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Biofilm of Antibiotics Resistant *Salmonella Typhimurium* and *Salmonella Enteritidis* Against Detergents

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ABSTRACT

Salmonella is able to produce biofilm which is more resistant toward disinfectants and antibodics than its planktonic form. *Salmonella typhimurium* from beef and *Salmonela Enteritidis* from raw vegetables isolates were tested for their susceptibility using 18 different antibiotics. *Salmonella typhimurium* isolate was resistant toward Streptomycin, Sulfamethoxazole, Penicillin, Erythromycin, Tetracyclin, Ampicillin, Rifampicin and Clarithromycin while *Salmonella enteritidis* was resistant toward Amikacin, Streptomycin, Penicillin, Cipfoflaxin, Erythromycin, Ampicilin, Tetracyclin, Rifampicin, Cepthalothin, Amikacin, Chloramphenicol and Clarithromycin. Both of *Salmonella* isolates showed MAR index > 0.2, indicating that these isolates might be originated from high risk sources. Out of the five detergents, Detergent 3 (D3) (Linear alkyl Sulfonic acid was found to be the most effective. The Minimum Inhibition Concentrations (MICs) and Minimal Bactericidal Concentration (MBCs) was ranged from 6250 – 25,000 µg/ml and 25,000 to > 50000 µg/ml, respectively. Biofilm-producing ability of antibiotics -resistant *Salmonella typhimurium* and *Salmonella enteritidis* were inhibited at 12,500 – 25,000 µg/ml and eradicated at >50000 µg/ml. Therefore, Detergents showed potential antimicrobial activity against *Salmonella.*

Introduction

Foodborne illness is a major international public health concern (Carl *et al.*, 2003) and this is proven by the microbial contamination affecting most foodstuffs consumed in the world (Concina *et al.*, 2008). *Salmonella* are among one of the most important causes of foodborne gastroenteritis world-wide. The infection of *Salmonella* is known as Salmonellesis. They are gram-negative, facultative anaerobes and inhabit the intestinal tract of animal (Chia *et al.*, 2009).

Antibiotics are the only effective therapy for the food-borne infections (Mao *et al.*, 2007). One of the most important food safety concerns is the increasing antibiotic resistance of food-borne pathogens. Recently, many aerobic and anaerobic bacteria were reported to show antibiotic resistance

(Yong *et al.*, 2004). Emergence of antibiotic resistance strains of *Salmonella* has become a serious threat in the food industries (Tendencia and Pena, 2001).

According to the National Institute of Health (NIH) in the United States, 80 % of microbial can form biofilm and this includes food borne pathogens, such as *Salmonella* spp., *Klebsiella* spp., *Pseudomonas* spp., *Campylobacter* spp., *Escherichia coli*, and *Listeria* spp. *Salmonella* has the ability to form biofilms on food-processing surfaces including plastic, potentially leading to food product contamination. The organism has the capability to adhere and form biofilms on surfaces such as plastic, glass, stainless steel or rubber surfaces (Joseph *et al.*, 2001). The biofilms, when formed on these contact surfaces, could be a continuous source of contamination and lead to serious implications in industrial, environmental, public health and medical situations (Hall-Stoodley *et al*., 2004).

In the food industry, the use of detergents is an important part of the manufacturing practices to prevent aggregation regime and subsequent microbial biofilm formation. However, various detergents which are extensively used in food industries may not be really effective against some microorganisms especially in biofilm form. Bacterial colonization of food processing equipment and facilities is the main concern and is a potential source of contamination of foods that may lead to spoilage or transmission of food borne pathogens. Therefore, this study investigated the effects of detergents on the growth of antibiotics resistant *Salmonella typhimurium* and *Salmonella enteritidis* in single cells until biofilm is formed.

Methodology

Antibiotic susceptibility test of Salmonella

Each of *Salmonella typhimurium* and *Salmonella enteritidis* was isolated from beef and raw vegetables and confirmed through Polymerase Chain reaction .Then, they were used for antibiotics susceptibility test. Antibiotic susceptibility of the isolates was determined through disc diffusion tests according to the guidelines of Clinical and Laboratory Standards Institute (CLSI),(2003). Using sterile non-toxic cotton swab, *Salmonella* cultures were uniformly swabbed on Mueller-Hinton (MH) agar (Merck, Darmstadt, Germany) plates and left to dry for 3-5 minutes. *E. coli* (ATCC 25922) was used as control.

