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Listeria monocytogenes response to sublethal sodium hypochlorite induced oxidative stress on its biofilm forming ability and antibiotic resistance

By

Mohit Bansal

A Thesis
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Master of Science
in Agriculture
in the Department of Poultry Science

Mississippi State, Mississippi

December 2017

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Listeria monocytogenes response to sublethal sodium hypochlorite induced oxidative stress on its biofilm forming ability and antibiotic resistance

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Listeria monocytogenes response to oxidative stress by sublethal sodium hypochlorite was investigated in this study. Continuous exposure of sublethal chlorine influenced biofilm formation and stress adaptation (homologous and heterologous) in *L. monocytogenes*. The biofilm forming ability of oxidative stress adapted and control cells were investigated on polystyrene surface at 22°C and 37°C. The oxidative stress adapted cells were found to form less biofilm in the presence of chlorine ($p < 0.10$) when compared to non-treated control cells at both the temperatures. In addition, the biofilm forming ability of *L. monocytogenes* was reduced significantly at higher sublethal chlorine concentrations ($p < 0.10$). In conclusion, oxidative stress adapted *L. monocytogenes* has developed tolerance to chlorine and some of the antibiotics. However, oxidative stress those cells did demonstrate an antibiofilm effect. This demonstrates that oxidative stress reduces *L. monocytogenes* biofilm formation but can also increase antibiotic resistance

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DEDICATION

To my family, teachers and God.

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CHAPTER I

INTRODUCTION

Food borne pathogens causes 48 million illnesses and 3,000 deaths annually in the United States (Scallan et al., 2011). Among them, *Listeria monocytogenes* an important foodborne pathogen which causes "listeriosis, in susceptible population such as the elderly, immunocompromised, infants and pregnant women. Listeriosis accounts for 19% of all deaths related to food borne diseases in the United States (Scallan et al., 2011). *L. monocytogenes* is an environmental resistant organism and can grow slowly at refrigeration temperatures (4°C). It can also survive in high salinity environments and in a wide range of pH`s. Due its ubiquitous nature, different food products like fish, meat, cole slaw, cantaloupes (Scallan et al., 2011), unpasteurized milk (Latorre et al., 2011) and cream were implicated as sources of infection (Cartwright, 2013).

Ready to eat products such as deli meats and hotdogs have also been implicated as a potential sources of *L. monocytogenes* in previous major outbreaks (CDC 2017). Turkey deli meat was responsible for listeriosis outbreaks in the US in 2002 (Gottlieb et al., 2006) and Canada in 2008 (Currie et al., 2015). There were 111 illness, 32 adult deaths and 3 fetal deaths associated with these two major listeriosis outbreaks. Since *L. monocytogenes* has the ability to survive and multiply at low temperature (4°C), it can contaminate food at any point in the processing plant including refrigerated storage. Despite multiple outbreaks from different food products, the transmission pathway and

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true reservoir of *L. monocytogenes* is still unclear. *L. monocytogenes* is regularly isolated from poultry processing and retail environments. The biofilms of *L. monocytogenes* act as a persistent source of cross contamination to the raw or cooked poultry products (Muhterem-Uyar et al., 2015). A previous study reported the prevalence of *L. monocytogenes* in poultry processing environments and concluded that 16.4 -20% of the samples collected were found positive for *L. monocytogenes* (Chiarini et al., 2009). As per reported by the FDA, 83% of listeriosis cases and deaths were associated with deli meats (Quesenberry et al., 2010). Due to its severe infection, the USDA has a zero tolerance policy for *L. monocytogenes* in ready-to-eat (RTE) food products since the 1980s. The USDA-FSIS zero tolerance policy is applied to all RTE products irrespective of distinction between foods contaminated at high or low levels of *L. monocytogenes*.

L. monocytogenes can attach to food or non-food contact surfaces such as polystyrene, stainless steel or rubber to form a biofilm (Borucki et al., 2003; Chavant et al., 2002). A biofilm can be defined as a "structured multicellular bacterial communities adherent to surfaces in man-made or natural environments" (Aguilar et al., 2012). These biofilms act as persistent sources of food contamination in the food processing plant (Folsom and Frank, 2007). These biofilms can also act as a shield to the organism, and provide protection from food processing environmental conditions such as desiccation, antimicrobials and water shear forces (Chmielewski and Frank, 2003).

Foodborne microorganisms are often exposed to different concentrations of antimicrobials, while in the presence of organic matter, at the processing plant. The sub-lethal concentrations of these antimicrobials (or biocides) can influence the bacterial

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response, creating environmental adaptations and at the same time decreasing the sensitivity of the bacteria to the antimicrobials.

Microorganisms develop their biofilm in the processing plant in response to environmental conditions, such as a lack of nutrients, a low moisture level or due to a wide range of pH. Bacteria in biofilms are found to be more resistance towards biocides than when in a planktonic state (Araújo et al., 2011). Sublethal stresses can induce physiological changes in bacterial physiology and morphology (Giotis et al., 2007), which in turn may influence the biofilm formation in pathogenic bacteria such as *L. monocytogenes*. Previous studies found *L. monocytogenes* is unable to form biofilms in the presence of plant derived antimicrobials such carvacol, thymol, trans-cinnamaldehyde, eugenol (Upadhyay et al., 2013) and morin (Sivaranjani et al., 2016). However, there is no information available describing whether or not the widely used chlorine compound, sodium hypochlorite, can also influence the biofilm forming ability of L monocytogenes.

Antibiotic such as ampicillin, gentamicin and cephalosporin are drugs of choice for listeriosis treatment (Schlech III, 2006) however, in the absence of confirmatory diagnosis, these cases are treated only according to symptoms. There is an increase in the number of reports related to antibiotic resistance by *L. monocytogenes*. Few strains of *L. monocytogenes* have third generation cephalosporin resistance and treatment of them with resistant drugs can lead to fatal conditions (Kose and Yakupogullari, 2015).

Antibiotic resistance can be intrinsic or acquired (Martins et al., 2013). Some bacteria have intrinsic or natural resistance against selective antibiotics because of an impermeable cell membrane or a lack of receptors, while some bacteria acquire resistance

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from the environment such as vancomycin resistance in Gram-negative bacteria (Lee, 1994; Nelson, 1999). Acquired antibiotic resistance could be genetic or phenotypic (Corona and Martinez, 2013). Genetic antibiotic resistance is well defined and it can spread in susceptible bacterial population. These population can develop resistance by mutation or acquisition of entire genes through plasmids or transposons (Kelly et al., 2009).

However, phenotypic antibiotic resistance has not been well studied. Changes in morphological properties such as cell surface or size also is thought to induce antibiotic resistance (Levin and Rozen, 2006). Selective sublethal stress can induces changes in cell morphology and physiology, which could cause antibiotic resistance. Food processing and other environmental stresses can induce the phenotypic antibiotic resistance in *L. monocytogenes* (McMahon et al., 2007)

The USDA has approved various cleaners, sanitizers and other antimicrobials for cleaning and disinfection purposes. Sodium hypochlorite is one of the commonly used chlorine compound for disinfection purposes in the poultry processing plant (Gray et al., 2014). Sodium hypochlorite dissociates into hypochlorous acid (HOCl) in its aqueous solution and acts as a source of chlorine. This HOCl is a powerful oxidant and highly reactive to a plethora of biomolecules in a cell such as the cell membrane, proteins and DNA which can cause further oxidative damage at lethal doses (Gray et al., 2014). However, bacterial cells can adapt to this oxidative stress after repeated exposure to sublethal concentrations.

The sublethal chlorine used in the disinfection process can induce the oxidative stress response in *L. monocytogenes*, which may influence its biofilm formation and

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biocide tolerance development. Along with that, residual chlorine can also induce the same adaptive response in *L. monocytogenes*

Therefore, the objectives of this thesis was to investigate: (i) the effect of a widely used chlorine compound , sodium hypochlorite, at sublethal concentrations, on its ability to influence *L. monocytogenes* biofilm formation; (ii) to determine whether sublethal sodium hypochlorite stress leads to the development of chlorine tolerance by measuring changes in minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) and changes in antibiotic resistance by measuring changes in zones of inhibition and MIC to commonly used antibiotics.

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References

Araújo, P., Lemos, M., Mergulhão, F., Melo, L., Simões, M., 2011. Antimicrobial resistance to disinfectants in biofilms 826–834.

