CHAPTER III

3.1 Materials and methods

3.1.1 Samples

A total of nine samples of fermented food yogurt (Nestle Yogurt, Fresh Yogurt, Syria Yogurt, Assafi Yogurt, and Homemade Yogurt), fermented belacan (Belacan Penang, Belacan Pankor, Belacan Talyor Malaysia) and fermented durian were obtained from the market around Bandar Baru Bangi. Cultures of *Bacillus* spp. (*B. cereus*, *B. subtilis*, *B. spizizenii)* were obtained from the Microbiology Laboratory, Faculty of Science and Technology, Universiti Sains Islam Malaysia (USIM).

3.1.2 Isolation and purification of lactic acid bacteria from fermented food

Ten grams of each sample (except curd 10 ml) was mixed with 90 ml of MRS broth (Oxoid) and incubated at 37°C for 48 h in an aerobic condition. A 10 ml of the was mixed with 90 ml pepton water and vortexed. Serial dilution of the sample (10^{-2}, 10^{-3}, $10^{-4,}$ 10^{-5} and 10^{-6}) was prepared. A 1.0 ml of each dilution was inoculated to the Petri plates and 15 mL of sterile and cooled molten MRS agar (Oxoid), thoroughly mixed and the agar was allowed to solidify. All plates were incubated at 37 ^{o}C for 24 h. The plates were observed for appearance of colonies. Bacteria was purified by streaking on MRS agar and incubated at 37 ^{o}C for 24 h, then transferred to MRS agar slants maintained in the refrigerator at 4 ^{o}C till further analysis (Pundir et al., 2013).

3.1.3 Preparation of spore suspensions

An overnight *Bacillus* spp. culture in nutrient incubated at 30 ºC for 24 h was spread plated on nutrient agar (Merck). Then the plates were incubated at 37 ºC for seven days to get more than 90% of the spores produced. Sporulation process was monitored by staining the bacterial smears with crystal violet and observed at 100x magnification using a light microscope (Olympus). Free spores were visible on the 3rd day and almost 90% of bacteria were in spore phase on the 7th day. On the seventh day, 3 to 5 mL of cooled (4ºC) sterile distilled water was added to the plate and gently the surface was scraped to collect the spores and put in the sterile Eppendorf. Then the tubes were centrifuged at 4ºC, at 9000 rpm for 15 minutes (centrifuge Epend). This step was repeated twice to remove impurities. Then, spores were suspended in 1 ml of sterile water, heated for 20 minutes in water bath 80ºC to kill the vegetative forms. Finally, spores suspension was kept at 4ºC until treatment with the LAB supernatants (Dabiri & Karbasizade, 2014).

3.1.4. Screening for antimicrobial activity of isolated LAB against *Bacillus* spp.

3.1.4.1 LAB cells spot method

Antagonistic activity screening was investigated against test bacteria by agar spot test and well diffusion assay as described by Schillinger and Luche (1989). Agar spot test experiments were conducted by spotting a loopful of an overnight LAB cultured on the surface of a MRS agar plate and incubated at 37°C for 24 to 48 h. These plates were then overlaid with 8 mL of soft agar (0.75% agar) seeded with 8 ml of a test bacteria culture (approximately 10^7 stationary-phase cells). After overnight incubation at 37°C, the plates were examined for zones of inhibition in the test bacteria (Kivanc et al., 2011).

3.1.4.2 Well method

LAB cultures were inoculated to MRS broth incubated at 37ºC for 24 to 48 h on shaker. The broth culture was centrifuge 7100 rpm for 15 min at 12 ºC. The supernatants were collected and

filter sterilized using 0.2 µm filter (Minisart®, Sartorius stedim). Bacillus spp. were grown in nutrient broth incubated at 37 °C and was diluted to a turbidity equivalent to that of a 0.5 McFarland standard (Pundir et al., 2013) and 0.1 mL was spread on the nutrient agar plates. Wells of 7 mm in diameter were cut into the plates, 0.2 ml supernatant inside the wells. Finally, the plates were incubated aerobically at 37^{o}C for 24 h. Antagonist effect resulting in the appearance of the clear zone around the wells of the growth of the putative inhibitor strain was inspected. (Darsanaki et al., 2012).

3.1.5 Effect of pH on antibacterial activity of LAB supernatant

LAB isolates were cultivated in their MRS broth incubated at 37 ^{o}C for 24 to 48 h on shaker. The broth culture was centrifuge (Combi 514R) at 7100 rpm for 15 min at 12 ^{0}C (Darsanaki et al., 2012). The supernatants were collected and filter sterilized using 0.2 µm filter. The pH of supernatant was adjusted to pH 4, 5, 6 with 0.2 NaOH to exclude the antimicrobial effect of organic acids. (Aween et al., 2012). Bacillus spp. were grown in nutrient broth incubated at 37 °C and was diluted to a turbidity equivalent to that of a 0.5 McFarland standard (Pundir et al., 2013) and 0.1 mL was spread on the nutrient agar plates. Wells of 7 mm in diameter were cut into the plates, 0.2 ml supernatant inside the wells Finally, the plates were incubated aerobically at 37oC for 24 h. Antagonist effect resulting in the appearance of the clear zone around the wells of the growth of the putative inhibitor strain was inspected. (Darsanaki et al., 2012).

3.1.6 Effect of enzymes on LAB supernatant against *Bacillus* spp.

Lactic acid bacteria isolate was grown in MRS broth overnight at 37°C. The broth culture was centrifuge 7100 rpm for 15 min at 12 ^{o}C. Supernatants were collected and filter sterilized using 0.2 µm filters. The supernatants were treated with Proteinase K and RNase I separately. 1µl of each enzyme was inoculated to 1 ml of supernatant in universal tubes and left for 1 h at room temperature. After that, the supernatant was tested against target bacteria in microtiter plates followed by incubation at 37°C for 48 h, supernatant without treat use as control, and bacterial growth was monitored using Optical Density (OD) 560 nm using BioTek EL×800 ELISA reader (Lash, Mysliwiec, & Gourama, 2005). The percentage growth of *Bacillus* spp. was measured using the equation (Aween et al., 2012).

$$\text{Percent growth of } \textit{Bacillus} \text{ spp.} = \left[\frac{OD_{560\text{ nm after 24h or 48 h}} - OD_{560\text{ nm after 0h}}}{OD_{560\text{ nm after 0}}} \right] \times 100$$

3.1.7 Detection of protein hydrolysis

3.1.7.1 Preparation of skim milk agar and cultures

Skim milk agar was made as follows: 25 g of skim milk was reconstituted with 250 ml of distilled water. The mixture was stirred thoroughly and autoclaved at 110°C for 10 min. Likewise, 500 ml of 2.5% agar solution was sterilized. For plating, skim milk and agar solutions were held in a water bath at 50°C and then the skim milk was poured into the agar bottle and mixed thoroughly. The skim milk agar was poured quickly into plates (Abubakr et al., 2012).

3.1.7.2 Measurements of protein hydrolysis

To detect protein hydrolysis, the selected LAB were inoculated on skim milk agar plates and were incubated at 37 ± 1 °C for 48 h in an anaerobic chamber followed by cooling in a refrigerator (4°C) for 3 days. Protein hydrolysis was observed by the production of clear halos surrounding isolated colonies. Duplicate trials were conducted and all results were averaged and reported as diameter in mm (Abubakr et al., 2012).

3.1.8 Determination of antibiotic resistance of the isolates

In this study, the 7 antibiotic discs were used to determine antibiotic resistance of *lactobacilli* strains. These antibiotic discs were Penicillin G (PI), Gentamicin (CN), Chloramphenicol (C), Vancomycin (VA), Streptomycin (S), and Tetracycline (TE). The susceptibility tests for each isolates were performed using disc diffusion method. The discs were placed on the solidified agar surface. The plates were incubated aerobically for 24 h at 37 ^{0}C. The resistances were determined according to the zone formation (Saranya & Hemashenpagam, 2011).

3.1.9 Statistical analysis

All measurements performed in duplicate were calculated for standard deviation, and results were presented as mean value ± S.D. and were analysed by two way variance (ANOVA) for significant mean $p < 0.005$ to evaluate the significant differences between groups.

3.2 Results

3.2.1 Isolation of LAB from food samples

A total of 45 bacterial cultures were isolated from yogurt, fermented shrimp paste belacan and fermented durian "tempoyak" and all the isolates were catalase negative, and Gram positive, thus assured they are LAB, These LAB isolate showed clear zone on the modified MRS agar with 0.8% $CaCO_3$ (figure 6 and table 4).

