CHAPTER 4

CHARACTERIZATION OF PROBIOTIC PROPERTIES OF LACTIC ACID BACTERIA ISOLATED FROM MALAYSIAN FERMENTED FOOD

4.1 Introduction

The most widely accepted definition of probiotics across global is "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO 2002). Probiotics provide numerous benefits, including improved health, a balanced ratio of healthy flora in the intestine, and increased host resistance to pathogen invasion (Tripathi and Giri, 2014). Probiotics have also been widely studied for their potential to lower serum cholesterol, alleviate lactose intolerance, prevention of allergies, and production of bioactive metabolites such as vitamins (Bhat and Bajaj, 2019).

Many studies have been done to discover potential probiotics in food, but FAO/WHO (2002) has set out a systematic approach in the evaluation of potential probiotics. It includes the ability of the species to resist the destructive level of gastric acidity and the bile acid produced in the digestive tract. Besides, the potential probiotics must be capable of producing antimicrobial activity towards common pathogenic bacteria in the digestive system and must also have resistance to antibiotics. Apart from that, their hemolytic properties must also be proved as non-β-haemolytic. They should also be capable of autoaggregating among themselves and could adhere to hydrocarbons, which could reflect their ability to adhere to the intestinal cells.

As probiotic foods obtained attention as health-promoting foods and several fermented foods were reported to contain potential probiotics, the situations enticed the attention of researchers to study various other types of fermented foods and discover new probiotic strains. It is also driven by the demand for non-dairy probiotics to overcome the increasing number of lactose intolerance cases around the globe (Argyri et al., 2013). The most investigated probiotics isolated from fermented foods are lactic acid bacteria (LAB), as they are the dominant microflora or fermented food and are also involved in the fermentation processes (Abushelaibi et al., 2017). LABs are classified as GRAS organisms and are dominant in the small intestine (Agaliya and Jeevaratnam, 2012). Numerous studies have reported that LAB isolated from fermented food possessed probiotic potential (Gupta and Tiwari, 2014; Angmo et al., 2016; Abid et al., 2018; Ahmad et al., 2018; Masalam et al., 2018).

In spite of the extensive use of belacan and budu in Malaysian meal as traditional condiments, limited research has been done to characterize the probiotic potentials of its isolates. To date, a few attempts have been made to characterize the bacteria isolated from budu and belacan, with certain drawbacks. For example, Liasi et al. (2009) only discussed the antimicrobial activity and antibiotic sensitivity of LAB isolated from budu. Meanwhile, Sim et al. (2012) reported the resistance profile of strains isolated from budu towards low pH and bile salts, as well as the inhibitory characteristics against foodborne pathogens, but lacked other probiotic characteristics. Another study by Khalil et al. (2018) concentrated on the probiotic characteristics of exopolysaccharides producing *Lactobacillus* isolated from budu. In the case of belacan sample, the study by Haitham (2017) focused on LAB, which grows in a strict anaerobic environment, with no evidence of haemolytic properties, resistance patterns towards antibiotics and autoaggregation ability. However, as bosou is one of the traditional fermented food natives for the Kadazandusun tribe of East Malaysia (Lajius, 2014), no work has been reported that explore the probiotic potential of strains isolated from bosou. Therefore, the objective of this study was to characterize the probiotic characteristics of LAB isolated from belacan, bosou, and non-cooked budu. The investigations include the acidic pH and bile tolerance abilities, haemolytic activities, antagonism properties towards four different pathogens, resistance patterns towards selected antibiotics, cell surface hydrophobicity, and autoaggregation ability.

4.2 Materials and Methods

4.2.1 Determination of Acidic pH Tolerance

The isolates were examined for its tolerance to acidic pH following Zhang et al. (2016) with slight modification. In brief, 100 µL overnight culture of LAB grown in MRS broth equivalent to 0.5 McFarland standard or 1.5 x 10^8 colony forming units/ml (CFU/mL) was transferred into 900 µL MRS broth adjusted to pH 2.5, pH 3, and pH 6.2 (control) using hydrochloric acid (HCl) and incubated at 37°C for 3 h. The survival ability of isolates towards different pH after 3 h were determined, respectively, by serially diluting and inoculating the culture on MRS agar plate and incubated at 37°C for 48 h. Colonies formed were enumerated, documented as $\log_{10}$ values of CFU/mL and compared with the control. The experiment was performed in duplicates with three repetitions, and *Lacticaseibacillus casei* strain Shirota (Yakult, Japan) was used as a reference strain. The percentage resistance to acidic pH was calculated using the equation below:

$$\boxed{\text{Percentage resistance (\%)} = (\log_{10T}/\log_{10C}) \times 100}$$

Where:

$\log_{10T}$: $\log_{10}$ CFU/ml at pH 2.5 or pH 3;

$\log_{10C}$: $\log_{10}$ CFU/ml at pH 6.2.

Isolates showing resistance above 90% at pH 3 considered acid-tolerant strains and were selected for further investigation (Zhang et al., 2016).

4.2.2 Determination of Bile Tolerance

The tolerance of each isolate towards bile were evaluated following Kumar and Kumar (2015) with some alterations. Initially, 25 μL overnight-grown bacterial suspensions equivalent to 0.5 McFarland standard were inoculated into 1 mL MRS broth without bile salt (control) and with bile salt (Oxoid, UK) adjusted to 0.3% (w/v), respectively. All isolates were incubated at 37°C, and the optical density (OD) was recorded at wavelength 600 nm after 7 h and 24 h incubation. The absorbance of a culture grown in MRS broth with 0.3% bile salt was compared to a control culture with the absence of bile salt. The experiments were performed in three independent repetitions with duplicates for each experiment, and *L. casei* strain Shirota was used as a reference strain. The percentage of resistance to bile was calculated using the following equation:

$$\boxed{\text{Percentage resistance (\%)} = (OD_T/OD_C) \times 100}$$

Where:

OD_T: absorbance of isolate in MRS broth with 0.3% bile salt at 7 h or 24 h;

OD_C: absorbance of isolate in MRS broth without bile salt at 7 h or 24 h.

Isolates with survival rates above 50% at 0.3% bile salt after 7-h incubation period was considered as bile tolerant and selected for further investigation (Kumar and Kumar, 2015).

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4.2.3 Determination of Haemolytic Activity

The selected isolates which show promising acidic pH and bile tolerance were assessed for their haemolytic activity. The overnight culture of each isolate, as well as the reference strain *L. casei* strain Shirota grown in MRS broth, was streaked on the surface of 5% sheep blood agar plates (Oxoid, UK) before incubated for 48 h at 37°C. The agar was observed for signs of β-haemolysis (clear zones around colonies), α-haemolysis (green-hued zones around colonies) or γ-haemolysis (no zones around colonies). The experiment was done in triplicates, and only isolates with α-haemolysis and γ-haemolytic properties were evaluated for the next characteristics (Argyri et al., 2013).

4.2.4 Determination of Antagonism Properties

The antagonistic interaction of selected isolates against two Gram-positive bacteria (*Bacillus cereus* (ATCC® 11778™ and *Staphylococcus aureus* NCTC 6571) and two Gram-negative bacteria (*Escherichia coli* ATCC® 25922™ and unknown strain of *Salmonella* Typhimurium) were determined by agar spot antimicrobial assay following the method described by Shokryazdan et al. (2014) with slight modifications. Overnight cultures grown in MRS broth were adjusted to the 0.5 McFarland standard and 2µL of each culture was spotted on MRS agar plates. The plates were air-dried for 30 min at 27°C prior to incubation at 37°C for 24 h. Colonies developed were overlaid with 10 mL molten MH agar (Muller Hinton; Merck Millipore, US) seeded with 1% pathogenic bacteria (10^6 CFU/ml) and were further incubated for another 24 h at 37°C. Ampicillin disc 10 µg (Oxoid, UK) was used as a positive control, whereas MRS broth was used as a negative control. *L. casei* strain Shirota was used as a reference strain. The inhibition zones produced were measured from the outward edge of isolates to the outward edge of the transparent region, and a reading of more than 1 mm around the spot was considered positive (Jacobsen et al., 1999).

4.2.5 Determination of Antibiotic Resistance Patterns

The antibiotic resistance patterns of the seventeen strains and the reference strain *L. casei* strain Shirota were determined semi-quantitatively using disc diffusion method as described by Angmo et al. (2016) with slight modifications. MRS agar plates were spread with 100 µl of overnight-grown bacterial suspension equivalent to 0.5 McFarland standard, respectively using a sterile cotton swab and were air-dried. Then, different classes of antibiotic discs (Oxoid, UK) containing ampicillin (10 µg), bacitracin (10 µg), chloramphenicol (10 µg), nalidixic acid (30 µg), penicillin G (10 units), streptomycin (10 µg), tetracycline (30 µg), and vancomycin (30 µg) were placed on inoculated plates under sterile conditions using ethanol dipped and flamed forceps. All plates were incubated for 48 h at 37°C before the diameter (mm) of inhibition zone was measured.

