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OPTIMIZED SAMPLE PREPARATION TECHNIQUE FOR VISUALISATION OF THE ADHERENCE OF LACTOBACILLACEAE SP. TO HUMAN COLORECTAL ADENOCARCINOMA CELL LINE HT-29 BY FIELD EMISSION SCANNING ELECTRON MICROSCOPE

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Abstract

The objective of this study was to compare and determine the optimal sample preparation techniques to observe the adherence of probiotic bacteria, Lactobacillaceae sp. to human colorectal adenocarcinoma cell line HT-29. Different fixation time and coating thickness were compared to evaluate the integrity and quality of image observed using field emission scanning electron microscope (FESEM). Based on the qualitative assessments, the one-hour fixation was proposed as a promising fixation period as it is better than overnight fixation in terms of showing clearer parts and the adhesive features on the surface of both bacteria and HT-29 cells. Platinum sputter-coating step is recommended at shorter time to increase conductivity of sample and reduce electron beam damage. Thicker platinum coat could obscure fine structural visualization although it helps in eliminating charging during the imaging process. This finding provides an important sample preparation optimization to enable better structural integrity and quality of image captured using FESEM.

INTRODUCTION

The human gastrointestinal tract comprises numerous bacterial populations known as gut microbiota, for example *Candida, Actinomyces, Rothia* and *Lactobacillus* species [1]. The microbiota is distributed along three main locations, which are in the stomach with a bacterial population of approximately 10^2 colony-forming units (cfu)/mL, the ileum with a bacterial population of approximately 10^2 to 10^3 cfu/mL and the large intestine with a bacterial population of approximately 10^{10} to 10^{12} cfu/mL [2]. Disruption of the microbial niches in the gastrointestinal tract can increase the host's tendency to get several different diseases. Probiotics, including bacteria isolated from food, could impact the host by sharing their genes and metabolites, supporting threatened gut flora, and affecting epithelial and immune cells directly [3]. As probiotics can improve the condition of a disease or prevent its occurrence by restoring the composition of the microbiome in the gastrointestinal tract, modulation of the gut microbiota using probiotic approach has caught the attention of researchers to find a cure for these diseases [4].

The definition of probiotic is "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" [5]. Probiotics are valuable living microorganisms, that have been used for periods due to its various health benefits. The interesting fact about probiotics, for example Lactobacillaceae, is it can compete with

pathogens in the intestinal environments for adhesion sites before they could exert various health effects to the host such as preventing and/or treating intestinal inflammation and lactose maldigestion, as well as regulate the transport of cholesterol [6-7]. The most investigated probiotic groups are lactic acid bacteria (LAB) and Bifidobacteria [4]. Generally, LAB are classified as 'generally recognised as safe' (GRAS) and has been involved in food fermentation for centuries, where this food is considered as nutritious foods with various therapeutic values [6, 8]. LAB are suitable as sources of exogenous probiotics because these microorganisms are also important components of the endogenous microbiota in the human ileum, jejunum, and colon [1-2]. The greatest effect of probiotics can be achieved if the probiotics withstand the gastric acidity and bile salt action and can attach to the cells of the intestinal mucosa. The ability to adhere to the intestinal cells is important because it acts as a prerequisite before microorganisms can colonize the area and express their therapeutic properties. The attachment can be evidenced by the image captured by field emission scanning electron microscope or FESEM, which proved the ability of isolates to remain attached to the cells, respectively, even after undergoing various cell treatments [9]. FESEM has a high resolution from 50 to 100 nm and could image with a magnification up to 30,000×, thus allowing a detailed 3D image analysis and morphological findings [10]. The process of fixation and coating of the sample made before the sample is observed becomes an important parameter to obtain high quality images, further helping in interpreting the characteristics of bacterial adhesion to intestinal cells. This study aimed to optimize the sample preparation technique for visualisation of the adherence of Lactobacillaceae to cell line by FESEM. In this study, human colorectal adenocarcinoma cell line HT-29 was used as the intestinal cell model.

