

## Culture Condition for *Listeria monocytogenes* 1421 Biofilm Formation and the Effect of Kimchi on Biofilm

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*Listeria monocytogenes*, a fatal food-borne pathogenic bacteria, can form a biofilm on many different supports. The biofilm gives *L. monocytogenes* more viability and resistance to disinfectants and sterilization procedures. *L. monocytogenes* formed biofilms on various culture vessels tested in this experiment and showed the maximum amount of biofilm when it was cultured for 4 days at 30°C in BHI broth. In this study, biofilm formation was stimulated or inhibited by addition of different Kimchi samples. That was not in accordance with the effect of Kimchi on the growth of *L. monocytogenes*.

**Key words** : *Listeria monocytogenes*, food-borne pathogen, biofilm, Kimchi

### Introduction

*Listeria monocytogenes* is a Gram positive, non-spore forming, facultative anaerobic bacteria which cause severe food-borne infections in both humans and animals. It showed over 25% of fatality rate every year in United States [24]. The hazard of *L. monocytogenes* as a food-borne pathogen is very important because it tolerates extreme conditions such as low temperature, low pH and microaerobic environment [26]. And *L. monocytogenes* can survive long periods of time in many different places including soil, water and variety of food resources [12,30]. Food materials and products are contaminated by *L. monocytogenes* through the soil carried on workers' shoes, clothes and equipments. And the growth of *L. monocytogenes* is enhanced when the place has enough water and nutrients. *L. monocytogenes* can grow and survive easily on the surface of working equipments and area in food processing plants. Therefore, Food products from those food plants have higher possibility of contamination by *L. monocytogenes*. Among those variety of foods including meat products, dairy products, vegetables and ready to eat food has the highest risk of *L. monocytogenes* contamination, because it is usually kept in refrigerator for a long periods of time to grow the microorganism. And the threat of *L. monocytogenes* will be increased according to the growth of meat product and ready to eat food consumption.

A biofilm is a single or mixed microbial species that at-

tached to a surface and embedded within extracellular polymeric substances [39]. Biofilms are occurred on various kinds of surface materials such as glass, stainless steel and rubber. And the growth of biofilms can be promoted on the surfaces containing nutrients, ionic salts and organic materials [5]. Therefore, biofilms can be developed working area in food processing plants and contaminate food products. In general, bacteria initiate to form biofilms in response to specific environmental signals such as temperature, pH, nutrients and oxygen concentration [27-29,31,37]. Biofilms formed by Gram-positive and Gram-negative bacteria have been studied including *Pseudomonas fluorescences*, *Escherichia coli*, *Vibrio cholerae*, *Staphylococcus epidermidis*, *Staphylococcus aureus* and many other species. Once the biofilm is developed on the surfaces, it is very difficult to remove, because biofilm enhances bacterial resistance to cleaning and sanitation reagents by preventing the reagents get into biofilm. Bacteria within biofilms are more resistance to antibiotics, disinfectants, environmental stress and sterilization procedures [8,23,29,35,38]. *L. monocytogenes* can develop biofilm on both abiotic surface such as glass, rubber, stainless steel and plastics, and biotic surface from plant and animal [1,4,9,35]. Therefore, *L. monocytogenes* forms biofilms on the food processing equipments in food processing plants, and cause food contamination and food-borne diseases.

Kimchi was selected as one of the world-wide health foods because of the high nutrition value and preventive effects on various pathogenic microorganisms by 'Health' in 2006. Many researches had been reported antimicrobial effects of Kimchi on pathogenic microorganisms including *Salmonella typhimurium*, *Staphylococcus aureus*, *E. coli* O157

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and *L. monocytogenes* [11,14-21,32-34]. However, the effect of Kimchi on biofilm and *L. monocytogenes* biofilm formation have not been studied. In this study, the culture conditions for biofilm formation of *L. monocytogenes* were optimized and studied the effect of Kimchi on *L. monocytogenes* biofilm formation.