4.2.6 Determination of Cell Surface Hydrophobicity

Cell surface hydrophobicity is generally defined as the ability of bacteria to adhere to hydrocarbons and was determined as described by Divya et al. (2012) with slight modification. Each overnight isolate and the reference strain *L. casei* strain Shirota grown in MRS broth were centrifuged at 10,000 rpm for 15 min, and the pellets were washed twice with phosphate-buffered saline (PBS) (Oxoid, UK). The collected cells were resuspended again using similar buffer solution and were adjusted to OD in between 0.7 to 0.9 at 600 nm, which consisted of about 10^8 CFU/mL of bacteria. The readings were recorded as OD 0 min. Next, 2 mL of hexadecane (J.T. Baker, US) was added into 2 mL of suspension before the mixture was vortexed for 60 sec and incubated at 27°C for 30 min or until the aqueous and organic phases were entirely separated. The aqueous phase was pipetted out, and the absorbance is measured again (OD 30 min). The experiments were performed in three independent repetitions with triplicates for each experiment. The percentage of bacterial adhesion to hydrocarbon (BATH) was calculated using the following equation:

$$\boxed{\text{Percentage of BATH (\%)} = [1 - (OD_{30\ min}/OD_{0\ min})] \times 100}$$

Where:

$OD_{0\ min}$: absorbance of isolate at 0 min;

$OD_{30\ min}$: absorbance of isolate at 30 min.

4.2.7 Determination of Autoaggregation Ability

Autoaggregation ability of the isolates was carried out according to Mallappa et al. (2019) with slight modification. Overnight grown isolates and the reference strain *L. casei* strain Shirota were centrifuged at 10,000 rpm for 15 min and washed twice with PBS. The pellets were resuspended in the same buffer, mixed homogenously and adjusted to obtain an OD equivalent to 0.5 McFarland standard at 600 nm. The readings were recorded as OD 0 h. The bacterial suspensions (4 mL) were vortexed for 10 sec before it was allowed to stand for 5 h at 37°C without agitation. After the incubation period, 1 mL of the suspension was collected carefully, and the absorbance of isolates was measured again and recorded as OD 5 h. The experiments were performed in triplicates with three independent repetitions. The percentage of bacterial autoaggregation was calculated using the following equation:

$$\boxed{\text{Percentage of autoaggregation (\%)} = [1 - (OD_{5\ h}/OD_{0\ h})] \times 100}$$

Where:

$OD_{0\ h}$: absorbance of isolate at 0 h;

$OD_{5\ h}$: absorbance of isolate at 5 h.

4.2.8 Statistical Analysis

All results were analysed following the methods mentioned in Section 3.2.6.

4.3. Results

4.3.1 Determination of Acidic pH Tolerance

The ecosystem of the gastrointestinal tract is harsh and challenging for the survival of microorganisms, which includes the high-acidic condition during transit in the stomach. All 59 LAB isolates were examined for its resistance after 3 h of incubation in MRS broth adjusted to pH 3 and pH 2.5, reflecting the gastric residence time (Maurer et al., 2015). Figure 4.1 shows the effect of different pH values on the survival of BE isolates, BO isolates, BUM isolates, and the reference strain *L. casei* strain Shirota after 3 h incubation at 37°C.

Out of the 59 LAB isolates, only seventeen isolates (28.8%) showed excellent resistance with values above 90% at pH 3 whereas only fifteen isolates (25.4%) survived with the same percentage at pH 2.5. The percentage of viable cells tolerated at pH 3 and pH 2.5 ranged in between 90.5 to 99.4% and 87.4 to 97.5%, respectively. Meanwhile, the survival percentage of the commercial probiotic *L.casei* strain Shirota was 95.7% at pH 3 and 94.9% at pH 2.5. The result showed BE7 (98.2% at pH 3; 91.6% at pH 2.5), BO16 (93.8% at pH 3; 87.4% at pH 2.5), and BUM18 (93.2% at pH 3; 90% at pH 2.5) isolates' survival dropped significantly ($P < 0.05$) when exposed to pH 2.5, whilst no significant difference ($P > 0.05$) was observed for the remaining isolates and *L. casei* strain Shirota resistance when pH decreases from pH 3 to pH 2.5.

In another perspective, although the resistance percentage of all seventeen strains are above 90% in pH 3, there are varieties for some isolates that are significantly different between one another. For instance, BO1 recorded the highest (99.4%) tolerance and it was significantly different ($P < 0.05$) compared to BO16 and most of the BUM isolates (excluding BUM 5, BUM6, and BUM7). BO8 tolerance is similar amongst the highest (98.8%) and was significantly higher compared to the same BUM isolates as stated above. Meanwhile, BUM15 survival rates (90.5%) were the lowest in pH 3 and was significantly different compared to BE7, BE16, BO1, BO8, BO10, BUM 5, BUM6, BUM7, and the reference strain *L. casei* strain Shirota.

Stronger acid concentration (pH 2.5) had also caused a significant difference in survival capacities to the isolates, especially isolates from bosou sample. The results revealed that BO16's (87.4%) tolerance to pH 2.5 was significantly lower ($P < 0.05$) compared to BE16, BO1, BO8, BO10, BUM5, BUM6, BUM7, and *L. casei* strain Shirota. In contrast, the BO8 (97.5%) survival rate was significantly higher ($P < 0.05$) compared to BE7, BO16, BUM12, BUM15, BUM18, BUM22, and BUM23. BO1 (96.1%) also showed significantly higher resistance value ($P < 0.05$) compared to BO16, BUM15, BUM18, and BUM23, while BO10 (96.7%) tolerance was significantly higher ($P < 0.05$) compared to BO16, BUM15, BUM18, BUM22, and BUM23.

