ANTIMICROBIAL RESISTANCE AND BIOFILM FORMATION POTENTIAL OF ENTEROTOXIGENIC Staphylococcus aureus AND Escherichia coli FROM CUTTING BOARD SURFACES

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By

SHU'AIBU ISA

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

May 2019

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DEDICATION

This thesis is dedicated to my late parents Mallam Isa Salihu and Safiya Abdullahi and to my uncle Alhaji Mustapha Ismahu for his steady and firm support in seeing me educated since my childhood. May Allah reward him with the best of Jannah.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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May 2019

Chairman : Associate Professor Nor Ainy Mahyudin, PhD
Faculty : Food Science and Technology

Survival of multidrug resistant enterotoxigenic *S. aureus* and *E. coli* with biofilm forming potential (BFP) in commercial food premises is a possible danger to the health of consumers especially in societies where most of the population depend on these premises for their daily meals. These organisms can serve as sources of outbreaks leading to morbidity and mortality particularly in persons with compromised immunity. Isolates obtained from selected food premises in Selangor Malaysia were subjected to Gram's staining and various biochemical identifications and later confirmed by polymerase chain reaction (PCR) using specific primers. Antimicrobial susceptibility testing was conducted to ascertain the multidrug resistant (MDR) isolates and PCR was undertaken using the primers for the genes of most widespread toxins implicated in food poisoning outbreaks as staphylococcal enterotoxin (SE) A and D as well as heat-labile (LT) and heat-stable enterotoxin genes of *E. coli*. The MDR and non-MDR isolates were further subjected to biofilm formation.The MDR isolates of both *S. aureus* and *E. coli* with strong BFP were challenged with chemical sanitizers, peracetic acid (PAA), sodium hypochlorite (NaOCl) and benzalkonium chloride (BAC) using minimum biofilm eradication concentration (MBEC) device to determine the sessile minimum inhibitory concentration (SMIC) and MBEC. Cutting boards similar in chemical composition (polyethylene) to those used in the food premises were contaminated with both MDR and non-MDR isolates with strong BFP and tested with the sanitizers and hot water at various concentrations and temperatures respectively, at various times of contact. Twenty four isolates were confirmed to be *S. aureus* and also 24 were confirmed to be *E. coli*. All the *S. aureus* isolates (100%) possess the SEA and SED genes while only 37.5% (n=9) of the confirmed *E. coli* possessed LT genes. The MDR and non-MDR *S. aureus* isolates were 87.5% (n=21) while 66.7% (n=16) were the non-MDR *E. coli* and 57% (n=12) of the MDR and non-MDR *S. aureus* had strong BFP while 75% (n=12) of the non-MDR *E. coli* had strong BFP. PAA proved to be the most effective of all the sanitizers although there were resistance by isolates tagged SA016

and SA022 of the *S. aureus*. Hot water had higher activity than the sanitizers and there was still resistance by SA016. On the cutting boards, the results of the sanitizers action was promising with PAA being significantly higher ($P<0.05$) in activity than the rest of the sanitizers. Hot water had the highest activity even at smaller contact time of antimicrobial action than the sanitizers reducing the logarithmic counts of the strongest biofilm forming isolates by 5.05 Log CFU/ 10 cm^2. All isolates were reduced to acceptable limits or totally eliminated by the sanitizers at 5 min and 10 min contact times. The findings in this study will give suggestions on the appropriate sanitizing agents and their concentrations to be used on cutting boards and other food contact surfaces for sanitization so as to reduce the risk/incidence of outbreaks due to these organisms.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

KERINTANGAN ANTI-MIKROBIAL DAN POTENSI PEMBENTUKAN BIOFILEM OLEH *Staphylococcus aureus* DAN *Escherichia coli* ENTEROTOKSIGENIK DARI PERMUKAAN PAPAN PEMOTONG

Oleh

SHU'AIBU ISA

Mei 2019

Pengerusi : Profesor Madya Nor Ainy Mahyudin, PhD
Fakulti : Sains dan Teknologi Makanan

