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# Evaluation of cells integrity using different fixation time by scanning electron microscopy

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## Abstract

The study aims to evaluate the integrity and structural changes of two intestinal cells adhered with *Lactobacillus* sp. in different fixation time. HT-29 and CCD-18Co intestinal cells with *Lactobacillus* sp. were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer for two, four, six and 12 hours at room temperature. Our study revealed that the different fixation time affects and change the integrity, viability and durability of cells. HT-29 cells structure remain intact even after 12 hours fixation while CCD-18Co cells remain intact at two and four hour fixation time whereas at six hour cells also remain intact except cilia structure not clearly seen on surface of the cell. However, at 12 hours fixation, CCD-18Co cells were completely broken and degraded. *Lactobacillus* sp. cells remains stable in both cell lines, showing that the bacteria cells were unaffected with various fixation time. The present study suggested fixation time is important as an aware different cell has different endurance and structural integrity.

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## Introduction

Ability of the bacteria to adhere and attach to cells as a host at epithelial surface is a complex process of non-specific and specific interactions (Ann & Friedman, 2000). According to Chang *et al.* (2004), adhesion is important because it is one of the strategies used by bacteria to maintain stable at gastrointestinal surroundings by competing for nutrients with other bacteria. Electron microscopy study of cells and tissue is one of the most cost-intensive approaches in biomedical research in order to interpret biological structures accurately (Eddie *et al.,* 2010). Scanning electron microscopy (SEM) is use to investigate the pattern of adhesion, changes in shape and membrane integrity of cells. Mahta and Michelle (2010) used SEM to determine adhesion ability of *Lactobacillus* strain with *Propionibacterium jensenii* 702 in co-culture system on human colon adenocarcinoma cell line Caco-2. SEM micrographs revealed that *Lactobacillus* contributed an antagonistic action on growth of *P. jensenii* 702. The study of Gomide Jr. *et al.* (2004) in the diagnosis of intestinal mucosa integrity introduced SEM as a routine method and technique acceptable to characterize and quantify the damages in the intestinal epithelium of broilers. Furthermore, the utilization of SEM showed qualitatively and quantitatively the loss of intestinal epithelium in broilers after exposed to prolonged feed deprivation and post hatching water.

To date, fixation is a critical step to interpret properly the ultra structure of cells and keep the cellular components cells as 'lifelike'. The purpose of fixation is to fix molecules and structures of organelles thus to stop metabolism and to preserve substructures of the cells by crosslinking and arresting many molecules in the cells (Eddie *et al.,* 2010). In the fixation process, the fixative agent has to penetrate by diffusion to the centre of the specimen and then adequate time has to be allowed for the reactions of fixation to take place. Both diffusion time and reaction time depend on the type, origin and thickness of the specimen and type of fixative agent as well. Besides that, important rudiments in fixation process need to be considered are temperature of incubation, pH and buffers during their application.

Fluids involved in the fixation process also should be isotonic to the cells as physiological as possible because cell is made up from phospholipids membranes that are easily damaged by extremely hypotonic or hypertonic solutions (Nedela *et al.,* 2012).

Fixation step at different incubation time will cause slight damages to internal structures of samples and might give misinterpretation results. On the other hand, the choice of the right fixation method is crucial to produce the best quality of electron micrograph for the right interpretation of results. Hence, the aim of this study is to evaluate the integrity and structural changes of two intestinal cells; HT-29 and CCD-18Co cells adhered with *Lactobacillus* sp. after exposure to various fixation time.

## Materials and methods

*Bacteria growth condition*

*Lactobacillus* sp. (GenBank accession number KT591874) were grown at 37°C for 48 hr in MRS agar (deMan, Rogosa and Sharpe) (Oxoid, Australia) before being transferred to the MRS broth (Oxoid, Australia) for overnight incubation at 37°C.

