



Resistance of pathogenic biofilms on glass fiber filters formed under different conditions

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Received: 5 February 2020/Revised: 5 May 2020/Accepted: 6 May 2020/Published online: 27 May 2020
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Abstract This study investigated the survivals of two pathogens (*Escherichia coli* O157:H7 and *Staphylococcus aureus*) in different adhered forms on glass fiber filters (GFFs) at 43 and 68% relative humidity (RH). Efficacies of chemical sanitizers at reducing pathogenic biofilms on GFFs were also evaluated. Inoculated GFFs were incubated at 28 °C in TSB (type I), on TSA (type II), or on TSA under 100% RH (type III) to produce biofilms. When GFFs were incubated at 43 or 68% RH for 7 days, type III biofilms were less than 2 log₁₀ CFU/filter reduction whereas type I and type II biofilms were 4–6 log₁₀ CFU/filter reduction. Additionally, type III biofilms were highly resistant to sanitizing treatment compared than other biofilms (type I and II). Therefore, the method to produce biofilms used in this study could be used to produce highly resistance pathogenic biofilms in the laboratory for related experiments.

Keywords Biofilm · Glass fiber filter · Adhered form · Chemical sanitizer · Relative humidity

Introduction

Microorganisms are widely present in our surroundings as biofilms. Biofilms is a community of microorganisms surrounded in hydrated extracellular polymeric substances (EPSs) that attach to surfaces (Decho and Gutierrez, 2017). Biofilm formation is dependent upon many factors, including microbial species, surface structure, nutrients, environment, temperature, and relative humidity (RH) (Phillips, 2016). In addition, biofilm formation properties of pathogens and their resistance differ significantly in response to different surface materials (Giaouris et al., 2014). For example, more pathogenic bacterial cells attach to hydrophobic surfaces (paper and plastics) than to hydrophilic surfaces (stainless steel and glass) (Jo et al., 2010). Also, *Escherichia coli* O157:H7 produced significantly high levels of biofilm on wooden compared than on stainless-steel, plastic, and glass surfaces (Bang et al., 2014). Rose et al. (2003) reported that two strains of *Yersinia pestis* exhibited significantly greater survival capabilities on paper than other tested surfaces.

Pathogenic biofilm contaminated on the food contact surface can act as a major cause of cross-contamination of pathogenic bacteria to food (Giaouris et al., 2014). Beside biofilms are strongly tolerant against environmental stresses including desiccation and treatment with sanitizers and antimicrobial compounds, compared to planktonic cells (Phillips, 2016). Therefore, pathogens can survive sanitizing treatment in food industry when they form biofilms, ultimately contributing to foodborne illnesses through cross-contamination (Bang et al., 2014). Accordingly, identification of effective methods for controlling pathogenic biofilms on material surfaces in the food industry is critical. There were many researches that have investigated physical or chemical control methods to eliminate

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pathogenic biofilms on material surfaces including ultrasound, ultraviolet (UV) irradiation, peracetic acid, chlorine, hydrogen peroxide, ozone, and so on (Bae et al., 2012; Park et al., 2012). However, the tolerance of pathogenic biofilms or adhered cells on material surfaces to various control methods can differ depending on the adhered form or type of biofilm produced under various conditions. Indeed, pathogenic biofilms produced in hydric environment (100% RH) were highly resistant on the survivals at dry conditions compared than biofilms produced in broth, which was a commonly used method for producing biofilms in the laboratory in previous studies (Bae et al., 2012). Aerosolized sanitizer was less effective for reducing pathogenic biofilms produced under 100% RH compared than biofilms formed in laboratory broth on polyvinyl chloride and stainless steel (Park et al., 2012). Based on these results, biofilms formed under hydrophilic conditions, such as by immersion in laboratory broth, may not possess greater resistance than that of adhered cells or planktonic cells, as expected. Therefore, the effects of sanitizing methods on the inactivation of biofilms formed on material surfaces may have been overestimated in previous studies, as these studies evaluated the effects of sanitizing methods on biofilms produced on material surfaces using the immersion method in laboratory broth (Nguyen and Yuk, 2013; Oliveira et al., 2009). In fact, as food processing environments are known to possess high humidity, biofilms present in real systems may be more resistant to various stresses than are biofilms tested in laboratories. The relative humidity for processing environment range represent 4–75% or over 75% in relatively dry to wet food production environments (Vogel et al., 2010). Therefore, it is necessary to perform laboratory tests using highly resistant forms of biofilms for the evaluation of sanitizing methods to inactivate bacterial biofilms on the surface of materials. Bacteria can form highly resistant biofilms on rough surfaces, microstructures, or filamentous structures than they can on smooth or even surfaces of materials, as they can produce biofilms possessing tightly packed forms or structures. In support of this, a previous study used glass fiber filters (GFFs) to produce a highly resistant form of biofilm, and this allowed for the investigation of the effectiveness of bactericide solutions on the inhibition of bacterial biofilms (Lebert et al., 2007).

Given these previous findings, in this study, we manufactured two pathogenic biofilms (*E. coli* O157:H7 and *S. aureus*) on GFFs produced by different methods, including immersing in broth and placing on agar without or with incubation under humid conditions (100% RH), and we tested their survivals at dry conditions (43% and 68% RH) and against treatments with various chemical sanitizers to determine a means to produce highly resistant forms of pathogenic biofilm in the laboratory. For sanitizing

treatments, five chemical sanitizers including alcohol-, chlorine-, hydrogen peroxide-, iodophor-, and quaternary ammonium-based sanitizers were evaluated to determine an effective sanitizing method to control pathogenic biofilms.

