CHAPTER IV

RESULTS AND DISCUSSION

4.1 Characteristics bee bread samples

The colour of bee bread depends on the colour of pollen collected by the bees. The colour of the bee bread obtained from three different stingless bee farms varied with type of stingless bees and sources where they were obtained. The bee bread obtained from *Thorasica* spp. was a mixture of brown, yellow and orange colour, while those from *Itama* spp. was yellow and brown in colour. *Terminata* spp. was yellow in colour (Figure 1 and Appendix A).

A

Bee bread from *Thorasica* spp. Sangai Merab

B

Bee bread from *Itama* spp. Sangai Merab

Figure 1: Colour of bee bread samples from different stingless bees (A) *Itama* spp. and (B) *Thorasica* spp.

The pH of bee bread also varied with the bees and sources ranging from 3.31 to 4.2 (Table 1). The samples from Negeri Sembilan tend to have higher pH value of 4.12 compared to from other sources. It was observed that LAB could be isolated from the

yellow coloured bee bread produced by *Thorasica* spp. farmed at Sungai Merab, Kajang, Selangor and *Thorasica* spp., *Itama* spp. and *Terminata* spp. farmed at Negeri Sembilan.

Table 1: Colour characteristics, pH and detection of LAB in bee bread samples obtained from different sources

Bee Bread						
Source	**Bee spp.**	**Colour**	**pH**	**Gram stain**	**Catalase**	**Temperature and Media**
Sangai Merab, Kajang/ Selangor	*Itama*	Brown	3.5 6	ng	ng	No growth at different temperature on MRS and TJ agar with $CaCO_3$.
		Yellow	3.5 8	ng	ng	
Kedah	*Itama*	Yellow	4.1 2	ng	ng	No growth at different temperature on MRS and TJ agar with $CaCO_3$.
Terengganu	*Itama*	Brown	4.0 1	ng	ng	
Sangai Merab, Kajang/ Selangor SM.	*Thorasica*	Brown	3.3 8	ng	ng	No growth at different temperature on MRS and TJ agar with $CaCO_3$.
		Orange	3.3 5	ng	ng	
		Yellow	3.3 1	+	_	Good growth at 37°C on MRS agar with $CaCO_3$
Negeri Sembilan NS.	*Thorasica*	Yellow	4.1 2	+	_	Good growth at 37°C on MRS agar with $CaCO_3$
	Itama	Yellow	4.2 0	+	_	Good growth at 37°C on MRS agar with $CaCO_3$
	Terminata	Yellow	4.1 2	+	_	Good growth at 37°C on MRS agar with $CaCO_3$

Note: Samples were analysed on 4 April 2016. ng = no growth

The bee bread contains mixtures of pollen, nectar and salivary gland secretions and fermented by LAB coming from the stomach of bees. The low pH of bee bread samples was within the range as reported by Vásquez et al., (2009) which was between pH 3.8 to 4.3 in bee bread produced by bees. A higher acidity of bee bread is due to the organic

acids produced by lactic acid bacteria and other metabolites that could maintain bee bread from spoilage by harmful microorganisms. It was observed that colour of the bee bread affected the isolation of LAB; fifteen LAB were isolated from yellow colour bee bread. LAB could not be isolated from the orange and brown bee bread.

4.2 Isolation of Lactic Acid Bacteria from bee bread

Good growth of LAB was observed using the tomato juice agar (Oxoid; Basingstoke, UK) and De Man Rogosa (MRS) agar (Merck; Darmstadt, Germany). This result was similar to that of Aween et al. (2012) who reported that LAB could be isolated from fresh honey by using MRS agar with 0.8% $CaCO_3$ and gave the highest number of isolates. MRS with 1% glucose supported growth of LAB from some honey samples obtained from different area, except for honey from Malaysia, and LAB were isolated on TJA with 0.8% $CaCO_3$ (Aween et al., 2012). The LAB could be isolated and viable in both the bee pollen and the two week old bee bread (Vásquez et al., 2009) and reported that different nectars have different impacts on the LAB growth. This may explain the differences in numbers and the inability to isolate LAB from Kedah and Terengganu. The age and colour component present in bee bread may have affected the viability of LAB in the sample.