## Materials and Methods

### Bacterial strain, medium and culture condition

*L. monocytogenes* 1421, which forms biofilm on 96-well polystyrene (PVC) plate, was kindly provided by Dr. Glenn M. Young, University of California, Davis. The strain was stored at -70°C in Brain Heart Infusion (BHI) broth medium (Becton, Dickinson and Company) containing 25% glycerol. *L. monocytogenes* 1421 was cultured at 30°C on BHI agar medium, and transferred to fresh medium every other week. Liquid seed-culture of *L. monocytogenes* 1421 was performed in BHI broth for 24 hr at 30°C without agitation.

In order to form biofilm, a droplet of *L. monocytogenes* 1421 glycerol stock stored at -70°C was streaked on BHI medium plate and cultured over night at 30°C. One colony isolated on the BHI plate was inoculated to 0.8 ml BHI broth in 1.5 ml microcentrifuge tube (Eppendorf, German) and cultured for 24 hr at 30°C as a seed culture. To quantify the biofilm amount, the main culture medium was inoculated with seed cultures at 1%, and cultured at different conditions according to variations.

### Preparation of kimchi sample

Three different home-made and two commercial Kimchi samples were used in this experiment. The pellets of each Kimchi samples were discarded after centrifuged at 8,000 rpm for 10 min, and the supernatants were stored at -70°C. The supernatants were boiled for 20 min at 100°C, then sterilized through 0.22 µm membrane (Milipore Co., USA) filtration, and kept in refrigerator. Kimchi sample was added to main culture at 2%.

### Biofilm quantification

In general, the PVC microtiter plate assay was used to determine the amount of *L. monocytogenes* 1421 attachment which provides an assessment of surface attach biomass [7]. However, the assay method was modified in this research, because the volume of each PVC plate well was not suitable to study the effect of Kimchi on *L. monocytogenes* 1421 biofilm

formation.

For the quantification of biofilm, the planktonic cells in main culture were removed by aspiration. The culture tube was rinsed twice with distilled water and allowed to air dry thoroughly. Then, the materials and cells remaining in the culture tube were stained with a solution of 1% crystal violet for 20 min. The samples were washed several times with distilled water and allowed to air dry thoroughly. The remaining crystal violet was extracted using 95% ethanol and quantified by measuring OD at 595 nm.

The effect of Kimchi on *L. monocytogenes* 1421 biofilm formation was analyzed by comparing OD values of each sample with negative control which contains BHI medium only.

## Results and Discussion

### Culture vessel

In order to test different culture vessels, *L. monocytogenes* 1421 was cultured for 2 days at 30°C in glass tube, falcon tube (screw cap, Corning), round tube (snap cap, Fisher) and microcentrifuge tube. *L. monocytogenes* 1421 formed biofilm in all culture tubes, as reported by other researches, and showed different amounts of biofilm in each culture vessel [2,3,22,26,39]. Although, *L. monocytogenes* 1421 formed similar biofilm amount in glass tube, round tube and microcentrifuge tube, microcentrifuge tube was selected as culture vessel, because of the easy preparation and constant results (Table 1).

### Culture medium

Optimum culture medium was selected by comparison of the biofilm amount after cultivation of *L. monocytogenes* 1421 for 2 days at 30°C in BHI, LB, Nutrient broth (NB) and Tryptose broth (TB). Although, *L. monocytogenes* 1421 was grown and developed biofilm in all media, BHI broth, generally using for listeria culture, showed the highest level of

Table 1. The amount of *L. monocytogenes* 1421 biofilm on different culture vessels

Culture vessel	Relative amount of biofilm (%)
Glass tube	100
Falcon tube (Corning, 15ml screw cap)	49.4
Round tube (Fisher, 15ml snap cap)	77.4
Microcentrifuge tube	97.0

\*Relative amount of biofilm represents average value obtained by multiple experiments.

