Caco-2 cells monolayer as an in-vitro model for probiotic strain translocation

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ABSTRACT

Background: Caco-2 cells monolayer is one of in vitro models to evaluate the translocation capacity of Lactobacillus spp probiotic strains. The translocation is influenced by mucosa permeability of enterocytes as shown by increasing transepithelial resistance (TER) and formation of tight junction proteins. The pore size of the supported permeable membrane used in in vitro assay was one of the crucial factors in performing bacterial translocation assay. Almost no study has been conducted using Caco-2 cells monolayer grown on 8-μm pore size polycarbonate membrane for evaluating probiotics translocation. Therefore this study aimed to determine whether the Caco-2 cells monolayer model was suitable as an in vitro translocation model.

Methods: Caco-2 cells monolayer was seeded onto 8-μm collagen-coated polycarbonate membrane insert Transwell®. Differentiation of Caco-2 cells was detected by measuring the TER, while the ZO-1 protein (the tight junction proteins) was detected by immunofluorescence. H2O2 was used as a tight junction disruptive agent. Data were analyzed using SPSS version 23 software to compare the mean of TER measurement between untreated and H2O2-treated Caco-2 cells monolayer.

Results: The result showed that the TER of Caco-2 cells monolayer was gradually increasing until day 14, reaching more than 800 Ω.cm². Furthermore, the ZO-1 protein was successfully detected, indicated the tight junction formation. TER value of H2O2-treated cells showed significantly lower than that of untreated cells (P<0.05), indicating a disturbance of cells monolayer integrity. Lactobacillus rhamnosus FB881 was used for validating the translocation. There was no translocation observed; however, translocation was observed in H2O2-treated cells.

Conclusion: Altogether suggests that Caco-2 cells grown on 8 μm-pore size permeable filters could be considered as a suitable in vitro model for probiotics strains translocation.

Keywords: Caco-2 cells, translocation, transepithelial resistance (TER), ZO-1, hydrogen peroxide


INTRODUCTION

Probiotics are live microorganisms when administered with an adequate amount will give a beneficial effect to host. 1 Beside the probiotic’s properties; the candidate strains must be assessed for their functional activities and safety aspect. One of the safety assessments of the probiotic strain is the translocation of the strain into extraluminal. 1, 2 The safety of probiotics strains is necessary to be evaluated since several studies reported the clinical cases associated with probiotic strains, although all cases occurred in immunocompromised hosts. 3, 4 Performing a safety assessment of the strain should be considered before further application of the strain to hosts, especially to human. One of safety assessment assays is the possibility of translocation of the probiotic strain. Translocation could be the initial process of movement of the bacterial strain to the extraluminal and then enter bloodstream causes bacteremia. Many studies have been conducted to detect translocation possibility of the probiotics strains and mostly were in-vivo studies. 5-14 The in-vivo study could be used or evaluating translocation; however, many confounding factors have to be considered causing difficulties in interpreting the results. Furthermore, it is also related to the ethical issue of animal involvement. In vitro study is one of the methods that can be used for detection of the translocation capacity of the probiotic strains since it is simpler than in vivo study. Nevertheless, there is a lack of study has been performed to evaluate the translocation of probiotics strain that use in vitro study. Caco-2 cell monolayer is one of in vitro models that could be used in assessing the translocation of probiotic strains, including Lactobacillus since this cell can differentiate into enterocyte-like cells, therefore, can be used for performing permeability studies. Most of the previous studies reported that the Caco-2 cell monolayer system could be used as an in vitro model for drug absorption study, Shiga toxin translocation and botulinum toxin translocation, as well as validating bacterial-enterocytes interactions and bacterial translocation of Cronobacter sakazakii and Campylobacter. 15-19
Caco-2 cells, the human colon carcinoma cell line, when seeded on permeable supports (insert Transwell® system, for example) will transform into polarized epithelial cells monolayer, showing enterocytes phenotypes. The polycarbonate membrane is coated with collagen type I (rat tail collagen) to prevent detachment of the cells during the differentiation process.\textsuperscript{20} Differentiation of Caco-2 cells has been known to be inter-lab variation.\textsuperscript{21} Review by Lea \textit{et al} showed that the differentiation of the cells takes approximately 14-21 days under standard culture condition.\textsuperscript{20} The impact of insert membrane pore size was found to be important in the assay.\textsuperscript{21} The previous study conducted by Lechanteur and group validated that the choice of insert membrane pore size is critical in permeability assay in drug absorption studies.\textsuperscript{23} Besides, a previous study evaluating translocation of \textit{C. sakazakii} used 3 μm-pores sizes insert Transwell® for detecting the bacteria translocation.\textsuperscript{18} Since \textit{Lactobacillus} spp has a different size (~5 μm length) with that of Gram-negative bacteria, it is speculated that bigger pore size of polycarbonate membrane, the platform of Caco-2 cell monolayer growth, is needed for determining translocation of \textit{Lactobacillus} spp. There is a lack of study performed to evaluate the translocation of lactic acid bacteria using \textit{in vitro} model. Therefore, the objective of this study was to validate Caco-2 cells monolayer seeded on 8 μm-pore sizes insert Transwell® membrane as an \textit{in vitro} model for probiotic strain translocation.

