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Effect of Bacteriophage Application on the Formation and Removal of *Listeria monocytogenes* Biofilms

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Abstract

Biofilm is a serious problem in food industry and *L. monocytogenes* can develop biofilm on the surfaces of food processing plants. In this study, the effect of lytic bacteriophage application on the formation and removal of biofilm by *Listeria monocytogenes* were investigated. For these purposes, *Listeria monocytogenes* ATCC 19111 (serotype 1/2a) and LMP-M117 were used as bacterial and lytic bacteriophage strain, respectively. The analysis was performed in 96-well microplate and biofilm amount were measured by spectrophotometer. In the study, three different concentrations (10^2, 10^4 and 10^6 cfu/ml) of *L. monocytogenes* was used for the formation of biofilm in the 96-well microplate. For the formation and removal of biofilm lytic bacteriophage with a concentration of 10^9 pfu/ml was used. According to the analysis, biofilm formation was increased when the initial contamination of *L. monocytogenes* decreased and the incubation period was extended from 24 to 48 hours. Additionally, 10^9 pfu/ml of bacteriophage application was reduced the biofilm formation of *L. monocytogenes*. However, no effect was observed on the removal of biofilm by bacteriophage treatment. In conclusion, it was detected that, lytic bacteriophage treatment can reduce the biofilm formation of *L. monocytogenes* on surfaces.

Keywords: *L. monocytogenes*; Bacteriophage; Biofilm; Microplate

Introduction

Biofilms can be defined as community of microorganisms exists on biotic or abiotic surfaces which enclosed in a self-produced extracellular polymeric substance [1]. When biofilms are formed, bacterial cells become more resistant to antimicrobial agents and harsh conditions. *L. monocytogenes* is one of the most popular bacteria that is known for its ability to produce biofilms on surfaces like stainless steel, glass, rubber, polymers [2]. Persisting in such food processing environments by forming biofilms makes *L. monocytogenes* a very significant source of contamination in the food industry. The worst listeriosis outbreak caused by cantaloupe in 2011 is a very good example for how *L. monocytogenes* can survive in processing plants and can result in death [3].

Although chemicals have been the most tested sanitizers to eradicate biofilms, they are not practically suitable to implement for all surfaces in facilities, their impacts depend on their concentration and repeated exposures especially to a single agent can end up with resistance [1,4]. Also some physical and biological methods even quorum sensing inhibition have been used to prevent biofilm formation so far [5]. Apart from these treatments, the use of bacteriophages is one of the most promising approaches to combat biofilms lately.

Bacteriophages are widely distributed viruses that only infect bacteria for propagation and harmless to living beings because of its high specificity. They have been used for various purposes including phage therapy, biocontrol, biopreservation and biosanitation [6]. There are commercially available phage preparations today and most of the biofilm removal studies for *L. monocytogenes* focused on effectiveness of these phages [1,4,5,7,8]. Previously, we isolated a lytic phage from a poultry slaughterhouse wastewater called LMP-117, and here in this study, we aimed to investigate LMP-117 efficacy against *L. monocytogenes* biofilms formed in polystyrene microplate wells.

Materials and Methods

Strains used in the study

In the study, *L. monocytogenes* ATCC 19111 was used as the bacterial strain. Serial dilutions of *L. monocytogenes* suspensions were prepared to determine optical densities (OD_{600}), and plate colony counts of *L. monocytogenes* were compared on Modified Oxford Agar (MOX, Oxoid CM856, SR140). *L. monocytogenes* was diluted in TSB (Tyriptic Soy Broth, Oxoid CM0129) for expected concentrations of 10^2, 10^4, and 10^6 cfu/ml according to previously determined OD_{600} values [9].

Bacteriophage LMP-M117 that showed the broadest lytic activity on *L. monocytogenes* isolates in a previous study was used for the control of formation and removal of biofilms in 96-well microplate. LMP-117 was enriched in *L. monocytogenes* (10^9 pfu/ml) just prior to use [9].

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The first line of 96-well microplate was used for the formation and phage removal of biofilm. Two wells were determined as positive control, four wells were determined as negative control and two wells were used for the test. For the analysis, 200 µl 10^7 cfu/ml of *L. monocytogenes* strain was inoculated to the positive control wells. For the negative control only 200 µl TSB was used.

The second line of the 96-well microplate was used to measure the biofilm formation ability of the bacteria in the presence of lytic phage. Two positive control and two negative control wells were used for each concentration (10^6, 10^4 and 10^2 cfu/ml). For this purpose, 100 µl of *L. monocytogenes* suspension and 100 µl TSB were added to the positive control wells. On the other hand, 100 µl *L. monocytogenes* and 100 µl phage were added to the test wells.

After 24 and 46 h of incubation at 37°C, the plate was stained with 200 µl of 1% crystal violet solution for 15 min for the quantification of the biofilms. All of the wells were washed two times with 200 µl of sterile physiological saline before staining. After the incubation period to remove the residual crystal violet stain, plate was washed six times by sterile physiological saline. Then, 200 µl of ethanol-acetone (80:20) was added to each well for the solubilization of bounded crystal violet from the stained biofilms. After this step, plate was read at OD_{562} for the quantification of biofilm.

Results and Discussion

In this study, a strain belongs to serotype 1/2a was used. In

the measurement of the biofilm by spectrophotometer; increased biofilm formation was detected as the level of *L. monocytogenes* contamination decreased (10^6 <10^4 <10^2 cfu/ml). This may be explained by the fact that the bacteria produce more biofilm to protect and to maintain its permanence when the number of bacteria is low. Also, the biofilm formation was increased when incubation time was extended from 24 hours to 48 h as expected. Soni and Nannapaneni [4] reported that, among 21 *L. monocytogenes* strains representing 13 different serotypes strains of serotype 1/2a showed maximum biofilm formation.

In our study, it was determined that 10^9 pfu/ml bacteriophage LMP-M117 inhibited the formation of biofilm on the polystyrene plate surface. This phage concentration is similar with the studies done with commercial *L. monocytogenes* phages [1,4,5,7]. The microtiter plate assay is a commonly used method in the biofilm studies to visualize adhesion of bacterial strains and understand growth conditions within each experiment [10]. This assay is frequently used as a first step to obtain data of biofilm forming ability of the strains before evaluating the effectiveness of bacteriophages [4].

It was observed that bacteriophage application was not effective for the removal of biofilm. This situation was also addressed by Gutiérrez D, et al. [5], indicating that although bacteriophage P100 was succeed in reducing *L. monocytogenes* biofilms, the complete removal of was not achieved. The researchers observed biofilms after 8 h of treatment, and viable cells were present after 48 h. For total removal of *L. monocytogenes* biofilms other sanitation methods can be used in combination.

As a result, bacteriophage application can reduce biofilm formation of *L. monocytogenes* on the surfaces. It is concluded that bacteriophage LMP-M117 can be used for the control of biofilm formation on food processing surfaces as well as biocontrol of *L. monocytogenes* in foods.

Conflicts of Interest

All the authors declared that they have no conflict of interest.

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