CHAPTER 7

TRANSCRIPTOMIC PROFILE ANALYSIS ON GENES INVOLVES IN CHOLESTEROL METABOLIC PATHWAY IN HUMAN COLORECTAL ADENOCARCINOMA CELL LINE HT-29 AFTER BEING TREATED WITH POTENTIAL PROBIOTIC LACTIC ACID BACTERIA ISOLATED FROM MALAYSIAN FERMENTED FOOD

7.1 Introduction

Hypercholesterolemia is known to be the major risk factor for cardiovascular disease (CVD). The high mortality rates associated with CVD even with the widespread use of drugs, for example, statins suggests that this method is not sufficiently effective and alternative options to overcome this are much needed (Michael et al., 2017). Homeostasis of cholesterol involves the regulation of the intake of dietary cholesterol, the synthesis of cholesterol by the body, the absorption of cholesterol into cells and the excretion of cholesterol out of the body (Badi et al., 2020). Besides the liver, another main organ involved in this process is the intestine. Early theories that said that cholesterol was absorbed into the intestine through passive transport were refuted by the finding that cholesterol transport was more effective than other sterols, which may be mediated by protein (Skov et al., 2011).

There are several cholesterol transporters involved in the regulation of cholesterol in the intestine. For instance, Nuclear Receptor Subfamily 1 Group H Member 3 (NR1H3), also known as Liver X Receptors (LXR) α plays a key role in the metabolism

of cholesterol. The transcriptional activity of this gene regulates the expression of genes involved in cholesterol metabolisms such as absorption, transport, efflux and excretion, as well as the conversion of cholesterol to bile acids (Wang and Tontonoz, 2018). Meanwhile, Niemann-Pick C1-Like 1 (NPC1L1) is the transport protein localised mainly at the enterocytes' apical membrane and is responsible for the uptake of available cholesterol from the intestinal lumen (Yu et al., 2019). Other proteins responsible for the transport of cholesterol across the enterocytes are the integral membrane glycoproteins named Scavenger Receptor Class B Member 1 (SCARB1) (Altmann et al., 2002) and Cluster of Differentiation 36 (CD36), also known as fatty acid translocase (FAT) (Nassir et al., 2007).

The administration of functional microorganisms has been shown to promote health by improving the intestinal environment and modulating cholesterol levels (Park et al., 2018). Studies of messenger RNA (mRNA) expression levels of the related transport protein have been previously done using multiple models to further understand the impact of the presence of probiotics on the modulation of cholesterol in the intestinal environment (Park et al., 2018; Wa et al., 2019; Badi et al., 2020). In a recent study, it was found that the expression of genes associated with lipid metabolisms in hyperlipidemic rats was more effective when the group received a single probiotic treatment than a combined probiotic treatment, giving the insight that the effect of probiotics on gene expression was strain-dependent (Wa et al., 2019).

The ability of the potential probiotic LAB, *L. plantarum* strain BE7, *L. plantarum* strain BO1, and *L. paracasei* strain BUM6 to lower cholesterol in broth media has previously been demonstrated; however, the mechanisms by which the strains can successfully exert their beneficial effects on the host cell remains unknown. Thus, this study was conducted with the aim of evaluating the effects of three potential probiotics

in relation to the modulation of expression of genes including NPC1L1, CD36, SCARB1 and NR1H3, which are involved in cholesterol homeostasis. The study was carried out using the HT-29 cell line as a human intestinal epithelial model.

7.2 Materials and Methods

7.2.1 HT-29 Cells Treatment With Potential Probiotic LAB

The HT-29 cells were seeded in a six-well plate at a concentration of 3×10^5 cells/well and were subjected to overnight incubation at 37°C to allow cell growth and attachment to the well. The next day, the supernatant was removed, and each well was washed thrice with PBS to eliminate unbound cell. Meanwhile, the overnight *L. plantarum* strain BE7, *L. plantarum* strain BO1 and *L. paracasei* strain BUM6 cultures were centrifuged and washed with PBS. The pellets were resuspended in McCoy's 5A medium without FBS and penicillin-streptomycin (10^7 CFU/mL), with or without the presence of water-soluble cholesterol (200 µg/mL), respectively (Gorenjak et al., 2014).

HT-29 cells were subjected to no treatment (cell only), treatment with 10^7 CFU *L. plantarum* strain BE7, treatment with 10^7 CFU *L. plantarum* strain BO1, treatment with 10^7 CFU *L. paracasei* strain BUM6, exposure to 200 µg water-soluble cholesterol, exposure to 200 µg water-soluble cholesterol and treatment with 10^7 CFU *L. plantarum* strain BE7, exposure to 200 µg water-soluble cholesterol and treatment with 10^7 CFU *L. plantarum* strain BO1, as well as, exposure to 200 µg water-soluble cholesterol and treatment with 10^7 CFU *L. paracasei* strain BUM6, respectively (Figure 7.1). The plates were then incubated for 6 h (Lim et al., 2017) and 24 h in a 5% CO_2 incubator at 37°C.

Following the incubation period, the culture medium was discarded, and the cells were gently washed with PBS. Each well was treated with 1 mL trypsin-EDTA solution and was left for approximately 5 to 10 min at 37°C to disrupt the adhered cells. McCoy's

5A medium was added to inhibit the activity of trypsin before the cells were harvested for total RNA extractions. Each treatment was done in triplicate and repeated thrice.