MATERIALS AND METHODS

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). Subculturing was performed when the cells reached approximately 70% confluent. 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.

Preparation of Bacteria

The bacteria were streaked on MRS agar (de Man, Rogosa, Sharpe; Oxoid, UK) containing 0.8% calcium carbonate ($CaCO_3$) and was incubated for 48 hours at 37°C. A single colony was then cultured in MRS broth (de Man, Rogosa, Sharpe; Oxoid, UK) overnight at 37°C.

Field Emission Scanning Electron Microscopy Sample Preparation

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 5000 ×g for 10 min at room temperature and washed with phosphate buffered saline. 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 was 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 24 h and 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 (Jeol, US) at 10 mA for 0 sec (no coating), 10 sec and 60 sec. 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.

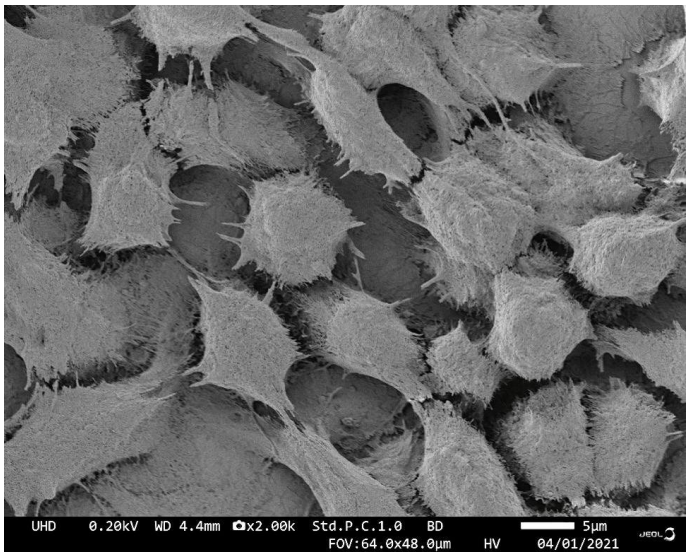
RESULTS AND DISCUSSION

The 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 [11]. The advancement of imaging technique such as scanning electron microscope provides a better way to observe the attachment of bacteria, as better resolution and magnification aid in observation of ultrastructural morphology of the samples [12]. The fixation

agent used was 2.5% glutaraldehyde in 0.1 M phosphate buffer which could efficiently crosslink with proteins and inhibits the activities of enzymes [13]. In the present study, we compared the effect of different fixation time and presence of coating on the micrograph image of bacterial adhesion to HT-29 cells produced by FESEM.

Figure 1 shows the FESEM image when the cells were fixed with 2.5% v/v glutaraldehyde for 24 h and coated with platinum for 60 sec. Overnight fixation is not uncommon as it is generally used to prepare biological samples for observation using electron microscopy [14-15]. The bacterial attachment onto the HT-29 cells could be observed, thus proving that fixation and dehydration process prior to FESEM visualisation does not affect the adhesion ability of the microorganisms. However, the common structure of the cell such as the ciliaric structures which covered the cell surfaces, and the intestinal crypts can't be seen clearly. The observations were contradicted with the ability of FESEM which could produce realistic view of the cell's three-dimensional structure, the exact shaping and the arrangement of cells [16]. Although free from charging effects, the images also lack data in terms of cell integrity and any possible damage following bacterial attachment. This condition may be due to the fixation process for 24 h was too long, as well as the platinum coating being too thick. Similarly, the CCD-18Co cells were found completely degraded with a smooth cell surface and damaged cilia structures when the cells were fixed at longer duration [13].

