CHAPTER 6

SCREENING OF CHOLESTEROL LOWERING PROPERTIES AND ADHESION ABILITY OF POTENTIAL PROBIOTIC LACTIC ACID BACTERIA ISOLATED FROM MALAYSIAN FERMENTED FOOD ON HUMAN COLORECTAL ADENOCARCINOMA CELL LINE HT-29

6.1 Introduction

Cholesterol is a biological molecule that has multiple responsibilities in the human body. The presence of cholesterol as a component of cell membrane's structure balances the need to maintain the integrity and fluidity of the cell. Besides, cholesterol is also involved in the production of vitamin D and acts as a precursor for steroid hormones such as testosterone and oestrogen (Li et al., 2019a). However, when there is too much cholesterol in the blood, it could block the blood vessels and lead to numerous cardiovascular complications. Elevated cholesterol concentrations in the blood at approximately 1 mmol above the normal value may increase the risk of ischemic heart disease by nearly 35% the risk of coronary death by 45% (Liong and Shah, 2005a).

Aside from changing one's diet and level of fitness, there are several types of prescription drugs available for hypercholesterolemia patients. Although drug use has a positive impact on the management of cholesterol in the blood, its effectiveness is also in line with other challenges. For example, statins are among the well-established and most frequently used drugs to treat hypercholesterolemia. However, this drug remains out of reach for many low- and middle-income countries (Lew et al., 2018). Its application was also associated with various side effects such as liver toxicity, diabetes risk, myopathy and rhabdomyolysis (Sirtori, 2014). Because the absorption of cholesterol that enters the body through foods contributes to the management of plasma cholesterol levels, controlling the cholesterol absorption and excretion process in the intestinal lumen could be a fantastic approach to lower overall cholesterol levels (Lim et al., 2017). A non-pharmacological approach has been continuously explored in the search for therapy to control or lower plasma cholesterol levels, with the aim of minimising adverse reactions brought by drug therapy.

Recent studies reported that probiotic strains with cholesterol-lowering properties could be used as an alternative in the management of hypercholesterolemia. Several studies have been conducted to examine the potential of probiotics isolated from food to lower cholesterol, focusing mainly on production of bile salt hydrolase (BSH) enzyme and cholesterol assimilation (Rapsang and Joshi, 2013; Huang et al., 2019; Nami et al., 2019). The target strain must also be able to attach to the intestinal epithelial cell and colonise the area before expressing its therapeutic abilities without causing cytotoxic effects to the enterocytes. As probiotic strains do not ordinarily inhabit the human digestive tract, the microorganisms could act as a suitable candidate for inducing a long-term hypocholesterolemic effect via constant intake (Tsai et al., 2014).

The behaviour and role of the probiotic lactic acid bacteria (LAB) isolated from Malaysian fermented foods in the intestinal tract remains undiscovered. Considering the insufficiency of information described above, the goal of the present research was to investigate the cholesterol-lowering properties of *Lactiplantibacillus plantarum* strains isolated from belacan and bosou, as well as *Lacticaseibacillus paracasei* strains isolated from non-cooked budu. The capability of the potential probiotic strain to produce BSH enzyme and remove cholesterol in experimental broth was investigated. In order to assess the ability of selected potential probiotic LAB strains to perform their function in the digestive tract, the strains were also evaluated for their ability to adhere to intestinal epithelial cells of the human digestive tract using the HT-29 cell line and their cytotoxicity properties.

6.2 Materials and Methods

6.2.1 Bile Salt Hydrolase Assay

Bile salt hydrolase (BSH) activity of *L. plantarum* strains isolated from belacan and bosou, and *L. paracasei* strains isolated from non-cooked budu, was determined using the plate screening assay (Zago et al., 2011). The MRS medium was modified by adding 0.37 g/L calcium chloride ($CaCl_2$) and 0.5% sodium salt of taurodeoxycholic acid (TDCA) or 0.5% sodium salt of glycodeoxycholic acid (GDCA) (Merck Millipore, US). The 7µL of overnight cultures and the reference strain *Lacticaseibacillus casei* strain Shirota (10^8 CFU/mL) were spotted on MRS agar plates supplemented or not supplemented with sodium salts and were incubated at 37ºC for 48 h. Each culture spot was observed for its ability to grow and the presence of visible halos or white opaque around the colonies. Isolates were also inoculated on MRS agar lacking $CaCl_2$ and sodium salt supplementation and served as the negative control.

6.2.2 Cholesterol Removal Ability by Potential Probiotic LAB

The cholesterol removal ability of the growing potential probiotic LAB was analysed according to Tomaro-Duchesneau et al. (2015) with slight modification. MRS broth was added with 100 µg/mL water-soluble cholesterol (Cholesterol-PEG 600; Sigma, US) and 1% of overnight isolates (10^8 CFU/ml) before it was incubated at 37°C for 24 h. Uninoculated modified MRS broth was set under the same conditions and used as a control. Following the incubation period, bacterial cells were removed by centrifugation at 10000 rpm for 15 min, and the remaining cholesterol in the cell-free supernatant was determined calorimetrically by using the cholesterol quantitation kit (MAK043; Sigma, US).

In brief, cholesterol standard solutions at concentrations of 0 (blank), 1, 2, 3, 4 and 5 µg/well were added into a 96-well plate. The 30 µL uninoculated modified MRS broth and the filtered cell-free supernatant were also inserted into the well plate, respectively. The final volume of each well was brought to a final volume of 50 µL with cholesterol assay buffer. Each well was added with 50 µL reaction mix containing the reagent listed in Table 6.1.

Table 6.1: Reaction Mixes Used in The Cholesterol Assimilation Assay

Reagent	Volume (µL)
Cholesterol assay buffer	44
Cholesterol probe	2
Cholesterol enzyme mix	2
Cholesterol esterase	2

6.2.3 Principal Component Analysis

Principal component analysis (PCA) was used to discriminate between the LAB strains with probiotic properties and cholesterol-lowering abilities. The discriminating variables were tolerance to acidic pH and bile, antagonism properties towards pathogenic bacteria, hydrophobicity and autoaggregation ability, as well as bile salt hydrolase and cholesterol assimilation properties. The results of the bile salt hydrolase assay were converted into coded values (0, 1, 2, and 3) and used as input data. PCA was carried out using SPSS (Statistical Package for the Social Sciences) version 26.0.

6.2.4 Cell Culture and Maintenance

Human colorectal adenocarcinoma cell line HT-29 (ATCC® HTB-38™) was obtained from Makmal Bioserasi, Universiti Kebangsaan Malaysia, Bangi, Selangor, and cultured in the Cell Culture Room, Faculty of Medicine and Health Sciences, Universiti Sains Islam Malaysia, Nilai, Negeri Sembilan. The cells were maintained in McCoy's 5A Medium (Sigma-Aldrich, Germany) supplemented with 10% (v/v) foetal bovine serum (FBS; Biosera, France) and 1% (v/v) penicillin-streptomycin (Merck Millipore, US). The cell line was routinely cultured in a 25 cm^2 cell culture flask (SPL Life Sciences, Korea) at 37°C in a humidified atmosphere with 5% carbon dioxide (CO_2). The morphology, health, confluency, and risk of contamination of cells were observed using an inverted microscope. The medium was replaced with fresh medium every alternate day.

Subculturing was performed when the cells reached approximately 70% confluent. The old medium was discarded without disturbing the adhered cells. Then, the flask was washed twice with phosphate-buffered saline (PBS; Oxoid, UK) and removed. The adhered cells were trypsinised by adding a 2 mL trypsin-EDTA solution (Sigma-Aldrich, US). The flask was gently swirl before it was incubated for approximately 5 to 10 min. The cells were examined using an inverted microscope to ensure complete detachment. The action of trypsin was inactivated by adding 5 mL of culture medium into the flask. The medium was transferred into a 15 mL falcon tube and centrifuged at 900 rpm for 10 minutes. The supernatant was discarded, and the cell pellet was resuspended with fresh medium.