**MATERIAL AND METHODS**

**Caco-2 cells monolayer**

Caco-2 cell was kindly provided by Professor Yukako Fujinaga (Department of Bacteriology, Graduate School of Medical Sciences, Kanazawa University, Japan). Cells, the human colon adenocarcinoma-derived cell line, were maintained and grown in a 5% CO\textsubscript{2}, humidified incubator at 37°C with Dubelco’s Modified Eagles Medium (DMEM) (Fujifilm, Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 20% heat-inactivated fetal bovine serum (FBS) and 1% (v/v) L-glutamine (FBS) (Fujifilm, Wako Pure Chemical Industries, Ltd., Osaka, Japan) [23]. After passage, the cell was seeded onto 8 μm-pore size polycarbonate membrane of insert Transwell® (Corning®, Corning, NY, USA). The bacterium was cultured in de Mann Rogosa Sharpe (MRS) agar plate (Oxoid) at 37°C for 24-48 hours anaerobically. The strain was then cultured in MRS broth (Oxoid) at 37°C for overnight, anaerobically. It was used for further experiment.

**Bacterial strain**

\textit{Lactobacillus rhamnosus} FBB81, a lactic acid bacteria strain isolated from the human that showed probiotic properties, was used for translocation validation of the \textit{in vitro} translocation assay system. The bacterium was cultured in de Mann Rogosa Sharpe (MRS) agar plate (Oxoid) at 37°C for 24-48 hours anaerobically. The strain was then cultured in MRS broth (Oxoid) at 37°C for overnight, anaerobically. It was used for further experiment.

**In vitro cell model for probiotic strain translocation**

**In vitro** cell model for translocation used 24-well 8 μm-pore size Transwell® polycarbonate membrane inserts with area 0.33 cm\textsuperscript{2} (Corning® Inc., Corning, NY, USA). The membrane was coated with rat-tail collagen type I (Corning® Inc., Corning, NY, USA) with final concentration 50 μg/ml. After 1 h incubation, the membrane was washed with sterile phosphate buffer saline. Caco-2 cells (4 × 10\textsuperscript{4} cells/ml) in DMEM supplemented with 20% heat-inactivated fetal bovine serum (FBS) and 1% (v/v) L-glutamine were applied onto apical (upper) chamber of double chamber model (Figure 1). The cells were maintained at 37°C, 5% CO\textsubscript{2}. The cell medium was changed every two days.

Transepithelial resistance (TER) measurement

In order to determine the differentiation of Caco-2 cells, the transepithelial resistant (TER) was measured by using Millicell ERS2 voltohmeter (Merck, Millipore). All plates containing Caco-2 cells were equilibrated to room temperature before TER measurement. The voltohmeter was checked and equilibrated using cell medium based on the manufacturer’s instruction (Merck Millipore). Control untreated- and H\textsubscript{2}O\textsubscript{2}-treated cells were measured for their TER day by day. All the TER measurements were conducted in triplicates.