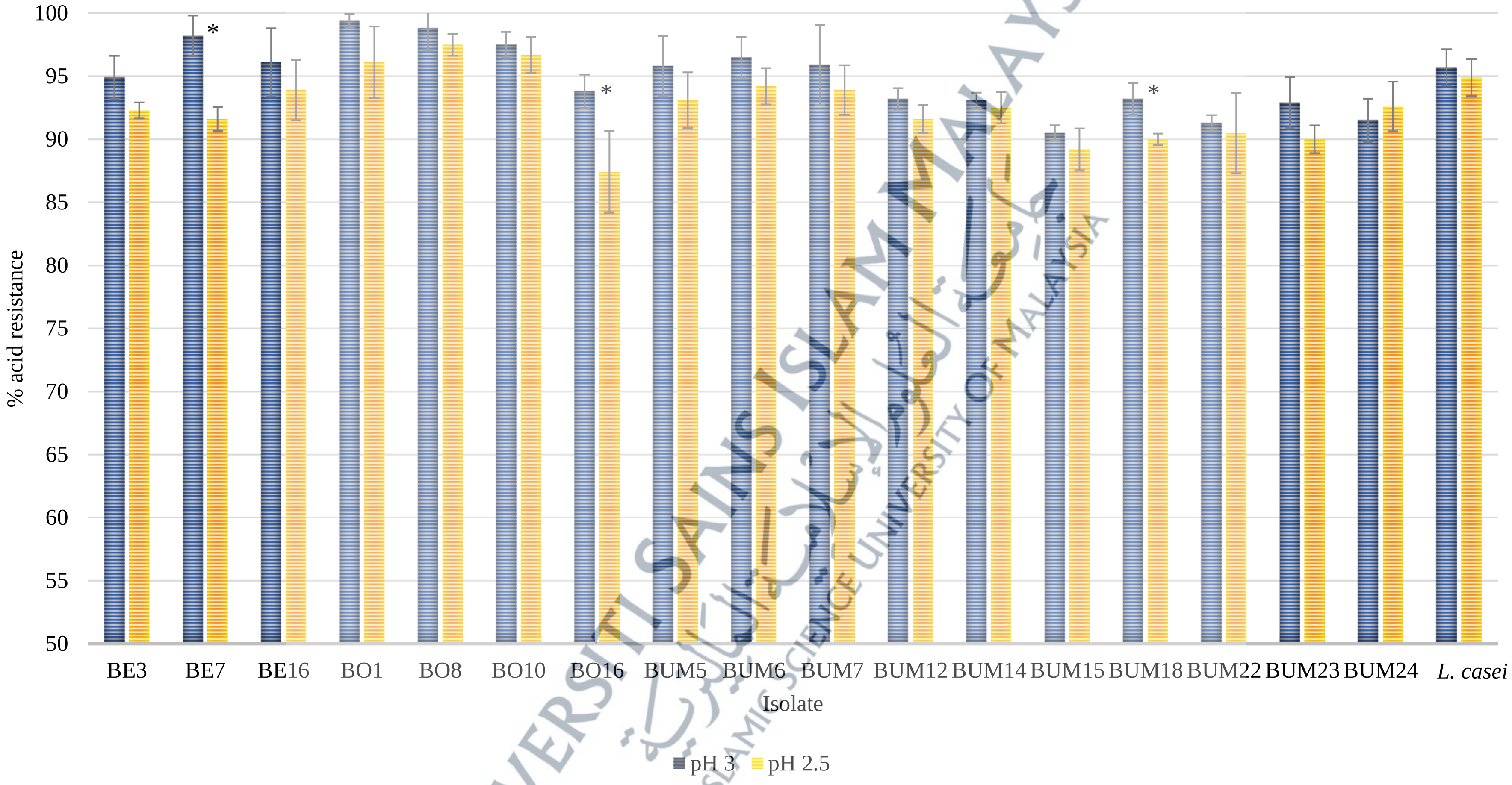

Figure 4.1: Effect of Different pH Values on Survival of BE Isolates, BO Isolates, BUM Isolates and *L. casei* Strain Shirota After 3 h Incubation At 37^{o}C

* Percentage resistances are significantly different (P<0.05) among both pH

4.3.2 Determination of Bile Tolerance

The seventeen isolates which survived acidic pH were further tested for their bile tolerance at 0.3% bile salts concentration. In this study, the data was recorded at 7 h; reflecting the condition in the intestines (Fallingborg et al., 1990; Maurer et al., 2015) and at 24 h. Figure 4.2 shows the effect of 0.3% bile salt on the survival of BE isolates, BO isolates, BUM isolates, and the reference strain *L. casei* strain Shirota after 7 h and 24 h incubation at 37°C. Generally, all seventeen isolates survived with a percentage above 50% after 7h incubation period in MRS broth containing 0.3% bile salt. However, only BE and BO isolates grew well, with values above 50% compared to controls after being incubated in the altered MRS broth for 24 h. Despite not reaching 50% survival in comparison to controls, BUM isolates were also growing, which was confirmed by the increases of absorbance reading when compared to 7 h. The percentage of resistant isolates ranged from 51.1% and 103.5% and from 33.9% to 82.2% after 7 h and 24 h incubation period, respectively. The commercial probiotic *L. casei* strain Shirota persisted in the 0.3% bile salt environment almost entirely, with resistance values of 94.7% after 7 h and 82.3% after 24 h interval.

At 7 h, BO16 (51.1%) recorded the lowest tolerance to bile and is significantly lower ($P < 0.05$) compared to most BUM isolates, BE3, and *L. casei* strain Shirota. On the other hand, BUM5 survived the highest with significantly different ($P < 0.05$) compared to all BO isolates, BE7, BE16, BUM12, BUM22, and BUM24. At 24 h, 80% of BUM isolates (excluding BUM5 and BUM6) survived significantly ($P < 0.05$) lower than at least three other isolates, namely *L. casei* strain Shirota, BE16, BO8. In contrast, BE and BO isolate survival capacities were not significantly differed ($P > 0.05$) between one another as well as *L. casei* strain Shirota.

In fact, all BUM isolates' survival percentage dropped significantly ($P < 0.05$) when exposed for a longer duration (24 h) to 0.3% bile salts. BE3 (99.1% at 7 h; 77.1% at 24 h) also showed significantly ($P < 0.05$) reducing effect as BUM isolates when compared among 7 h and 24 h. However, BO1 (71.5% at 7 h; 79.4% at 24 h), BO8 (65.1% at 7 h; 80.3% at 24 h), and BO16 (51.1% at 7 h; 78.1% at 24 h) resistance patterns appeared contradictory with significant ($P < 0.05$) increments at 24 h.

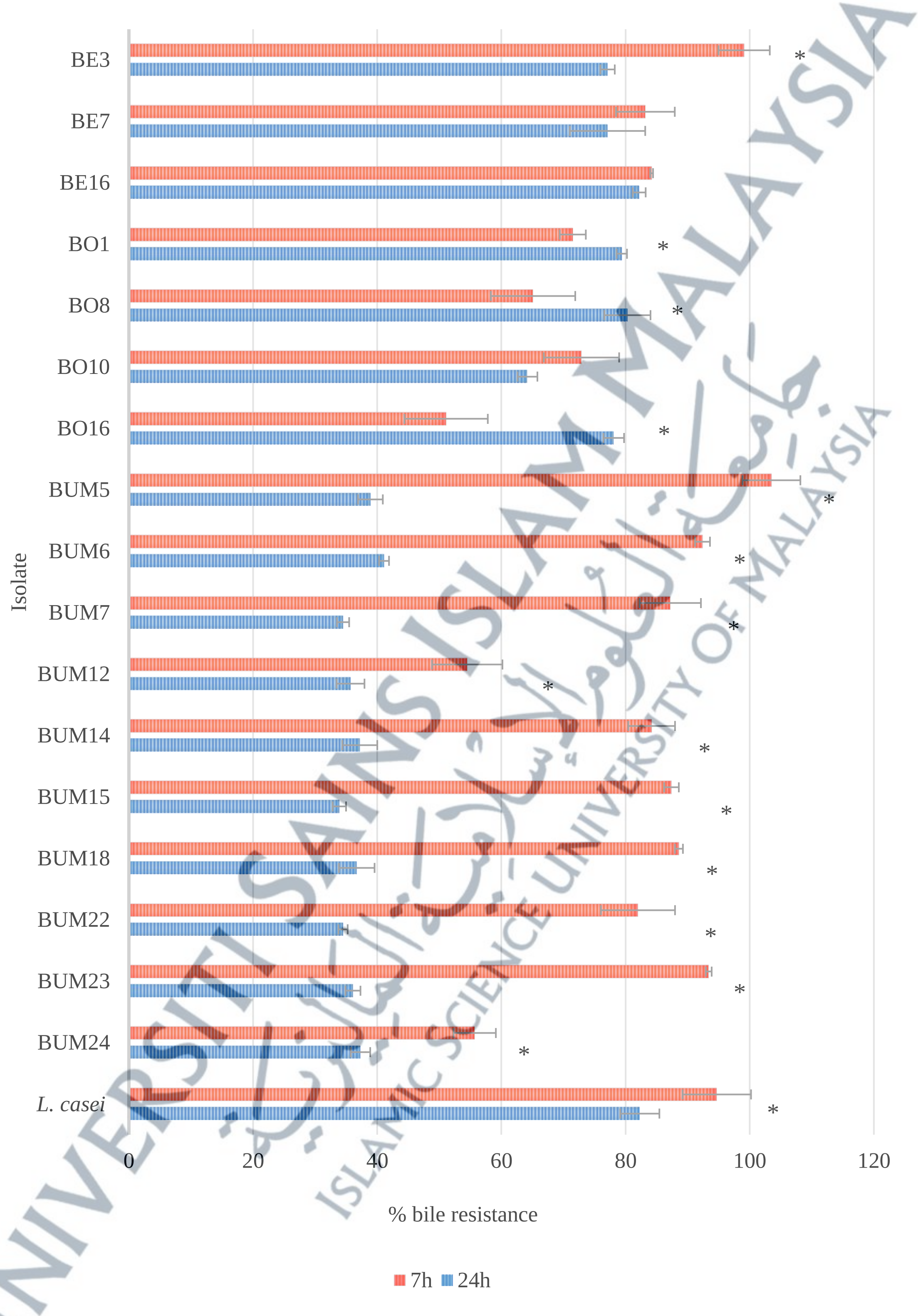

Figure 4.2: Effect of 0.3% Bile Salt on Survival of BE Isolates, BO Isolates, BUM Isolates and *L. casei* Strain Shirota After 7 h and 24 h Incubation At 37^{o}C

***** Percentage resistances are significantly different (*P*<0.05) among both incubation period

4.3.3 Determination of Haemolytic Activity

The haemolytic properties of the seventeen isolates were tested using 5% sheep blood agar plates. Haemolysis of blood agar is the destruction of red blood cells components, which could be due to production of haemolytic proteins by bacteria. The result of this study indicates that all isolates (BE3, BE7, BE16, BO1, BO8, BO10, BO16, BUM5, BUM6, BUM7, BUM12, BUM14, BUM15, BUM18, BUM22, BUM23, and BUM24) and the commercial probiotic *L. casei* strain Shirota showed α-haemolysis, which is partial haemolysis and did not demonstrate undesirable β-haemolytic or complete haemolytic activities (Figure 4.3).