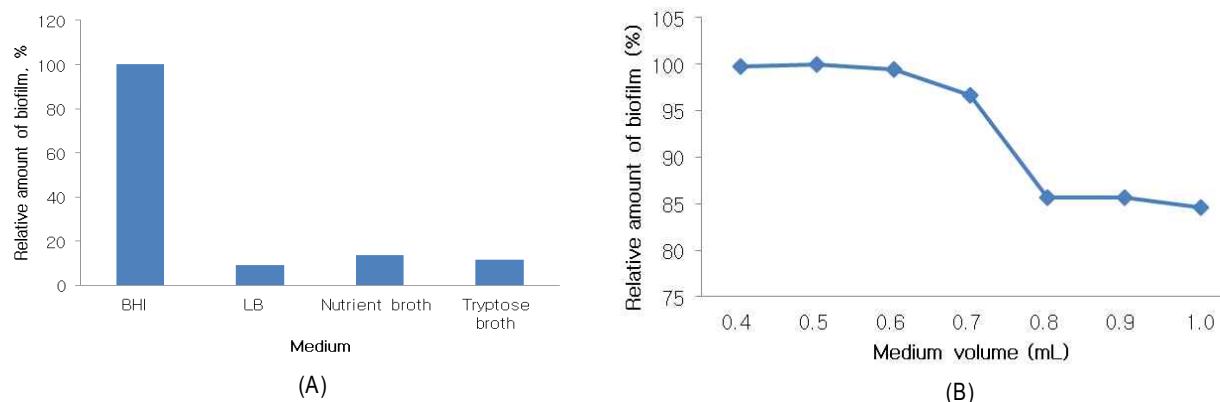


Fig. 1. Effect of culture medium (A) and medium volume (B) on the amount of *L. monocytogenes* 1421 biofilm. Relative amount of biofilm represents average value obtained by multiple experiments.

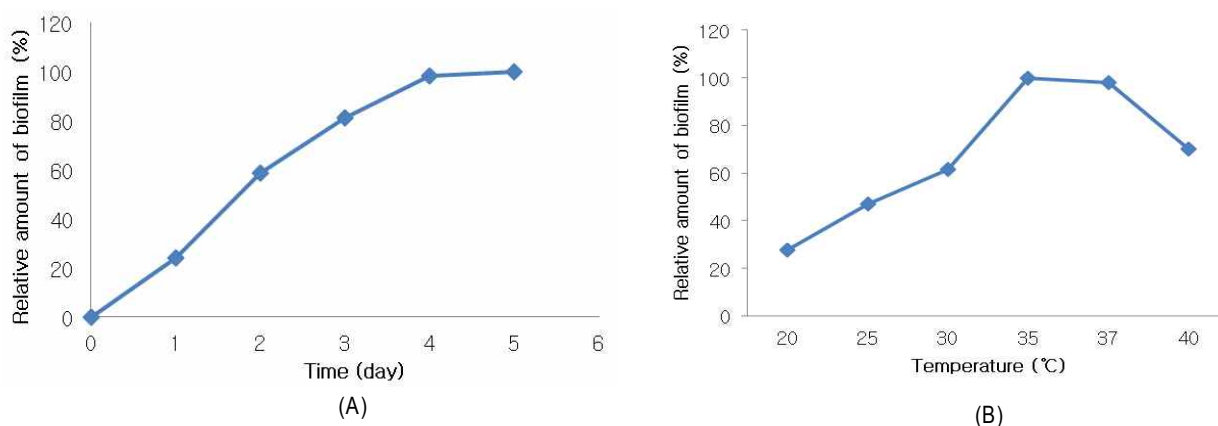


Fig. 2. Effect of culture time (A) and temperature (B) on the amount of *L. monocytogenes* 1421 biofilm. Relative amount of biofilm represents average value obtained by multiple experiments.

biofilm amount (Fig. 1).

#### Culture volume

Because of the aerobic property of *L. monocytogenes*, optimum culture volume was determined by comparison of the biofilm amount after cultivation of *L. monocytogenes* 1421 for 2 days at 30°C in different volume of BHI broth. The biofilm amount was increased against culture volume until 0.6 ml, then showed similar amount after 0.6 ml (Fig. 1).

