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# Combined effects of selected food additives on adhesion of various foodborne pathogens onto microtiter plate and cabbage leaves

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ABSTRACT

The study was conducted to examine the adhesion inhibition and antibacterial activities by combined use of some selected food additives such as Sucrose Fatty Acid Ester (SE) C18, Gardenia Yellow (GY), Monascus Pigment (MP), Protamine (PT), ε-polylysine (PL) and Milk Serum Protein (MSP), against *Salmonella* Enteritidis, *Salmonella* Typhimurium, *Listeria monocytogenes*, *Pseudomonas aeruginosa,* and *Staphylococcus aureus*. The adhesion of those pathogenic bacteria was reduced by several combination of food additives compared to that of each of the single use. The combinations decreased the relative adhesion more than 10% compared to that of each of the single use were taken into consideration. The following combinations such as SE18 & GY, PT & MSP, and PL & MSP were effective in inhibiting the attachment of *S.* Enteritidis onto microtiter plate compared to that of each of the single use. In case of *S.* Typhimurium, the combination of MSP & MP, SE18 & GY, SE18 & PT, SE18 & PL, SE18 & MP, SE18 & MSP, MP & PT, MSP & PL, GY & PL, and MSP & PT were effective in adhesion inhibition. The combination of SE18 & GY, GY & MSP, and PL & MSP were effective in inhibiting the attachment of *P. aeruginosa* onto microtiter plate compared to that of each of the single use. The combination of GY & MSP, MSP & PT or PL and MP & PL were effective to inhibit the attachment of *L. monocytogenes*. The adhesion of *S. aureus* was reduced by combined use of SE18 & PT, GY & PT, GY & PL, MSP & GY, MSP & PL, SE18 & GY, SE18 & MP, and MSP & PT compared to that of each of the single use. On the other hand, there were no such significant changes in viable counts of all pathogenic bacteria tested by using combination of food additives compared to that of each of the single use. However, the viable counts of *S.* Enteritidis and *P. aeruginosa* decreased drastically in the presence of PL (0.01%) and PT (1%), respectively and reached to the lower detection limit. In addition, the viable counts of *L. monocytogenes* decreased around 4 Log in the presence of PL. These results clearly showed that the combination of MSP and PT or PL effectively reduced adhesion of all the Gram-positive and negative pathogens tested on the microtiter plate. The pretreatment of cabbage leaves with combination of PL & MSP was also effective to reduce the viable counts of secondary-contaminated *S.* Enteritidis on the cabbage leaves by washing with water compared to that of untreated cabbage leaves.



## 1. Introduction

Foodborne illness is a major public health problem worldwide in terms of numbers of affected people and in resultant economic losses. There is increasing concern in the food sector with the growth and presence of the foodborne pathogen in processing plants. In the last few years, outbreaks of foodborne illness linked to the consumption of fresh produce have rapidly increased (Warriner, Huber, Namvar, Fan, & Dunfield, 2009). Among the pathogenic bacteria, *Escherichia coli* O157:H7 and *Salmonella* are the major pathogens causing the outbreaks of foodborne illness associated with fresh produce (Buck, Walcott, & Beuchat, 2003; Food and Drug Administration (FDA), 1998; Warriner et al., 2009).

Since vegetables and fresh-cut produce are typically consumed as raw without heat treatment or sterilization processes, the intervention methods to maintain the safety of those produces is

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very much essential (Schuenzel & Harrison, 2002). Secondary microbial contamination can occur at various stages during production, harvesting, processing, and transportation and this contamination can arise from environmental, animal or human sources (Food and Drug Administration (FDA), 2001; World Health Organisation/Food and Agriculture Organization (WHO/FAO), 2008). Further, cutting, slicing or peeling can cause tissue damage leading to leakage of nutrients and result in promotion of the growth of inhabitant and secondary contaminated bacteria (Harris et al., 2003).

Post-harvest decontamination of fresh produce, usually with sodium hypochlorite, is an important method for pathogen reduction (Warriner et al., 2009). However, there are several factors limiting its effectiveness include the hydrophobicity of plant surfaces, internalization of pathogens within plant tissue as well as biofilm formation by bacteria (Burnett & Beuchat, 2000; Whipps, Hand, Pink, & Bending, 2008).

Biofilm is an assemblage of the microbial cells adherent to each other and/or to a surface and usually enclosed in a matrix of exogenous polymers secreted from the microbes (Costerton, Stewart, & Greenberg, 1999). Attached microorganisms are not easily removed by washing with water or antibacterial agents. Several chemical agents such as sodium or calcium hypochlorite, ethanol, hydrogen peroxide, chlorine dioxide, and a variety of detergents partially can reduce the bacterial populations (Beuchat, 1997; Gandhi, Golding, Yaron, & Matthews, 2001). These chemical sanitizers are generally unable to remove most of the bacteria in biofilm due to the protection by the matrix of exopolymers.

Many pathogenic bacteria including *Salmonella*, *Bacillus*, *Listeria*, *Staphylococcus* and *Escherichia* are capable of adhering and forming biofilm on metal, glass, plastic or rubber surfaces (Elhariry, 2008; Ryu, Kim, & Beuchat, 2005; Sinde & Carballo, 2000). However, there are some investigations that involved in adhering and forming biofilm on the surface of fresh vegetables. *Salmonella enterica* was shown to form biofilms on several surfaces including plants leaves (Joseph, Otta, Karunasagar, & Karunasagar, 2001; Lapidot, Romling, & Yaron, 2006; Sinde & Carballo 2000).