Then, 100 µL of cell suspension was pipetted out and mixed gently with an equal volume of Trypan Blue (Sigma-Aldrich, Germany) until homogenized. The cell suspension was slowly pipetted into the haemocytometer chamber and viewed under an inverted microscope using 20X magnification for cell counting. The cell suspension was split into new culture flasks containing pre-warmed fresh medium following appropriate cell concentration. Cells free from contamination and with a viability exceeding 90% were used for the experiments.

6.2.5 Adhesion Ability of Potential Probiotic LAB to HT-29 Cells

The adherence of selected potential probiotic LAB strains to the HT-29 cell line was examined quantitatively as described previously by Fonseca et al. (2020) with a few modifications. Monolayers of HT-29 cells were prepared by seeding 1 mL cell at a concentration of 10^5 cells/well in a six-well plate. The medium in the wells was replaced with fresh medium every alternate day until the cells achieved 90% to 100% confluency (approximately 12 days). Prior to experiments, the bacterial cultures were grown overnight and harvested by centrifugation. The pellet was washed with PBS and resuspended in culture medium without FBS and penicillin-streptomycin to a concentration of approximately 10^8 CFU/mL.

Subsequently, monolayers were washed thrice with PBS to eliminate the penicillin-streptomycin. Then, 1 mL of each bacterial suspension was added into the respective well containing the monolayer cells. The plate was then incubated for 90 min in a 5% CO_2 incubator at 37°C. After incubation, the culture medium was discarded, and the monolayers were washed thrice with PBS to remove the non-attached bacteria. To disrupt the adhered cells, the HT-29 monolayers were trypsinised by treatment with 1 mL trypsin-EDTA solution for approximately 5 to 10 min at 37°C. The cell suspension was serially diluted, and appropriate dilutions were plated on MRS agar medium. The bacterial cells primarily inserted into each well of the six well plates were also undergoing serial dilution and plated on the same agar. The plates were incubated at

37°C for 48 h to explicate the number of viable adherent bacteria. The adhesion assay was performed in duplicate and repeated thrice. The probiotic *L. casei* strain Shirota was employed as a reference strain. The adherence percentage was calculated as follows:

$$\text{Adherence percentage (\%)} = (CFU_T/CFU_C) \times 100$$

Where:

CFU_T: bacterial cells adhered to HT-29 cells;

CFU_C: bacterial cells added to HT-29 cells.

6.2.6 Adherence Study of Potential Probiotic LAB to HT-29 Cells by Field Emission Scanning Electron Microscopy (FESEM)

Field emission scanning electron microscopy (FESEM) was employed to observe qualitatively the adhesion capacity of a potential probiotic LAB strain to HT-29 cells. The HT-29 cells were grown and maintained on sterile glass cover slips placed in a six-well plate by seeding 1 mL of cells at a concentration of 10^5 cells/well. After the monolayer was formed, each well was washed three times to get rid of antibiotics. Meanwhile, the overnight culture of potential probiotic LAB strains was centrifuged at 900 rpm for 10 min and washed with PBS. The HT-29 cells were treated with 1 mL of LAB strains suspended in McCoy's 5A medium without FBS and penicillin-streptomycin (10^9 CFU/mL) and incubated for 90 min at 37°C in a 5% CO_2 incubator. Following incubation, the supernatant was removed and each well washed three times with PBS to remove unbound bacteria.

Later, cells were prepared for FESEM analysis. Fixation was performed with 2.5% v/v glutaraldehyde (Sigma, US) in 0.1 M PBS for 1 h at ambient temperature. The cells were then washed with PBS, followed by dehydration in an ascending gradation of ethanol starting with 30%, followed by 50%, 60%, 70%, 80%, 90%, and 95% v/v for 10 min per step. The cells were finally dehydrated with absolute ethanol thrice (15 min for each session) and were dried in a critical point dryer (Leica EM CPD 300, Austria). The glass cover slips were mounted on stubs and sputtered with a thin layer of platinum using a smart coater at 10 mA for 10 sec (Jeol, US). The samples were then imaged using a JSM-IT800 Field Emission Scanning Electron Microscope (Jeol, US) at Faculty of Science and Technology, Universiti Sains Islam Malaysia, Nilai, Negeri Sembilan, Malaysia.

6.2.7 Cytotoxicity Activity of Potential Probiotic LAB Against HT-29 Cell Line by MTT Assay

The cytotoxic potential of selected potential probiotic LAB strains and the reference strain was assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as per Ardestani et al. (2019) with slight modifications. Briefly, a 100 µL aliquot containing 5×10^4 HT-29 cells was seeded into 96 well plates and the cells were subjected to overnight culture at 37°C to allow cell growth and attachment to the well. Subsequently, the medium was removed, and the cells were gently washed twice with PBS. After discarding PBS, 100 µL of different concentrations of LAB (10^4, 10^5, 10^6, 10^7, 10^8, and 10^9 CFU/ml) prepared in culture media without FBS and penicillin-streptomycin were added to the respective wells. Wells containing HT-29 cells without the presence of LAB strains were used as the negative control for the experiment, while hydrogen peroxide was used as a positive control. The LAB was also inserted into wells without HT-29 cells to withdraw the reading interference from the bacteria itself. The results were consequently analysed after 24 h.

Prior to the analysis, 20 µL of tetrazolium dye MTT at a concentration of 5mg/ml were added into each well, and the plates were further incubated in the dark at 37°C for 4 h. Next, 100 µL dimethyl sulfoxide (DMSO; Fisher, UK) was added to the wells to solubilize the purple formazan crystal formed. After 30 min of incubation in an orbital shaker, the absorbance was determined spectrophotometrically at 570 nm. The experiment was repeated three times with triplicate samples. The results were reported as percentage of cell viability and half maximal lethal concentration (LC_{50}) values. The percentage of viable cells was calculated as the following equation:

$$\text{Cell viability percentage (\%)} = (OD_T/OD_C) \times 100$$

Where:

OD_T: absorbance of wells containing HT-29 cells and potential probiotic LAB strain;

OD_C: absorbance of wells containing HT-29 cells only.