#### Culture time

Optimum culture time was determined by comparison of the biofilm amount after cultivation of *L. monocytogenes* 1421 in BHI broth at 30°C during different culture time. The biofilm amount was increased till 4 days according to culture time, and showed static level after 4 days (Fig. 2).

Although, the inconsistency among studies focusing on

planktonic cell attachment to a substratum, attachment was initiated very quickly and the mature biofilm complex was developed within 24 hr [3,5,13,25]. However, the biofilm amount was increased after 24 hr in this study. The difference between these results due to different culture system. Biofilm was formed by standing culture in this study, however, others used flow cell system.

#### Temperature

Optimum culture temperature was determined by comparison of the biofilm amount after cultivation of *L. monocytogenes* 1421 in BHI broth for 4 days at different temperatures. The maximum amount of biofilm was obtained at 30°C, the general culture temperature for *L. monocytogenes*, and the amount was decreased in higher or lower temperature (Fig. 2).

Table 2. Effect of Kimchi on the formation of *L. monocytogenes* 1421 biofilm

Kimchi sample	Relative amount of biofilm (%)
None ( <i>Listeria</i> only)	100
A-home made (radish roots Kimchi)	51.7
B-home made (cabbage Kimchi)	149.4
C-home made (cabbage Kimchi)	138.7
D-commercial (cabbage Kimchi)	78.8
E-commercial (cabbage Kimchi)	71.7

\*Relative amount of biofilm represents average value obtained by multiple experiments.

#### Effect of Kimchi on biofilm formation

In order to study the effect of Kimchi on biofilm formation, three different home-made and two commercial Kimchi samples were added to *L. monocytogenes* 1421 culture in optimum culture condition. The experiments were performed at least 5 times, and the mean values were obtained. Although, many researches had been reported antimicrobial effects of Kimchi on *L. monocytogenes*, each sample showed different effect on *L. monocytogenes* 1421 biofilm formation (Table 2) [14,15,18,21,33,34]. According to many different materials and methods to make Kimchi, it is hard to find the factor which affects the different results of Kimchi on antimicrobial effects and biofilm formation.

Therefore, more researches should be performed to see the effect of Kimchi on the biofilm formation of pathogenic microorganisms including *L. monocytogenes*.