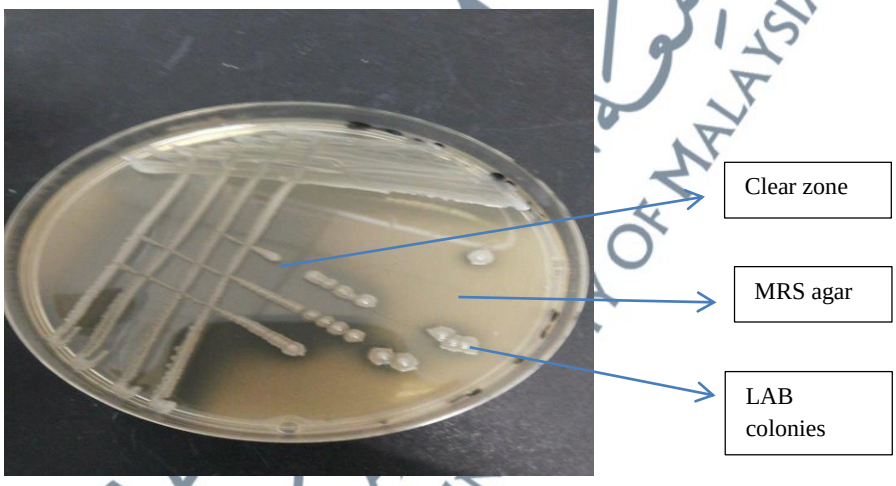


Figure 6: Growth of LAB isolates on modified MRS with 0.8% $CaCO_3$ showing clear zones around the colonies

Table 3: Gram stain and catalase test of LAB isolated from different food samples

Food samples	Code of Isolate	Catalase test	Gram stain
Nestle Yogurt	M1	Negative	Positive
	M2	Negative	Positive
	M3	Negative	Positive
	M4	Negative	Positive
	M4	Negative	Positive
	M5	Negative	Positive
Fresh Yogurt	F1	Negative	Positive
	F2	Negative	Positive
	F3	Negative	Positive
	F4	Negative	Positive
	F5	Negative	Positive
Syria Yogurt	S1	Negative	Positive
	S2	Negative	Positive
	S3	Negative	Positive
	S4	Negative	Positive
	S5	Negative	Positive
Assafi Yogurt	N1	Negative	Positive
	N2	Negative	Positive
	N3	Negative	Positive
	N4	Negative	Positive
	N5	Negative	Positive
Homemade Yogurt	H1	Negative	Positive
	H2	Negative	Positive
	H3	Negative	Positive
	H4	Negative	Positive
	H5	Negative	Positive
Belacan Penang	BP1	Negative	Positive
	BP2	Negative	Positive
	BP3	Negative	Positive
	BP4	Negative	Positive
	BP5	Negative	Positive
Belacan Pankor	BK1	Negative	Positive
	BK2	Negative	Positive
	BK3	Negative	Positive
	BK4	Negative	Positive
	BK5	Negative	Positive
Belacan Talyor Malaysia	BT1	Negative	Positive
	BT2	Negative	Positive
	BT3	Negative	Positive
	BT4	Negative	Positive
	BT5	Negative	Positive
Fermented Durian	D1	Negative	Positive
	D2	Negative	Positive
	D3	Negative	Positive
	D4	Negative	Positive
	D5	Negative	Positive

3.2.2 Antimicrobial activity of LAB against growth of vegetative *Bacillus* spp. as determined by agar spot assay and well-diffusion method

The antimicrobial activity of LAB cells against growth of vegetative cells of *Bacillus* spp. was evaluated by two methods: agar spot assay and well-diffusion method. The diameter of inhibition zone was rated as strong (>13 mm), moderate (13 to 9 mm) and weak (<9 mm) (Sumathi, 2012).

3.2.2.1 Agar spot assay

The results of agar spot assay against vegetative cell of *Bacillus* spp. are shown in figure 7 and table 5. Strong Significant difference was observed between and among all the samples ($p < 0.05$). All the 45 LAB cells strongly inhibited (98%) the growth of *B. cereus* compared to 78% against *B. spizizenii* and 51% against *B. subtilis*. The inhibition zone demonstrated by the LAB isolates was variable against the targeted *Bacillus* spp. It was observed that isolate BP4 showed 28 mm inhibition zone against *B. subtilis*, BK1 showed 30 mm inhibition zone against *B. cereus* and BT5 showed 25 mm inhibition zone against *B. spizizenii*, Low zone inhibition was recorded for LAB isolates BK2, H5,F3, F5 against *B. subtilis* (10 mm), BP3 against *B. cereus* (12 mm) and S3,F4, F5 against *B. spizizenii* (12 mm).

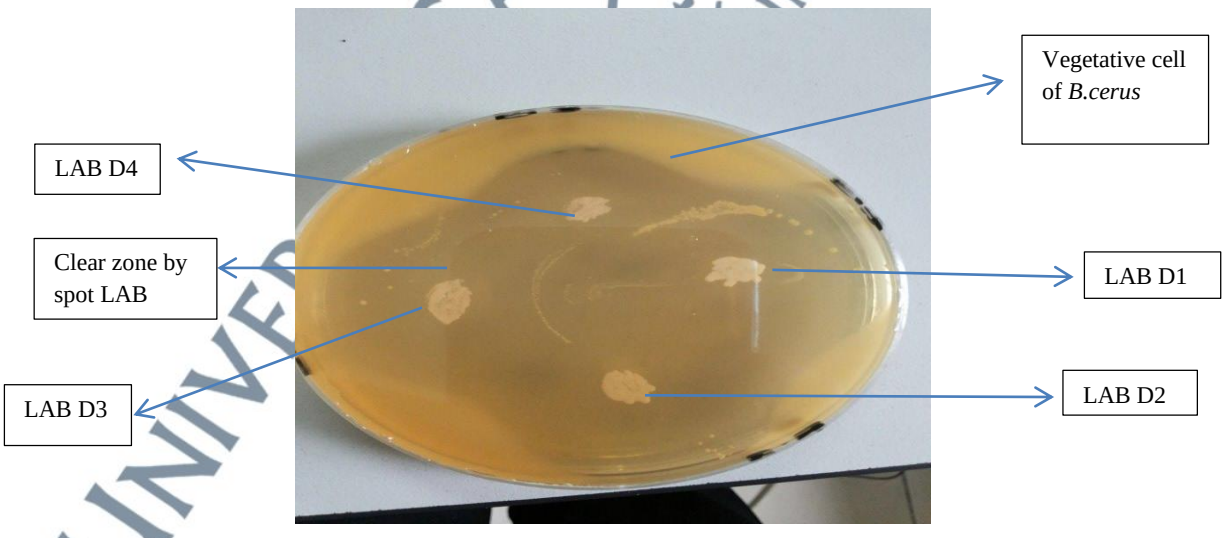


Figure 7: Antimicrobial activity of LAB against vegetative cell *Bacillus spp.* using *agar* spot assay

Table 4: Antimicrobial activity of LAB against vegetative cell *Bacillus spp.* using agar spot assay

Food sample	Code of isolate	*B. subtilis*	*B. cereus*	*B. spizizenii*
Nestle yogurt	M1	14.00±1.414	24.00±1.414	16.00±2.828
	M2	11.00±1.414	28.00±1.414	13.00±1.414
	M3	18.00±1.414	22.00±.000	18.00±4.243
	M4	11.00±1.414	26.00±1.414	17.00±1.414
	M5	15.00±2.828	18.00±1.414	13.00±1.414
Fresh Yogurt	F1	12.00±8.485	28.00±2.828	18.00±2.828
	F2	13.00±1.414	28.00±1.414	18.00±1.414
	F3	10.00±2.828	22.00±2.828	17.00±2.828
	F4	13.00±.000	26.00±1.414	10.00±4.243
	F5	10.00±1.414	25.00±2.828	10.00±.000
Syria Yogurt	S1	12.00±1.414	26.00±1.414	15.00±2.828
	S2	11.00±.000	25.00±.000	17.00±1.414
	S3	10.00±1.414	18.00±2.828	10.00±.000
	S4	11.00±1.414	15.00±1.414	14.00±1.414
	S5	11.00±2.828	20.00±1.414	12.00±2.828
Assafi Yogurt	N1	16.00±1.414	19.00±1.414	14.00±2.828
	N2	13.00±1.414	15.00±1.414	12.00±1.414
	N3	12.00±2.828	21.00±2.828	15.00±2.828
	N4	13.00±2.828	16.00±2.828	12.00±1.414
	N5	25.00±2.828	17.00±1.414	15.00±2.828
Homemade Yogurt	H1	13.00±2.828	22.00±1.414	17.00±4.243
	H2	11.00±1.414	15.00±1.414	15.00±1.414
	H3	11.00±1.414	17.00±2.828	15.00±.000
	H4	14.00±.000	19.00±1.414	15.00±2.828
	H5	10.00±2.828	15.00±.000	17.00±1.414
Belacan Penang	BP1	22.00±1.414	22.00±2.828	18.00±2.828
	BP2	12.00±2.828	20.00±2.828	22.00±2.828
	BP3	27.00±1.414	12.00±1.414	14.00±.000
	BP4	28.00±2.828	27.00±2.828	12.00±1.414
	BP5	18.00±2.828	15.00±.000	21.00±2.828
Belacan Pankor	BK1	11.00±2.828	30.00±4.243	22.00±4.243
	BK2	11.00±1.414	17.00±2.828	16.00±2.828
	BK3	23.00±1.414	21.00±1.414	19.00±1.414
	BK4	11.00±1.414	20.00±1.414	17.00±4.243
	BK5	12.00±.000	18.00±1.414	17.00±1.414
Belacan Talyor Malaysia	BT1	20.00±2.828	16.00±.000	22.00±4.243
	BT2	17.00±1.414	28.00±2.828	16.00±2.828
	BT3	25.00±4.243	23.00±2.828	12.00±1.414
	BT4	11.00±1.414	21.00±2.828	13.00±.000
	BT5	12.00±1.414	27.00±2.828	25.00±4.243
Fermented Durian	D1	16.00±1.414	25.00±2.828	12.00±.000
	D2	11.00±4.243	23.00±1.414	17.00±2.828
	D3	12.00±.000	21.00±1.414	12.00±1.414
	D4	25.00±2.828	22.00±2.828	22.00±1.414
	D5	21.00±1.414	26.00±4.243	19.00±1.414

Diameter of growth inhibitory zone against vegetative cell *Bacillus* spp. was measured in(mm) after 24 h using spot assay method the result was expressed as mean±standrad deviation of value obtained from duplicate experiment , Mean ±SD.

32.2.2 Well-diffusion method

The results of antimicrobial activity of LAB cell determined by well-diffusion method against vegetative cell of *Bacillus* spp. are shown in Table 6 and Figure 8. Strong Significant difference was observed between and among the samples ($p < 0.05$). Strong inhibitory activity was observed against *B. cereus* (58%), followed by *B. spizizenii* (44.5%) and *B. subtilis* (31%). The diameters of inhibition zones using well-diffusion method were lower than by agar spot assay and ranged between 8 to 15 mm against *B. subtilis*, 8 to 18 mm against *B. cereus* and 8 to 16 mm against by *B. spizizenii*. Highest zone of inhibition against the vegetative cell was observed for CFS of BK3, BT1, D5 with 15 mm against *B. subtilis*, H1,BK1 with 18 mm against *B. cereus* and S2,H5 with 16 mm against *B. spizizenii*. Lowest zone inhibition was recorded by CFS of M4, F2, F3, BP2, BP3, BK5, D2 with 8 mm against *B. subtilis*, BP4 with 8 mm against *B. cereus* and BP3, BT4,D1 with 8 mm against *B. spizizenii*.

