Food Research International 137 (2020) 109432

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Thermal and non-thermal treatment effects on *Staphylococcus aureus* biofilms formed at different temperatures and maturation periods

Woo-Ju Kim[a,c,1], Soo-Hwan Kim[a,1], Dong-Hyun Kang[a,b,*]

[a] Department of Food and Animal Biotechnology, Department of Agricultural Biotechnology, Center for Food and Bioconvergence, and Research Institute for Agricultural and Life Sciences, Seoul National University, Seoul 08826, Republic of Korea
[b] Institutes of Green Bio Science & Technology, Seoul National University, Pyeongchang-gun, Gangwon-do 25354, Republic of Korea
[c] Department of Food Science and Technology, The Ohio State University, Columbus 43202, USA

ARTICLE INFO

Keywords:
Biofilm
EPS
Steam
NaOCl
Food safety

ABSTRACT

The objective of this study was to investigate the effect of temperature and maturation period on the resistance of *Staphylococcus aureus* biofilms to thermal and non-thermal treatments. First, biofilm development was compared at three different temperatures (15, 25, and 37°C) for 5 days. The cell population at 15 and 25°C remained relatively consistent approximately at 6.3 log CFU/cm^2, whereas 37°C resulted in the highest cell population on day 1 (7.6 log CFU/cm^2) followed by a continual decline. Then, biofilm resistance to steam and sodium hypochlorite (NaOCl) treatments was evaluated. Obtained results highlighted that biofilms had different resistance to both treatments depending on development conditions. Specifically, steam treatment of 10 s eliminated 4.1 log CFU/cm^2 of the biofilm formed at 25°C for 5 days. The same treatment inactivated over 5 log population of biofilms developed in other temperature and maturation period conditions. Treatment with NaOCl reduced approximately 1 log CFU/cm^2 of biofilm cells developed at 25°C for 5 days. However, inactivation was found to be over 2 log CFU/cm^2 under other development conditions. An extracellular polymeric substances (EPS) quantification using 96-well plates and stainless steel coupons was conducted. In the 96-well plate experiment, it was found that the highest amount of polysaccharide was secreted at 25°C ($p < 0.05$), while total biomass and protein contents were greatest at 37°C ($p < 0.05$). No significant difference in EPS content was observed for stainless steel, but the results displayed a similar trend to the 96-well plate. In particular, biofilms developed at 25°C tended to secret the highest amount of polysaccharide, which aligned with the current literature. This finding indicated that polysaccharide was the main contribution to the enhanced resistance of *S. aureus* biofilms. Overall, it was shown that biofilms formed at 25°C for 5 days exhibited the greatest resistance to thermal and nonthermal treatments due to the elevated exopolysaccharide secretion. This study demonstrates that temperature and maturation period significantly affect the resistance of *S. aureus* biofilms to thermal and non-thermal treatments.

1. Introduction

Foodborne disease is a growing concern in developed countries as well as developing nations (Park & Yoon, 2019). Every year, many cases of multistate foodborne illnesses outbreak have been reported in the US by the CDC (CDC, 2017, 2018). In response to the need for a proper food safety, systems such as Hazard Analysis and Critical Control Point (HACCP) have been implemented. Among many pathogenic species, *Staphylococcus aureus* is one of the most common microorganisms that raises food safety concerns. *S. aureus* is a gram-positive bacteria that produces enterotoxin, a toxin known to be the leading contributor of food poisoning (Le Loir, Baron, & Gautier, 2003; Troeman, Van Hout, & Kluytmans, 2019). Foodborne outbreaks caused by *S. aureus* are prevalent around the globe through various types of food (Asao et al., 2003; Hennekinne, De Buyser, & Dragacci, 2012; Kérouanton et al., 2007). This is inherently to do with the microorganism's capacity to grow in a wide range of temperatures and pH levels and high resistance to osmotic pressure (Sutherland, Bayliss, & Roberts, 1994). It is also known that *S. aureus* is able to form biofilms on the abiotic surfaces used during food processing under various stress conditions (Asao et al., 2003; Hennekinne et al., 2012; Kérouanton et al., 2007; Xu et al., 2019)

Biofilm is a well-constructed structure in which bacterial cells are

* Corresponding author at: Department of Agricultural Biotechnology, Seoul National University, Seoul 08826, Republic of Korea.
E-mail address: kang7820@snu.ac.kr (D.-H. Kang).
[1] Co-first-authors.

https://doi.org/10.1016/j.foodres.2020.109432
Received 2 February 2020; Received in revised form 30 May 2020; Accepted 9 June 2020
Available online 12 June 2020

embedded in the matrix of extra polymeric substance (EPS), secreted by biofilm-generating microorganisms. EPS consists of many types of substances such as polysaccharides, lipids, glycopeptides, and other biomolecular materials (Cortés, Bonilla, & Sinisterra, 2011). In comparison to planktonic cells, this special structure found in bacterial cells provides an increased resistance to environmental stressors such as pH, UV radiation, and antimicrobial substances (Gupta, Sarkar, Das, Bhattacharjee, & Tribedi, 2016; Simões, Simões, Machado, Pereira, & Vieira, 2006). An important issue concerning biofilms is that they can be developed in various environmental conditions and types of surfaces including abiotic and biotic surfaces (Cortés et al., 2011; Kim, Jeong, Cheon, & Kang, 2019; Parsek & Singh, 2003). In addition, biofilms on food-contact surfaces have been linked with bacterial cross-contaminations in food products (Simoes, Simoes, & Vieira, 2010). In particular, *S. aureus* species with their biofilm formation capability can be a source of cross-contamination (Plaza-Rodríguez, Kaesbohrer, & Tenhagen, 2019). Gibson, Taylor, Hall, and Holah (1999) reported a high risk of contamination due to *S. aureus* biofilms on food-contact surfaces. Thus, it is paramount that biofilms formed on food processing surfaces as well as foodstuffs are efficiently removed (Chen, Rossman, & Pawar, 2007; Frank, Ehlers, & Wicker, 2003; Jessen & Lammert, 2003).

It has been reported that sanitization processes might not be enough to entirely eliminate adhered pathogens due to crevices or cracks on processing surfaces (Menon, 2016). The remaining cells still possess metabolic activity leading to biofilm formation and contaminations on food contact surfaces (Poulsen, 1999; Wirtanen, 2019). Remaining cells on abiotic surfaces are exposed to various sub-lethal conditions, from processing to storage, transportation, and distribution of the food product. (Jørgensen, Mørk, Høgåsen, & Rørvik, 2005; Pagedar, Singh, & Batish, 2010). This indicates that the biofilm structures and properties may be different depending on the surrounding conditions, giving rise to a different degree of resistance and thereby generating food safety issues.

Several factors may influence biofilm formation in food facilities. Di Bonaventura et al. (2008) reported that temperature affects the hydrophobicity of cell membranes, resulting in different biofilm formation ability. It has also been reported that pH and water activity are important factors that impact biofilm formation (Giaouris, Chorianopoulos, & Nychas, 2005). Specifically, *S. aureus* biofilm formation was found to be facilitated at pH and water activity close to optimal growth conditions (Tango et al., 2018). Hydrophobicity of the surface also influences biofilm formation. *S. aureus* showed an enhanced biofilm formation ability on a polypropylene coupon compared to a more hydrophilic abiotic surface (Pagedar et al., 2010). These factors may affect the resistance of *S. aureus* to various environmental stresses. Several studies reported that sessile cells have different resistance to antimicrobial substances depending on the aforementioned factors or attachment form (Bae, Baek, & Lee, 2012; Chavant, Gaillard-Martinie, & Hébraud, 2004). Reynoso, Ferreyra, Durantini, and Spesia (2019) also reported that biofilm appears to have different resistance depending on media conditions. However, no study regarding the effect of temperature and maturation period on the resistance of *S. aureus* biofilms to thermal and non-thermal treatments has been reported.

