacteria by dual agar overlay method

All the six LAB isolates showed different inhibitory activities against the target bacteria by the dual agar overlay method. *S. aureus* was greatly inhibited by all LAB isolates as shown by the inhibitory zone greater than 25 mm while *S. epidermis* and *B. subtilis* were inhibited but to a lesser inhibitory effects (Table 5, Figure 1). LAB H009-G isolated from manuka honey, New Zealand showed the highest inhibitory activity, followed by LAB H009-F from Saudi Arabia, then LAB H006-A from Al-Seder honey, Libya and LAB H008-E from Cameron Highland, Malaysia against *S. aureus*.

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Table 5: Growth inhibition zone of target bacteria by LAB isolated from honey by dual agar overlay method[a]

Pathogenic bacteria	LAB					
	H006-A	H006-C	H008-D	H008-E	H009-F	H010-G
S. aureus	28.0±0.25	25.0±0.20	26.0±0.25	28.0±0.25	30.0±2.82	32.0±0.25
S. epidermis	15.5±9.19	18.5±4.94	21.5±2.12	20.0±2.82	18.5±3.53	14.0±1.41
B. subtilis	16.5±0.70	11.5±0.70	16.5±2.12	16.5±0.70	13.5±0.70	18.5±3.53
*S.*Typhimurium	29.3±4.24	23.2±0.30	25±2.82	30.3±0.60	30.3±1.41	23.5±0.70
E. coli	17.5±0.70	7.5±3.53	12.5±0.70	16.2±0.81	14±2.82	18±2.82
E. aerogenes	12.5±3.53	9.5±1.41	13.5±2.12	15.5±0.20	17.5±1.41	16.5±1.41
S. marcescens	17.5±1.41	7.5±4.24	12.5±2.82	16.0±0.20	14.0±0.70	18.0±0.70
K. pneumoniae	15.0±0.20	19.5±3.53	21.5±2.12	20±2.82	17.5±2.12	13.5±0.70
S. sonnei	14.0±1.41	14.5±7.77	19±8.48	22±1.41	15±5.65	9.0±4.24

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

Figure 1: 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. aureus* | Inhibitory zone of LAB against *S. epidermis* | Inhibitory zone of LAB against *B. subtilis*

4.3.3 Growth inhibition of target bacteria in microtiter plate

Percentage growth of target bacteria were reduced in the range of 40 to 80% by the supernatant of all LAB isolates compared to control within 24 h incubation

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(Table 6). Supernatant H008-E caused complete inhibition of all target bacteria, H010-G inhibited *S. epidermis* and *B. subtilis*, and H008-D also inhibited the growth of *S. aureus* during 72 h incubation. However, some LAB supernatant allowed growth of the target bacteria as shown by H009-F against *S. aureus* after 24 h incubation and H010-G against *S. epidermis* after 48 h incubation.

Table 6: Percentage growth of target bacteria in the presence of LAB supernatant in microtiter plate incubated at 30 °C for 72 h[a]

Target bacteria	Time (h)	LAB						
		H006-C	H009-F	H010-G	H008-D	H008-E	H006-A	Control
S. aureus	24	24.51	0.30	3.51	2.49	NG	2.01	77.04
	48	5.06	NG	2.20	0.24	7.17	2.41	120.75
	72	9.51	1.71	NG	NG	81.49	2.81	151.57
S. epidermis	24	3.74	6.79	9.14	NG	6.75	NG	49.70
	48	4.44	7.22	6.38	NG	24.60	NG	76.16
	72	5.49	6.91	7.73	NG	17.68	5.11	98.68
B. subtilis	24	4.41	10.11	8.94	7.52	8.22	NG	88.58
	48	1.33	2.81	5.90	NG	25.21	NG	129.30
	72	3.62	26.22	7.22	NG	18.65	NG	157.75

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

4.3.4 Effect of heat treatment on antimicrobial activity of supernatant

Heating the supernatant at 90 and 121 °C for 1 h resulted in growth reduction of all the target bacteria compared to control after 24 h incubation (Tables 7 and 8). It seems that heating resulted in better inhibitory activity of supernatants H006-C, H008-D, H008-E, H009-F and H010-G against *S. aureus*. *B. subtilis* was inhibited by supernatant H009-F and H010-G after heating at 90 and 121°C for 1 h. However, heating at 121 °C destroyed the inhibitory activity of H006-C but generated inhibitory activity in H008-E against *B. subtilis*.