Figure 4.3: α-hemolysis Pattern of BUM1 Isolate and *L. casei* Strain Shirota on 5% Sheep Blood Agar Plate

4.3.4 Determination of Antagonism Properties

One of the crucial aspects of probiotic is the ability to fight and eliminate pathogens. The antagonistic properties of the BE isolates, BO isolates, BUM isolates, and the reference strain *L. casei* strain Shirota was assessed using agar spot antimicrobial assay against four pathogenic strains, and the results are shown in Table 4.1. The results found that all seventeen isolates were able to inhibit the growth of all pathogens in varying degrees and are strain-dependent (Kumar and Kumar, 2015; Chopade et al., 2019). The isolates exhibited an inhibition zone against *B. cereus*, *S. aureus*, *E. coli*, and *S. typhimurium* with a range in between 5.50 to 10.67 mm, 7.33 to 10.60 mm, 8.00 to 14.17 mm and 7.83 to 13.20 mm, respectively. The results found that isolate BE3 exhibited the most robust inhibitory activity against *E. coli* (14.17 mm), while BUM23 recorded the weakest antagonism properties against *B. cereus* (5.50 mm).

Although all isolates obtained lower inhibition zones against *B. cereus* compared to other pathogens, the majority of the isolates' activities were significantly higher compared to a positive control ($P < 0.05$). Meanwhile, all BUM isolates excluding BUM15 inhibited the growth of *S. aureus* significantly less ($P < 0.05$) than the positive control. In terms of growth inhibition of *E. coli*, all BE and BO isolates, as well as four BUM isolates (BUM5, BUM14 BUM15, and BUM24) exhibited better ($P < 0.05$) antagonistic activities against *E. coli* than Ampicillin (10µg). The results also showed that only BUM7 and BUM22 showed significantly lower inhibition capacities ($P < 0.05$) towards *S. typhimurium* when compared with the positive control, whilst the others are not significantly dissimilar ($P > 0.05$).

When compared with the reference strain, all BE and BO isolates inhibited *B. cereus* growth significantly higher ($P < 0.05$) than *L. casei* strain Shirota, but only BE3 and BE7 showed significantly higher inhibition zones ($P < 0.05$) against *S. aureus*. Other than that, BE3, BO1, BO8, and BO16 also produced significantly greater inhibition zone ($P < 0.05$) than *L. casei* strain Shirota towards the growth of *E. coli* whilst BE7 is significantly better in inhibiting the growth of *S. typhimurium* compared to *L. casei* strain Shirota.

From the different viewpoint of comparison, BO10 and BUM7 antagonism activities against all tested pathogens are not significantly different ($P > 0.05$) from one another whereas BE3 inhibition patterns towards these four pathogens were significantly dissimilar ($P < 0.05$).

Table 4.1: The Mean of The Inhibition Zone of BE Isolates, BO Isolates, BUM Isolates and *L. casei* Strain Shirota Against Pathogenic Bacteria

	Inhibition zone (from outward edge of isolates to outward edge of clear region) (mm)			
Isolates	*B. cereus*	*S. aureus*	*E. coli*	*S.* Typhimurium
BE3	8.83 ± 0.75	10.17 ± 0.98	14.17 ± 0.75	12.00 ± 0.63
BE7	8.00 ± 0.63	10.60 ± 1.14	11.50 ± 2.51	13.20 ± 1.64
BE16	8.33 ± 0.82	10.33 ± 1.97	11.83 ± 2.48	11.80 ± 1.64
BO1	8.67 ± 0.52	9.33 ± 0.82	13.17 ± 0.75	10.00 ± 1.41
BO8	8.67 ± 0.52	9.17 ± 0.98	13.67 ± 2.66	12.17 ± 1.17
BO10	10.67 ± 1.63	9.50 ± 0.55	10.83 ± 1.94	12.17 ± 1.60
B016	8.40 ± 0.55	9.67 ± 1.03	12.67 ± 1.03	11.83 ± 0.75
BUM5	6.67 ± 1.21	7.33 ± 1.03	11.50 ± 0.55	10.00 ± 0.89
BUM6	5.67 ± 1.03	7.80 ± 0.45	9.17 ± 0.98	10.80 ± 0.84
BUM7	6.00 ± 0.89	7.80 ± 1.64	8.00 ± 2.00	7.83 ± 0.75
BUM12	6.33 ± 0.52	7.67 ± 1.21	9.33 ± 0.52	11.83 ± 3.12
BUM14	6.67 ± 0.82	8.33 ± 0.82	10.83 ± 1.47	11.33 ± 2.50
BUM15	7.33 ± 1.21	10.00 ± 2.76	9.50 ± 2.88	12.33 ± 2.25
BUM18	6.33 ± 1.37	8.67 ± 2.66	9.00 ± 1.26	10.67 ± 2.42
BUM22	6.33 ± 0.82	7.67 ± 1.21	9.17 ± 0.98	9.00 ± 0.71
BUM23	5.50 ± 0.55	8.17 ± 2.14	9.33 ± 1.03	10.50 ± 2.26
BUM24	6.33 ± 1.21	8.00 ± 1.55	9.67 ± 0.81	11.00 ± 2.68
L. casei	6.17 ± 0.75	8.20 ± 1.48	9.67 ± 2.34	10.17 ± 1.17
Ampicillin (10ug)	1.50 ± 0.55	11.00 ± 0.71	6.83 ± 0.75	11.50 ± 0.84

Values represents the means of two independent experiments, each in triplicates ± SD.

4.3.5 Determination of Antibiotic Resistance Patterns

The isolates were tested for their antibiotic resistance patterns against eight different antibiotics using a disc diffusion assay, and the results are shown in Table 4.2. Diameters of 20 mm or greater were considered susceptible, diameters of 15 mm to 19 mm were considered as intermediate, and diameters of 14 mm or less were considered resistant (Sharma et al., 2016). Based on the findings, the resistant patterns of BE isolates are strain-dependent. Whilst all three LAB strains were susceptible to ampicillin and chloramphenicol, as well as resistant to nalidixic acid, streptomycin and vancomycin, it showed different resistance patterns towards bacitracin, penicillin G, and tetracycline.

Meanwhile, three out of four BO isolates were sensitive to half of the antibiotics used in the assay (ampicillin, chloramphenicol, penicillin G, and tetracycline), but showed intermediate response towards bacitracin and were resistant to nalidixic acid, streptomycin, and vancomycin. BO8 showed a different profile as it was also resistant to bacitracin. In contrast, all BUM isolates exhibited similar patterns of antibiotic resistance profiles between each other, and the reference strain *L. casei* strain Shirota. The isolates were susceptible to ampicillin, bacitracin, chloramphenicol, penicillin G and tetracycline but resistant to nalidixic acid, streptomycin, and vancomycin.

Table 4.2: Assessment of Antibiotic Resistance Patterns of BE Isolates, BO Isolates, BUM Isolates and *L. casei* Strain Shirota to Eight Different Types of Antibiotics

	Antibiotic							
Isolates	AMP	BAC	CHL	NAL	PEN	STR	TET	VAN
BE3	S	I	S	R	S	R	I	R
BE7	S	R	S	R	I	R	I	R
BE16	S	I	S	R	S	R	S	R
BO1	S	I	S	R	S	R	S	R
BO8	S	R	S	R	S	R	S	R
BO10	S	I	S	R	S	R	S	R
B016	S	I	S	R	S	R	S	R
BUM5	S	S	S	R	S	R	S	R
BUM6	S	S	S	R	S	R	S	R
BUM7	S	S	S	R	S	R	S	R
BUM12	S	S	S	R	S	R	S	R
BUM14	S	S	S	R	S	R	S	R
BUM15	S	S	S	R	S	R	S	R
BUM18	S	S	S	R	S	R	S	R
BUM22	S	S	S	R	S	R	S	R
BUM23	S	S	S	R	S	R	S	R
BUM24	S	S	S	R	S	R	S	R
L. casei	S	S	S	R	S	R	S	R

Degree of inhibition: Susceptible (S): inhibition zone ≥ 20 mm; Intermediate (I): inhibition zone 15–19 mm; Resistance (R): inhibition zone ≤ 14 mm (Sharma et al., 2016).
AMP: Ampicillin; BAC: Bacitracin; CHL: Chloramphenicol; NAL: Nalidixic acid; PEN: Penicillin G; STR: Streptomycin; TET: Tetracycline; VAN: Vancomycin

4.3.6 Determination of Cell Surface Hydrophobicity

Hydrophobicity is one of the vital features to measure the potential of bacteria as future probiotics. Further analysis was done to measure the adhesion of the seventeen isolates to hydrocarbons (hexadecane) using the phase separation method. As shown in Figure 4.4, the hydrophobicity of the strains was varied with values that ranged from 16.62 to 19.26%, 11.66 to 25.62%, and 3.63 to 7.09% to BE isolates, BO isolates, and BUM isolates, respectively. The results obtained showed that BO1 had the highest percentage of hydrophobicity (25.62%) with a significant difference ($P < 0.05$) to all BUM isolates and the reference strain *L. casei* strain Shirota (5.53%). In fact, BUM isolates showed quite low hydrophobicity values and were fairly similar among them as well as *L. casei* strain Shirota. However, no significant difference ($P > 0.05$) in hydrophobicity was observed among strains isolated from similar samples, respectively, except for a significant difference ($P < 0.05$) in hydrophobicity percentage in between BUM7 and BUM18.