There are very few sanitizers available in the market are permissible to use for the decontamination of vegetables and fruits and most of them are unable to eliminate the pathogenic bacteria adhered to the vegetables and fruits surfaces, therefore, food additives could be the alternative for the decontamination of vegetables. Food additives are safe to use in the viewpoint of eliminating potential biofilm bacteria from vegetable surfaces. The commonly used microtiter plate method for determining bacterial adhesion to and biofilm formation on plastic surface and the effects of food additives was applied to understand the adhesion and inhibition process of biofilm bacteria (Rode, Langsrud, Holck, & Møretrø, 2007). This research result has been used for the selection of particular food additives that can effectively inhibit the initial attachment of biofilm forming bacteria on vegetables.

In the previous study, adhesion inhibitory effects of several food additives, such as Polylysine (PL), Sucrose fatty acid Ester (SE) with fatty acid of C8 to C18, Gardenia Yellow (GY), Monascus Pigment (MP), Protamine (PT) and Whey Protein (WP) on several pathogenic bacteria onto microtiter plate have been shown (Miyamoto et al., 2011).

Therefore, in this study, we have examined adhesion inhibition and antibacterial activities by combination of some selected food additives against *Salmonella* Enteritidis, *Salmonella* Typhimurium, *Listeria monocytogenes*, *Pseudomonas aeruginosa,* and *Staphylococcus aureus* onto microtiter plate. In addition, we also investigated the effects of pretreatment with the combination of Milk Serum Protein (MSP) & PL on the adhesion of *S.* Enteritidis on cabbage leaf.

## 2. Materials and methods

### 2.1. Bacterial strain and culture condition

*Salmonella* Enteritidis NBRC3313, *Salmonella* Typhimurium NBRC12529, *P. aeruginosa* NBRC13275 and *S. aureus* NBRC13276 were obtained from NITE Biological Research Center (NBRC), Kazusakamatari, Kisarazu-shi, Chiba, Japan. *L. monocytogenes* no.185 was kindly provided by Public Health Center, Saku, Nagano, Japan. *Salmonella* Enteritidis IFO3313 was electrophoretically transformed with a plasmid vector, pEGFP (BD Biosciences, USA), carrying a gene for an enhanced green fluorescent protein (EGFP). The transformed strain was designated S. Enteritidis-EGFP.

Luria–Bertani broth (LB, Becton, Dickinson and Company) was used to culture *S.* Typhimurium and *S.* Enteritidis. Tryptic Soy Broth (TSB, Becton, Dickinson and Company) was used to culture *L. monocytogenes* and *P. aeruginosa*. These bacteria were cultured overnight at 30 °C with shaking at 130 rpm to obtain cells in stationary phase of growth. *S. aureus* was cultured overnight in TSB supplemented with 2% NaCl at 37 °C with shaking at 130 rpm to obtain cells in stationary phase of growth.

For adhesion or adhesion inhibition tests, 0.1% Bacto-Soytone (Difco Laboratories) was used for *S.* Typhimurium and *S.* Enteritidis, Brain Heart Infusion broth (BHI, OXOID) for *L. monocytogenes*, 1/5 BHI broth for *P. aeruginosa*, and 1/5 TSB supplemented with 2% NaCl for *S. aureus* (Miyamoto et al., 2011).

For the test using cabbage leaf, a loopfull of *S.* Enteritidis from the stock culture stored at 4 °C in TSA plate was inoculated into 5 mL of Luria–Bertani broth (LB, Becton, Dickison and Company) supplemented with 500 ng ampicillin and incubated at 30 °C overnight with shaking at 130 rpm. Then, 10 μL of 1000-fold diluted culture broth was transferred again into 5 mL of LB supplemented with 500 ng ampicillin. These bacteria were cultured at 30 °C for 21–24 h with shaking at 130 rpm to obtain cells in stationary phase of growth. The bacterial cells were harvested by centrifugation for 5 min at 13,000 rpm and 4 °C. The cells were suspended in sterile water. The optical density at 660 nm ($OD_{660}$) of the cell suspension was adjusted to 0.12 with sterile water. The suspension was used for contamination of cabbage leaf.

### 2.2. Adhesion inhibition test

Adhesion inhibition test was done according to the method of Miyamoto et al., (Miyamoto, Kawagichi, Shimotsu, Kawagishi, & Honjoh, 2009). Each of the bacterial cultures was adjusted to $OD_{660} = 0.7$ with a suitable medium mentioned above. After 150 μL of the bacterial suspension were inoculated into a well of 96-well microtiter plate (made of polystyrene, SANPLATEC Co., Ltd., Osaka, Japan), 50 μl of adhesion inhibitor was added to the well. The mixture was incubated at 30 °C for 24 h for *S.* Enteritidis, *S.* Typhimurium and *L. monocytogenes*, at 30 °C for 48 h for *P. aeruginosa*, at 37 °C for 24 h for *S. aureus*.

Among the food additives, Sucrose Fatty Acid Ester (SE) C18 were obtained from Mitsubishi-Kagaku Foods Corporation, Tokyo, Japan; Gardenia Yellow (GY) & Monascus Pigment (MP) from Wako Pure Chemical Co. Ltd., Tokyo, Japan; Protamine (PT) and Milk Serum Protein (MSP) from Asama Chemical Co., Ltd., Tokyo, Japan; ε-polylysine (PL) from Chisso Corporation, Tokyo, Japan. The fatty acid esters were dissolved in water and sterilized in an autoclave at 121 °C for 20 min. The other additives were dissolved in water and filter-sterilized with EB-DISK 25 (pore size 0.2 μm, Kanto Chemical