Ketahanan rintangan terhadap pelbagai jenis dadah (MDR) oleh bakteria enterotoksigenik *Staphylococcous aureus* dan *Escherichia coli* di premis makanan komersial memberi kesan kepada kesihatan pengguna terutamanya di dalam masyarakat yang bergantung pada premis ini sebagai tempat mendapatkan makanan seharian. Organisma-organisma ini boleh menjadi sumber kepada wabak yang memberi kesan kepada morbiditi dan mortaliti terutamanya kepada mereka yang mempunyai tahap imuniti yang rendah. Isolat bakteria yang telah diperoleh daripada premis makanan terpilih di Selangor, Malaysia telah dibuat pewarnaan Gram dan pelbagai pengenalan biokimia dan kemudiannya disahkan melalui tindak balas rantai polimerase (PCR) menggunakan primer-primer spesifik. Ujian kecenderungan anti-mikrobial telah dijalankan untuk menentukan isolat yang rintang kepada pelbagai jenis dadah (MDR) dan ujian PCR juga telah dijalankan menggunakan primer-primer untuk gen-gen yang mempunyai toksin yang menyumbang kepada wabak keracunan makanan seperti enterotoksin syaphylococcal (SE) A dan D serta enterotoksin *E. coli* yang labil pada haba (LT) dan stabil pada haba. Isolat MDR dan bukan MDR seterusnya diuji kepada pembentukan biofilem. Kedua-dua isolat MDR *S. aureus* dan *E. coli* yang mempunyai potensi pembentukan biofilem (BFP) yang kuat telah diuji dengan bahan sanitasi kimia asid perasetik (PAA), natrium hipoklorit (NaOCl) dan benzalkonium klorida (BAC) dengan menggunakan peranti kepekatan pembasmian biofilem minimia (MBEC) untuk menentukan kepekatan perencat minima (SMIC) dan MBEC. Papan pemotong yang mempunyai komposisi bahan kimia yang sama (polietilena) seperti yang digunakan di premis makanan telah dicemari dengan isolat MDR yang mempunyai BFP yang kuat, dan seterusnya diuji dengan menggunakan bahan sanitasi dan air panas pada pelbagai kepekatan, suhu dan masa. Dua puluh empat isolat telah disahkan sebagai *S. aureus* dan juga 24 disahkan sebagai *E. coli*. Semua isolat *S. aureus* (100%) mempunyai gen SEA dan SED manakala hanya 37.5% (n=9) *E. coli* disahkan memiliki gen LT. MDR dan bukan MDR *S. aureus* adalah 87.5% (n=21) manakala 66.7% (n=16) adalah bukan

MDR *E. coli* dan 57% (n=12) adalah MDR dan bukan MDR *S. aureus* yang mempunyai BFP yang kuat manakala 75% (n=12) MDR mempunyai BFP yang kuat. PAA terbukti paling berkesan dari semua bahan sanitasi walaupun terdapat rintangan oleh isolat SA016 dan SA022 dari *S. aureus*. Air panas mempunyai aktiviti yang lebih tinggi daripada bahan sanitasi dan masih terdapat rintangan oleh SA016. Hasil tindakan bahan sanitasi PAA ke atas papan pemotong adalah amat memberangsangkan dan signifikan ($p<0.05$) berbanding penggunaan bahan sanitasi yang lain. Air panas mempunyai aktiviti anti-mikrobial yang paling tinggi walaupun masa pendedahan yang diberikan adalah lebih singkat berbanding bahan sanitasi, iaitu dapat mengurangkan jumlah logaritma pembentukan biofilem daripada isolat sebanyak 5.05 Log CFU/10 cm^2. Semua isolat telah dikurangkan kepada had yang boleh diterima atau disingkirkan sepenuhnya apabila terdedah kepada bahan sanitasi selama 5 min dan 10 min. Hasil kajian ini memberi cadangan kepada penggunaan bahan sanitasi yang sesuai berserta kepekatannya untuk digunakan semasa sanitasi papan pemotong dan permukaan menyentuh makanan yang lain dan seterusnya mengurangkan risiko wabak disebabkan oleh bakteria ini.

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Declaration by graduate student

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LIST OF ABBREVIATIONS

AOAC	Association of Official Analytical Chemists
AML	Amoxycillin
BAC	Benzalkonium Chloride
BFP	Biofilm Formation Potential
BHI	Brain Heart Infusion
BPA	Baird Parker Agar
CAZ	Cefatzidime
CBD	Calgary Biofilm Device
CDC	Centre for Disease Control
CF	Colonization Factor
CFTR	Cystic Fibrosis Transmembrane Regulator
cGMP	Cyclic Guanosine Monophosphate
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standard Institute
CN	Gentamicin
CPS	Coagulase Positive Staphylococci
CRO	Ceftriaxone
CTX	Cefotaxime
DHFR	Dihydroxyfolate Reductase
DNA	Deoxyribonucleic Acid
EAST-1	Enteroaggregative Thermostable Toxin 1
ECM	Extracellular Matrix
EDTA	Ethylene Diamine Tetra Acetic Acid
EMB	Eosin Methylene Blue
EPS	Extracellular Polymeric Substance

