International Food Research Journal 20(2): 987-990 (2013)
Journal homepage: *http://www.ifrj.upm.edu.my*

Assessing biofilm formation by *Listeria monocytogenes*

[1*]Fouladynezhad, N., [1]Afsah-Hejri, L., [1]Rukayadi, Y., [1]Abdulkarim, S.M., [2]Marian, M.N. and [1]Son, R.

[1]Food Safety Research Center (FOSREC), Faculty of Food Science and Technology, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia.
[2]Faculty of Applied Sciences, Universiti Teknologi MARA Malaysia, 40450 Shah Alam, Selangor, Malaysia

Article history

Received: 1 August 2012
Received in revised form: 27 September 2012
Accepted: 5 October 2012

Keywords

Biofilm
Listeria monocytogenes
microtitre plate assay

Abstract

Listeria monocytogenes (*L. monocytogenes*) is a serious food-borne pathogen for immunocompromised individuals. *L. monocytogenes* is capable of producing biofilm on the surface of food processing lines and instruments. The biofilm transfers contamination to food products and impose risk to public health. Transfers contamination to food products, and impose risk hazard to public health. The aim of this study was to investigate biofilm producing ability of *L. monocytogenes* isolates. Microtitre assay was used to measure the amount of biofilm production by ten *L. monocytogenes* isolates from minced chicken / meat, sausages and burgers. Results showed that all 10 *L. monocytogenes* isolates were able to form biofilm after 24 h at 20˚C on polystyrene surface (the common surface in food industries). Some strains were capable of forming biofilm more than the others. All strains showed a slight raise in the quantities of attached cells over 48 and 72 h. *L. monocytogenes* strains isolated from minced chicken, minced meat and burgers were better biofilm-producers comparing to the strains isolated from sausages.

Introduction

Listeria monocytogenes (*L. monocytogenes*) is a gram-positive, facultative anaerobic, non-spore-forming, rod-shaped bacterium with optimal growth temperature range of 30 - 37˚C. *L. monocytogenes* is a food-borne pathogen causing disease in human and animals (Chen *et al*., 2010; Pagadala *et al*., 2012) . *L. monocytogenes* is widely distributed in different environments and can be found in water, soil, animal fecal matter and sewage (Vaid *et al*., 2010). Discovery of *L. monocytogenes* goes back to 1924, when for the first time it was isolated as the etiological agent of a septicaemic disease (causing death of rabbits and guinea pigs) (Murray *et al*., 1926; Swaminathan and Gerner-Smidt, 2007). Among eight species of *Listeria* genus, only two are considered pathogen (Barbuddhe *et al*., 2012). *L. ivanoii* is pathogens for animals, when *L. monocytogenes* is pathogenic for both animals and humans (Brugère-Picoux, 2008).

L. monocytogenes, is considered a serious food-borne pathogen in immunocompromised individuals such as cancer and HIV patients, elderly and pregnant women. Ingestion of *L. monocytogenes* can cause listeriosis in human and animals (Chen *et al*., 2010; Pagadala *et al*., 2012). Listeriosis can lead to gastroenteritis, septicaemia, perinatal infections, stillbirth, abortion, meningitis and meningoencephalitis in immunocompromised individuals (Barbuddhe *et al*., 2012). Listeriosis affects a wide variety of mammals including monogastric, ruminants (mostly sheep) and human with morality rate of 20-30% (Chaturongkasumrit *et al*., 2011).

Despite efforts of industries and governmental agencies for controlling *L. monocytogenes* in the food supply, number if victims by this microorganism is increasing. In 2008, consumption of contaminated sliced chilled luncheon meats with *L. monocytogenes* resulted in 56 confirmed cases with 22 deaths (Weatherill *et al*., 2009). After a large outbreak of listeriosis with high fatality rate in Canada, *L. monocytogenes* was recognized as a dangerous food-borne pathogen and human health concern (Swaminathan and Gerner-Smidt, 2007). Human listeriosis occurs in several forms depending on the degree of invasiveness of *L. monocytogenes*. Liver, cerebral spinal fluid, spleen and blood are the most common parts of body affected by *L. monocytogenes*. The usual symptoms in healthy adults are diarrhea

*Corresponding author.
Email: *ninafouladynezhad@gmail.com*

and fever. In pregnant women symptoms are fever, diarrhea, abortion or stillbirth; neonates get sepsis, meningitis and pneumonia (Barbuddhe *et al*., 2012).

