Recent studies in lactic acid bacteria (LAB) biofilm formation have reported that the functional properties of LAB are associated with biofilm formation. In this study, *Pediococcus acidilactici* and *Lactobacillus plantarum* showed the ability to form a biofilm on a solid surface under static conditions. The morphology of LAB on MRS-CR agar showed that both *P. acidilactici* and *L. plantarum* could produce exopolysaccharide (EPS) on the cell surface which mainly contributed to forming the biofilm of single and co-culture strains on solid surfaces. Under the flow condition, cell motility played an important role in supporting the cell to overcome the repulsive force to reach and interact with the surface. As a result, the *L. plantarum* and co-culture cells with higher motility caused a noticeably thicker biofilm on the glass surface compared to the *P. acidilactici* strain. Moreover, the antagonistic activity assay showed that co-culture biofilm formation improved the antagonistic effects of LAB against *Pseudomonas aeruginosa* and *Salmonella typhimurium* compared to those in planktonic culture. It suggested that co-culture biofilm could be an effective model to apply useful LAB as potential probiotics and to control pathogen in food chain, especially under the flow condition.

**Keywords:** *Lactobacillus plantarum; Pediococcus acidilactici; biofilm; co-culture; food pathogens*

1. **Introduction**

Lactic acid bacteria (LAB) are an important part of the microbiota in various types of foods. LAB are defined as a group of lactic-acid-producing, non-spore-forming, gram-positive rods and cocci. Many studies have shown that LAB strains isolated from traditional fermented foods have probiotic characteristics, which include tolerance to acid and bile and antagonistic activity toward intestinal pathogens. Several studies have also reported that some traditional fermented foods contain LAB that can inhibit pathogenic microorganisms. For example, *Lactobacillus plantarum* isolated from kimchi, a traditional fermented Korean vegetable food showed antimicrobial activity against several foodborne pathogenic microorganisms and target pathogens such as *Pseudomonas aeruginosa, Salmonella enterica,* and *Staphylococcus aureus*. Aoudia et al. (2016) demonstrated that *Lactobacillus plantarum* and *Lactobacillus fermentum* can inhibit food pathogens. *Pediococcus acidilactici* and *Lactobacillus plantarum* produced biosurfactants with antimicrobial, anti-adhesive, and anti-biofilm action against *Staphylococcus aureus*.[3]

Biofilm is a term that refers to stable bacterial communities on abiotic or biotic surfaces. Although the whole process is still incompletely understood, it is widely accepted that biofilm formation consists of four main stages: initial cell attachment, early colonial development, biofilm maturation, and dispersion of freely suspended cells.[4] During the first stage of biofilm formation, the planktonic cells use their motility to interact with and approach the surface. In the later stages, the attached cells on the surface produce exopolysaccharide (EPS) to strongly adhere together and to the surfaces.[4] Recent studies investigating the biofilm formation of LAB revealed that the LAB cells in biofilm structures are protected from temperature, bile, gastric pH, and mechanical force in the animal gut compared to the planktonic cells.[5] A mature biofilm has greater antibacterial activity and tolerance to gastric pH than attached cells on the surface.[6] Furthermore, the biofilm formation of LAB is
associated with antagonistic effects against pathogens and is bacterial strain dependent[2].

In nature, bacterial cells live together in stable communities containing multiple species. The interaction of bacterial cells in biofilms and the effect of stress conditions on biofilms containing multiple species is still unknown. In this study, the effect of the flow condition on co-culture biofilm of Lactobacillus plantarum VTCC 10890 and Pediococcus acidilactici VTCC 10800 was investigated. Furthermore, the contribution of cell motility and EPS on biofilm formation under static and flow conditions was analyzed. We also examined the antagonistic effects against food pathogens of LAB biofilm cultures compared to planktonic cultures.

2. Materials and Methods

2.1 Bacterial strains and media

Lactobacillus plantarum VTCC 10890, Pediococcus acidilactici VTCC 10800, Pseudomonas aeruginosa VTCC 12273 and Salmonella typhimurium VTCC 12271 strains used in this study were obtained from the Vietnam Type Culture Collection (VTCC), Hanoi, Vietnam. L. plantarum and P. acidilactici cells were cultured in MRS broth (CM0359, Oxoid, UK). Pseudomonas aeruginosa VTCC 12273 and Salmonella typhimurium VTCC 12271 were cultured in lysogeny broth (LB) medium (10 g/L Polypeptone, 5 g/L Bacto-yeast extract and 10 g/L NaCl). Prior to inoculation, all test cultures of L. plantarum and P. acidilactici strains were incubated in MRS media overnight at 30°C, and then diluted in fresh MRS medium to obtain an OD660 of 1.0. The inoculated cells were stood for 2 h to facilitate their adherence onto the glass bottom surface of the flow cell. Fresh medium was then pumped through the flow cell at a constant rate of 0.15 mL/min by using a peristaltic pump, the rate of flow being determined to ensure that dilution rate surpasses the specific growth rate of LAB cells so that all planktonic cells are washed out from the flow cell under the chosen conditions. After 24 h of incubation, a confocal laser scanning microscope (CLSM) (Model AZ-C1, Nikon, Japan) was employed to observe the cells. Before this observation, the flow cell was gently washed with 0.9% (w/v) NaCl solution to remove medium and planktonic cells. The biofilm cells were stained with 5 mL of acridine orange. Coverage and layer thickness of biofilm on the surface were determined using COMSTAT 2 image-processing software[9] (i).