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Table 7: Growth percentage of Gram-positive pathogenic bacteria with LAB supernatant after heat treatment at 90 °C measured in microtiter plate incubated at 30 °C for 72 h[a]

Target bacteria	Time (h)	LAB						
		H006-C	H009-F	H010-G	H008-D	H008-E	H006-A	Control
S. aureus	24	7.89	2.49	2.54	1.56	0.26	NG	221.32
	48	16.20	NG	NG	NG	NG	NG	249.12
	72	12.46	NG	NG	NG	NG	74.59	315.03
S. epidermis	24	5.72	6.49	5.06	5.03	6.49	8.30	219.13
	48	13.34	13.74	10.45	9.75	19.98	26.09	348.44
	72	15.33	21.77	16.73	15.47	34.40	171.82	428.10
B. subtilis	24	NG	NG	4.31	4.63	0.14	NG	108.16
	48	1.58	NG	0.93	1.14	NG	NG	262.58
	72	0.34	NG	0.14	0.21	NG	NG	311.06

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

Table 8: Growth percentage of Gram-positive pathogenic bacteria with LAB supernatant after heat treatment at 121 °C measured in microtiter plate incubated at 30 °C for 72 h[a]

Target bacteria	Time (h)	LAB						
		H006-C	H009-F	H010-G	H008-D	H008-E	H006-A	Control
S. aureus	24	7.25	1.61	NG	0.21	NG	NG	220.31
	48	12.54	NG	NG	NG	NG	NG	258.33
	72	8.72	NG	NG	NG	NG	NG	296.52
S. epidermis	24	4.83	4.14	4.98	12.66	2.88	NG	211.44
	48	13.51	8.23	8.02	5.69	11.94	10.23	353.87
	72	14.94	14.80	10.85	8.03	16.81	36.90	425.58
B. subtilis	24	2.28	1.85	1.38	0.04	NG	69.38	2.287
	48	0.30	6.90	2.19	NG	NG	NG	314.28
	72	0.30	6.17	1.70	NG	NG	NG	304.59

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

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4.3.5 Enzymes sensitivity of LAB supernatant

Generally, treatment of supernatants with enzymes proteinase K and RNase II resulted in different antimicrobial activity against target bacteria. Treating supernatant with proteinase K and RNaseII did not destroy the antimicrobial activity of H006-A against *S. aureus* indicating that the supernatant H006-A contained protein-like antimicrobial compounds against this bacteria (Tables 9 and 10). The antimicrobial activity against *B. subtilis* was diminished in all RNase II treated supernatants; while the antimicrobial activity against *S. epidermis* was diminished by similarly treated supernatant H006-A, H008-D, H008-E and H010-G. The result from this study indicates that different strains of *L. acidophilus* present in honey produce different types of antimicrobial compounds and with different killing mechanisms against the target bacteria.

Table 9: Growth percentage of Gram-positive pathogenic bacteria with LAB supernatant after treatment with Proteinase K measured in microtiter plate incubated at 30 °C for 72 h[a]

Target bacteria	Time (h)	LAB						
		H006-C	H009-F	H010-G	H008-D	H008-E	H006-A	Control
S. aureus	24	7.23	3.03	3.67	6.13	NG	NG	35.74
	48	7.85	NG	NG	NG	NG	NG	42.54
	72	NG	NG	NG	NG	NG	15.33	72.23
S. epidermis	24	NG	NG	NG	NG	NG	NG	288.66
	48	NG	NG	NG	NG	NG	NG	320.07
	72	NG	NG	NG	NG	NG	6.65	324.44
B. subtilis	24	NG	NG	NG	NG	NG	NG	242.10
	48	NG	NG	NG	NG	NG	NG	327.81
	72	NG	NG	NG	NG	NG	NG	425.31

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

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Table 10: Growth percentage of Gram-positive pathogenic bacteria with LAB supernatant after treatment with RNase II measured in microtiter plate incubated at 30 °C for 72 h[a]

Target bacteria	Time (h)	LAB						
		H006-C	H009-F	H010-G	H008-D	H008-E	H006-A	Control
S. aureus	24	3.11	NG	0.37	1.65	NG	NG	109.04
	48	7.69	NG	NG	0.08	NG	NG	145.24
	72	4.91	NG	NG	NG	NG	1.06	175.79
S. epidermis	24	1.71	NG	8.58	18.17	NG	NG	262.16
	48	6.34	NG	6.36	14.11	NG	6.95	289.15
	72	5.83	1.34	3.93	8.65	NG	12.36	300.99
B. subtilis	24	0.23	0.09	0.09	0.20	0.65	NG	302.35
	48	0.71	0.78	1.66	0.70	0.98	0.14	405.64
	72	0.15	0.19	0.58	NG	0.32	NG	464.47

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

4.3.6 pH sensitivity of supernatant on antimicrobial activity

Adjusting the pH of crude supernatant to pH 3 inhibited the growth of *S. aureus* and *S. epidermis* compared to the control during 72 h incubation. Growth of *B. subtilis* was completely inhibited by all the supernatant at pH 3 (Table 11). Growth inhibition at pH 5 was not observed by all supernatants except H008-E and H009-F which inhibited the growth of *B. subtilis* and *S. aureus* and *B. subtilis* after 72 h incubation (Table 12).