CDC (2017) Center for disesae control and prevention. Listeria prevention, 2017. https://www.cdc.gov/listeria/prevention.html. Accessed July 6, 2017.

Chiarini, E., Tyler, K., Farber, J.M., Pagotto, F., Destro, M.T., 2009. Listeria monocytogenes in two different poultry facilities: Manual and automatic evisceration. Poult. Sci. 88, 791–797. doi:10.3382/ps.2008-00396

Chmielewski, R. a N., Frank, J.F., 2003. Biofilm Formation and Control in Food Processing Facilities. Compr. Rev. Food Sci. Food Saf. 2, 22–32. doi:10.1111/j.1541-4337.2003.tb00012.x

Corona, F., Martinez, J., 2013. Phenotypic Resistance to Antibiotics. Antibiotics 2, 237–255. doi:10.3390/antibiotics2020237

Currie, A., Farber, J.M., Nadon, C., Sharma, D., Whitfield, Y., Gaulin, C., Galanis, E., Bekal, S., Flint, J., Tschetter, L., Pagotto, F., Lee, B., Jamieson, F., Badiani, T., MacDonald, D., Ellis, A., May-Hadford, J., McCormick, R., Savelli, C., Middleton, D., Allen, V., Tremblay, F.-W., MacDougall, L., Hoang, L., Shyng, S., Everett, D., Chui, L., Louie, M., Bangura, H., Levett, P.N., Wilkinson, K., Wylie, J., Reid, J., Major, B., Engel, D., Douey, D., Huszczynski, G., Di Lecci, J., Strazds, J., Rousseau, J., Ma, K., Isaac, L., Sierpinska, U., 2015. Multi-Province Listeriosis Outbreak Linked to Contaminated Deli Meat Consumed Primarily in Institutional Settings, Canada, 2008. Foodborne Pathog. Dis. 12, 645–652. doi:10.1089/fpd.2015.1939

Daniell, H., 2012. NIH Public Access 76, 211–220. doi:10.1007/s11103-011-9767-z.Plastid

Giotis, E.S., Blair, I.S., McDowell, D.A., 2007. Morphological changes in Listeria monocytogenes subjected to sublethal alkaline stress. Int. J. Food Microbiol. 120, 250–258. doi:10.1016/j.ijfoodmicro.2007.08.036

Gottlieb, S.L., Newbern, E.C., Griffin, P.M., Graves, L.M., Hoekstra, R.M., Baker, N.L., Hunter, S.B., Holt, K.G., Ramsey, F., Head, M., Levine, P., Johnson, G., Schoonmaker-Bopp, D., Reddy, V., Kornstein, L., Gerwel, M., Nsubuga, J., Edwards, L., Stonecipher, S., Hurd, S., Austin, D., Jefferson, M.A., Young, S.D., Hise, K., Chernak, E.D., Sobel, J., 2006. Multistate outbreak of Listeriosis linked to turkey deli meat and subsequent changes in US regulatory policy. Clin. Infect. Dis. 42, 29–36. doi:10.1086/498113

Gray, M.J., Wholey, W., Jakob, U., 2014. NIH Public Access 141–160. doi:10.1146/annurev-micro-102912-142520.Bacterial

المنارة للاستشارات
www.manaraa.com

Kelly, B.G., Vespermann, A., Bolton, D.J., 2009. Horizontal gene transfer of virulence determinants in selected bacterial foodborne pathogens. Food Chem. Toxicol. 47, 969–977. doi:10.1016/j.fct.2008.02.007

Kose, A., Yakupogullari, Y., 2015. A rapidly fatal sepsis caused by listeria monocytogenes type-4b in a patient with chronic renal failure. Jundishapur J. Microbiol. 8, e19980. doi:10.5812/jjm.19980

Lee, M.S., 1994. Vancomycin-Resistant Leuconostoc mesenteroides and Lactobacillus 176, 260–264.

Levin, B.R., Rozen, D.E., 2006. Non-inherited antibiotic resistance. Nat. Rev. Microbiol. 4, 556–562. doi:10.1038/nrmicro1445

Martins, A., Hunyadi, A., Amaral, L., 2013. Mechanisms of resistance in bacteria: an evolutionary approach. Open Microbiol. J. 7, 53–8. doi:10.2174/1874285801307010053

McMahon, M.A.S., Xu, J., Moore, J.E., Blair, I.S., McDowell, D.A., 2007. Environmental stress and antibiotic resistance in food-related pathogens. Appl. Environ. Microbiol. 73, 211–217. doi:10.1128/AEM.00578-06

Muhterem-Uyar, M., Dalmasso, M., Bolocan, A.S., Hernandez, M., Kapetanakou, A.E., Kuchta, T., Manios, S.G., Melero, B., Minarovičová, J., Nicolau, A.I., Rovira, J., Skandamis, P.N., Jordan, K., Rodríguez-Lázaro, D., Stessl, B., Wagner, M., 2015. Environmental sampling for Listeria monocytogenes control in food processing facilities reveals three contamination scenarios. Food Control 51, 94–107. doi:10.1016/j.foodcont.2014.10.042

Nelson, R.R.S., 1999. Intrinsically vancomycin-resistant Gram-positive organisms: Clinical relevance and implications for infection control. J. Hosp. Infect. 42, 275–282. doi:10.1053/jhin.1998.0605

Schlech III, W.F., 2006. Epidemiology and clinical manifestations of Listeria monocytogenes infection, in: Gram-Positive Pathogens, Second Edition. American Society of Microbiology, pp. 601–608.

Sivaranjani, M., Gowrishankar, S., Kamaladevi, A., Pandian, S.K., Balamurugan, K., Ravi, A.V., 2016. Morin inhibits biofilm production and reduces the virulence of Listeria monocytogenes ??? An in vitro and in vivo approach. Int. J. Food Microbiol. 237, 73–82. doi:10.1016/j.ijfoodmicro.2016.08.021

Quesenberry, H.H., Gallagher, D., Endrikat, S., LaBarre, D., Ebel, E., Schroeder, C., Kause, J., 2010. FSIS Comparative Risk Assessment for Listeria monocytogenes in Ready-to- eat Meat and Poultry Deli Meats.

المنارة للاستشارات

www.manaraa.com

Upadhyay, A., Upadhyaya, I., Kollanoor-Johny, A., Venkitanarayanan, K., 2013. Antibiofilm effect of plant derived antimicrobials on Listeria monocytogenes. Food Microbiol. 36, 79–89. doi:10.1016/j.fm.2013.04.010

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CHAPTER II

LITERATURE REVIEW

Deli meat as a source of *L. monocytogenes* outbreaks in the USA and Canada

The first foodborne listeriosis case from meat was reported in England in 1988. Cooked chicken was found as the source of infection (K. Kerr et al., 1988; K. G. Kerr et al., 1988). Later, in the USA the first foodborne *L. monocytogenes* case was reported in 1989, contaminated turkey frankfurter was implicated as the source of the outbreak (Wenger et al., 1990). *L. monocytogenes* serotype 1/2a was isolated from the contaminated turkey frankfurter and from the infected patient. The largest poultry associated listeriosis outbreak was reported in 1998-99. This outbreak was responsible for 108 listeriosis cases with 14 deaths and 4 stillbirths. It was found to originated from contaminated turkey deli meat. *L. monocytogenes* serotype 4b was found to be associated with this outbreak (Mead et al., 2006).

In 2008, one of the largest deli meat listeriosis outbreaks occurred in Canada. This multi-province outbreak resulted in 57 confirmed cases and 24 deaths. Later, this same outbreak was linked to *L. monocytogenes* serotype 1/2a which contaminated sliced turkey deli meat, ham, salami, roast beef and corned beef.

Defective deli-meat handling, cutting, and insufficient sanitation practice can result in *L. monocytogenes* contamination. Previous prevalence studies showed *L. monocytogenes* is a frequent contaminating agent of chicken and its different parts. Few of the poultry related *L. monocytogenes* outbreaks are listed in Table 2.1.