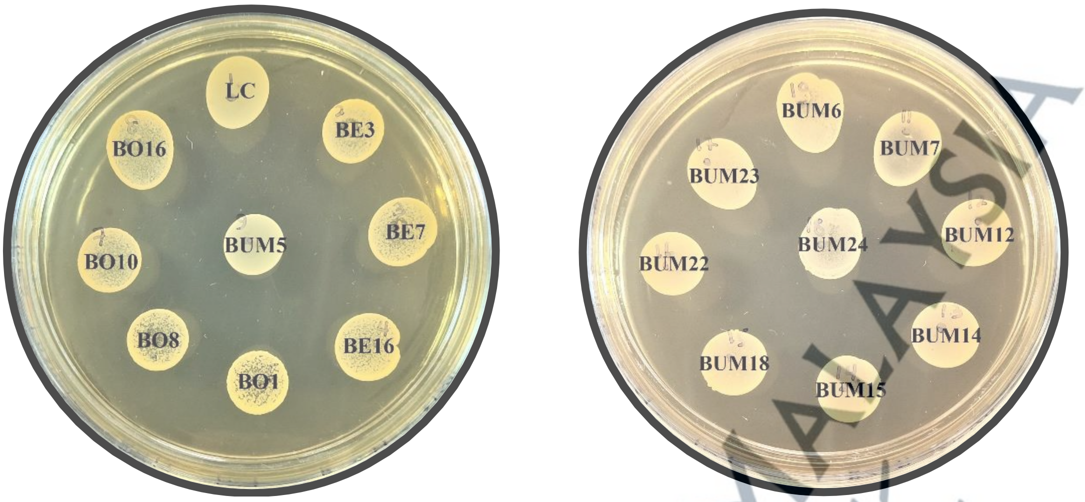
6.2.8 Statistical Analysis

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

6.3. Results

6.3.1 Bile Salt Hydrolase Assay

The ability of bacteria to grow and hydrolyse conjugated bile salts has been included as one of the selection criteria for probiotics associated with hypercholesterolemia management, using TDCA and GDCA, which have been recognised as good indicators for this criterion (Allain et al., 2018). In this study, the *L. plantarum* strains isolated from both belacan and bosou were shown to be tolerant and were able to grow on MRS agar supplemented with 0.5% TDCA, similarly as in the negative control plate (Figure 6.1 and Table 6.2). In contrast, the reference strain, *L. casei* strain Shirota, and all *L. paracasei* strains' growth was reduced when inoculated on the same modified agar, proving that the TDCA bile salt was more inhibitory to these species. When the isolates were inoculated on MRS agar supplemented with 0.5% GDCA, the bile salt inhibited most of the potential probiotic strains examined. The growth of all *L. paracasei* and *L. casei* strain Shirota was inhibited entirely, whilst the development of all *L. plantarum* was very much lessened. No hydrolase activity against both TDCA and GDCA could be observed from all 17 strains.



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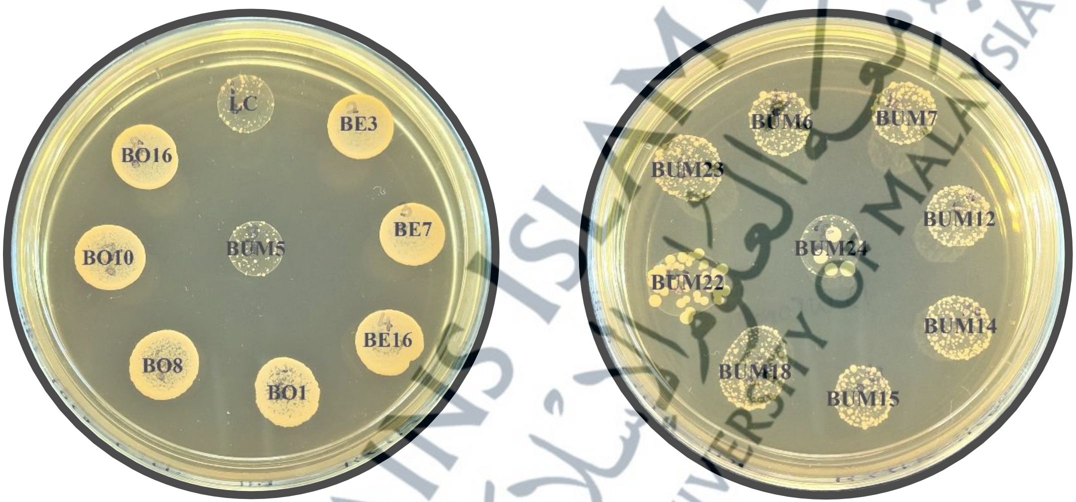


(b)

(c)

Figure 6.1: *L. casei* Strain Shirota, *L. plantarum* Strains, and *L. paracasei* Strains Growth Patterns on a) MRS Agar, b) 0.5% TDCA – MRS agar and c) 0.5% GDCA – MRS Agar

Table 6.2: *L. plantarum* Strains, *L. paracasei* Strains and *L. casei* Strain Shirota Growth Characteristics on Bile Salt Supplemented MRS Agar

Isolates	Growth on MRS agar	Growth on 0.5% TDCA – MRS agar	0.5% TDCA – MRS agar hydrolase activity	Growth on 0.5% GDCA – MRS agar	0.5% GDCA – MRS agar hydrolase activity
L. plantarum strain BE3	+++	+++	ND	+	ND
L. plantarum strain BE7	+++	+++	ND	+	ND
L. plantarum strain BE16	+++	+++	ND	+	ND
L. plantarum strain BO1	+++	+++	ND	+	ND
L. plantarum strain BO8	+++	+++	ND	+	ND
L. plantarum strain BO10	+++	+++	ND	+	ND
L. plantarum strain B016	+++	+++	ND	+	ND
L. paracasei strain BUM5	+++	++	ND	-	ND
L. paracasei strain BUM6	+++	++	ND	-	ND
L. paracasei strain BUM7	+++	++	ND	-	ND
L. paracasei strain BUM12	+++	++	ND	-	ND
L. paracasei strain BUM14	+++	++	ND	-	ND
L. paracasei strain BUM15	+++	++	ND	-	ND
L. paracasei strain BUM18	+++	++	ND	-	ND
L. paracasei strain BUM22	+++	++	ND	-	ND
L. paracasei strain BUM23	+++	++	ND	-	ND
L. paracasei strain BUM24	+++	++	ND	-	ND
L. casei strain Shirota	+++	++	ND	-	ND

Values are mean ± standard error of triplicates.

Growth characteristics: -: no growth; +: very reduced growth; ++: reduced growth; +++: normal growth; ND: not detected.

6.3.2 Cholesterol Removal Ability by Potential Probiotic LAB

The removal of cholesterol from the MRS medium during the 24 h of growth of the LAB strains is depicted in Figure 6.2. All the seventeen LAB strains were able to remove cholesterol from the medium, but the concentration of cholesterol eliminated was diverse among strains and ranged from 44.6% to 89.7% in comparison with the uninoculated control MRS broth. In general, all *L. plantarum* strains isolated from both belacan and bosou showed a significantly higher capability ($P < 0.05$) to remove cholesterol, with cholesterol reduction values ranging from 82.1% to 89.66%. Meanwhile, all *L. paracasei* strains isolated from non-cooked budu were found to reduce cholesterol levels by approximately half, with values falling in between 44.6% and 52.4%. The reference *L. casei* strain Shirota reduced the cholesterol by 51.3% compared to control MRS broth. The capability of different strains in both species to lower the cholesterol level was not significantly different ($P > 0.05$) between one another, respectively. The highest percentage of cholesterol removal was observed in media inoculated with *L. plantarum* strain BE3, whilst the lowest value was traced in *L. paracasei* strain BUM5.