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Table 11: Growth percentage of Gram-positive pathogenic bacteria with LAB supernatant after adjusting pH to 3 measured 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. aureus	24	8.88	1.83	4.62	7.81	4.93	6.03	78.20
	48	7.48	1.94	4.16	3.02	3.70	4.24	105.57
	72	6.17	1.55	3.56	5.14	3.22	3.49	115.54
S. epidermis	24	1.96	1.52	2.25	1.76	2.33	2.03	310.83
	48	2.31	2.34	2.18	2.08	2.66	1.72	385.60
	72	2.94	2.59	2.63	2.56	2.25	1.40	365.94
B. subtilis	24	NG	NG	NG	NG	NG	NG	449.83
	48	NG	NG	NG	NG	NG	NG	662.79
	72	NG	NG	NG	NG	NG	NG	681.06

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

Table 12: Growth percentage of Gram-positive pathogenic bacteria with LAB supernatant after adjusting pH to 5 measured in microtiter plate incubated at 30 °C for 24, 48 and 72 h[a]

Target bacteria	Time (h)	LAB						
		H006-C	H009-F	H010-G	H008-D	H008-E	H006-A	Control
S. aureus	24	233.41	7.38	199.92	NG	NG	7.10	68.45
	48	279.88	73.49	273.73	9.95	NG	191.80	100.67
	72	329.15	196.67	298.57	194.04	193.96	209.68	100
S. epidermis	24	68.31	10.92	68.5	10.44	11.47	22.24	308.66
	48	95.37	10.92	76.1	7.86	11.91	24.01	391.33
	72	77.36	13.01	73.3	2.72	6.43	20.47	361.76
B. subtilis	24	NG	35.27	3.55	NG	NG	NG	297.35
	48	8.32	35.92	49.50	NG	NG	0.086	640.59
	72	178.35	52.59	64.92	NG	NG	274.93	708.91

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

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4.4 Discussion

The occurrence of MAR bacterial strains is of public health concern because the bacteria are not easily killed by normal antibiotics used for health therapy. *S. aureus* ATCC25923 and *S. epidermis* ATCC12228 used in this study demonstrated MAR to tetracycline, naladixic acid, polymyxin B and chloramphenicol, and *S. epidermis* was also resistant to gentamycin. All the target bacteria were not completely inhibited by cephalothin. Some strains of *Staphylococcus* species were resistant to several antibiotics (Salvatore *et al*. 2010); *S. aureus* was resistant to ampicillin, kanamycin and oxytetracycline, while *S. epidermis* showed resistant to ampicillin, ceftriaxone, ceftriaxone, kanamycin, cloxacillin, ofloxacin and oxytetracycline. Both the cells and supernatants of LAB isolated from honey samples could inhibit the growth of the MRA bacteria. The antimicrobial activity in this study was carried out longer than 24 h than that normally carried by other researchers (Oliveira *et al.* 2008) to determine the bacteriostatic or bactericidal effect. *L. acidophilus* supernatant showed bactericidal effect against the MAR target bacteria especially *S. aureus*. LAB isolates from New Zealand honey (H010-G) and Saudi Arabia honey (H009-F) showed good inhibition against the growth of *S. aureus* with diameter of inhibition zone between 30 and 32 mm. Marie-Hé Lé Ne *et al*., (1997) observed that *L. acidophilus* LB isolated from human stool specimen produced antibacterial activity against *S. aureus* and *B. cereus,* but did not inhibit lactobacilli and bifidobacteria. Similarly, Mohammed, (2010) observed that *L. acidophilus* isolated from stock culture of yogurt in Iraq showed antimicrobial activity against Gram-positive bacteria; *S. aureus*, *S. epidermis* and bacilli that cause burn wound infections. 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).

The effect of heat on the antimicrobial activity of bacteriocin produced by *L. acidophilus* varies with strains. In this study, the antibacterial activities of all the supernatants were not affected by heating at 90 °C for 1 h, but slightly affected by heating at 121 °C for 1 h (Tables 7 and 8). Similarly, Marie-He´Le`Ne *et al*. (1997) reported that antimicrobial activity of supernatant from human *L. acidophilus* LB was

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not affected by heat treatment at 110 °C for 1 h, while *L. acidophilus* 11088 produced bacteriocin Lactacin F that was more heat resistant and exhibits a broader spectrum of antimicrobial activities (Muriana & Klaenhammer, 1991; Zaheer, *et al*. 2009). In contrast, *L. acidophilus* LAPT 1060 produces heat-labile bacteriocin contrary to Lactacin B and Lactacin F (Toba *et al*. 1991). *L. acidophilus* strain isolated from a commercial dairy produced a bacteriocin, acidocin CH5 and was relatively heat stable at 121°C for 20 min but75% of the activity was lost by the heat treatment (Chumchalova *et al*. 1995; Zaheer, *et al*. 2009). Similarly, Oliveira *et al*. (2008) reported that lactic acid bacteria from vacuum packaged beef and the cell free supernatant of most of the isolates had antimicrobial activity after heating at 100 °C for 10 min.