*Intestinal cell cultures*

Human adenocarcinoma cell lines HT-29 (ATCC® HTB38™) and human colonic fibroblast CCD-18Co (ATCC® CRL-1459™) were cultured in McCoy's 5A Medium (Sigma, USA) and Eagle's Minimum Essential Medium (Sigma, USA), respectively. Both media were supplemented with 10 % (v/v) fetal bovine serum (FBS; Sigma, USA) and 100 µg $ml^{-1}$ of penicillin–streptomycin (Sigma, USA). Both cells were cultured in Nunc™ tissue culture flasks (Thermo Fisher Scientific, USA) at 37°C in a 5 % $CO_2$ using a humidified incubator (Binder, USA). The cell culture medium was replaced with fresh medium every alternate day.

*Specimen preparation*

Coverslips (13 mm) (Sarstedt, USA) were placed at the bottom of the 24-well tissue culture plates (Thermo Fisher Scientific, USA). Both the HT-29 and CCD-18Co cells (about $2 \times 10^5$ cells $ml^{-1}$) were seeded and incubated at 37°C in 5 % $CO_2$. The cell monolayers were washed twice with PBS (pH 7.2) before a 900 µl antibiotic-free medium was added.

Overnight cultures of *Lactobacillus* strains in MRS broth were centrifuged for 10 min at 1008 × *g*, and the pellets were re-suspended in an antibiotic-free medium to a final concentration of $10^8$ CFU $ml^{-1}$. The wells containing monolayer cells and 100 µl bacteria suspensions were incubated for 4 hr at 37°C in a humidified incubator supplemented with 5% $CO_2$. After incubation, the HT-29 and CCD-18Co cell monolayers were washed three times with 0.1 M phosphate buffer (pH 7.2) to release any unbound bacteria. The cells were fixed with 2.5% (v/v) glutaraldehyde and 4% (v/v) formaldehyde (Sigma Aldrich, USA) in 0.1 M phosphate buffer for two, four, six and 12 hr at room temperature. Then, the cells were dehydrated in a graded ethanol series (50% v/v, 70% v/v, 80% v/v, 90% v/v, and 95% v/v) for 15 min each session, followed by the dehydration step (twice) in 100% ethanol for 30 min. The cover slips containing the cells were air dried at room temperature for 30 min, mounted on stubs, and coated with gold for 15 s. The specimens were then examined through SEM (Live Stereoscopic VPSEM; Hitachi, Japan).

**Results and discussion**

Scanning electron microscopy technique was chosen to evaluate the adherence ability of *Lactobacillus* sp. on HT-29 and CCD-18Co cells as it provides a large depth of focus which allows a broad area of the cells structure to be examined (Samarayanake, 1996) and SEM also allow rapid and easy preparation. In the present study, the ultra structural results revealed the optimal time for fixation vary between cellular materials (Table 1).

**Table 1.** Optimum fixation time of HT-29 cells, CCD-18Co cells and *Lactobacillus* sp.

| Type of cells | Optimum time (hour) |
|---|---|
| HT-29 | 2 – 12 |
| CCD-18Co | 2 |
| *Lactobacillus* sp. | Remain stable at any variable time |

Besides different fixation times were used in this study, HT-29 cells were also exposed to different fixation buffer; 2.5% glutaraldehyde and 4% formaldehyde in 0.1 M phosphate buffer, respectively to see the difference effect on both intestinal cells. Glutaraldehyde and formaldehyde are non-coagulants fixing agents which are useful for ultrastructural studies. However, there is no difference on the cell structures between both buffers (Fig. 1.).