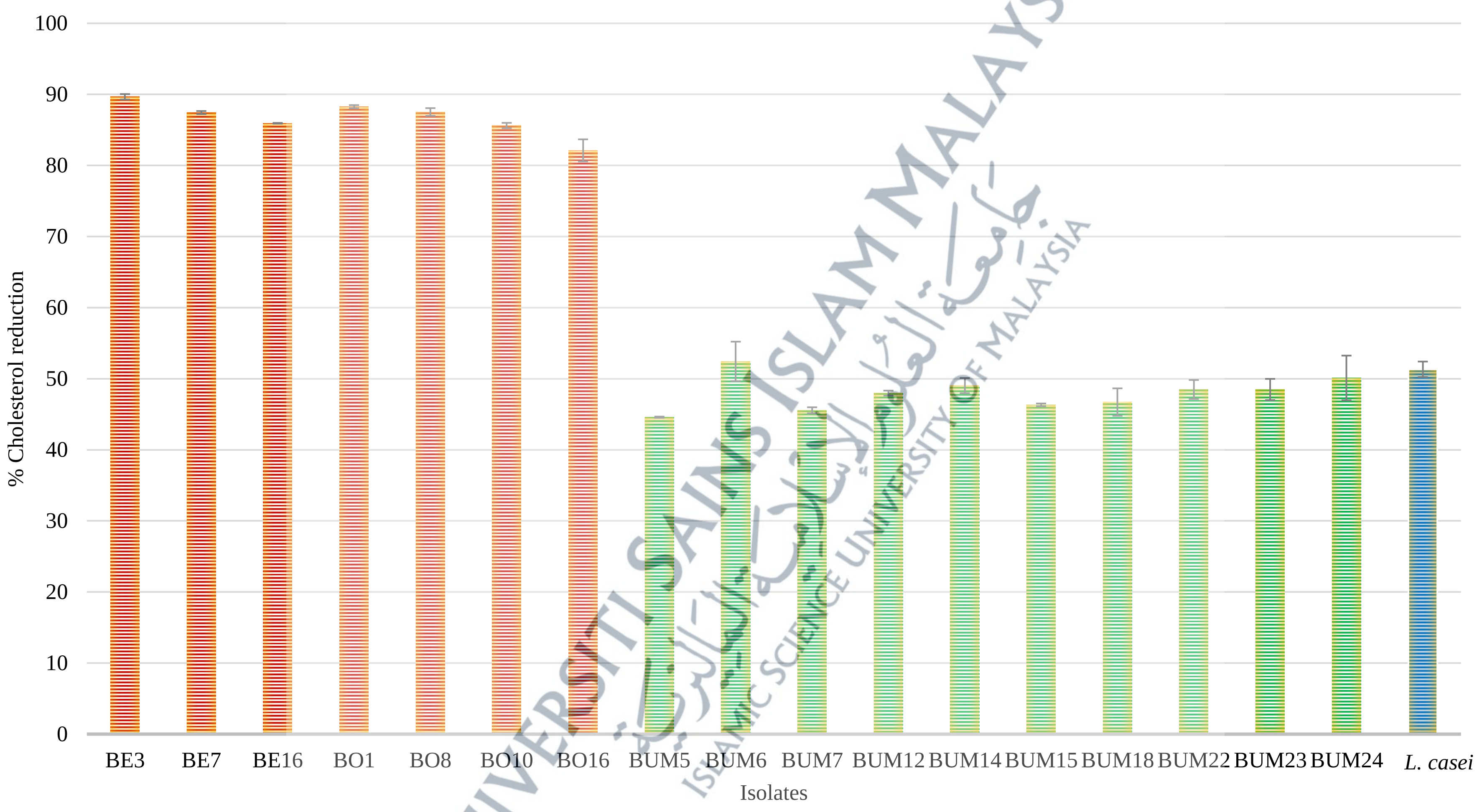


Figure 6.2: The Cholesterol Reduction (%) of *L. plantarum* Strains (red) *L. paracasei* Strains (green) and *L. casei* Strain Shirota (blue) Incubated in MRS Broth Supplemented With 100 µg/mL Water-soluble Cholesterol For 24 h

6.3.3 Principal Component Analysis

The results of multiple assays were subjected to a Principal Component Analysis (PCA) to evaluate the similarity and variability between the potential probiotic strains in various probiotic phenotypes. The study of the contribution of variables to factors showed that principal component (PC) 1 (64.60% of variance) was related to the tolerance of isolates to 0.3% bile at 24 h, their growth on both 0.5% GDCA and 0.5% TDCA, cholesterol removal and autoaggregation ability, cell surface hydrophobicity, and antagonism properties to all pathogens, namely *B. cereus*, *S. aureus*, *E. coli*, and *S. typhimurium*, respectively. PC 2 (14.13% of variance) was linked to isolates' tolerance to both pH 3 and pH 2.5, as well as tolerance to 0.3% bile at 7 h. These two components accounted for 78.73% of the total variation. The projection of LAB isolates in two-dimensional space of the PC1 and PC2 loading factors revealed three main clusters (Figure 6.3).

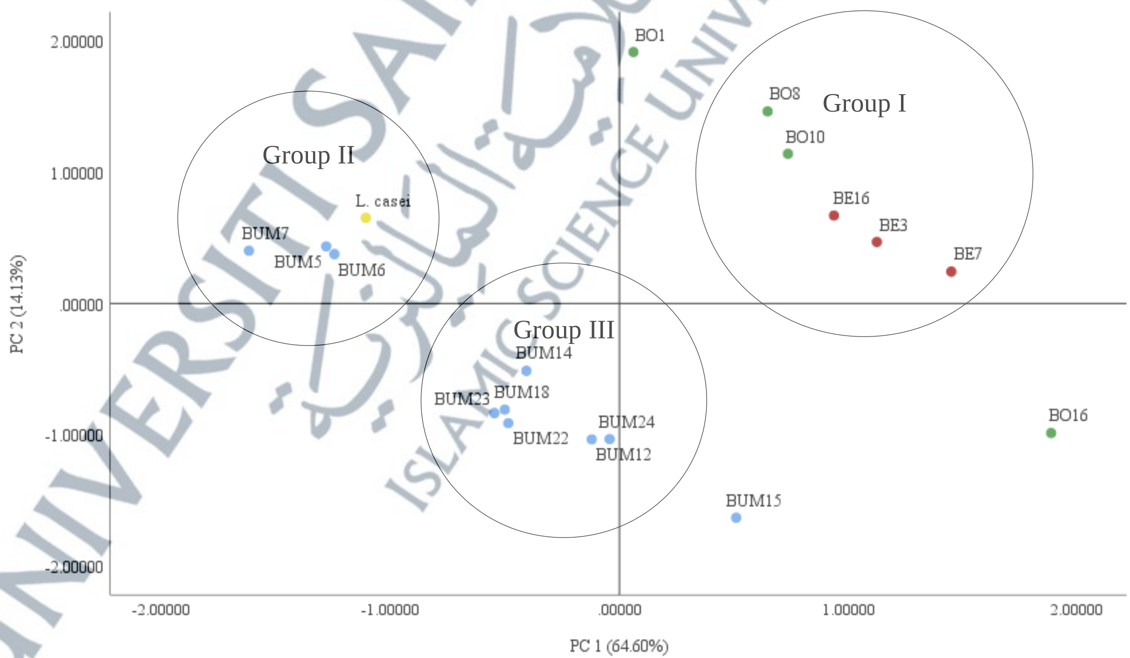


Figure 6.3: Principal Component Analysis (PCA) of LAB Isolates Based on Probiotic Characteristics and Cholesterol Lowering Properties

Group I consist of *L. plantarum* strain BE3, BE7, BE16, BO8, and BO10 isolates, which have the highest probiotic characteristics. Meanwhile, group II includes *L. paracasei* strains BUM5, BUM6, BUM7, and the reference strain *L. casei* strain Shirota, which showed the least tolerance to bile salts, specifically at longer duration, lowest hydrophobicity, and cholesterol removal percentage, as well as the smallest autoaggregation ability and antagonism properties. However, these strains could tolerate acidic pH better than the other *L. paracasei* isolates. Group III comprised of the remaining BUM isolates (*L. paracasei* strain BUM12, BUM14, BUM18, BUM22, BUM23, and BUM24) that have the lowest tolerances to acidic pH, though tolerate better to bile salts and have slightly higher hydrophobicity, autoaggregation, and antagonistic abilities than isolates in group II.

The *L. plantarum* strains BO1 and BO16, as well as *L. paracasei* strain BUM15, were the isolated strains other than the remaining *L. plantarum* and *L. paracasei* strains. BO1 has the highest coping values for acidic pH, which is the first challenging barrier that each probiotic must encounter when entering the gastrointestinal tract. Vice versa, despite having a solid profile for other probiotic traits, both BO16 and BUM15 were unable to tolerate the acidic pH and caused them to be the weakest strains for this characteristic, among the other strains in a similar species, respectively.

According to PCA analysis, three isolates, namely *L. plantarum* strain BE7, *L. plantarum* strain BO1 and *L. paracasei* strain BUM6, were chosen to be further investigated for their interactions with the human colorectal adenocarcinoma cell line HT-29. The selection was based on the source of each strain, different patterns of probiotic characteristics, and cholesterol-lowering properties exhibited by each isolate, respectively.