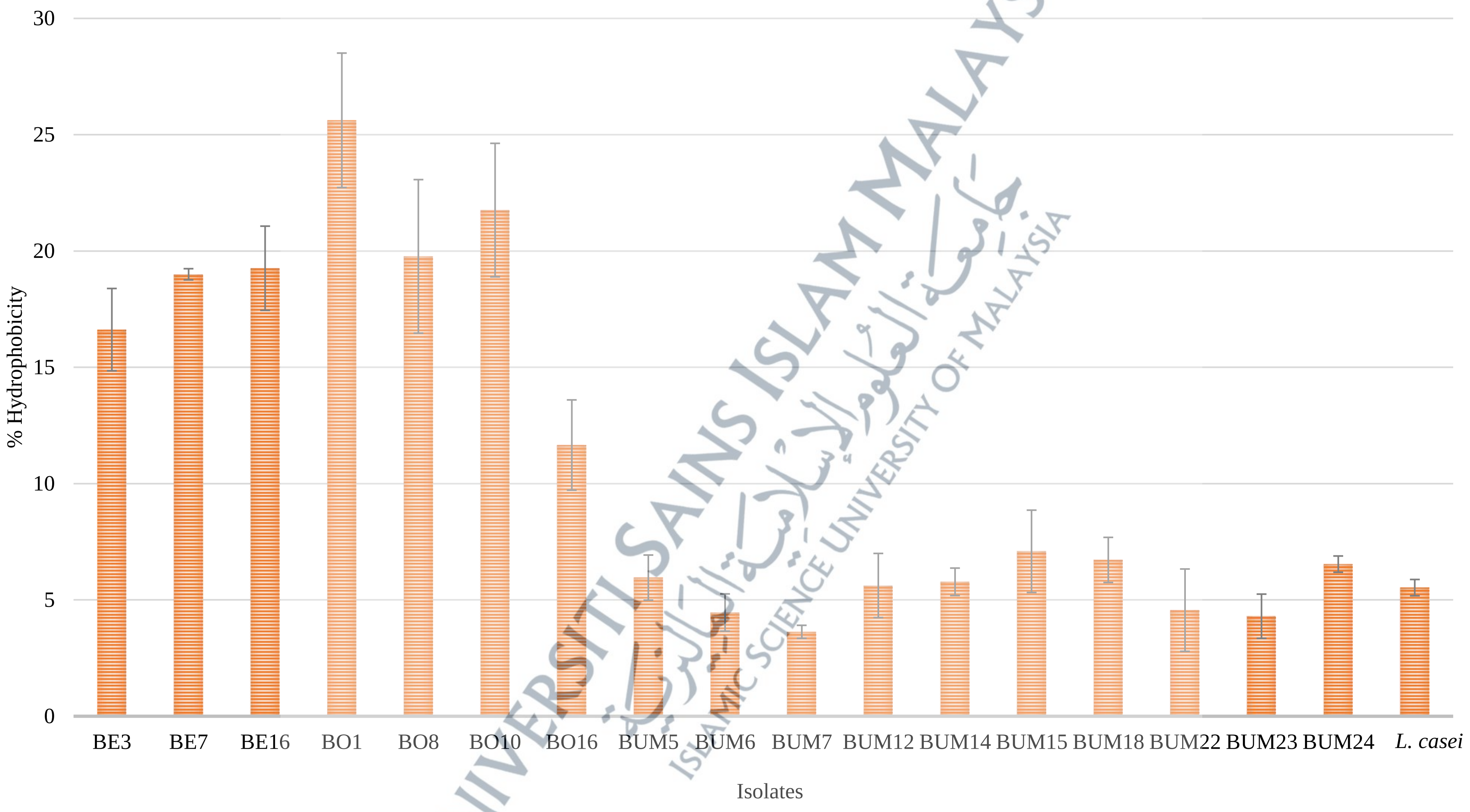

Figure 4.4: Surface Hydrophobicity of BE Isolates, BO Isolates, BUM Isolates and *L. casei* Strain Shirota by Their Bacterial Adherence to Hexadecane

4.3.7 Determination of Autoaggregation Ability

In addition to the hydrophobic character, the ability of a probiotic candidate to adhere and colonise the intestinal cells was measured by their capacity to autoaggregate with each other. The autoaggregation capabilities guarantee the high concentration of bacterial cell in the digestive tract, which may contribute to better adhesion mechanism (de Melo Pereira et al., 2018). The autoaggregation ability of BE isolates, BO isolates, BUM isolates, and the reference strain *L. casei* strain Shirota are shown in Figure 4.5. All seventeen isolates exhibited autoaggregation potential of 25.15 to 42.87% after 5 h of the incubation period. The most autoaggregative isolate was BE7 (42.87%), which autoaggregated significantly higher ($P < 0.05$) compared to BO1 and all BUM isolates excepting BUM22 and BUM24. Indeed, BUM22 (35.11%) and BUM24 (33.24%) autoaggregation ability were not significantly different ($P > 0.05$) to any of the isolates as well as *L. casei*. The autoaggregative capacity of all BUM isolates was also not significantly different ($P>0.05$). Meanwhile, BE3 (37.91%), BE16 (37.91%), BO8 (38.9%), BO10 (37.69%), and BO16 (39.66%) isolates recorded quite a similar percentage of autoaggregation that were significantly greater ($P < 0.05$) than at least two other isolates, namely BUM6 and BUM18.