A total of 15 LAB were isolated from bee bread of three stingless bee species from two different sources in Malaysia by a combination of pre-incubation in MRS broth followed by plating in two microbiological media. All the isolates were catalase negative and Gram- positive bacteria (Figure 2). MRS agar with 0.8 % $CaCO_3$ showed the highest number of isolates compared with tomato juice agar (Table 1). MRS with 0.8% $CaCO_3$ supported the growth of LAB isolated from four bee bread samples belonging to three stingless bee species namely, *Thorasica* spp. from Sangai Merab, Kajang, Selangor (SM) and *Thorasica* spp, *Itama* spp, *Terminata* spp from Negeri Sembilan (NS). TJA with 0.8% $CaCO_3$ did not support the growth of LAB from all bee bread samples. LAB coded as A-T-SM, B-T-SM, C-T-SM, D-T-SM, E-T-SM and F-T-SM were isolated from *Thorasica* spp. that was collected from Sangai Merab, Kajang, Selangor (SM). LABs

isolated from bee bread obtained from Negeri Sembilan (NS) were coded as follows: G-T-NS, H-T-NS and I-T-NS were isolated from bee bread of *Thorasica* spp. J-I-NS, K-I-NS and L-I-NS were isolated from bee bread of *Itama* spp and M-Te-NS, N-Te-NS and O-Te-NS were isolated from bee bread of *Terminata* spp. LAB was not isolated from bee bread of *Itama* spp. from Kedah and *Itama* spp. from Terengganu. All these LAB isolates were selected for antimicrobial, probiotic properties and antibiotic resistant study.

Figure 2: Typical growth characteristics of LAB isolate on MRS agar medium with $CaCO_3$ (A) and microscopic view of the isolates when Gram stained (B)

4.3 Antimicrobial activity of LAB isolates against target bacteria

4.3.1 Dual agar overlay method

Lactic acid bacteria are well known producer of antibacterial substances mainly organic acids among others and many studies revealed the success of the LAB isolates to prevent the growth of pathogenic and foodborne bacteria *in vivo* and *in vitro* (Aween et al., 2012). From the 13 LAB strains isolated from the bee gastro-intestinal tract of *A. mellifera* L. in America it was observed that only *Lactobacillus* was able to inhibit *S. aureus* ATCC 29213 and *E. coli* O157 (Audisio et al., 2011). LABs are known producers of antibacterial components; therefore, it is known that the traits, qualities and compounds produced by LAB are species- and strain-dependent (Olofsson et al., 2014).

The LAB coded as A-T-SM, B-T-SM, C-T-SM, D-T-SM, E-T-SM and F-T-SM obtained from bee bread of *Thorasica* (SM) and G-T-NS, H-T-NS, I-T-NS from beebread of *Thorasica* spp. (NS), J-I-NS, K-I-NS and L-I-NS isolated from beebread of *Itama* spp. (NS) and M-Te-NS, N-Te-NS and O-Te-NS isolated from beebread of *Terminata* spp. (NS) showed strong and significant ($p<0.05$) inhibitory activity against the target bacteria by the dual agar overlay method (Figure 3-B and Appendix C). All LAB isolates greatly inhibited *S.* Typhimurium ATCC 13311 as shown by the inhibitory zone ranging between 23.1 to 37.6 mm. A, B, C, D, E and F-T-SM LAB isolated from Sungai Merab showed higher inhibition activity against *S. aureus* ATCC 25923 than LAB isolated from NS and the inhibitory zone ranged between 14.0 to 25.8 mm. In contrast *E. coli* ATCC 25922 was inhibited but to a lesser effect by LAB isolated from SM with inhibition zone ranging from 17 to 39.6 mm. *S. aureus* and *E. coli* was greatly inhibited by LAB G, H and I-T-NS isolated from *Thorasica* spp. (NS), J, K and L-I-NS from *Itama* spp. (NS) and M, N and O-Te-NS from *Terminata* spp. (NS) (Figure 3-A). LAB H-T-NS showed the highest inhibitory affect against *S. typhimurium*, and O-T-NS showed the highest inhibitory affect against *E. coli*, while E-T-SM showed the highest inhibitory affect against *S. aureus* in comparison to other LAB isolates (Appendix B).