6.3.4 Adhesion Ability of Potential Probiotic LAB to HT-29 Cells

Adhesion of bacteria to the intestinal mucosa is listed as one of the most important selection criteria for probiotics as this ability is associated with their documented positive health impacts on the host (Ouwehand and Salminen, 2003). For a better indication of the potential intestinal adhesion, the attachment of the *Lactobacillaceae* strains to the HT-29 cells was determined quantitatively by growing the isolates on MRS agar at 37°C for 48 h. All isolates tested were able to adhere to HT-29 cells with an adhesion percentage ranging from 24.7% to 39.6%. They all showed a much higher percentage of adherence than the reference strain *L. casei* strain Shirota. The greatest adherence percentage was recorded by *L. plantarum* strain BO1 (39.6%), followed by *L. paracasei* strain BUM6 (33.5%) and then *L. plantarum* strain BE7 (24.7%) (Figure 6.4). Meanwhile, *L. casei* strain Shirota showed the lowest adhesion index of 0.33%. Results found that the adhesion ability was strain-specific and varied within the same species, as the percentage was significantly different between one another ($P < 0.05$).

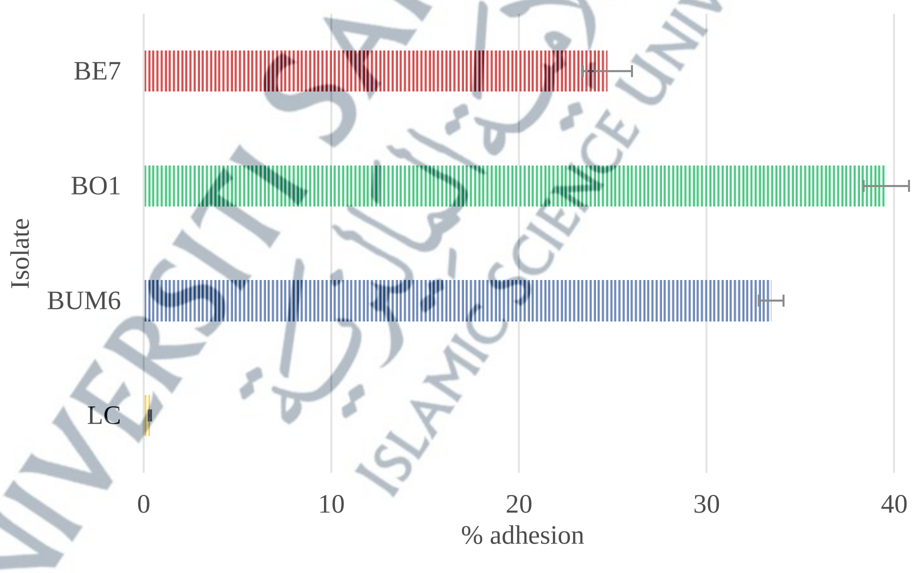


Figure 6.4: Adhesion Ability of *L. plantarum* Strain BE7, *L. plantarum* Strain BO1, *L. paracasei* Strain BUM6 and *L. casei* Strain Shirota (LC) to HT-29 Cells

6.3.5 Adherence Study of Potential Probiotic LAB to HT-29 Cells by Field Emission Scanning Electron Microscopy (FESEM)

Adhesion of *L. plantarum* strain BE7, *L. plantarum* strain BO1 and *L. paracasei* strain BUM6 to HT-29 cells was further illustrated by qualitative FESEM observations (Figure 6.5). Micrographs showed the spatial arrangement of bacteria on the exterior of HT-29 cells and good inter-cell interactions between bacteria and the intestinal cells. Bacteria cells exhibited strong attachment and did not cause any damage to the exact structure of the epithelial cells. This claim is proved by the structure of the HT-29 cells treated by the bacteria that remain complete without any damage, similar to the appearance of cells with the absence of bacteria. Each treated HT-29 cell displayed a typical apical brush border, or the microvilli, and a protruding tight junction between cells (Martínez-Maqueda et al., 2015). Mucous secretion can also be observed in some of the images.

Qualitatively, all bacteria showed remarkable intra-cell interactions by forming a cluster among themselves and appearing in aggregated form. Besides that, the bacteria were also seen to be solidly bound in a diffuse pattern throughout the examined area, either in short chains or in pairs on the HT-29 cell surface. In contrast to *L. plantarum* species, *L. paracasei* strain BUM6 was found adhered singly. The micrographs of the HT-29 intestinal cells treated with *L. plantarum* strain BE7, *L. plantarum* strain BO1 and *L. paracasei* strain BUM6 showed excellent adherence to the enterocytes, which signified their ability to endure the environment in the intestinal lumen without causing the abnormal changes in the tissue’s morphology.