In most food manufacturing plants, cleaning processes are conducted on a daily basis using sanitizers (Chmielewski & Frank, 2003). Many types of chemical agents can be utilized to disinfect food-contact surfaces. Most sanitizers are formulated with chlorine, hydrogen peroxide, quaternary ammonium, and acidic compounds (Chmielewski & Frank, 2003; Park, Kim, & Kang, 2018). Among them, sodium hypochlorite (NaOCl), a chlorine-based sanitizer, is the most commonly used cleaning agent in food processing (De Beer, Srinivasan, & Stewart, 1994). This sanitizer removes bacterial loads by means of oxidation. It was reported that chlorine sanitizers were able to efficiently eliminate EPS materials attached to stainless steel surfaces (Ronner & Wong, 1993). Specifically, Norwood and Gilmour (2000) reported that 20 ppm of NaOCl removed approximately 3 log population of *Staphylococcus xylosus*. In addition, a recent study illustrated that NaOCl was found to be more effective in removing biofilms than chlorhexidine (Röhner et al., 2020).

Thermal treatments are also widely utilized to ensure safety in food manufacturing processes (Park & Yoon, 2018; Park, Yoo, Jung, & Yoon, 2019). Among many thermal treatments, steam is a promising thermal technology used for biofilm inactivation (Park & Yoon, 2018; Park et al., 2019). Steam has several advantages over other conventional thermal treatments. First, steam has higher heat transfer capabilities when it is condensed. Also, it can be operated in oxygen-free environments (Bari et al., 2010). Furthermore, steam can easily penetrate the crevices or cracks on surfaces that cells often adhere to for their protection, hence is capable of efficiently eliminating foodborne pathogens (Morgan, Goldberg, Radewonuk, & Scullen, 1996). It is reported that steam pasteurization is an effective method to inactivate foodborne pathogens at rapid rates due to its high heat capacity (Chang, Han, Reyes-De-Corcuera, Powers, & Kang, 2010). Steam above boiling temperature at a constant pressure is called superheated steam (Cenkowski, Pronyk, Zmidzinska, & Muir, 2007). This type of steam has a higher capability to inactivate foodborne pathogens. Previously, Ban, Yoon, and Kang (2014) showed the possibility of steam and superheated steam in inactivating various foodborne pathogens that are capable of developing biofilms on stainless steel surfaces. Kim et al. (2019) also showed that steam treatment is very effective in biofilm inactivation from various abiotic surfaces.

Several studies have investigated effects of temperature and time on the trend of *S. aureus* biofilm formation (Pompermayer & Gaylarde, 2000; Rode, Langsrud, Holck, & Møretrø, 2007). However, to the best of our knowledge, no study has related thermal and non-thermal treatments to temperature and biofilm maturation period for their change biofilm resistance. Thus, in this study, steam and sodium hypochlorite were applied as thermal and non-thermal treatments, respectively, and resistance of *S. aureus* biofilms on stainless steel surfaces were evaluated. In addition, the amount of EPS contents including carbohydrates and protein was measured in order to explain different degree of biofilm resistance between biofilm growth conditions.

2. Materials and methods

2.1. Bacterial cultures and cell suspension

Three strains of *S. aureus* (ATCC 25923, ATCC 27213 and ATCC 29213) were obtained from the bacterial culture collection of the Department of Food Science and Biotechnology at the Seoul National University (Seoul, South Korea). Each strain (maintained at −80°C frozen stocks) was streaked for isolation onto tryptic soy agar (TSA; Difco, Becton, Dickinson, Sparks, MD) and incubated for 24 h at 37°C. A single colony of each strain was transferred to 5 ml of tryptic soy broth (TSB; Difco) and incubated for 24 h at 37°C. Cells of each strain were collected by centrifugation at 4000*g* for 20 min at 4°C, then washed three times with phosphate-buffered saline (PBS, pH 7.4; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2PO_4, and 1.8 mM KH_2PO_4). The final pellets were resuspended in sterile PBS and collected as strain cocktail, corresponding to approximately 10^7-10^8 CFU/ml

2.2. Coupons preparation

2 × 5 × 0.1 cm of type 304 stainless steel coupons with no. 4 finish (STS no.4; Ian industry, Korea) were used. Stainless steel coupons were immersed in 70% ethanol for 60 min to disinfect the surface and rinsed with sterile distilled water before further sterilization by autoclaving at 121°C for 15 min. Subsequently, the coupons were dried and stored in a dry oven at 50°C. For EPS quantification, 1 × 1 × 0.1 cm of stainless steel coupons were prepared using the same sterilization procedure.

2.3. Biofilm formation assay

The biofilm formation method was adapted and modified from Ban et al., 2014. Each prepared coupon was immersed in a sterile 50 ml conical centrifuge tube (BD Falcon™, USA) containing 30 ml suspensions of *S. aureus* in PBS (approximately10^7–10^8 CFU/ ml). Coupons in bacterial cell suspensions were incubated at 4°C for 24 h to facilitate the attachment of cells. Each coupon was then removed with sterile forceps, immersed in 1300 ml of sterile distilled water (22 ± 2°C), and gently stirred for 5 s in order to remove loosely attached cells. Rinsed coupons were deposited in 50 ml conical centrifuge tubes containing 30 ml of TSB, then incubated at 15, 25, and 37°C for 1 to 5 days. When forming biofilms on a 96-well plate for EPS quantification, 200 μl suspensions of *S. aureus* were added to the 96-well plate and incubated at 4°C for 24 h. Suspensions were removed and the plate was washed twice with 250 μl of PBS. Then, 200 μl of TSB was added to each well and incubated at the given temperatures for 1 or 5 days.

2.4. Steam treatment

The steam generator used in this research was described in a previous study conducted by Ban and Kang (2016). Coupons were removed from the tubes and rinsed, then dried at room temperature using a fan for 30 min. Dried samples were exposed to saturated steam of 1.2 m/s on both sides for 2, 4, 7, or 10 s total. The distance between the coupons and the nozzle of the steam generator was 7 cm. During the steam treatment, the temperature was controlled using a k-type temperature sensor and an on–off system.

2.5. Sodium hypochlorite (NaOCl) treatment

After the drying step previously described, the NaOCl treatment was carried out. The concentration of NaOCl was 10 ppm throughout the whole experiment. Coupons were immersed in 30 ml of NaOCl in a 50 ml conical centrifuge tube for 5, 10, 20 and 30 s. After that, to wash out residues of NaOCl, coupons were washed two times by immersing in 30 ml of sterile distilled water for 5 s.

2.6. Bacterial enumeration

After the steam or NaOCl treatment, coupons were immediately deposited in sterile 50-ml conical centrifuge tubes containing 30 ml of peptone water (PW; Difco) and 3 g of sterile glass beads (425–600 μm; Sigma Aldrich, St. Louis, MO, USA), then agitated with a benchtop vortex mixer set at maximum speed for 1 min. Cell suspensions in tubes were tenfold serial diluted in PW, then 0.1 ml of undiluted cell suspension or diluents were spread-plated onto Baird Parker Agar (BPA; MB cell) to enumerate the number of *S. aureus* biofilm cells. When low bacterial numbers were anticipated, 1 ml of undiluted cell suspension was plated onto four plates of the medium. The plates were incubated at 37°C for 24–48 h and colonies were counted. For injured cells of *S. aureus*, the overlay method was utilized (Kim, Jeong, & Kang, 2017; Kim, Park, & Kang, 2018). TSA was used as a nutrition medium to resuscitate injured cells. The same amount of diluent was spread-plated onto TSA, then incubated for 2 h at 37°C and overlaid with 10 ml of BPA. After solidification, overlaid plates were transferred to an incubator and kept for 22–46 h at 37°C.