Eighteen antibacterial agents were used in this study includes Trimethoprim (W, 5 µg), Amoxicillin (AML, 25 µg), Streptomycin (S, 10 µg), Sulfamethoxazole (RL, 25 µg), Penicilin (P, 10 µg), Ciproflaxin (CIP, 5 µg), Erythromycin (E, 15 µg), Ampilcilin (AMP, 10 µg), Tetracyclin (Te, 30 µg), Nalidixic Acid (NA, 30 µg), Rifampicin (RD, 25 µg), Cephalothin (RT, 30 µg), Amikacin (AMC, 30 µg), Sulphamethozaxole Trimethoprim 19:1 (SXT, 30 µg),Chloramphenicol (C, 10 µg), Gentamicin (CN, 10 µg), Kanamycin (K, 30 µg)**,** clarithromycin(CR, 15 µg**).** Antibiotic discs (8 mm diameter) were supplied by Oxoid (Hamphire, United Kingdom) (Table 1). Then, Antibiotic discs were placed on the inoculated plates and incubated at 37°C overnight.

Multiple Antibiotic Resistance (MAR) index

Multiple antibiotic resistances (MAR) index of *Salmonella* isolates was determined based on the index a/b, where 'b' represents the number of multiple antibiotics to which *Salmonela* isolates are exposed and 'a' the number of multiple antibiotics to which *Salmonella* isolates are resistant (Gwendellynne *et al.,* 2005).

Preparation of detergent assay as antibacterial agent

The stock solution was prepared according to Rukayadi *et al.* (2009) with some modifications. Detergents were diluted in the DMSO (100%) to get the final 10% stock solution as it was the minimum concentration which can inhibit the growth of *Salmonella.* Standard control, Chlorhexidine (CHX)(1,1-hexa-methylenebis (5-p-chlorphenyl biguanide) was purchased from Sigma Chemical (St Louis, MO, USA), and dissolved in sterile-distilled water for 10 000 µg/ml (1% stock solution). The following abbreviations were used for 5 detergents commonly used in cleaning process in Malaysia: Detergent D1 (Acidified sodium chlorite), Detergent D2 (Chlorine dioxide), Detergent D3 (Linear alkylbenzene Sulfonic acid), Detergent D4 (Hydrogen peroxide) and Detergent D5 (Sodium Lauryl Ether) generally used in dishwashing and cleaning purpose.

In vitro susceptibility test for detergents

The standard paper blank disc-diffusion assay (CLSI, 2009) was used to test the susceptibility of *Salmonella* isolates to detergents. One ml of *Salmonella* from TSB was transferred to a new plate and added with 15 ml of TSA. Sterile filter paper discs (6 mm diameter) (Schleicher and Schuell, Dassel, Germany), were placed on TSA plates and 20 µl of 10% stock solution of each detergent was loaded on the discs. A negative control (10% of DMSO) and standard control were included in the assay. The plates were observed for clear zones after 24 h incubation at 37 °C. All experiments were conducted in duplicates. The method proposed by Rukayadi *et al.* (2009) modified as follows: antibacterial was diluted in 10% DMSO followed by 2-fold dilutions in the test wells; thus, the final concentration of DMSO was serially decreased. The effect of DMSO has been examined on the growth and viability of resistant strains tested. DMSO at < 10% was found not to affect growth or viability of the strain tested. These results suggested that DMSO has no effect on activity and all measured antimicrobial activity was due to local detergents.

Minimum Inhibitory Concentrations (MICs) and Minimal Bactericidal Concentrations (MBCs) Determination

MICs and MBCs tests were performed in 96-well microtiter plates according to the method described in the CLSI M7-A6 guidelines. MICs for *Salmonella* isolates were determined using McFarland standard (5×10^6 CFU ml^{-1}) by diluting 1:1000 using TSB. Each antibacterial agent was diluted 1:10 in TSB containing 5×10^3 CFU ml^{-1} inoculums. Dilutions started from wells in column 12 of the microtiter plates. Therefore, column 12 of microtiter plates contained the highest concentration of antibacterial and column 3 contained the lowest concentration of detergent. Column 2 served as the positive control (antimicrobial agent-free wells, only medium and inoculum), and column 1 was the

negative control (only medium, no inoculum, no antibacterials agent). After 24 h incubation at 37 °C, the MIC was measured as the lowest concentration of antimicrobial agent resulting in complete inhibition of visible growth.