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#### References

- Amy, C. 1998. Biofilm in food processing environments. *J. Dairy Sci.* **81**, 2765-2770.
- Beresford, M. R., Andrew, P. W. and Shama, G. 2001. *Listeria monocytogenes* adheres to many materials found in food-processing environments. *J. Appl. Microbiol.* **92**, 556-565.
- Blackman, I. C. and Frank, J. E. 1996. Growth of *Listeria monocytogenes* as a biofilm on various food-processing surfaces. *J. Food Prot.* **59**, 827-831.
- Chae, M. S. 2001. Cell viability of *Listeria monocytogenes* biofilms. *Food Microbiol.* **18**, 103-112.
- Chae, M. S. and Schraft, H. 2000. Comparative evaluation of adhesion and biofilm formation of different *Listeria monocytogenes* strains. *Int. J. Food Microbiol.* **62**, 103-111.
- Chae, M. S. and Schraft, H., Truelstrup, L. H. and Mackereth, R. 2006. Effect of physicochemical surface characteristics of *Listeria monocytogenes* strains on attachment to glass. *Food Microbiol.* **23**, 250-259.
- Djordjevic, D., Wiedmann, M. and McLandsborough, L. A. 2002. Microtiter plate assay for assessment of *Listeria monocytogenes* biofilm formation. *Appl. Environ. Microbiol.* **68**, 2950-2958.
- Drenkard, E. and Ausubel, F. M. 2002. Pseudomonas biofilm formation and antibiotic resistance are linked to phenotypic variation. *Nature* **416**, 740-743.
- Gorski, L., Palumbo, J. D. and Mandrell, R. E. 2003. Attachment of *Listeria monocytogenes* to radish tissue is dependent upon temperature and flagella mobility. *Appl. Environ. Microbiol.* **69**, 258-266.
- Helke, D. M. and Wong, A. C. I. 1994. Survival and growth characteristics of *Listeria monocytogenes* and *Salmonella typhimurium* on stainless steel and buns-n rubber. *J. Food Prot.* **57**, 963-968.
- Hong, J. S. 2007. Biochemical characterization of bacteriocin produced by lactic acid bacteria isolated from kimchi. M. S. Thesis, Ajou University, Suwon, Korea.
- Juntilla, J. R., Niemela, S. I. and Hirn, J. 1988. Minimum growth temperatures of *Listeria monocytogenes* and non-haemolytic *Listeria*. *J. Appl. Bacteriol.* **65**, 321-327.
- Kalmokoff, M. L., Austin, J. W., Wan, X. D., Sanders, G., Banerjee, S. and Farber, J. M. 2001. Adsorption, attachment and biofilm formation among isolates of *Listeria monocytogenes* using model conditions. *J. Appl. Microbiol.* **91**, 725-734.
- Kang, C. H., Chung, K. O. and Ha, D. M. 2002. Inhibitory effect on the growth of intestinal pathogenic bacteria by Kimchi fermentation. *Korean J. Food Sci. Technol.* **34**, 480-486.
- Kang, S. Y. and Han, M. J. 2005. Effect of Kimchi ingredients on the growth of pathogenic and lactic acid bacteria. *Korean J. Cookery Sci.* **21**, 838-843.
- Kim, H. J. and Chang, H. C. 2006. Isolation and characterization of exopolysaccharide producing lactic acid bacteria from Kimchi. *Kor. J. Microbial. Biotechnol.* **34**, 196-203.
- Kim, H. J., Hwang-Bo, M. H., Lee, H. J., Yu, T. S. and Lee, I. S. 2005. Antibacterial and anticancer effects of Kimchi extracts prepared by *Monascus purpureus* Koji paste. *Korean J. Food Sci. Technol.* **37**, 618-623.
- Kim, J. H. 1995. Inhibition of *Listeria monocytogenes* by bacteriocin(s) from lactic acid bacteria isolated from Kimchi. *Agri. Chem. Biochem.* **38**, 302-307.
- Kim, M., Lee, S. J., Seul, K. J., Park, Y. M. and Ghim, S. Y. 2009. Characterization of antimicrobial substance produced by *Lactobacillus paraplantarum* KNUC25 isolated from Kimchi. *Kor. J. Microbial. Biotechnol.* **37**, 24-32.
- Lee, S. G., Han, K. S., Jeong, S. G., Oh, M. H., Jang, A., Kim, D. H., Bae, I. H. and Ham, J. S. 2010. A study on the sensory characteristic of yogurt and antimicrobial activity of *Lactobacillus plantarum* LHC52 isolated from Kimchi. *Korean J. Food Sci. Ani. Resour.* **30**, 328-335.