Inhibition Zone of LAB-A isolated from SM

LAB coded I, G, H isolated from NS source against *E. coli*

Figure 3-A: Growth inhibition zone of LAB isolates against pathogenic bacteria by dual agar overlay method after 24 h incubated at 37◦C.

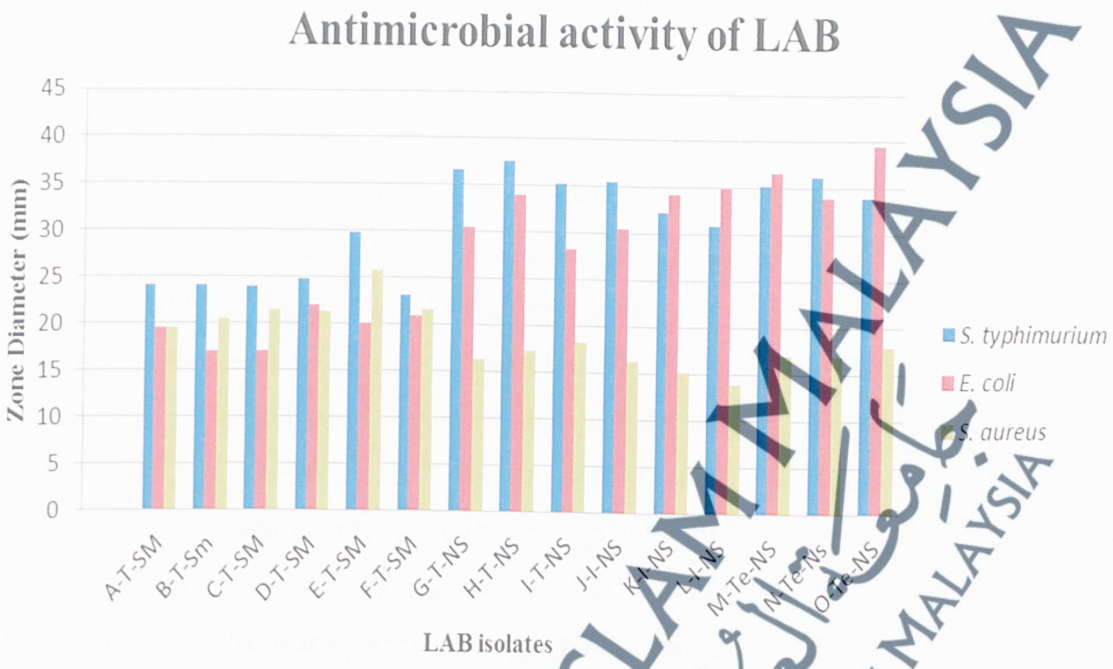


Figure 3-B: Antimicrobial activity of isolated LAB against targeted pathogens by dual agar overlay method