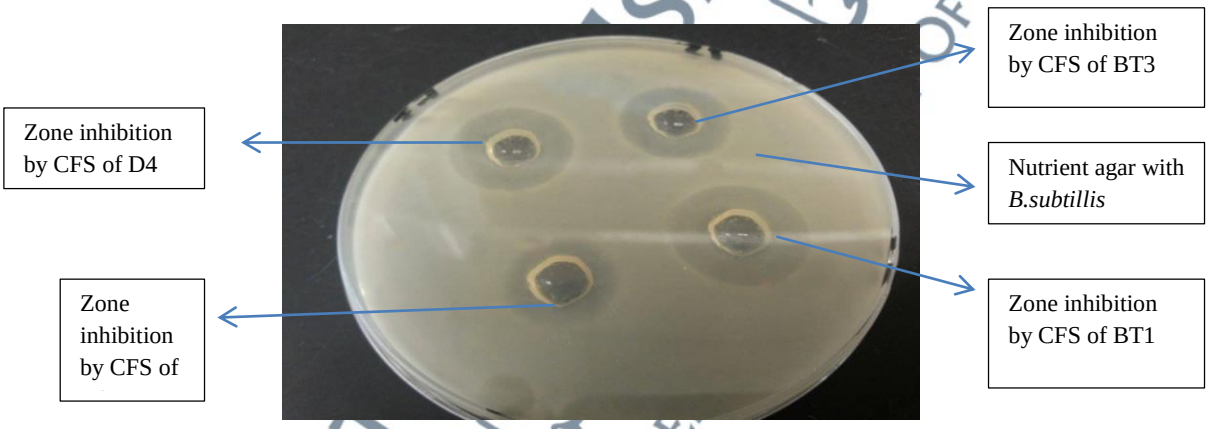


Figure 8: Plate showing growth inhibition of vegetative cell *Bacillus* spp. By CFS of LAB using well-diffusion method incubate at 37 ^{o}C for 24 h

Table 5: Antimicrobial activity of LAB supernatant against vegetative cell of *Bacillus* spp.

Using well diffusion method

Food sample	Code of Isolate	*B. subtilis*	*B. cereus*	*B. spizizenii*
Nestle yogurt	M1	10.00±1.414	14.00±1.414	11.00±1.414
	M2	10.00±.000	12.00±1.414	13.00±.000
	M3	9.00±1.414	11.00±1.414	14.00±2.828
	M4	8.00±.000	12.00±1.414	11.00±2.828
	M5	9.00±2.828	12.00±2.828	10.00±.000
Fresh yogurt	F1	10.00±.000	13.00±1.414	12.00±1.414
	F2	8.00±.000	12.00±.000	10.00±2.828
	F3	8.00±1.414	13.00±2.828	12.00±.000
	F4	9.00±.000	10.00±.000	10.00±1.414
	F5	10.00±2.828	11.00±1.414	12.00±2.828
Syria yogurt	S1	11.00±1.414	11.00±1.414	10.00±.000
	S2	14.00±.000	15.00±2.828	16.00±1.414
	S3	10.00±1.414	15.00±1.414	13.00±2.828
	S4	13.00±4.243	16.00±1.414	15.00±1.414
	S5	14.00±1.414	17.00±.000	14.00±.000
Assafi Yogurt	N1	13.00±1.414	16.00±2.828	13.00±1.414
	N2	11.00±2.828	15.00±4.243	13.00±.000
	N3	11.00±1.414	14.00±2.828	13.00±.000
	N4	11.00±3.536	14.00±1.414	13.00±2.828
	N5	11.00±1.414	15.00±2.828	12.00±1.414
Homemade Yogurt	H1	11.00±.000	18.00±2.828	14.00±2.828
	H2	10.00±1.414	15.00±2.828	12.00±2.828
	H3	10.00±.000	15.00±.000	14.00±1.414
	H4	9.00±2.828	13.00±1.414	11.00±2.828
	H5	13.00±4.243	15.00±2.828	16.00±2.828
Belacan Penang	BP1	13.00±.000	13.00±.000	12.00±1.414
	BP2	8.00±2.828	15.00±1.414	13.00±.000
	BP3	8.00±1.414	11.00±2.828	8.00±.000
	BP4	14.00±2.828	8.00±.000	14.00±1.414
	BP5	13.00±4.243	9.00±.000	13.00±2.828
Belacan Pankor	BK1	9.00±1.414	18.00±2.828	15.00±2.828
	BK2	9.00±2.828	14.00±1.414	13.00±1.414
	BK3	15.00±2.828	10.00±.000	12.00±1.414
	BK4	10.00±1.414	9.00±.000	14.00±4.243
	BK5	8.00±1.414	11.00±1.414	11.00±2.828
Belacan Talyor Malaysia	BT1	15.00±1.414	18.00±2.828	13.00±.000
	BT2	13.00±1.414	16.00±.000	14.00±2.828
	BT3	11.00±.000	14.00±1.414	13.00±2.828
	BT4	9.00±.000	13.00±4.243	8.00±1.414
	BT5	10.00±2.828	15.00±2.828	13.00±1.414
Fermented Durian	D1	13.00±2.828	14.00±1.414	8.00±2.828
	D2	8.00±.000	11.00±1.414	13.00±1.414
	D3	11.00±.000	16.00±4.243	9.00±.000
	D4	14.00±2.828	12.00±.000	14.00±1.414
	D5	15.00±1.414	16.00±2.828	13.00±2.828

Diameter of growth inhibitory zone against vegetative cell of *Bacillus* spp. was measured in(mm) after 24 h using well diffusion method, the result was expressed as mean±standrad deviation of value obtained from duplicate experiment , Mean ±SD

3.2.3 Antimicrobial activity of LAB cells and supernatant on *Bacillus* spp. spore germination by agar spot assay and well-diffusion method

3.2.3.1 Agar spot assay

The results of agar spot assay against spore germination of *Bacillus* spp. are shown in Table 7 and Figure 9. Strong Significant difference was observed between and among the samples ($p < 0.05$). It was observed that all the 45 LAB cells inhibited strongly spore germination of *B. subtilis* (89%) greater than *B. cereus* (75.5%) and *B. spizizenii* (73.3%). Highest zone of inhibition against spore germination using spot agar test was shown by F3 with 25 mm against *B. subtilis*, F1 with 28 mm against *B. cereus* and BP1 with 26 mm against *B. spizizenii*. The lowest inhibition zone was recorded by BT1, M4, F2, S3 with 12 mm against *B. subtilis*, BT5, BP5, H2, N4, M4 with 11 mm against *B. cereus* and BT2, M3, M1, F4, S3 with 11 mm against *B. spizizenii*.

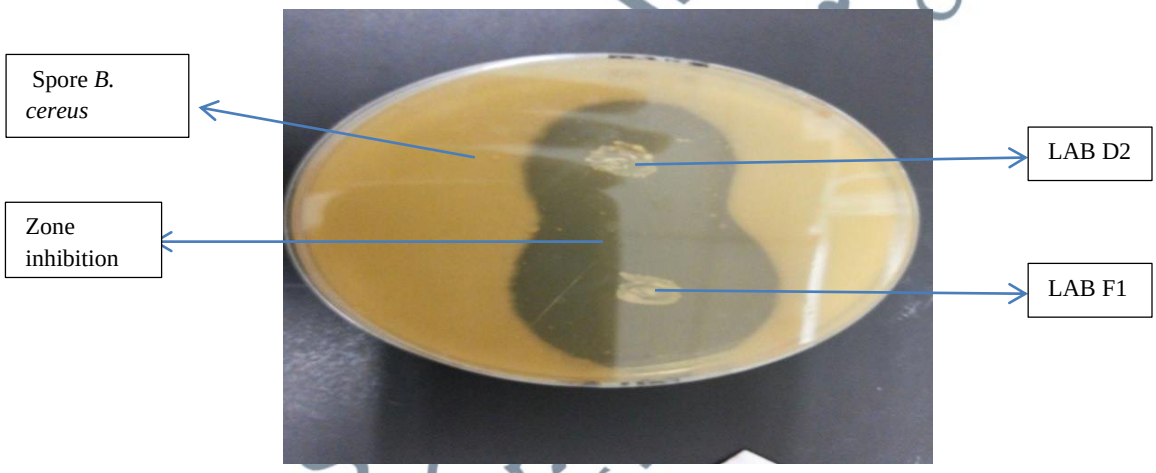


Figure 9: Antimicrobial activity of LAB cells against spore germination of *Bacillus* spp. using agar spot assay

Table 6: Antimicrobial activity of LAB against spore germination of *Bacillus spp.* using agar spot assay