2.2 Biofilm formation under static conditions

The biofilm formation on plastic surfaces under static conditions was as described by O'Toole et al. (1999) with some minor modifications[7]. After preculture overnight, the respective cells were suspended in fresh MRS medium to obtain an OD660 of 1.0. The single LAB strain biofilm was prepared with 2% single cell suspension. Co-culture biofilm was prepared with 1% P. acidilactici and 1% L. plantarum cell suspension. Aliquots of the cell suspension (175 μL) were inoculated into 96-well microtiter plates (Nalge Nunc International, Roskilde, Denmark). After 24 h of incubation at 30°C, the culture broth containing planktonic cells was removed from each well and remaining cells were stained by incubation with 200 μL of 50 mg/L crystal violet solution for 20 min at room temperature, followed by washing 5 times with water. The wells were allowed to dry at room temperature for 2 h. The dye with biofilm cells on the well surface was solubilized by adding 200 μL of 20% (v/v) acetone in ethanol and scrubbing the well surface by using a spatula. The solubilized dye sample was collected from four wells under a given condition to obtain a sufficient volume for measurement. An index of biofilm formation under static conditions (I_{590}) was represented as the absorbance of the dye solution measured at 570 nm by a microtiter plate reader (Benchmark Plus, Bio-Rad, Tokyo, Japan).

2.3 Biofilm formation under the flow condition

The flow cell experiment was performed as described in our previous work with some modifications[8]. Precultured LAB cells were diluted to obtain OD660 of 0.02 and injected into the flow cell by means of a syringe. For co-culture biofilm, L. plantarum cells were mixed with an equal amount of the P. acidilactici cells to get the final OD660 of 0.02. The inoculated cells were stood for 2 h to facilitate their adherence onto the glass bottom surface of the flow cell. Fresh medium was then pumped through the flow cell at a constant rate of 0.15 mL/min by using a peristaltic pump, the rate of flow being determined to ensure that dilution rate surpasses the specific growth rate of LAB cells so that all planktonic cells are washed out from the flow cell under the chosen conditions. After 24 h of incubation, a confocal laser scanning microscope (CLSM) (Model AZ-C1, Nikon, Japan) was employed to observe the cells. Before this observation, the flow cell was gently washed with 0.9% (w/v) NaCl solution to remove medium and planktonic cells. The biofilm cells were stained with 5 mL of acridine orange. Coverage and layer thickness of biofilm on the surface were determined using COMSTAT 2 image-processing software[9] (i).

2.4 Observation of biofilm with scanning electron microscope

Biofilm of LAB cells was formed on glass by incubation with MRS medium at 30°C for 24 h under static conditions. The preparation of biofilm cells was described by Ojima et al.[10] with some modifications. Briefly, the biofilm cells on glass pieces were fixed with 2.5% glutaraldehyde and then subjected to vacuum for 20 min. The prepared specimen was observed under a scanning electron microscope (SEM) (Model JSM–7600F, Jeol, Tokyo, Japan).
2.5 Congo red binding assay
EPS formed on LAB cell surfaces was detected by the inoculation of cells on an MRS-CR agar (MRS medium with 40 mg/L Congo red and 20 mg/L Coomassie brilliant blue). Overnight culture was spot-inoculated onto an MRS-CR agar plate and incubated at 30°C for 24 h. Binding of Congo red to EPS resulted in the development of a red color in the bacterial colonies[11].

2.6 Soft agar motility rate assay
For the assay of apparent motility rate of LAB cells, the soft agar method was used as described by Ojima et al. (2012)[10]. Cell suspensions with OD₆₆₀ of 0.001 were spread on MRS plates (1.5% agar) and incubated at 30°C for 24–28 h. A single colony of each strain was spot-inoculated onto an MRS plate (0.3% agar) using sterilized toothpicks. The changes in radius of colonies at 30°C during 24 h was analyzed to calculate the apparent motility rate.