L. monocytogenes is able to produce biofilm. Biofilm production is a way to help microorganism to survive and grow for an extended period of time (Swaminathan and Gerner-Smidt, 2007). The biofilm that is produced on the surface of food processing lines and instruments can be transferred to food products, causing hazards to public health and contaminate environment (Chaturongkasumrit *et al*., 2011). *L. monocytogenes* frequently exists of food processing industries and is the main causing agent of many food poisonings associated with contaminated ready-to-eat (RTE) food product. Department of Agriculture Food Safety and Inspection Service (FSIS) reported that 90% of human Listeriosis cases reported in United States were associated with consumption of contaminated RTE deli meat products (Pradhan *et al*., 2010). Post-contamination after heat treatment in Ready-To-Eat (RTE) foods may occur through several ways but the most common way is when the product come into direct contact with processing machinery (Truelstrup Hansen and Vogel, 2011). Any post-process contamination can decrease the shelf life of food products and impose economic burden on producers due to broad product recalls.

Direct transfer of microorganisms from processing surface to product depends on the number of microorganisms, composition of food, temperature, type of surface material, and presence of other bacteria biofilms (Truelstrup Hansen and Vogel, 2011). Since a psychrotolerant bacterium such as *L. monocytogenes* is capable of multiply during storage, even its low initial number can contaminate food and cause disease (Nilsson *et al*., 2011). According to Harvry *et al*. (2007), younger bacteria form more biofilm comparing to the older ones (Harvey *et al*., 2007). Contamination of finished products with *L. monocytogenes* is considered a significant threat in food safety and quality. Study the survival of *L. monocytogenes* in food processing line can improve the intervention strategies and reduce health hazards. The aim of this study was to investigate biofilm producing ability of ten *L. monocytogenes* isolates.

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.

Bacterial strain and culture conditions

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

Preparation of L. monocytogenes *cultures to study formation of biofilm*

L. monocytogenes test strains were inoculated into 5ml of TSB and incubated at 30˚C for 18h. After incubation period, 20 µl of cultures was transferred to 5 ml of TSB and incubated for another 18h at 20˚C. Finally 125 µl of over-night culture was transferred to 5 ml of TSB. After mixing for 1 min, suspension was transfered to microtitre plate wells (100 µl for each wells) (Harvey *et al*., 2007).

Microtitre plate assay

To quantify biofilm formation by *L. monocytogenes* strains, the microtitre plate biofilm screening assay was used (Harvey *et al*., 2007). A 100 µl of the mentioned bacterial mixture was transferred into a well of sterile polystyrene microtitre plate (six wells for each strain). Plates were incubated at 20˚C for 24, 48 and 72 h. Each plate included six positive and negative controls. Negative wells comprised of 100 ml of un-inoculated TSB. At the end of incubation period, cell densities were measured at 595 nm. Each microtitre plate well was washed three times with 150 µl of sterile water (after removing the cultures in order to omit loosely associated bacteria). After drying wells at 30˚C for 30 min, 150µl 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.

Results and Discussions

Results showed that all 10 *L. monocytogenes* strains isolated from sausages, burgers, minced chicken and minced meat were able to form biofilm over 24 h at 20˚C on polystyrene surface (the common surface in food industries). Some strains (strains No 7, 8, 9, and 10) were capable of forming

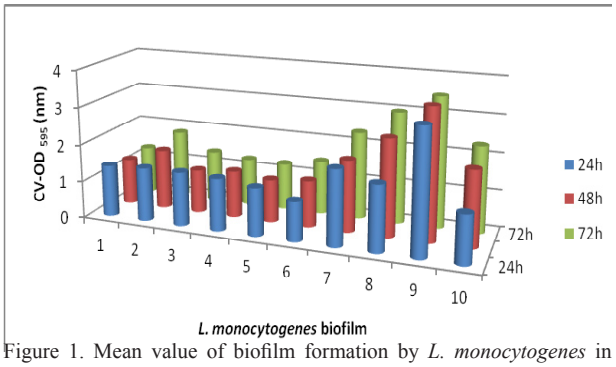

Figure 1. Mean value of biofilm formation by *L. monocytogenes* in polystyrene microtitre wells (OD_{595} at 24, 48 and 72 h)

biofilm more than others but there was no significant difference between the amounts of biofilm formed by each strains ($P< 0.05$). All the strains showed a slight raise in the quantities of attached cells over 48 and 72 h (Figure 1). No reduction in the amount of biofilm was observed during testing hours.