Materials and methods

Bacterial cell preparation

Each three strains of *E. coli* O157:H7 (ATCC 43889, ATCC 43890, and ATCC 35150) and *S. aureus* (ATCC 12600, ATCC 49444, and ATCC 12692) from Chung-Ang University (Anseong-si, Korea) were used in this study. Cultures stored at -80°C in tryptic soy agar (TSB; Difco Laboratories, Detroit, MI, USA) with 20% glycerol were cultured on tryptic soy agar (TSA; Difco) at 37°C for 24 h. Next, colonies on TSA were cultured in TSB at 37°C for 24 h. Cell pellets were harvested by centrifugation at $13,000\times g$ for 3 min at 4°C , washed and resuspended in phosphate-buffered saline (PBS; pH 7.2). Cell suspension (ca. 10^{5-6} CFU/mL) were prepared by dilution with PBS and used in further experiments.

Preparation of GFFs

Type A/E GFFs (Pall Corporation, Mexico; 25 mm diameter, 1270 μm thickness, and 1 μm pore size) were used in this study. GFFs were sterilized before use.

Formation of biofilm on GFFs

GFFs were transferred into sterilized Petri dishes and inoculated with each pathogen using 0.5 mL of each pathogenic suspension of three-strain mixture by depositing droplets. Inoculated GFFs were dried for 2 h at 22°C in a laminar flow biosafety hood to facilitate cell attachment. And then, inoculated GFFs were placed in 50 mL tubes containing TSB (30 mL) and incubated at 28°C . Alternatively, the same inoculated GFFs were placed on TSA in a desiccator containing distilled water to produce humid condition (100% RH) and incubated at 28°C . Each set of for 2 h was removed from TSB or TSA after 0, 1, 3, or 5 days using sterile forceps, washed with PBS, and dried in a hood for 2 h. Following incubation, the numbers of pathogen on GFFs were enumerated as described below.

Preparation of different adhered forms on GFFs

In this study, adhered cells or three different types were prepared on the surfaces of GFFs by of biofilm by biofilm formation stages (bacterial attachment, microcolony

formation, and bacterial biofilm maturation). These included adhered cells, biofilms produced in TSB (type I; bacterial biofilm maturation stage), biofilms produced on TSA (type II; microcolony formation stage), and biofilms produced on TSA under 100% RH (type III; bacterial biofilm maturation stage). Culture cocktails (0.5 mL) of the two pathogens were prepared as described above and inoculated onto the GFF. Following adherence of each pathogen onto GFFs, inoculated GFFs used as adhered cells following drying in a hood for 2 h. Inoculated GFFs were incubated in 50 mL tubes containing TSB (30 mL) at 28 °C for 3 days. Following incubation, GFFs were washed with PBS and dried for 2 h, and these GFFs were used as type I biofilms (produced in TSB). The same inoculated and dried GFFs described above were alternatively placed on TSA and then incubated at 28 °C for 1 day. Following drying for 2 h, these GFFs were considered as type II biofilms (produced on TSA). Additionally, the same inoculated and dried GFFs described above were incubated on TSA in a desiccator containing distilled water to produce humid condition (100% RH) at 28 °C for 3 days. 1 day. Following drying for 2 h, these GFFs were considered as type III biofilms (produced on TSA under 100% RH condition).

Survivals of pathogens in different adhered forms on GFFs

Prepared GFFs were transferred to sterile Petri dishes and then stored under 43% or 68% RH to investigate bacterial survivals in biofilm on GFFs at dry condition. The two RH conditions (43% and 68% RH) were prepared in a desiccator containing 200 mL of potassium carbonate (Samchun Pure Chemical Co., Ltd., Gyeonggi-do, Korea) or lithium acetate (Samchun Pure Chemical Co., Ltd., Gyeonggi-do, Korea), respectively. Survived numbers of pathogens on GFFs in the desiccator were enumerated during incubation for 7 days at 28 °C.

Treatment with chemical sanitizers

For treatment with chemical sanitizers, four types of adhered cell or biofilms on GFFs were treated with distilled water (control) or commercial sanitizers including alcohol-based (70% ethanol, Fisher Scientific Korea, Seoul, Korea; 99.9% ethyl alcohol, 0.02% water), chlorine-based (Clean Shot, Chemical Leader Co., Seoul, Korea), hydrogen peroxide-based (P3-oxonia active, Deasung CNS Co., Korea; 22% hydrogen peroxide, 6% acetic acid, 4% peroxy acetic acid, 0.6% bisphosphoric acid), iodophor-based (Mikroklene DF, Ecolab Inc., USA; 6.5% phosphoric acid, 1.8% 2-butoxy-ethanol, 12.8% polyalkylene colichol butoxy monoether, 2% iodophor, 1.9% sodium iodide), and

quaternary ammonium-based sanitizers (Quat Plus, 3 M, USA; di-n-alkyl dimethylammonium chloride, alkyl dimethyl benzyl ammonium chloride, 6% alkyl dimethyl ethylbenzyl ammonium chloride, ethyl alcohol, sequestering agent). All chemicals were prepared using distilled water according to the manufacturer's instruction before each experiment. GFFs were immersed in 30 mL of each sanitizer or distilled water at 22 °C for 10 min as treatments or control, respectively.

Bacterial quantification

For quantification of the tolerance of pathogens on the surfaces of GFFs, GFFs in a stomacher bag (3 M) stomached for 90 s (Bagmixer 400; Interscience, France) with 20 mL D/E neutralizing broth (Difco). After serial dilution in 0.2% peptone water (PW; Difco), samples were surface plated onto TSA and plates were incubated at 37 °C for 24 h. The number of colonies on TSA were enumerated.

