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Efficiency of four Malaysian commercial disinfectants on removing *Listeria monocytogenes* biofilm

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Abstract

Listeria monocytogenes (*L. monocytogenes*) is a gram positive food-borne pathogen that is able to form biofilm on food factory surfaces. Formation of biofilm makes the bacteria much more resistance to environmental stresses such as disinfectant. The extracellular polymeric matrix (biofilm structure) which is mostly comprised of sticky extracellular polysaccharides (EPS) and proteins can protect bacteria in a harsh condition. The efficiency of four disinfectants on removing *L. monocytogenes* biofilm was investigated. Five concentration levels (100, 50, 25, 12.5, and 6.25%) of disinfectants were tested. In the microtitre assay, the optical density at 595 nm CV-OD_{595} value, was used to measure the amount of remained biofilm after 24 h. Results showed that disinfectants did not have significant effect on removing *L. monocytogenes* biofilm. Formation of *L. monocytogenes* biofilm significantly decreased the efficiency of disinfectants. Biofilm produced by strain number 9 showed higher resistance to disinfectant. Low concentrations (<50%) of disinfectants did not show significant effect on removing *L. monocytogenes* biofilm.

Introduction

Biofilm or sessile microbial cell is a structured community of microorganisms' cells stuck in a self-producing matrix and attach to both inert and living surfaces. Generally, two form of bacterial life style can be observed in nature; 1) the living form as individual free floating organisms (planktonic) and 2) biofilm consisting of attached cells to the surfaces (sessile), single or as a network structure (Carpentier and Cerf, 1993). Formation and development of biofilms in the food processing environment enhance resistance of cells to the environmental stresses and defend cells against disinfectants and sanitizers. Bacterial strains in the form of biofilm are hard to remove. Some routine cleaning procedures in food industry cannot remove biofilms and microorganisms in the form of biofilm remain on food industrial equipments. The protective nature of biofilm reduces the efficiency of disinfectants. Among various disinfectants used in food industries, only a few are effective in removing biofilm from the processing surfaces.

Biofilms attach to the surfaces in the form of three-dimensional multi-cellular structures. The biofilm matrix is produced by accumulation and attachment of bacterial cells. Biofilm formation occurs through a sequence of procedures: attachment of individual microorganism cells to a surface, cell growth and aggregation into microcolonies, matrix formation, and cell withdrawal. The interaction of microbial cells with the surfaces and also each other initiate the biofilm production. The biofilm matures during the producing of extracellular matrix which is mostly comprised of sticky extracellular polysaccharides (EPS) and proteins. Biofilm cells are much more resistant than planktonic cells to environmental stresses (such as sanitizers and disinfectants) and they may compromise up to 80% of total microbial population exist on the plant surfaces (Ölmez and Temur, 2010). Microorganisms cells embedded within a biofilm are able to tolerate nutrient deprivation, pH changes, oxygen radicals, disinfectants, detachment, and antibiotics (Florjanič and Kristl, 2011).

Listeria monocytogenes (*L. monocytogenes*) is a gram positive food-borne pathogen. *L. monocytogenes* attachment to food industry/processing environments and biofilm formation is a serious potential source of life-threatening food-borne disease listeriosis (Oliveira *et al.*, 2010). *L. monocytogenes* is a resistant bacterium to heat and high osmotic pressure which

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is able to grow under refrigeration temperatures and form biofilm (Oliveira *et al*., 2010). Center for Disease control and Prevention (CDC) reported occurring of about 1,600 cases of listeriosis in the United States. Within a period of 1996-1998 to 2003, the incidence of listeriosis decreased about 38%, but still several cases of illnesses and deaths are being reported. According to the CDC between 1998 to 2008, 2.4 outbreaks were reported each year. The most serious outbreak happened in 2002 due to consumption of *L. monocytogenes* contaminated turkey deli meat which resulted in 54 illnesses and 8 deaths. A listeriosis outbreak was reported in Canada in 2008, which resulted in 57 total confirmed cases and 23 deaths. *L. monocytogenes* is a constant concern in food industries. Food processing equipments, utensils and surfaces are made from stainless steel, polypropylene, polyvinylchloride and polystyrene. Such surfaces are the common places for *L. monocytogenes* attachment and biofilm formation. Despite normal cleaning procedures *Listeria* can survive and adhere to different surfaces in food industries. This attachment introduces an important challenge in assuring the microbiological safety in food industries.