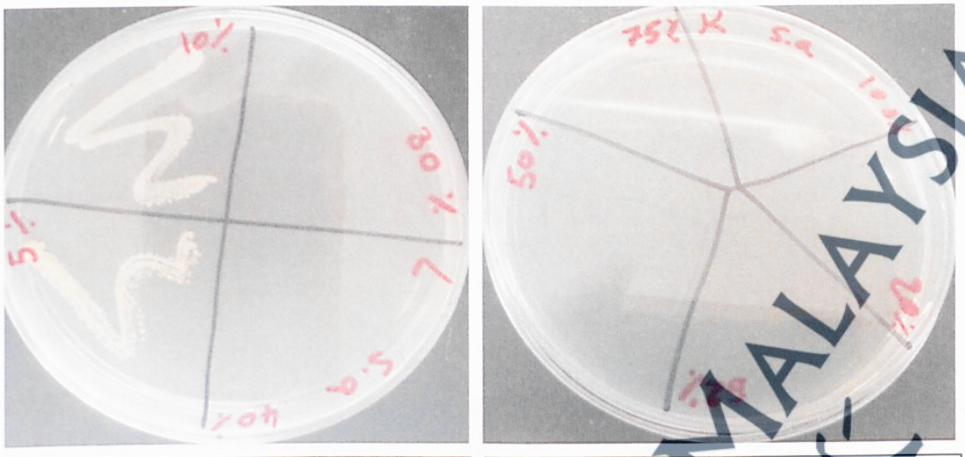
4.3.2 Growth inhibition of target bacteria in microtiter plate

Earlier studies revealed that LABs isolated from honey samples have very good antimicrobial efficiency against many Gram negative bacteria. The cells and cell free supernatants of *L. acidophilus* strain isolated from honey samples showed antibacterial activity toward the tested Gram negative bacteria *S. typhimurium, E. coli* and *E. aerogenes* (Aween et al., 2012). *Lactobacillus* spp. cell-free supernatant contained different antimicrobial substances. The production of these effective components by intestinal microflora is considered an essential traits for a probiotic and therefore, it is important to check the antimicrobial property of the LAB isolates as potential candidates for use as probiotic (Bilkova et al., 2011). Antibacterial activity is considered as one of the most significant selection criteria for probiotics. Compounds that contribute to the antibacterial effect of LAB include organic acids (lactic, acetic, propionic acids), carbon

dioxide, hydrogen peroxide, diacetyl, low molecular weight antibacterial materials and bacteriocins (Dunne et al., 2001).

It was observed that the percentage growth of target bacteria was decreased in the range of 50 to 100% by the all the LAB cell free supernatants compared to control within 24 h incubation (Figure 4). The antibacterial activity varies with LAB. Supernatant of all LAB from NS and SM bee breads caused significant ($p<0.05$) inhibition of all target bacteria at 50% dilution (Appendix D-H). Supernatant J-I-NS, K-I-NS, L-I-NS from NS *Itama* spp. caused significant inhibition of *S. aureus* at up to 20% dilution. Similarly, supernatant M-Te-NS, N-Te-NS, O-Te-NS from *Terminata* spp. showed inhibition at 20% dilution and showed inhibited significantly ($p<0.05$) the growth of *S.* Typhimurium and *E. coli* (Appendix J). However, the supernatant of LAB A-T-SM, B-T-SM, C-T-SM, D-T-SM, E-T-SM and F-T-SM from *Thorasica* spp. (SM) and G-T-NS, H-T-NS, I-T-NS from *Thorasica* spp (NS) allowed growth of the target bacteria at 50% dilution after 24 h incubation.

Percentage growth of *E. coli* with different concentration of CFS of M-Te-NS isolate after 24 h incubation



Percentage growth of *S. aureus* with different concentration of CFS of L-I-NS isolate after 24 h

Percentage growth of *S. aureus* with different concentration of CFS of K-I-NS isolate after 24 h

Figure 4: Percentage growth of targeted bacteria with different concentration of cell free supernatant of LAB isolates after 24 h incubation using microtiter plates method