FE-SEM observation

The micro-structures of pathogenic biofilms on GFFs (adhered cells or biofilms) were analyzed using Field emission-scanning electron microscopy (FE-SEM; SIGMA; Carl Zeiss, UK). GFFs were fixed in 2% glutaraldehyde for 4 h and washed twice with 0.05 M sodium cacodylate buffer (pH 7.2). Next, GFFs were immersed in 1% osmium tetroxide in 0.05 M sodium cacodylate buffer for 2 h. Fixed GFFs were dehydrated in graded ethanol series (30, 50, 70, and 100%). Following dehydration, GFFs dried, sputter coated with gold were used for FE-SEM analysis.

Curve fitting

The Weibull distribution was used for fitting the survival curves of pathogens in GFFs: $S(t) = e^{-t/a^n}$ where t is the storage days, and a and n are the scale and shape parameters, respectively (Buzrul and Alpas, 2007). The n parameter accounts for the upward concave curve ($n < 1$), a linear survival curve ($n = 1$), and downward concave curve ($n > 1$) (Fernandez et al., 2009). The least-squares criterion in Graph Pad PRISM (Graph Pad Software, San Diego, CA, USA) was utilized for determining the a and n values. The model fitting performances were statistically analyzed using R -square values in order to ensure the model suitabilities.

Statistical analysis

The data was converted to \log_{10} CFU/filter and averages values of three replicated experiments were evaluated in this study. D -values of pathogens were calculated from the

linear regression of the \log_{10} of bacterial surviving cells. The data was analyzed using the Statistical Analysis System (Version 9.4; SAS Institute, Cary, NC, USA) for analysis of variance (ANOVA) and Duncan's multiple range to determine significant differences ($P \leq 0.05$) among treatment groups.

Results and discussion

Figure 1 represents pathogenic levels on the surfaces of GFFs in TSB or on TSA at 100% RH during incubation at 28 °C for 5 days. Initial counts of pathogens were approximately 4–5 \log_{10} CFU/filter. Pathogen levels on the surface of GFFs increased significantly, reaching approximately 8–10 \log_{10} CFU/filter after 1 day of incubation.

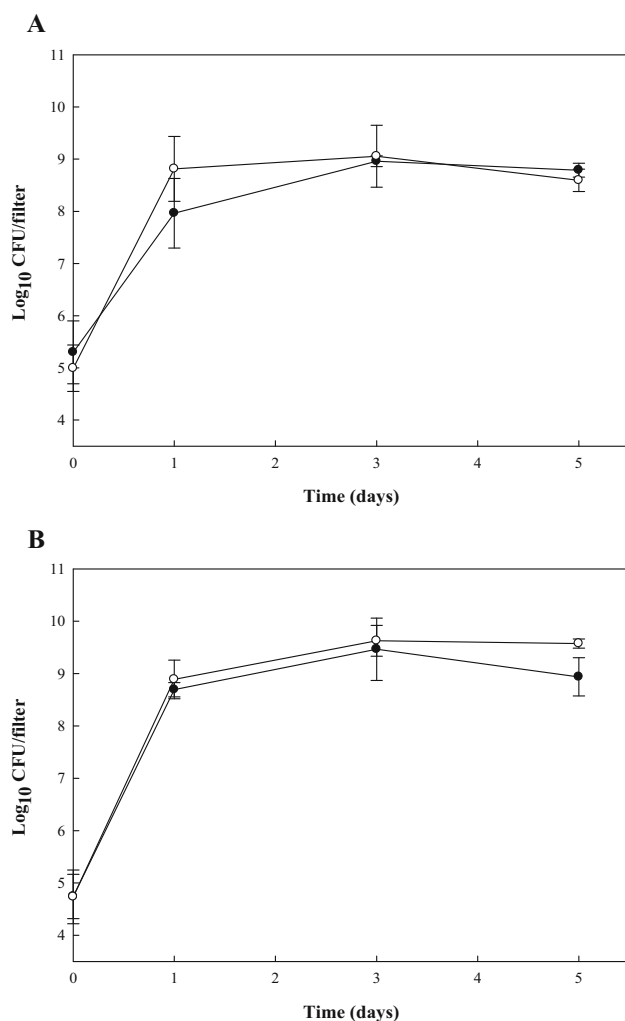


Fig. 1 Growth (\log_{10} CFU/filter) of *Escherichia coli* O157:H7 (filled circle) and *Staphylococcus aureus* (open circle) contaminating glass fiber filters as biofilms formed in TSB incubated at 28 °C for 5 days (A) or biofilms formed on TSA under 100% RH at 28 °C for 5 days (B)

These levels were maintained for 5 days of incubation. From these results, *E. coli* O157:H7 and *S. aureus* grew and formed biofilms on GFFs in TSB (hydrophilic condition) and at 100% RH (hydrophobic condition). Several studies have investigated the pathogenic survivals on the surface of materials under various RH conditions. Levels of *C. sakazakii* in biofilms formed in infant formula and M9 medium were maintained at 100% RH (Kim et al., 2008) Choi et al. (2011) studied the numbers of total aerobic bacteria recovered from spinach leaves stored at RHs of 43%, 85%, and 100% for up to 120 h, and they found that populations increased significantly when stored at 100% RH for 120 h. Other study (Iturriaga et al., 2007) also observed the levels of biofilm formation on a tomato surface were increased with the increase of RH over 10 days of storage. Through the results of these studies, we can conclude that hydrated environments, such as environments possessing 100% RH with nutrients, can enhance the numbers of bacteria and biofilm formation on material surfaces.