2.7. EPS quantification

EPS quantification methods were adapted and modified from Stiefel et al. (2016). In this study, EPS quantification was performed in two different ways. First, EPS was quantified for the biofilms formed in a 96-well plate. Secondly, EPS contents were analyzed for biofilms developed on stainless steel coupon. In the former method, a diluted cell suspension was added to a 96-well plate to form biofilms. After biofilms formation, the plate was washed twice with 250 μl of PBS and staining dye was directly added to the 96-well plate. The latter method was performed by the following procedure. After adding 1 ml of the bacterial solution and stainless steel coupons to a 15-ml tube, biofilms were formed as described above. The biofilms developed onto stainless steel were removed from the tubes and rinsed to removed loosely attached cells. After rinsing, each coupon was transferred to the staining solution to extract EPS. Then, the extracted solution was analyzed on a 96-well plate.

2.7.1. Crystal violet assay (Total biomass contents)

250 μl of 0.1% crystal violet solution was added to each well and incubated for 30 min at room temperature. After incubation, the stained solution was removed and washed three times with 0.9% NaCl solution. 200 μl of 30% acetic acid was used for dissolving the crystal violet solution bound to the biofilm and transferred to another 96-well plate. Absorbance was measured at 595 nm using a spectrofluorophotometer (Spectramax M2e; Molecular Devices).

2.7.2. Calcofluor white staining (Polysaccharide contents)

250 μl of 1 mg/ml calcofluor white staining solution (Sigma Aldrich; USA) was added to each well and incubated 30 min in the dark at room temperature. After incubation, the stained solution was removed and washed three times with 0.9% NaCl solution. 200 μl of 95% EtOH was added per well and transferred. Fluorescence was measured at an excitation wavelength of 360 nm and an emission wavelength of 460 nm using a spectrofluorophotometer.

2.7.3. FITC staining (Protein contents)

250 μl of 20 μg/ml fluorescein isothiocyanate in dH2O (FITC solution) was added to each well and incubated for 30 min in the dark at room temperature. After the incubation, the stained solution was removed and washed three times with 0.9% NaCl solution. 200 μl of dH2O was added per well and transferred. Fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 528 nm using a spectrofluorophotometer.

2.8. Statistical analysis

Microbial reductions and EPS quantification were given as means ± standard deviations of three independent determinations with duplicate samples at each trial. Obtained Data were analyzed by analysis of variance (ANOVA) accompanied with separation of means by Duncan's multiple range test using the Statistical Analysis System (SAS Institute, Cary, NC, USA). Statistical analyses were performed at a probability level of $p < 0.05$.

3. Results and discussion

3.1. Biofilm formation at different temperatures

In this experiment, temperatures ranging from 15 to 37°C and maturation periods from 1 to 5 days were chosen as experimental conditions for the biofilm development. This temperature range was selected due to its relevancy to food processing (15°C) and infectious disease (37°C) (Di Ciccio et al., 2015; Piercey, Hingston, & Hansen, 2016). Previously, planktonic cells have shown to demonstrate a robust resistance to thermal treatment as growth temperature increases from 20°C to 37°C (Cebrián, Condón, & Mañas, 2019). However, many researchers have selected 25°C for suboptimal conditions to promote biofilm formation (Herrera, Cabo, Gonzalez, Pazos, & Pastoriza, 2007; Pagedar et al., 2010). Cleaning practice is often implemented once a week depending on the processing conditions (Al-Akhras, Al Shorman, Masadeh, Aljarrah, & Ababneh, 2018; López-Gálvez, Truchado, Tudela, Gil, & Allende, 2020). The maturation period (5 days) was chosen based on this assumption. Thus, the biofilm was developed for 5 days (2 days

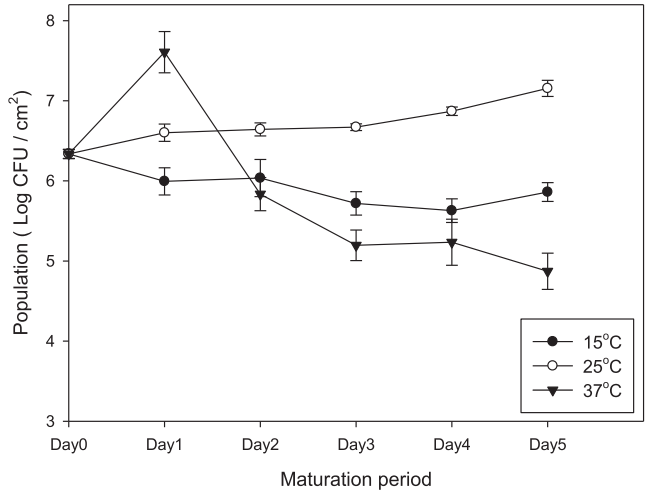

Fig. 1. The population curve of *S. aureus* biofilm on stainless steel for 5 days at 15°C (●), 25°C (○) and 37°C (▼).

extra to account for the initial cell growth and attachment) with the initial population of 6.34 log CFU/cm^2. As Fig. 1 depicts, the biofilm formed at 15 and 25°C showed the relatively constant population, though the latter treatment resulted in a higher population than the former. Specifically, the cell population of *S. aureus* at 15 and 25°C on day 1 was 5.99 and 6.60 log CFU/cm^2 while that of the day 5 maintained at 15 and 25°C was 5.86 and 7.16 log CFU/ cm^2. The observed constant cell population of *S. aureus* was also documented by Rode et al. (2007) who reported that no additional biofilm formation was detected during the maturation period at 20 and 25°C. Also, it was found that the temperature range of 7–28°C did not influence the biofilm cell population (da Silva Meira, de Medeiros Barbosa, Athayde, de Siqueira-Júnior, & de Souza, 2012). A similar trend was observed in another study (Webber et al., 2019).

In contrast, the population of biofilm cells at 37°C sharply increased on day 1 and showed a gradual decline, resulting in the lowest cell population on day 5 ($p < 0.05$). While the cell population on day 1 was 7.61 log CFU/ cm^2, that of day 5 was significantly reduced (4.87 log CFU/cm^2) as illustrated in Fig. 1. The initial increase at 37°C could be partially attributed to a higher extent of hydrophobicity of the cell membrane. A temperature increase from 20 to 37°C leads to an increase in hydrophobicity of the cell membrane, facilitating planktonic cell adhesion to surfaces (Jama, Abdallah, Boukherroub, Faille, & Chihib, 2017). The decline following the initial increase at 37°C is, however, inconsistent with previous findings. In previous studies, the biofilm at 37°C showed a relatively constant cell population (Rode et al., 2007). Abdallah et al. (2014) also found no significant change in the cell population during biofilm maturation at 37°C However, it should be noted that those experiments were conducted only for 48 h while the maturation period in this study was 5 days. The observed decline in the cell population of *S. aureus* biofilms at 37°C was attributed to a presumably higher metabolic rate. Specifically, *S. aureus* grows optimally at 37°C, which would increase its metabolic rate of nutrition consumption and consequently expedite a build-up of its metabolic wastes. Planktonic cell population also showed a similar trend (data not shown). Similar observations were shown where the population of *S. aureus* declined at 37°C (Al-Akhras et al., 2018; Luppens, Rombouts, & Abee, 2002). The previous study also showed that *S. aureus* population was significantly lower after 60 h at 37°C (Luppens et al., 2002).

Table 1
Comparison of log reduction [a] of *S. aureus* induced by steam treatment in selective media and resuscitative media for biofilms developed at ((A) 15°C, (B) 25°C, (C) 37°C).