The use of disinfectant and sanitizers in food industries is incorporated into good manufacturing practices regimes to stop the accumulation of microorganism cells and consequently biofilm formation. Different categories of disinfectant and sanitizers are used in the food industries (such as quaternary ammonium compounds (QACs), chlorine and iodofors). There has been some reports of disinfectant and sanitizers to be effective against *L. monocytogenes* cells in planctonic form (Aarnisalo *et al.*, 2007). Compared to bacterial cells in suspension, sessile microbial cells are much more resistance to disinfecting or sanitizing agents. Generally, presence of sessile *L. monocytogenes* cells and organic material on the surfaces can decrease the efficiency of disinfectants.

The cell wall, cytoplasmic membrane, and cytoplasm of bacteria are the main targets for biocide interactions of disinfectants or chemical biocides. The biocide activity of a compound depends on the cell morphology, extracellular material and cellular chemical composition. Intrinsic resistance of bacterial can be explained by phenotypic variation (Denyer and Stewart, 1998). Correlation between the concentrations (at which bacteriostatic or bactericidal effect starts) and falling specific biochemical or physiological changes, is the classical move toward determination of disinfectant mechanism of action (Denyer and Stewart, 1998). In this study, microtitre plate assay was used to evaluate the effectiveness of different concentration level of four disinfectants in removing *L. monocytogenes* biofilm.

Materials and Methods

Media and chemicals

Palcam Agar (Merck, Darmstadt, Germany) and Tryptic Soy Brath (TSB) (Merck, Darmstadt, Germany) and Crystal violet (1%) (Merck, Darmstadt, Germany) were used in this study. Four Malaysian commercial disinfectants AJAX FLOOR CLEANER, LEBAH, LEO and KIWI with different active ingredients in five different concentrations were tested. Disinfectants were diluted in reverse osmosis (RO) sterile water.

Bacterial strain and culture conditions

Ten *L. monocytogenes* strains were supplies by Food Safety Research Centre (FOSREC). The strains were isolated from Malaysian minced chicken and meat, burgers and sausages. *L. monocytogenes* isolates were maintained at -4^{o}C in Palcam Agar.

Disinfectants

In this study, four commercial disinfectants with four different active ingredients were used at five concentrations were used. All disinfectants were purchased from Malaysian market and diluted with reverse osmosis (RO) sterile water immediately before use. Diluted disinfectants were used within 10 min of preparation (Cruz and Fletcher, 2012).

Preparation of microtitre plate for L. monocytogenes *biofilm formation*

L. monocytogenes test strains were inoculated into 5 ml of TSB and incubated at 30^{o}C for 18 h. After incubation period, 20 µl of cultures was transferred to 5 ml of TSB and incubated for 18 h at 20^{o}C. Finally 125 µl of over-night culture was transferred to 5 ml of TSB. After mixing for 1 min, 100 µl of suspension was transferred to each well of microtitre plate (Greiner Scientific, Sigma-Aldrich, Australia) (Harvey *et al*., 2007).

Determination removing curve efficiency of disinfectants on L. monocytogenes *in biofilm*

The microtitre plate assay proposed by Harvey *et al*. (2007) was used for preparing cultures of *L. monocytogenes* biofilm (Harvey *et al.*, 2007). Disinfectants were diluted in sterile water at five different concentration levels (100, 50, 25, 12.5, and 6.25%). The microtitre plates were incubated at 20^{o}C for 24 h in order to biofilm formation.

After removing TSB from the wells which contains planktonic form of *L. monocytogenes*,

biofilm attached to the bottom and walls of the wells was used for the rest of the study. Diluted disinfectants (20 µl/well) were added to wells comprising *L. monocytogenes* biofilm. The plate was gently tapped three times on the side to make distribution of disinfectants solution easier and let it stay for 15 min under static condition (Contact time was according to previous researches), and then wells were washed with 150 µL distilled water 3 times to omit loosely associated bacteria (Oliveira *et al*., 2010). Each plate included eight positive and negative controls. Negative wells comprised of 100 µl of un-inoculated TSB plus four concentrations of disinfectants with duplicate. For the positive wells which contained inoculated TSB, disinfectants did not be added.