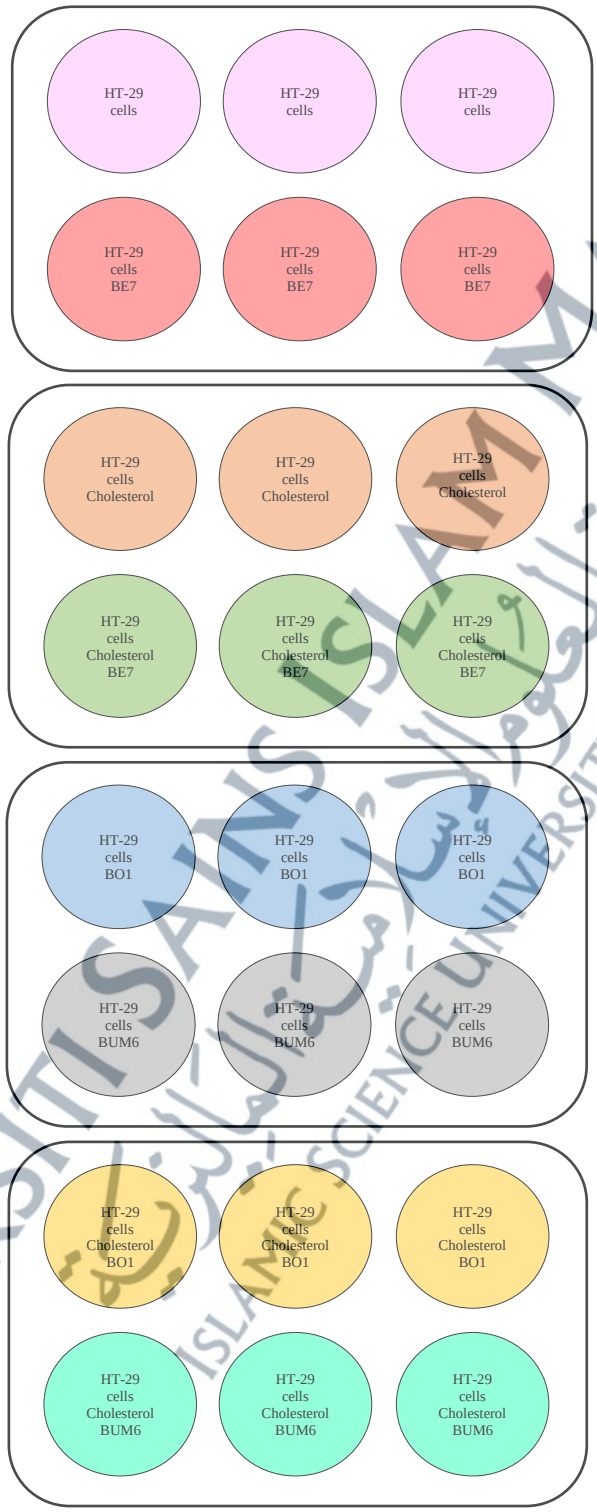

Figure 7.1: Design for HT-29 Cells Treatment with Potential Probiotic LAB

7.2.2 RNA Extraction

Total RNA was extracted from cells by using NucleoSpin® RNA (Macherey-Nagel, Germany), as instructed by the manufacturer. Briefly, buffer and β-mercaptoethanol were added to the tube and homogenised to lyse the cells. The lysed cells were then filtered and centrifuged for 1 min. The lysate was added with 70% ethanol to adjust the RNA binding conditions before pipetting it into the NucleoSpin® RNA column in a collection tube. The column was centrifuged for 30 sec, and it was then placed into a new tube. The membrane desalting buffer was applied to desalt the silica membrane before the addition of the DNase reaction mixture into the column to digest the DNA. The column was then washed using buffer and the RNA was finally eluted in 60 µl RNase-free H_2O. The concentration and purity of extracted RNA were estimated using the NanoPhotometer® P 300 (Implen, US). Pure RNA usually yields a 260/280 ratio of ~2.0 (Desjardins and Conklin, 2010). The collection tubes containing pure RNA were labelled and kept at -80°C until further use.

7.2.3 cDNA Synthesis

The RNA extract was reverse transcribed to cDNA by reverse transcription using ReverTra Ace™ qPCR RTMaster Mix with gDNA Remover (Toyobo, Japan) according to the manufacturer's instructions. A quantity of 0.3 µg of each RNA sample was used as the template for the 10 µL reaction. The RNA sample was mixed with Master Mix, gDNA remover, and nuclease-free water and incubated at 37°C for 5 min. After that, Master Mix for reverse transcription was added and the reaction mixture was placed in the T100 thermal cycler (Bio-Rad, US). The reaction mixture was incubated at 37°C for 15 min, followed by 50°C for 5 min and finally 98°C for 5 min. The cDNA was diluted

two times using nuclease-free water for use as a template for the qPCR and stored at -20°C until further use.

7.2.4 Sequence Retrieval of Target and Reference Genes and Primer Design

The list of primers used in this study is shown in Table 7.1. The sequence of primers was designed and aligned using the IDT Oligo Analyzer (https://sg.idtdna.com/calc/analyzer) and Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). The potential for secondary structure formation, self-dimer, and hetero-dimer of each primer was also evaluated. The sequences were converted into their reverse-complement counterparts using Reverse Complement (https://www.bioinformatics.org/sms/rev_comp.html). All primers were assessed for specificity against the mRNA database using NCBI BLAST and were tested by qPCR standard curve method to determine the efficiency and linear range.