To determine MBCs, wells with no visible growth were used. The medium (approximately of 100µl) of each well was removed and was spread onto agar plates supplemented with 3%NaCl and incubated at 37 °C for 24 h (or until visible growth in the positive control). The positive controls in column 2, (antimicrobial agent-free wells), and growth-negative controls in column 1, were included in the MBC test. MBC was defined as the lowest concentration of antimicrobial agent at which *Salmonella* in the culture were killed or the lowest concentration with no visible growth on TSA plates.

Assessment of in vitro biofilm formation

Both of *Salmonella typimurium* and *Salmonella enteritidis* isolates were allowed to form biofilm in the wells of presterilized, polystyrene flat-bottomed 96-well microtiter plates, according to Sandoe *et al.* (2007). Briefly, the wells of microtiter plates were filled with 100 µl of TSB. To generate biofilms, 100 µl of the standard inoculum was transferred into each well. The plates were covered and sealed with parafilm and incubated at 37°C for 24h. The medium was then discarded and non-adherent cells were removed through washing the biofilm with sterile phosphate buffered saline (PBS). The washing step was repeated 3 times and plates were inverted to remove residual medium.

Sessile Minimum inhibitory concentration (SMICs) and Minimum Eradication bactericidal concentration (MBECs)

To measure SMICs and MBECs of detergents, washed adherent cells in the 96-well microtiter plates were filled with 200 ml of the stock solution in TSB, ranging from 97.656 - ≥ 50,000 µg/ml. Dilutions started from the wells in column 12 of the microtiter plate, meaning that column 12 of the microtiter plate contained 100,000 µg/ml of stock solution and column 3 contained 97.656 µg/ml of stock solution. Column 2 served as the positive control (medium and inoculum) and column 1 was the negative control (only medium). The plates were incubated at 37°C for 24h and biofilms were then washed and stained, as described above. The optical density (OD_{650}) was measured after the incubation. The SMIC was defined as the lowest concentration where no growth occurred in the supernatant fluid, confirmed by no increase in OD_{650} compared to the initial reading.

To determine MBECs, the biofilms at the bottom of treated wells were rinsed and then scarred with a metal loop and spread over the surface of TSA plates. Plates were incubated at 37°C for 24h and the MBEC was determined as the lowest concentration at which no bacterial growth was observed on the TSA plates. All experiments were performed in triplicates.

Result and Discussion

According to Table 1, *Salmonella typhimurium* from beef was resistant toward Streptomycin, Sulfamethoxazole, Penicillin, Erythromycin, Tetracyclin, Ampicillin, Rifampicin and Clarithromycin. This finding is in agreement with Johanna *et al.* (1998) who reported that *Salmoenlla*

typhimurium was resistant toward streptomycin, rifampicin, and nalidixic acid. Then, Benacer *et al.* (2010) reported that *Salmonella typhimurium* showed high resistance rates to tetracycline, streptomycin, ampicillin, kanamycin and chloramphenicol. The highest resistance toward tetracyclines can be explained by the fact that this antibiotic is used in two thirds of the therapeutic regimens applied in veterinary medicine.

Table1. The antibiotic resistance profile patterns and Multiple Antibiotic Resistance (MAR) index of *Salmonella typhimurium*

Pattern	Strain No.	Antibiotic Resistant Profiles[a]	MAR Index
I	*S. typhimurium*	S10RL25P10E15AMP10RD5CR15	0.38
II	*S. typhimurium* ATCC 1331	S10RL25E15RD5CR15	0.28

Tested for S10: Streptomycin : RL25 : sulfamethoxazole ; P10 : Penicillin ; E15 : Erythromycin ; Te30: Tetracyclin : AMP10: Ampicillin : RD25 : Rifampicin ; CR15 : Clarithromycin