Next, we investigated the desiccation tolerance of pathogenic biofilms possessing different adhered forms on GFFs using various methods. Different forms of adhered cells on GFFs including (1) adhered cells, (2) biofilm produced in TSB (type I), (3) biofilm produced on TSA (type II), and (4) biofilm produced on TSA under 100% RH (type III) were tested in this study. Figure 2 shows the survival of *E. coli* O157:H7 and *S. aureus* on the surfaces of GFFs at 43% and 68% RH, respectively. Initial counts of *E. coli* O157:H7 on GFFs before storage at 43% and 68% RH were approximately 6–8 and 7–9 \log_{10} CFU/filter, respectively (Fig. 2A and B), while those of *S. aureus* on GFFs before storage at 43% and 68% RH were approximately 7–9 and 8–10 \log_{10} CFU/filter, respectively (Fig. 2C and D). Types I and II biofilms exhibited survival patterns similar to those of adhered cells, indicating similar levels of tolerance under dry conditions. Type III biofilms, however, showed high tolerance under dry conditions for both pathogens relative to that of other types of adhered cells on GFFs.

Table 1 shows the decimal reduction time and n -value index of *E. coli* O157:H7 and *S. aureus* survivals on GFFs when stored at 43% and 68% RH for 7 days. When GFFs were incubated at 28 °C for 7 days, levels of *E. coli* O157:H7 and *S. aureus* on GFFs were not significantly different between conditions of 43% and 68% RH ($P > 0.05$) based on D - and n -values. Populations of *E. coli* O157:H7 and *S. aureus* on GFFs produced under 100% RH (type III) were maintained at high levels (> 6 and > 8 \log_{10} CFU/filter, respectively) for 7 days of storage, respectively, and therefore n -values were not determined for type III biofilms for both pathogens. D -values of type III biofilms were significantly higher than other adhered

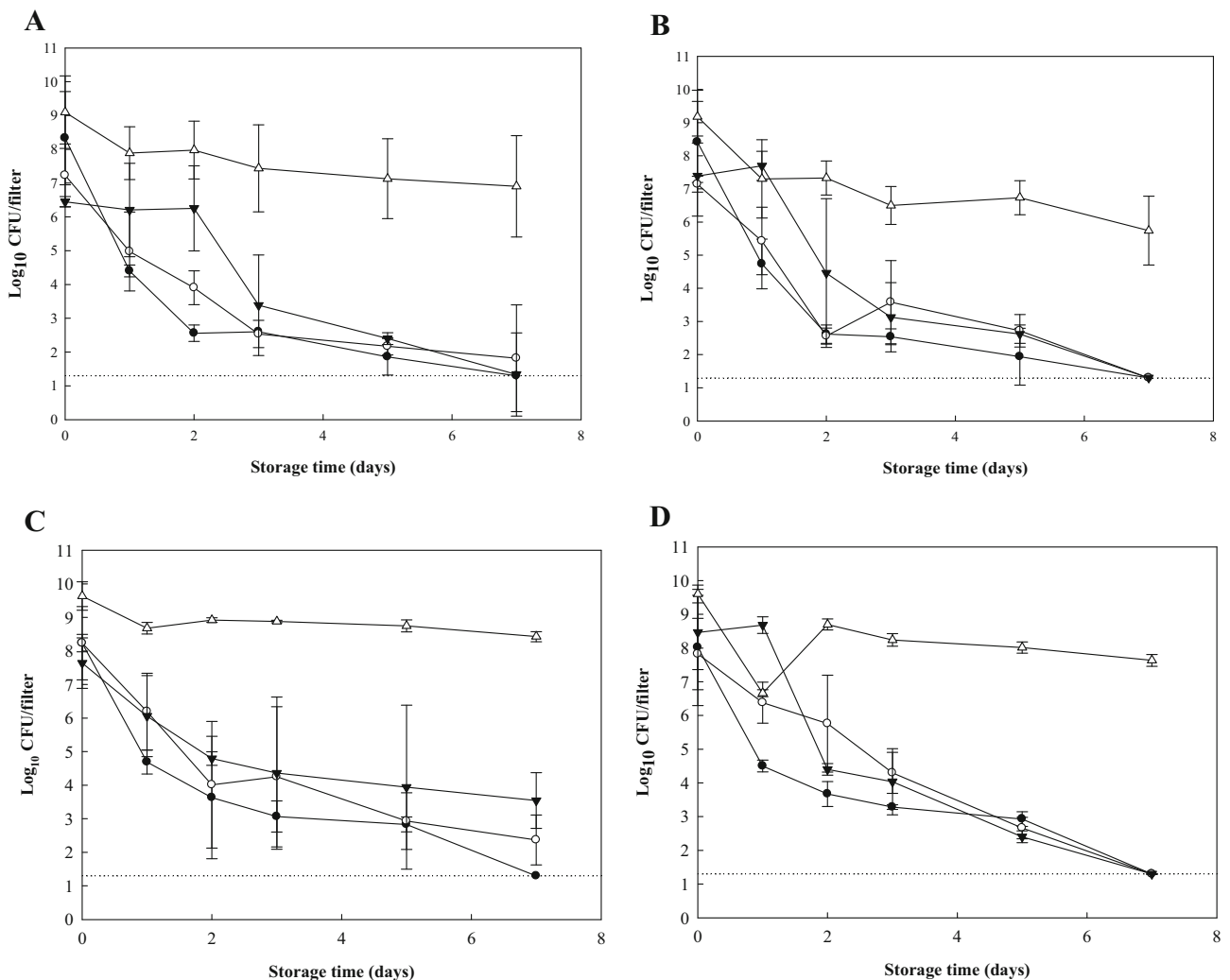


Fig. 2 Numbers (\log_{10} CFU/filter) of *Escherichia coli* O157:H7 (A and B) and *Staphylococcus aureus* (C and D) surviving on the surfaces of glass fiber filters as adhered cells (filled circle), biofilms formed at 28 °C for 3 days in TSB (type I) (filled inverted triangle),