Table 7.1: Forward and Reverse Primers of Reference Gene and Target Genes

Gene	Gene names	Accession no.	Primer Sequence 5' to 3'
NPC1L1	Homo sapiens NPC1 like intracellular cholesterol transporter 1	NM_013389.3	F = CTG GTA TCA CTG GAA GCG AGT R = CAC GCG GGT CAC ATT GAT GA
SCARB1	Homo sapiens scavenger receptor class B member 1	NM_005505.5	F = GAG CCA AGA GAA ATG CTA TTT R = ACC CTA CAG TTT TGC TTC CTG C
CD36	Homo sapiens CD36 molecule	NM_000072.3	F = CAA TTA AAA AGC AAG TTG TCC TCG A R = ATC ACT TCC TGT GGA TTT TGC A
NR1H3	Homo sapiens nuclear receptor subfamily 1 group H member 3	NM_005693.4	F = GTC GCA AGT GCC AGG AGT G R = GAC GAG CTT CTC GAT CAT GCC
β-actin	Homo sapiens actin beta	NM_001101.5	F = CCT GGC ACC CAG CAC AAT R = GCC GAT CCA CAC GGA GTA CT

7.2.5 Gene Expression Analysis by qPCR Reactions

The qPCR reaction contained a 10 µL reaction mix comprising 5 µL PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, US), 1 µl of 5 µM forward and reverse primers each, 15ng cDNA template, and pure water. The qPCR run for each primer was performed separately in triplicate using the QuantStudio™ 5 Real-Time PCR Systems (Applied Biosystems, US). A non template control was included in each batch of qPCR runs and served as a negative control. The amplification conditions for β-actin, NPC1L1, CD36, and NR1H3 primers were optimised as follows: one step of UDG activation and Dual-Lock™ DNA polymerase at 50°C for 2 min and 95°C for 2 min, respectively, followed by 40 cycles of denaturation at 95°C for 15 sec and annealing/extension at 60°C for 1 min, and lastly, the melt curve stage. Meanwhile, for the SCARB1 primer, the annealing temperature is 58°C for 15 sec. The data was obtained from the instrument as threshold cycle (C_t) values. The relative fold change in gene expression of each target gene was normalised to β-actin expression, which acts as the housekeeping gene, and the data were calculated using the Pfaffl method (Pfaffl, 2001).

7.2.6 Statistical analysis

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

7.3. Results

7.3.1 Primer Efficiency and Specificity

The primers were evaluated for their amplification efficiency and specificity by making fold dilutions and running the qPCR reactions. The standard curve of each primer was constructed, and the efficiency (E) value was determined using the equation (10 ^ (-1 / slope value) – 1) × 100. All primers were discovered to have efficiencies ranging form 103% to 109%, which is within the recommended range (Abdel Nour and Pfaffl, 2020). Meanwhile, the specificity of primer annealing was assessed by melt curve analysis. The curve for each primer displayed a single distinct peak with no shouldering, which reflects the amplification of a single product with no primer dimer formation or nonspecific PCR amplification.

7.3.2 Analysis of Influence of Potential Probiotic LAB in the Expression of Genes Involved in Cholesterol Metabolic Pathway in HT-29 cells

To address the transcriptomic profile of genes involved in the cholesterol metabolic pathway in the human colorectal adenocarcinoma cell line HT-29 after being treated with potential probiotic LAB, the mRNA levels were assayed by qPCR. HT-29 cells were treated with 10^7 CFU of *L. plantarum* strain BE7, *L. plantarum* strain BO1, and *L. paracasei* strain BUM6 for 6 h and 24 h, with or without the presence of water-soluble cholesterol. Cells that did not receive any treatment act as negative controls.

7.3.2.1 Evaluation of Relative NPC1L1 Gene Expression Level by HT-29 Cells After Being Treated with Potential Probiotic LAB For 6 h and 24 h With or Without Presence of Water-soluble Cholesterol

Figure 7.2 shows the relative NPC1L1 gene expression level in HT-29 cells co-cultured with the potential probiotic strains, with or without the presence of water-soluble cholesterol (200 µg/mL) for 6 h and 24 h. Both *L. plantarum* strain BO1 and *L. paracasei* strain BUM6 upregulated the NPC1L1 expression in HT-29 cells after 6 h of treatment without the presence of water-soluble cholesterol. On the contrary, the addition of *L. plantarum* strain BE7 to the same treatment condition caused a decrease in the gene expression level when compared with the control cells. The expression of NPC1L1 gene also exhibited a statistically significant decrease in comparison with the untreated cells ($P < 0.05$) after 6h of treatment with all the potential probiotic LAB, in the presence of water-soluble cholesterol.

Compared to 6 h treatment, the presence of potential probiotic strains for 24 h had an inverse effect on NPC1L1 gene expression in almost all treatment conditions, excluding HT-29 cells treated with *L. paracasei* strain BUM6 in the presence of water-soluble cholesterol. A significant upregulation of NPC1L1 mRNA level ($P<0.05$) was observed in the HT-29 cells treated with *L. plantarum* strain BE7 for 24 h in the presence of water-soluble cholesterol.