After drying wells at 30°C for 30 min, 150 µl of aqueous 1% crystal violet solution was added to each well and incubated at 20°C for 45 min. After removing crystal violet solution, wells were washed three times with sterile water (150 µl) and air-dried at 30°C for 30 min. Destaining the biofilm was performed by adding Alcohol 95% (100 µl) to each wells and concentration of crystal violet was ascertained by measuring the optical density at 595 nm (CV-OD_{595} value). Assays were performed duplicate for each test strain and mean CV-OD_{595} values and standard deviations were calculated. The average of CV-OD_{595} value obtained for the negative control was subtracted from the average CV-OD_{595} value of each test strain.

Statistical analysis

The data obtained from the microtitre reader were subjected to two ways analysis of variance (ANOVA) to determine the significant differences among the four disinfectants defined at $p < 0.05$. The corresponding variables will be more significant ($p < 0.05$) if the absolute F ratio becomes larger and the p-value becomes smaller (Table 1 and 2). The type of disinfectants (Table 1) and the level of concentrations (Table 2) were considered as the response variable in this study. All measurements were carried out in triplicate and reported as the mean ± standard deviation (SD) of independent trials. Data analysis was carried out using the Minitab 15 statistical package (Minitab Inc., State College, PA, USA).

Results and Discussion

The effectiveness of the different concentration level of disinfectant on removing biofilm was measured using microtitre plate assay. Results showed that the commercial disinfectants used in this study do not have adequate efficiency on removing biofilm from polystyrene surfaces especially at low concentrations (<50%). D1 showed efficiency on removing *L. monocytogenes* biofilm at the concentration > 50% (Figure 1). Surprisingly, D1 at the concentration < 50% showed no efficiency on the removing biofilm for strain number 9 isolated from minced meat (Figure 1). This is in contrast with removing efficiency of D1 as it showed prohibiting effect even at its lowest concentration. Sessile minimum inhibitory concentration (SMIC) for D1 was reported to be 6.25% in our previous study. Effective compounds of D1 were calcium carbonate, sodium dodecylbenzenesulfonate, and trichlorocyanuric acid. Trichlorocyanuric acid is an organic compound which functions as oxidants and chlorinating agents. Trichloroisocyanuric acid (TCCA) is used as industrial disinfectant, bleaching agent, and bactericide. TCCA is known as an important efficient source of the electrophilic chlorenium ion (Cl^+), which has functioned for chlorination of alkenes and carbonyl compounds, preparation of esters, chlorofluoro compounds, N-chloro compounds, and in diverse oxidation reactions (Mendonça *et al*., 2011). In addition, TCCA compared to several similar N-chlorinated compounds (e.g.: 1, 3-dichloro-5,5dimethyl hydantoin, NCS, N-chlorosaccharine, and chloramines-T) shows great advantages since it can transfer three equivalents of chlorine atoms to the substrate up to 45.5% of its mass.

D2 showed high efficiency on removing biofilm at concentration > 50%, but similar behaviour was observed for strain number 9 as its biofilm was more resistant compared to other strains. At concentration

Table 1. Effect of independent variable (type of strain and disinfectant) and their interaction on killing efficiency

		killing efficiency		
Concentration (%)		Type of strain	Disinfectant	Interaction
100	P value*	0.000	0.000	0.000
	F ratio	141.68	4.99	10.35
50	P value*	0.000	0.000	0.000
	F ratio	5.31	58.19	7.08
25	P value*	0.000	0.000	0.000
	F ratio	11.42	12.78	4.25
12.5	P value*	0.000	0.000	0.000
	F ratio	7.21	22.94	6.89
6.25	P value*	0.000	0.000	0.000
	F ratio	8.89	30.80	8.01

* p-value < 0.05; for type of the strain and disinfectant: significant difference within the group, p-value < 0.05 for interaction: significant difference between the groups.

Table 2. Effect of concentration level and type of strain on removing curve efficiency

		Removing efficiency		
Disinfectant		Strain	Concentration	Interaction
1	P value	0.000	0.000	0.000
	F ratio	47.99	98.23	5.42
2	P value	0.000	0.000	0.000
	F ratio	16.21	144.32	4.75
3	P value	0.000	0.000	0.000
	F ratio	11.25	104.98	2.87
4	P value	0.000	0.000	0.000
	F ratio	9.78	111.33	4.55

* p-value < 0.05; significant difference in term of removing biofilm.