biofilms formed at 28 °C for 1 days on TSA (type II) (open circle), or biofilms formed at 28 °C for 3 days on TSA under 100% RH (type III) (open triangle) and stored at 43% (A and C) or 68% (B and D) RH for 7 days. Detection limit (dotted line) is 1.30 log CFU/filter

forms ($P \leq 0.05$) for both pathogens. D -values of *E. coli* O157:H7 type III biofilms on GFFs were 2.73 and 3.56 days at 43% and 68% RH, respectively, while those of other adhered cells were less than 1.08 days. Additionally, D -values of *S. aureus* type III biofilms were 6.42 and 6.16 days at 43% and 68% RH, respectively, while those of other adhered cells were less than 1.59 days. Pathogenic biofilms produced under hydrated conditions on TSA under 100% RH (type III) exhibited the most tolerance against desiccation among the different adhered forms of pathogens on GFFs, including adhered cells or biofilms formed under hydrophilic conditions in TSB (type I). Another study also evaluated the tolerances of different forms of biofilms on material surfaces under dry conditions. In the study of Bae et al. (2012), biofilms of five pathogens including *E. coli* O157:H7, *L. monocytogenes*, *S.*

Typhimurium, *S. aureus*, and *C. sakazakii* on stainless steel surfaces produced under 100% RH condition showed strong resistance on the survivals at dry environment compared to other adhered forms, including adhered cells and biofilms produced in TSB, which is consistent with the current results. For both pathogens, D -values among different adhered forms, with the exception of type III biofilms ($P > 0.05$) were not significantly different ($P > 0.05$). For type III biofilms, *S. aureus* exhibited smaller reductions than *E. coli* O157:H7 during storage for 7 days at 43% and 68% RH. Additionally, the resistance of *E. coli* O157:H7 and *S. aureus* biofilm on GFFs produced under 100% RH were much higher compared than those of biofilms on stainless steel under the same condition when the result of this study was compared with previous study (Bae et al., 2012). In particular, reduction of *E. coli*

Table 1 *D*-values (days)^a and *n*-values (shape) of *Escherichia coli* O157:H7 and *Staphylococcus aureus* contaminated as biofilms formed on the surfaces of glass filters and stored under two different RHs for 7 days

Strain	Adhered form ^b	<i>D</i> -value		Weibull distribution			
				<i>n</i> -value		<i>R</i> ^{2c}	
		43% RH	68% RH	43% RH	68% RH	43% RH	68% RH
<i>Escherichia coli</i> O157:H7	Adhered cell	0.83 ± 0.14B ^d a ^c	0.51 ± 0.36Ba	0.31 ± 0.07Ba	0.29 ± 0.11Ba	0.99 ± 0.01	0.96 ± 0.02
	Biofilm (type I)	0.94 ± 0.11Ba	0.76 ± 0.13Ba	0.69 ± 0.12Aa	0.86 ± 0.26Aa	0.89 ± 0.09	0.86 ± 0.02
	Biofilm (type II)	1.08 ± 0.10Ba	0.98 ± 0.14Ba	0.28 ± 0.06Ba	0.44 ± 0.13Ba	0.96 ± 0.03	0.84 ± 0.03
	Biofilm (type III)	2.73 ± 0.86Aa	3.56 ± 1.72Aa	ND ^f	ND	ND	ND
<i>Staphylococcus aureus</i>	Adhered cell	0.87 ± 0.13Ba	0.93 ± 0.20Ba	0.39 ± 0.23Ba	0.34 ± 0.10Ba	0.97 ± 0.02	0.97 ± 0.02
	Biofilm (type I)	1.59 ± 0.96Ba	0.64 ± 0.06Ba	0.52 ± 0.19Aa	0.82 ± 0.24Aa	0.89 ± 0.29	0.87 ± 0.03
	Biofilm (type II)	0.98 ± 0.38Ba	0.80 ± 0.19Ba	0.48 ± 0.23Aa	0.83 ± 0.08Aa	0.78 ± 0.09	0.95 ± 0.02
	Biofilm (type III)	6.42 ± 2.15Aa	6.16 ± 2.54Aa	ND	ND	ND	ND

^aData represent the means ± standard deviations of three measurements

^bAdhered cells were prepared by drying GFFs for 2 h following inoculation; type I biofilms were formed by immersing inoculated GFFs in TSB for 3 days at 28 °C; type II biofilms were formed by cultivation on inoculated GFFs on TSA for 1 days at 28 °C; type III biofilms were formed by cultivation on inoculated GFFs on TSA under 100% RH for 3 days at 28 °C

^c*R*² values range from 0 to 1 and the closer the values are to the maximum value the better the fit of the model

^dMeans with the same letter within a column for each pathogen are not significantly different (*P* > 0.05)

^eMeans with the same letter within a row for *D*-value and *n*-value are not significantly different (*P* > 0.05)

^fNot determined

O157:H7 biofilms on stainless steel produced under 100% RH were ca. 4–5 log₁₀ CFU/coupon after storage for 5 days at 68% and 43% RH (Bae et al., 2012), while reduction of *E. coli* O157:H7 biofilms on GFFs under same condition (RH 100%) were < 3 log₁₀ CFU/filters after storage for 7 days at 68% and 43% RH. From these results, more resistant forms of pathogenic biofilms against dry conditions could be produced using GFFs in the laboratory relative to those produced using other materials such as stainless steel.