2.7 Antagonistic activity
The antagonistic activity of LAB biofilm was measured by the method of delayed antagonism in solid nutrient medium[12]. Briefly, 50 μL of cell-free supernatant from LAB biofilm cultures under static or flow conditions was dropped into wells (4.5 mm diameter) of MRS plates inoculated with suspensions of pathogenic bacterial strains (OD₆₆₀ = 0.01). The plates were incubated for 24 h at 37°C. The antagonistic activity was detected as a zone of inhibition of the pathogens’ growth. In separate experiments, LAB cells were cultured in 75 mL of MRS medium at 30°C, 120 rpm for 24 h. The cell-free supernatant was collected after centrifugation at 10,000 rpm for 10 min. The cell-free supernatant of each liquid culture was then applied to inoculated agar plates to determine the antagonistic activity.

3. Results and discussion
3.1 Biofilm formation of LAB strains
The biofilm of Lactobacillus plantarum VTCC 10890 and Pediococcus acidilactici VTCC 10800 under static conditions on plastic surfaces was shown in Fig. 1. The indices of biofilm formation, I₀ of P. acidilactici and L. plantarum were 0.44±0.08 and 0.59±0.06, respectively. Interestingly, the co-culture noticeably enhanced biofilm formation capacity of P. acidilactici and L. plantarum with an I₀ of 0.62±0.1. In addition, the SEM images supported this finding (Fig. 2). The bacilli of L. plantarum adhered to the cocci of P. acidilactici to form the matured
biofilm colonies on the glass surface. In order to understand the effect of cell growth on the difference in biofilm formation of LAB strains, the growth kinetics of target cells were examined at 30°C in MRS medium shaken at 120 rpm. The growth curve of *L. plantarum* and *P. acidilactici* were similar and reached the stationary phase at 16 h (data not shown). The specific growth rate of *L. plantarum* and *P. acidilactici* were 0.54 h⁻¹ and 0.53 h⁻¹, respectively. This suggests that the similar growth rate of *L. plantarum* and *P. acidilactici* may not be related to the enhancement of co-cultured biofilms compared to those of pure strains.

### 3.2 Effect of flow condition

In animal intestines, the bacteria are continuously influenced by the flow condition. The formation of self-biofilm enhanced the survival period of the bacterial cells in the gut without washing away at the end of the gastrointestinal tract. In this study, we examined the effect of the flow condition on biofilm formation of LAB strains by using a flow cell FC81. Although the biofilm formation of *L. plantarum* and *P. acidilactici* was not significantly different under static conditions, *P. acidilactici* biofilm was much lower than that of *L. plantarum* under the flow condition (Fig. 3A). The CLSM images showed that the cells of *P. acidilactici* formed small clusters on a glass slide while *L. plantarum* cells formed a matured biofilm structure. The biofilm coverage analysis supported these findings (Fig. 3B). The maximum coverage of *P. acidilactici* cells was 1.4% while *L. plantarum* cells covered up to 34%. The co-culture of *L. plantarum* and *P. acidilactici* significantly enhanced biofilm formation with the coverage reaching 39%. This result suggested that the factors supporting the adherence of *L. plantarum* and *P. acidilactici* cells in co-culture biofilm were species-dependent.

### 3.3 The contribution of cell motility and EPS

In biofilm formation, factors such as cell motility and exopolysaccharide (EPS) production play an important role in early and later stages, respectively [10, 11, 13, 14]. Without the stress of the flow condition, the cells can conveniently overcome the repulsive force to reach the abiotic surface and produce the EPS to form tiny colonies of single or multiple species [15, 16]. Previous study showed that the EPS of *L. platarum*, composed of ribose, rhamnose, arabinose, xylose, mannose, glucose and galactose, played an important role in its interaction to the abiotic and biotic surfaces [6, 17]. On the other hand, the EPS of *P. acidilactici* was composed of glucan and had antioxidant activity [18]. Our result showed that the colonies of *P. acidilactici* and *L. plantarum* strains on

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**Fig. 3** (A) CLSM images of *L. plantarum*, *P. acidilactici*, and co-culture biofilm on glass surfaces cultured for 24 h. The whole bacterial cells were stained with acridine orange. The scale bars indicate 150 μm. (B) Distributions of bacterial cells in biofilms. (C) The biomass of bacterial biofilms. The corresponding CLSM images in panels A were analyzed by the COMSTAT software.

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Congo red agar plates were red, indicating abundant EPS on the cell surfaces (Fig. 4). Our result indicated that the high biofilm formation on the plastic and glass surfaces were observed in *P. acidilactici*, *L. plantarum* and co-culture under static conditions. Besides, the motility rate of *P. acidilactici* and *L. plantarum* cells on soft agar plates were 225.5±17 and 333.3±16.9 μm/h, respectively indicating that the motility of *L. plantarum* cells was higher than that of *P. acidilactici* cells. It suggested that the number of *L. plantarum* cells approached that of *P. acidilactici* cells, resulting in the higher biofilm formation of *L. plantarum* strain.