Food sample	Code of Isolate	*B. subtilis*	*B. cereus*	*B. spizizenii*
Nestle Yogurt	M1	18.00±2.828	12.00±1.414	11.00±.000
	M2	19.00±4.243	16.00±2.828	18.00±1.414
	M3	22.00±2.828	17.00±1.414	11.00±.000
	M4	12.00±.000	11.00±.000	15.00±2.828
	M5	15.00±1.414	20.00±4.243	16.00±2.828
Fresh Yogurt	F1	15.00±4.243	28.00±4.243	12.00±1.414
	F2	12.00±.000	20.00±4.243	16.00±1.414
	F3	25.00±2.828	21.00±4.243	15.00±2.828
	F4	24.00±1.414	19.00±2.828	11.00±1.414
	F5	14.00±1.414	23.00±2.828	16.00±4.243
Syria Yogurt	S1	17.00±2.828	19.00±5.657	12.00±.000
	S2	19.00±4.243	17.00±1.414	15.00±2.828
	S3	12.00±.000	12.00±2.828	11.00±2.828
	S4	22.00±4.243	19.00±2.828	17.00±2.828
	S5	21.00±.000	21.00±4.243	18.00±2.828
Assafi Yogurt	N1	21.00±4.243	18.00±2.828	14.00±2.828
	N2	17.00±1.414	15.00±2.828	21.00±1.414
	N3	19.00±2.828	18.00±2.828	17.00±1.414
	N4	17.00±2.828	11.00±.000	23.00±2.828
	N5	21.00±2.828	22.00±2.828	15.00±.000
Homemade Yogurt	H1	24.00±5.657	21.00±2.828	12.00±1.414
	H2	16.00±4.243	11.00±4.243	18.00±4.243
	H3	18.00±3.536	12.00±2.828	12.00±.000
	H4	14.00±5.657	21.00±2.828	12.00±4.243
	H5	16.00±1.414	23.00±1.414	21.00±4.243
Belacan Penang	BP1	17.00±1.414	22.00±4.243	26.00±2.828
	BP2	20.00±4.243	17.00±2.828	18.00±1.414
	BP3	16.00±1.414	15.00±1.414	18.00±4.243
	BP4	20.00±2.828	21.00±4.243	20.00±1.414
	BP5	13.00±.000	11.00±2.828	15.00±4.243
Belacan Pankor	BK1	13.00±2.828	14.00±2.828	19.00±1.414
	BK2	23.00±4.243	17.00±2.828	12.00±1.414
	BK3	21.00±2.828	24.00±2.828	16.00±1.414
	BK4	15.00±4.243	12.00±.000	21.00±1.414
	BK5	24.00±1.414	12.00±4.243	19.00±2.828
Belacan Talyor Malaysia	BT1	12.00±2.828	17.00±4.243	17.00±1.414
	BT2	18.00±.000	22.00±4.243	11.00±2.828
	BT3	16.00±.000	21.00±1.414	18.00±4.243
	BT4	21.00±5.657	12.00±.000	20.00±2.828
	BT5	15.00±1.414	11.00±1.414	19.00±2.828
Fermented Durian	D1	18.00±2.828	16.00±2.828	17.00±4.243
	D2	22.00±1.414	25.00±5.657	17.00±2.828
	D3	12.00±1.414	18.00±2.828	23.00±1.414
	D4	20.00±.000	18.00±2.828	12.00±.000
	D5	17.00±4.243	15.00±1.414	20.00±4.243

Diameter of growth inhibitory zone against spore germination of *Bacillus* spp. was measured in(mm) after 24 h using spot assay method , the result was expressed as mean±standrad deviation of value obtained from duplicate experiment , Mean ±SD.

3.2.3.2 Well-diffusion method

The results of antimicrobial activity against spore germination of *Bacillus* spp. as determined by well-diffusion are shown in Table 8 and Figure 10. Significant difference was observed between and among the samples ($p < 0.05$). In contrast to LAB cells, the CFS of LAB tends to give lower activity against spore germination of the *Bacillus* spp. studied. It was observed 24.4% of the CFS prevent germination of *B. subtilis*, 29% for *B. cereus* and 15.5% against *B. spizizenii*. Highest zone inhibition against spore germination was observed for H1, BT1 with 16 mm against *B. subtilis*, H5 with 16 mm against *B. cereus* and D4 with 15 mm against *B. spizizenii*. The lowest zone inhibition was recorded by BT4 with 8 mm against *B. subtilis*, M1, F4, BK4 with 8 mm against *B. cereus* and F1,F4, F5, S2, S3, H3 with 8 mm against *B. spizizenii*.

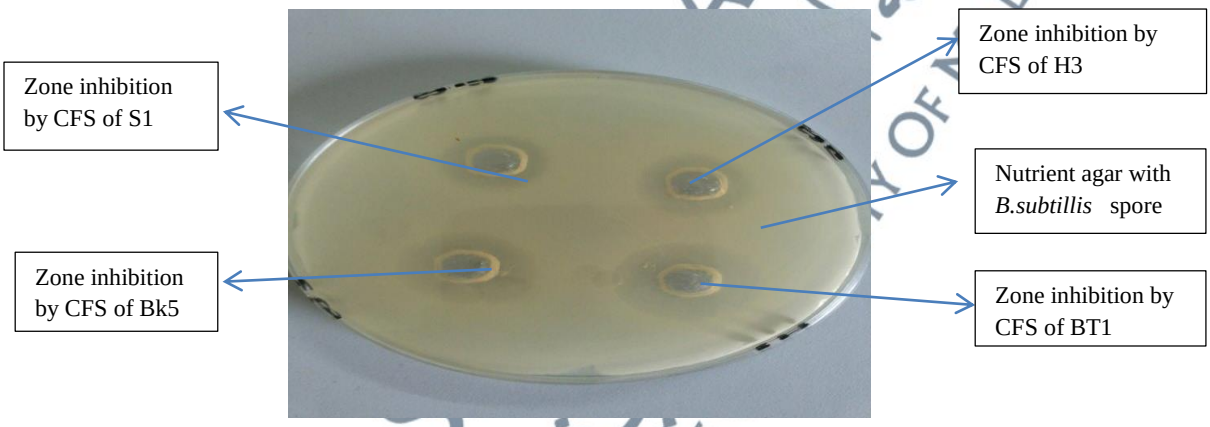


Figure10. Plate showing inhibition zone of *Bacillus* spp. spore germination by well-diffusion method incubate at 37 ºC for 24 h

Table 7: Antimicrobial activity of lactic acid bacteria supernatant against spore germination of *Bacillus* spp. using well diffusion method

Food sample	Code of Isolate	*B. subtilis*	*B. cereus*	*B. spizizenii*
Nestle Yogurt	M1	11.00±2.828	8.00±.000	9.00±1.414
	M2	12.00±4.243	9.00±1.414	9.00±2.828
	M3	11.00±1.414	9.00±.000	9.00±4.243
	M4	9.00±2.828	10.00±.000	12.00±1.414
	M5	11.00±1.414	10.00±2.828	11.00±2.828
Fresh Yogurt	F1	9.00±1.414	10.00±2.828	8.00±.000
	F2	9.00±2.828	10.00±1.414	9.00±4.243
	F3	11.00±1.414	9.00±.000	10.00±1.414
	F4	12.00±2.828	8.00±.000	8.00±.000
	F5	12.00±.000	9.00±1.414	8.00±.000
Syria Yogurt	S1	12.00±1.414	10.00±.000	10.00±2.828
	S2	14.00±2.828	11.00±2.828	8.00±.000
	S3	9.00±2.828	9.00±1.414	8.00±.000
	S4	15.00±2.828	11.00±.000	11.00±2.828
	S5	11.00±1.414	15.00±2.828	10.00±1.414
Assafi Yogurt	N1	14.00±2.828	14.00±2.828	11.00±1.414
	N2	14.00±1.414	11.00±2.828	10.00±.000
	N3	15.00±2.828	11.00±1.414	11.00±1.414
	N4	14.00±.000	11.00±1.414	9.00±4.243
	N5	14.00±1.414	11.00±.000	9.00±4.243
Homemade Yogurt	H1	16.00±2.828	11.00±1.414	10.00±.000
	H2	11.00±2.828	9.00±2.828	10.00±2.828
	H3	11.00±2.828	15.00±1.414	8.00±1.414
	H4	10.00±1.414	15.00±4.243	9.00±1.414
	H5	9.00±.000	16.00±2.828	10.00±1.414
Belacan Penang	BP1	15.00±1.414	9.00±.000	14.00±2.828
	BP2	11.00±1.414	10.00±.000	11.00±2.828
	BP3	12.00±1.414	14.00±4.243	9.00±1.414
	BP4	14.00±.000	13.00±2.828	9.00±1.414
	BP5	9.00±2.828	11.00±.000	13.00±2.828
Belacan Pankor	BK1	11.00±1.414	10.00±.000	11.00±2.828
	BK2	12.00±1.414	13.00±2.828	12.00±2.828
	BK3	9.00±.000	11.00±2.828	9.00±4.243
	BK4	10.00±.000	8.00±1.414	9.00±2.828
	BK5	14.00±1.414	13.00±1.414	9.00±.000
Belacan Talyor Malaysia	BT1	16.00±4.243	14.00±2.828	10.00±.000
	BT2	10.00±1.414	11.00±.000	13.00±2.828
	BT3	9.00±2.828	9.00±.000	9.00±1.414
	BT4	8.00±.000	10.00±2.828	11.00±4.243
	BT5	11.00±1.414	12.00±1.414	11.00±.000
Fermented durian	D1	14.00±2.828	13.00±4.243	14.00±1.414
	D2	11.00±.000	14.00±1.414	13.00±2.828
	D3	9.00±.000	11.00±1.414	14.00±2.828
	D4	10.00±1.414	13.00±1.414	15.00±2.828
	D5	14.00±.000	11.00±2.828	10.00±1.414

Diameter of growth inhibitory zone against spore germination of *Bacillus* spp. was measured in(mm) after 24 h using well diffusion method, the result was expressed as mean±standrad deviation of value obtained from duplicate experiment, Mean ±SD.

3.2.4 Effect of pH of LAB cell free supernatants on growth inhibitory activity of *Bacillus* spp. vegetative cells and spore germination

Variable inhibitory activity against vegetative cells and spore germination was observed when the pH of cell free supernatants was adjusted to pH 4, 5 and 6.5 (Table 9). The supernatants of all LAB isolates showed good inhibitory activity against vegetative cells at pH 4 of which 46.5 % of CFS was effective against *B. cereus* compared to 31% against *B. subtilis* and 37.8 % against *B. spizizenii*. However, the isolate from yogurt (S1-S5, F1-F5, M1-M5, N1-N5, H1-H5), lost its inhibitory activity when the pH was adjusted to 5 and 6.5. While isolates from belacan (BP1-BP5, BK1-BK5, BT1-BT5) and fermented durian (D1-D5) showed activity at pH 5 and 6.5, 15.5% against *B. subtilis*, 20% against *B. cereus* and 2% against *B. spizizenii*.