According to Carlson *et al.* (1999), the incidence of antibiotics resistant remained low in *Salmonella enteritidis* compared to *Salmonella typhimurium*. However, this statement is not compatible with the result in Table 2 which showed *Salmonella enteritidis* isolate was resistant toward 11/18 (61.11%) antibiotics which are Amikacin, Streptomycin, Penicillin, Cipfoflaxin, Erythromycin, Ampicilin, Tetracyclin, Rifampicin, Cepthalothin, Chloramphenicol, Clarithromycin. The resistance toward Amikacin is in agreement with report from Rouahi *el al.* (2000). Amikacin is most often used for treating severe, hospital-acquired infections with multidrug-resistant or gram-negative bacteria and to treat non-tubercular mycobacterial infections and tuberculosis.

Table 2. The antibiotic resistance profile patterns and Multiple Antibiotic Resistance (MAR) index of *Salmonella enteritidis*

Pattern	Strain No.	Antibiotic Resistant Profiles[a]	MAR Index
I	*S.enteritidis* ATCC13076	AmL25P10CIP5E15AMP10Te30RD5RF30AmC30C10CR15	0.55
II	*S. enteritidis*	AML25S10CIP5E15AMP10Te30RD5RF30AmC30C10CR15	0.55

Tested for AmL25 : Amikacin ; S10 : Streptomycin ; P10 : Penicillin ; E15 : CIP5 : Cipfoflaxin ; E10 : Erythromycin ; AMP10 : Ampicilin ; Te30 : Tetracyclin ; RD25 : Rifampicin ; RF30 : Cepthalothin ; AmC30 : Amikacin ; C10 : Chloramphenicol ; CR15 : Clarithromycin

The MAR index for both isolates (*Salmonella typhimurium* 0.38 and *Salmonella enteritidis* 0.55) which was more than > 0.2 are considered to be originated from high risk sources of contamination and such high risk sources include human and farm animals such as poultry, swine and dairy cattle that are frequently exposed to antibiotics. As shown in Table 2, MAR index of *Salmonella enteritidis* is higher than *Salmonella typhimurium. Salmonella enteritidis c*an be transmitted to humans by contaminated foods of animal origin, predominantly eggs.

Raw eaten or undercooked eggs that have been infected in the hen's ovaries can cause gastroenteritis. According to Nillian *et al*. (2011), vegetables which have a close contact with soil may have a higher possibility of contamination. The contaminated irrigation water, animal waste fertilizers and postharvest washing can be the sources of contamination in vegetables. The results of this study served to provide useful information in finding safe and efficient antibiotics. In addition, it can provide some insights for the problems faced by the Agriculture industry. This can be a key element to provide the latest information on the magnitude and the trends in resistance and susceptibility of bacterial infection related to *Salmonella.*

Clean and disinfected food contact surfaces are of the utmost importance in the food industry to control the risk of microbiological contamination in the processing line. Although the isolates have a high resistance toward antibiotics, nevertheless, the isolates of both *Salmonella typhimurium* and *Salmonella enteritidis* can be killed by the detergents. According to the MICs and MBCs results shown in Table 3, detergents agent in 96 wells microtitre plates can inhibit the growth of resistant strains in the concentrations ranging from 97.656 µg/ml to ≥ 50,000 µg/ml.

Table 3. Results of antibacterial susceptibility testing formed by resistant Salmonella isolates against detergents as antibacterial.

***Salmonella* isolates**	**Detergent 1 (µg/ml)**		**Detergent 2 (µg/ml)**		**Detergent 3 (µg/ml)**		**Detergent 4 (µg/ml)**		**Detergent 5 (µg/ml)**	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
S. typhimuirum ATCC1331	12500	25000	12500	25000	6250	25000	25000	25000	25000	25000
S. typhimurium	12500	25000	12500	25000	6250	25000	25000	25000	25000	25000
S. enteritidis ATCC13076	12500	25000	12500	25000	6250	25000	25000	25000	25000	25000
S. enteritidis	12500	25000	12500	25000	6250	25000	25000	25000	25000	25000

As seen in Table 3, Detergent 3 (Linear alkyl Sulfonic (LAS) acid) can inhibit the bacterial at 6250 µg/ml and killed both isolates at 25,000 µg/ml. This is in agreement with Nillian *et al.* (2013) who used Linear alkyl Sulfonic (LAS) acid to killed the multi antibiotics resistant *V. parahaemolyticus* isolates from seafood. Thus, it can be concluded that LAS detergent has a high potential to eliminate the food borne pathogen in the future. However, other detergents demonstrated MICs at 12,500 µg/ml and MBC for both *Salmonella* Typhimurium and *Salmonella enteritidis* were at 25,000 µg/ml. Herein, the detergents showed antibacterial activity against antibiotics resistant *Salmonella enteritidis* and *Salmonella typhimurium* isolates.