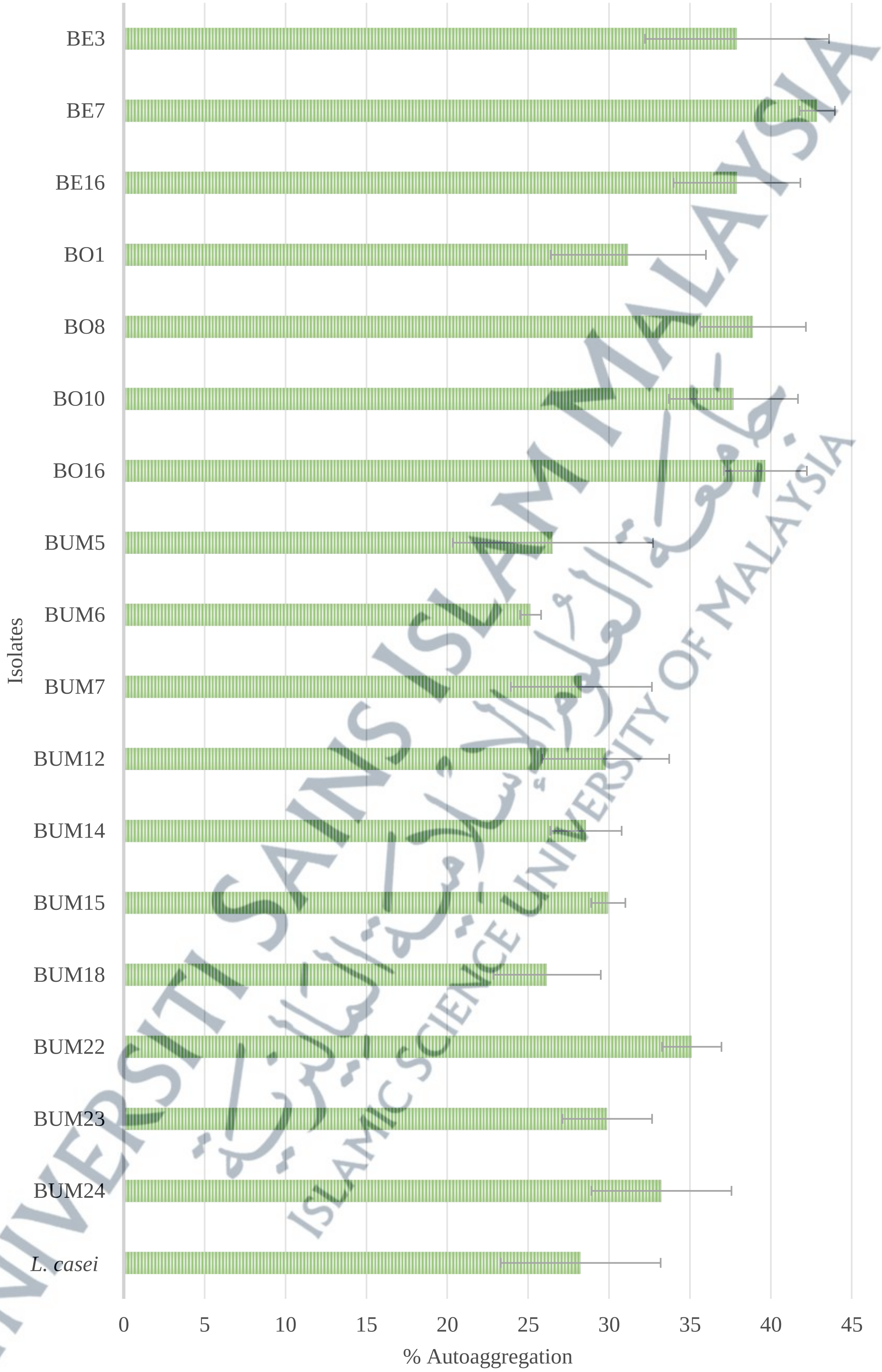

Figure 4.5: Autoaggregation Ability of BE Isolates, BO Isolates, BUM Isolates and *L. casei* Strain Shirota

4.4 Discussion

Probiotics have been extensively researched for their potential as an alternative therapy for human disease and as food preservatives (Mulaw et al., 2019). Due to its high demands, the search of novel probiotic strains has been ongoing, with sources including milk, faecal samples containing microorganisms from the gut, and fermented foods (Maqsood et al., 2013; Nami et al., 2015; Ida Muryany et al., 2017), The investigated characteristics include their tolerance to harsh environment, mimicking the condition in the human digestive system, their safety profiles and antagonistic properties, as well as hydrophobicity and autoaggregation ability. In this study, the 59 lactic acid bacteria strains isolated from belacan, bosou and non-cooked budu, were evaluated for these parameters under laboratory conditions.

The transit through the acidic surroundings of stomach represents a primary defence mechanism that all ingested microorganisms must deal with, including pathogens as well the beneficial probiotics. The isolates were assessed for their acid tolerance following 3 h incubation in acidic environments at pH 3 and pH 2.5. Generally, acid resistance capacities of isolates are strain and species-dependent, with general decrements below pH 3 (Corcoran et al., 2005; Nami et al., 2019). This theory validates our preliminary study of acid tolerance, in which the isolate cannot withstand pH 2 and below (data not shown).

At pH 3, only seventeen isolates or 28.8% from the total isolates, showed excellent resistance with values above 90%, which was slightly higher than the percentage of 25% observed in a study by Zhang et al. (2016). The high survival cut-off percentage (90%) was chosen as it is crucial to determine the only LAB that has the potential to withstand the first barrier, which is a harsh acidic environment, without losing its viability (Castorena-Alba et al., 2018). The patterns of discovery are also nearly comparable to the findings of Yu et al. (2013), who reported that seven *Lactobacillus plantarum* strains could tolerate acidic conditions at pH 3, but their growths is strongly influenced at pH 2. In another study by Sagdic et al. (2014), *L. plantarum* strains were able to grow at pH 2.5, but several *L. casei*, *L. brevis*, and *L. buchneri* strains failed to survive. Meanwhile, Haitham et al. (2017) found that from 166 LAB strains isolated from belacan samples, only thirteen strains survived acidic environment in between pH 2 to pH 4, but with decreasing capabilities following the pH acidity.

Referring to the meaning of probiotic as stated by FAO/WHO (2002), the potential microorganism must be alive for it to confer a health benefit on the host. The survival capability during gastric transit is one of the required criteria of a probiotic candidate. The LAB must withstand the robust acidic environment which varies in between pH 1.5 and pH 3 before they can reach, colonise, and perform their benefits in the intestinal tract (Corcoran et al., 2005; Liong and Shah, 2005a). The pH could also be altered as high as pH 6 after food ingestion. However, the values generally range in between pH 2.5 to pH 3.5. Meanwhile, the food transit time in the stomach ranging from less than one hour to up to four hours depending on various factors such as the individual itself and the nature of the food consumed (Guo et al., 2012). The high acidity creates significant stress, which could prevent overgrowth and suppress the colonisation of microorganisms (Haitham et al., 2017).

The high survival rates of these seventeen isolates compared to the remaining 42 isolates tested could be due to the ability to adjust their internal pH (Ng et al., 2015). In general, LAB can induce an acid tolerance response under acidic stress, resulting in pH homeostasis and a repair process that will eventually cause them resist low pH (Aarti et al., 2017). The mechanisms include cell membranes structures that are highly impermeable and modulation of membrane channel size to restrict the flow of protons inside the cell; creating chemiosmotic gradients through potassium ATPases to bounce the inflow of protons, pumping out the excess proton by proton pump; and modulation of fatty acid structure to sustain the integrity and fluidity of cell membranes (Guan and Liu, 2020).

Defeating the inhospitable acidic environment is worthless if the microorganisms to be applied as probiotic is unable to survive in the intestine. As bile salts are naturally destructive towards bacteria, their presence becomes the next hurdle for the isolates to endure before they can remain active, grow, and colonise the intestinal tract (Song et al., 2015). In this study, the growth of isolates in MRS broth containing 0.3% bile concentration was compared with MRS broth without the presence of bile at 7 h and 24 h. Although the commonly proposed bile salt concentrations to evaluate the probiotic characteristics are between 0.15% and 0.5%, isolates showing above 50% survival rates at 0.3% bile salts were adequate to be considered as bile resistant (Kumar and Kumar, 2015; Papadimitriou et al., 2015). High percentages of survival indicated higher possible recovery of the isolates during passage through the small intestine (Song et al., 2015).

All seventeen strains were resistant to bile with a percentage above 50% after incubated for 7 h, which agreed with Ruiz et al. (2013) who proposed that strains that are resistant to other stress condition such as acidic pH are also resistant towards bile. The observations in this study were in agreement with results reported by Gharbi et al. (2019), revealing their *Lactobacillus* strains exhibited tolerance to bile conditions with survival rate in between 53 and 108%. Most isolates showed lower survival percentages at 24 h compared to 7 h, while vice versa for some strains such as BO1, BO8, and BO16, suggesting that the bile resistance properties may be strain specific. The high growth of these isolates at more extended incubation period may be due to their capacity to accommodate the stress condition over a long period of incubations (Sahadeva et al., 2011). The findings are supported by Chopade et al. (2019) and Mulaw et al. (2019), who reported survival percentage above 90% for their LAB isolates incubated in 0.3% bile environments after a 24 h incubation period. Meanwhile, an earlier study by Kumar and Kumar (2015) showed diversity in the survival percentage of *Lactobacillus* isolates after being incubated for 24 h, with a bigger yet lower range of 35% and 72%.

Bile is synthesised in the liver, stored in the gall bladder, and released into the duodenum for the period of digestion activities. Bile acts as biosurfactants, aiding in the solubilisation and absorption of dietary fats (Sulin et al., 2019). Bile could also function as a bactericidal agent to fight against potential pathogens that might cause foodborne illnesses (Merritt and Donaldson, 2009). Bile can cause destruction of bacteria by breaking the bacterial cell integrity, causing leakage of bacterial components and cell death (Sahadeva et al., 2011). Besides, the unconjugated forms of bile are weak acid that could diffuse passively into the cell and cause bacterial cytoplasm to acidify (Ruiz et al., 2013).