Similarly, the cell free supernatant of all LAB inhibited spore germination at pH 4; 26% against *B. cereus*, 29% against *B. subtilis* and 13.3% against *B. spizizenii* (Table 10). The cell free supernatant of isolate from yogurt (S1-S5, F1-F5, M1-M5, N1-N5, H1-H5), lost the ability to prevent spore germination when pH of supernatant was adjusted to 5 and 6.5. It is interesting to note that the isolates from belacan (BP1-BP5, BK1-BK5, BT1-BT5) and fermented durian(D1-D5) showed activity at pH 5 and 6.5; of 13.3 % prevent spore germination of *B. cereus* 11% against *B. subtilis* and 13.3% against *B. spizizenii*.

Table 8: Antimicrobial activity of LAB cell free supernatants against vegetative cell of *Bacillus* spp. after pH adjusted as determined by well-diffusion method incubated at 37 ºC for 24 h

LAB	pH:4			pH:5			pH:6,5		
Isolate	*B. cereus*	*B. subtilis*	*B. spizizenii*	*B. cereus*	*B. subtilis*	*B. spizizenii*	*B. cereus*	*B. subtilis*	*B. spizizenii*
S1	10	10	11	N.I	N.I	N.I	N.I	N.I	N.I
S2	15	13	14	N.I	N.I	N.I	N.I	N.I	N.I
S3	13	11	13	N.I	N.I	N.I	N.I	N.I	N.I
S4	15	10	13	N.I	N.I	N.I	N.I	N.I	N.I
S5	14	13	14	N.I	N.I	N.I	N.I	N.I	N.I
F1	12	10	10	N.I	N.I	N.I	N.I	N.I	N.I
F2	12	8	10	N.I	N.I	N.I	N.I	N.I	N.I
F3	11	8	11	N.I	N.I	N.I	N.I	N.I	N.I
F4	10	10	10	N.I	N.I	N.I	N.I	N.I	N.I
F5	12	9	12	N.I	N.I	N.I	N.I	N.I	N.I
M1	14	8	12	N.I	N.I	N.I	N.I	N.I	N.I
M2	10	10	11	N.I	N.I	N.I	N.I	N.I	N.I
M3	11	8	11	N.I	N.I	N.I	N.I	N.I	N.I
M4	13	9	11	N.I	N.I	N.I	N.I	N.I	N.I
M5	11	9	10	N.I	N.I	N.I	N.I	N.I	N.I
N1	16	11	12	N.I	N.I	N.I	N.I	N.I	N.I
N2	13	10	14	N.I	N.I	N.I	N.I	N.I	N.I
N3	12	11	12	N.I	N.I	N.I	N.I	N.I	N.I
N4	13	13	13	N.I	N.I	N.I	N.I	N.I	N.I
N5	15	12	10	N.I	N.I	N.I	N.I	N.I	N.I
H1	15	11	14	N.I	N.I	N.I	N.I	N.I	N.I
H2	13	10	11	N.I	N.I	N.I	N.I	N.I	N.I
H3	15	11	15	N.I	N.I	N.I	N.I	N.I	N.I
H4	13	10	11	N.I	N.I	N.I	N.I	N.I	N.I
H5	12	10	13	N.I	N.I	N.I	N.I	N.I	N.I
BP1	8	14	14	9	13	13	8	11	11
BP2	10	11	12	11	11	12	10	11	10
BP3	13	13	10	12	12	11	9	10	9
BP4	13	14	9	13	14	9	10	12	8
BP5	10	10	12	11	11	10	9	9	9
BK1	10	11	13	9	11	12	9	10	12
BK2	14	13	10	13	12	11	11	11	10
BK3	11	9	11	11	11	9	12	12	10
BK4	9	10	9	10	10	12	11	12	12
BK5	14	13	9	12	12	9	13	12	9
BT1	15	15	9	15	13	8	13	14	10
BT2	10	11	14	11	11	13	12	12	12
BT3	10	11	9	10	11	11	12	12	9
BT4	10	8	10	10	9	9	12	11	8
BT5	14	11	12	13	12	12	10	11	10
D1	13	15	14	14	13	12	11	12	11
D2	15	11	13	14	12	13	13	13	13
D3	13	9	14	11	8	15	11	9	13
D4	12	10	15	12	9	12	14	10	12
D5	11	14	10	10	13	9	11	13	9

Note: the diameter of inhibition zone was measured as diameter in mm. N.I:no inhibition

Table 9: Antimicrobial activity of pH adjusted LAB cell free supernatants against vegetative cell of *Bacillus* spp. as determined by well-diffusion method incubated at 37 ºC for 24 h

LAB Isolate	pH:4			pH:5			pH:6.5		
	B. ereus	*B. subtilis*	*B. spizizenii*	*B .cereus*	*B. subtilis*	*B. spizizenii*	*B. cereus*	*B. subtilis*	*B. spizizenii*
S1	8	12	10	N.I	N.I	N.I	N.I	N.I	N.I
S2	11	13	8	N.I	N.I	N.I	N.I	N.I	N.I
S3	8	10	8	N.I	N.I	N.I	N.I	N.I	N.I
S4	11	13	11	N.I	N.I	N.I	N.I	N.I	N.I
S5	13	11	10	N.I	N.I	N.I	N.I	N.I	N.I
F1	10	9	9	N.I	N.I	N.I	N.I	N.I	N.I
F2	9	9	9	N.I	N.I	N.I	N.I	N.I	N.I
F3	11	11	10	N.I	N.I	N.I	N.I	N.I	N.I
F4	9	11	8	N.I	N.I	N.I	N.I	N.I	N.I
F5	9	12	10	N.I	N.I	N.I	N.I	N.I	N.I
M1	8	10	8	N.I	N.I	N.I	N.I	N.I	N.I
M2	9	12	10	N.I	N.I	N.I	N.I	N.I	N.I
M3	10	11	11	N.I	N.I	N.I	N.I	N.I	N.I
M4	10	10	11	N.I	N.I	N.I	N.I	N.I	N.I
M5	10	11	10	N.I	N.I	N.I	N.I	N.I	N.I
N1	13	13	12	N.I	N.I	N.I	N.I	N.I	N.I
N2	11	14	11	N.I	N.I	N.I	N.I	N.I	N.I
N3	11	13	11	N.I	N.I	N.I	N.I	N.I	N.I
N4	11	14	11	N.I	N.I	N.I	N.I	N.I	N.I
N5	11	14	9	N.I	N.I	N.I	N.I	N.I	N.I
H1	10	16	9	N.I	N.I	N.I	N.I	N.I	N.I
H2	9	12	10	N.I	N.I	N.I	N.I	N.I	N.I
H3	15	11	9	N.I	N.I	N.I	N.I	N.I	N.I
H4	13	9	8	N.I	N.I	N.I	N.I	N.I	N.I
H5	14	9	10	N.I	N.I	N.I	N.I	N.I	N.I
BP1	8	14	14	8	14	14	8	13	14
BP2	10	11	11	9	11	10	9	11	10
BP3	13	12	9	12	13	9	12	13	9
BP4	13	13	9	13	12	9	13	12	9
BP5	10	10	14	9	10	13	9	13	13
BK1	10	10	12	10	10	11	10	10	11
BK2	14	12	10	14	12	9	14	12	9
BK3	11	10	9	11	10	10	10	10	10
BK4	9	10	8	9	10	9	9	10	9
BK5	13	13	10	13	12	10	13	12	10
BT1	14	15	10	14	15	10	14	15	10
BT2	10	10	13	9	9	13	10	9	12
BT3	10	9	9	10	9	9	10	10	9
BT4	10	9	11	10	9	10	10	9	10
BT5	12	11	11	11	11	11	11	11	11
D1	13	12	13	13	13	13	13	12	13
D2	15	10	13	15	11	12	15	10	12
D3	12	8	15	12	8	15	12	8	15
D4	12	11	15	12	11	14	12	10	14
D5	11	14	9	11	13	10	11	13	10

Note: the diameter of inhibition zone was measured as diameter in mm. N.I:no inhibition

3.2.5 Detection of proteolytic activity of selected LAB isolates

It was observed that 20 LAB isolates produced clear zones when grown on skim milk agar. The diameter of hydrolysis varied with LAB isolates ranging from 2.5 to 3 mm (Figure 11 and Table 11). Fifteen LAB isolated from different sources of belacan samples and five from fermented durian produce extracellular proteolytic enzyme that hydrolyzed casein. This may be indicative that peptides are produced by these LAB.