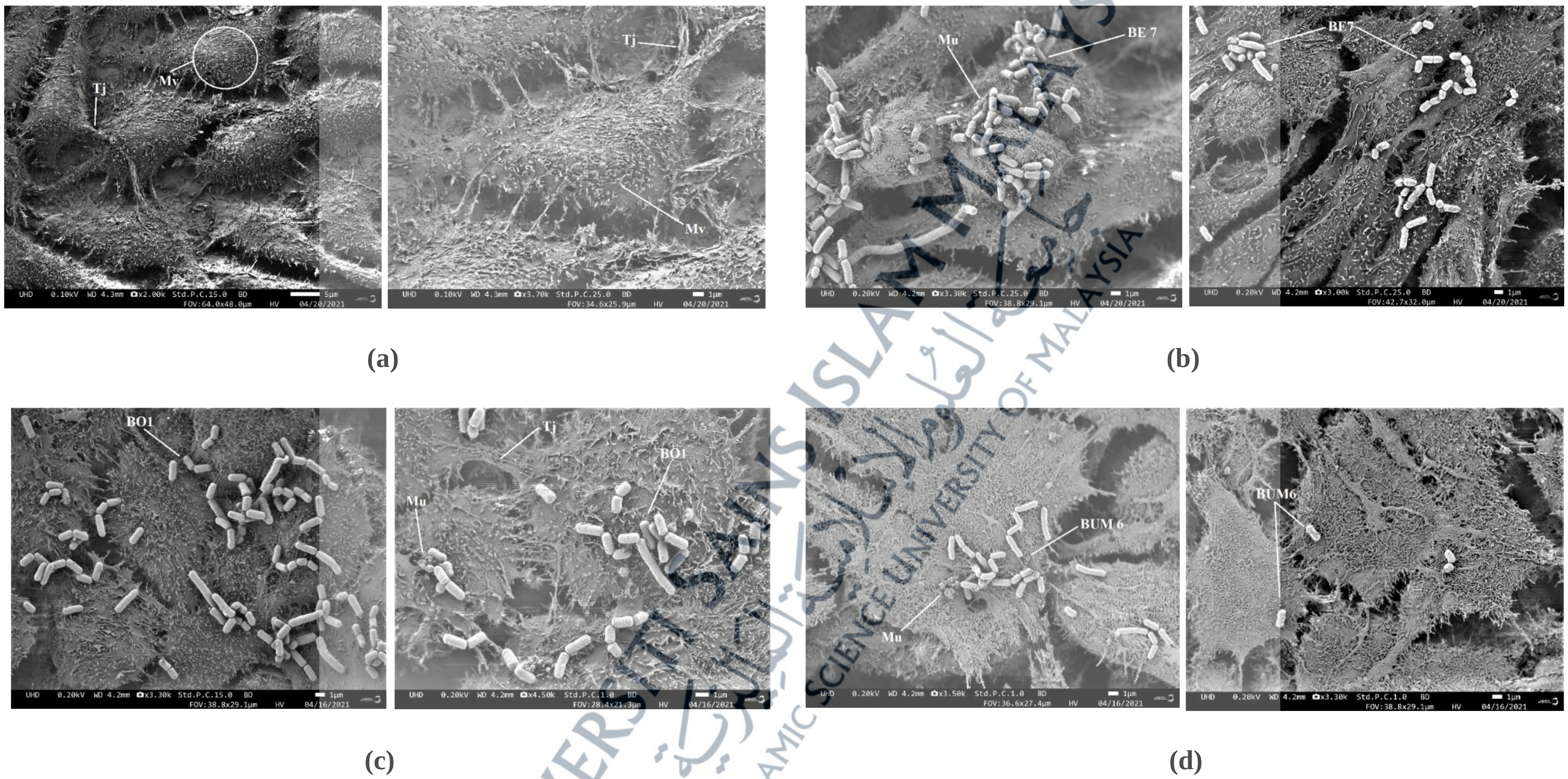


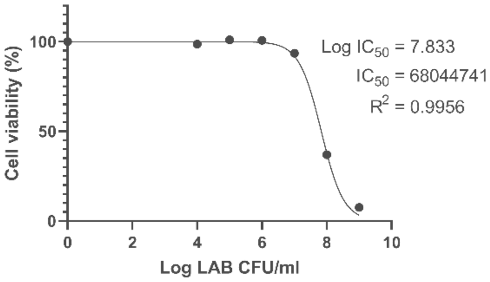
Figure 6.5: FESEM Images of a) Untreated HT-29 Cells, and HT-29 Cells Treated With b) *L. plantarum* Strain BE7, c) *L. plantarum* Strain BO1 and d) *L. paracasei* Strain BUM6

Abbreviations: Mu: mucous; Mv: microvilli; Tj: tight junction.

6.3.6 Cytotoxicity Activity of Potential Probiotic LAB Against HT-29 Cell Line by MTT Assay

In order to investigate the potential cytotoxic effects of bacterial isolates on cell viability, the HT-29 cells were treated with different concentrations of *L. plantarum* strain BE7, *L. plantarum* strain BO1 and *L. paracasei* strain BUM6 for 24 h. The effects of isolates on cell viability were evaluated using the MTT assay, and were compared with the negative control, which was the wells containing only HT-29 cells and incomplete growth medium. Based on the MTT assay results, the readings of the cell's optical density were transformed into a plotted graph showing the impacts of treatment with different bacterial concentrations on HT-29 cell viability.

Figure 6.6 indicates the non-dose-dependent effects of increasing concentrations of bacterial isolates on the viability percentage of HT-29 cell lines. When lower bacterial concentrations (10^4 to 10^7 CFU/mL) were inoculated in the well containing the HT-29 cells, each isolate could hardly produce a cytotoxic effect on the HT-29 cells. However, with increasing concentrations of LAB isolates at 10^8 and 10^9 CFU/mL, a steep decline in cell viability percentage was observed. Among the isolates, *L. plantarum* strain BO1 exhibited the most potent cytotoxic effect against HT-29 cells with an IC_{50} of 1.48×10^7 CFU/mL after 24 h of incubation, followed by *L. paracasei* strain BUM6 and *L. plantarum* strain BE7, with IC_{50} values of 2.44×10^7 CFU/mL and 6.80×10^7 CFU/mL, respectively. The reference isolate *L. casei* strain Shirota also recorded quite a parallel IC_{50}, which was 2.51×10^7 CFU/mL. Meanwhile, the cytotoxic potential of the positive control, hydrogen peroxide, followed a dose-dependent order, with an IC_{50} of 5.191 mM.



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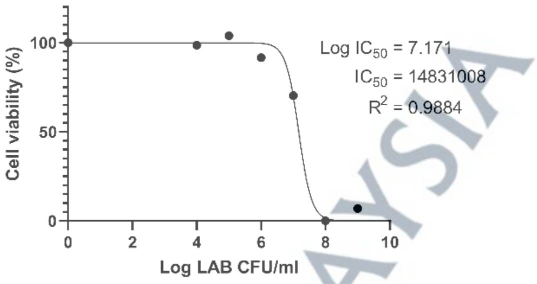

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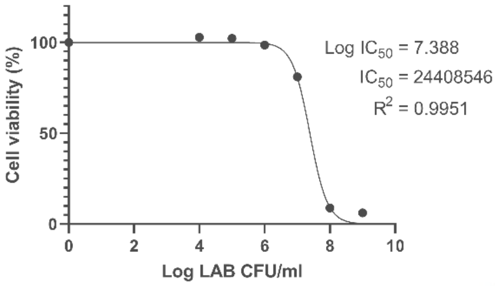

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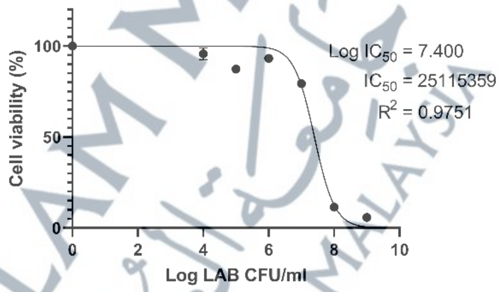

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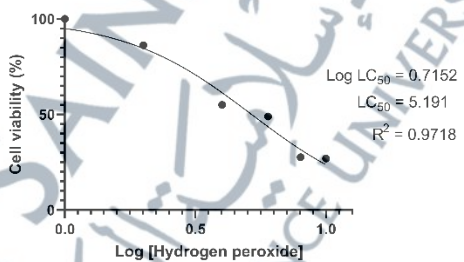

(e)

Figure 6.6: Viability of HT-29 Cells Exposed To Multiple Concentration of a) *L. plantarum* Strain BE7, b) *L. plantarum* Strain BO1, c) *L. paracasei* Strain BUM6, d) *L. casei* Strain Shirota and e) Hydrogen Peroxide For 24 h

6.4 Discussion

Awareness of proper diet selection as well as its impact on better health has been growing in the community. Nowadays, diets not only supply basic nutrients for the growth and metabolic processes of the human body, but people also choose diets that work to provide added value to human health (Bhat and Bajaj 2019), for example, foods containing probiotics. Among the examples of areas of concern related to the exploration of probiotic potential is its ability to modulate cholesterol, as an elevated level of serum cholesterol has always been linked with an increased risk of coronary heart disease (Liong and Shah, 2005a). It is guided by the fact that, besides pharmaceutical methods, probiotics therapy has shown a potential effect in total cholesterol regulation (Wang et al., 2018). In addition, the study is also supported by the fact that therapeutic drugs to treat hypercholesterolemia are causing various side effects, thus encouraging researchers to seek more natural and safe alternatives (Liu et al., 2013). In this study, the potential probiotic LAB strains with multiple probiotic characteristics were evaluated for their cholesterol lowering properties before the best three isolates were chosen based on principal component analysis. The selected isolates were then evaluated for their ability to adhere to intestinal cells and their potential cytotoxic threat using the human colorectal adenocarcinoma HT-29 cell line.

Initially, seven *L. plantarum* strains and ten *L. paracasei* strains were compared for their growth in the presence of two main conjugated bile salts of the human body, namely TDCA and GDCA, and their BSH activity. The BSH activity in probiotics has been agreed to be one of the mechanisms associated with the reduction of cholesterol levels (Choi et al., 2015). Cholesterol is the precursor for primary bile acids, which are conjugated to either glycine or taurine and stored as conjugated bile salt (Geng and Lin, 2016). When LAB produces BSH, it may act as a shield against the toxification of host bile salt by deconjugating the bile salt into free amino acid residues and unconjugated bile acids. Because the unconjugated bile acids are less soluble, they are less likely to be reabsorbed by the small intestine and thus excreted (Liong and Shah, 2005b). This situation would contribute to reduced intestinal absorption of cholesterol and decrements of serum cholesterol to produce more primary bile salt (Begley et al., 2006). Although the ability to produce BSH is a desirable attribute to aid in reducing the host cholesterol level, it is not an essential characteristic of probiotics (Pinto et al., 2020).