4.3.3 Antimicrobial Activity of pH-adjusted Cell Free Supernatant using Agar Well Diffusion Assay

It was well established that cell free supernatant of LAB contains many inhibitory components, like organic acids, oxygen catabolites, proteinaceous substancess, fat and amino acids metabolites and another different compounds (Valerio et al., 2004). Cell free supernatant of eight LAB isolates presented good antimicrobial activity against 23 serotypes of MAR's *Salmonella* associated with food poisoning diseases in Malaysia with almost 100% broad spectrum antibacterial actions (Salleh et al., 2014). Irish et al. (2008) reported that there is considerable antibacterial action of stingless bee honey from *T. carbonaria* evaluated by using the agar diffusion method, whilst other publication reported that there was no antimicrobial activity by using similar method (Kimoto et al., 2008). Similarly, Boorn et al. (2010) suggested that there are many limitations of the agar diffusion assay which have been noted by many authors when investigating honey or other natural antimicrobial substances and reported that little activity was seen by agar well diffusion assay.

It was observed that the supernatant of LAB isolated from the Malaysian bee bread SM and NS did not show antibacterial effect against *S. typhimurium*, *E. coli* and *S. aureus* when the acidity of the supernatant were adjusted to 5, 6, 7, and 8 pH (Figure 5). This suggests that the antimicrobial activity of the CFS of LAB isolates from bee bread samples could be from the organic acids produced by the LAB.

Figure 5: Antimicrobial activity of adjusted cell free supernatant of LAB against *E. coli* (A), *S. aureus* (B) by well diffusion assay at pH 5 and 6 and *S. typhimurium* (C) at pH 7 and 8.

4.4 Probiotic properties

4.4.1 pH tolerance

It was observed that all LAB isolates from SM and NS sources were able to maintain good growth and significant multiplication ($p<0.05$) at less than pH 3 in MRS broth (Appendix F1). Figure 6 and Appendix E show that growth of all LAB isolates declined significantly with increased acidity of MRS broth from 4 to 2. LAB isolated from NS source were more susceptible to the increase acidity of the MRS broth while LAB isolated from SM were more resistant. LAB E-T-SM isolated from *Thorasica* spp. SM showed high resistant to pH 3 followed by all other LAB isolated from the same source. On the other hand, all LAB isolated from SM and NS sources were susceptible to increase

acidity to pH 2 but not B-T-SM that was isolated from *Thorasica* spp. SM which showed the highest and significant multiplication ($p<0.05$) in MRS broth with pH 2 (Figure 6).

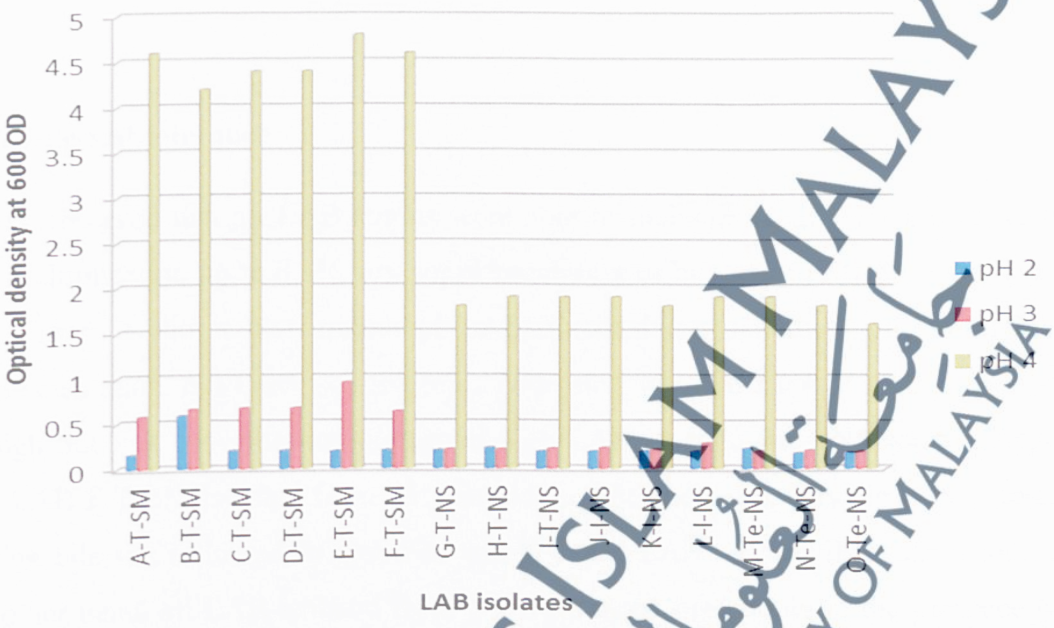


Figure 6: pH tolerance of LAB isolated from bee bread reading by Bio-photometer OD_{600nm}