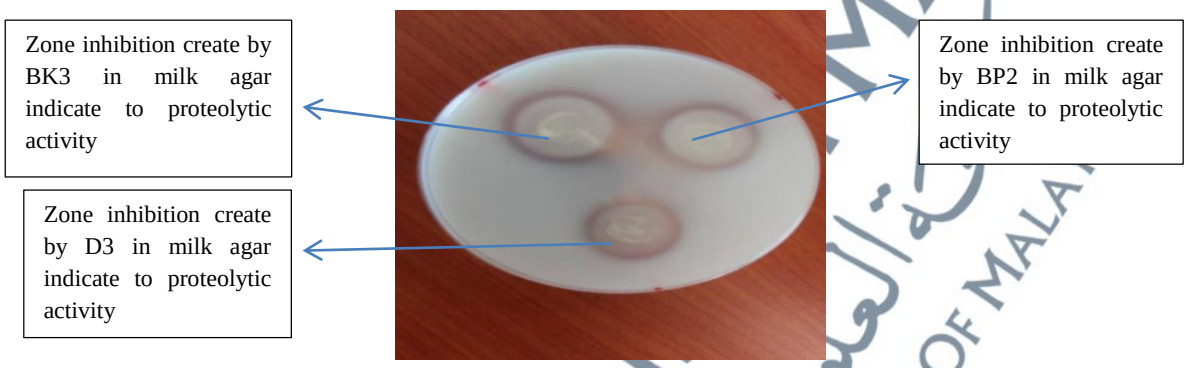


Figure 11: Clear zone surrounding the colonies indicate proteolysis activity

Table 10: Protoylitic activity of LAB isolates on skim milk agar

LAB isolates	Clear zone (mm)
BP1	2.5
BP2	2.5
BP3	3
BK1	3
BK2	3
Bk3	3
BT1	3
BT2	2
BT3	3
BT4	2
D1	3
D2	2
D3	3
D4	3
D5	2

3.2.6 Effect of enzymes treated LAB cell free supernatant on antimicrobial activity

Treatment of proteinase K and RNase enzymes to the selected 20 LAB supernatants showed that four isolates BP2, BK4, BT1 from belacan and D2 from fermented durian resulted in decrease in their antimicrobial activity against vegetative cell of *Bacillus* spp. compared to untreated CFS. Even though growth of *B. cereus*, *B. subtilis* and *B. spizizenii* was observed in the titer plates, the increase in growth was less than control indicating that supernatant contain antimicrobial peptide (Figure12, 13 and 14 and table 12 and 13).

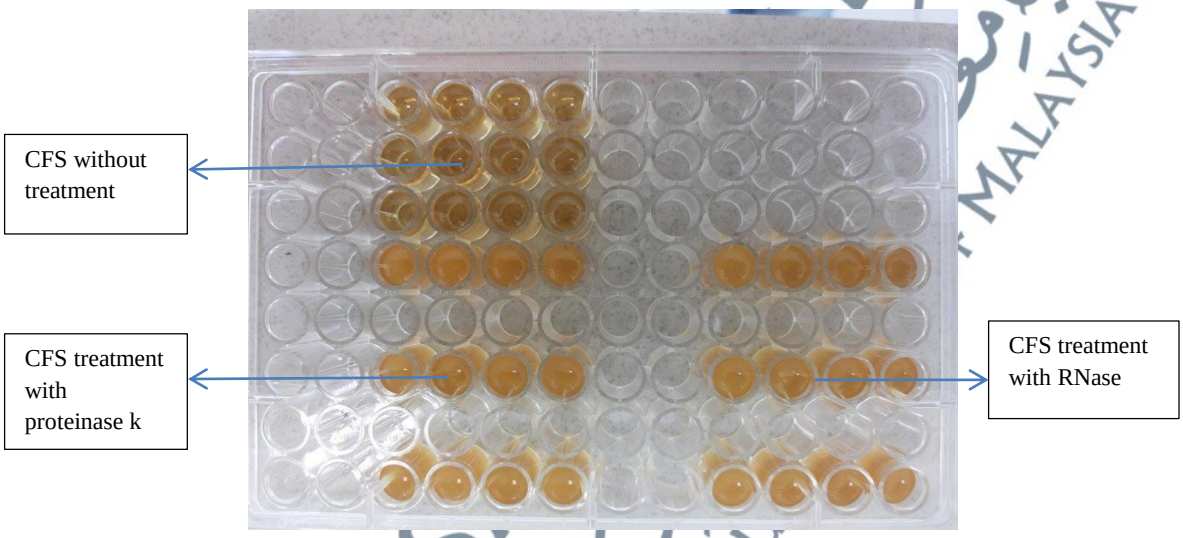


Figure 12: Effect of enzymes on LAB supernatant against *Bacillus* spp.

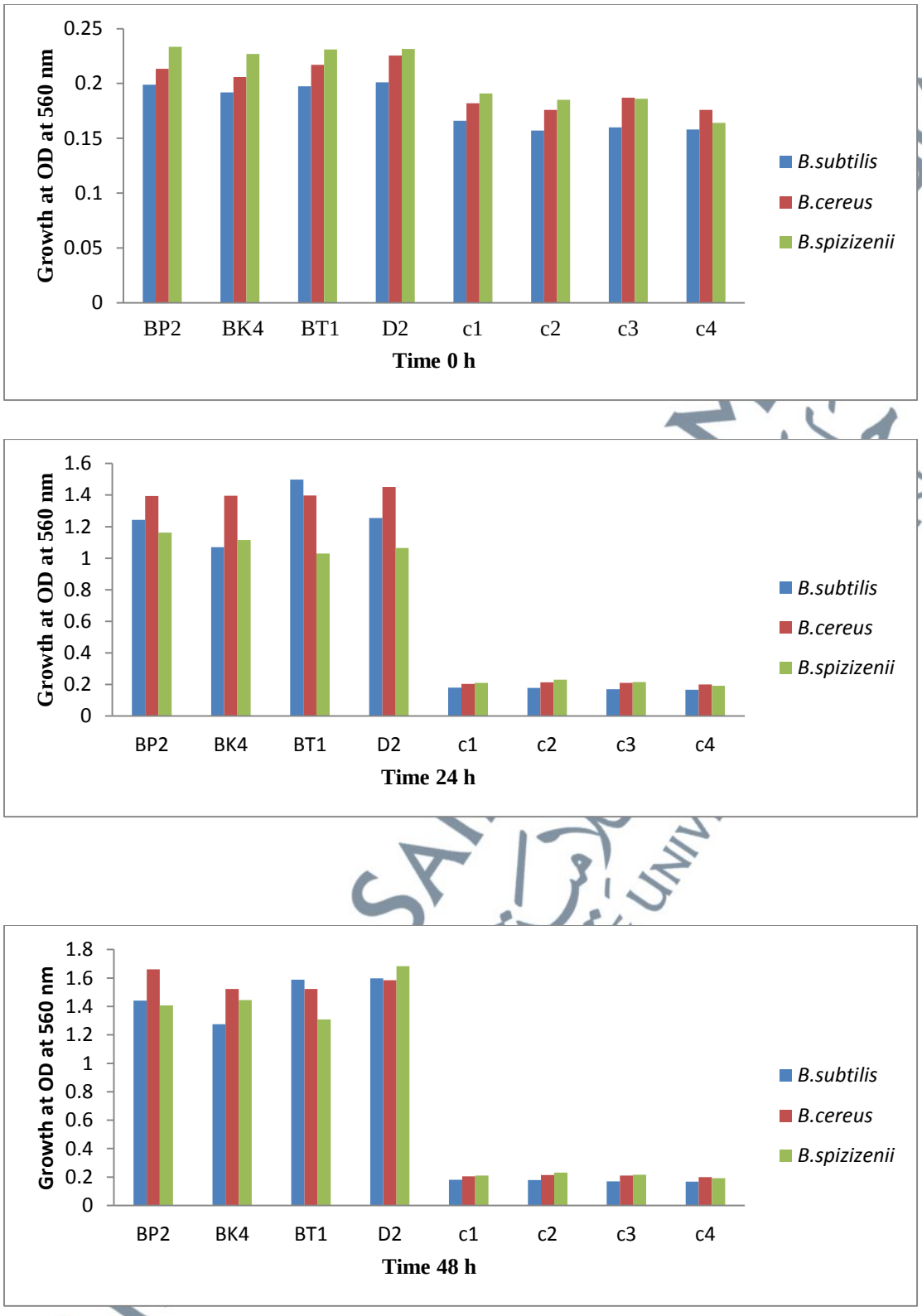
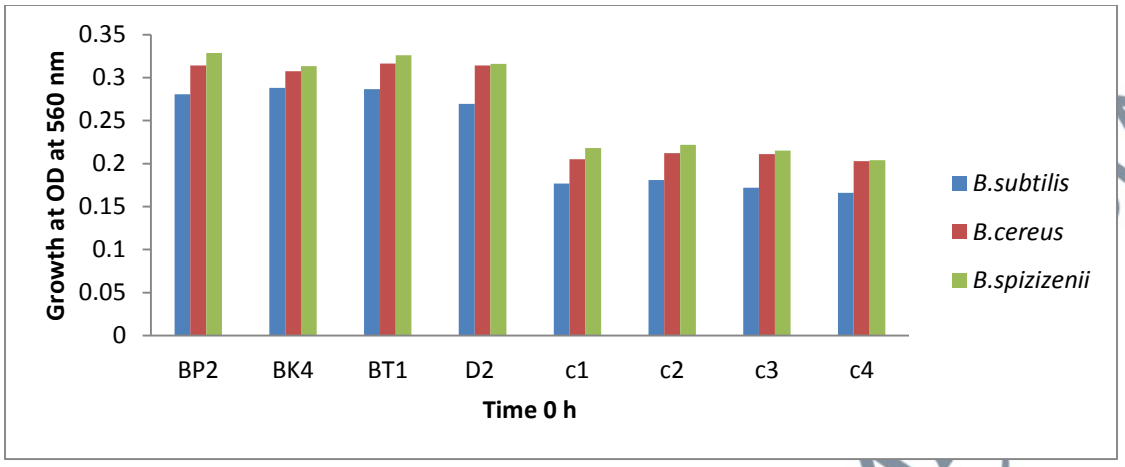
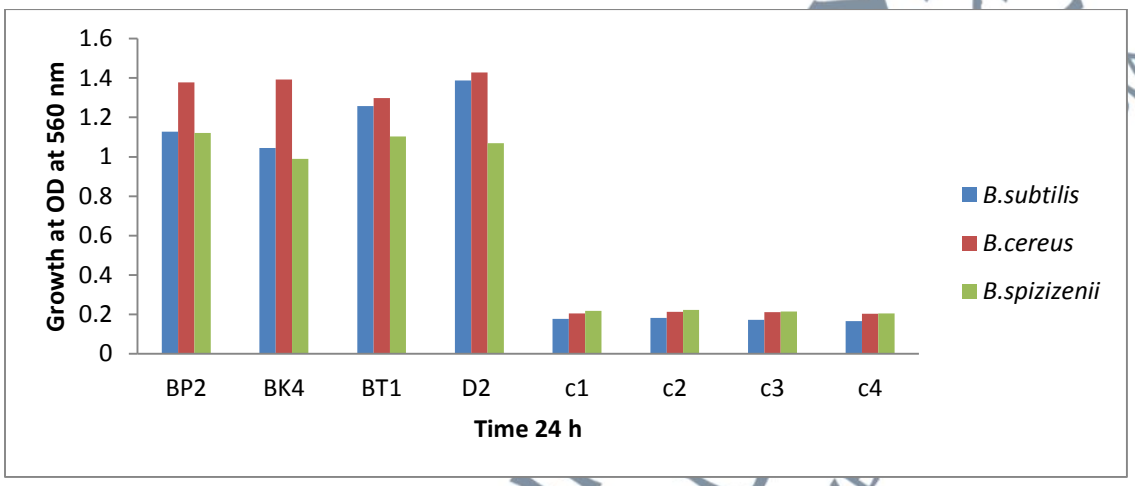