All tested strains did not demonstrate both TDCA and GDCA hydrolase activities, as evidenced by the absence of visible halos or white opaque around the colonies. The growth ability of the strains also agreed with Allain et al. (2018), which stated that more isolates could grow on TDCA that is less bactericidal than GDCA, with *L. casei* and *L. paracasei* strains being more vulnerable than *L. plantarum* strains (Xiong et al., 2017). Although BSH is common in *Lactobacillus*, the LAB's ability to produce BSH was correlated to its natural habitat (Begley et al., 2006). Probiotic bacteria with BSH activity are more frequently isolated from bile salt environments, such as strains isolated from the gastrointestinal tract (Sedláčková et al., 2015). The results of this study are consistent with Pinto et al. (2020), who also reported negative BSH activity for all their LAB strains, though it contradicts some published studies (Agaliya and Jeevaratnam, 2012; Huang et al., 2019), which reported the presence of BSH activity in LAB isolated from environments in which bile salts are absent. In fact, the screening of 800 LAB strains showed less than 3% having positive BSH results (Tsai et al., 2014).

According to Vinderola and Reinheimer (2003), some strains were able to grow in the presence of bile without producing BSH, as the ability to express BSH is not associated with the ability to withstand the toxicity of bile salt. Hence, the bacteria may not be able to express the cholesterol-lowering properties through this mechanism.

Although it seems unbeneficial in terms of cholesterol management, the absence of BSH in these seventeen strains might be fortunate to the host due to the fact that deconjugation of bile is also associated with multiple detrimental effects such as gallstone formation and higher risks of colon cancer (Choi et al., 2015).

Undeniably, the ability of LAB to reduce cholesterol concentration is deemed an essential parameter of a probiotic with health benefits. Probiotics could play a preventive role in various diseases caused by hypercholesterolemia if they could prevent the absorption of cholesterol in the hosts' digestive systems (Sakandar et al., 2019). For the cholesterol removal evaluation, the initial cholesterol concentration was set at 100 µg/mL as it was the ideal concentration for the cholesterol uptake analysis by *Lactobacillus* strains (Singhal et al., 2011). The findings showed that all seventeen strains displayed cholesterol reduction of at least half of the total water-soluble cholesterol inserted into the MRS medium, compared with the control MRS broth. This means the removal of cholesterol by these strains would make cholesterol less available for absorption into the circulation.

The outcomes from this study were somewhat more excellent than the *L. plantarum* strain isolated from fermented kimchi, which could reduce the cholesterol concentration by almost 50% compared to the control (Huang et al., 2019). Another study also confirmed that some LAB strains isolated from fermented shrimp have various cholesterol reduction levels, but with a lower percentage ranging from 12.5 up to 27% (Le and Yang, 2019a). Although many studies stated that cholesterol lowering only occurred when cultures were grown in the cholesterol-modified media with presence of bile salts (Castorena-Alba et al., 2018; Huang et al., 2019; Majeed et al., 2019), recent findings agreed with Lim et al. (2017), who stated that the presence of bile salt is not compulsory to evaluate cholesterol reduction in-vitro. Hojjati et al. (2020)

who investigated the cholesterol assimilation ability of *Lactobacillus brevis* gp104 reported that the strain was able to assimilate cholesterol from cholesterol-added medium both in the presence and absence of bile. The results of BSH activity and cholesterol removal in this study were also contrary to the outcomes reported by Lakra et al. (2020), who found a direct association between BSH activity and the cholesterol reduction capability of the LAB strains.

There are several mechanisms by which the BE, BO, and BUM isolates could reduce the cholesterol in the MRS medium, which suggests that when the bacteria are ingested, they could help to regulate the cholesterol level by performing similar roles in the intestine. The mechanisms involve incorporating the cholesterol into their cell membrane during growth or binding the cholesterol to the live or dead bacterial cell surface (Kimoto et al., 2002; Wang et al., 2014). The growth status of the bacteria affects the significant removal of cholesterol, with growing cells having a higher ability to reduce cholesterol compared to resting and dead cells (Miremadi et al., 2014; Ma et al., 2019). Besides, the cholesterol could also be assimilated into the bacteria, go through enzymatic reduction by cholesterol reductase, or precipitated with the free bile salts in the media (Liong and Shah, 2005b; Tomaro-Duchesneau et al., 2015; Abushelaibi et al., 2017). However, the latter may not be correlated to our findings as the procedures did not include the presence of bile salts.

A PCA was subsequently performed to further analyse the data for probiotic characteristics and cholesterol lowering properties of the strains. PCA was applied to identify the main factors among the multiple characters, then map each strain based on their performance. This analysis is used in exploratory science due to its capability to project a relationship of inter-samples and inter-variables graphically, thus helping to reduce the complexity of the data (Sidira et al., 2017). Multiple studies have applied the analysis (Solieri et al., 2014; Montoro et al., 2016; Cai et al., 2019) to optimise the process of selecting the most suitable nominee out of many strains for advanced investigations.

In this study, three strains, namely *L. plantarum* strain BE7, *L. plantarum* strain BO1 and *L. paracasei* strain BUM6, were chosen as potential probiotic candidates based on their own unique profile against the tested variable, as pictured by a comprehensive PCA of 13 descriptors. The selection of the best strains was almost similar to Chopade et al. (2019), who decided to choose the probiotics plotted furthest on the x-axis, as well as probiotics plotted in contrasting axis. Meanwhile, *L. paracasei* strain BUM6 was selected due to the location of its plot that is closest to the *L. casei* strain Shirota, similar to the approach taken by Angmo et al. (2016). The plot proved its high resemblance of probiotic activities with the reference strain, thus allowing comparisons to be made between isolates with distinct characteristics and isolates with characteristics almost similar to the common probiotics used commercially. For example, as the characteristics of the chosen isolates were different from one another, it is noteworthy to excavate more information about each strain, for example, their interactions with the intestinal cells.

The probiotics' ability to attach to intestinal cells is crucial when taking into account the factor of high flow rates in the lumens that could wash off these microorganisms from the intestinal tracts (Ouwehand and Salminen, 2003). For example, the probiotics' attachment onto the intestinal mucosa allows potential protection against pathogens through competitive exclusion for nutrients and binding sites, but this temporary colonisation is also important for the probiotic bacteria to exert their expected favourable effects, for example, cholesterol lowering properties and modulation of immune system (Tomaro-Duchesneau et al., 2015; Monteagudo-Mera et al., 2019). Earlier, Alizadeh Behbahani et al. (2019) reported that based on both qualitative and quantitative analysis, the *L. plantarum* strain L15 was observed in aggregated features on the Caco-2 cell lines with an adhesion value of 12.2% and was able to inhibit *E. coli* adhesion in the intestine. Hojjati et al. (2020) suggested that there is a direct correlation between a good percentage of in-vitro adhesion and the exact colonisation of probiotics in the human intestine. In this study, the HT-29 cell line was used to study the adhesion ability of the selected *Lactobacillaceae* strains. Because the characteristics of HT-29 cells are comparable to those of mature enterocytes in terms of their features such as membrane potential, ion conductance, and permeability properties, this cell has been widely used in studies of intestinal properties, transportation, and metabolism (Lim et al., 2017).

