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Effect of Selected Food Additives on Biofilm Formation by Foodborne Pathogens on Stainless Steel

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The effects of food additives, including sucrose fatty acid ester with C18 (SFE C18) and monoglycerin fatty acid ester with C18 (MFE C18), gardenia yellow pigment (GY), *monascus* pigment (MP), milk serum protein (MSP), protamine (PT) and polylysine (PL) on biofilm formation by pathogenic and spoilage bacteria on stainless–steel were investigated. *Salmonella* Enteritidis, *S*. Typhimurium, *Pseudomonas aeruginosa*, *P. fluorescens, Listeria monocytogenes* and *Staphylococcus aureus* were cultivated with food additives at different concentrations, followed by evaluation of biofilm formation and viable counts after 24 or 48 h. SFE C18 inhibited biofilm formation by *S*. Enteritidis, *S*. Typhimurium and *L. monocytogenes* at 0.005% (w/w). The inhibition of biofilm formation by MFE C18 was weaker than that of SFE C18. GY reduced more than 50% of biofilm formation by *S*. Typhimurium even at 0.01%. MP greatly decreased biofilm formation of *S*. Enteritidis at 0.1%. MSP inhibited biofilm formation of *S*. Enteritidis and *S. aureus* at 0.0025%, but not those of *S*. Typhimurium, *P. aeruginosa* and *L. monocytogenes* at 0.025%. PT greatly reduced the biofilm formation of *S*. Enteritidis, *S*. Typhimurium and *S. aureus* with increasing PT concentration from 0.001 to 0.1%. PL inhibited biofilm formation on stainless steel by *S*. Enteritidis, *S*. Typhimurium, and *L. monocytogenes* with increasing PL concentration from 0.001 to 0.1%. In contrast, Biofilm formation of *P. aeruginosa* was promoted by GY, PT, and PL, that of *S. aureus* by MP, and *P. fluorescens* by PT at the concentrations effectively decreased those of the other bacteria. Especially, 0.1% PT and 0.01% PL killed planktonic cells of *P. aeruginosa* though increased the biofilm formation of the bacterium.

Key words: biofilm, food additives, foodborne–pathogens, stainless steel

INTRODUCTION

Biofilms are sessile and complex communities where bacterial cells are embedded in a matrix of extracellular polymeric substance (EPS) produced by the microorganisms. Compared with their planktonic counterparts, bacteria in the biofilms are far more difficult to eradicate because of the enhanced resistance to antimicrobials. Various pathogenic bacteria such as *Salmonella, Listeria, Pseudomonas* and *Staphylococcus* have been linked to foodborne disease outbreaks from consumption of dairy products, chicken, fruits and packaged salads. According a report regarding global foodborne burden published by the WHO, pathogenic bacteria accounted for over 50 percentage of the approximately 600 million cases of foodborne illness globally in 2010 (World Health Organization, 2015). Therefore, biofilm formation by food–borne pathogens during food processing is always a great concern especially in fresh produce safety, as biofilms on food processing equipment and contact surfaces can be continuous sources of contamination and lead to cross–contamination of bacteria in the food processing environments (Beuchat, 2002).

Because of abundant nutrients and water in food processing lines, pathogenic and spoilage bacteria are more prone to form biofilms on surfaces of utensils and equipment, it is of particular importance for food manufacturing industry to depend on regular cleaning and disinfection procedure for the purpose of ensuring food safety and quality. However, biofilms located in the inaccessible areas of some food processing equipment can easily evade cleaning treatments (Diaz *et al*., 2016). In addition, commonly used chemical disinfectant, such as sodium hypochlorite and its derivatives are not preferred especially during fresh–cut industry owing to the increasing concerns of their toxicity and safety issues (Meireles *et al*., 2016).

Thus, developing alternative disinfectants has always been a hot spot in the area of reducing prevalence of foodborne pathogens in food industry. Previously, we have reported that several selected food additives, such as Sucrose fatty acids ester (SFE) with fatty acid of C8 to C18, Monoglycerin fatty ester (MFE) with fatty acid of C8 to C18, Gardenia yellow pigment (GY), *Monascus* pigment (MP), Protamine (PT), ε-Polylysine (PL), and Milk serum protein (MSP) were effective to inhibit the initial attachment of several common pathogenic bacteria onto plastic surfaces of microtiter plate (Miyamoto *et al*., 2011; Islam *et al*., 2014). Also, the inhibition effect can be enhanced by combination of some selected food additives on microtiter plate and proved to be useful in reducing secondary–contami-

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nated *Salmonella* on cabbage leaf, lettuce and radish sprouts (Islam *et al*., 2014, 2016).