However, under the flow condition, cell motility is necessary for the bacterial cells to reach and adhere to the abiotic surface[13, 16]. Our result showed that the low motility of *P. acidilactici* cells (225.5±17 μm/h) caused a low biofilm formation on the glass surface (Fig. 5). The biomass of *P. acidilactici* cells on the slide glass was 51.2±4.4 μm²/μm² (Fig. 3C). However, the *L. plantarum* cells with higher motility rate (333.3±16.9 μm/h) were able to locate on the abiotic surface. The motility rate of the co-culture was slightly higher than that of *L. plantarum* alone, suggesting that the motility of *P. acidilactici* and *L. plantarum* cells in co-culture was independent. In the later phase of biofilm formation, the adhered cells on the solid surface reproduced to form newborn cells or captured the same species or different species cells from the liquid environment to form colonies[10, 16]. This explained the finding that the biomass of *L. plantarum* and co-culture biofilm was 83.6±17.2 and 128.1±7.1 μm²/μm², respectively, which were significantly higher compared to that of *P. acidilactici* (Fig. 3C). It suggested that the enhancement of LAB biofilm formation by co-culture was possible to facilitate the good properties of LAB strains[5, 6, 19].

3.4 Antagonistic effects of LAB biofilm on pathogen growth

In this work, we compared the effect of various supernatants obtained either from biofilm or liquid cultures of LAB strains on the growth of several food pathogens: *Pseudomonas aeruginosa* VTCC 12273 and *Salmonella typhimurium* VTCC 12271. The supernatants of *P. acidilactici* planktonic culture had no effect to *P. aeruginosa* and *S. typhimurium* growths (Table 1). No inhibition zone appeared on the agar inoculated with pathogenic cells. However, the biofilm supernatants of *P. acidilactici* had an antagonistic effect against all pathogens under both static and flow conditions. Moreover, *L. plantarum* biofilm culture slightly enhanced the antimicrobial activities against all tested pathogens compared to the planktonic cells (Table 1). Our results showed that the antagonistic effects of co-culture biofilm on the same pathogenic bacteria were statistically different from this of *L. plantarum* and *P. acidilactici* under static condition. For example, the zones of inhibition of *P. acidilactici*, *L. plantarum*, and co-culture biofilm under static conditions against *S. typhimurium* were 9.6±0.5; 10.4±0.5 and 12.2±0.4 mm, respectively. Under flow condition, the biofilms of all tested strains had antimicrobial activities against both *P. aeruginosa* and *S. typhimurium*. It seems that the co-culture enhanced slightly the antagonistic effect against pathogens, compared to those of pure cul-

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**Fig. 4** Morphology of LAB colonies on MRS-CR agar. The LAB cells were cultured at 30°C for 24 h.

**Fig. 5** Values of the motility rate of each strain. The open, black and gray bars show the motility rate of *L. plantarum*, *P. acidilactici*, and co-culture, respectively. The data are the means of measurements obtained from three independent experiments. The statistical significance among the data sets was assessed by Student’s t-test (*p<0.05).
tature biofilms under the flow condition.

Gomez et al. (2016) reported that biofilm of non-bacteriocinogenic and bacteriocinogenic LAB are able to inhibit Listeria monocytogenes, Escherichia coli O157:H7 and Salmonella typhimurium biofilm formation. Also, Perez-Ibarreche et al. (2014) showed that biofilm on abiotic surfaces of bacteriocinogenic Lactobacillus sakei are able to control food pathogen adhesion[20]. In this study, the effect against pathogen growth was observed in bacteriocinogenic LAB biofilm formation through the contribution of cell motility and EPS, especially under flow conditions.[5, 19, 22]. Besides, the LAB biofilm may trap and kill the pathogen cells inside their 3D structure[22].

In conclusion, we found that the co-culture enhanced the LAB biofilm formation through the contribution of cell motility and EPS, especially under flow conditions. The abundance of EPS on P. acidilactici and L. plantarum cell surfaces mainly contributed to adherence and formation of biofilm colonies under static conditions. Under the flow condition, the co-culture caused a noticeably thicker biofilm through a higher motility rate compared to those of P. acidilactici. Moreover, our results showed that biofilm formation improved the antagonistic effects against Pseudomonas aeruginosa, and Salmonella typhimurium. It suggested that the cell interaction of multiple species of LAB resulted in the enhanced biofilm which facilitated the antimicrobial activity against pathogenic bacteria, especially under the flow condition such as animal intestines.

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