Generally, it is accepted that bacterial biofilms are greater tolerance to treatment with sanitizers than planktonic cells (Cadena et al., 2019). The increased tolerance of biofilms to sanitizers may be explained by EPSs such as cellulose and curli (Phillips, 2016). The EPSs produced by attached bacteria may protect sessile cells from sanitizers therefore it is highly difficult to eliminate bacterial biofilms on food contact surfaces in food industry (Decho and Gutierrez, 2017). Treatments with various kinds of sanitizers such as iodophor (Ban and Kang, 2016), chlorine (Olmez and Temur, 2010), hydrogen peroxide (Choi et al., 2012), peracetic acid, quaternary ammonium compound (Nguyen and Yuk, 2013), ozonated water (Olmez and Temur, 2010), and sodium hypochlorite (Meira et al., 2012) have been investigated for reducing bacterial biofilms on material surfaces in published studies. Efficacies of chemical sanitizers on inhibiting biofilm on material surfaces could, however, differ depending on their adhered

forms. To obtain accurate results for the identification of effective sanitizing methods that are able to eliminate pathogenic biofilms on material surfaces, sanitizing methods should be evaluated using highly resistant forms of biofilm. Therefore, in this study, various sanitizing methods were evaluated using different types of biofilms on GFF produced by the different methods described. Table 2 shows populations of *E. coli* O157:H7 and *S. aureus* on GFFs before and after treatment with five different chemical sanitizers. Initial counts of *E. coli* O157:H7 and *S. aureus* on GFFs were 8–10 and 9–10 log₁₀ CFU/filter, respectively. Levels of *E. coli* O157:H7 and *S. aureus* on GFFs reduced by water treatment (control) were < 1 log₁₀ CFU/filter, while chemical sanitizers were effective to reduce both pathogenic biofilms with the exception of iodophor-based sanitizers. Among tested sanitizers, alcohol-, chlorine-, hydrogen peroxide-, and quaternary ammonium-based sanitizers were effectively reduced adhered cells and biofilms of *E. coli* O157:H7 on GFFs. Also, populations of *S. aureus* on GFFs after treatments with alcohol and chlorine-based sanitizers were significantly decreased (*P* ≤ 0.05). Reduction of pathogens by treatments with chemical sanitizers were significantly different depending on their adhered forms (*P* ≤ 0.05). For example, when alcohol-based sanitizer was used to treat different adhered forms of *E. coli* O157:H7 and *S. aureus* on GFFs, levels of these pathogens were 3.5, 5.4, 7.9, and 8.9 log₁₀ CFU/filter for *E. coli* O157:H7 and 5.6, 5.2, 5.8,

Table 2 Population (\log_{10} CFU/filter)^a of *Escherichia coli* O157:H7 and *Staphylococcus aureus* as biofilms formed on the surfaces of glass filters before and after treatment with water or chemical sanitizers

Strains	Adhered form ^b	None	Distilled water	Type of chemical sanitizer				Hydrogen peroxide-based	Iodophor-based	Quaternary ammonium-based
				Alcohol-based	Chlorine-based	Hydrogen peroxide-based	Iodophor-based			
<i>Escherichia coli</i> O157:H7	Adhered cell	8.08 ± 1.08 ^{c,d}	6.50 ± 0.17Db	3.45 ± 0.62Cd	4.78 ± 0.48Cc	4.45 ± 0.15Cc	7.82 ± 0.19Ca	4.70 ± 0.35Bc		
	Biofilm (type I)	10.03 ± 0.48Ba	9.58 ± 0.10Cab	5.42 ± 0.86Bcd	4.54 ± 0.24Cd	6.25 ± 1.65Bc	8.45 ± 0.38Bb	5.37 ± 0.78Bcd		
	Biofilm (type II)	10.26 ± 0.28ABa	10.85 ± 0.49Aa	7.89 ± 0.17Ac	7.64 ± 0.92Bc	7.59 ± 1.63ABc	9.58 ± 0.16Aab	8.76 ± 0.64Abc		
<i>Staphylococcus aureus</i>	Biofilm (type III)	10.76 ± 0.03Aa	10.22 ± 0.38Bb	8.88 ± 0.23Ad	9.34 ± 0.34Acd	9.40 ± 0.27Ac	9.34 ± 0.28Acd	9.12 ± 0.06Acd		
	Adhered cell	9.12 ± 1.08ABa	8.45 ± 0.31Bab	5.60 ± 0.37Db	5.22 ± 0.48Db	6.48 ± 1.18Bcd	8.82 ± 0.18Bab	5.50 ± 1.11Bbc		
	Biofilm (type I)	8.69 ± 0.15Ba	8.83 ± 0.98Ba	5.19 ± 0.47Bc	5.71 ± 0.29Bbc	6.44 ± 0.70Bb	7.98 ± 0.41Ca	8.18 ± 0.41ABa		
	Biofilm (type II)	9.17 ± 0.86ABa	8.81 ± 0.11Ba	5.81 ± 0.95Bb	5.73 ± 2.41Bb	7.70 ± 2.03ABab	8.71 ± 0.29Ba	7.76 ± 0.87Bab		
	Biofilm (type III)	10.36 ± 0.19Aa	10.25 ± 0.32Aa	7.88 ± 1.30Ba	9.63 ± 0.20Aa	9.81 ± 0.14Aa	9.62 ± 0.40Aa	9.32 ± 0.36Aa		

