UNIVERSITI PUTRA MALAYSIA

BIOFILM FORMATION OF MULTIDRUG-RESISTANT Staphylococcus aureus (MDRSA) ON STAINLESS STEEL SURFACES AND THE EFFECT OF DISINFECTANTS IN ELIMINATING THE BIOFILM

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By

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Staphylococcus aureus is a pathogenic bacterium capable of adhering and forming a biofilm on food processing surfaces, thus causing the cross-contamination of foods. The adhered biofilm can detach from the surface and easily contaminate food as it passes through the surface and leaves toxins. In Malaysia, multidrug-resistant *Staphylococcus aureus* (MDRSA) has been studied in the context of the foodservice environment but the findings on the survival of MDRSA in forming biofilm on stainless steel surfaces and the resistance towards commercial disinfectant are limited. The objectives of this study were 1) to isolate and characterize *S. aureus* from food contact surfaces for the antibiotic-resistant properties, 2) to study the adherence and biofilm formation of the MDRSA on stainless steel surfaces at 25ºC and 37ºC, and 3) to examine the effect of disinfectants in eliminating the biofilm produced by MDRSA on stainless steel surface at 37ºC. A total of 38 *S. aureus* isolated from food contact surface by biochemical test were tested for the antibiotic resistance by using five classes of antibiotics; Penicillin (I), Cephalosporins (II), Amino-glycosides (III), Quinolones Fluoroquinolone (IV), and Sulphonamide (V) by the standard procedures of Kirby-Bauer disc diffusion method. The adherence and biofilm assay performed using 23 MDRSA cultures placed on stainless steel discs at 25°C and 37°C for (24, 48, and 72) hours in adherence and (3, 6, 9, 12, and 15) days in biofilm. The elimination of biofilm was completed on three MDRSA cultures at 37ºC on 9 days using three concentrations of peracetic acid and sodium hypochlorite; 0.01%, 0.02%, and 0.03%. As a result, all the MDRSA can adhere on stainless steel with a minimum 4.00 log CFU/mL. The adherence of MDRSA on stainless steel during 24, 48 and 72 hours ranged from 4.11 to 6.55 log CFU/mL and 4.25 to 6.86 log CFU/ml at 25ºC and 37ºC, respectively. The highest adherence was found on 48 hours at both temperatures. The biofilm formation of MDRSA on 3, 6, 9, 12, and 15 days ranged from 3.56 to 6.80 log CFU/mL and 3.74 to 7.05 log CFU/mL at 25ºC and 37ºC, respectively. SA18 exhibited the best biofilm formation on Day 9 at both temperatures because of the

highest viable cell (log CFU/ml) that formed on the stainless steel surfaces. The MDRSA cultures revealed a high capacity to adhere and form biofilm on stainless steel at 37ºC. As for the disinfectants, sodium hypochlorite was found to be more effective than peracetic acid at eliminating the biofilm, with a log reduction ranging from 2.48–3.54 log CFU/mL in all concentrations. In conclusion, the MDRSA cultures can strongly adhere and form biofilm on stainless steel at their optimum growth temperature. Nevertheless, the biofilm can be eliminated using sodium hypochlorite, which could be a sufficient disinfectant for reducing biofilm formation in the food industry.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PEMBENTUKAN BIOFILM OLEH *Staphylococcus aureus*YANG RINTANG KEPADA PELBAGAI UBAT (MDRSA) PADA PERMUKAAN KELULI TAHAN KARAT DAN KESAN DISINFEKTAN DALAM MENGHAPUSKAN BIOFILM