(A)

Processing time (s)	Day1		Day2		Day5	
	BPA	OV-BPA	BPA	OV-BPA	BPA	OV-BPA
2	0.84 ± 0.59 Aa [b]	0.43 ± 0.29 Aa	1.01 ± 0.56 Aa	0.70 ± 0.50 Aa	0.45 ± 0.35 Aa	0.47 ± 0.38 Aa
4	2.17 ± 0.46 Ba	2.13 ± 0.20 Ba	2.48 ± 0.40 Ba	2.55 ± 0.45 Ba	1.88 ± 1.16 Aa	1.52 ± 1.03 Aa
7	> 5.74	4.50 ± 0.68C	> 5.60	4.78 ± 0.66C	> 5.70	5.01 ± 0.49B
10	> 5.74	> 5.77	> 5.60	> 5.28	> 5.70	> 5.68

(B)

Processing time (s)	Day1		Day2		Day5	
	BPA	OV-BPA	BPA	OV-BPA	BPA	OV-BPA
2	0.68 ± 0.38 Aa	0.65 ± 0.47 Aa	0.29 ± 0.19 Aa	0.21 ± 0.16 Aa	0.46 ± 0.48 Aa	0.56 ± 0.70 Aa
4	2.31 ± 1.39 ABa	2.30 ± 1.49 ABa	1.47 ± 0.21 Ba	1.65 ± 0.27 Ba	0.67 ± 0.03 Aa	0.84 ± 0.06 Ab
7	4.13 ± 1.06 Ba	3.52 ± 1.60 Ba	3.94 ± 0.86 Ca	3.84 ± 0.23 Ca	2.52 ± 0.36 Ba	2.64 ± 0.60 Ba
10	> 5.97	> 5.72	> 5.92	> 5.87	4.10 ± 0.53 Ca	5.22 ± 1.63 Ca

(C)

Processing time (s)	Day1		Day2		Day5	
	BPA	OV-BPA	BPA	OV-BPA	BPA	OV-BPA
2	0.55 ± 0.27 Aa	0.38 ± 0.48 Aa	0.53 ± 0.22 Aa	1.03 ± 0.98 Aa	0.25 ± 0.10 Aa	0.44 ± 0.16 Aa
4	1.76 ± 0.34 Ba	1.56 ± 0.34 Aa	1.47 ± 0.38 Aa	1.44 ± 0.31 Aa	1.25 ± 0.29 Ba	1.40 ± 0.19 Ba
7	3.90 ± 0.67 Ca	2.89 ± 0.63 Ba	3.53 ± 1.06 Ba	3.77 ± 1.20 Ba	3.49 ± 0.64 Ca	4.18 ± 0.82 Ca
10	5.71 ± 0.55 Da	5.39 ± 1.08 Ca	> 4.81	> 4.53	> 4.88	> 4.67

[a] The Unit of log reduction is Log CFU/cm^2.
[b] Values in the same column followed by the same uppercase letter are not significantly different ($p > 0.05$) and Values in the same row followed by the same lowercase letter are not significantly different ($P > 0.05$)

3.2. Comparison of S. aureus biofilm resistance against thermal and non-thermal treatment

Inactivation experiments were also conducted to compare the effects of temperature and maturation period on the biofilm resistance to thermal and non-thermal treatments. It has been reported that biofilm age has a significant effect on the resistance to sanitizers (Belessi, Gounadaki, Psomas, & Skandamis, 2011). Nguyen and Yuk (2013) researched the effect of aging conditions on the resistance of *Salmonella* Typhimurium biofilms to disinfectants. Specifically, the authors noted that biofilm aging resulted in enhanced resistance, though the extent of this improvement was highly dependent on development conditions (Nguyen & Yuk, 2013). In another study, incubation time for 12 days at 4°C significantly increased the resistance of *Listeria monocytogenes* against quaternary ammonium compound and peroxyacetic acid (Belessi et al., 2011). The effect of temperature on biofilm resistance was also documented in a study by Pang, Wong, Chung, and Yuk (2019) who showed that biofilms of *L. monocytogenes* exhibited higher resistance to quaternary ammonium compound at 15°C when compared to 4°C. The biofilms of *Pseudomonas aeruginosa* were found to become more resistant as temperature increased (Khelissa, Abdallah, Jama, & Chihib, 2019). A similar trend was observed in our study.

As shown in Table 1, the steam treatment for 10 s was sufficient to inactivate biofilms over 4.8 log CFU/cm^2 in most of the tested conditions, except for the biofilm at 25°C on day 5. For the biofilm at 25°C on day 5, the steam treatment only resulted in 4.1 log reduction. This result indicated a significant increase in the resistance of the biofilm matured at 25°C. On the other hand, the biofilm formed at 15°C was most vulnerable to thermal treatment (Table 1-(A)). Specifically, no viable cell was detected even after 7 s of the treatment regardless of the maturation period. However, no cross effect between temperature and maturation period was observed in the steam treatment ($p > 0.05$).

Similar trends were documented for the NaOCl treatment as reported in Table 2. The biofilm

of *S. aureus* at 37°C showed over a 2-log reduction after 20 s exposure to NaOCl, but no significant difference was observed among the maturation periods ($p > 0.05$). In contrast, the maturation period of biofilm development played a significant role on the biofilm resistance enhancement at 15 and 25°C ($p < 0.05$) (Table 2). The same treatment only resulted in an approximately 1 log reduction for the biofilms developed at 25°C for 5 days. However, Table 2-(B) clearly indicated that the biofilms formed at 25°C for 1 and 2 days were more vulnerable to a NaOCl treatment of 20 s ($p < 0.05$). A similar trend was also observed at the biofilm maintained at 15°C. The log reduction of the 5 day biofilm after 30 s treatment was 1.7 log CFU/cm^2, which was significantly lower than day 1 and day 2 ($p < 0.05$). Contrary to the steam treatment, the cross effect between temperature and maturation period was significant for the NaOCl treatment ($p < 0.05$). Overall, our results aligned with a previous study that showed enhanced *S. aureus* biofilm stability at 25°C, leading to a greater amount of viable bacterial cells (Pagedar et al., 2010).

We also investigated injured bacterial cells after the thermal and non-thermal treatments. An injured cell can be defined as a cell that is sub-lethally injured while still capable of performing metabolic activity (Kim et al., 2018). As an injured cell retains its metabolic activity, it can resuscitate and recover its toxicity in a favorable environment (Bozoglu, Alpas, & Kaletunç, 2004). Table 1 represents the comparison of log reduction of cell populations from the inoculation in selective and non-selective media after the steam treatment. Overall, the steam treatment did not show a significant difference between the two media ($p > 0.05$). However, for the biofilm developed at 15°C, it took 10 s to eliminate the cell population over 5.5 CFU log/cm^2 in non-selective media, whereas 7 s was enough in selective media. Table 2 also demonstrates the comparison of the reduction level in both media after the NaOCl treatment. No significant difference between the two media was observed in any tested conditions. This implied that both

Table 2
Comparison of log reduction [a] induced by NaOCl treatment in selective media and resuscitative media for biofilms developed at ((A) 15°C, (B) 25°C, (C) 37°C).