Figure 13: Growth of *Bacillus* spp. in treated supernatant with proteinase k as measured by optical density 560 nm in 96 wells titer plates





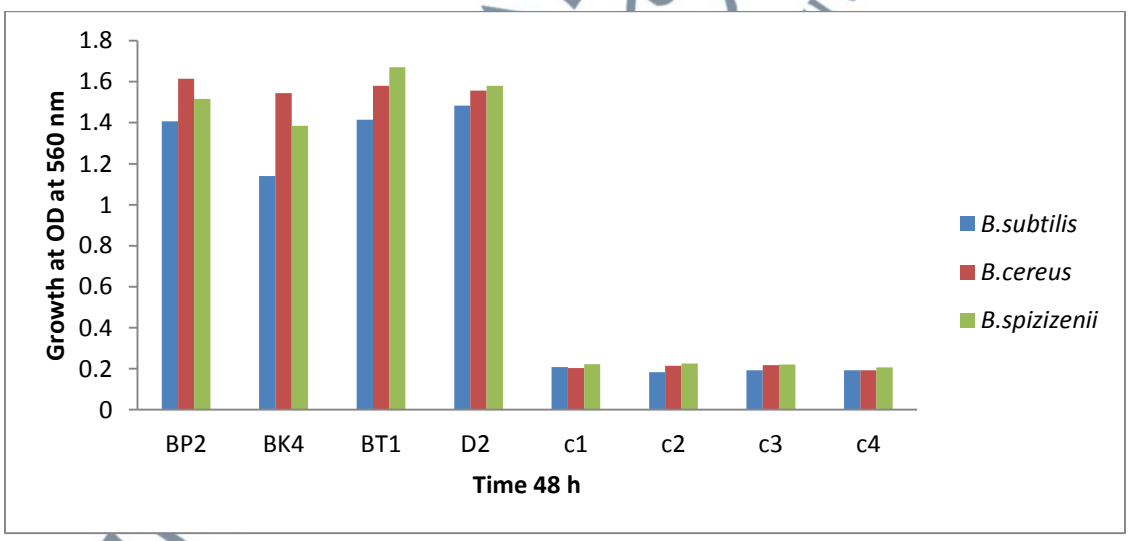


Figure 14: Growth of *Bacillus* spp. in treated supernatant with RNase as measured by optical density 560 nm in 96 wells titer plates

Table 11: Growth percentage of *Bacillus* spp. with LAB supernatant after treatment with Proteinase K measured in microtiter plate incubated at 30 ◦C for 48 h

Bacillus spp.	Time (h)	BP2	Control BP2	BK4	Control BK4	BT1	Control BT1	D2	Control D2
B. subtilis	24 h	3.01	N.G	2.62	N.G	3.39	N.G	4.14	N.G
	48 h	4.01	0.17	2.91	0.016	3.93	0.122	4.50	0.16
B. cereus	24 h	3.38	N.G	3.52	N.G	3.09	N.G	3.52	N.G
	48 h	4.1	N.G	4.02	0.014	3.99	0.028	3.95	N.G
B. spizizenii	24 h	2.41	N.G	2.12	N.G	2.38	N.G	2.38	N.G
	48 h	3.63	0.018	3.41	0.014	4.12	0.027	4.00	0.14

Growth was measured as OD at 560 nm, NG: No growth.

Table 12: Growth percentage of *Bacillus* spp. with LAB supernatant after treatment with RNase I measured in microtiter plate incubated at 30 ◦C for 48 h

Bacillus spp.	Time (h)	BP2	Control BP2	BK4	Control BK4	BT1	Control BT1	D2	Control D2
B. subtilis	24 h	5.24	0.084	4.54	0.14	6.5	0.062	5.24	0.05
	48 h	6.23	0.29	5.64	0.15	7.04	0.20	6.95	0.31
B .cereus	24 h	5.52	0.12	5.77	0.21	5.43	0.12	5.43	0.13
	48 h	6.78	0.11	6.39	0.42	6.01	0.11	6.02	0.13
B. spizizenii	24 h	3.9	0.099	3.20	0.24	3.46	0.16	3.60	0.16
	48 h	5.02	0.10	5.33	0.26	4.66	0.17	4.95	0.15

Growth was measured as OD at 560 nm, NG: No growth.

3.2.7 Antibiotic susceptibility of LAB isolates

The 20 isolates that maintain activity at PH 5 and 6.5 showed variable susceptibility to six antibiotics (penicillin, gentamicin, streptomycin, chloramphenicol, vancomycin and tetracycline), evaluated by agar diffusion method. All the isolates were highly susceptible to PI (penicillin,), C (chloramphenicol) and TE (tetracycline) but less resistant to GN (gentamicin). However, all the isolates were resistant to VA (vancomycin) and S (streptomycin) (Table 12 and Figure 15).

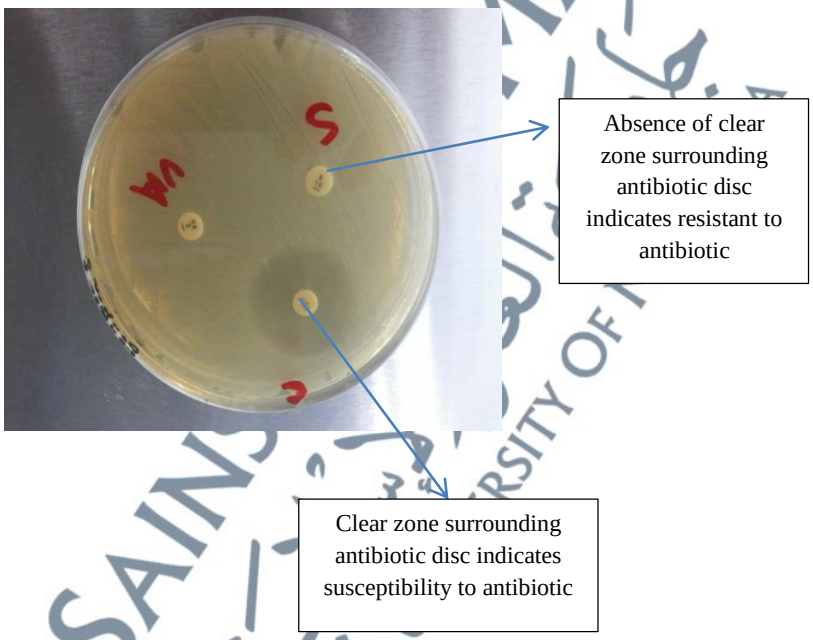


Figure 15: Antibiotic susceptibility assay of the LAB isolates using antibiotic disc method

Table 13: Antibiotic resistance of the LAB isolates to different antibiotics as measured by diameter of inhibition zones

LAB Isolate	Antibiotic(clear zone inhibition mm)					
	PI	GN	C	VA	S	TE
BP1	15	4	17	R	R	14
BP2	14	4	19	R	R	15
BP3	14	4	21	R	R	16
BP4	14	4	19	R	R	17
BP5	14	4	20	R	R	17
BK1	16	4	21	R	R	17
BK2	14	4	21	R	R	17
BK3	14	4	22	R	R	17
BK4	14	4	20	R	R	17
BK5	15	4	21	R	R	17
BT1	10	4	21	R	R	14
BT2	14	4	21	R	R	18
BT3	13	4	21	R	R	17
BT4	11	4	18	R	R	14
BT5	16	4	21	R	R	17
D1	11	4	23	R	R	17
D2	14	4	21	R	R	16
D3	14	4	19	R	R	14
D4	14	4	21	R	R	16
D5	14	4	21	R	R	14

PI (penicillin,), C (chloramphenicol), TE (tetracycline), GN (gentamicin), VA (vancomycin) and S (streptomycin), R (resistant)

3.3 Discussion

Lactic acid bacteria are one of the most important groups of microorganisms that use in the production of valuable foods including fermented milk products (cheese, yogurt, kefir, cereals and vegetables) due to ability of LAB to produce different compound such as such as organic acids, di-acetyl, hydrogen peroxide and bacteriocin during lactic fermentations (Darsanaki et al., 2012). The use of antimicrobial compounds produce by LAB is one method that can be used to control of microorganisms (Djadouni & Kihal, 2012).

In this study the cells and the cell free supernatant of LAB isolated from different fermented food available in Malaysian market (yogurt, belacan and fermented durian) showed variable ability in their ability to inhibit the growth of the vegetative cells or spore germination, Using spot assay method, it was observed that growth of vegetative cells of *B. subtilis*, *B. cereus* and *B. spizizenii* was inhibited by the cells of LAB isolated from belacan BP4, BK1 and BT5, respectively. The antagonistic effect against spore germination was observed for cells of isolate H1 and BK5 against *B. subtilis*, D2 against *B. cereus* and BP1 against *B. spizizenii*. In contrast, the cell free supernatants of BT1, H1 and S2 were observed affective to inhibit the growth of vegetative cell and spore germination of *B. subtilis*, *B. cereus* and *B. spizizenii*, respectively.