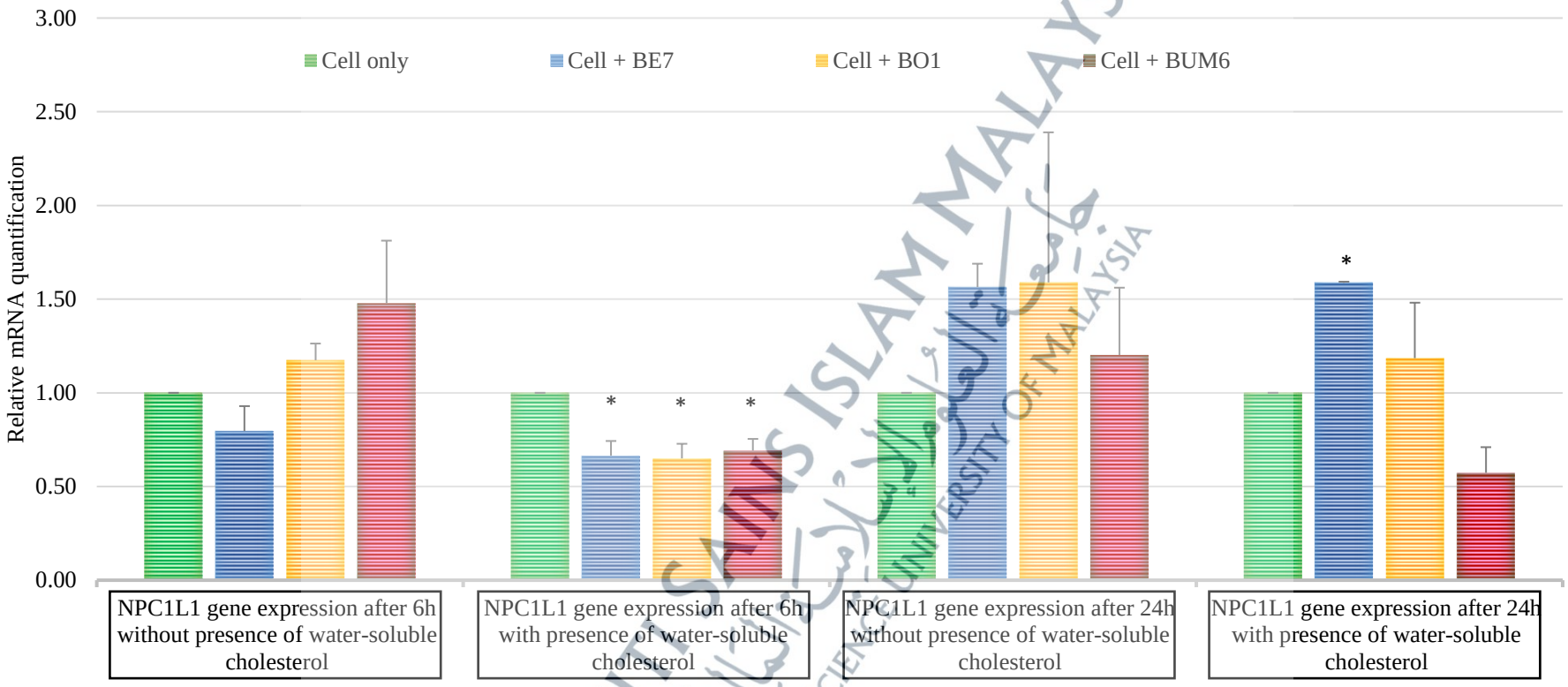

Figure 7.2: Analysis of NPC1L1 Gene Expression Level in HT-29 Cell Co-Cultured with *L. plantarum* Strain BE7, *L. plantarum* Strain BO1 and *L. paracasei* Strain BUM6, With or Without Presence of Water-Soluble Cholesterol (200 µg/mL) for 6 h and 24 h. The Expression of Gene Was Normalised to β-actin (housekeeping gene)

*Significant (P<0.05) in relative to control

7.3.2.2 Evaluation of Relative CD36 gene Expression Level by HT-29 Cells After Being Treated with Potential Probiotic LAB For 6 h and 24 h With or Without Presence of Water-soluble Cholesterol

Figure 7.3 shows the relative CD36 gene expression level in HT-29 cells co-cultured with the potential probiotic strains, with or without the presence of water-soluble cholesterol (200 µg/mL) for 6 h and 24 h. Nearly all treatment cells showed downregulated patterns of gene expression after being treated for 6 h. There was a significant decrease in the CD36 gene expression level in HT-29 cells after being treated for 6 h with *L. plantarum* strain BO1, without the presence of water-soluble cholesterol ($P < 0.05$) in comparison with the untreated control.

A longer treatment period (24 h) resulted in fluctuations in the relative level of CD36 gene expression. The gene was downregulated in cells receiving *L. plantarum* strain BE7 and *L. paracasei* strain BUM6 treatment, without the presence of water-soluble cholesterol. Conversely, incubation with *L. plantarum* strain BO1 in the absence of water-soluble cholesterol and *L. paracasei* strain BUM6 in the presence of water-soluble cholesterol caused an increase of CD36 gene expression level. In fact, a significant upregulation ($P < 0.05$) of CD36 gene expression was observed in the HT-29 cells after being treated with *L. plantarum* strain BE7 and BO1, respectively, with the presence of water-soluble cholesterol for 24 h, compared to the control cells.