(A)

Processing time (s)	Day1		Day2		Day5	
	BPA	OV-BPA	BPA	OV-BPA	BPA	OV-BPA
5	1.00 ± 0.74 Aa [b]	0.98 ± 0.51 Aa	1.34 ± 0.16 Aa	1.26 ± 0.34 Aa	1.41 ± 0.49 Aa	1.45 ± 0.45 Aa
10	1.41 ± 0.49 Aa	1.11 ± 0.55 Aa	1.55 ± 0.37 Aa	1.39 ± 0.50 Aa	1.70 ± 0.27 ABa	1.81 ± 0.10 ABa
20	2.48 ± 0.29 Ba	2.13 ± 0.29 Ba	1.96 ± 0.51 Aba	1.65 ± 0.48 Aa	2.08 ± 0.18 Ba	2.16 ± 0.26 Ba
30	3.48 ± 0.27 Ca	3.25 ± 0.18 Ca	2.88 ± 0.25 Ba	2.61 ± 0.20 Ba	1.70 ± 0.27 ABa	1.79 ± 0.39 ABa

(B)

Processing time (s)	Day1		Day2		Day5	
	BPA	OV-BPA	BPA	OV-BPA	BPA	OV-BPA
5	0.81 ± 0.30 Aa	0.91 ± 0.33 Aa	0.92 ± 0.57 Aa	0.93 ± 0.37 Aa	0.37 ± 0.39 Aa	0.41 ± 0.22 Aa
10	1.21 ± 0.22 Ba	1.29 ± 0.19 Aa	1.28 ± 0.42 Aa	1.26 ± 0.42 Aba	0.75 ± 0.26 Aba	0.87 ± 0.24 Ba
20	1.80 ± 0.09 Ca	1.85 ± 0.08 Ba	2.10 ± 0.95 Aa	2.38 ± 0.72 Ca	0.88 ± 0.16 Ba	1.04 ± 0.13 Ba
30	2.40 ± 0.09 Da	2.56 ± 0.15 Ca	2.10 ± 0.67 Aa	2.23 ± 0.58 BCa	0.95 ± 0.16 Ba	1.09 ± 0.18 Ba

(C)

Processing time (s)	Day1		Day2		Day5	
	BPA	OV-BPA	BPA	OV-BPA	BPA	OV-BPA
5	0.42 ± 0.52 Aa	0.26 ± 0.60 Aa	0.79 ± 0.47 Aa	1.11 ± 1.00 Aa	1.05 ± 0.31 Aa	0.89 ± 0.57 Aa
10	1.17 ± 0.57 Aa	1.32 ± 0.69 ABa	1.08 ± 0.82 Aa	1.61 ± 1.10 Aa	1.00 ± 0.40 Aa	0.89 ± 0.31 Aa
20	2.14 ± 0.20 Ba	2.20 ± 0.71 BCa	2.33 ± 0.16 Ba	1.88 ± 0.34 Aa	2.04 ± 0.05 Ba	1.77 ± 0.53 Aa
30	2.72 ± 0.44 Ba	2.94 ± 0.89 Ca	2.03 ± 0.14 Ba	2.03 ± 0.49 Aa	2.76 ± 0.30 Ca	2.70 ± 0.43 Ba

[a] The Unit of log reduction is Log CFU/cm^2.
[b] Values in the same column followed by the same uppercase letter are not significantly different ($p > 0.05$) and Values in the same row followed by the same lowercase letter are not significantly different ($P > 0.05$)

Table 3
Comparison of cell mass (crystal violet), polysaccharide (Calcofluor white staining), protein (FITC) for day 1 and day 5 of biofilm developed on 96 well plates.

Temperature	Crystal violet		Calcofluor white staining		FITC	
	Day 1	Day 5	Day 1	Day 5	Day 1	Day 5
15°C	0.15 ± 0.01Aa [a]	0.15 ± 0.01Aa	637 ± 15Aa	1300 ± 61Ab	276 ± 28Aa	800 ± 22Ab
25°C	0.19 ± 0.01Aa	0.42 ± 0.08Bb	635 ± 27Aa	2191 ± 169Bb	123 ± 5Ba	1902 ± 134Bb
37°C	0.76 ± 0.04Ba	0.82 ± 0.05Ca	795 ± 152Aa	1457 ± 182Ab	173 ± 2Ca	3306 ± 241Cb

[a] Data represents mean ± standard deviation. The values followed by same uppercase letter in the same column and same lowercase letter in the same row are not significantly different ($P > 0.05$).

treatments effectively inactivated *S. aureus* biofilms without the generation of injured bacterial cells.

3.3. EPS quantification and comparison among different conditions

To elucidate the improved the resistance of biofilm formed at 25°C, we conducted an experiment for the total biomass and EPS quantification using 96-well plates and stainless steel coupons. The rationale for this experiment was that EPS may serve as a means of protection to lethal environmental conditions for bacterial cells embedded in its structure (Stiefel et al., 2016). Table 3 shows changes in total biomass, polysaccharide, and protein content in terms of varying temperatures and maturation periods. It was shown that the cell mass increased at 25°C ($p < 0.05$), and the highest mass was observed at 37°C on day 5 ($p < 0.05$). Also, the polysaccharide content increased for all temperatures. Even though there was no significant difference between the biofilms at 15 and 37°C ($p > 0.05$), the highest polysaccharide content was observed at 25°C ($p < 0.05$) as reported in Table 3. In addition, the protein content increased in all conditions, but *S. aureus* secreted the most amount of protein at 37°C ($p < 0.05$).

A similar trend was observed for the total biomass and EPS contents of biofilm formed onto stainless steel, but there was no significant difference among varying conditions. The highest cell mass was observed for biofilms after 5 days of maturation at 37°C (Table 4). In addition, bacterial cells at 25°C secreted the largest amounts of polysaccharides, though no significant difference was observed between 25 and 37°C ($p > 0.05$). Likewise, the greatest amount of protein was observed at 5 days at 37°C, but there was no significant difference between 25 and 37°C ($p > 0.05$). Our results showed that the amount of EPS increased significantly as the biofilm aged in the given temperature ranges. In contrast to the inactivation results, it was observed that the incubation at 37°C resulted in the highest value in the CV (Total biomass) and FITC staining (Protein). The CV method stains not only EPS, but also adhered cells so that we can evaluate the total biomass constructing the biofilm (Flemming, Neu, & Wingender, 2016). This finding indicated that the biofilm formed at 37°C contained a substantial amount of biomass. As previously mentioned, this could be due to the optimal *S. aureus* metabolism at 37°C (Le Loir et al., 2003). Previous work has demonstrated that *S. aureus* exhibited the highest metabolic activities at 37°C in the temperature range of 25–65°C (Miao et al., 2019). The biofilm matrix consists of viable cells, EPS, and dead cells (Flemming & Wingender, 2010; Tang et al., 2018; Webb et al., 2003). As discussed earlier, the biofilm cell population decreased from day 1 to day 5 due to depletion of nutrients and accumulation of metabolic waste, resulting in an increase in the number of dead cells. Thus, we can assume that the higher CV value at 37°C was mainly due to the dead cells. In addition, most of the dead cells are located in the lower part of the biofilm structure (Klinger-Strobel, Stein, Forstner, Makarewicz, & Pletz, 2017). This implies that viable cells cannot be protected by dead cells from outer lethal stress. Regarding the observed increased EPS content in the biofilm developed at 25°C, it was attributed to the secreted polysaccharide instead of total protein content. Specifically, poly-N-acetylglucosamine (PNAG) and polysaccharide intercellular adhesin (PIA) have shown to be two major EPS components produced by *S. aureus* (Cramton, Gerke, Schnell, Nichols, & Götz, 1999; Maira-Litrán et al., 2002; Rode et al., 2007). Ravaioli et al. (2020) also showed that exopolysaccharide accounts for greater contribution to *Staphylococcus* spp. biofilm compared to protein or eDNA. In fact, certain studies using microscopy techniques have clearly shown that *S. aureus* biofilm was well developed around 28°C (da Silva Meira et al., 2012; Makovcova et al., 2017). However, Jia, Xue, Duan, and Shao (2011) reported that only several cell clusters were presented and EPS was hardly observed in the SEM image of the biofilm formed at 37°C. Considering these findings, the increased tolerance to both thermal and non-thermal treatments at 25°C was likely due to the greater amount of secreted polysaccharide.