Before reaching the intestinal tract, the LAB to be used as probiotic bacteria must first survive passing through the stomach, of which the pH can be as low as 1.5 to 2 (Mourad et al., 2006). Many researchers reported that acids like the hydrochloric acid exist in human stomach which interrupt the biomolecules of cells such as proteins, vitamins, fatty acids and DNA (Hassanzadazar et al., 2012). The acidic environment can inhibit the metabolism and reduce the multiplication and viability of lactobacillus. The impact of pH range from 2.0 to 7.0 on the survivability of the 54 selected strains was studied by Vasiee et al. (2014). For a bacteria to be used as probiotic it should withstand at least pH 3.0 (Barakat et al., 2011) and the researchers mentioned that high resistance to the acidic condition may due to production polysaccharides on the cell wall. When the pH increased to 3.0, more than 40% of LAB isolates showed survivability higher than 75%, but when the pH increased up to 7.0, all isolates exhibited survivability 100% (Allameh et al.,

2012). The most significant standard for selection of LAB as probiotic is the potential ability of maintain good growth at acidic condition, thus pH 3 is considered as a standard for acid resistant as reported by Sahadeva et al. (2011).

4.4.2 Bile-salt tolerance

It was observed that all LAB strains were able to maintain significant growth ($p<0.05$) and multiplication up to 0.3% w/v supplementation of bile-salt in MRS broth (Appendix F2). Figure 7 shows that growth of LAB declined gradually with increased bile-salt supplementation. All LAB isolated from *Thorasica* spp. SM showed significant resistant to high bile salt concentration compared with LAB isolated from NS source (Appendix E), LAB F-T-SM isolated from *Thorasica* spp. SM showed highly significant tolerance to high bile salt followed by D-T-SM and B-T-SM LAB isolated from same source. On the other hand, all LAB isolated from NS were more susceptible to the presence of ox-bile, isolate O-Te-NS showed lesser resistant to high bile salt followed by I-T-NS isolated from the same source. The results indicate that the isolate F-T-SM, D-T-SM, B-T-SM followed by E-T-SM, C-T-SM and A-T-SM (SM) may have probability to be used as probiotic bacteria.