As biofilm has a protective effect on microorganism, *L. monocytogenes* resistance can increase after formation of biofilm. Formation of biofilm makes the bacterium 1000 times more resistance to environment stresses (Chapman, 2003). Bacteria in the form of biofilm will be protected against the disinfectant. Since the efficiency of disinfectant decline in the present of biofilm, sanitation faces serious difficulties in food industries (Chapman, 2003).

Truelstrup Hansen and Vogel (2011), studied the survival of *L. monocytogenes* biofilm during desiccation (43% RH and 15˚C) on stainless steel coupons and its transfer to salmon products. Their research showed formation of biofilm after 2 days at 100% RH and 15˚C would extend the survival of *L. monocytogenes* cells during desiccation period. This study indicates the protective influence of biofilm matrix against environmental stresses.

Chaturongkasumrit *et al*. (2011) studied the use of polyesterurethane (PSU) conveyor belt as the surface and reported the formation of biofilm by two different *L. monocytogenes*. The conveyor belts belonged to a frozen RTE chicken meat product factory in Thailand. In the mentioned studies, formations of biofilm by *L. monocytogenes* on variety of surfaces were investigated while current research mostly focuses on the differences in ability of forming biofilm.

It is important to note that physicochemical properties of the surface (like: surface charge, hydrophobicity, temperature, pH and nutrient composition) have influence on the adherence and biofilm formation of microorganisms (Chmielewski and Frank, 2006). Relatively to the materials used as the surface in food industries, different degree of biofilm would form. As an example, salmonella and *listeria* prefer to attach to hydrophobic surfaces than to hydrophilic; besides the presence of food residue on the surface would enhance microorganism adherence and biofilm formation (Chmielewski and Frank, 2006). Hydrophobic materials are widely used in food processing instruments (like: gaskets, trays, conveyer belts and cutting boards).

In microtitre plate assay using polystyrene microtitre plates, optical density at 595 nm (OD_{595}nm) was used for assessing biofilm formation. Using crystal violet for staining, the cells and biofilm became visible as purple rings or purple mass. To measure the amount of biomass in biofilm, alcohol 95% was used as destaining solution. The observed OD_{595}nm values ranged from 1 to 3.5 nm after 72 h. For all the strains during growth period (24, 48 and 72 h at 20˚C in TSB), rising in the quantities of biofilm formation was observed. However the growth was slightly lower in the incubation period beyond 24 h.

Biofilm form of *L. monocytogenes* is much more resistance to environment stresses and can stay for long time on the surfaces. According to Rodríguez *et al*. (2008), the most important factor that contaminates food products during processing, transport, retail and preparation is direct contact of foods with contaminated surfaces (Rodríguez *et al*., 2008). Since food is in direct contact with food processing line surface, cross-contamination of final products with *L. monocytogenes* is a serious hazard for human health.

Adhesion is the first step for biofilm formation. *L. monocytogenes* inoculated cells grew in TSB and seek for appropriate surface for attachment. By Introducing cells to the surface, some time is required to adapt to the new situation. At the adaptation stage, some bacterial cells might diffuse to the pre-conditioned substratum. Next step for successfully absorbed cells is surrounding themselves with polysaccharides to stick to the polystyrene surface. They remain on the substratum till the complete occupation of the sites would occur. The exopolysaccharide help the cells to stay on the surface.

The *L. monocytogenes* strains belonging to minced chicken, minced meat and burgers were better biofilm-formers (up to 3.5 nm) comparing to the strains belonging to sausages (up to 1.5 nm). Although the amount of biofilm formed by strains belong to sausages is lower, but it is more important as the heat treatment used for sausages before consumption is not enough for killing bacteria. Even in the same environmental condition (surface, temperature, time) different strains of the same bacteria can form different amount of biofilm. The amount of biofilm formed by all the strains were almost the same

since all strains were isolated from meat products. To explain the differences between the amounts of biofilm formation by different *L. monocytogenes* strains further investigation is required.