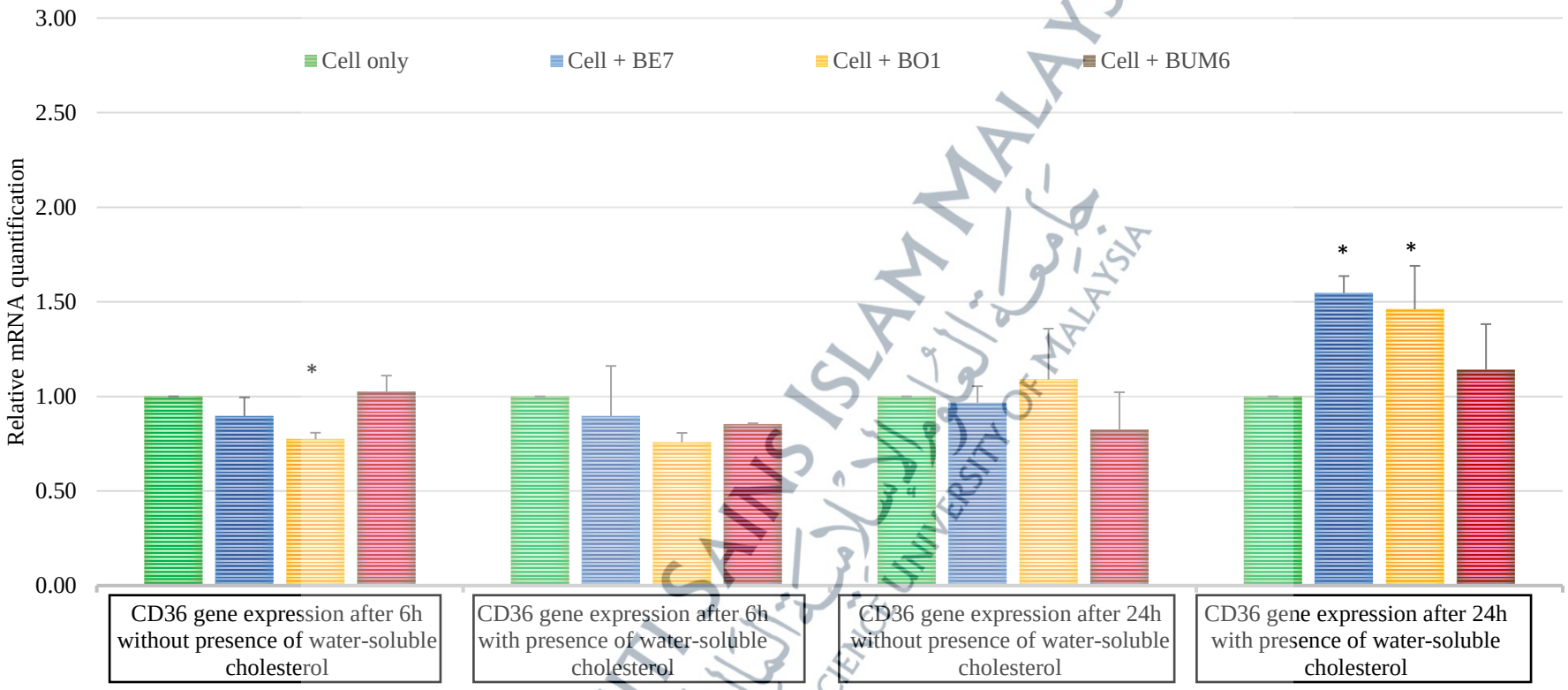

Figure 7.3: Analysis of CD36 Gene Expression Level in HT-29 Cell Co-Cultured with *L. plantarum* Strain BE7, *L. plantarum* Strain BO1 and *L. paracasei* Strain BUM6, With or Without Presence of Water-Soluble Cholesterol (200 µg/mL) for 6 h and 24 h. The Expression of Gene Was Normalised to β-actin (housekeeping gene)

*Significant (P<0.05) in relative to control

7.3.2.3 Evaluation of Relative SCARB1 gene Expression Level by HT-29 Cells After Being Treated with Potential Probiotic LAB For 6 h and 24 h With or Without Presence of Water-soluble Cholesterol

The data on Figure 7.4 illustrate the relative SCARB1 gene expression level in HT-29 cell co-cultured with the potential probiotic strains for 6 h and 24 h, with or without the presence of water-soluble cholesterol (200 µg/mL). Expression of SCARB1 gene in HT-29 cells was significantly upregulated by treatment with *L. paracasei* strain BUM6 for both 6 h and 24 h ($P < 0.05$) in the absence of water-soluble cholesterol. However, treatment with L. *plantarum* strain BE7 and BO1 in similar conditions did not cause a significant influence on the increase of SCARB1 gene expression levels when compared with cells that did not receive any treatment.

The treatment of all potential probiotic LAB also caused variation in the expression level of SCARB1 gene in HT-29 cells in the presence of water-soluble cholesterol. In a shorter treatment period (6 h), the expression of SCARB1 gene exhibited a slight increment in comparison with the untreated cells. In contrast, the reduction of SCARB1 gene expression level was observed when the cells were treated with the potential probiotic LAB for 24 h.