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Staphylococcus aureus merupakan bakteria patogen yang mampu melekat dan membentuk biofilm pada permukaan pemprosesan makanan yang boleh menyebabkan kontaminasi makanan. Biofilm yang melekat boleh terlepas dari permukaan dan mudah mencemari makanan apabila ia melalui permukaan dengan toksin. Di Malaysia, *Staphylococcus aureus* yang rintang kepada pelbagai ubat (MDRSA) telah dilaporkan dari persekitaran khidmat makanan tetapi hasil kajian dalam kemandirian MDRSA terhadap disinfektan komersial masih terhad. Tujuan kajian ini adalah 1) untuk mengasingkan dan mencirikan *S. aureus* dari permukaan sentuhan makanan untuk sifat rintang antibiotik, 2) untuk mengkaji keupayaan pelekatan dan pembentukan biofilm MDRSA pada permukaan keluli tahan karat pada 25ºC dan 37ºC, dan 3) untuk menguji kesan disinfektan dalam menghapuskan biofilm yang dihasilkan oleh MDRSA pada permukaan keluli tahan karat. Sebanyak 38 *S. aureus* yang diasingkan dan dikenalpasti dari permukaan sentuhan makanan dengan ujian biokimia telah diuji untuk rintangan antibiotik dengan menggunakan lima kelas antibiotik; Penisilin (I), Cephalosporins (II), Amino-glycosides (III), Quinolones Fluoroquinolone (IV), dan Sulphonamide (V) oleh kaedah resapan cakera piawai Kirby-Bauer. Ujian pelekatan dan biofilm dilakukan menggunakan 23 MDRSA kultur yang diletakkan pada cakera keluli tahan karat pada 25 °C dan 37 °C untuk (24, 48, dan 72) jam dalam pematuhan dan (3, 6, 9, 12, dan 15) hari dalam biofilm. Penghapusan biofilm pula dilakukan ke atas tiga kultur MDRSA pada 37ºC untuk 9 hari menggunakan asid peracetic dan natrium hipoklorit dengan tiga kepekatan; 0.01%, 0.02%, dan 0.03%. Hasilnya dinyatakan dalam log CFU/mL. Sebagai hasil, semua MDRSA dapat melekat pada keluli tahan karat dengan minimum 4.00 log CFU/mL. Pelekatan MDRSA pada keluli tahan karat selama 24, 48 dan 72 jam adalah dari 4.11 hingga 6.55 log CFU/mL dan 4.25 hingga 6.86 log CFU/mL pada 25ºC dan 37ºC, masing-masing. Pelekatan tertinggi ditemui pada 48 jam pada kedua-dua suhu.

Pembentukan biofilm MDRSA pada 3, 6, 9, 12, dan 15 hari adalah antara 3.56 hingga 6.80 log CFU/mL dan 3.74 hingga 7.05 log CFU/mL pada 25^{o}C dan 37^{o}C, masing-masing. SA18 menunjukkan pembentukan biofilm tertinggi semasa hari ke-9 pada kedua-dua 25^{o}C dan 37^{o}C dengan 6.80 dan 7.05 log CFU/mL, masing-masing. MDRSA mendedahkan kapasiti tinggi untuk mematuhi dan membentuk biofilm pada keluli tahan karat pada suhu 37^{o}C. Dalam menghapuskan biofilm, natrium hipoklorit didapati paling berkesan dengan pengurangan log antara 2.48 hingga 3.54 log CFU/mL dalam semua kepekatan. Sebagai kesimpulan, MDRSA boleh melekat dan membentuk biofilm pada keluli tahan karat pada suhu pertumbuhan optimum, seterusnya ia dapat disingkirkan oleh natrium hipoklorit yang boleh menjadi disinfektan yang memadai bagi mengurangkan pembentukan biofilm dalam industri makanan.

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LIST OF ABBREVIATIONS

%	Percentage
/	Per
±	Plus-minus sign
µm	Micrometer
CFU	Colony forming unit
Cm	Centimetre
et al	et alia or and others
G	Gram
h	Hour
L	Liter
Log	Logarithm
MDR	Multidrug-resistant
Min	Minute
mL	Mililiter
N	Number of sample
^{o}C	Degree Celcius
pH	Potential of Hydrogen
S	Second
×g	Gravity forces
PAA	Peracetic acid
QAC	Quarternary Ammonium Compoundd
SE	Staphylococcal enterotoxin
SEA	Staphylococcal enterotoxin A
SEE	Staphylococcal enterotoxin E
SEG	Staphylococcal enterotoxin G
SEH	Staphylococcal enterotoxin H

SEl	Staphylococcal-like
SER	Staphylococcal enterotoxin R
SET	Staphylococcal enterotoxin T
STF	Staphylococcal food poisoning
MHA	Mueller Hinton Agar
BHI	Brain Heart Infusion
eDNA	Environmental DNA
SPW	Sterile Peptone Water
MSA	Mannitol Salt Agar
NB	Nutrient Broth
BPA	Baird Parker Agar
MSSA	Methicillin Susceptible *Staphylococcus aureus*
MRSA	Methicillin Resistant *Staphylococcus aureus*
MDRSA	Multidrug Resistant *Staphylococcus aureus*
PCA	Plate Count Agar
EPS	Extracellular polymeric substance
PBS	Phosphate buffered saline
TSB	Tryptic Soy Broth
PIA	Polysaccharide intercellular adhesion
DVLO	Derjaguin, Verwey, Landau and Overbeek
NEO	Northeast Ohio
NaOCl	Sodium hypochlorite
AISI	American Iron and Steel Institute
SA	Staphylococcus aureus
SEM	Scanning Electron Microscope