The present study aimed to investigate the inhibitory effect of these food additives including SFE C18, MFE C18, GY, MP, PT, PL and MSP on biofilms by *S*. Enteritidis, *S*. Typhimurium, *Pseudomonas aeruginosa*, *P. fluorescens, Listeria monocytogenes*, and *Staphylococcus aureus* on stainless–steel surfaces.

MATERIALS AND METHODS

Bacterial strains and culture condition

The 4 different bacterial species used in this study and their primary incubation conditions are listed in Table 1. *Salmonella* Enteritidis NBRC3313, *S*. Typhimurium NBRC12529, *Pseudomonas aeruginosa* NBRC13275 and *Staphylococcus aureus* NBRC13276 were obtained from NITE Biological Research Center (NBRC), Kazusakamatari, Kisarazu–shi, Chiba, Japan. *Pseudomonas fluorescens* FHC was isolated from lettuce and identified with RiboPrinter system in our laboratory. *Listeria monocytogenes* No. 185 was kindly offered by Public health center, Saku, Nagano, Japan. The stock cultures were maintained on Tryptic soy agar (TSA; Becton Dickinson, Franklin Lakes, NJ, USA) slants (TSA with 2% NaCl for *S. aureus*) at 4°C and each strain was activated twice by transferring a loopful of bacteria to 5 mL of Luria–Bertani broth (LB; Becton Dickinson, Franklin Lakes, NJ, USA) or Tryptic soy broth (TSB; Becton Dickinson, Franklin Lakes, NJ, USA), and incubated overnight at 30°C or 37°C with shaking at 130 rpm to obtain cells in stationary phase of growth. Then, 10 μL of 1000–fold diluted culture broth was transferred and incubated at same condition. The cells were harvested by centrifugation (6,000×*g* for 5 min at 4°C) and the final pellets were resuspended with sterile water to an OD_{660} of 0.7, corresponding to approximately 10^9–10^{10} colony–forming units (CFU)/mL.

Preparation of stainless-steel washers and food additives solutions

Stainless–steel washers (SUS304, inner diameter 2.3 mm, outer diameter 6 mm, thickness 0.4 mm, unpolished) were used in this study. Prior to the experiments, washers were soaked in 10% (v/v) alkaline detergent (DKS Co., Ltd., Kyoto, Japan) and ultrasonic cleaned by SONO Cleaner 100a (Kaijo Corporation, Matsumoto, Nsgano, Japan) for 30 min. Washers were rinsed with distilled water for 3 times, followed by immersion in 70% alcohol and air dried in clean bench.

Protamine (PT) and Milk serum protein (MSP) ASAMA were provided by Asama Chemical Co., Ltd., Tokyo, Japan, Polylysine (PL) was purchased from Chisso Corporation, Tokyo, Japan, Sucrose fatty acid esters (SFE) was purchased from Mitsubishi–Kagaku Foods Corporation, Tokyo, Japan. *Monascus* pigment (MP) and Gardenia yellow (GY) were purchased from Wako pure Chemicals Co., Ltd, Tokyo, Japan. Monoglycerin fatty acid esters (MFE) were the products of Taiyo Kagaku Co., Ltd., Tokyo, Japan.

Food additive solutions were prepared as described by Miyamoto *et al*. (2011). For SFE C18 and MFE C18, two concentrations (0.005%, 0.05%) were prepared by dissolving in pure water and autoclaved at 121°C for 20 min, for GY & MP (0.01%, 0.1%), PT & PL (0.001%, 0.01%, 0.1%) and MSP (0.0025%, 0.025%), they were dissolved in water and filter–sterilized with EB–DISK 25 (pore size 0.2 μm, Kanto Chemical Co., Ltd., Tokyo, Japan).

Biofilm inhibition test on stainless-steel washers

Adhesion or adhesion inhibition tests were done in 0.1% Bacto–Soytone (BS, Becton-Dickinson, Franklin Lakes, NJ, USA) for *S.* Typhimurium, Brain Heart Infusion Broth (BHIB, Oxoid Ltd., Hampshire, UK) for *L. monocytogenes*, 1/5 BHIB for *P. aeruginosa* and *P. fluorescens*, and 1/5 TSB supplemented with 2% NaCl for *S. aureus*. Prepared stainless–steel washers were transferred to sterile round–bottom 96–well microtiter plates (SANPLATEC corp., Osaka, Japan) containing 100 μL of media prepared at twice the concentration. Then, 50 μL of prepared cell suspension and 50 μL of food additive solutions were added to the well. For the control samples, 50 μL food additive solutions were replaced with sterile water. Eight wells were used for every concentration of food additive solution. The plates were subsequently incubated under static conditions. The media and conditions for biofilm formation are shown in Table 1.