Minimum inhibitory concentration is an important factor to be considered while choosing a detergent as MIC shows the effectiveness of detergents toward pathogenic microorganism (Andrew, 2001). MBC values are defined as the lowest concentration of detergents required to kill a particular

bacteria. As shown in Table 3, all MBCs values were higher than MICs values (less than 4 times) meaning that the tested detergents were able to kill and inhibit growth of antibacterial-resistant *Salmonella* isolates. This concurs with French (2006) who stated that antimicrobial agents can be regarded as bactericidal if the MBC value is not more than four times higher than MIC value. Therefore, D3 (Linear alkylbenzene sulfonic based) was the most effective in inhibition of the antibacterial antibiotics resistant *Salmonella* growth.

In food industry, biofilms may create a persistent source of product contamination, leading to serious hygienic problems and also economic losses due to food spoilage (Brooks and Flint, 2008). Therefore, cleaning and sanitizing procedures must be a part of the standard operating procedures that makes up food safety program. Improperly cleaned and sanitized surfaces would allow harmful microorganisms to be transferred from contaminated surface onto food products.

In addition, the SMICs and MBECs for Detergent 3 is the lowest among other detergents showing results at 12,500 µg/ml and > 50,000 µg/ml while the others detergents were in the range of 25,000 µg/ml to > 50,000 µg/ml respectively. This is because Detergent 3 which is alkyl benzene based was an effective detergent to inhibit the growth of antibiotic resistant *Salmonella* isolates due to its linear alkylbenzene Sulfonic (LAS) base structure. Linear alkyl benzene sulphonic acids are commonly used as cleaning agents (household and personal care products). It was reported to be able to remove biofilm in river system (Boeije *et al.*, 2000).

Table 4. Results of Biofilm antibiotics resistant *Salmonella* against detergents

***Salmonella* isolates**	**Detergent 1 (µg/ml)**		**Detergent 2 (µg/ml)**		**Detergent 3 (µg/ml)**		**Detergent 4 (µg/ml)**		**Detergent 5 (µg/ml)**	
	SMIC	**MBEC**	**SMIC**	**MBEC**	**SMIC**	**MBEC**	**SMIC**	**MBEC**	**SMIC**	**MBEC**
S. typhimurium ATCC1331	25000	>50000	25000	>50000	12500	>50000	25000	>50000	25000	>50000
S. typhimurium	25000	>50000	25000	>50000	12500	>50000	25000	>50000	25000	>50000
S. enteridis ATCC13076	25000	>50000	25000	>50000	12500	>50000	25000	>50000	25000	>50000
S. enteritids	25000	>50000	25000	>50000	12500	>50000	25000	>50000	25000	>50000

This study provided a deeper insight on the effectiveness of detergents (especially detergents as antibacterial agents) as growth inhibitors for biofilm of antibiotic-resistant *Salmonella* strains at the beginning of cleaning process. Future studies are suggested to investigate the effect of mentioned factors to find the best formulation and method for elucidation of *Salmonella* biofilms. Support of regulatory agencies for application of anti-biofilm detergents is highly needed. Therefore, from this study, the concern on the cleaning phase is the most important stage for minimizing microbial colonization and for removing attached microorganisms. Every detergent has different effects; hence, effective detergents should be used to produce effective cleansing, save labor, and low in cost for cleaning the processing line (FSIS, 2012).

Conclusion

This study showed the antibacterial activity of detergents against biofilm growth of antibiotics resistant *Salmonella enteritidis and Salmonella typhimurium*. The finding demonstrates the importance of choosing an appropriate and an effective detergent in the operations in food processing line for the efforts to mitigate the formation of biofilm as rapidly as possible in any food contact surfaces and processing units in future.

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