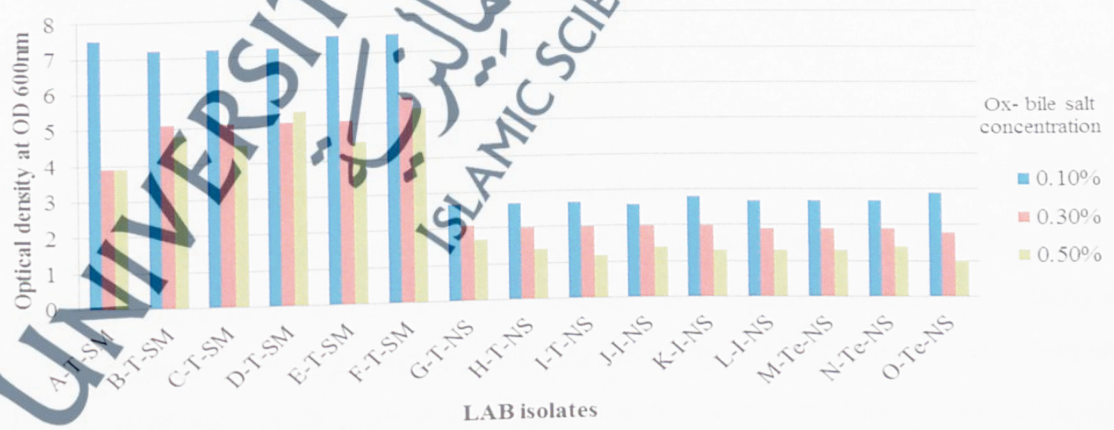


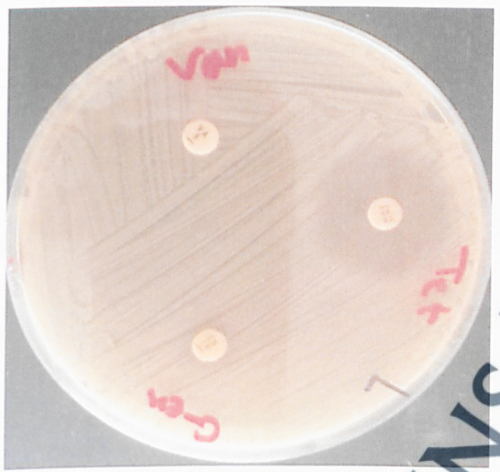
Figure 7: Tolerance of LAB isolated from bee bread to ox- bile salt concentration determined by using Bio-photometer OD_{600nm}

Bile-salt and pH tolerance of the isolates were conducted to determine whether the isolates possess probiotic properties. After 60 min of digestion the physical rate of bile salt in the intestine become around 0.3%. Many publications have mentioned that the resistances to bile salts greatly differ between LAB species and even among strains. The resistant to bile-salt of some strains is due to the presence of specific enzyme activity, bile salt hydrolase (BSH) that supports hydrolyse conjugated bile, hence reducing its toxic effect, Therefore, when estimating the probable effective probiotics of an organism it is considered important to evaluate their capability to tolerate the effects of bile salts (Mourad et al., (2006). Oxgall is a normal dried bovine bile substance including both conjugated and unconjugated bile salts (Barakat et al., 2011). While screening for resistant isolates, 0.3% is considered to be crucial concentration for bile-tolerance (Zhou et al., 2007). Resistant to bile salt has not been related to a particular mechanism but likely to a complicated regulation of gene expression (Ruiz et al., 2013). The bile salts which is in protonated (non-dissociated) form cause separation of lipid bilayer and integral protein of cell membranes, leading in bacterial content leakage and lastly cell death (Mandal et al., 2006). Reduction of viable LAB cells gradually was noted when the concentration of bile acid was increased to 1.0% (Sahadeva et al., 2012). The protective impact of food matrix could prohibit the bacteria from bile exposure and thus, giving rise to the increased ability of LAB to tolerate bile salts (Vasiee et al., 2014).

4.5 Antibiotic resistant test of LAB isolates

It was observed that all LAB isolated from SM and NS source showed significant ($p<0.05$) and totally resistant to vancomycin and streptomycin (Table 2, Appendix G). All LAB isolated from SM source were highly significant ($p<0.05$) in resistance to gentamycin while LAB isolated from NS source were slightly resistant to gentamycin with the inhibition zone of 2 mm (Figure 8). Additionally, all LAB isolated from SM and NS sources of bee bread were more susceptible to chloramphenicol, LAB from SM source showed less susceptibility with 16 to 18 mm than LAB isolated from NS source with 17 to 21 mm inhibition zone. All LAB showed high sensitivity towered tetracycline

(10-15 mm inhibition zone) and penicillin G (8 to 15 mm inhibition zone). Isolate F-T-SM from *Thorasica* spp. SM was less sensitive to penicillin G followed by E-T-SM and D-T-SM from the same source with 8 and 9 mm inhibition zone, respectively, while LAB isolated from NS source showed 10 to 15 mm inhibition zone. The findings from this study was similar to that reported by Muhialdin et al. (2012) the LAB they isolated from food sources were sensitive to chloramphenicol.