ETEC	Enterotoxigenic *E. coli*
FBD	Foodborne Diseases
FDA	Food and Drug Administraion
GC	Guanylate Cylase
GIT	Gastrointestinal Tract
Gyr	Gyrase
HGC	Horizontal Gene Transfer
INCSS	International Nomenclature Committee for Staphylococcal Superantigens
KF	Cephalothin
LT	Heat Labile (Thermo-labile)
LB	Luria Bertani
MAR	Multiple Antibiotic Resistance
MBC	Minimum Bactericidal Concentration
MBEC	Minimum Biofilm Eradication Concentration
MDR	Multidrug Resistant
MHA	Mueller Hinton Agar
MHC	Major Histocompatibility Complex
MRSA	Methicillin Resistant *S. aureus*
MRVP	Methyl Red-Voges Proskauer
MSA	Mannitol Salt Agar
NA	Nalidixic Acid
NaOCl	Sodium Hypochlorite
OD	Optical Density
P	Penicillin G
PAA	Peracetic Acid
PCR	Polymerase Chain Reaction

PE	Polyethylene
PKA	Protein Kinase A
PP	Polypropylene
PT	Pyrogenic Exotoxin
QAC	Quaternary Ammonium Compounds
QRDR	Quinolone Resistance Determining Regions
RNA	Ribonucleic Acid
rRNA	Ribosomal RNA
S	Streptomycin
SE	Staphylococcal Enterotoxin
SEl	Staphylococcal Enterotoxin-like
SFP	Staphylococcal Food Poisoning
SF	Sulphafurazole
SIM	Sulphide Indole Motility
ST	Heat Stable (Thermostable)
TAE	Tris-acetate EDTA
TD	Traveller's Diarrhoea
TE	Tris-EDTA
THM	Trihalomethane
TSS	Toxic Shock Syndrome
US	United States
VISA	Vancomycin Intermediate *S. aureus*
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

1.1 Background

Diseases of food origin are the main concern for public health globally (Balaban and Rasooly, 2000; Le Loir et al., 2003; Kadariya et al., 2014). Foodborne disease (FBD) was defined by the World Health Organization (WHO) as a toxic or infectious disease that is known or thought to have been caused by food and/or water consumption (Le Loir et al., 2003). Annually, foodborne diseases lead to millions of cases of ailments throughout the globe (Cooke, 1990; Todd, 1992; Mead et al., 1999; Luppens et al., 2002). One of the major ways through which food becomes contaminated with the foodborne pathogens is via contact with equipment in the food establishments where foods are processed.

Among the major problems the food industry faces is the survival of microorganisms that are of food spoilage or pathogenic origin as a result of inadequate disinfection or sanitization of instruments or surfaces that are directly or indirectly in touch with foods (Carpentier and Cerf, 1993; Fuster et al., 2008). In the course of production, such bacteria that adhere to the surfaces at some point in time become detached thereby contaminating foods when they are processed on the surface. Consequently, this contamination greatly affects the safety and quality of the food being processed which in turn poses a potential hazard to the health of consumers, especially if such a contamination takes place sequel to the step of bactericidal treatment (Bagge-Ravn et al., 2003; Fuster et al., 2008).

Cutting boards, tables and knives kitchen equipment and instruments are termed "food contact surfaces".Also included are surfaces onto which food may drip, drain, or splash, such as the inside of a microwave oven or refrigerator (Cosby et al., 2008). If the process of cleaning these instruments is not properly and efficiently done and/or that the dishes and equipment are not efficiently and properly dried when washed, they possibly serve as direct contamination sources. In reducing the occurrence of foodborne ailments and cross-contamination, efficient hygienic and cleaning techniques are measures so effective to propose (Begani et al., 2012; Kim et al., 2017). Owing to their importance in the control of outbreaks due to pathogenic microbes, the surfaces in contact with food in the establishments where foods are processed are of major concern (Konecka-Matyjek et al., 2012).