The protective mechanism in LAB towards bile could be due to several mechanisms. For example, the production of bile salt hydrolase enzyme (BSH) which hydrolyse the bile and lowers the lethal effect (Kumar and Kumar, 2015). However, some strains were also able to grow in the presence of bile without producing BSH, as the ability to express BSH is not associated to the ability to withstand the toxicity of bile salt (Vinderola and Reinheimer, 2003). Besides, the isolates could also modify their composition of the cell membrane and performing active efflux of bile to neutralise the deadly effect of bile (Ruiz et al., 2013).

Besides tolerance to acidic pH and bile, FAO/WHO (2002) recommended a haemolysis test to ensure the safeness of potential probiotics as haemolytic ability must be absence in probiotics. Pathogens use the haemolytic capability to supply iron for their survival, thus triggering anaemia and oedema in the host (Vesterlund et al., 2007). The findings in this study showed that all seventeen isolates and the reference strain *L. casei* strain Shirota exhibited α-haemolysis or partial haemolysis.

There are some arguments for considering the types of haemolytic activity that are considered safe to develop as probiotics. For instance, Chopade et al. (2019) did not consider their isolates that exhibited partial haemolysis or α-haemolytic properties to be further investigated for probiotic potential. The same opinion is also expressed by Abushelaibi et al. (2017), who rejected the LAB isolates with α-haemolysis for further identification.

Meanwhile, a few studies recommended that only strain with β-haemolysis was considered destructive and vice versa for α-haemolytic bacterial strains (Argyri et al., 2013; Touret et al., 2018; Nami et al., 2019), as they may harbour very minimal virulence ability and are common among lactobacilli from foods and dairy products (Halder et al., 2017). This is due to the fact that α-haemolysin is sensitive to trypsin which is the enzyme found in the small intestine that breakdown proteins and are activated in the presence of calcium ion (Aghemwenhio et al., 2017). These strains are accepted as harmless and have the potential in probiotics development and food applications (Masalam et al., 2018). In fact, the reference strain *L. casei* strain Shirota used in this study is comparable to the approach by González-Vázquez et al. (2015), which reported no haemolysis for this strain.

The ability to produce antimicrobial compounds is also crucial for probiotics to compete, inhibit the growth, or kill pathogens (Sakandar et al., 2019). In line with the current situation, the lowering of antibiotic efficacy has become a global alarm for public health due to an increase in microbial resistance (Balouiri et al., 2016). Thus, discovering another source of antimicrobial compounds, one of which is isolated from prokaryotic bacteria, is indispensable. Consumption of fermented food containing these beneficial microbes could offer options for preventing or curing related gastrointestinal disorders due to its ability to defeat drug-related challenges such as drug-resistant strains, chronic toxicity, and disappearance of healthy microbiota (Coman et al., 2014).

All isolates presented a broad inhibitory spectrum and managed to inhibit the growth of all four common foodborne pathogens used as indicators. The results were in the same way as previous research by Shokryazdan et al. (2014), in which all *Lactobacillus* isolates demonstrated antagonistic property against tested pathogens. Sim et al. (2012) have also reported the antagonism properties of *L. plantarum* (strain LP1 and LP2) and *Lactococcus lactis* subp *lactis* strain LL2 isolated from budu towards *S. aureus*, *E. coli*, *Salmonella typhimurium*, and *Listeria monocytogenes* but with different capabilities. Meanwhile, the study by Khalil et al. (2018) reported that *Lactobacillus fermentum* strains BU11 and BU14 isolated from budu were able to inhibit the growth of indicator pathogens such as *Salmonella typhimurium*, *E. coli*, *Pseudomonas aeruginosa*, methicillin-resistant *S. aureus*, *S. aureus*, and *Listeria monocytogenes* with different levels of inhibitory activity.

However, this pattern contrast with that of Hajar and Hamid (2013), who found that some of their isolates did not show any antagonistic activity towards several pathogens tested. In their findings, none of the isolates inhibits the growth of *Bacillus subtilis*, while different isolates were incapable of inhibiting the growth of *S. aureus*, *E. coli*, and *Salmonella typhimurium*. In fact, Chopade et al. (2019) also reported that some of their cultures could not impede the growth *S. aureus*, *E.*

coli, *Salmonella typhimurium*, and one of the isolates were unable to inhibit any of the pathogen tested.

Probiotics can form molecules that behave antagonistically towards pathogens and spoilage microorganisms. The antagonistic properties of LAB strains towards the pathogenic bacteria may be ascribed to the battle for nutrients constraint, attachment site, or the production of antimicrobial substances such as bacteriocin-like molecules, organic acids, and hydrogen peroxide (Shokryazdan et al., 2014; Chopade et al., 2019). The probiotic's ability to produce these substances is one of the essential attributes for competing with pathogens and expressing their advantageous effects that favour the host. Bacteriocins are protein produced by bacteria, which could inhibit the growth of similar or closely related strains in the same species or across genera (Yang et al., 2018). Bacteriocins are distinguished by their non-toxicity to humans, stability in low pH environments, and heat stability.

Meanwhile, organic acids produced by metabolisms in LAB, for example, lactic acid, acetic acid, and formic acid, decrease the pH of the environment, thus inhibiting the growth or kill the pathogens. The organic acids could also diffuse through the pathogen's cell membrane, dissociate, and release hydrogen ions, thus causing the cytoplasm's acidification, which leads to cell death. (Tharmaraj and Shah, 2009). The ability of LAB to lower the pH and survive well in that environment is advantageous as it helps to sustain the general health of the digestive tract (Kaushik et al., 2009).

The other common antimicrobial substance of LAB is hydrogen peroxide, which is produced in the presence of oxygen via the action of flavoprotein oxidases or nicotinamide adenine dinucleotide (NADH) peroxidases. The antimicrobial capacity of hydrogen peroxide may be due to its robust oxidising impact on the bacterial cell, causing the denaturation of cell proteins and the peroxidation of membrane lipids, which increases membrane permeability (Lindgren and Dobrogosz, 1990; Hwanhlem et al., 2011). An example of antimicrobial substances' impact on pathogenic cell structures has been previously described by Yong et al. (2015). Interactions between LAB and pathogens may disrupt the membrane integrity, leading to cell lysis or the formation of pores on the cell membrane, causing the influx of extracellular components inside the cells, leading to swelling of the cells. The results in this study, however, did not inspect further the possible substance produced, which cause the growth inhibition of all pathogens tested.

Besides the production of antimicrobial substances, the isolates' antagonistic properties are also correlated to the methods used (Coman et al., 2014). In this study, the antagonistic interactions of isolates against the tested strain were determined by using spotted colonies that grew beneath the MH agar seeded with pathogens. A preliminary study using the well plate diffusion method and the disc diffusion method failed to give positive results. Previous research by Rahimifard et al. (2016) suggested the effectiveness of probiotic's cell-free supernatant compared to the cell sediments in displaying the inhibition zone towards tested pathogens. This condition may be due to greater diffusion of supernatant into agar or destruction of cells through centrifugation. The agar spot antimicrobial assay used in this study was a suitable technique to be used in antagonism properties research. It allows the colonies to grow while also producing inhibitory substances that disperse deeply into the pathogenic seeded agar, inhibiting growth (Çadirci and Çitak, 2005).

The isolates were also evaluated for their antibiotic resistance towards selected antibiotics. In this study, the isolates were completely (100%) sensitive to ampicillin and chloramphenicol, 94% were sensitive to penicillin G, and 88% were sensitive to tetracycline. These findings concur with those reported by Goswami et al. (2017), which their LAB strains isolated from Kahudi, a traditional fermented food, were susceptible to most of the common antibiotics that are ampicillin, tetracycline, chloramphenicol, and penicillin G. Ampicillin and penicillin G are β-lactams, which inhibit the synthesis of bacterial cell walls, while chloramphenicol and tetracycline act by inhibiting the protein synthesis of bacteria (Shaikh et al., 2015).

The isolates were also completely (100%) resistant to nalidixic acid, streptomycin, and vancomycin but showed various resistance patterns against bacitracin. These findings were in agreement with current results of Khalil et al. (2018) who found out that all of their strains isolated from different Malaysian fermented foods showed resistance to vancomycin and nalidixic acid, besides another antibiotic, namely ciprofloxacin, but were either susceptible or resistant towards bacitracin. These results were also comparable with the previous report by Sharma et al. (2016), where their lactobacilli isolates were resistant to similar types of antibiotics (nalidixic acid, streptomycin and vancomycin) but display lower resistant profile or sensitive towards ampicillin, chloramphenicol, penicillin and tetracycline. Much earlier, Liasi et al. (2009) reported the resistant profile of LAB isolates isolated from budu to several antibiotics including streptomycin, bacitracin, and nalidixic acid. Streptomycin is an aminoglycosides which is supposed to inhibit protein synthesis, while bacitracin is a polypeptides that inhibits the synthesis of the bacterial cell wall. On the other hand, nalidixic acid is a quinolone that interferes with the synthesis of nucleic acid in Gram-negative bacteria, which proves its resistance to the Gram-positive LAB (Shaikh et al., 2015).