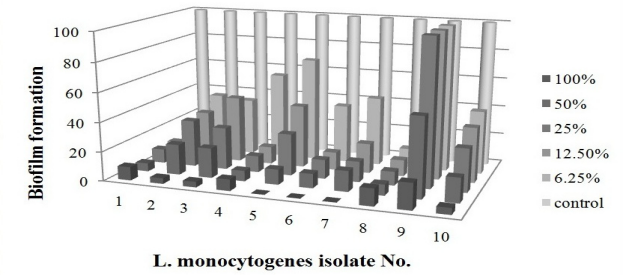

Figure 1. Efficiency of D1 on removing *L. monocytogenes* biofilm from the polystyrene surface

100%, D2 removed biofilm from the polyvinyl surfaces completely. Sodium Percarbonate was the effective compound of D2 (Figure 2).

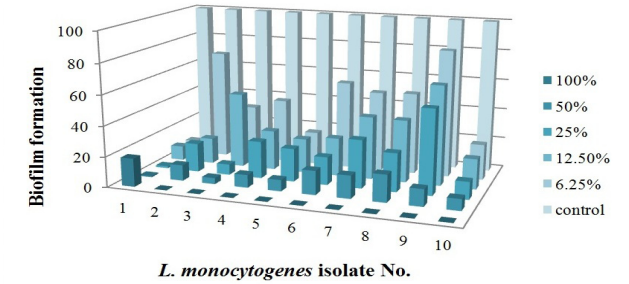

Figure 2. Efficiency of D2 on removing *L. monocytogenes* biofilm from the polystyrene surface

Sodium percarbonate or sodium carbonate peroxyhdarte disunites into sodium, carbonate and hydrogen peroxide, making a strong oxidation reaction which destruct algal cell membranes and chlorophyll. Sodium percarbonate-based disinfectants act rapidly and biodegrade entirely by addition of 13% bio-available oxygen to water in 60 sec of application. Cellular damage happens through a chain of oxidative reactions. These reactions will be initiated with removal of hydrogen from fatty acid by oxygen radical. This removal leads to creating a carbon-centered radical inside the fatty acid in which carbon reacts with oxygen and produce a peroxy radical. This final peroxy radical reacts with other fatty acids or protein in the cell (Peroxide and Acid, 1976; DeQueiroz and Day, 2007; Wagner *et al*., 2012).

According to the results reported for D3, we can sum up no significant differences was observed in efficiency of different concentration < 100% (Figure 3). The highest removing efficiency rate of D3 was reported at its highest concentration level (100%). Comparing the results of D3, a sodium percarbonate-based disinfectant, to D1 and D2 was illustrated D3 is less effective in removing biofilm than other two disinfectants. The same result was obtained in comparing biofilm prohibition efficiency of these three disinfectants.

Results clearly showed that sodium percarbonate based disinfectants were effective for food factories since they showed high efficiency on biofilm removing and sodium percarbonate based disinfectantshave no adverse effect on the aquatic ecosystem. Sodium percarbonate-based disinfectants are environment friendly disinfectants as carbonate can be neutralized by organic waste water treatment plant to bicarbonate. Besides, sodium also does not have a high toxicity and the passed out amount of sodium form food factories using such disinfectants is moderately low, meaning that it does not have reverse effect on the environmental organisms imposed to or receiving drain.

Table 3. Effect of different concentration level of on removing efficiency of each disinfectant

Disinfectant	Concentration (%)	F ratio	P value
1	100	10.28	0.000
	50	15.86	0.000
	25	16.32	0.000
	12.5	17.90	0.000
	6.25	10.78	0.000
2	100	18.51	0.000
	50	4.44	0.003
	25	8.38	0.000
	12.5	6.27	0.000
	6.25	6.94	0.000
3	100	1.19	**0.354**
	50	3.99	0.005
	25	2.40	0.049
	12.5	3.70	0.007
	6.25	10.23	0.000
4	100	23.44	0.000
	50	8.17	0.000
	25	3.06	0.018
	12.5	6.79	0.000
	6.25	3.82	0.006

*p-value > 0.05; no significant difference between the strains in term of biofilm production after treatment with each concentration level of disinfectant (at confident level of 95%), p-value < 0.05: significant difference.