Most work reported the antimicrobial activity of LAB on vegetative cells of Gram positive and Gram negative bacteria (Aween et al., 2012; Kivanc et al., 2011; Li et al., 2015; Djadouni and Kihal , 2012) as well as against growth of fungi mycelia and germination of fungal spores (Muhialdin & Hassan, 2011).

However, there are limited reports on antimicrobial activity of LAB against both vegetative cells and spore germination of *Bacillus* spp. Digaitiene et al. (2012) reported that supernatant of *L. sakei* KTU05-06, *P. acidilactici* KTU05-7, *P. pentosaceus* KTU05-8, KTU05-9 and KTU05-10 isolated from sourdoughs produced bacteriocin-like inhibitory substances (BLIS) that were able to inhibit growth of activated spores of *B. subtilis* in varying degree. The metabolites produced by *L. johnsonii* CRL1647 and *Enterococcus faecium* SM21 produce lactic acid , acetic acid and bacteriocin that had the ability to reduce the number of vegetative cells and spores of the *B. cereus* strains (Soria & Audisio, 2014). Similarly, Pepe et al., (2003) reported that *L. plantarum* E5 and *Leuconostoc mesenteroides* A27 had anti-rope activity against

B. subtilis, B. licheniformis, *B. cereus, B. clausii* and *B. firmus* that produce ropiness in bread slices, and suggested that these LAB to be used as starters for bread-making. Klewicka and Libudzisz (2004) found the *L. acidophilus* had inhibitory activity against vegetative cells and spores of *B. mycoides* and *B. subtilis* besides *E. coli, P. fluorescens, P. aeruginosa, S. aureus* and suggested that the inhibitory effect was due to the ability of *L. acidophilus* to produce lactic and acetic acids as well as hydrogen peroxide.

It was observed that the supernatant of LAB that was isolated from fermented yogurt loss its antimicrobial activity against cell growth and spore germination of *Bacillus* spp. when the pH was adjusted to 5 and 6.5 but not at pH 4. Similar observation was reported by Aween et al. (2012) whereby the antimicrobial activity of *L. acidophilus* supernatant isolated from honey remained at pH 3, but the antimicrobial activity was lost at pH 5 for supernatant H006-A and H008-D. This suggests that the inhibition activity of LAB could not be attributed to lactic acid alone but also to some as yet uncharacterized substances effective at pH values below 4.5 in the presence of lactic acid and also secretion of organic acids which depends strongly on the pH (Kivanc et al., 2011; Laref & Guessas, 2013).

Laref and Guessas (2013) suggested that LAB produce organic acid that might also activate other antifungal compounds such as peptides by lowering the pH, this peptide was stable at pH values between 3.0 and 4.5 but rapidly decreased between 4.5 and 6.0. These researchers also observed that the antifungal activity of *L. plantarum* LB52 and *L. plantarum* LB20 strains remained at pH 6.0 and 7.0 due to dissociate organic acids. Zeineb et al. (2013) reported that the antimicrobial peptide produced by LAB is active in a pH range between 2 and 11. The supernatant of *L. plantarum* strains isolated from the traditional butter made from camel milk were stable between pH 2 and 6, but lost activity at pH 8 (Maurad & Meriem, 2008). Saad et al. (2015) Noted that the antimicrobial activity of crude supernatant was stable at wide range of pH (2-8) and may be useful in acidic as well as non-acidic foods.

It was observed that the selected 20 LAB strains showed proteolytic activity suggesting these LAB produced extracellular proteolytic enzymes that hydrolyzed casein protein present in the milk agar as shown by the emergence of clear zones around the colony. Atanasova et al.(2014) reported that *L. lactic* subs. *lactis* (strain 1598), *S. thermophilus* (strains t3D1, Dt1, t39, t38), *L. delbrueckii* subsp. *lactis* (strains 1043) and *L. delbrueckii* subsp. *bulgaricus* (strains b38,

b122 and b24) utilize milk proteins as their prime source amino acids for growth and generated proteolytic and bioactive peptides after the peptidase hydrolysis of long oligopeptides during milk fermentation. In another study, *L. plantarum* CRL 775 and *P. pentosaceus* CRL 792 isolated from sourdough was reported had the ability to degrade α-gliadin fragments, and this indicates that these LAB contain peptidase enzyme responsible for this degradation (Gerez et al., 2008). Similarly, Yelnetty et al. (2014) reported that *L. plantarum* and *L. pentosus* isolated from fermented local goat milk produced clear zone in skim milk agar indicating that the isolates could degrade protein or had proteolytic activity. The proteolytic activity of dairy lactic acid bacteria *Lactococcus lactis* subsp. *lactis, E. faecalis, E. faecium, E. durans, L. paracasei* subsp. *paracasei, L. plantarum* and *L. rhamnosus* is essential for the bacterial growth in milk and production of high quality fermented dairy products due to peptidase and amino acids formed (Hassaïne et al. 2007).

Four strain of LAB BP2, BK4, BT1and D2 were treated with different enzyme (proteinase K, RNase I), and all these isolates lost the antimicrobial activity compared with the untreated sample (control). The sensitivity of the supernatant to proteinase K and RNase I indicates the presence of proteinaceous compounds. Similar results were reported by Saad et al. (2015) and Taheri et al. (2012). The antibacterial compounds produced *L. pentosus*2MF8 and 8CF, *L. plantarum* 4DE, 3DM and *Lactobacillus* spp. CS1 were inactivated by proteolytic enzymes proteinase K, protease B and trypsin (Corsetti et al., 2004). Loss of anti-bacterial activity of the bacteriocin produce by *L. curvatus, L. delbrueckii, L. fermentum, E. faecium,* and *P. acidilactici* was due to treatment with trypsin, protease E, and proteinase K (Tomé et al. (2009), These enzyme cause peptides to degrad in the intestinal tract and so will be easily digested without affecting the intestinal flora. Anas et al. (2008) observed that the chymotrypsin and trypsin enzyme reduced totally the antimicrobial activity of the *Lactobacillus* strain *(L. rhamnosus, L. plantarum, L. casei, L. paracasei* subsp. *paracasei, L. acidophilus, L. delbrueckii* subsp. *lactis, L. fermentum, L. paraplantarum* and *L. sakei* subsp. *Sakei*) against *S. aureus*, The activity of strain supernatant was lost after the treatment with proteolytic enzyme indicating that active component was proteinaceous and growth inhibition was caused by bacteriocin. On the contrary LAB isolated from sourdoughs produce BLIS which were active against spore germination and vegetative outgrowth of *B. subtilis*, these BLIS had little or no effect on the

antimicrobial activities after treatment with four different baking enzymes hemicellulase, lipase, amyloglucosidase and amylase (Narbutaite et al., 2008).

The susceptibility to antibiotic varies with strains of LAB evaluated. In this study a total of 20 isolates were tested for their antibiotic susceptibility. All isolates were susceptible to penicillin, gentamicin, chloramicin and streptomycin but resistant to chloramphenicol, vancomycin. The antibiotic susceptibility of LAB is important because bacteria used as probiotics can provide antibiotic resistant genes transfer to the pathogenic bacteria (Tigu et al. 2016). Previously, Dessalegn & Ashenafi (2010) reported that strains of *Lactobacillus, Lactococcus, Pediococcus* and *Leuconostoc* are resistant to vancomycin and lactobacilli are usually sensitive to antibiotic such as penicillin and ampicillin. Uroić et al. (2014) noted that *Lactobacillus, Leuconostoc pseudomesenteroides* ZGBP4-14 isolated from artisanal fresh soft and white pickled cheeses had phenotypic resistance to vancomycin and susceptible to 11 antibiotics (penicillin G, ampicillin, bacitracin, erythromycin, gentamicin, clindamycin, chloramphenicol, streptomycin, neomycin, tetracycline and novobiocin). Zhou et al. (2012) mention that *L. bulgaricus* and *S. thermophiles* isolated from Chinese yogurts showed resistance to ampicillin, chloramphenicol, chlortetracycline, tetracyclines, lincomycin, streptomycin, neomycin, and gentamycin while *S. thermophilus* was susceptible to penicillin G and roxithromycin.

3.4 Conclusion

Lactic acid bacteria (LAB) isolated from fermented food (yogurt, belcan, durian) had good antimicrobial activity against foodborne pathogens *Bacillus* spp. Lactic acid bacteria have been demonstrated in this study to produce antimicrobial compound against growth cell and spore germination *B. cereus*, *B. subtilis* and *B. spizizenii* such as lactic acid, acetic acid, hydrogen peroxide and bactriocin. All Cell free supernatant (CFS) produce by LAB enhance their antimicrobial activity at PH 4, 5, 6.5 except LAB isolate from yogurt lost their antimicrobial activity at PH 5, 6.5, however, the CFS lost their antimicrobial activity after treatment with proteinase k and RNase I this indicate that the CFS produce by LAB from fermented belcan, durian had ability to release peptide compound can inhibit growth and spore germination of *Bacillus* spp. The proteolytic activity of 20 of the LAB provide proteolytic enzymes cause degraded of casein protein generated proteolytic and bioactive peptides. The antibiotic susceptibility of LAB is important because LAB used as probiotics can provide antibiotic resistant genes transfer to the pathogenic bacteria .

3.5 RECOMMENDATIONS

The result of this study showed that LAB isolate from fermented food (of yogurt, belcan, durian) produce some compound had antimicrobial activity against growth cell and spore germination of *Bacillus* spp. that often cause food spoilage and food born disease. Future studies are needed as listed below:

- Identify the responsible compounds for the antimicrobial activities of these LAB.
- Study the effectiveness of these compound on other pathogenic microorganisms.