CHAPTER 1

INTRODUCTION

Staphylococcus aureus is a Gram-positive bacterium with a coccus shape that forms irregular clusters. It is famously known as the main human pathogen to cause widespread infection. *S. aureus* is capable of forming biofilms mainly on food processing surfaces; thus opening up pathways for food cross-contamination (Ciccio, Vergara, & Festino et al., 2015). This bacterial pathogen can also form biofilms on various medicinal plants and host tissues (Boles & Horswill, 2008; Khatoon, McTiernan, Suuronen, et al., 2018). Most bacteria generally live by adhering to surfaces and then forming populations called biofilms (Malheiros, dos Passos, & Casarin, et al., 2010; Dang & Lovell, 2016). Biofilms are groups of microorganisms embedded within an extracellular polymeric substance (EPS) that attaches to actual surfaces. Azeredo et al. (2017) stated that the ability of bacteria to adhere and develop on inert surfaces and the resistance of the resultant cells have been the subject of continuous and extensive studies. Since biofilm bonds with EPS during bacterial infection, the biofilm can be eliminated via antibiotic treatment and the host's immune response (Batoni, Maisetta, & Esin, 2016). If left untreated, however, these covered cells could develop into chronic infection and cause fatality.

There are several unknown factors relating to the adherence and biofilm-forming ability of *S. aureus* or its limitations at the early phases of bacterial adsorption (Mafu, Plumety, & Deschênes et al., 2011). The corrosion impact on compact materials must also be measured since it could indicate the formation and growth of hollows (Mafu et al., 2011). This material, in turn, becomes a trigger location for food pathogens, thus emphasising the importance of cleaning and disinfecting procedures. As mentioned by Jeronimo et al. (2012) and Meira et al. (2012), *S. aureus* has a high capability of adhering and forming biofilm on stainless steel and polypropylene surfaces. Food contact surfaces that have biofilm presence are possibly a health threat (Ciccio et al., 2015). The features of the cell surface and the different environments (e.g. pH, temperature, organic materials, and water activity) of the food industry could influence bacterial adhesion and biofilm formation (Galie et al., 2018; Mafu et al., 2011; Vázquez-Sánchez, Habimana, & Holck, 2013). Recently, *S. aureus* has been found highly resistant to disinfectants (Rode, Langsrud, Holck, & Møretrø, 2007; Pagedar, Singh, & Virender, 2010).

1.1 Problem statement

S. aureus is considered the third most important cause of disease in the world. In Malaysia, an incidence of food poisoning was reported, with *S. aureus* being one of the common causes besides *Vibrio parahaemolvticus* and *Salmonella.* The lack of hygienic practices amongst food handlers particularly during preparation can contaminate food-contact surfaces such as cutting boards and these are the major factors causing *S. aureus* contamination in food products. Plus, the survival of

Staphylococcus aureus on food contact surfaces enhances the capacity of transmission of the bacterial to foods. The bacterial cells could produce toxins and contaminate food as it passes and transfers from the surface. These bacteria are heat-stable and currently show resistance to commercial disinfectants. Therefore, the strategies to control and prevent *S. aureus*-related food contamination in Malaysia must be improved. As the surviving biofilms on surfaces can become potential sources of food contamination, it is crucial to identify the fundamental conditions under which *S. aureus* cells can survive, multiply on food contact surfaces, form biofilms and be removed. The findings of this study will provide fundamental knowledge for forming potential strategies to develop effective cleaning measures and prevent food contamination.

1.2 Objectives

i. To isolate and characterize *S. aureus* from food contact surfaces for the antibiotic-resistant properties.
ii. To study the adherence and biofilm formation of the MDRSA on AISI-304 stainless steel surfaces at 25^{o}C and 37^{o}C.
iii. To examine the effect of disinfectants in eliminating the biofilm produced by MDRSA on AISI-304 stainless steel surface at 37^{o}C.

1.3 Hypothesis

Cleaned food contact surfaces could still contain *Staphylococcus aureus,* which carries antimicrobial resistant properties and able to form the biofilm. The ability of *S. aureus* to adhere and form biofilm on food contact surface such as stainless steel surfaces caused the food contamination and it is influenced by the temperature and time. The bacteria normally have the highest growth at the optimum temperature and time. Furthermore, *S. aureus* has now become less susceptible to disinfectants and hot water, which both are usually used in a food processing environment.

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