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Table 2.1 *L. monocytogenes* in poultry and poultry products in USA and Canada

Year	Country	Product	No. positive/ No. cases	Reference
1988	USA	Poultry	7/22	(McClain and Lee, 1988)
1989	Canada	Chicken legs	9/16	(Farber et al., 1989)
1989	USA	Broiler carcasses	21/90	(Bailey et al., 1989)
1989	USA	Fresh wings, legs, livers	17/100	(Genigeorgis et al., 1989)
1990	USA	Fresh turkey parts	27/90	(Schonberg and Gerigk, 1991)

Characteristics of *Listeria monocytogenes*

Listeria taxonomy

Listeria monocytogenes is a Gram-positive non-spore forming, rod-shaped food borne pathogen. (Scallan et al., 2011). It is a facultative intracellular bacterium, often found in different kinds of food as well as environments. It is responsible for severe nervous infections (Invasive listeriosis) and mild illness (listerial gastroenteritis) especially among elderly people, pregnant women, children and immunocompromised patients (Ooi and Lorber, 2005).

E. G. D. Murray first described *Listeria monocytogenes* as *Bacterium monocytogenes* in rabbits and guinea pigs. (Murray et al., 1926). Later, in 1927, H. H. Pirie isolated the same organism from wild gerbils in South Africa and renamed it as *Listeria hepatolytica* (Pirie et al., 1927). It was finally given its present name in 1940 (Gray and Killinger, 1966). The first human case was described in 1929 (Vázquez-

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Boland et al., 2001). Later, several zoonotic listeriosis cases were reported from workers coming in contact with infected animals. Food borne listeriosis cases were reported in the mid- 1980s in North America and Europe and were linked to different kind of food such as coleslaw (Schlech et al., 1983), milk (Fleming et al., 1985), and soft cheeses (Bille and Glauser, 1988; Linnan et al., 1988).

Despite the low incidence of 0.25/million listeriosis cases, *L. monocytogenes* contamination was considered as a notifiable disease (Schlech et al., 1983). Further research revealed that *Listeria* can survive in a food processing environment and has a tolerance towards bacteriostatic preservation systems (De Abrew Abeysundara et al., 2016; Taormina and Beuchat, 2001)

***Listeria* microbiology**

Listeria is short rod shaped (0.4-0.5 by 1-2 µm) organism, that is non-spore forming but motile. *Listeria* has a characteristic tumbling motility, expressed only in a narrow temperature range. This motility is characteristic of its peritrichous flagella. *L. monocytogenes* flagellin is expressed and produced between 20 - 25°C, and less so at 37°C (Peel et al., 1988). *Listeria* is widely present in biotic and abiotic surfaces including plants, soil, and surface water samples. It has also been isolated from human and animal feces, silage, normal and contaminated milk (Vasseur et al., 1999).

Based on the DNA homology and 16S rRNA cataloging results, the Listeria genus is classified into the following 7 species., *L. monocytogenes, L. innocua, L. seeligeri, L. welshimeri, L. ivanovii, L. grayi,* and *L. murrayi* (Seeliger, 1984). All species of Listeria are motile with five to six peritrichous flagella. Extensive research over the past few decades has revealed that the flagella contribute to virulence in many foodborne

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pathogens, either as the effectors of motility, colonization and invasion of the intestine, or as mediators of toxin secretion inside the host (O'Neil and Marquis, 2006). In addition, recent evidence suggests that the flagella participate in processes such as biofilm formation and modulation of the host immune response (Duan et al., 2013). In *L. monocytogenes*, flagella aid in biofilm formation on surfaces of the external environment (Lemon et al., 2007) and facilitate intestinal epithelial cell invasion in the host (Bigot et al., 2005; Dons et al., 2004).

***L. monocytogenes* control measures**

Cross contamination of deli meat from raw product and mechanical cutting devices such slicers and dicers are potential source of L. monocytogenes. Proper cleaning and sanitation is key to prevent cross contamination of ready to eat meat products. As per the risk-assessment study, proper cleaning and sanitation of all cross contamination points particularly, slicers, would decrease the predicted risk of listeriosis by approximately 34% (FSIS, 2017). Chemical disinfection is one of the commonly used method for cleaning and sanitation. Chlorine is widely used antimicrobial compound for disinfection of food contact surfaces. However, inadequate chlorine (sodium hypochlorite) concentration application would not help in prevention of cross contamination rather repeated exposure to such low concentrations might aid in *L. monocytogenes* adaptation.

Sodium hypochlorite and oxidative stress

Chlorine compound, sodium hypochlorite, is a widely used, low cost, antimicrobial compound for disinfection and sanitation. Chlorine is a potent chemical

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oxidant and can transform or react with inorganic and organic compounds (Gray et al., 2014). Various species of chlorine (HOCl, ClO^-, Cl_2, etc) may be present in solution depending upon the pH of the solution, however, HOCl (hypochlorous acid) is the major reactive chlorine species (Deborde and von Gunten, 2008). At neutral pH, sodium hypochlorite will form 50% HOCl and 50% ClO^- (Figure 2.1). The other chlorine reactive species do not have significant effects on microorganisms, as they are present in very low concentration in the solution. Hypochlorous acid induces structural changes in parent organic compound and lead to the formation of chlorinated or oxidized compounds (Deborde and von Gunten, 2008). Chlorine reacts with peptides and amino acids (only terminal amines) present in solutions which leads to the formation of chloramines (oxidized proteins) (How et al., 2017).

The HOCl and ClO^- compounds react with organic compounds in broth solution like TSB which then leads to the formation of Chloramines. These chloramines are also considered reactive chlorine species, as they are also capable of chlorinating and oxidizing compounds (Gray et al., 2014). However, chloramines are less active (four-five orders less) than HOCl and appear to have a higher affinity for cysteine and methionine oxidation. Oxidative stress by reactive chlorine species is generated when the concentration of these compounds (HOCl and chloramines etc) increase inside the bacteria and the bacteria is not capable of displacing them (Sies, 1997). These chlorine reactive species induce the expression of the soxRS locus (superoxide or nitric oxide) promoter reporter system (Figure 2.2). The soxRS region acts collectively to prevent oxidative damage or induces the repair system by different mechanisms such as scavenging for oxidants (superoxide dismutase), DNA repair (endonuclease IV), re-

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reduction of oxidized metals in prosthetic groups (flavodoxin and ferredoxin reductase), reconstitution of the NADPH pool (glucose-6-phosphate dehydrogenase), reduced permeability (micF) and excretion of toxicants (efflux pumps). The soxRS activation can also induce heterologous adaptation by increasing the tolerance limits of macrophage-generated nitric oxide (NO), as well as increasing the bacteria's tolerance to antibiotics (Chou et al., 1993; Greenberg et al., 1990; Pomposiello and Demple, 2001).

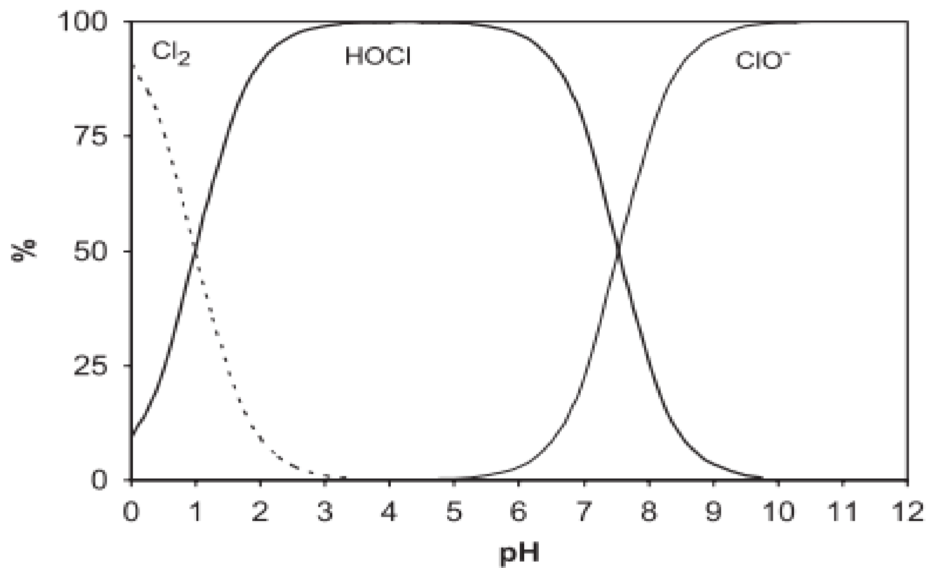

Figure 2.1 Relative distribution of main aqueous chlorine species as a function of pH at 25°C.