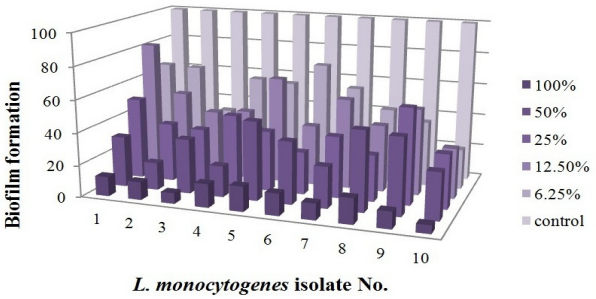

Figure 3. Efficiency of D3 on removing *L. monocytogenes* biofilm from the polystyrene surface

The effectiveness of D4 was similar to other three disinfectants and it has high efficiency for all strains above than 50% at all concentrations. Surprisingly 50% concentration of D4 showed lower efficiency than the concentration < 50%. All the strains demonstrated almost the same level of sensitivity to the concentration levels below 50% with removing up to 75% (Figure 4). According to the results, choosing a suitable disinfectant for use in food line processing plants is an important issue. Besides, an efficient cleaning program must be used to ensure prevention

or removal of sessile *L. monocytogenes* cells.

Table 4. Effect of concentration level of disinfectants on removing efficiency of strains

Disinfectant	Strain	F ratio	P value*
1	1	0.67	0.627
	2	15.69	0.000
	3	6.79	0.007
	4	81.32	0.000
	5	18.27	0.000
	6	8.5	0.003
	7	12.18	0.001
	8	2.21	0.141
	9	73.52	0.000
	10	10.70	0.001
2	1	23.16	0.000
	2	20.07	0.000
	3	13.03	0.001
	4	24.7	0.000
	5	23.77	0.000
	6	43.32	0.000
	7	9.36	0.002
	8	10.15	0.002
	9	50.20	0.000
	10	12.85	0.001
3	1	9.47	0.002
	2	38.8	0.000
	3	41.09	0.000
	4	12.83	0.000
	5	5.56	0.013
	6	9.53	0.002
	7	257.09	0.000
	8	19.5	0.000
	9	14.78	0.000
	10	7.23	0.005
4	1	59.27	0.000
	2	22.43	0.000
	3	6.97	0.006
	4	26.31	0.000
	5	11.86	0.001
	6	11.04	0.001
	7	5.85	0.011
	8	11.09	0.001
	9	9.61	0.002
	10	17.28	0.000

*p-value > 0.05; no significant difference between the strains in term of biofilm production by changing the concentration level of each disinfectant (at confident level of 95%), p-value < 0.05: significant difference.

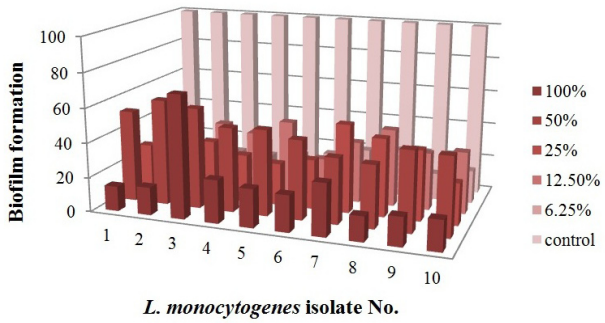

Figure 4. Efficiency of D4 on removing *L. monocytogenes* biofilm from the polystyrene surface

Conclusion

The results showed the susceptibility of different strains of *L. monocytogenes* biofilm varies based on the concentration level of disinfectants. Bacteria in the form of biofilm are much more resistance to different kind of environmental stresses (such as disinfectant). Some strains, such as strain number 9 (isolated from minced meat) showed higher resistance to disinfectants. D1 did not have any significant effect on removing *L. monocytogenes* biofilm (strain No.9) at the concentration <50%. Some *L. monocytogenes* strains showed high susceptibility to D1 even after the formation of biofilm. High concentration (50% and 100%) of all disinfectants (except D3) had the highest efficiency in removing biofilm from the polystyrene surfaces. Since disinfectants at 100% concentration make a serious stress to bacterial cells and even biofilm, more than 95% of *L. monocytogenes* were removed (except for D4). While a disinfectant is not able to completely remove bacteria, the surviving bacteria become much more resistance to the disinfectant. For food factories, is suggested to do sanitization procedure every 24 h and make sure that both forms of bacteria (biofilm and planktonic) are removed from the surface in each cleaning procedure.

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