The resistant ability of the bacterial strain to several antibiotics could be due to various reasons, either related to the antibiotics or the bacteria itself (Shaikh et al., 2015; Abid et al., 2018). In terms of the antibiotics, it involves the nature of the antibiotics and their ability to interfere with the production of the bacterial cell wall, inhibit bacterial metabolic pathway, synthesis of bacterial proteins and synthesis of nucleic acid, as well as modifies the bacterial cell membrane. When considering the factors related to the bacteria, it includes their cell wall structure, the permeability of the membrane, as well as their efflux mechanism. The bacterial resistance gene to the antibiotic can be either intrinsic or acquired (Nawaz et al., 2011). The intrinsic resistance is natural, inherited, and not transferred horizontally; thus, it does not develop any risk. Meanwhile, acquired resistance might be spread horizontally to other bacteria, and it could happen due to bacterial genome's mutations or acquisition of genetic components, for example plasmids or transposons.

To date, there are growing concerns about the ability of LAB isolated from food to act as a reservoir for antibiotic resistance genes and transfer them to human pathogenic bacteria either during the manufacturing of food or during passage through the digestive tract (Ammor et al., 2007). For the LAB to pass its antibiotic resistance genes to other bacteria, they must have communications between each other through the aid of plasmids and transposons. Plasmids are commonly present in enterococci, lactococci, leuconostocs, pediococci, lactobacilli and bifidobacterial while transposons are found in enterococci, lactococci, and streptococci (Mathur and Singh, 2005).

Different antibiotic resistance patterns of probiotic bring benefits and drawbacks. The former relates to bacteria's survival in the gastrointestinal tract of people receiving antibiotic treatment, while the latter involves the potential transfer of acquired resistance genes to pathogenic bacteria found in the intestine (Jose et al., 2015). However, the extensive use of LAB in fermented foods has a good long record of safety and is recognised as GRAS as most of the strain contains intrinsic resistance genes that are not transferable horizontally (Ammor et al., 2007; Jose et al., 2015). Thus, the risk of infection derived from ingestion of these bacteria is extremely limited. According to Abriouel et al. (2015), vancomycin- and chloramphenicol- resistance genes in *Lactobacillus* species are highly conserved in the chromosome of each species and probably non-transferable to other bacteria.

The investigation of cell surface hydrophobicity of the seventeen isolates was done using the BATH methods and hexadecane as the solvent. The BATH test has been broadly applied by many researchers to evaluate the cell surface hydrophobicity of bacterial strains that could potentially develop as probiotics (Angmo et al., 2016; Gharbi et al., 2019; Sakandar et al., 2019). The use of hexadecane to determine the bacterial adhesion properties is a legitimate qualitative phenomenological method which suggests the ability of the isolates to adhere to intestinal cells (Vinderola and Reinheimer, 2003).

The seventeen LAB isolates exhibited comparatively lower hydrophobicity (3.63 to 25.62%) when compared with Haitham et al. (2017) (51.45 to 62.61%), but similar or slightly higher to those reported by Abushelaibi et al. (2017) (0.6 to 16.2%), against similar solvent. The range of hydrophobicity percentage observed was also narrower than that described previously by Ng et al. (2015) and Angmo et al. (2016), who recorded values ranging from 12.00 to 55.14% and 5.81 to 46.59%, respectively. However, results obtained for the reference strain *L. casei* strain Shirota were almost analogous to those reported by Melgar-Lalanne et al. (2013) using the same solvent. It is believed that the cell hydrophobicity values of LAB isolates are strain-dependent.

Bacterial cell surface characteristics are thought to be one of the markers of its ability to attach to the digestive tract before colonising and causing harm to the host (Haitham et al., 2017). Bacterial adhesion could also help to avoid the attachment of pathogenic bacteria by occupying the binding sites of the intestinal cells

(Ashayerizadeh et al., 2017). Indeed, the BATH test using hexadecane reflects the bacterial cell surface hydrophobicity while the other hydrocarbons may signify different mechanisms, such as chloroform related to the electron donor or acceptor characteristics. Though the bacteria have the low hydrophobic surface, they could adhere to cells via electron donor characteristics as bacteria have various compositions, structures, and organisations of the cell wall (Melgar-Lalanne et al., 2013).

Moreover, the combination of hydrophobic and hydrophilic appendages, as well as other macromolecule components, might produce complex cell surface mosaic, hence affecting the hydrophobicity values toward hydrocarbons (Angmo et al., 2016). The adhesion ability of bacteria to host cells could also involve specific ligand-receptor mechanisms besides the non-specific mechanism such as hydrophobicity (Agaliya and Jeevaratnam, 2012). This theory was supported by another study by Tomaro-Duchesneau et al. (2015), who discovered that although their isolate had an excellent cell surface hydrophobicity (70.3%), the strain was non-adhesive to Caco-2 intestinal cell lines, suggesting that adherence mechanisms with cells may be based on specific binding and not through non-specific electrostatic interactions. Therefore, low hydrophobicity values do not necessarily lead to low adhesion properties to the host (Zago et al., 2011).

The autoaggregation ability, or the clumping of identical bacterial strains, is a valuable property of probiotics that aids in adhering to and colonising the human digestive tract (Mallappa et al., 2019). In the present study, all seventeen strains displayed autoaggregation proficiencies to varying degree (25.15 to 42.87%). The percentages were slightly similar or a bit lower than some of the previous investigations of aggregation values, for example 16.3 to 61.5% by Lee et al. (2014) for LAB isolated from salted and fermented Korean seafood, the percentage above 10% but below 60%

for probiotic candidates isolated from Indonesian fermented food (Emmawati et al., 2016), and 87.74% as reported by Guan et al. (2017) for their *L. plantarum* HLX37 strains.

Nevertheless, the results on aggregation capability of the reference strain *L. casei* strain Shirota supported the earlier reports by Romero-Luna et al. (2020), which recorded 34% aggregation for the strain. Autoaggregation percentage greater than 40% was considered high autoaggregation; percentages between 10 and 40% were considered moderate autoaggregation; and percentages less than 10% were considered low autoaggregation (Wang et al., 2010). Hence, apart from BE7, all the other sixteen isolates expressed moderate autoaggregation properties.

One of the crucial properties of potential probiotics is their ability to adhere to epithelial cells (Collado et al., 2008). It is widely agreed that the percentage of autoaggregation is closely related to the ability to adhere to intestinal cells, with the view that a higher autoaggregation percentage is associated with better adherence (Ng et al., 2015; Le and Yang, 2019a; Sakandar et al., 2019). Contradict to this opinion, the study by Li et al. (2015) showed that significant correlations did not existed for their LAB strains in terms of autoaggregation percentage and adhesion ability, possibly because both characteristics are strain-specific. Therefore, bacteria with higher aggregative values do not necessarily adhere better to the intestinal cells.

Though autoaggregation abilities are undesirable for pathogens, it is expected to exist in probiotics as it brings defensive impact to the bacteria in terms of resistance to the challenging condition in the gastrointestinal tract. Not only that, the development of bacterial biofilm over the host tissue also protects the host system from the colonisation of pathogens and stimulates the immune system (Tareb et al., 2013). Aside from strain specificity, the broad spectrum of aggregation percentage of LAB isolates may be due to their binding ability, which requires precise type and volume of surface layer proteins, as well as other factors such as ions or exopolysaccharide production (Nikolic et al., 2010; Ng et al., 2015). During autoaggregation, bacteria may excrete antimicrobial substances such as acids, bacteriocin-like substances, or hydrogen peroxide, which has been linked to probiotic antagonism (Choi et al., 2018).

4.5 Conclusion

Out of 59 strains, it was found that only seventeen strains showed strong resistance to the acidic physiological environment and were further tested for the next probiotic characteristics. The results also revealed that the isolates exhibited sufficient bile tolerance abilities towards physiological bile concentrations, while none of the isolates exhibited β-haemolysis on blood agar. All seventeen isolates also exhibited antagonistic activities with a broad inhibitory spectrum towards pathogenic indicator strain and possessed various antibiotic resistance patterns depending on antibiotics used. These isolates showed slightly low hydrophobicity values and moderate autoaggregation ability. The results suggest the reasonably good probiotic potential of all seventeen strains. All isolates were identified using the molecular approach in the next study.