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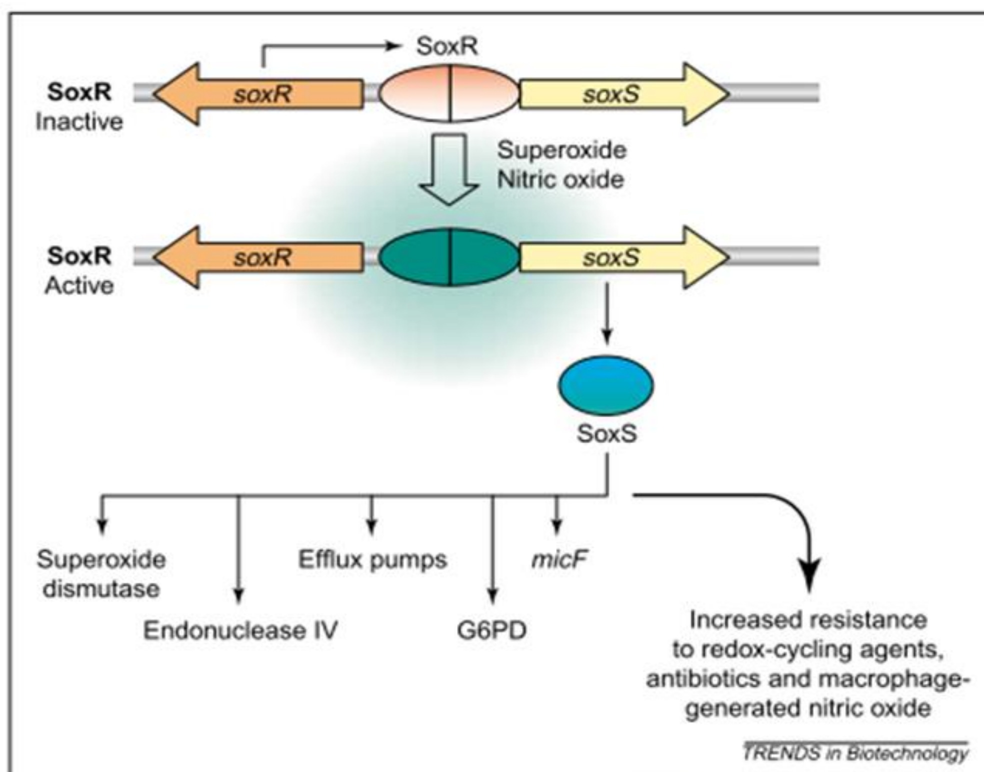

Figure 2.2 The soxRS regulon, composed of soxR and soxS divergently transcribed gene.

The soxR protein is activated in the presence of superoxide generating agents or nitric oxide. This activated soxR enhances expression of soxS. SoxS activates transcription of genes that increase the resistance to oxidants, antibiotics and macrophage-generated NO.

The Biofilm Concept

Bacteria prefer to live in communities or colonies under natural environmental conditions (Palková, 2004). They can attach to different surfaces and protect themselves from cidal environmental conditions. The microbes that can freely float in liquid medium are considered as planktonic cells, while those which are attached to any surfaces are referred to as biofilms (Palková, 2004). Biofilms are comprised of a cellular and

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extracellular casing which includes; extra polymeric compounds, proteins and extracellular DNA (Vu et al., 2009). Zobell and Anderson (1936) revealed the role of surfaces like glass and sand, which enhance bacterial growth and activity. Later Heukelekian and Heller (1940) supported (Zobel's, 1943) findings revealing the supportive role of a solid surface in providing a concentrated form of nutrients found in liquid medium.

Zobell, in 1943, identified that surface attachment of bacteria is a two-phase process; reversible attachment and irreversible attachment. Marshall (1971) observed the role of polymeric fibers in *Pseudomonas* biofilm formation. Later Costerton (1970) stated that bacteria are encased in a glycocalyx matrix and that biofilms can attach to surfaces, interfaces, and to each other. Thereafter a new terminology was utilized, 'biofilmology' which is a multidisciplinary branch of science involving the study of surface, extracellular, cellular and intracellular components.

The biofilm term was first introduced in the early 1980s by Characklis (Characklis, 1973). Later, biofilm was defined as "immobilized cells that grow, reproduce, and produce an extracellular polymer substance (EPS) that frequently extends from the cell, forming a tangled mass of fibers lending structure to the entire assemblage" (Characklis and Cooksey, 1983)

Biofilm formation and stages

Biofilm matrix

The majority of biofilms are composed of cells, which is approximately 10% dry mass, and its matrix which is around 90% (Flemming and Wingender, 2010). The matrix or extracellular component of biofilm, is produced by cells that are entrenched and is

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termed extracellular polymeric substances (EPS). Previously, EPS was known as “the dark matter of biofilms” because of its complex and difficult to analyze structure. This EPS consists of different kinds of biopolymer compounds such as polysaccharides, proteins, phospholipids, teichoic acids and extracellular DNA (Flemming et al., 2007). The EPS component of a biofilm provides physical strength for development of the three-dimensional structure of a biofilm. In addition, EPS enhances the cell-cell interaction and acts as a protection for the cells against bactericidal factors such as antibiotics, biocides, desiccation, UV rays and water shear force.

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Table 2.2 Biofilm chemical composition and their functions

Components of EPS	Functions	Reference
eDNA	Hydrated hydrophilic molecules. Acts as an intercellular connector.	(Flemming and Wingender, 2010)
Surfactants and lipids	Hydrophobic molecules, initial micro colony formation; surface associated bacterial migration; prevents colonization of channels and biofilm dispersion.	(Donlan, 2002)
Extracellular proteins Enzymes	Biopolymers degrade and promote detachment of bacteria from biofilm to low-molecular-mass products. They act as virulence factors.	(Colagiorgi et al., 2016)
Structural proteins	Cell surface (Lectins) and extra cellular; biofilm associated protein (Bap) and appendages such as pili, fimbriae and flagella.	(Fong and Yildiz, 2015)
Exopolysaccharides	Major fraction of the EPS matrix and structural component.	(Donlan, 2002)

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Steps in biofilm formation

Biofilm formation begins with planktonic cell attachment to a abiotic surface. It involves five main steps: reversible attachment, irreversible attachment, micro-colony development, production of exopolysaccharides (EPS), and dispersal. Surface conditioning with organic matter contamination can enhance the biofilm formation. Organic matter contamination reduces the hydrophobicity of surfaces and induces cell attachment. The reversible stage (when bacteria are loosely attached to surfaces by different physical forces such as van der Waals and gravitational forces) of attachment is short term (5-10 seconds) (Mittelman, 1998). Thus, the initial attachment of planktonic cells can be controlled by many surface charges such as the electrical charge of the bacteria, Van der Waals forces, electrostatic factors, hydrophobic interactions, and Brownian motion (Mittelman, 1998; Donlan & Costerton, 2002). Following reversible attachment, the irreversible attachment (when bacteria firmly attaches to the surfaces by exopolysaccharides and other compounds) may take a longer time, 20 min to 4 hours (Mittelman, 1998). After the period of irreversible attachment, cells establish their microcolonies and produces EPS in larger amounts which helps in the formation of a three-dimension structure of biofilm. After complete formation, cells may disperse from biofilm and may repeat production at another location.

Relation of phylogeny of *L. monocytogenes* strains with its biofilm formation and outbreaks

Biofilm acts as persistent source of bacteria survival, multiplication, and transmission. It also has been hypothesized that the ability of *L. monocytogenes* to colonize both food or nonfood contact surfaces may be a reason for the differences in

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prevalence of the various serotypes. (Valderrama, 2012). In other words, the isolates with a higher ability to colonize surfaces are likely to be found during environmental sampling, while those with a lower colonizing ability may be found less often.

L. monocytogenes has been classified into two major and one minor phylogenetic divisions or lineages. Lineage I is comprised of serotype 1/2b and 4b and lineage II is comprised of serotypes 1/2a and 1/2c. A third lineage has not been well characterized yet (Wu et al., 2015). Many studies have investigated the possible relationship of different serotypes, or lineages, with the prevalence of *L. monocytogenes* in the food processing environment with their colonizing and biofilm forming ability. However, as of yet, there is no clear relationship between Listeria phylogeny prevalence and its biofilm formation ability (Doijad et al., 2015). Lineage I (mostly serotype 4b) has been found to be the cause of most listeriosis outbreaks while lineage II serotypes 1/2a, 1/2b and 1/2c are commonly isolated in the processing plants (Thévenot et al., 2005; Wagner et al., 2006; Latorre et al., 2007; Swaminathan and Gerner-Smidt, 2007; Meloni et al., 2009).