Quantification of biofilm cells on stainless–steel washers

After incubation for 24 or 48 h, the stainless–steel washers were transferred to a new round-bottom plate and rinsed with 200 μL phosphate-buffered saline (PBS,

Table 1. Bacterial strains, pre–culture and biofilm formation condition used in this study

Strains	Incubation conditions	Biofilm formation condition
Salmonella Enteritidis NBRC3313	LB[a], 30°C	0.1% BSb[c], 30°C, 24 h
Salmonella Typhimurium NBRC12529	LB[a], 30°C	0.1% BSb[c], 30°C, 24 h
Pseudomonas aeruginosa NBRC13275	TSB[b], 30°C	1/5 BHI[d], 30°C, 48 h
Pseudomonas fluorescens FHC	TSB[b], 30°C	1/5 BHI[d], 30°C, 48 h
Listeria monocytogenes No.185	TSB[b], 30°C	BHI[d], 30°C, 24 h
Staphylococcus aureus NBRC13276	TSB[b] with 2% NaCl, 37°C	1/5 TSB[b] with 2% NaCl, 37°C, 24 h

[a] Luria Broth (LB, Becton-Dickinson, Sparks, MD, USA), [b] Tryptic soy broth (TSB, Becton–Dickinson, Sparks, MD, USA), [c] Bacto–soytone (BSb, Becton–Dickinson, Sparks, MD, USA), [d] Brain heart infusion (BHI, Oxoid Ltd., Hampshire, UK)

1.47 mM KH_2PO_4, 8.10 mM Na_2HPO_4, 2.68 mM KCl, 137 mM NaCl, pH 7.4) to remove planktonic cells and loosely attached bacterial cells. After drying on paper tissues (Nippon Paper Crecia Co., Ltd., Tokyo, Japan), the washers were transferred to another new round–bottom plate containing 100 μL of 99% (v/v) ethanol and immersed for 5 min. The washers were dried again and then stained with 100 μL of 1% crystal violet (AMRESCO, Ohio, USA) for 5 min in a new round–bottom plate. The stained washers were gently rinsed twice using distilled water and dried in air. The total of 8 washers of each sample were collected and transferred to 2 mL test tube containing 300 μL of 99% (v/v) ethanol, crystal violet was solubilized by ultrasonic agitation at room temperature for 30 min. One–hundred and fifty of the mixture was transferred to a new flat–bottom plate, and its optical density was determined at 595 nm (OD_{595}; model 450, Bio–Rad Laboratories Japan, Tokyo, Japan).

Enumeration of the planktonic cells

Viable counts of bacteria were performed using conventional plating method as previously described by Miyamoto *et al*. (2009). After incubation with the food additives, 100 μL of the cell suspension was pipetted from the wells, and serially diluted using sterile PBS. The serially diluted sample (100 μL) was plated on TSA (TSA with 2% NaCl for *S. aureus*) and incubated at 37°C (30°C for *P. aeruginosa* and *P. fluorescens*) for 24 h before colony counting.

Statistical analysis

The relative adhesion of biofilm on the washers is expressed as the percentage of the non–treatment control formed biofilm. The viable cell counts are expressed as log values. The results of relative adhesion of biofilm on the washers and viable cell count are the average of two experimental replicates. Statistical analyses were performed using Microsoft Excel for Mac build 16.36 (Microsoft, Redmond, WA, USA).

RESULTS

Effects of sucrose fatty acid ester

The effects of SFE C18 on biofilm formation on stainless steel and viability of bacteria are shown in Figure 1. Biofilm formation of *S*. Enteritidis, *S*. Typhimurium and *L. monocytogenes* were strongly inhibited in the presence of 0.005% SFE C18 by nearly 70%, 85% and 40%, respectively (Fig. 1A, B and E). In contrast, SFEC18 did not inhibit biofilm formation of *P. aeruginosa*, and *S. aureus* at all the concentrations tested (Fig. 1C and F). In the case of *P. fluorescens*, biofilm formation was reduced by about 50% at 0.05% (Fig. 1D). Viable bacterial counts of the planktonic cells did not change largely in the presence of SFE C18 even at 0.05%.