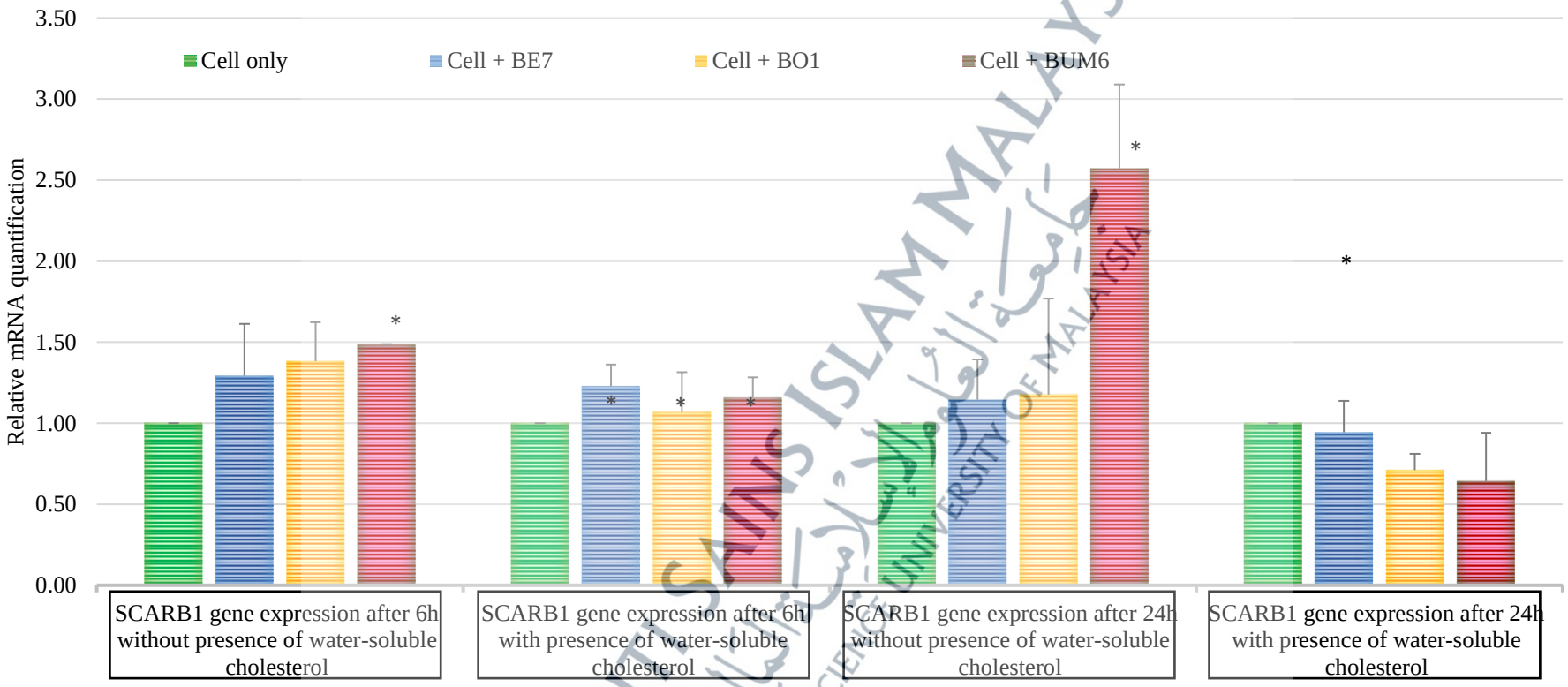

Figure 7.4: Analysis of SCARB1 Gene Expression Level in HT-29 Cell Co-Cultured with *L. plantarum* Strain BE7, *L. plantarum* Strain BO1 and *L. paracasei* Strain BUM6, With or Without Presence of Water-Soluble Cholesterol (200 µg/mL) for 6 h and 24 h. The Expression of Gene Was Normalised to β-actin (housekeeping gene)

*Significant (P<0.05) in relative to control

7.3.2.4 Evaluation of Relative NR1H3 gene Expression Level by HT-29 Cells After Being Treated with Potential Probiotic LAB For 6 h and 24 h With or Without Presence of Water-soluble Cholesterol

The relative NR1H3 gene expression levels in HT-29 cells co-cultured with the potential probiotic strains with or without the presence of water-soluble cholesterol (200 µg/mL) for 6 h and 24 h are shown in Figure 7.5. Treatment with all potential probiotic LAB for 6 hours in the presence of water-soluble cholesterol resulted in downregulated NR1H3 gene expression in HT-29 cells. Nevertheless, similar treatment but with the non-existence of water-soluble cholesterol did not produce overt results on the gene expression in treated cells, in comparison to the control cells.

Results from the qPCR assay also revealed that the NR1H3 gene expression was significantly decreased ($P < 0.05$) in the HT-29 cells treated with all potential probiotic LAB without the presence of water-soluble cholesterol for 24 h in comparison with the control cells. The addition of *L. plantarum* strain BE7 in cell cultures for 24 h, with the presence of water-soluble cholesterol also deeply impacted the level of NR1H3 expression. Nevertheless, the strain significantly upregulated the gene ($P < 0.05$) compared to control cells. The NR1H3 gene expression level was also increased when treated with *L. plantarum* strain BO1 and *L. paracasei* strain BUM6 in the same treatment condition, although the expression was less pronounced compared to treatment with *L. plantarum* strain BE7.

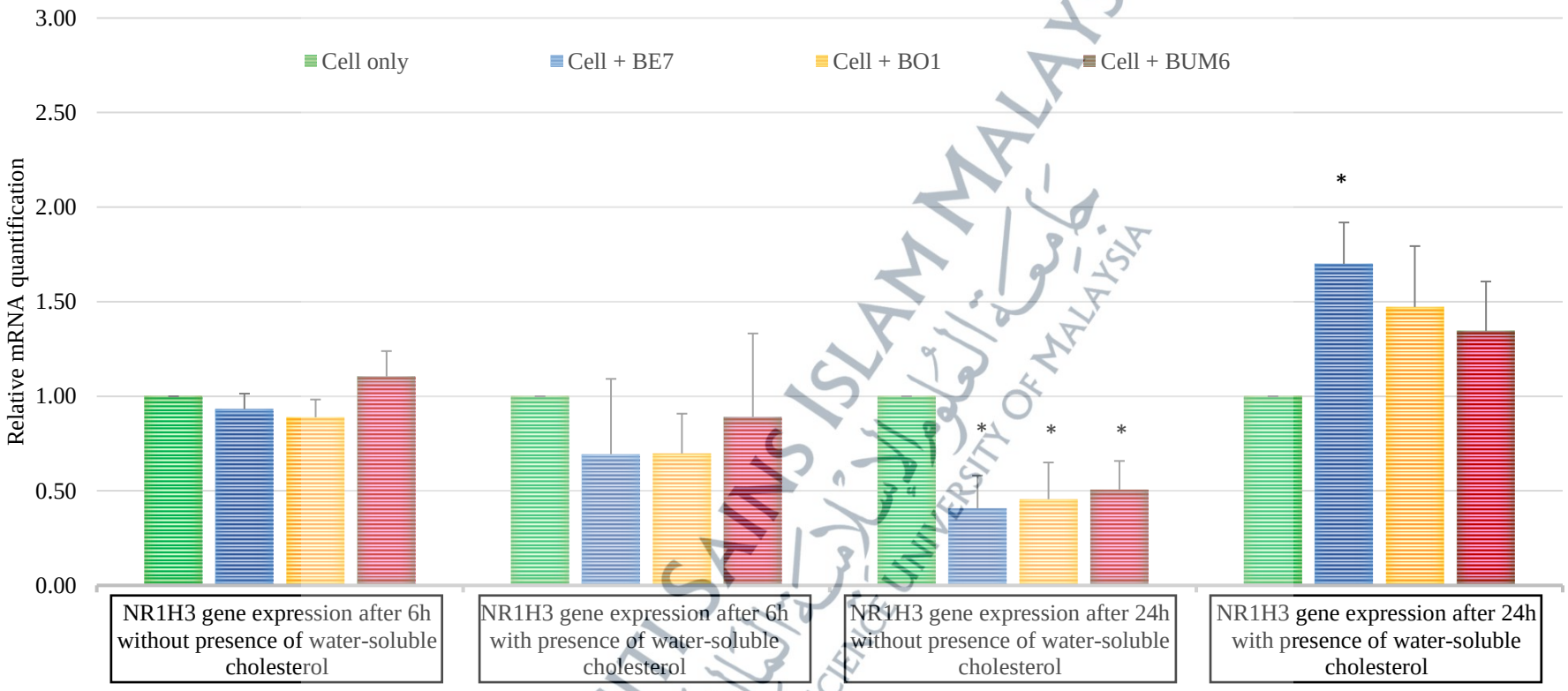

Figure 7.5: Analysis of NR1H3 Gene Expression Level in HT-29 Cell Co-Cultured with *L. plantarum* Strain BE7, *L. plantarum* Strain BO1 and *L. paracasei* Strain BUM6, With or Without Presence of Water-Soluble Cholesterol (200 µg/mL) for 6 h and 24 h. The Expression of Gene Was Normalised to β-actin (housekeeping gene)