L. monocytogenes are exposed to various environmental conditions in the processing plant such as desiccation, lack of nutrients, moisture, and intermittent exposure to different antimicrobials (Taormina and Beuchat, 2001). Various studies have used these conditions to study the biofilm formation ability of *L. monocytogenes* under laboratory conditions and have found differences in the relationship between lineage and their ability to form biofilm (Borucki et al., 2003).

L. monocytogenes biofilm forming ability was found to be strongly correlated with its lineages in the presence of ample amounts of nutrients. Lineage II (serotypes 1/2a and 1/2c) were found to form stronger biofilms than lineage I (serotypes 4b and 1/2b) on

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polyvinyl chloride plates (Borucki et al., 2003). Nonetheless, no association could be establish based on serotypes. (Borucki et al., 2003; Doijad et al., 2015).

Nutrient availability can influence the biofilm formation ability of the different lineages. Under limited nutritional conditions, lineage II was found to produce stronger biofilm than linage I (Harvey et al., 2007). Nutrient availability in the processing plant could be a probable factor for high prevalence of lineage II. The results of various conditions demonstrate that listeria's biofilm forming ability is greatly influenced by environmental conditions.

Influence of environmental factors on *L. monocytogenes* biofilm formation

The formation of a biofilm is thought to be a stress response by bacterial cells (Oliveira et al., 2015). Along with lineages, the environmental conditions can influence the bacterial surface attachment and biofilm formation ability. These factors can be abiotic (hydrodynamics, temperature, pH, ionic composition, or nutrient availability) and/or biotic (other organisms, flagellation, motility and disparity of cell structure). These factors can influence biofilm development individually or synergistically. *L. monocytogenes* are exposed to different antimicrobials in a processing plant. Along with other factors, antimicrobials can also influence the ability of biofilm formation.

Influence of temperature:

Temperature can modulate the virulence and environmental genes in *L. monocytogenes*, which can then induce changes in its cell surface (Liu et al 2002). *Listeria* spp. surface attachment is influenced by temperature on various surfaces, significantly higher attachment was observed at 37°C on three surfaces such as

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polystyrene, glass and steel when compared to 4, 12 and 22°C (Di Bonaventura et al., 2008). Significantly higher biofilm formation was observed on the glass and stainless steel surfaces than on polystyrene at 37°C (Di Bonaventura et al., 2008).

Influence of pH:

Previous studies demonstrated that pH and ions can also influence the growth of planktonic listeria cells. Sodium chloride and acidic pH significantly increased the lag and growth phase (>250 hours) of Listeria Scott A (Bereksi et al., 2002). Increased concentrations of NaCl (10%) along with a pH 5.0 has also been found to induce morphological changes in Listeria Scott A. However at a pH of 5.0 with a 5% concentration of NaCl, short filaments were observed under the electron microscope (Bereksi et al., 2002). These properties showed *L. monocytogenes* can grow in altered environmental conditions but with modified surface properties. In addition, acidifying the pH of the basal growth medium can increase *Listeria* attachment to stainless steel and Buna N rubber (Mafu et al., 1991; Duffy and Sheridan, 1997; Briandet et al., 1999, Smoot and Pierson, 1998).

Influence of nutrients:

Biofilm forming ability is influenced by nutrient availability in the food processing plant. Serotype 4b strains produced stronger biofilms than serotype 1/2a strains in Tryptic Soy Broth (TSB) while, serotype 1/2a strains formed stronger biofilms than 4b in 1/10 TSB (i.e. under low nutrient conditions) over 24 hours at 32°C (Folsom et al., 2006). These results suggest a probable reason of the lower prevalence of serotype 4b strains in the food processing plant where limited nutrients might be available.

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In addition to nutrient availability, growth medium can also influence the biofilm forming ability in *L. monocytogenes.* Most of strains showed strong biofilm producing ability on PVC plates in modified Welshimer's broth when compare to TSB at 32°C (Moltz et al 2005).

Influence of antimicrobials:

Pre-exposure of sub inhibitory concentrations (SIC) of a plant-derived antimicrobial, "morin", can inhibit the biofilm forming ability of *listeria* planktonic cells (Sivaranjani et al., 2016). This antibiofilm activity of morin against planktonic cells was observed to be dependent on concentration (6.25, 12.5, 25.0 µg/ml), temperature 32 °C and time (48 h) (Sivaranjani et al., 2016). *L. monocytogenes* flagellar mediated motility is essential for initial attachment to abiotic surfaces (Lemon et al., 2007; Tresse et al., 2006). SICs of morin; modulate the motility (swimming and swarming) and cell to surface and cell to cell interactions of *L. monocytogenes*, which can further inhibit the formation of biofilm. However, morin is found to be inefficient in the eradication of mature biofilms at both sub MIC and MIC (minimum inhibitory concentration) (Sivaranjani et al., 2016).

In another study, SIC of plant derived antimicrobials (PDA) such as trans-cinnamaldehyde, carvacrol, thymol and eugenol inhibited the biofilm forming ability of *L. monocytogenes* on both polystyrene and stainless steel surfaces at 37, 25 and 4°C (Upadhyay et al., 2013). The authors found that SIC of PDAs down regulate the genes responsible for the initial biofilm forming step (quorum sensing, initial attachment and motility). However, limited information is available on the effect of chlorine antimicrobials such as sodium hypochlorite on *L. monocytogenes* biofilm forming ability.

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L. monocytogenes biofilm formation in food processing plants

L. monocytogenes is a ubiquitous saprophytic organism and it can enter a food processing plant at any place or point. *L. monocytogenes* can survive in their niche or harborage sites, which can provide protection from disinfectants (Mureddu et al., 2014). These sites are usually difficult to clean and sanitize. The niche also provides necessary optimal conditions such as temperature, organic content, and moisture which are all needed for multiplication and biofilm formation.

The presence of organic matter and availability of moisture on food or nonfood contact surfaces in processing plants provide a suitable microbial habitat for development of microbial biofilms. These surfaces are cleaned regularly and disinfect with antimicrobials to avoid contamination of food.

Listeria monocytogenes is well known for surface attachment and biofilm formation in the food processing plant (Pan et al., 2006; Rodriguez et al., 2008) however, there is no direct evidence available of the role of biofilms in food borne outbreaks (Valderrama, 2012). The possible reasons for this could be that the isolation and characterization of biofilms has not been a part of food borne outbreak investigations, or its definition is not yet clear.

Antibiotic Resistance in *Listeria* spp.

Bacteria are the most primitive organisms on this earth and are capable of surviving in a range of environmental conditions, from extreme high temperatures (60-73°C) in hot springs to the extreme low temperatures of Antarctica (Pearce et al., 2009). Bacteria such as *Bacillus simplex* and *Staphylococcus pasteuri* have been even isolated from the stratosphere (Wainwright et al., 2003). Biocidal compounds have been used for

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centuries for food preservation, wound dressings and sanitation. Biocides can be defined as “active substances and preparations containing one or more substances intended to destroy, deter, render harmless, prevent the action of, or otherwise exert a controlling effect on any harmful organism by chemical or biological means”

Use of biocides in the food-processing environment:

Use of biocides have increased with increasing demand for food of animal origin. Disease control and maintenance of adequate sanitation is an integral part of safe food production. Biocides are commonly used for cleaning and disinfection of food contact and nonfood contact surfaces in the food processing plant (Verraes et al., 2013). Along with production plant hygiene, biocides are also widely used directly with the animals (for example hoof and udder management). High concentration of biocides can prevent spoilage and growth of pathogenic microorganisms, which can increase the products shelf life. However, sub-lethal biocide concentrations may induce adaptation in bacteria (Capita et al., 2014; Forbes et al., 2014).

Use of biocides in health care:

The increase in reports of antibiotic resistance in microorganisms emphasizes the importance of biocides in disinfection and infection control practices. Biocides like carboxylic acids and hydrogen peroxides are widely used for skin infection management. Many biocides are used as topical applications for controlling infection. Biocides are also used as an alternative for chemical sterilization or disinfection of heat sensitive devices (WHO, n.d.).

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Use of biocides in consumer products:

Biocides are widely used as preservatives in cosmetic products to avoid contamination while in use. Increase in demand of cosmetic products with biocide became the practice in the 1960s and 1970s when 24% of unopened and up to 49% of used products were found to be contaminated with microorganisms (Siti Zulaikha et al., 2015). Biocides are also used for disinfection of swimming pools, sewage, and drinking water (National Academy of Sciences, 1977).