Effects of monoglycerin fatty ester

The effects of MFE C18 on biofilm formation on stainless steel and viability of bacteria are shown in Figure 2. MFE C18 inhibited biofilm formation of *S*. Enteritidis and *P. aeruginosa* with the increase of the concentration. At 0.05% MFE C18, biofilm mass of *S.* Enteritidis and *P. aeruginosa* was reduced to about 50% and 60%, respectively (Fig. 2A and C). Similarly, MFE C18 at 0.005 and 0.05 % caused about 50% reduction of

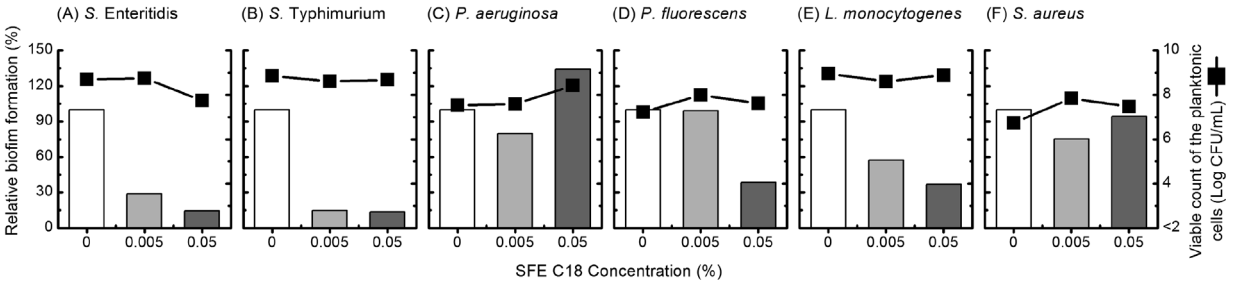

Fig. 1. Effect of SFE C18 on biofilm formation on stainless–steel. Bars and lines show the relative biofilm formation and viable counts of planktonic cells. (A) *S.* Enteritidis, (B) *S.* Typhimurium, (C) *P. aeruginosa*, (D) *P. fluorescens*, (E) *L. monocytogenes*, (F) *S. aureus*.

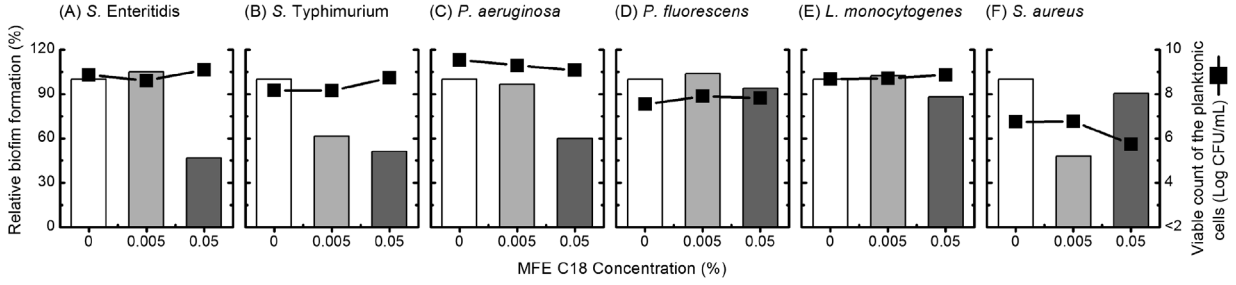

Fig. 2. Effect of MFE C18 on biofilm formation on stainless–steel. Bars and lines show the relative biofilm formation and viable counts of planktonic cells. (A) *S.* Enteritidis, (B) *S.* Typhimurium, (C) *P. aeruginosa*, (D) *P. fluorescens*, (E) *L. monocytogenes*, (F) *S. aureus*.

biofilm formation of *S*. Typhimurium (Fig. 2B). In the case of *S. aureus*, MFE C18 inhibited biofilm formation at 0.005%, but not at 0.05%. In contrast, viable counts of *S. aureus* decreased by about 1–log in the presence of 0.05% MFE C18 (Fig. 2F). On the other hand, biofilm formation of *P. fluorescens* and *L. monocytogenes* did not change in the presence of the MFE (Fig. 2D and E).

Effects of gardenia yellow pigment

The effects of GY on biofilm formation on stainless steel and viability of bacteria are shown in Figure 3. GY was effective to inhibit biofilm formation of *S*. Enteritidis, *S*. Typhimurium and *S. aureus* (Fig. 3A, B and F). Specifically, GY was more effective on *S*. Typhimurium than the other two strains, showing 70% decrease in biofilm formation at 0.01% (Fig. 3B). Instead, GY did not affect biofilm formation of *P. fluorescens* and *L. monocytogenes* at all the concentrations tested (Fig. D and E). However, it promoted biofilm formation by *P. aeruginosa* (Fig. 3C). Viable bacterial counts were slightly decreased by about 1 log as the increasing concentration of GY in the case of *S*. Enteritidis, *S*. Typhimurium, *P. aeruginosa*, and *S. aureus*.