21. Lee, S. H., Kim, M. K. and Frank, J. F. 1995. Growth of *Listeria monocytogenes* during Kimchi fermentation and in the presence of Kimchi ingredients. *J. Food Protec.* **58**, 1215-1218.
22. Mafu, A. A., Roy, D., Goulet, J. and Magny, P. 1990. Attachment of *Listeria monocytogenes* to stainless steel, glass, polypropylene, and rubber surfaces after short contact time. *J. Food Prot.* **53**, 742-746.
23. Mah, T. F. and O'Toole, G. A. 2001. Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol.* **9**, 34-39.
24. McCarthy, S. A. 1990. *Listeria* in the environment, pp. 25-29, Foodborne Listeriosis. Elsevier Science Publishing, Inc. New York.
25. Mittelman, M. W. 1998. Structure and functional characteristics of bacterial biofilms in fluid processing operations. *J. Dairy Sci.* **81**, 2760-2764.
26. Moretro, T. and Langsrud, S. 2004. *Listeria monocytogenes* biofilm formation and persistence in food-processing environments. *Biofilms* **1**, 107-121.
27. O'Toole, G., Kaplan, H. B. and Kolter, R. 2000. Biofilm formation as microbial development. *Annu. Rev. Microbiol.* **54**, 49-79.
28. Palmer, R. Jr. W. D. 1997. Developmental biology of biofilms: implications for treatment and control. *Trends Microbiol.* **5**, 431-437.
29. Pan, Y., Breidt, F. Jr. and Kathariou, S. 2006. Resistance of *Listeria monocytogenes* biofilms to sanitizing agents in a simulated process environment. *Appl. Environ. Microbiol.* **72**, 7711-7717.
30. Petran, R. 1989. A study of factors affecting growth and recovery of *Listeria monocytogenes*. *J. Food Sci.* **54**, 458-460.
31. Prett, L. A. and Kolter, R. 1998. Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, mobility, chemotaxis and type I pili. *Mol. Microbiol.* **30**, 285-293.
32. Sheo, H. J. and Seo, Y. S. 2003. The Antibacterial Action of Chinese Cabbage Kimchi Juice on *Staphylococcus aureus*, *Salmonella enteritidis*, *Vibrio parahaemolyticus* and *Enterobacter cloacae*. *J. Korean Soc. Food Sci. Nutr.* **32**, 1351-1356.
33. Shin, S. M., Park, J. Y. and Hahn, Y. S. 2005. Antimicrobial effect of Kimchi ingredients of methanol extract on pathogenic microorganisms. *Kor. J. Food Cook Sci.* **21**, 53-63.
34. Song, Y. J., Park, S. H., You, J. Y., Cho, Y. S. and Oh, K. H. 2009. Antibacterial activity against food-poisoning causing bacteria and characterization of *Lactobacillus plantarum* YK-9 isolated from Kimchi. *KSBB J.* **24**, 273-278.
35. Stepanovic, S., Cirkovic, I., Ramin, L. and Svabic-Vlahovic, M. 2004. Biofilm formation by salmonella spp. and *Listeria monocytogenes* on plastic surface. *Lett. Appl. Microbiol.* **38**, 428-432.
36. Stewart, P. S., Rayner, J., Roe, F. and Rees, W. M. 2001. Biofilm penetration and disinfection efficiency of alkaline hypochlorite and chlorosulfamates. *J. Appl. Microbiol.* **91**, 525-532.
37. Watnick, P. I. and Kolter, R. 1999. Steps in the development of a *Vibrio cholerae* El Tor biofilm. *Mol. Microbiol.* **34**, 586-595.
38. Whitely, M., Banger, M. G., Bumgarner, R. E., Parset, M. R., Teitzel, G. M., Lory, S. and Greenberg, E. P. 2001. Gene expression in *Pseudomonas aeruginosa* biofilms. *Nature* **413**, 860-864.
39. Wong, A. 1998. Biofilms in food processing environments. *J. Dairy Science* **81**, 2765-2770.

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초록 : Biofilm 형성을 위한 *Listeria monocytogenes* 1421의 배양 조건과 김치에 의한 영향

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식품으로부터 유래되어 치명적인 질병을 유발하는 *Listeria monocytogenes*는 다양한 지지체 위에 biofilm을 형성할 수 있다. 이러한 biofilm은 여러 가지 소독제나 살균과정으로부터 *L. monocytogenes*의 생존력이나 저항성을 증가시켜 준다. 본 연구에서도 *L. monocytogenes*는 다양한 배양용기에서 biofilm을 형성하였으며, BHI를 배지로 하여 30℃에서 4일 동안 배양하였을 때 최대의 biofilm을 형성하였다. *L. monocytogenes*의 biofilm의 형성에 미치는 김치의 효과를 살펴본 결과 김치를 첨가함에 따라 biofilm의 양이 줄어들기도 하고 늘어나기도 하여, 김치에 따라 다른 영향을 미치는 것으로 여겨졌으며, 이러한 결과는 *L. monocytogenes*의 생육에 미치는 김치의 효과와는 차이가 있었다.