The antimicrobial activity of LAB supernatants are affected by enzyme treatment (Marie-He´Le`Ne *et al*. 1997; Kacem and Kaid, 2008). It was observed that supernatant H006-A, H008-D and H008-E contained compounds that were sensitive to RNase II (Table 10). The antimicrobial activity of all the supernatant was completely lost when treated with proteinase K except supernatant H006-A and H010-G (Table 9) indicating that other forms of antimicrobial compounds were produced by different LAB isolates. The protein-like compounds present in the supernatant H006-A, H008-D and H008-E demonstrated growth inhibition to *S. epidermis* while H006-A was inhibitory only to *S. aureus*. Similarly, Kacem and Kaid (2008) reported that the antibacterial activity of *L. plantarum* SH24 and *L. plantarum* SH12 isolated from butter varies with the enzyme treatment; complete loss of antibacterial activity was observed when the supernatant was treated with proteinase K and chymotrypsin, but partially inactivated after treatment with pronase E and trypsin. Marie-He´Le`Ne *et al*. (1997) observed that human *L. acidophilus* showed antimicrobial activity against *S. aureus* and the activity decreased slightly when treated with trypsin, pronase, and proteinase K.

A broad antibacterial spectrum substance produced by *L. acidophilus*, lactocidin was reported by Vincent *et al*. as early as 1959. Since then, proteinaceous bacteriocin and bacteriocin-like compounds have been identified produced by *L. acidophilus* and have been found to have potent antimicrobial activities toward closely harmful microorganisms (Zaheer *et al*. 2009). Additionally, LABs are known to produce

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carbon dioxide, lactic acid, acetic acid, caproic acid, 3-hydroxy fatty acids, phenyllactic acid, hydrogen peroxide, reuterin, fungicins, cyclic dipeptides and diacetyl that have antimicrobial activity against different microorganisms (Messenes and De Vuyst, 2002; Lindgren and Dobrogosz, 1990).

pH adjustment influences the antimicrobial activity of LAB supernatant. It was observed that adjusting the supernatant to pH 3 maintains the antimicrobial activity (Table 11). The antimicrobial activity was lost at pH 5 for supernatant H006-A and H008-D but variable activity for the other supernatants (Table 12), nevertheless, complete loss of activity was observed at pH 6. Similarly, Nirunya *et al.* (2008) observed that lactic acid bacteria strains of *Pediococcus pentosaceus* SL4, *Enterobacter faecium* SF and *Pediococcus pentosaceus* LM2 isolated from gastrointestinal tracts of fish, shellfish and shrimp showed antimicrobial activity against *L. monocytogenes* and *S. aureus*, but the activity was lost when pH of the supernatant was adjusted to 6.5 to 7.0. In other study, Kacem and Kaid (2008) also reported that lactic acid bacteria strains of *L. plantarum* from Algerian camel milk butter produced antimicrobial compounds that were stable between pH 2 to 6, but the activity was totally lost at pH 8 against indicator strain of *Lactococcus lactis* B8. In contrast, Oliveira *et al.* (2008) observed that the antimicrobial activity of cell free supernatant of lactic acid bacteria isolated from vacuum packaged beef was stable between pH 4 to 9 against indicator strain *L. acidophilus*. This further supports that LAB produce different antimicrobial compounds that function best at different pH. The cell free supernatant of H006A (from Libya) H008-D and H008-E (from Malaysia) produced antimicrobial compounds that are protein-like and stable at pH 3 and heating at 90 ºC for 1 h.

4.5 Conclusion

This study observed that LAB from different sources of honey have varying antibacterial activity against MAR gram positive bacteria. Growth of MAR bacteria especially *S. aureus* was readily inhibited by either the cells or cell-free supernatants of all the LAB isolates. Additionally, LAB isolates from Libyan and Malaysian honey produced antimicrobial compounds that are stable to heating, enzymes treatment and pH adjustments. Antimicrobial compounds produced by these naturally present LAB may play important role in enhancing the antimicrobial properties and the medicinal

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benefit and value of honey. The presence of LAB in honey could be one of the criteria worth considering in the selection of honey for pharmaceutical, health care and food product applications.

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