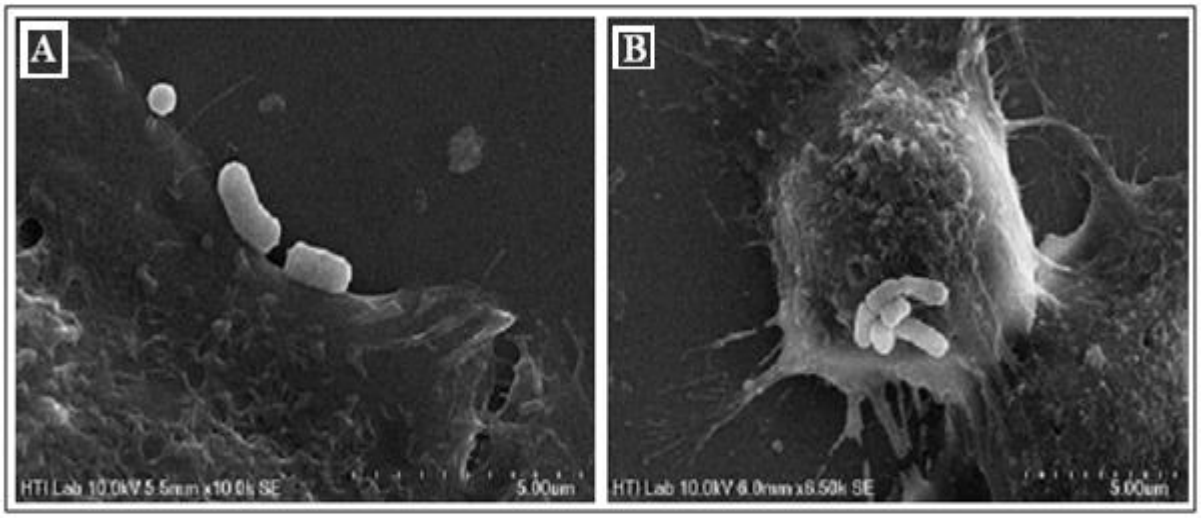


**Fig. 1.** SEM micrographs of HT-29 cells after fixed 12 hr with (A) 4% formaldehyde in 0.1 M phosphate buffer and (B) 2.5% glutaraldehyde in 0.1 M phosphate buffer. Original magnification X10 000 (bar=5 µm).

Thereby, we used 2.5% glutaraldehyde in 0.1 M phosphate buffer as a fixing agent because glutaraldehyde is a much more efficient cross-linker for proteins and also inhibits enzyme activity more than formaldehyde (Eltoum *et al.,* 2001). Additionally, phosphate buffer was used because it is safer and almost similar to cytoplasmic environments of most biological samples (Hayat, 2000).