Effects of monascus pigment

The effects of MP on biofilm formation on stainless steel and viability of bacteria are shown in Figure 4. Generally, MP decreased biofilm formation of *S*. Enteritidis and *P. aeruginosa* on stainless–steel washers by about 70% (Fig. 4A) and 50% (Fig. 4C) at 0.1% without affecting viability. Contrary, MP promoted biofilm formation by *S. aureus* (Fig. 4F). It did not affect biofilm formation of *L. monocytogenes* and *P. fluorescens* (Fig. 4D and E).

Effects of milk serum protein

The effects of MSP on biofilm formation on stainless steel and viability of bacteria are shown in Figure 5.

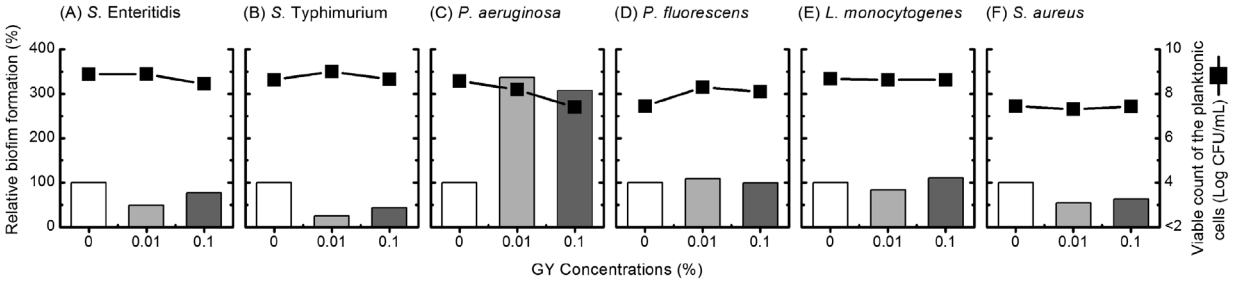

Fig. 3. Effect of GY on biofilm formation on stainless–steel. Bars and lines show the relative biofilm formation and viable counts of planktonic cells. (A) *S*. Enteritidis, (B) *S*. Typhimurium, (C) *P. aeruginosa*, (D) *P. fluorescens*, (E) *L. monocytogenes*, (F) *S. aureus*.

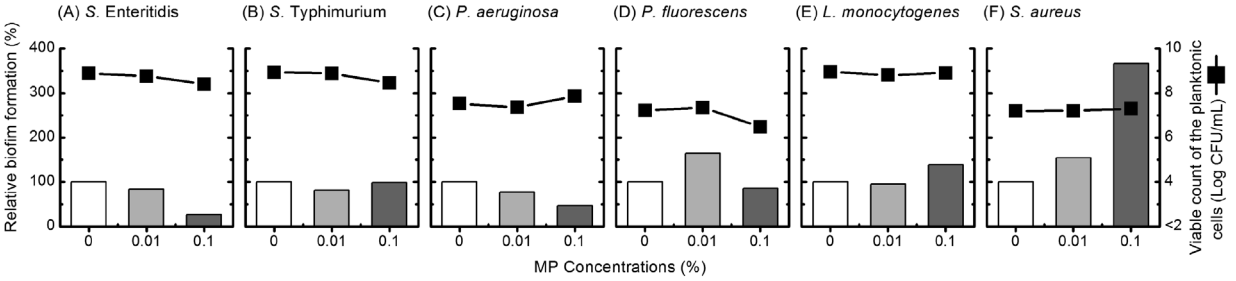

Fig. 4. Effect of MP on biofilm formation on stainless–steel. Bars and lines show the relative biofilm formation and viable counts of planktonic cells. (A) *S*. Enteritidis, (B) *S*. Typhimurium, (C) *P. aeruginosa*, (D) *P. fluorescens*, (E) *L. monocytogenes*, (F) *S. aureus*.