**Immunofluorescence microscopy of ZO-1 protein**

The confluence of the monolayers was observed with confocal microscopy after 14 days of passage. Differentiation of Caco-2 cells was also shown as the formation of the tight junction. One of the tight junction proteins is ZO-1 protein. The existence of this protein was detected using immunofluorescence. Caco-2 cells, 4 × 10\textsuperscript{4} cells/ml (0.6 cm\textsuperscript{2} /well) were seeded onto collagen type I-coated flexiPERM\textsuperscript{a} (SARSTEDT AG & Co.KG, Numbrecht, Germany) chamber attached to cover glass slides (Matsunami Glass). The cells medium was changed every two days. Fourteen-day post confluence Caco-2 cells (untreated and 10 mM H\textsubscript{2}O\textsubscript{2}-treated) were fixed with 1% of paraformaldehyde in PBS for 15 minutes and then washed three times with PBS-Tween (PBS-T). The cells were blocked with 1 drop each well of Blocking One Histo (Nacalai Tesque, Kyoto) and incubated for 15 minutes at room temperature. The plate was then washed three times with PBS-Tween. Specific primary antibody, ZO-1 anti-rabbit
monoclonal antibody (rabbit monoclonal antibody, 1:400, cat no. ab96594, Abcam) in Blocking One (Nacalai Tesque, Kyoto) and PBS-Tween was added into well, and the plate was incubated at 4°C, overnight. After PBS-T washing for five times, secondary antibody consisted of the Alexa Fluor™ 488 (goat anti-rabbit IgG (Invitrogen, Carlsbad, CA) with Hoechst 33342 (hydrochloride) (Cayman Chemical) in blocking buffer were added into wells, and the plate was incubated at room temperature, dark place for 1 hour. The plate was then rinsed with PBS-Tween for five-times and the fixation was done by applying 1-2 drops Fluoromount/Plus (Diagnostic Biosystems, Pleasanton, Calif., USA). The experiments were performed in triplicates. The fluorescence was visualized using a fluorescence microscope, Biozero (Keyence, Osaka, Japan). Images (60x) were representative of more than 10 images taken for each condition in more than three experiments.

**Bacterial translocation**

Validation of translocation assay system was evaluated using untreated- (control) and H2O2-treated Caco-2 cell monolayer. Since H2O2 is an oxidative agent, therefore it was mimicking inflammatory bowel diseases (IBD) in nature. DMEM containing 20% heat-inactivated FBS of the Caco-2 cells media was replaced with DMEM without FBS before the validation of translocation assay. For the H2O2-treated system, H2O2 (10 mM) was added into the basolateral chamber and incubated at 37°C 5% CO2 for 2-3 hours. About 106 CFU/ml of L. rhamnosus FBB81 (local probiotic strain) was applied onto the apical chamber and incubated for 1 hour. Fifty microliters of the basolateral chamber were streaked on MRS agar and incubated at 37°C anaerobically for 24-48 hours. Any visible colony grown on MRS agar was indicated as translocation. The colonies were then confirmed as lactic acid bacteria (L. rhamnosus FBB81) by Gram stain, microscopic observation of the cell form and shape following the catalase test. All experiments were performed in triplicates.

**Statistical analysis**

Data were reported as the mean SEM. Also, unpaired t-test analysis using SPSS version 23 software was used to compare the mean of TER measurement between untreated and H2O2-treated Caco-2 cells monolayer. A p-value of < 0.05 was considered statistically significant.

**RESULTS**

**Trans epithelial Resistance (TER) and ZO-1 protein detection of Caco-2 cell monolayer**

Most of the studies used animal models for evaluating the strain translocation; however, it faced many confounding factors and ethical issue. Specific *in vitro* system for detecting probiotic strain translocation has not been widely investigated. Caco-2 cell is a carcinoma colon cell that is established used for in vitro permeability assay. These cells can differentiate into mature enterocytes cells, therefore, mimicking human gut mucosa. To validate the system used for probiotic strain translocation *in vitro*, 8-µm pore size polycarbonate membrane of insert Transwell® used for this study.

The formation of tight junction determined differentiated Caco-2 cell showed as the increasing of transepithelial resistance (TER) and detection of tight junction protein, ZO-1. Measurement of TER is commonly used for evaluating epithelial barrier function, including the uniformity of the cell monolayer on the insert membrane and the integrity of tight junctions [20]. The TER can be measured using portable voltohmometer (Militcel-ERS Voltmeter, Merck, Millipore or EVOM2, Epithelial Voltohmeter, World Precision Instruments Inc, Sarasota, FL). Hence it is convenient and straightforward. In this study, the TER value of the Caco-2 cell was found to be stable to increase after day 6 of the passage. The TER reached higher value (more than 500 ohm.cm²) from day 10 and increasing until day 14 that reached 844.4 ± 58.8 ohm.cm² (Figure 2).

The TER of Caco-2 cell monolayer that was treated with 10 mM H2O2 was significantly lower than that of untreated (before treatment was 692.9 ± 36.89, while after treatment was 207.5 ± 10.37; p = 0.000). The ZO-1 protein immunofluorescence supported these results. Since the ZO-1 protein is one of the tight junction proteins, the changes of this protein structure will alter the integrity of cells monolayer. The cells were grown until confluence (14 days) flexiPERM® and stained with primary antibody of anti-rabbit ZO-1 protein. The ZO-1 protein of control untreated cells showed smooth and regular edge (Figure 3a); on the other hand, that of H2O2-treated cells showed dramatic displacement (zig-zag pattern) as compared with the untreated cells (Figure 3b).