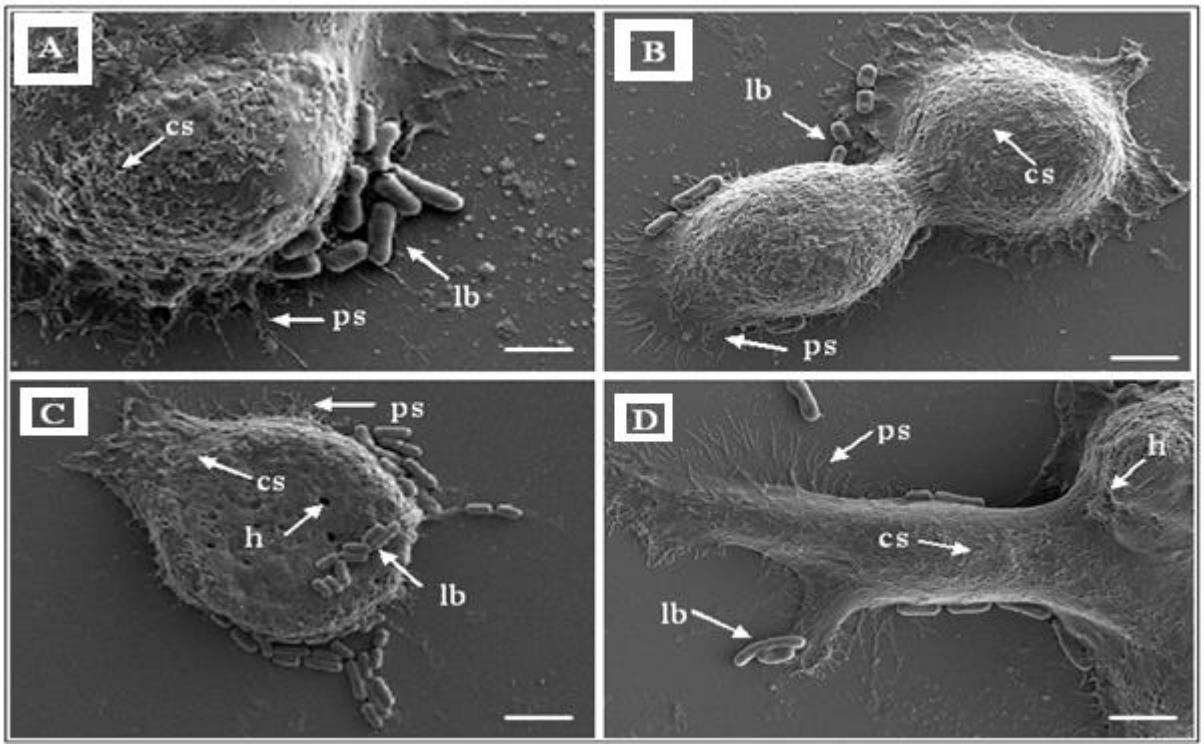


**Fig. 2.** SEM micrographs of HT-29 cells after incubation in different fixation time. (A) 2 hr, (B) 4 hr, (C) 6 hr, and (D) 12 hr. Arrows indicate the attachment of *Lactobacillus* sp. on the surface and side of the cell (lb), holes (h) and the ciliaric structures on the surface of the cell (cs) and protruding structures from the side of cell (ps). Original magnification X10 000 (bar=5 µm).

In this study, electron micrograph revealed undoubtedly the attachment of *Lactobacillus* sp. onto intestinal cells and they were remains stable on both cells (Fig. 1., Fig. 2., and Fig. 3.), showing that the bacteria cells were unaffected with various fixation time. Furthermore, Fig. 2. shows HT-29 cells remained intact from two until 12 hr of fixation time. Ciliaric projections can be clearly seen protruding from the side of cell while abundant of ciliaric structures cover the surface of cell. Holes also known as intestinal crypts which function to absorb water can be seen obviously on the surface of cells. Contradictory with SEM images obtained from CCD-18Co cells where this cell was intact at two and four hr fixation time whereas at six hr cells became flattened while the cilia structure was not clearly seen on the surface of cell (Fig. 3).

Furthermore, at 12 hr fixation, the structures of CCD-18Co cells were completely broken and degraded. The cell shrinked, epithelium was lost and the surface of the cell became smooth and collapses while all cilia structures were totally damaged. This observation is in good agreement with Bozzola and Russel (1999) that stated, incubation of the fixed samples for very long periods should be avoided as degradation of the samples may occur.

The different in the integrity of cells might be influenced by the diverse size and shape of cells. Wurzinger-mayer *et al.* (2014) reported the thickness and dimension of the sample determines the fixation time because it influenced the penetration of fixatives in every specimens.

In our study, structure and shape of *Lactobacillus* sp. remained steady and ideal because we believed using fresh microbial cultures and avoid death phase of their growth curve or overgrown cultures would give better results. On the other hand, the specimens should not leave in a refrigerator or at room temperature for long period of times because this would produce unsatisfactory results in the SEM such as autolysis, drying effects and may damage the ultra structural and integrity of the tissues (Allen, 1983).

We have shown that both intestinal cells have different optimum fixation time and obviously, fixation time is very important to obtain accurate and valid results. Therefore, this study revealed that different cell has different fixation time and this finding suggested different cell has different endurance and integrity depending on its passive forces, electrostatic interaction, hydrophobicity and specific cellular surface components which act as defense system and control the movement of substances in and out of the cells thus leading to the usage of different fixation time for fragile cells.