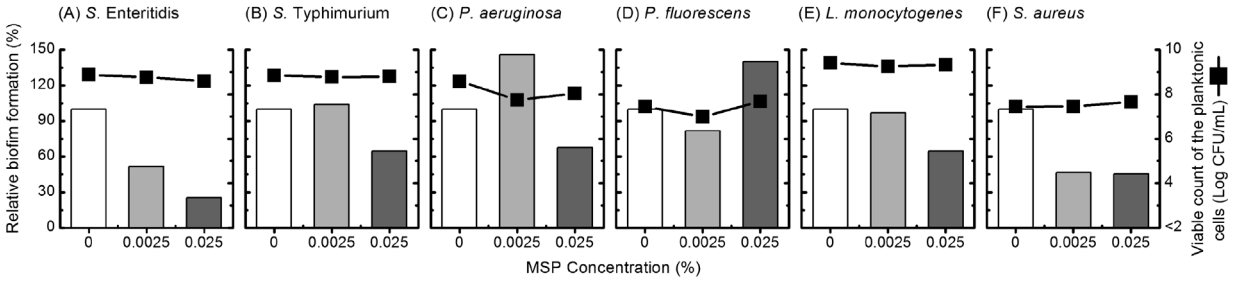

Fig. 5. Effect of MSP on biofilm formation on stainless–steel. Bars and lines show the relative biofilm formation and viable counts of planktonic cells. (A) *S*. Enteritidis, (B) *S*. Typhimurium, (C) *P. aeruginosa*, (D) *P. fluorescens*, (E) *L. monocytogenes*, (F) *S. aureus*.

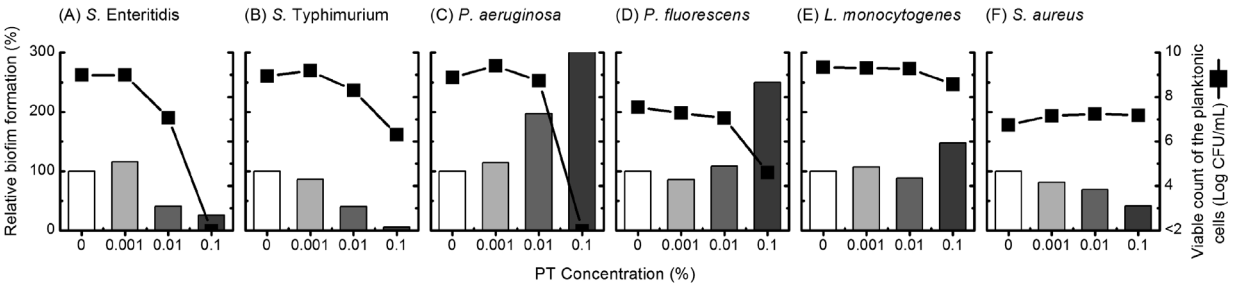

Fig. 6. Effect of PT on biofilm formation on stainless–steel. Bars and lines show the relative biofilm formation and viable counts of planktonic cells. (A) *S*. Enteritidis, (B) *S*. Typhimurium, (C) *P. aeruginosa*, (D) *P. fluorescens*, (E) *L. monocytogenes*, (F) *S. aureus*.

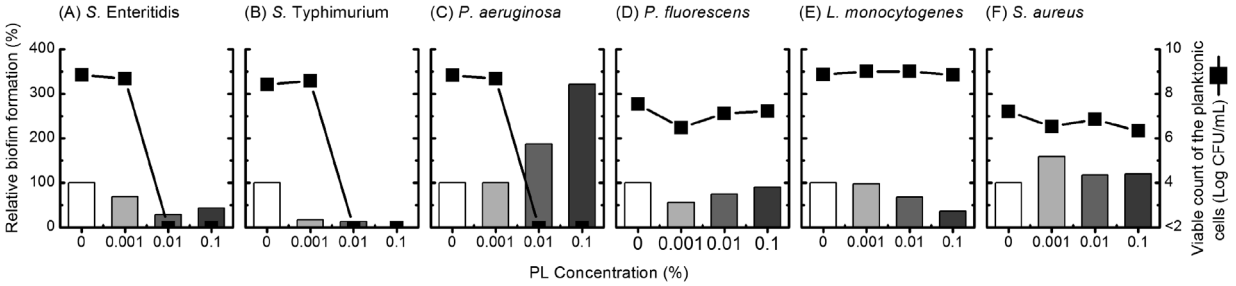

Fig. 7. Effect of PL on biofilm formation on stainless–steel. Bars and lines show the relative biofilm formation and viable counts of planktonic cells. (A) *S*. Enteritidis, (B) *S*. Typhimurium, (C) *P. aeruginosa*, (D) *P. fluorescens*, (E) *L. monocytogenes*, (F) *S. aureus*.

MSP showed inhibitory effect on biofilm formation of bacteria tested except for *P. fluorescens* (Fig. 5D). Biofilm mass of *S*. Enteritidis, *S*. Typhimurium, *P. aeruginosa* and *L. monocytogenes* were decreased with increasing concentration of MSP. Especially in *S*. Enteritidis, biofilm mass was decreased more than 70% in the presence of 0.025% MSP (Fig. 5A). For *S. aureus*, nearly 55% reduction of biofilm formation was observed at 0.0025% and 0.025% (Fig. 5F). MSP did not decrease the viability of all the tested bacteria even at 0.025%.