*Significant (P<0.05) in relative to control

7.4 Discussion

Intestinal cells play a significant role in cholesterol modulation due to the existence of multiple cholesterol transporters. The presence of bacteria in the intestinal environment, whether the original gut microbiota or the ingested probiotics, also helps with cholesterol management (Badi et al., 2020). A number of studies have reported their findings on the positive effect of the presence of beneficial bacteria on the modulation of intestinal cholesterol in cell line models (Huang and Zheng, 2010; Huang et al., 2013; Lim et al., 2017), with the cholesterol lowering activities being mostly strain-specific. Moreover, Tian et al. (2022) proved that treating hypercholesterolemic rats with *L. plantarum* N-1 induced a significant reduction of total cholesterol and low-density lipoprotein cholesterol levels in serum when compared to the control group. In fact, the decreases were almost similar to those seen in the treatment group receiving simvastatin, signifying the potential of using probiotic treatment as a functional food supplement to treat hypercholesterolemia.

In this regard, this study aimed to determine the effect of supplementation of *L. plantarum* strain BE7, *L. plantarum* strain BO1 and *L. paracasei* strain BUM6 isolated from Malaysian fermented food on the relative gene expression levels of genes involved in modulation of cholesterol, specifically NPC1L1, CD36, SCARB1 and NR1H3 in HT-29 cells. Reverse transcription of mRNA into complimentary DNA (cDNA) and followed by quantitative polymerase chain reaction (qPCR) is the technique used to analyse mRNA expression obtained from countless sources because it is a very sensitive method that can measure the level of gene expression with high accuracy and can detect any small changes in gene expression (Pfaffl, 2001). The human colorectal adenocarcinoma cell line HT-29 is commonly used in research on transport and

metabolism of drugs and nutrients in the intestinal environment as this cell represents many characteristics of enterocytes (Thomson et al., 1997).

The treatments were done for 6 h and 24 h in the absence or presence of water-soluble cholesterol (200 µg/mL). Changes in gene expression in HT-29 cells treated with potential probiotic LAB were evaluated by qPCR using the SYBR method as well as relatively calculated using the Pfaffl method. In this method, relative gene expression is calculated based on the ratio of target gene expression versus reference gene expression, that is β-actin, considering the actual primer efficiencies (Pfaffl, 2001). The reference gene, also known as the housekeeping gene, is used as the control for normalisation of gene expression in qPCR as it is present in all nucleated cells as well as expressed at a stable rate regardless of any treatment condition because it is essential for cell survival (Gorenjak et al., 2014; Lim et al., 2017; Le and Yang, 2019b).

Theoretically, NPC1L1 transmembrane protein might be sensitive to the presence of cholesterol as its structure includes a sterol-sensing domain (Skov et al., 2011). NPC1L1 will act as the cholesterol transporter that will absorb the cholesterol in the intestinal lumen into the enterocyte. This mechanism has caused NPC1L1 to be one of the important targets in managing hypercholesterolemia caused by diet (Badi et al., 2020). In this study, the impact of the presence of water-soluble cholesterol in the treatment medium seems to be suppressed by the presence of potential probiotic LAB as it fails to increase the expression of the gene. In fact, there was a significant reduction ($P<0.05$) in NPC1L1 geneexpression in the group of cells treated with all three potential probiotic LAB for 6 h in the presence of water-soluble cholesterol, when compared to the control cells.

The reduction of NPC1L1 gives the insight that these potential probiotic strains are not only capable of lowering cholesterol by assimilation, but the presence of the

isolates along with the host cells can aid the cholesterol modulation. This theory was supported by Huang et al. (2014), which concluded that the administration of *Lactobacillus acidophilus* ATCC 4356 could protect the apolipoprotein E-knockout (ApoE/) mice against atherosclerosis by inhibiting NPC1L1-mediated absorption. Earlier studies reported that 6 h of treatment with *Lactobacillus acidophilus* ATCC 4356 is capable of reducing the expression of NPC1L1 in Caco-2 cells (Huang and Zheng, 2010). Downregulation of NPC1L1 mRNA expression in Caco-2 cells and hypercholesterolemic rats following the *L. plantarum* Lp27 treatment has also been described by Huang et al. (2013). The consistency of these findings indicates that most Lactobacillaceae may modulate cholesterol transport through regulation of this gene.

The presence of potential probiotic LAB or their metabolites may also function similarly to ezetimibe, which causes the deformation of the sterol-sensing domain of the NPC1L1 protein and the destruction of the stable cholesterol structural cluster, thus blocking cholesterol transport into the cells (Hu et al., 2021). Additionally, although the addition of potential probiotic LAB in cell treatment using cholesterol-free medium has resulted in an upregulation of the NPC1L1 gene expression, it has no impact because there is no supply of cholesterol to be transported into the cells via the NPC1L1 transport protein.