Use of biocides in the poultry processing plant:

In a study, *Salmonella* was found positive in 90.9% of sampled farms and 94.5% of processing plants. Campylobacter was also a commonly isolated pathogen with 63.6% of farms flock and 87.3 of processing plants being positive (Beghaus et al., 2013). *L. monocytogenes* is a commonly found foodborne pathogen in poultry processing plant. Previous study showed that the prevalence of *L. monocytogenes* in the poultry processing environments was 16.4 -20% positive (Chiarini et al., 2009). The USDA FSIS has recommended intervention practices to control pre- and post-harvest pathogen contamination in broiler chicken production. For example, stringent biosafety and biosecurity steps as well as strict sanitation practices, along with the use of chlorinated water for immersion chilling has been recommended (USDA 2010). Apart from chlorine, other compounds for example organic acids (lactic and acetic acids), trisodium phosphate, cetylpyridinium chloride and hydrogen peroxide have been approved for use in poultry processing plants (Capita et al., 2002).

Despite these stringent measures, food borne pathogens are still prevalent in processing plants. These pathogens are exposed to different processing plant stresses for

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example biocides, which may induce antimicrobial resistance in poultry associated pathogens (Mbata 2005)

Classification of biocides and their mechanism of action

Generally, biocides have multiple strategies to kill microorganisms at bactericidal concentrations (the lowest concentration of a biocide, which will kill all bacteria) however; in present food preservation systems, bacteriostatic concentrations (the lowest concentration of a biocide, which will inhibit the bacterial growth) are commonly used. Used at bacteriostatic concentrations biocides have specific actions, similar to antibiotics, on specific target sites such as the cytoplasmic membrane, proteins, DNA, RNA and other cytosolic components (Denyer, 1995).

Antibiotic resistance can be developed by its improper use in medical and veterinary and livestock production practices (Sahoo et al., 2010). Antibiotics provide selection pressure for development of antibiotic resistance (Kolar et al., 2001). In addition to antibiotics, biocides may also provide selection pressure for antibiotic resistance in bacteria. Understanding of the mechanism of action of a biocide essential to know the antibiotic resistance development. The mechanism of action of commonly used biocide is listed in Table 2.3.

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Table 2.3 Biocides and their mechanism

No.	Biocides	Mechanism of action	Other attributes	References
1.	Aldehydes	Cross links DNA, RNA, and proteins	Bacteria, bacterial spores, fungi, and viruses	(Tretyakova et al., 2015)
2.	Chlorine based Compound	Proteins and enzymes (thiol groups are especially regarded as targets), and DNA-Membranes	Highly oxidative - bacteria, viruses and spores	(Mcdonnell and Russell, 1999)
3.	Peroxygen compounds	Cellular constituents such as membrane lipids, DNA, ribosomes, enzymes, and proteins	Strong oxidizing compound	(Mcdonnell and Russell, 1999)
4.	Phenols and bisphenols	Fatty acid synthesis	Antibacterial, antifungal, and antiviral properties	(Escalada et al., 2005)
5.	QAC	Membrane active compounds	Antibacterial, antifungal, and antiviral properties	(Ioannou et al., 2007)
6.	Alcohols	Denaturation membrane proteins	Antibacterial, antifungal	(Tóth et al., 2014)

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Antibiotics

An antibiotic or antimicrobial compound can be defined as "a drug that at low concentrations exerts an action against microbial pathogens and exhibits selective toxicity towards them" (EFSA 2008). Generally, antibiotics are used at a level close to minimal inhibitory concentration (MIC) for therapeutic purposes. Antibiotics can act as support for the immune system, where they initially inhibit bacterial growth and then later the immune system controls the infection.

Unlike biocides, antibiotics compounds have specific mechanisms and act on specific physiological processes as described in Table 2.4

Table 2.4 Antibiotics and their mechanism of actions

No.	Antibiotics	Target sites	Action	Special attribute	Reference
1.	β-lactams, Glycopeptides	Transpeptidase enzyme	Inhibition of cell wall synthesis	Bactericidal	(Nikolaidis et al., 2014)
2.	Tetracyclines (T), Aminoglycosides (A)	T- blocks tRNA A- 30s Ribosomes	Inhibition of protein synthesis	T- Bacteriostatic A-Bactericidal	(Chopra and Roberts, 2001)
3.	Macrolides, Chloramphenicol	Anti-50S Ribosomes	Inhibition of protein synthesis	Bacteriostatic	(Vannuffel and Cocito, 1996)
4.	Sulphonamides	para-aminobenzoic acid (PABA)	Inhibition of Folic acid Synthesis	Bacteriostatic	(Hammoudeh et al., 2013)
5.	Quinolones	DNA gyrase enzyme	Inhibition of DNA Synthesis	Bactericidal	(Aldred et al., 2014)

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Antibiotic resistance

Bacterial strains have intrinsic natural resistance against selective and specific groups of antibiotics. Antibiotic resistance in bacteria can be classified broadly into two categories; intrinsic and acquired. Intrinsic antibiotic resistance could be attributed to bacterial structural properties (phenotypic) such as thickened cell wall or cell membrane and lack of antibiotic targets (Genotypic) (Martins et al., 2013); some bacterial strains have intrinsic (natural) resistance against specific groups of antibiotics. Other bacteria can acquire antibiotic resistance in the presence of selective pressure (Munita et al., 2016) which can result in mutation of cellular or plasmid DNA. Inaccurate usage, for example sub-lethal dosages of antibiotics and biocides, can exert selective pressure which can the lead to the evolution of tolerant bacteria populations or sub populations (McMahon et al., 2007).

Type of antibiotic and biocide resistance

Genetic or inherited antibiotic resistance

Bacteria have the ability to mutate (point mutations, deletions and insertions) at their chromosomal loci which can increase their toleration of inhibitory or bactericidal compounds (McMahon et al., 2007). These mutations are usually genetically stable and disseminate through populations by horizontal gene transfer (HGT). Existence of bacterial tolerance to antibiotics was unknown until after the discovery of penicillin. Modulation of bacterial physiology and genetics in changed environmental conditions increased their antibiotic tolerance. Mutation of chromosomal DNA and subsequent horizontal gene transfer (carried by plasmids or transposon) was the key genetic basis of acquired antibiotic resistance (Bennett, 2009). The genetic method acts through different

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mechanisms and is responsible for acquired antibiotic resistance (Munita et al., 2016; Seier-Petersen, 2013; Spratt, 1994). They are as follows:

I. An increase in efflux pump activity
II. Enzymatic modification and degradation of the antibiotic,
III. Acquisition of alternative metabolic pathways to those inhibited by the drug,
IV. Modification of antibiotic targets such as a change in the penicillin binding protein

Horizontal gene transfer can occur in three ways (i). conjugation (gene transfer by means of plasmids) (ii). transduction (gene transfer mediated by bacteriophages) and (iii). transformation (the uptake of free DNA from the environment) (Von Wintersdorff et al., 2016). Plasmid integrons and trasnsposons act as mobile genetic vehicles for conjugation and transduction (Frost et al., 2005). Among these mechanisms of HGT, conjugation is the most common mechanism responsible for the dissemination of antibiotic resistance genes.

Non-inherited resistance

Bacteria also have the ability to develop transient resistance against antibiotics in the absence of mutational change. These populations or sub populations of bacteria are generally genetically unaltered and have uniform susceptibility against antibiotics, but develop transient antibiotic resistance, due to phenotypic changes (McMahon et al., 2007). Non-inherited antibiotic resistance can be attributed to their physiological state (log or stationary phase) and physical and structural properties. Drug indifference, persistent cells and changes in bacterial permeability to antibiotics are commonly

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responsible for non-inherited antibiotic resistance in planktonic cells (Corona and Martinez, 2013; Levin and Rozen, 2006).

Drug indifference:

Non-dividing, resting, or slow growing bacterial cells can be partially or completely refractory to antibiotics of major classes. Recent studies revealed that such stationary phase (not dividing) cells are completely refractory to antibiotics such as ampicillin and tetracycline and less so to other antibiotics such as ciprofloxacin and streptomycin as compared to log phase bacteria (Anderl et al., 2003; Wiuff et al., 2005). Drug indifference is reported in both in vitro and in vivo studies, and has shown that it's not an arbitrary mechanism limited to in vitro studies only (Levin and Rozen, 2006). Bacterial growth in a host has many growth limiting factors such as location of infection, availability of nutrients, delay of antibiotic treatment, or desired antibiotic concentrations at the location of infection. These factors can induce phenomenon of drug indifference.