Another interesting finding is that the prolonged treatment of sodium hypochlorite treatment at 25°C for 5 days did not induce further microbial inactivation, as shown in Table 2. This was consistent with previous works that reported similar results (Abdallah et al., 2014; da Silva Meira et al., 2012). This may be because EPS acts as a physical barrier and prevents the chemical sanitizers from interacting with the bacterial cells, leading to an increase in tolerance against disinfectants (Bridier, Dubois-Brissonnet, Greub, Thomas, & Briandet, 2011).

4. Conclusion

This study showed that the biofilm resistance to both thermal and non-thermal treatments was highly dependent on the temperature and biofilm maturation period. In particular, the biofilm of *S. aureus* under the 25°C condition showed the greatest extent of resistance due to the formation of the greatest exopolysaccharide content. Though the sodium hypochlorite treatment reduced only 1 log population of biofilm cells formed at 25°C for 5 days, the steam treatment was more effective

Table 4
Comparison of cell mass (crystal violet), polysaccharide (Calcofluor white staining), protein (FITC) for day 1 and day 5 of biofilm developed on stainless steel coupon.

Temperature	Crystal violet		Calcofluor white staining		FITC	
	Day 1	Day 5	Day 1	Day 5	Day 1	Day 5
15°C	0.17 ± 0.03Aa [a]	0.07 ± 0.01Ab	268 ± 74Aa	221 ± 26Aa	234 ± 28Aa	3180 ± 790Ab
25°C	0.01 ± 0.00Ba	0.14 ± 0.01Ab	182 ± 11Aa	475 ± 98Bb	501 ± 69Ba	7253 ± 1689ABb
37°C	0.21 ± 0.02Aa	0.36 ± 0.12Ba	181 ± 15Aa	276 ± 102ABa	358 ± 13Ca	12534 ± 4899Bb

[a] Data represents mean ± standard deviation. The values followed by same uppercase letter in the same column and same lowercase letter in the same row are not significantly different ($P > 0.05$).

in eliminating the pathogen, by > 4 log CFU/cm^2. Thus, this study highlights the need of careful evaluations of the environment so that the food sanitization treatments can be optimized. However, it is important to note that there are many other environmental conditions such as pH and nutritional composition, both of which can affect the resistance of *S. aureus* biofilm. Hence, future studies must evaluate the combination effects of these factors on the build-up of bacterial biofilm resistance.

CRediT authorship contribution statement

Woo-Ju Kim: Conceptualization, Methodology, Validation, Investigation, Writing - original draft. **Soo-Hwan Kim:** Methodology, Formal analysis, Investigation, Writing - original draft. **Dong-Hyun Kang:** Writing - review & editing, Supervision, Project administration.

Acknowledgment

This work was supported by a National Research Foundation of Korea (NRF) grant funded by the South Korean government (grant NRF-2018R1A2B2008825).

References

Abdallah, M., Chataigne, G., Ferreira-Theret, P., Benoliel, C., Drider, D., Dhulster, P., & Chihib, N.-E. (2014). Effect of growth temperature, surface type and incubation time on the resistance of *Staphylococcus aureus* biofilms to disinfectants. *Applied Microbiology and Biotechnology, 98*(6), 2597–2607.

Al-Akhras, M.-A. H., Al Shorman, M., Masadeh, M. M., Aljarrah, K., & Ababneh, Z. (2018). Modeling the effect of Rose Bengal on growth and decay patterns of *Pseudomonas aeruginosa*. *Escherichia coli and Staphylococcus aureus: Vol. 305*, (pp. 012004–). IOP Publishing.

Asao, T., Kumeda, Y., Kawai, T., Shibata, T., Oda, H., Haruki, K., ... Kozaki, S. (2003). An extensive outbreak of staphylococcal food poisoning due to low-fat milk in Japan: Estimation of enterotoxin A in the incriminated milk and powdered skim milk. *Epidemiology and Infection, 130*(1), 33.

Bae, Y.-M., Baek, S.-Y., & Lee, S.-Y. (2012). Resistance of pathogenic bacteria on the surface of stainless steel depending on attachment form and efficacy of chemical sanitizers. *International Journal of Food Microbiology, 153*(3), 465–473.

Ban, G.-H., & Kang, D.-H. (2016). Effectiveness of superheated steam for inactivation of *Escherichia coli* O157: H7, *Salmonella* Typhimurium, *Salmonella* Enteritidis phage type 30, and *Listeria monocytogenes* on almonds and pistachios. *International Journal of Food Microbiology, 220*, 19–25.

Ban, G.-H., Yoon, H., & Kang, D.-H. (2014). A comparison of saturated steam and superheated steam for inactivation of *Escherichia coli* O157: H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* biofilms on polyvinyl chloride and stainless steel. *Food Control, 40*, 344–350.

Bari, L., Nei, D., Sotome, I., Nishina, I. Y., Hayakawa, F., Isobe, S., & Kawamoto, S. (2010). Effectiveness of superheated steam and gas catalytic infrared heat treatments to inactivate *Salmonella* on raw almonds. *Foodborne Pathogens and Disease, 7*(7), 845–850.

Belessi, C.-E. A., Gounadaki, A. S., Psomas, A. N., & Skandamis, P. N. (2011). Efficiency of different sanitation methods on *Listeria monocytogenes* biofilms formed under various environmental conditions. *International Journal of Food Microbiology, 145*, S46–S52.

Bozoglu, F., Alpas, H., & Kaletunç, G. (2004). Injury recovery of foodborne pathogens in high hydrostatic pressure treated milk during storage. *FEMS Immunology & Medical Microbiology, 40*(3), 243–247.

Bridier, A., Dubois-Brissonnet, F., Greub, G., Thomas, V., & Briandet, R. (2011). Dynamics of the action of biocides in Pseudomonas aeruginosa biofilms. *Antimicrobial Agents and chemotherapy*.

CDC (2017). Foodborne Outbreak Online Database.

CDC (2018). List of Selected Multistate Foodborne Outbreak Investigations.

Cebrián, G., Condón, S., & Mañas, P. (2019). Heat resistance, membrane fluidity and sublethal damage in *Staphylococcus aureus* cells grown at different temperatures. *International Journal of Food Microbiology, 289*, 49–56.

Cenkowski, S., Pronyk, C., Zmidzinska, D., & Muir, W. (2007). Decontamination of food products with superheated steam. *Journal of Food Engineering, 83*(1), 68–75.

Chang, S. S., Han, A., Reyes-De-Corcuera, J., Powers, J., & Kang, D. H. (2010). Evaluation of steam pasteurization in controlling *Salmonella* serotype Enteritidis on raw almond surfaces. *Letters in Applied Microbiology, 50*(4), 393–398.

Chavant, P., Gaillard-Martinie, B., & Hébraud, M. (2004). Antimicrobial effects of sanitizers against planktonic and sessile *Listeria monocytogenes* cells according to the growth phase. *FEMS Microbiology Letters, 236*(2), 241–248.

Chen, J., Rossman, M. L., & Pawar, D. M. (2007). Attachment of enterohemorrhagic Escherichia coli to the surface of beef and a culture medium. *LWT-Food Science and Technology, 40*(2), 249–254.

Chmielewski, R., & Frank, J. (2003). Biofilm formation and control in food processing facilities. *Comprehensive Reviews in Food Science and Food Safety, 2*(1), 22–32.

Cortés, M. E., Bonilla, J. C., & Sinisterra, R. D. (2011). Biofilm formation, control and novel strategies for eradication. *Science Against Microbial Pathogens: Communicating Current Research and Technological Advances, 2*, 896–905.