(a)

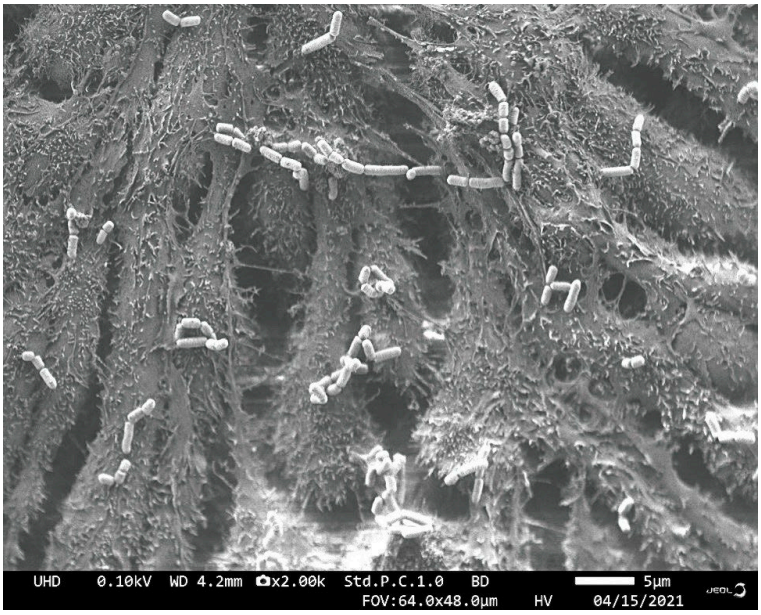


(b)

Figure 1. FESEM image of HT-29 cells after 24 h fixation with 2.5% v/v glutaraldehyde in 0.1 M PBS and coated with platinum for 60 sec. (a) The common structure of the cell such as the microvilli and tight junction can't be seen clearly. (b) Bacteria (BE 7) attached successfully onto the surface of HT-29 cells.

FESEM images become clearer when cells were fixed for 1 h only and not coated with platinum (Figure 2). However, the overall micrograph itself were not clean due to increased charging effect. The observation contradicts the study by Al Shehadat et al. [17] who claim that the sputter-coating step is not suggested because the image quality does not increase significantly. Biological samples usually require a metal coating to produce an electrically conductive surface for FESEM imaging. Coating the surface of nonconductive specimen with conductive material was done to prevent the accumulation of electrostatic charge that may cause scanning faults. Ultra-thin metal coating of the specimen provides higher resolution and improves image detail and contrast when using FESEM [18]. Nevertheless, fine ultrastructural details could be obscured if the thickness of the coating layer deposited is more than 20 nm [19]. Alternatively, charging effect can also be avoided by using ionic liquid staining [19].

(a)



(b)

Figure 2. FESEM image of HT-29 cells after 1 h fixation with 2.5% v/v glutaraldehyde in 0.1 M PBS and did not coat with platinum. (a) The common structure of the cell such as the microvilli and tight junction becomes clearer. The absence of metal coating produces background charging effect (in circle). (b) Higher magnification produces background charging effect (in circle).

The bacterial adhesion pattern on HT-29 cells could be observed clearly and best when the cells were fixed for 1 h and coat with platinum in shorter duration which in only 10 sec (Figure 3). The micrographs showed the spatial arrangement of bacteria on the exterior of HT-29 cells and good inter-cells interactions between bacteria and the intestinal cells. Bacteria cells exhibited good attachment and did not cause any damage to the exact structure of the epithelial cells. The findings agree with Joubert et al. [20] in which fixation of their *Aspergillus fumigatus* samples for 24 h caused damage to the ultrastructure, in contrast to fixation for only 1 h which resulted in better quality micrographs such as the structure of hyphae and extracellular matrix fibers. In the adhesion process, the characteristics of molecules found on the surface of bacterial cells play a very important role, for example the hydrophobicity features and surface charges [15]. The HT-29 cells also displayed a typical apical brush border or the microvilli, and a protruding tight junction between cells [21]. Mucous secretion can as well be observed in some of the images.