^aData represent the means ± standard deviations of three measurements^bAdhered cells were prepared by drying GFFs for 2 h following inoculation; type I biofilms were formed by immersing inoculated GFFs in TSB for 3 days at 28 °C; type II biofilms were formed by cultivation on inoculated GFFs on TSA for 1 days at 28 °C; type III biofilms were formed by cultivation on inoculated GFFs on TSA under 100% RH for 3 days at 28 °C^cMeans with the same letter within a column for each pathogen are not significantly different ($P > 0.05$)^dMeans with the same letter within a row for water and chemical sanitizer are not significantly different ($P > 0.05$)

and 7.9 \log_{10} CFU/filter for *S. aureus* after treatment for 10 min, respectively. Therefore, pathogenic biofilms showed similar results to those observed using adhered cells when biofilms on GFFs were produced in TSB for 3 days (type I). *E. coli* O157:H7 and *S. aureus* biofilms produced on TSA under 100% RH (type III), however, were highly resistant against sanitizing treatments. Given this, levels were maintained at high levels (> 8 and > 7 \log_{10} CFU/filter), respectively, following treatment with alcohol-sanitizers, indicating that biofilms produced in hydrated environments exhibited enhanced tolerance to sanitizing treatments (Table 2). In particular, alcohol-based sanitizers were highly effective at reducing pathogenic adhered cells and biofilms on GFFs. Other studies also reported that treatment with alcohol were highly effective to reduce pathogenic biofilms compared to treatment with chlorine (Bae et al., 2009; Lee et al., 2007). Bae et al. (2012) reported that alcohol-based sanitizers are highly effective to inhibit adhered pathogens on stainless steel. Additionally, pathogenic biofilms formed under hydrophilic conditions, such as by immersion in laboratory broth, do not show differences in tolerance to treatment with chemical sanitizers when compared with those observed using adhered cells or planktonic cells (Bae et al., 2012; Park et al., 2012). Sporing and Lewis (2001) reported that *Pseudomonas aeruginosa* biofilms formed under hydrophilic conditions in Mueller–Hinton broth were not different from planktonic cells in stationary phase in their tolerance to antibiotics and biocides. It was also reported by Park et al. (2012), who found increased resistance of pathogenic biofilms on two coupons under 100% RH relative to that of biofilms produced by immersion in TSB at 25 °C for 6 days. These results support the observation that biofilms produced on TSA under 100% RH (type III) are highly resistant against sanitizers compared to biofilms formed under hydrophilic conditions, such as by immersion in laboratory broth. Additionally, several studies have found that the tolerance of pathogenic biofilms to sanitizer treatments could be various depending on the surface type of material. *Salmonella* biofilm on stainless steel were more susceptible to chlorine- and iodine-based sanitizers than those biofilms on plastic. Also, *S. aureus* biofilms on glass surfaces are more resistant to treatment with sanitizers than biofilm formed on stainless steel (Marques et al., 2007).

Additionally, biofilm formation of pathogens and their resistance to survival under dry conditions or after treatment with sanitizers could differ depending on material types and surface structures. Generally, biofilms formed on hydrophobic surface such as paper, filter, and Teflon possessed higher resistance to dry conditions or sanitizers relative to resistance observed in biofilms formed on hydrophilic surfaces such as stainless steel and glass. Pan