Cramton, S. E., Gerke, C., Schnell, N. F., Nichols, W. W., & Götz, F. (1999). The intercellular adhesion (ica) locus is present in Staphylococcus aureus and is required for biofilm formation. *Infection and immunity, 67*(10), 5427–5433.

da Silva Meira, Q. G., de Medeiros Barbosa, I., Athayde, A. J. A. A., de Siqueira-Júnior, J. P., & de Souza, E. L. (2012). Influence of temperature and surface kind on biofilm formation by *Staphylococcus aureus* from food-contact surfaces and sensitivity to sanitizers. *Food Control, 25*(2), 469–475.

De Beer, D., Srinivasan, R., & Stewart, P. S. (1994). Direct measurement of chlorine penetration into biofilms during disinfection. *Applied and Environmental Microbiology, 60*(12), 4339–4344.

Di Bonaventura, G., Piccolomini, R., Paludi, D., D'orio, V., Vergara, A., Conter, M., & Ianieri, A. (2008). Influence of temperature on biofilm formation by *Listeria monocytogenes* on various food-contact surfaces: Relationship with motility and cell surface hydrophobicity. *Journal of Applied Microbiology, 104*(6), 1552–1561.

Di Ciccio, P., Vergara, A., Festino, A., Paludi, D., Zanardi, E., Ghidini, S., & Ianieri, A. (2015). Biofilm formation by Staphylococcus aureus on food contact surfaces: Relationship with temperature and cell surface hydrophobicity. *Food Control, 50*, 930–936.

Flemming, H.-C., Neu, T. R., & Wingender, J. (2016). *The perfect slime: Microbial extracellular polymeric substances (EPS)*. IWA Publishing.

Flemming, H.-C., & Wingender, J. (2010). The biofilm matrix. *Nature Reviews Microbiology, 8*(9), 623.

Frank, J. F., Ehlers, J., & Wicker, L. (2003). Removal of *Listeria monocytogenes* and poultry soil-containing biofilms using chemical cleaning and sanitizing agents under static conditions. *Food Protection Trends, 23*(8).

Giaouris, E., Chorianopoulos, N., & Nychas, G.-J. (2005). Effect of temperature, pH, and water activity on biofilm formation by *Salmonella enterica* enteritidis PT4 on stainless steel surfaces as indicated by the bead vortexing method and conductance measurements. *Journal of Food Protection, 68*(10), 2149–2154.

Gibson, H., Taylor, J., Hall, K., & Holah, J. (1999). Effectiveness of cleaning techniques used in the food industry in terms of the removal of bacterial biofilms. *Journal of Applied Microbiology, 87*(1), 41–48.

Gupta, P., Sarkar, S., Das, B., Bhattacharjee, S., & Tribedi, P. (2016). Biofilm, pathogenesis and prevention—a journey to break the wall: A review. *Archives of Microbiology, 198*(1), 1–15.

Hennekinne, J.-A., De Buyser, M.-L., & Dragacci, S. (2012). *Staphylococcus aureus* and its food poisoning toxins: Characterization and outbreak investigation. *FEMS Microbiology Reviews, 36*(4), 815–836.

Herrera, J., Cabo, M., Gonzalez, A., Pazos, I., & Pastoriza, L. (2007). Adhesion and detachment kinetics of several strains of *Staphylococcus aureus* subsp. aureus under three different experimental conditions. *Food Microbiology, 24*(6), 585–591.

Jama, C., Abdallah, M., Boukherroub, R., Faille, C., & Chihib, N.-E. (2017). Effect of incubation duration, growth temperature, and abiotic surface type on cell surface properties, adhesion and pathogenicity of biofilm-detached *Staphylococcus aureus* cells. *AMB Express, 7*(1), 191.

Jessen, B., & Lammert, L. (2003). Biofilm and disinfection in meat processing plants. *International Biodeterioration & Biodegradation, 51*(4), 265–269.

Jia, P., Xue, Y., Duan, X., & Shao, S. (2011). Effect of cinnamaldehyde on biofilm formation and sarA expression by methicillin-resistant Staphylococcus aureus. *Letters in Applied Microbiology, 53*(4), 409–416.

Jørgensen, H., Mørk, T., Høgåsen, H., & Rørvik, L. (2005). Enterotoxigenic *Staphylococcus aureus* in bulk milk in Norway. *Journal of Applied Microbiology, 99*(1), 158–166.

Kérouanton, A., Hennekinne, J., Letertre, C., Petit, L., Chesneau, O., Brisabois, A., & De Buyser, M. (2007). Characterization of *Staphylococcus aureus* strains associated with food poisoning outbreaks in France. *International Journal of Food Microbiology, 115*(3), 369–375.

Khelissa, S. O., Abdallah, M., Jama, C., & Chihib, N.-E. (2019). Actively detached *Pseudomonas aeruginosa* biofilm cell susceptibility to benzalkonium chloride and associated resistance mechanism. *Archives of Microbiology, 201*(6), 747–755.

Kim, W., Jeong, K.-O., Cheon, H.-L., & Kang, D.-H. (2019). Comparison of spindle and stomacher efficacy for detaching biofilms from stainless steel, PVC, and green leafy vegetable surfaces. *British Food Journal, 122*, 503–514.

Kim, W.-J., Jeong, K.-O., & Kang, D.-H. (2017). Inhibition of Initial Attachment of Injured *Salmonella* Typhimurium onto Abiotic Surfaces. *Journal of Food Protection, 81*(1), 37–42.

Kim, W.-J., Park, S.-H., & Kang, D.-H. (2018). Inactivation of foodborne pathogens influenced by dielectric properties, relevant to sugar contents, in chili sauce by 915 MHz microwaves. *LWT, 96*, 111–118.

Kim, S.-H., Park, S.-H., Kim, S.-S., & Kang, D.-H. (2019). Inactivation of *Staphylococcus aureus* biofilms on food contact surfaces by superheated steam treatment. *Journal of Food Protection, 82*(9), 1496–1500.

Klinger-Strobel, M., Stein, C., Forstner, C., Makarewicz, O., & Pletz, M. W. (2017). Effects of colistin on biofilm matrices of *Escherichia coli* and *Staphylococcus aureus*. *International Journal of Antimicrobial Agents, 49*(4), 472–479.

Le Loir, Y., Baron, F., & Gautier, M. (2003). *Staphylococcus aureus* and food poisoning. *Genetics and Molecular Research, 2*(1), 63–76.

López-Gálvez, F., Truchado, P., Tudela, J. A., Gil, M. I., & Allende, A. (2020). Critical points affecting the microbiological safety of bell peppers washed with peroxyacetic acid in a commercial packinghouse. *Food Microbiology, 88*, Article 103409.

Luppens, S., Rombouts, F., & Abee, T. (2002). The effect of the growth phase of *Staphylococcus aureus* on resistance to disinfectants in a suspension test. *Journal of Food Protection, 65*(1), 124–129.

Maira-Litrán, T., Kropec, A., Abeygunawardana, C., Joyce, J., Mark, G., Goldmann, D. A., & Pier, G. B. (2002). Immunochemical properties of the staphylococcal poly-N-acetylglucosamine surface polysaccharide. *Infection and Immunity, 70*(8), 4433–4440.

Makovcova, J., Babak, V., Kulich, P., Masek, J., Slany, M., & Cincarova, L. (2017).