The absence of water-soluble cholesterol in the treatment environment has caused a fluctuation of CD36 gene expression. However, the relative CD36 gene expressions showed similar patterns when HT-29 cells were treated with the potential probiotic strains in the presence of water-soluble cholesterol, that is downregulated following 6 h of treatment but upregulated after treated for 24 h. The decreased CD36 expression not only reduced the cholesterol uptake into the enterocytes but also reduced the cholesterol output into the lymph before it enters the bloodstream (Cifarelli and

Abumrad, 2018). CD36 is thought to be a novel nutrient-sensitive biomarker as dietary fatty acids may alter CD36 expressions in tissues involved in cholesterol regulation (Ulug and Nergiz-Unal, 2021). Given that CD36 expression has an "optimal protective window" in which both persistent up-regulation and CD36 deficiency may increase the risk of atherosclerosis (Zhao et al., 2018), minimal downregulation of relative CD36 gene expression after 6 h of treatment with potential probiotics suggests that probiotics supplementation may provide a positive effect in reducing cholesterol absorption without harming the host environment. It is also demonstrated that the expression of NPC1L1 in the distal intestine of CD36-knockout mice is significantly higher compared to wild type mice, suggesting the involvement of intestinal CD36 in the transportation of cholesterol via the NPC1L1 pathway (Nassir et al., 2007; Zhao et al., 2021).

Although SCARB1 is not the primary site for intestinal cholesterol absorption to occur, this protein does play its role in the uptake of dietary cholesterol (Altmann et al., 2002). SCARB1 could also act as an important regulator in preventing diet-induced weight gain and dyslipidaemia (Lino et al., 2015). This study showed that the SCARB1 gene expression pattern was contradicted by the expression level of NPC1L1 when co-cultured with the potential probiotic LAB. When compared to the control cells, it is found that the treatment of *L. plantarum* strain BE7, *L. plantarum* strain BO1, and *L. paracasei* strain BUM6 for 24 h in the presence of water-soluble cholesterol decreased the expression of SCARB1, whereas vice versa when the relative SCARB1 gene expression was calculated following 6 h treatments. Therefore, the results suggest that the improvement of cholesterol absorption through downregulation of SCARB1 may be time-dependent. However, this finding was inconsistent with a previous study by Lim et al. (2017), who reported a decrease in SCARB1 after 6 h of cholesterol exposure, signifying that cholesterol modulation by probiotics is strain-specific. Interestingly, the

level of SCARB1 transcription after 6 h treatment with the potential probiotics was upregulated, giving the impression that the non-parallel patterns of NPC1L1 and CD36 transcription could be due to the compensatory mechanisms of cellular cholesterol modulation (Michael et al., 2017). The increase of SCARB1 mRNA expression was somehow intriguing, as SCARB1 is recognised as the HDL receptor that could transport HDL cholesterol into the cells, and back into the liver for further decomposition (Liu et al., 2021).

Meanwhile, activation of the NR1H3 gene is driven by an increase in cellular cholesterol levels (Wang and Tontonoz, 2018). This is evidenced by the data obtained via qPCR assay that recorded low NR1H3 gene expression when the given medium did not contain cholesterol, but the expression levels increased significantly in the presence of cholesterol. Following 24 h of treatment with the presence of water-soluble cholesterol, supplementation of *L. paracasei* strain BUM6 caused activation of NR1H3 whilst reducing NPC1L1 gene expression, which may be capable of causing a decline in cholesterol absorption into the cells.

At the same time, though there is an increment in NPC1L1 expression level when the cells are co-cultured for 24 h with *L. plantarum* strain BE7 and *L. plantarum* strain BO1, respectively, the upregulation of the NR1H3 gene may also lead to cholesterol reduction. The improved expression of this gene could possibly activate other cholesterol transport proteins such as ABCG5 and ABCG8, which could excrete the cholesterol in the cells back into the intestinal lumen (Lo Sasso et al., 2010). Indeed, NR1H3 gene activation via synthetic ligands resulted in increased cholesterol efflux and a reduction of cholesterol influx, thus causing the decreasing cholesterol levels in the enterocytes (Lo Sasso et al., 2013). In line with this finding, other studies have previously demonstrated clear evidence of the correlation between NR1H3 activation

and protection of atherosclerosis by reducing intestinal cholesterol absorption through upregulation of ABCG5, ABCG8 and ABCA1, the genes involved in cholesterol efflux (Lo Sasso et al., 2010). Another previous study also showed that the increased expression of NR1H3 was strongly correlated to upregulation of ABCG5 and ABCG8, besides downregulation of NPC1L1 (Gorenjak et al., 2014).

Vice versa, the reduction of NR1H3 mRNA expression in HT-29 cells after a shorter treatment duration (6 h) suggests that the downregulation of NPC1L1 gene expression during the short treatment period was not related to the activation of the NR1H3 gene. The findings from this study contradict those of Hagita et al. (2018), who believe that NPC1L1 and NR1H3 are related as they can regulate and be regulated by one another.

Taken together, the compelling evidence showed that supplementing HT-29 cells with potential probiotics *L. plantarum* strain BE7, *L. plantarum* strain BO1, and *L. paracasei* strain BUM6 could regulate the expression of genes involved in cholesterol modulation. Therefore, the presence of these strains may benefit cholesterol transport across the enterocytes and contribute to alternative hypercholesterolemia treatment.

7.5 Conclusion

Based on the findings of this study, *L. plantarum* strain BE7, *L. plantarum* strain BO1, and *L. paracasei* strain BUM6 have the potential to be considered as potential probiotics that can help in the modulation of cholesterol in the host cell. The mechanisms involve the downregulation of CD36 and NPC1L1 gene expression following short exposure to the potential probiotic LAB for 6 h, as well as an increase of NR1H3 gene expression and a reduction of SCARB1 gene expression after a longer treatment duration, which is 24 h. The ability of these potential probiotics to regulate

the genes may exert a beneficial effect on the reduction of intestinal cholesterol absorption. Further studies should be conducted using animal models to ascertain the efficacy of these LAB strains in the regulation of cholesterol homeostasis in the digestive tract as well as to examine the possible synergism of the potential probiotics with the gut microbiota.