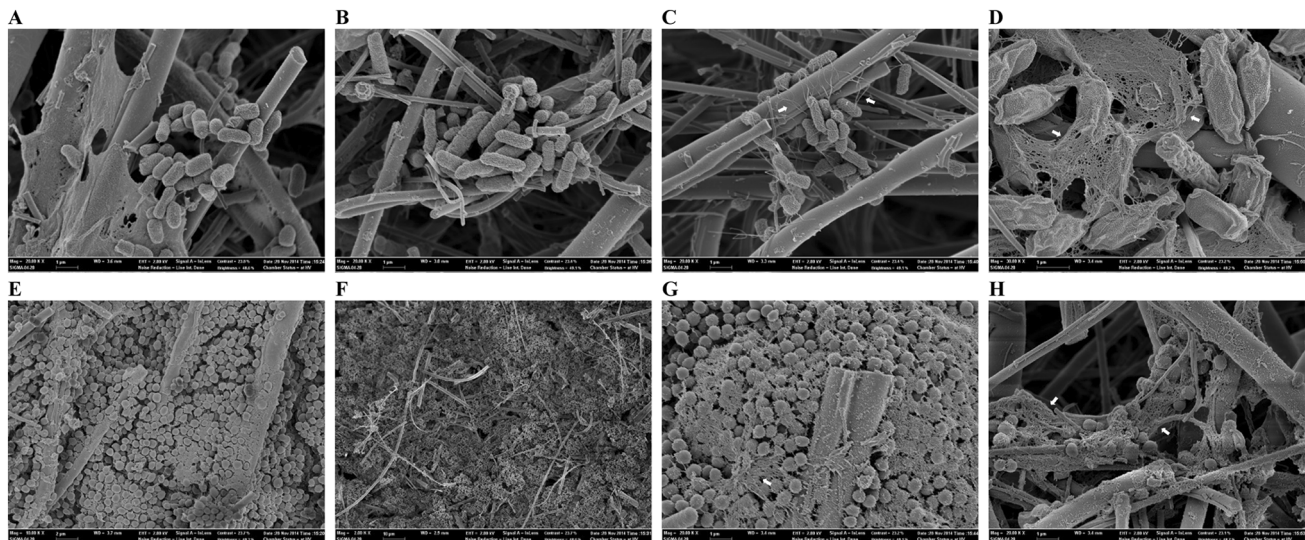


Fig. 3 Field-emission scanning electron microscopy (FE-SEM) micrographs of *Escherichia coli* O157:H7 (A–D) and *Staphylococcus aureus* (E–H) on the surfaces of glass fiber filters contaminated as adhered cells (A and E), type I biofilms formed by immersion of inoculated glass fiber filters in TSB for 3 days at 28 °C (B and F),

type II biofilms formed by cultivation of inoculated glass fiber filters on TSA for 1 days at 28 °C (C and G), or type III biofilm formed by cultivation of inoculated glass fiber filters on TSA under 100% RH for 3 days at 28 °C (D and H). Magnification: (A–C and E–G), $\times 20,000$; (D and H), $\times 30,000$

et al. (2006) reported that pathogenic biofilms on Teflon (hydrophobic) were more resistant to sanitizers than biofilms on stainless steel (hydrophilic). The structure of material surfaces could influence the resistance of pathogenic biofilms as well. A previous study indicated that biofilms formed on microstructures or filamentous structures using GFFs were highly resistant to treatment with bactericide solutions (Lebert et al., 2007). Indeed, treatment with alcohol-based sanitizers for 5 min reduced 5.73 and 6.71 log CFU/coupon of *E. coli* O157:H7 and *S. aureus* on stainless steel produced under 100% RH, respectively (Bae et al., 2012). In the present study, however, treatment with the same alcohol-based sanitizer for 10 min were 1.87 and 2.48 log₁₀ CFU/filter of *E. coli* O157:H7 and *S. aureus* on GFFs produced under the same conditions (100% RH), respectively. From the results of this study, the tolerance of biofilms formed on GFFs under a hydrated environment (100% RH) was much higher than that of biofilms formed on stainless steel under the same condition (100% RH) or on GFFs in laboratory media. Therefore, the method for the formation of biofilms on GFFs under 100% RH could be used to produce highly resistant forms of pathogenic biofilms against environmental stresses, and these could in turn be applicable to the development of effective sanitizing methods for inhibiting and eliminating bacterial biofilms in laboratories.

Figure 3 shows FE-SEM images of the four different adhered forms of *E. coli* O157:H7 and *S. aureus* biofilms on GFFs. Cells were surrounded under EPS within the formed biofilm, remaining adhered on the surface. Biofilms

produced on the surfaces of GFFs on TSA showed an EPS formation shielding the colonies (Fig. 3C and G), and slimy materials were also identified in the colonies for type III biofilms, which showed the most resistance to dry conditions and sanitizer treatment (Fig. 3D and H). Additionally, biofilm cells were observed as clump-like structures. This phenomenon was more prevalent in the biofilm produced under 100% RH on the surface of GFFs than it was in other adhered forms. Rodriguez et al. (2007) also observed that biofilms produced in this manner possess different structure and composition of EPS that could affect cell survival in various stress conditions than do biofilms produced under a highly hydrated environment. In this study, biofilms produced under 100% RH were more resistant in terms of survival on the GFF relative to biofilms produced in TSB. Therefore, the presence of a hydrated environment is an important factor to produce highly resistant biofilms on various material surfaces. Also, GFFs possessed a structure that promoted the formation of a fibrous or filamentary layer. Given this, the GFFs may provide a protective microenvironment for bacteria from desiccation. Tremoulet et al. (2002) observed that the *E. coli* O157:H7 cells on glass fibers and appeared encapsulated in polymeric matrix. Rose et al. (2003) also reported that paper coupons demonstrated an improved ability to support the bacteria cells relative to other surfaces.

In conclusion, biofilms formed on GFFs under hydrated conditions (100% RH) (type III biofilm) were highly resistant to dry conditions and chemical sanitizer treatment relative to other biofilms. Additionally, among the various

chemical sanitizers, alcohol-based sanitizers were the most effective for eliminating all types of biofilms; however, efficacies of the sanitizers were very different depending on the types of biofilms produced under different conditions. There was 1.8 and 2.5 log₁₀ CFU/filter reductions of *E. coli* O157:H7 and *S. aureus* in type III biofilms by treatments with alcohol-based sanitizer, respectively. These strong resistances exhibited by pathogenic biofilms produced on GFFs under 100% RH may be due to specific conditions such as GFF (filamentous structure) and hydrated conditions that result in the formation of biofilms that possess large amounts of EPS and slime around the cells. From the results of this study, the method of biofilm formation (biofilm produced under a highly hydrated environment on GFFs) could be used to produce highly resistant forms of pathogenic biofilms and could be applied to develop effective methods for eliminating bacterial biofilms on cooking equipment surfaces and other related environments.

Acknowledgements This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Korea government (MSIT) (NRF-2016R1A2B4014591) and the Chung-Ang University Excellent Student Scholarship in 2020.

Author contributions Hana Song collected data and drafted the manuscript. Sun-Young Lee designed the study, interpreted the results and revised the manuscript.

Compliance with ethical standards

Conflicts of interest The authors declare that there are no conflicts of interest regarding the publication of this paper.

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