The adhesion ability of *L. plantarum* strain BE7, *L. plantarum* strain BO1 and *L. paracasei* strain BUM6 was somewhat excellent, as it showed quite high percentage values with a range of 24.7% to 39.6%. The values are higher than the percentages reported by Benítez-Cabello et al. (2019) for their *Lactobacillus* strains, though the adhesion percentage of the reference strain *L. casei* strain Shirota was almost parallel to our results. Fonseca et al. (2020) also reported lower adhesion capacities of LAB strains, namely *L. plantarum*, *L. paracasei*, and *L brevis*, with values below 5%. If the obtained results are directly equated with the in-vivo situation, the number of all three isolates that need to be ingested is much less than that of commercial probiotics to obtain the same percentage of adhesion. Besides the cell counting method, the strong adhesion was further evidenced by the FESEM image, which proved the ability of all three bacterial isolates to remain attached to the surface of the HT-29 cell line, respectively, even after undergoing various cell treatments such as fixation, multiple stages of alcohol dehydration, and critical point drying.

Remarkably, based on our finding, the strain displaying the weakest adhesion ability belongs to the similar species that recorded the greatest adhesion, thus signifying the strain specificity phenomenon (Gharbi et al., 2019). The theory was supported by previous studies involving both *L. plantarum* and *L. paracasei*. Mathara et al. (2008) reported that their *L. paracasei* strains BFE 5979 and BFE 6175, isolated from fermented milk, possessed slightly better adhesive properties on HT-29 cells with a value of 42% and 39%, respectively. More recently, *L. plantarum* strain FB003 isolated from Korean salted-fermented shrimp showed a slightly lower adhesion percentage of 34.5% (Le and Yang 2018). No clear relation was observed between the adhesion of these isolates to hydrocarbons (hexadecane) and adhesion to HT-29 cells. Our previous data showed that the hydrophobicity percentage of *L. plantarum* strain BE7, *L. plantarum* strain BO1 and *L. paracasei* strain BUM6 was 19.0%, 25.6%, and 4.5%, respectively. Similar results were also found in previous work for other *Lactobacillus* strains (Tomaro-Duchesneau et al., 2015; Benítez-Cabello et al., 2019). Contrary to the results we obtained, Han et al. (2017) reported that their *Lactobacillus* strains with higher rates of adhesion to the experimental cell line also had higher hydrophobicity values.

The absence of this correlation is due to the adhesion of bacteria, which is a complex process that involves interaction between the cell membrane and cooperating surfaces. The interaction depends not only on hydrophobicity but also on various other factors such as Brownian movement, Van der Waals attraction, gravitational forces, and surface electrostatic charges. The diffuse pattern of bacterial adhesion onto the HT-29 cells was described earlier by Satish Kumar et al. (2011), which may be facilitated by the presence of mucin on the HT-29 apical side. Mucin secretion by enterocytes is important to create a mucus layer that plays a role in modulating the adhesion of microorganisms to its surface and to provide nutrients for bacterial growth (Martínez-Maqueda et al., 2015).

Besides, carbohydrate and proteinaceous compounds on the bacterial surface might be responsible for the adhesive properties, as inferred by Satish Kumar et al. (2011) and Gao et al. (2020) for their *L. plantarum* AS1 and *L. plantarum* Y44, respectively. Both studies showed that the adhesion percentage of treated isolates to HT-29 cells was significantly lower than that of untreated isolates. The ability to produce these elements is strain-specific, thus leading to different adherence percentage of bacteria to intestinal epithelial cells (Ida Muryany et al., 2018). In addition, the bacteria themselves are able to change their external characteristics, whether hydrophobic or hydrophilic, depending on environmental pressures such as temperature and nutrients (Benítez-Cabello et al., 2019). *Lactobacillus* also generates adhesins, a cell-surface component that facilitates adhesions of cells by adhering to receptor cells existing in the human intestinal tract, such as mucin, extracellular matrix, and lectin-like proteins (Hojjati et al., 2020).

The cytotoxicity profiles of *L. plantarum* strain BE7, *L. plantarum* strain BO1 and *L. paracasei* strain BUM6 on HT-29 cells were investigated using the MTT assay to determine the safety profiles of bacteria for the digestive tract and their potential as a functional supplement and an adjunctive treatment for hypercholesterolemia. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) is a water-soluble tetrozolium salt that can be reduced by mitochondrial succinate dehydrogenase to the insoluble MTT-formazon. MTT-formazon is insoluble and thus will accumulate in healthy living cells. Thus, the MTT assay reflects the ability of mitochondria in living cells to respire (Mosmann, 1983). In this experiment, the isolates were prepared in incomplete medium without the presence of FBS and antibiotics to prevent the growth of both HT-29 cells and bacteria. According to Forgue-Lafitte et al. (1989), HT-29 doubling time is one day with the existence of serum in the growth medium but a longer period without the presence of serum. Thus, incomplete medium is sufficient to assess the viability of cells, without considering their growth parameters.

The bacteria exerted inhibitory activity on more than half of HT-29 cells at a high concentration of 10^8 and 10^9 CFU/ml. This result is almost parallel to the findings reported by Ahmad et al. (2018) that bacterial cells and supernatant of *L. plantarum* isolated from Malaysian fermented durian were able to inhibit more than 50% of the growth of HT-29 cells at 10^8 CFU/ml. From the chemopreventive point of view, it is interesting to know that these potential probiotics were able to modulate the growth of colon cancer cells. Experimental evidence for anti-cancer activity of both viable and nonviable *L. paracasei* has also been documented on HGC-27 gastric and DLD-1 colon cancer cell lines (Orlando et al., 2012).

Some possible mechanisms that lead to cancer inhibition are secretion of bioactive metabolites by the probiotic LAB that induce the host's signalling pathway and immune response to react against the cancer cells, or induction of the apoptosis process to stop cancer progression (Nozari et al., 2019). Furthermore, another recent study conducted by Chuah et al. (2019) concluded that the post metabolites produced by six strains of *L. plantarum* possess promising anticancer activity on various human cancer cells but recorded low cytotoxicity to normal mammalian cells. It is noteworthy to mention that although HT-29 is used extensively in the exploration of probiotics due to its suitability, it is still a type of cancer cell that may benefit from the cytotoxic properties of high concentrations of *L. plantarum* strain BE7, *L. plantarum* strain BO1, and *L. paracasei* strain BUM6.

However, our results clarify that although these three potential probiotics displayed significant cytotoxic activities against HT-29 cells, based on the adhesion study, the concentration of bacteria that could safely adhere to the cell was much lower. In fact, the IC_{50} of these isolates was almost parallel with that of the commercial probiotic strain, *L. casei* strain Shirota. Thus, this condition assures that these potential probiotics are safe and might be beneficial to the intestinal environment. This could be supported by previous findings on fermented milk beverages containing live *L. paracasei* strain 01, which demonstrated significantly higher Caco-2 cell growth than blank substrate and the same beverage undergoing heat treatment, thus suggesting the essential role of the live bacteria in the protection of the intestine (Chen et al., 2016). The possible protective mechanisms include enterocytes' growth promotion and integrity enhancement, as well as downregulation of cytokines and chemokine production, leading to a reduction of inflammation.

6.5 Conclusion

The seventeen LAB strains isolated from belacan, bosou, and non-cooked budu, the fermented fish products of Malaysia, have been explored for their cholesterol-lowering ability. Although the strains did not show BSH activity, they were able to grow with varying abilities on the supplemented bile salt agar as well as remove cholesterol in MRS medium quite promisingly. Through PCA analysis, *L. plantarum* strain BE7, *L. plantarum* strain BO1, and *L. paracasei* strain BUM6 were chosen as the potential isolates to be further explored according to their distinctive properties in several probiotic tests and a cholesterol reduction assay. All three isolates showed remarkable adhesion ability to HT-29 cells quantitatively using the plate method and qualitatively using FESEM, as well as only causing cytotoxicity at exceptionally high concentrations.

This study suggests that *L. plantarum* strain BE7, *L. plantarum* strain BO1 and *L. paracasei* strain BUM6 may be suitable candidates for probiotic use in cholesterol management. However, more analysis, such as transcriptomic study, is required to further explore the influence of these potential probiotics on gene expression of the cells, which is related to modulation of cholesterol.