CHAPTER III

ISOLATION OF LACTIC ACID BACTERIA FROM HONEY MARKETED IN MALAYSIA

3.1 Introduction

Honey contains fructose (18.4%), glucose (30.3%), sucrose (1.3%), other carbohydrates (12%), minerals (0.169%), protein (169 mg/100g) with water content of about 17.2% (White *et al.*, 1962). pH of honey ranges from 3.4 to 6.1 with an average of 3.9, while the water activity varies between 0.5 and 0.6 (White *et al.* 1962). The high sugar content but a low water activity and acidity in honey are unfavorable for microbial growth. Honey can inhibit the growth of a wide range of bacteria, fungi, protozoa and viruses (Molan, 1992a; Cooper *et al.* 2002; Blair and Carter, 2005). It is suggested that the acidity and enzymatic generation of hydrogen peroxide (Molan, 1992b) and the aromatic acids and phenolic compounds present contribute to the antimicrobial activity of honey (Weston and Brocklebank, 1999; Weston *et al.*, 1999). The antibacterial properties of honey were reviewed in depth by Molan (1992a & b) and again briefly by Armstrong and Otis (1995), McCarthy (1995) and Molan (1995).

The microorganisms in honey has been reviewed by Snowdon and Cliver (1996), however, the microbiota associated with honey is still not fully understood. Several studies have reported the presence of lactic acid bacteria isolated in honey and honey bee. *Lactobacillus* and *Bifidobacterium* were isolated from stomach of honeybees (Olofsson & Vasquez, 2008; Eva *et al*, 2009). *Gluconobacter* and *Lactobacillus* were isolated from ripening honey (Ruiz and Rodriguez, 1975), and *Lactobacillus*, *Streptococcus*, *Leuconostoc* and *Pediococcus* were isolated from Ethiopian honey wine (Bekele *et al.* 2006). Recently, these LABs were also isolated in honey bee-gut (Audisio *et al*, 2011). These microorganisms possess interesting properties not only for the food industry but also for the benefit of health (Naidu *et al.* 1999).

Information on the possible role of LAB in honey is lacking. Therefore, this study reports the isolation of LAB from commercially available honey in Malaysia, this

study may provide an insight to the possible contribution of the naturally present LAB in honey.

3.2 Materials and methods

3.2.1 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) (Appendix A1). pH of honey was determined using pH meter (METTLER TOLEDO).

3.2.2 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 9 ml of MRS broth incubated at 30 °C for 24 to 48 h followed by serial dilution with peptone water (0.1 % w/v). 0.1 ml was spread plated on several modified media namely, MRS agar (De man *et al.*, 1960), MRS agar with 0.8 % $CaCO_3$ (Wanchai *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.

3.3 Results

3.3.1 Isolation of lactic acid bacteria using different media

Isolation of LAB from honey was possible by using modified media. A total of 32 isolates were catalase negative and Gram positive. Highest number of LAB was isolated from MRS agar with 0.8% $CaCO_3$ (Table 3). LAB present in Al-Sedar honey from Libya, pure honey from Malaysia, and Al-Shifaa honey from Saudi Arabia were isolated from MRS with 1 % glucose, while LAB from Madu bunga gelam from 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) was isolated from Al-Seder honey (pH 5.6), followed by Alshifa honey (> 10^4/ml, pH 3.66) and Monuka honey (> 10^4/ml, pH 4.38). It was observed that honey from Malaysia contained low number of LAB (>10/ml) and lower pH. Six of these LAB isolates were of short in shape (Appendix A2) and selected for antibacterial study against multiple antibiotic resistant (MAR) Gram negative bacteria.

Table 3: 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 + $CaCO_3$	+	-	10^1
				MRS + Glucose			NG
				TJA + $CaCO_3$	+	-	10^1
				TJA + Glucose			NG
H005	Melaka, Malaysia	Wild Honey	3.41	MRS agar			NG
				MRS + $CaCO_3$	+	-	10^1
				MRS + Glucose			NG
				TJA + $CaCO_3$			NG
				TJA + Glucose			NG
H006	Misurata, Libya	Al-Seder Honey	5.06	MRS agar			NG
				MRS + $CaCO_3$	+	-	10^5
				MRS + Glucose	+	-	10^3
				TJA + $CaCO_3$			NG
				TJA + Glucose			NG
H008	Cameron Highland, Malaysia	Pure Honey	3.61	MRS agar			NG
				MRS + $CaCO_3$	+	-	10^3
				MRS + Glucose	+	-	10^3
				TJA + $CaCO_3$			NG
				TJA + Glucose			NG
H009	Saudi Arabia	Al-Shifaa	3.66	MRS agar			NG
				MRS + $CaCO_3$	+	-	10^5
				MRS + Glucose			NG
				TJA + $CaCO_3$			NG
				TJA + Glucose			NG
H010	New Zealand	Monuka Honey	4.38	MRS agar			NG
				MRS + $CaCO_3$	+	-	10^5
				MRS + Glucose			NG
				TJA + $CaCO_3$			NG
				TJA + Glucose			NG

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

3.4 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 (Table 3). 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 Wanchai *et al.*, (2007) and Muhialdin *et al.*, (2011b).

Earlier reports detected the presence of lactic acid bacteria in raw honey (Eva *et al.* 2009; Ruiz & Rodriguez, 1975; Bekele *et al.* 2006; Hosny *et al.* 2009). Additionally, many studies reported the isolation of LAB from stomach of bee, flowers, and plants. Recently, Akihito *et al.*, (2009) isolated lactic acid bacteria from flowers and fruits, and identified them as *Lactobacillus kunkeei*, *Fructobacillus pseudoficulneus*, and *F. 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). In this study, 32 lactic acid bacteria were isolated from commercially available honey in Malaysia and six isolates were selected randomly for further study. To the best of our knowledge, this is the first report on the detection of lactic acid bacteria strains in commercial honey marketed in Malaysia.

3.5 Conclusion

This chapter demonstrates that isolation of LAB from honey requires modification of the normally used MRS media, and LAB could be isolated from honey.