Persistence:

Killing actively dividing bacterial cells is a time dependent phenomenon and declines with an increase in exposure time. A substantial fraction of actively dividing cells survive antibiotic killing after a certain period time and such bacterial survival can be termed 'bacterial persistence'(Balaban, 2004) or 'phenotypic tolerance' (Levin and Rozen, 2006). Persistence is different from drug indifference since it is concerned with bacterial antibiotic refractory action of actively dividing cells. Antibiotic resistance in persistent cells is non-inherited however; such cells are genetically regulated or expressed. Antibiotic resistance (ABR) of persistent cells is not a stable phenomenon. If

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persistent cells are cultured in the absence of antibiotic stress and are again exposed to the antibiotics, they become sensitive to antibiotics as their parent cells (Levin and Rozen, 2006; Wiuff et al., 2005).

Possibly, the transient inhibition of active replication or delayed replication, while repairing damaged DNA and SOS response of cells that transiently inhibit active bacterial replication, can trigger the increase in frequency of persistent cell formation (Debbia et al., 2001).

Reduced uptake:

Membranous transformation –

Bacterial cell walls act as its first line of defense. Modulation of the cell membrane can affect the transport or permeability of antibiotics. Changes in lipopolysaccharides (LPS) of the cell membrane (Delcour, 2009), increases in cell surface area (development of outer membrane vesicles) (Vellarikkal et al., 2013) can reduce antibiotic susceptibly of bacteria. Sublethal stresses induce modulation of the bacterial outer membrane and can induce reduction in uptake of biocides resulting in less susceptibility. Modulated cell membranes can reduce the influx of biocides thereby increasing bacterial tolerance.

Multi drug efflux pump –

Efflux pumps are responsible for expulsion of toxic substances, which could be structurally similar or dissimilar compounds from bacteria. These pumps can be substrate specific or nonspecific (i.e. multidrug efflux pump). Efflux pump expressions are regulated by the presence of external stimulators such as antibiotics**.** These efflux pumps

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are responsible for low or modest levels of reduced susceptibility to the antibiotic or biocide (Fernández and Hancock, 2012).

Efflux pumps can be classified into five different groups (or families) based on their composition, substrate transmembrane spanning regions, and energy source. They are as described as the major facilitator superfamily (MFS), the ATP-binding cassette (ABC) family, the resistance-nodulation-division (RND) family, the small multidrug resistance (SMR) family (now part of the drug/metabolite transporter (DMT) superfamily), and the multidrug and toxic compound extrusion (MATE) family. These pumps can be chromosomally or plasmid encoded. In *Listeria* two nonspecific efflux pumps are well-characterized Multi-Drug Resistance *Listeria (*MdrL) which can extrude the antibiotics (macrolides and cefotaxime), heavy metals, and ethidium bromide (EtBr) and the *Listeria* drug efflux (Lde) which expulse fluoroquinolone acridine orange and EtBr (Muenster et al., 2006).

The SOS response:

Bacteria have evolved many stress response systems to adapt to and survive many forms of environmental stressors. One of these systems is the SOS response which is activated by formation of single-stranded DNA (ssDNA). The SOS response is governed by the activator RecA and the repressor LexA. RecA recognizes and binds to ssDNA forming nucleoprotein complexes that stimulate the self-cleavage of the LexA repressor. This results in the de-repression of the SOS regulon which includes genes encoding error-prone DNA polymerases, and proteins involved in DNA repair and recombination (Kreuzer, 2013). In addition to the classical inducers of the SOS response; UV-light and mitomycin C, and a variety of additional factors such as exposure to a number of

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therapeutic antimicrobials, pH-extremes, high pressure, oxidative stress, and nutrient starvation have been found to trigger the SOS response.

In summary, *L. monocytogenes* is a food borne pathogen that causes life-threatening diseases in susceptible populations. The biofilm formation ability of *L. monocytogenes* helps in its persistence in the processing plant and acts as a cross contamination source in the deli meat plant. Despite the availability of specific antibiotics for listeriosis treatment, emerging antibiotic resistance in *L. monocytogenes* is a matter of concern. Inadequate application of antimicrobials such as chlorine in the processing plant can influence the *L. monocytogenes* biofilm formation and may create stress resistant subpopulations with changes in susceptibility to biocides and antibiotics. Therefore, measures should be taken to ensure application of appropriate concentrations of antimicrobials in deli and poultry processing plants that will prevent the development of tolerant *L. monocytogenes* populations.

المنارة للاستشارات
www.manaraa.com

References

Ahmed, D., Sadique, T., Sami, A.B., Abdullah, A., Nahid, M.A., Hossain, A., Mazumder, R., Rahaman, M.A., 2015. Listeria monocytogenes infection in a 56-year-old female cancer patient: a case report. JMM Case Reports 2, 2–5. doi:10.1099/jmmcr.0.000076

Al-Nabulsi, A.A., Osaili, T.M., Shaker, R.R., Olaimat, A.N., Jaradat, Z.W., Zain Elabedeen, N.A., Holley, R.A., 2015. Effects of osmotic pressure, acid, or cold stresses on antibiotic susceptibility of Listeria monocytogenes. Food Microbiol. 46, 154–160. doi:10.1016/j.fm.2014.07.015

Aldred, K.J., Kerns, R.J., Osheroff, N., 2014. Mechanism of Quinolone Action and Resistance. Biochemistry. doi:10.1021/bi5000564

Anderl, J.N., Roe, F., Stewart, P.S., 2003. Role of Nutrient Limitation and Stationary-Phase Existence in. Society 47, 1251–1256. doi:10.1128/AAC.47.4.1251

Araújo, P., Lemos, M., Mergulhão, F., Melo, L., Simões, M., 2011. Antimicrobial resistance to disinfectants in biofilms 826–834.

Attaran, B., Falsafi, T., 2017. Identifi cation of Factors Associated with Biofi lm Formation Ability in the Clinical Isolates of Helicobacter pylori. Natl. Inst. Genet. Eng. Biotechnol. 15, 58–66. doi:10.15171/ijb.1368

Bailey, J.S., Fletcher, D.L., Cox., N.A., 1989. Bailey, J. S., D. L. Fletcher, and N. A. Cox. 1989. Recovery and serotype distribution of Listeria monocytogenes from broiler chickens in southeastern United States. J. Food Prot. 52, 148–150.

Balaban, N.Q., 2004. Bacterial Persistence as a Phenotypic Switch. Science (80-.). 305, 1622–1625. doi:10.1126/science.1099390

Begley, M., Kerr, C., Hill, C., 2009. monocytogenes 4, 2–5. doi:10.1186/1757-4749-1-11

Bennett, P.M., 2009. Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria. Br. J. Pharmacol. 153, S347–S357. doi:10.1038/sj.bjp.0707607

Bereksi, N., Gavini, F., Bénézech, T., Faille, C., 2002. Growth, morphology and surface properties of Listeria monocytogenes Scott A and LO28 under saline and acid environments. J. Appl. Microbiol. 92, 556–565. doi:10.1046/j.1365-2672.2002.01564.x

Biofilms, M., Carolina, N., Carolina, N., 2009. Competition of Listeria monocytogenes Serotype 1 / 2a and 4b Strains in 75, 5846–5852. doi:10.1128/AEM.00816-09

المنارة للاستشارات

www.manaraa.com

Bogino, P.C., Oliva, M. de las M., Sorroche, F.G., Giordano, W., 2013. The role of bacterial biofilms and surface components in plant-bacterial associations. Int. J. Mol. Sci. 14, 15838–15859. doi:10.3390/ijms140815838

Borucki, M.K., Peppin, J.D., White, D., Loge, F., Call, D.R., 2003. Variation in biofilm formation among strains of Listeria monocytogenes. Appl. Environ. Microbiol. 69, 7336–7342. doi:10.1128/AEM.69.12.7336

Bos, J., Zhang, Q., Vyawahare, S., Rogers, E., Rosenberg, S.M., Austin, R.H., 2014. Emergence of antibiotic resistance from multinucleated bacterial filaments. doi:10.1073/pnas.1420702111