Dynamics of mono-and dual-species biofilm formation and interactions between *Staphylococcus aureus* and Gram-negative bacteria. *Microbial Biotechnology, 10*(4), 819–832.
Menon, K. V. (2016). Biofilm and food industry. *International Journal of Advanced Research in Biological Sciences, 3*(4), 137–142.
Miao, J., Lin, S., Soteyome, T., Peters, B. M., Li, Y., Chen, H., ... Xu, Z. (2019). Biofilm formation of *Staphylococcus aureus* under food heat processing conditions: First report on CML production within biofilm. *Scientific Reports, 9*(1), 1–9.
Morgan, A. I., Goldberg, N., Radewonuk, E. R., & Scullen, O. J. (1996). Surface pasteurization of raw poultry meat by steam. *LWT-Food Science and Technology, 29*(5–6), 447–451.
Nguyen, H. D. N., & Yuk, H.-G. (2013). Changes in resistance of *Salmonella* Typhimurium biofilms formed under various conditions to industrial sanitizers. *Food Control, 29*(1), 236–240.
Norwood, D., & Gilmour, A. (2000). The growth and resistance to sodium hypochlorite of *Listeria monocytogenes* in a steady-state multispecies biofilm. *Journal of Applied Microbiology, 88*(3), 512–520.
Pagedar, A., Singh, J., & Batish, V. K. (2010). Surface hydrophobicity, nutritional contents affect *Staphylococcus aureus* biofilms and temperature influences its survival in preformed biofilms. *Journal of Basic Microbiology, 50*(S1), S98–S106.
Pang, X., Wong, C., Chung, H.-J., & Yuk, H.-G. (2019). Biofilm formation of *Listeria monocytogenes* and its resistance to quaternary ammonium compounds in a simulated salmon processing environment. *Food Control, 98*, 200–208.
Park, S., Kim, W., & Kang, D. (2018). Effect of relative humidity on inactivation of foodborne pathogens using chlorine dioxide gas and its residues on tomatoes. *Letters in Applied Microbiology, 67*(2), 154–160.
Park, H. W., Yoo, J. S., Jung, H., & Yoon, W. B. (2019). Developing a sterilization processing and a grading system to produce a uniform quality of sterilized whole corn (Zea mays L. var. ceratina). *Journal of Food Engineering, 249*, 55–65.
Park, H. W., & Yoon, W. B. (2018). Computational fluid dynamics (CFD) modelling and application for sterilization of foods: A review. *Processes, 6*(6), 62.
Park, H. W., & Yoon, W. B. (2019). A quantitative microbiological exposure assessment model for *Bacillus cereus* in pasteurized rice cakes using computational fluid dynamics and Monte Carlo simulation. *Food Research International, 125*, Article 108562.
Parsek, M. R., & Singh, P. K. (2003). Bacterial biofilms: An emerging link to disease pathogenesis. *Annual Reviews in Microbiology, 57*(1), 677–701.
Piercey, M. J., Hingston, P. A., & Hansen, L. T. (2016). Genes involved in *Listeria monocytogenes* biofilm formation at a simulated food processing plant temperature of 15 C. *International Journal of Food Microbiology, 223*, 63–74.
Plaza-Rodríguez, C., Kaesbohrer, A., & Tenhagen, B. A. (2019). Probabilistic model for the estimation of the consumer exposure to methicillin-resistant *Staphylococcus aureus* due to cross-contamination and recontamination. *MicrobiologyOpen, 8*(11), Article e900.
Pompermayer, D. M., & Gaylarde, C. C. (2000). The influence of temperature on the adhesion of mixed cultures of *Staphylococcus aureus and Escherichia coli* to polypropylene. *Food Microbiology, 17*(4), 361–365.
Poulsen, L. V. (1999). Microbial biofilm in food processing. *LWT-Food Science and Technology, 32*(6), 321–326.
Ravaioli, S., Campoccia, D., Speziale, P., Pietrocola, G., Zatorska, B., Maso, A., ... Arciola, C. R. (2020). Various biofilm matrices of the emerging pathogen *Staphylococcus lugdunensis*: Exopolysaccharides, proteins, eDNA and their correlation with biofilm mass. *Biofouling, 36*(1), 86–100.
Reynoso, E., Ferreyra, D. D., Durantini, E. N., & Spesia, M. B. (2019). Photodynamic inactivation to prevent and disrupt *Staphylococcus aureus* biofilm under different media conditions. *Photodermatology, Photoimmunology & Photomedicine, 35*(5), 322–331.
Rode, T. M., Langsrud, S., Holck, A., & Møretrø, T. (2007). Different patterns of biofilm formation in *Staphylococcus aureus* under food-related stress conditions. *International Journal of Food Microbiology, 116*(3), 372–383.
Röhner, E., Jacob, B., Böhle, S., Rohe, S., Löffler, B., Matziolis, G., & Zippelius, T. (2020). Sodium hypochlorite is more effective than chlorhexidine for eradication of bacterial biofilm of staphylococci and *Pseudomonas aeruginosa. Knee Surgery Sports Traumatology, Arthroscopy,* 1–7.
Ronner, A. B., & Wong, A. C. (1993). Biofilm development and sanitizer inactivation of *Listeria monocytogenes* and *Salmonella* typhimurium on stainless steel and Buna-n rubber. *Journal of Food Protection, 56*(9), 750–758.
Simões, M., Simões, L. C., Machado, I., Pereira, M. O., & Vieira, M. J. (2006). Control of flow-generated biofilms with surfactants: Evidence of resistance and recovery. *Food and Bioproducts Processing, 84*(4), 338–345.
Simoes, M., Simoes, L. C., & Vieira, M. J. (2010). A review of current and emergent biofilm control strategies. *LWT-Food Science and Technology, 43*(4), 573–583.
Stiefel, P., Rosenberg, U., Schneider, J., Mauerhofer, S., Maniura-Weber, K., & Ren, Q. (2016). Is biofilm removal properly assessed? Comparison of different quantification methods in a 96-well plate system. *Applied Microbiology and Biotechnology, 100*(9), 4135–4145.
Sutherland, J., Bayliss, A., & Roberts, T. (1994). Predictive modelling of growth of *Staphylococcus aureus*: The effects of temperature, pH and sodium chloride. *International Journal of Food Microbiology, 21*(3), 217–236.
Tang, H., Zhang, Y., Ma, Y., Tang, M., Shen, D., & Wang, M. (2018). Regulation of nicotine tolerance by quorum sensing and high efficiency of quorum quenching under nicotine stress in *Pseudomonas aeruginosa* PAO1. *Frontiers in Cellular and Infection Microbiology, 8*, 88.
Tango, C. N., Akkermans, S., Hussain, M. S., Khan, I., Van Impe, J., Jin, Y.-G., & Oh, D. H. (2018). Modeling the effect of pH, water activity, and ethanol concentration on biofilm formation of *Staphylococcus aureus. Food Microbiology, 76*, 287–295.
Troeman, D., Van Hout, D., & Kluytmans, J. (2019). Antimicrobial approaches in the prevention of *Staphylococcus aureus* infections: A review. *Journal of Antimicrobial Chemotherapy, 74*(2), 281–294.
Webb, J. S., Thompson, L. S., James, S., Charlton, T., Tolker-Nielsen, T., Koch, B., ... Kjelleberg, S. (2003). Cell death in *Pseudomonas aeruginosa* biofilm development. *Journal of Bacteriology, 185*(15), 4585–4592.
Webber, B., Oliveira, A. P. D., Pottker, E. S., Daroit, L., Levandowski, R., Santos, L. R. D., ... Rodrigues, L. B. (2019). *Salmonella* Enteritidis forms biofilm under low temperatures on different food industry surfaces. *Ciência Rural, 49*(7).
Wirtanen, G. (2019). Biofilm risks in food processing.
Xu, Z., Xie, J., Soteyome, T., Peters, B. M., Shirtliff, M. E., Liu, J., & Harro, J. M. (2019). *Polymicrobial interaction and biofilms between Staphylococcus aureus and Pseudomonas aeruginosa: An underestimated concern in food safety. Current Opinion in.* Food Science.