Effects of protamine

The effects of PT on biofilm formation on stainless steel and viability of bacteria are shown in Figure 6. PT greatly reduced the biofilm formation of *S*. Enteritidis, *S*. Typhimurium and *S. aureus* with increasing PT concentration. In the presence of 0.1% PT, biofilm mass was decreased by about 74, 95, 55 % in *S*. Enteritidis, *S*. Typhimurium and *S. aureus*, respectively (Fig. 6A, B and F). On the contrary, biofilm mass increased in *P. aeruginosa*, *P. fluorescens* and *L. monocytogenes* in the presence of 0.1% PT (Fig. 6C, D and E). Viable counts of planktonic cells largely decreased in *S*. Enteritidis, *P. aeruginosa* and *P. fluorescens* with increasing PT concentration, but not in *L. monocytogenes* and *S. aureus*.

Effect of polylysine

The effects of PL on biofilm formation on stainless steel and viability of bacteria are shown in Figure 7. PL inhibited biofilm formation on stainless steel by *S*. Enteritidis, *S*. Typhimurium, and *L. monocytogenes* with increasing PL concentration. The inhibitory effect was most strongly exerted on *S*. Typhimurium, being nearly 100% inhibition in the presence of 0.1% PL (Fig. 7B). Biofilm formation of *P. fluorescens* was slightly decreased by PL (Fig. 7D). However, PL was not effective on the biofilm formation of *P. aeruginosa* and *S. aureus* (Fig. 7C and F). Biofilm mas increased in *P. aeruginosa* in the presence of 0.01 and 0.1% PL in spite of the significant decrease in the viability of the planktonic cells (Fig. 7C). Significant decrease in the viability at 0.01% PL was also observed in *S*. Enteritidis, and *S*. Typhimurium, but not in *P. fluorescens*, *L. monocytogenes*, and *S. aureus*.

DISCUSSION

Biofilm formation of foodborne and spoilage bacteria is a particular concern for food manufacturing industries, as the occurrence of undesirable biofilms during food processing and storage may lead to foodborne illness, as well as food spoilage (Srey *et al*., 2013; Whitehead and Verran, 2015). However, it still remains challenging to find ideal strategies to control biofilm in food processing environment. In this study, effects of some selected food additives, which were proved to be effective on plastic surfaces for inhibiting biofilm formation of some bacteria (Miyamoto *et al*., 2011), were investigated on bacterial biofilm formation on stainless steel.

It was early demonstrated that fatty acid especially those with more than 12 carbons had the potent antimicrobial properties against Gram–positive bacteria

(Kabara *et al*., 1972), and their derivatives by esterifying the fatty acid with polyhydric alcohols showed even stronger antimicrobial activity (Akoh, 1994). Based on these findings, more selective studies focused on antibacterial spectrum of their derivatives like sucrose fatty acid esters and monoglycerin fatty acid esters have been performed. For example, Conley and Kabara (1973) found that sucrose oleate and sucrose linoleate were effective against Gram–positive bacteria but not against Gram–negative bacteria. Furukawa *et al*. (2010) tested a wide range of food additives against biofilm formation by foodborne–pathogenic bacteria and observed that sugar fatty acid esters with 14–16 carbon chains possessed inhibitory effect on biofilm formation by *L. monocytogenes* at 0.01% (w/w), but not on bacterial growth. Similarly, Schlievert and Peterson (2012) reported that glycerol monolaurate inhibited biofilm formation by *S. aureus* and *Haemophilus influenzae* in microtiter plates. In this study, SFE and MFE were both effective in reducing biofilm formation by *S*. Enteritidis and *S*. Typhimurium on stainless steel (Fig. 1 and 2). In addition, SFE also inhibited biofilm formation of *P. fluorescens* and *L. monocytogenes* (Fig. 1). These results on stainless steel were basically in accordance with the previous observations on microtiter plates mentioned above. The inhibitory activity of SFE and MFE is thought to be attributed to their amphipathic properties, which usually lead to destabilization of cell membrane, increased cell permeability or even cell lysis (Yoon *et al*., 2018).

Gardenia yellow is a natural colorant extensively used in food industry. Crocetin derivatives like crocin is one of the major components in GY, and their unique carotenoid–like polyene structure was reported to prevent *S*. Enteritidis from binding to collagen (Miyamoto *et al*., 2003) and adhering to microtiter plate (Miyamoto *et al*., 2009). Also, gentiobiose (a component of crocin) is presumed to bind to the surface of bacterial cells because of its hydrophilicity, which consequently inhibits the interactions between bacterial cell and the surfaces (Miyamoto *et al*., 2011). The biofilm formation of *S*. Enteritidis, *S*. Typhimurium and *S. aureus* on stainless steel surface were weakly inhibited by GY in this study. These results were similar to those reported on plastic surface (Miyamoto *et al*., 2011).