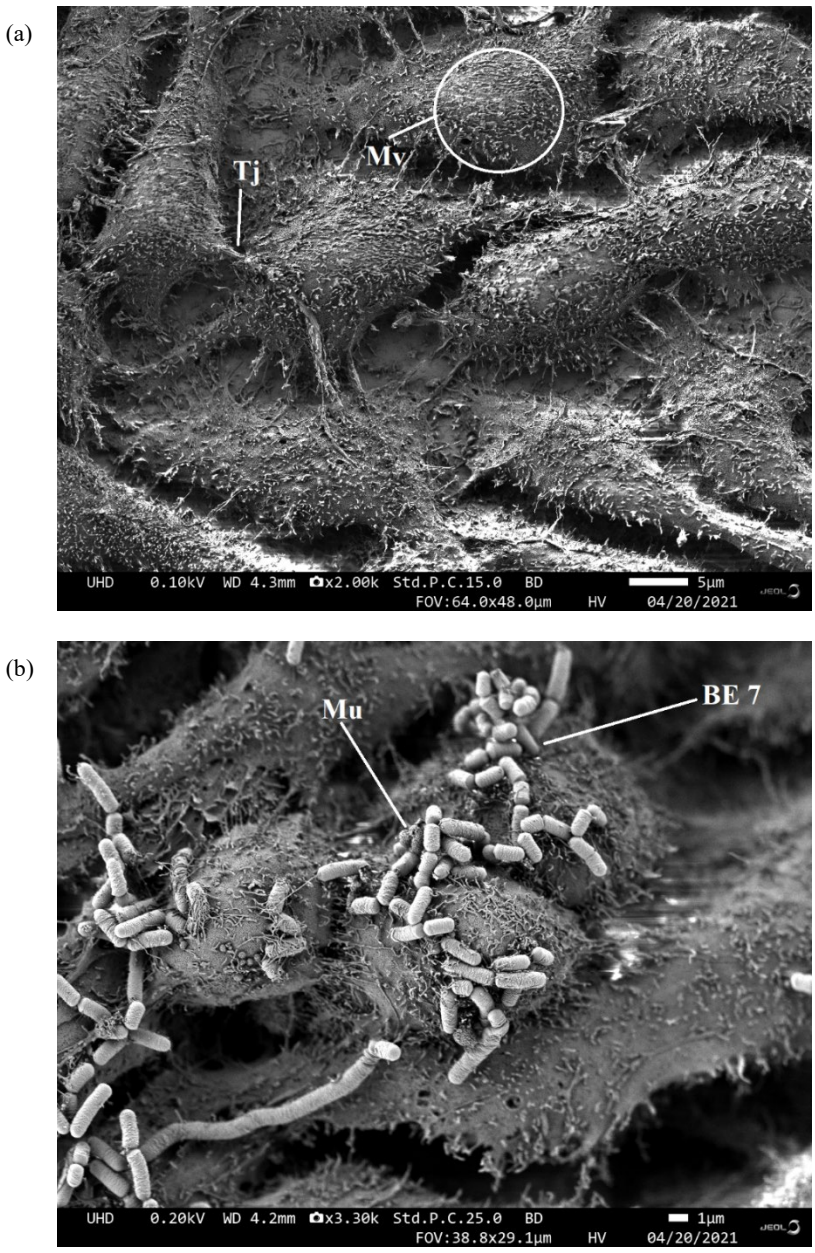


Figure 3. FESEM image of HT-29 cells after 1 h fixation with 2.5% v/v glutaraldehyde in 0.1 M PBS and coated with platinum for 10 sec. (a) The ultrastructural morphology of the intestinal cells such as mucous (Mu), microvilli (Mv) and tight junction (Tj) could be observed clearly. (b) The spatial arrangement and attachment of bacteria (BE 7) on HT-29 cells could be observed clearly.

CONCLUSION

Different fixation time and presence of platinum coatings shows remarkable bacterial adhesion ability on HT-29 cells when observed qualitatively using FESEM. However, the one-hour fixation was better than the overnight fixation in terms of showing clearer parts and the adhesive features on the surface of both bacteria and HT-29 cells. A thicker platinum coat could obscure the visualization of fine structures although it helps in eliminating charging during the imaging process. Hence, the platinum sputter-coating step is recommended at lower current charge and shorter time to increase conductivity of sample and reduce electron beam damage. This finding provides an important optimization of sample preparation to enable better structural integrity and quality of image captured using FESEM.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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