Occurrence of cross-contamination of food products as a result of their contacts with food contact surfaces in the food processing industries is perhaps a serious problem to the industries (Kusumaningrum et al., 2003). Suggestions have been put forward that the bacterial continued existence on the surfaces is linked to their binding to materials of the food contacts (Gravesen et al., 2005; Valeriano et al., 2012). Bacterial pathogens

could also attach to the surfaces of equipment thereby intensifying the dangers of disease transmissions (Silva et al., 2008; Belessi et al., 2011).

Staphylococcus aureus is a common pathogen associated with serious community and hospital acquired diseases and has for a long time been considered a major problem of public health (Pesavento et al., 2007). Furthermore, *S. aureus*is often present in foods (Normano et al., 2005; 2006) and it is among the leading causes of foodborne bacterial intoxications worldwide (Genigeorgis, 1989; Mead et al., 1999; Tauxe, 2002). Many factors of virulence associated with *S. aureus* pathogenicity were described in the past and these include thermonucleases, hyaluronidases, lipase and hemolysin (Sandel and McKillip, 2004; Kuroda et al., 2007; Normano et al., 2007) which are involved in tissue invasion of the host. Perhaps the most notable virulence factors associated with this microorganism are the heat-stable enterotoxins that cause the sporadic food-poisoning syndrome or foodborne outbreaks (Martin et al., 2003; Kérouanton et al., 2007).

Stapylococcus aureus is a ubiquitous pathogen associated with both human and animal diseases including mastitis, toxic shock syndrome (TSS) and staphylococcal food-poisoning (SFP) (Le Loir et al., 2003). SFP symptoms include sudden onset of nausea, vomiting, abdominal cramps and diarrhoea, and is caused by ingestion of food containing heat-stable staphylococcal enterotoxins (SETs) (Balaban and Rasooly, 2000).

Poisoning of food by staphylococcus is among the commonest global foodborne infections and results upon intake of food contaminated with coagulase-positive staphylococci (CPS) that are able to produce enterotoxins (Staphylococcal enterotoxins, SEs) especially *S. aurues* (Jablonski and Bohach, 1997). Such enterotoxins are also hardly found in other staphylococcal species such as *S. intermedius* (Genigeorgis, 1989; Khambaty et al., 1994).

Enterotoxigenic *Escherichia coli* (ETEC) bacteria are a diverse group of organisms that share the ability to secrete and effectively deliver heat-stable toxin (ST) and/or heat-labile toxin (LT) enterotoxins (Fleckenstein et al., 2010). Collectively, these organisms cause millions of infections and are one of the leading pathogens associated with death following moderate to severe diarrhea in young children (Qadri et al., 2005, 2007; Kotloff et al., 2013). In the classical paradigm for ETEC pathogenesis, these organisms adhere to the small intestinal mucosa via plasmid-encoded antigens known as colonization factors (CFs). Toxins are delivered at the place by ETEC to the receptors on the cells of epithelium. The concentrations of the cyclic nucleotide of the cells of the host are increased by the bound eventually making CFTR (cystic fibrosis trans-membrane regulatory) channel activated and finally ending in efflux of fluid into the lumen of the intestine (Fleckenstein et al., 2010).

According to the World Health Organization, enterotoxigenic *Escherichia coli* (ETEC) are responsible for 280 million to 400 million episodes of diarrhea, many of which lead to malnutrition, and about 380,000 deaths annually. Majority of the affected ones are children under 5 years of age from developing nations. This has qualified ETEC to be among the popular enteropathogenic organisms in the children of the poor. In addition, ETEC is regarded the commonest agent of traveler's diarrhea (WHO, 2006).

Antimicrobial resistance refers to reduced sensitivity or complete insensitivity of some microorganisms to one or more antimicrobial agents. This report is principally concerned with resistance to therapeutic antimicrobials. Therapeutic antimicrobials are compounds which, at low concentrations, can interact with and disrupt specific biochemical pathways in critical processes of particular bacteria (Leekha et al., 2011). Through this action, they kill the bacteria or prevent the bacteria from growing. Therapeutic antimicrobials ideally have little or no toxic effect on the critical functions of animal cells at the concentration that is needed to impact on target bacteria. This ability to target a particular process in bacteria is the basis for the selective toxicity of antimicrobial agents (McEwen & Fedorka-Cray 2002).