Conclusion

Bacteria in the form of biofilm can tolerate harsh environments more than their planktonic counterparts. *L. monocytogenes* isolated from food products are able to form biofilm. Biofilm formation is a complicated process influenced by some factors such as time, temperature, and growth medium, nevertheless the process is not still entirely understood. As microorganisms are able to survive on the food processing surfaces, *L. monocytogenes* can persist and re-contaminate food products and is considered an important issue in food safety. The regular cleaning and sanitation procedures are not efficient enough to remove Listeria from food machinery surfaces. Although the number of *L. monocytogenes* transfer to food product might be low, but this bacterium can grow at refrigerator temperature. Further research is required to find an effective way to clean the surfaces and effective sanitization methods to completely remove this bacterium.

References

Barbuddhe, S. B., Malik, S. V. S., Kumar, J. A., Kalorey, D. R. and Chakraborty, T. 2012. Epidemiology and risk management of listeriosis in India. International Journal of Food Microbiology 154(3): 113–118.

Brugère-Picoux, J. 2008. Ovine listeriosis. Small Ruminant Research 76(1–2): 12–20.

Chapman, J. S. 2003. Disinfectant resistance mechanisms, cross-resistance, and co-resistance. International Biodeterioration and Biodegradation 51(4): 271–276.

Chaturongkasumrit, Y., Takahashi, H., Keeratipibul, S., Kuda, T. and Kimura, B. 2011. The effect of polyesterurethane belt surface roughness on *Listeria monocytogenes* biofilm formation and its cleaning efficiency. Food Control 22(12): 1893–1899.

Chen, B.-Y., Pyla, R., Kim, T.-J., Silva, J. L. and Jung, Y.-S. 2010. Prevalence and contamination patterns of *Listeria monocytogenes* in catfish processing environment and fresh fillets. Food Microbiology 27(5): 645–652.

Chmielewski, R. A. N. and Frank, J. F. 2006. A predictive model for heat inactivation of *Listeria monocytogenes* biofilm on buna-N rubber. LWT - Food Science and Technology 39(1): 11–19.

Harvey, J., Keenan, K. P. and Gilmour, A. 2007. Assessing biofilm formation by *Listeria monocytogenes* strains. Food Microbiology 24(4): 380–392.

Murray, E.G.D., Webb, R.A. and Swann, M. B. R. 1926. No TitleA disease of rabbits characterised by a large mononuclear leukocytosis, caused by a hitherto undescribed bacillus Bacterium monocytogenes. Journal of Pathology and Bacteriology 7: 407–439.

Nilsson, R. E., Ross, T. and Bowman, J. P. 2011. Variability in biofilm production by *Listeria monocytogenes* correlated to strain origin and growth conditions. International Journal of Food Microbiology 150(1): 14–24.

Pagadala, S., Parveen, S., Rippen, T., Luchansky, J. B., Call, J. E., Tamplin, M. L. and Porto-Fett, A. C. S. 2012. Prevalence, characterization and sources of *Listeria monocytogenes* in blue crab (*Callinectus sapidus*) meat and blue crab processing plants. Food Microbiology 31(2): 263–270.

Pradhan, D., Mishra, D., Kim, D. J., Ahn, J. G., Chaudhury, G. R. and Lee, S. W. 2010. Bioleaching kinetics and multivariate analysis of spent petroleum catalyst dissolution using two acidophiles. Journal of Hazardous Materials 175(1–3): 267–273.

Rodríguez, F. L., Ibáñez, M. N., Rodríguez, J. P. and Rivero, S. S. 2008. The Credibility of the European monetary System: A Review. Cuadernos de Economía 31(86): 5–34.

Swaminathan, B. and Gerner-Smidt, P. 2007. The epidemiology of human listeriosis. Microbes and Infection 9(10): 1236–1243.

Truelstrup Hansen, L. and Vogel, B. F. 2011. Desiccation of adhering and biofilm *Listeria monocytogenes* on stainless steel: Survival and transfer to salmon products. International Journal of Food Microbiology 146(1): 88–93.

Vaid, R., Linton, R. H. and Morgan, M. T. 2010. Comparison of inactivation of *Listeria monocytogenes* within a biofilm matrix using chlorine dioxide gas, aqueous chlorine dioxide and sodium hypochlorite treatments. Food Microbiology 27(8): 979–984.

Weatherill, G.A. and Burton, P.W., 2009. Delineation of shallow seismic sources using Kmeans cluster analysis, with specific application to the Aegean region. Geophysical Journal International 176: 565–588.