Resistant of L-I-NS isolate to vancomycin, slightly resistant to gentamycin and sensitive to tetracycline

Resistant of G-T-NS isolate to streptomycin, sensitive to penicillin and chloramphenicol

Figure 8: Antibiotic susceptibility of LAB tested against selected antibiotics using disk diffusion test (DDT)

Table 2: Susceptibility of LAB isolates from bee bread toward antibiotics using disk diffusion test (DDT)

LAB isolates	Antibiotics					
	Vancomycin (5µm)	Tetracycline (30 µm)	Gentamycin (10 µm)	Penicillin G (10 µm)	Streptomycin (10 µm)	Chloramphenicol (30 µm)
A-T-SM	_	11±0.21	_	12±0.21	_	17±0.21
B-T-SM	_	10±0.35	_	11±0.21	_	16±0.14
C-T-SM	_	10±0.35	_	10±0.35	_	16±0.14
D-T-SM	_	10±0.35	_	9±0.28	_	16±0.14
E-T-SM	_	10±0.35	_	9±0.28	_	18±0.35
F-T-SM	_	11±0.21	_	8±0.21	_	17±0.21
G-T-NS	_	14±0.14	2±0.35	14±0.14	_	18±0.35
H-T-NS	_	15±0.35	2±0.35	15±0.35	_	19±0.35
I-T-NS	_	15±0.35	2±0.35	13±0.42	_	17±0.21
J-I-NS	_	15±0.35	2±0.35	12±0.21	_	19±0.35
K-I-NS	_	14±0.14	2±0.35	14±0.14	_	21±0.70
L-I-NS	_	15±0.35	2±0.35	14±0.14	_	21±0.70
M-Te-NS	_	15±0.35	2±0.35	13±0.42	_	21±0.70
N-Te-NS	_	14±0.14	2±0.35	13±0.42	_	19±0.35
O-Te-NS	_	14±0.14	2±0.35	11±0.21	_	17±0.21

Diameter of growth inhibition zone around the discs was measured in mm after 24 h incubation at 37 °C.

Limited study has been focusing on evaluating the antibiotic activity of LAB isolated from fresh bee bread in Malaysia. Klare et al. (2007) evaluated the antibiotic resistant among *Lactobacillus* spp. and showed that *Lb. acidophilus* was more sensitive to streptomycin than other strains. *Lactobacilli* and *bifidobacteria* isolated from some pharmaceutical and dairy products were clindamycin tolerance (Gad et al., 2014). Survivability of few fractions of bacterial cells after exposure to strict stress of antibiotics has been related to transient case of late or arrested multiplication of bacterial cells in the colony, which is varied from resistance (Martins et al., 2015). This provides an ideal guide for LAB evolution of "tolerance" to antibiotics. Nevertheless, this has not been improved yet; it is believed that LAB bacteria have all adapted to the antibiotic through tolerance and not resistance (Fridman et al., 2014). Results showed that *Lb. plantarum* strains (originate of fermented olives) were sensitive to the majority of antibiotics used and low resistances were detected. This is not similar with many reports indicating that LAB are normally have the ability to resist the principal antibiotics like penicillin G, ampicillin, vancomycin, chloramphenicol or ciprofloxacin (Coppola et al., 2005). Vancomycin is an antibiotic that belongs to glycopeptide antibiotics which prevents the peptidoglycan synthesis that is an important structural compound of the bacterial cell wall. Thus, lactic acid bacteria which is Gram-positive bacteria, are particularly vulnerable to vancomycin treating (Mourad et al., 2006). It was suggested that the resistant phenotype for streptomycin *Bifidobacterium bifidum* strains has not been related to the acquisition of particular antibiotic resistance gene but rather to chromosomal mutation on the rpsL gene for ribosomal protein S12 in *B. bifidum* and *B. breve* (Sato et al., 2010).