**Validation of translocation assay system**

To validate the translocation assay system, L. rhamnosus FBB81, local probiotic strain human origin, was used as a bacterial strain. The bacterial cells were applied onto the apical chamber, and then translocation was evaluated by culturing medium of the basolateral chamber on MRS agar. The result showed that no visible colony was observed from basolateral cell culture media of untreated Caco-2 cell monolayer, indicated that no translocation of probiotic strain through intact cell monolayer. In contrast, there were many visible typical colonies
were cultured from that of H2O2-treated cells, which was Gram-positive long-rod bacteria with a negative catalase test result that indicated the strain used for this study, suggesting that the system appropriately worked (Figure 4).

DISCUSSION

This study was performed to validate the in vitro system contained Caco-2 cell monolayer grown on 8-μm permeable filter membrane that used for translocation assay. Translocation is one of the safety assessments that must be evaluated for probiotic strains. In vitro system of translocation needs model that can mimic the natural condition of the gut mucosa. Caco-2 cell is a human colorectal carcinoma cell widely used as a model of gut mucosa since the cells can differentiate into enterocytes under specific circumstances. The differentiation of Caco-2 cell can be detected by increased TER and detection of ZO-1 protein, which is important for evaluating bacterial strain translocation. TER can be used to determine cell monolayer integrity since it is a good marker of tight junction formation. Differentiation of Caco-2 cell monolayer is inter-lab variable; therefore, to use this system for translocation assay needs further validation. Previous studies reported variation of time required for cell differentiation. Delie et al. reported that confluency of Caco-2 cells monolayer was reached after 5 days of initial seeding. Vachon and colleague found that there were three stages of Caco-2 cells in culture. Up to 20 days after confluency, the cells were heterogeneously polarized and differentiated. Furthermore, the fully differentiated cells were shown in > 30 days of confluency. The pore size of the membrane also influenced the confluence and polarization of Caco-2 cells grown on the filter membrane. Lechanteur et al. found that bigger of membrane pore size stabilized the TER sooner. When they used 1 μm–a pore size of filter membrane; the TER values were stagnant after days 12. In our study used 8-μm collagen-coated polycarbonate membrane support, showed that Caco-2 cell differentiation was started at day 6 as shown as increasing of TER. The TER gradually increased until day 14; furthermore, the result was in concordance with immunofluorescence staining of ZO-1 protein, the tight junction protein. The system was then needed to further evaluated especially if there is any disruption agent could break the tight junction protein. Addition of H2O2, an oxidative agent, that usually found in inflammatory bowel diseases (IBD) mimicking the original condition was found to be successfully disrupted the TJ protein as shown as dramatically decreased the TER of the cell, and strongly supported with ZO-1 protein staining that showed displacement of the protein. The previous study showed that the addition of H2O2 could reduce the integrity of tight junction of cells monolayer that was demonstrated as reducing of TER and displacement of ZO-1 protein.
8 μm-pore size polycarbonate membrane insert Transwell®. Bacteria, which are probiotic strains, must be assessed for their safety including their translocation capacity.\(^2,^3,^28\) Previous study used 3-μm pore size for the system, and found that no translocation of *Escherichia coli* into the basolateral chamber through intact Caco-2 cell monolayer.\(^29\) Another study for other bacteria translocation also used Caco-2 cells and successfully used as *in vitro* model for translocation assay. However, it also used a 3-μm pore size.\(^18\) Since the size of lactobacilli (probiotic strains) are much bigger than gram-negative bacteria, it is thought to use bigger pore size of polycarbonate membrane therefore based on the bacterial size, 8-μm pore size was chosen for the model in this study. As shown that no translocation of the experiment bacterial strain (*L. rhamnosus* FBB81) was observed through intact Caco-2 cell monolayer, while there was translocation from the disrupted-cell monolayer. It suggested that the system could support the *in vitro* translocation assay for other *Lactobacilli* spp. probiotics strain.

**CONCLUSION**

This study showed that 14-day post confluence Caco-2 cells grown on 8-μm pore size polycarbonate membrane coated with collagen type I could be used as *in vitro* models for evaluating translocation activity of probiotics strains, therefore it is a promising model for probiotics safety assessment assay *in vitro*, although the *in vivo* study is also needed for supporting the translocation event.

**CONFLICT OF INTEREST**

There is no competing interest regarding the manuscript.

**ETHICS CONSIDERATION**

Ethics approval has been obtained prior to the study being conducted by the Ethics Committee of Faculty of Medicine, Universitas Udayana, Bali, Indonesia

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**AUTHOR CONTRIBUTION**

All of the authors are equally contributed to the study from the conceptual framework, data gathering, data analysis, until reporting the results of the study.
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