When the efficacy of an antibiotic is reduced such that it cannot cure an infection or disease, such a phenomenon is termed 'resistance'. However, if the antibiotic or antimicrobial agent is not meant for killing or inhibiting a pathogenic microorganism, the term is thus referred to as drug tolerance or failure of dose. Organisms are termed multidrug resistant if they are able to resist the action of at least two drugs from different categories (Shaikh et al., 2015). Various mechanisms of resistance are present in various strains of bacteria.

Biofilms are matrix-enclosed microbial accretions that adhere to biological or non-biological surfaces (Hall-Stoodley et al., 2004). In other word, a biofilm is an aggregation of microbial cells and their associated extracellular polymeric substances (EPS), actively attached to, growing and multiplying on a surface (Flint et al., 1997). The extracellular polymeric substances (EPS), which are mainly polysaccharides, proteins, nucleic acids and lipids, are responsible for the morphology, structure and physicochemical features of these aggregates (Flemming and Wingender, 2010). Since biofilm is a universal phenomenon, i.e. microbes prefer to live on surfaces rather than in the liquid phase, it is very likely that most of the microbial contaminations of food products are biofilm-related (Brook and Flint, 2008). The biofilm formation mechanisms in a number of environments have been the subject of debate and were reviewed in many comprehensive and authoritative books and reviews (Hall-Stoodley et al., 2004; Costerton 2007). Biofilm-associated cells can be differentiated from their freely suspended counterparts, called planktonic microorganisms, by generation of EPS, reduced growth rates, the up- and down-regulation of specific genes and cell-to-cell communication (Donlan, 2002).

1.2 Problem Statements

According to the Malaysian Health Ministry, 14, 433 cases of food poisoning were reported in 2016 alone and as many as 60 cases of food poisoning were being investigated (Joe, 2018).Incidences of *S. aureus* and *E. coli* from various food sources implicated in food poisoning outbreaks in Malaysia have been reported in 1983(Rampal, 1983) and in 2005 by the National Public Health Laboratory and the Malaysian Ministry of Health (NPHL/MOH, 2005). In addition, researches on incidences of foodborne pathogens from cutting board surfaces have been recently investigated (Abdul-Mutalib et al., 2016) in Selangor Malaysia. Therefore, considering both *S. aureus* and *E. coli* among the major bacterial agents responsible for human foodborne infections globally, and that Malaysia is among the leading countries in Asia and the world at large where majority of the population patronize restaurants as the source of their meals, problems specifically justifying the achievement of the scientific objectives proposed in this PhD thesis include:

- That both *S. aureus* and *E. coli* are detected in food premises (restaurants) as a result of cross-contamination from food handlers and food contact surfaces.Improper cleaning and food handling practices lead to the emergence of these pathogenic bacteria. This has led to an increase in food borne infections associated with food contact surfaces in both developed and developing nations and poses a threat to the public.
- That both *S. aureus* and *E. coli* produce enterotoxins that induce emesis and cholera-like diarrhoea respectively and are responsible for food poisoning outbreaks due to these organisms
- Antimicrobial resistance of bacteria obtained from food contact surfaces to antibiotics and other antimicrobial agents increases frequently. This resistance of the bacterial pathogens to antibiotics increases their potential for causing food poisoning outbreaks when they are ingested with the food particles.
- Bacteria in biofilms increase resistance to multiple antibiotics and antimicrobial agents.The emergence of bacterial biofilms of *S. aureus* and *E. coli* on cutting board surfaces increases their tolerance to biocidal agents so high that it provides them with a long-lasting persistence within foods and food-related environments.
- The existence of *S. aureus* and *E. coli* on the already cleaned and washed cutting boards' surfaces must be counteracted by the use of chemical sanitizers and hot water so as to avoid possible outbreaks due to them.

1.3 Objectives

In this respect, coupled with the final goal for the improvement of the control of these pathogens in the food premises through their identification as well as the evaluation of the promising strategies of disinfection against them, the following objectives were achieved:

1. To reconfirm the *S. aureus* and *E. coli* obtained from cutting boards of some selected restaurants in Selangor, Malaysia and detect the presence of genes responsible for enterotoxin production.
2. To determine the antimicrobial resistant *S. aureus* and *E. coli* isolates to commonly used antibiotics.
3. To determine the biofilm formation potential (BFP) of the multidrug resistant *S. aureus* and *E. coli* isolates and determine the *in-vitro* susceptibility of their biofilms to hot water and chemical sanitizers.
4. To assess the effects of the chemical sanitizers and hot water on the *S. aureus* and *E. coli* biofilms developed on plastic cutting boards.

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