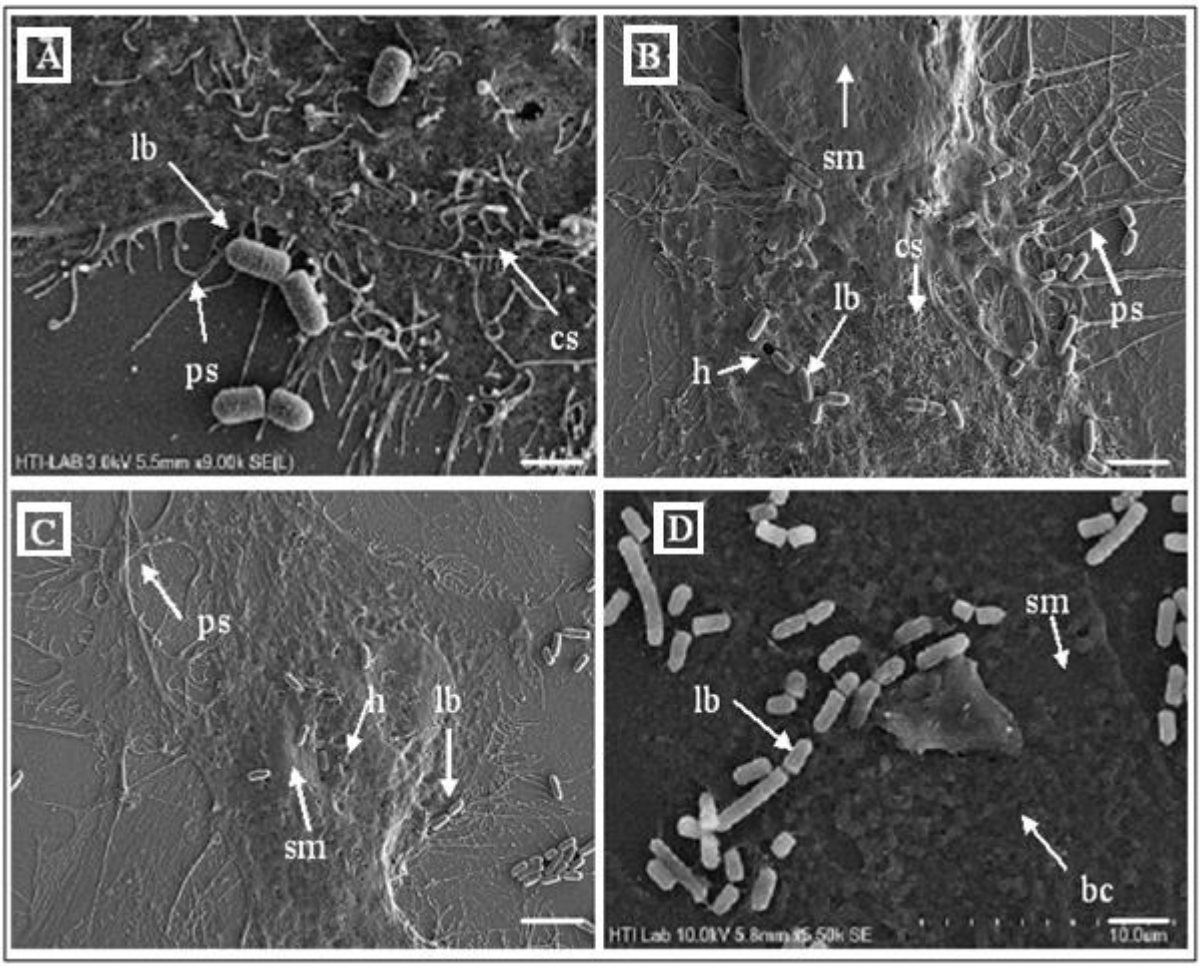


**Fig. 3.** SEM micrographs of CCD-18Co cells after incubation in different fixation time. (A) 2 hr, (B) 4 hr, (C) 6 hr, and (D) 12 hr. Arrows indicate the attachment of *Lactobacillus* sp. on the surface and side of the cell (lb), holes (h) and the ciliaric structures on the surface of the cell (cs) and protruding structures from the side of cell (ps). Arrows also indicate some of the smooth area of the cell (sm) and broken cell (bc) after exposed to the long fixation time. Original magnification X10 000 (bar=10 μm).

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