Monascus pigment has long been used as a natural food colorant in East Asia (Dufossé *et al*., 2005). It has been reported that MP has variety of biological activities including antimicrobial activities. Two orange constituents of MP, rubropunctatin and monascorubrin, were found to have strong antibiotic activities against *Bacillus subtills* and *Candida pseudotropicalis* (Martinkova, 1999). Natural pigments produced by *Monascus ruber* also showed antibacterial activity against *S. aureus*, *Escherichia coli* and *S*. Enteritidis (Vendruscolo *et al*., 2014). Biofilm formation of *S*. Enteritidis and *P. aeruginosa* was inhibited while that of *S. aureus* was promoted by 0.1% of MP, without affecting viability. The result on *S. aureus* is quite different from that by Miyamoto *et al*. (2011) reporting inhibition of the adhesion *S. aureus* on microplate by MP. It seems important to know the difference in the mechanism for biofilm formation of *S. aureus* on polystyrene and stainless–steel surfaces for developing an effective method for inhibiting biofilm formation by *S. aureus*.

Milk serum refers to the milk minus milk fat globules and casein micelles, and the soluble protein separated and concentrated from milk serum is called milk serum protein (Walstra, 1999). Because it is produced without exposure to enzymes or chemicals in the cheese–making process, it is regarded as "native" whey proteins (Evans *et al*., 2009). The whey protein includes bioactive compounds like β–lactoglobulin and α–lactalbumin, as well as some antibacterial peptides, which have been reported to control of microbial infections (Atanasova and Ivanova, 2010). In the present study, MSP reduced biofilm formation of *S*. Enteritidis and *S. aureus* but not those of *Pseudomonas*. To know the detailed inhibitory mechanism for biofilm formation, effects of each of the components of MSP will be further investigated.

Protamine is a broad–spectrum antimicrobial peptide existed in the sperm cells of vertebrates like fish (Truelstrup Hansen *et al*., 2001). Protamine is usually positively charged due to the high content of arginine. It attaches to negatively charged cell surface through electrostatic interactions, causing leakage of K^+, ATP and intracellular substances and exerting antibacterial action (Islam *et al*., 1987; Johansen *et al*., 1997; Stumpe and Bakker, 1997). Protamine has been studied to control pathogens including *E. coli* (Hansen and Gill, 2000), *L. monocytogenes* (Uyttendaele and Debevere, 1994), *P. aeruginosa* (Boussard *et al*., 1994) and *S.* Typhimurium (Aspedon and Groisman, 1996). Likewise, polylysine is also a natural antimicrobial peptide with positive charge, and has antibacterial activity against Gram–positive and Gram–negative bacteria, yeast and fungi (Hiraki, 2000). The antibacterial mechanism of polylysine is thought to be similar with protamine (Ye *et al*., 2013; Hyldgaard *et al*., 2014; Lin *et al*., 2018; Li *et al*., 2019), but its antibacterial activity is stronger than PT (Conte *et al*., 2007). In our previous study, PT and PL significantly inhibited the biofilm formation of the same bacteria tested in this study on plastic surface (Miyamoto *et al*., 2011). In this study, the biofilm formation on the surface of stainless steel was largely decreased in *S*. Enteritidis, *S*. Typhimurium and *S. aureus*, but increased in *P. aeruginosa*, *P. luorescens* and *L. monocytogenes* by 0.1% PT (Fig. 6). Meanwhile, the viable counts of planktonic cells of Gram–negative bacteria were significantly decreased by PT at the same concentration. Similar to PT, 0.01% PL inhibited biofilm formation of *S*. Enteritidis and *S*. Typhimurium but promoted that of *P. aeruginosa*. Almost all the planktonic cells of these 3 species were killed by PL at the same concentration (Fig. 7). These results on biofilm formation of *Pseudomonas* on stainless–steel surface were quite different from the results on plastic surface, suggesting that cellular components released from *Pseudomonas* cells killed by PT and PL attached and accumulated on the surface of stainless steel.

This study suggests that different pathogens have diverse response during their exposure to the food additives on the stainless-steel surface. Application of some of selective food additives is promising to control bacterial biofilm formation of pathogenic and spoilage bacteria in food processing environments though the selection of the additive suitable to the target bacteria is important.

AUTHOR CONTRIBUTIONS

C. SHEN performed the analysis and wrote the paper. C. MACHIDA performed the experiments and collected the data. Y. MASUDA and K. HONJOH commented on the manuscript. T. MIYAMOTO designed the study, supervised the work, and wrote the paper. All authors assisted in editing the manuscript and approved the final version.

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