Capita, R., Riesco-Peláez, F., Alonso-Hernando, A., Alonso-Calleja, C., 2014. Exposure of Escherichia coli ATCC 12806 to sublethal concentrations of food-grade biocides influences its ability to form biofilm, resistance to antimicrobials, and ultrastructure. Appl. Environ. Microbiol. 80, 1268–1280. doi:10.1128/AEM.02283-13

Capozzi, V., Fiocco, D., Amodio, M.L., Gallone, A., Spano, G., 2009. Bacterial stressors in minimally processed food. Int. J. Mol. Sci. 10, 3076–3105. doi:10.3390/ijms10073076

Centers for Disease Control and Prevention, 2011. Multistate outbreak of listeriosis associated with Jensen Farms cantaloupe--United States, August-September 2011. MMWR. Morb. Mortal. Wkly. Rep. 60, 1357–8. doi:mm6039a5 [pii]

Characklis, W.G., 1973. Attached microbial growths—II. Frictional resistance due to microbial slimes. Water Res. 7, 1249–1258. doi:http://dx.doi.org/10.1016/0043-1354(73)90002-X

Chau, T.T.H., Campbell, J.I., Schultsz, C., van Vinh Chau, N., Diep, T.S., Baker, S., Chinh, N.T., Farrar, J.J., Rogier van Doorn, H., 2010. Three adult cases of Listeria monocytogenes meningitis in Vietnam. PLoS Med. 7. doi:10.1371/journal.pmed.1000306

CHEN, Y., BURALL, L.S., MACARISIN, D., POUILLOT, R., STRAIN, E., DE JESUS, A.J., LAASRI, A., WANG, H., ALI, L., TATAVARTHY, A., ZHANG, G., HU, L., DAY, J., KANG, J., SAHU, S., SRINIVASAN, D., KLONTZ, K., PARISH, M., EVANS, P.S., BROWN, E.W., HAMMACK, T.S., ZINK, D.L., DATTA, A.R., 2016. Prevalence and Level of Listeria monocytogenes in Ice Cream Linked to a Listeriosis Outbreak in the United States. J. Food Prot. 79, 1828–1832. doi:10.4315/0362-028X.JFP-16-208

Chiarini, E., Tyler, K., Farber, J.M., Pagotto, F., Destro, M.T., 2009. Listeria monocytogenes in two different poultry facilities: Manual and automatic evisceration. Poult. Sci. 88, 791–797. doi:10.3382/ps.2008-00396

المنارة للاستشارات
www.manaraa.com

Chmielewski, R. a N., Frank, J.F., 2003. Biofilm Formation and Control in Food Processing Facilities. Compr. Rev. Food Sci. Food Saf. 2, 22–32. doi:10.1111/j.1541-4337.2003.tb00012.x

Chopra, I., Roberts, M., 2001. Tetracycline Antibiotics: Mode of Action, Applications, Molecular Biology, and Epidemiology of Bacterial Resistance. Microbiol. Mol. Biol. Rev. doi:10.1128/MMBR.65.2.232-260.2001

Chou, J.H., Greenberg, J.T., Demple, B., 1993. Posttranscriptional repression of Escherichia coli OmpF protein in response to redox stress : positive control of the micF antisense RNA by Posttranscriptional Repression of Eschenichia coli OmpF Protein in Response to Redox Stress : Positive Control of t 175, 1026–1031.

Colagiorgi, A., Di Ciccio, P., Zanardi, E., Ghidini, S., Ianieri, A., 2016. A Look inside the Listeria monocytogenes Biofilms Extracellular Matrix. Microorganisms 4, 22. doi:10.3390/microorganisms4030022

Condon, S., Raso, J., Virto, R., Man, P., 2005. Membrane Damage and Microbial Inactivation by Chlorine in the Absence and Presence of a Chlorine-Demanding Substrate 71, 5022–5028. doi:10.1128/AEM.71.9.5022

Conly, J.M., Johnston, B.L., 2008. Listeria: A persistent food-borne pathogen. Can. J. Infect. Dis. Med. Microbiol. 19, 327–328.

Corona, F., Martinez, J., 2013. Phenotypic Resistance to Antibiotics. Antibiotics 2, 237–255. doi:10.3390/antibiotics2020237

Currie, A., Farber, J.M., Nadon, C., Sharma, D., Whitfield, Y., Gaulin, C., Galanis, E., Bekal, S., Flint, J., Tschetter, L., Pagotto, F., Lee, B., Jamieson, F., Badiani, T., MacDonald, D., Ellis, A., May-Hadford, J., McCormick, R., Savelli, C., Middleton, D., Allen, V., Tremblay, F.-W., MacDougall, L., Hoang, L., Shyng, S., Everett, D., Chui, L., Louie, M., Bangura, H., Levett, P.N., Wilkinson, K., Wylie, J., Reid, J., Major, B., Engel, D., Douey, D., Huszczynski, G., Di Lecci, J., Strazds, J., Rousseau, J., Ma, K., Isaac, L., Sierpinska, U., 2015. Multi-Province Listeriosis Outbreak Linked to Contaminated Deli Meat Consumed Primarily in Institutional Settings, Canada, 2008. Foodborne Pathog. Dis. 12, 645–652. doi:10.1089/fpd.2015.1939

Daniell, H., 2012. NIH Public Access 76, 211–220. doi:10.1007/s11103-011-9767-z.Plastid

De Abrew Abeysundara, P., Nannapaneni, R., Soni, K.A., Sharma, C.S., Mahmoud, B., 2016. Induction and stability of oxidative stress adaptation in Listeria monocytogenes EGD (Bug600) and F1057 in sublethal concentrations of H2O2 and NaOH. Int. J. Food Microbiol. 238, 288–294. doi:10.1016/j.ijfoodmicro.2016.09.027

المنارة للاستشارات
www.manaraa.com

Debbia, E.A., Roveta, S., Schito, A.M., Gualco, L., Marchese, A., 2001. Antibiotic persistence: the role of spontaneous DNA repair response. Microb. Drug Resist. 7, 335–342. doi:10.1089/10766290152773347

Deborde, M., von Gunten, U., 2008. Reactions of chlorine with inorganic and organic compounds during water treatment-Kinetics and mechanisms: A critical review. Water Res. 42, 13–51. doi:10.1016/j.watres.2007.07.025

Delcour, A.H., 2009. Outer Membrane Permeability and Antibiotic Resistance. Biochim Biophys Acta. 1794, 808–816. doi:10.1016/j.bbapap.2008.11.005.Outer

Denyer, S.P., 1995. Mechanisms of action of antibacterial biocides. Int. Biodeterior. Biodegrad. 36, 227–245. doi:10.1016/0964-8305(96)00015-7

Di Bonaventura, G., Piccolomini, R., Paludi, D., D'Orio, V., Vergara, A., Conter, M., Ianieri, A., 2008. Influence of temperature on biofilm formation by Listeria monocytogenes on various food-contact surfaces: Relationship with motility and cell surface hydrophobicity. J. Appl. Microbiol. 104, 1552–1561. doi:10.1111/j.1365-2672.2007.03688.x

Djordjevic, D., Wiedmann, M., Mclandsborough, L.A., 2002. Microtiter Plate Assay for Assessment of Listeria monocytogenes Biofilm Formation 68, 2950–2958. doi:10.1128/AEM.68.6.2950

Doijad, S.P., Barbuddhe, S.B., Garg, S., Poharkar, K. V., Kalorey, D.R., Kurkure, N. V., Rawool, D.B., Chakraborty, T., 2015. Biofilm-forming abilities of listeria monocytogenes serotypes isolated from different sources. PLoS One 10, 1–14. doi:10.1371/journal.pone.0137046

Donlan, R.M., 2002. Biofilms: Microbial life on surfaces. Emerg. Infect. Dis. 8, 881–890. doi:10.3201/eid0809.020063

Drevets, D.A., Bronze, M.S., 2008. Listeria monocytogenes: Epidemiology, human disease, and mechanisms of brain invasion. FEMS Immunol. Med. Microbiol. 53, 151–165. doi:10.1111/j.1574-695X.2008.00404.x

Ernst, R.K., Guina, T., Miller, S.I., 2001. Salmonella typhimurium outer membrane remodeling: Role in resistance to host innate immunity. Microbes Infect. 3, 1327–1334. doi:10.1016/S1286-4579(01)01494-0

Escalada, M.G., Russell, A.D., Maillard, J., Ochs, D., 2005. Triclosan – bacteria interactions : single or multiple target sites ? 476–481. doi:10.1111/j.1472-765X.2005.01790.x

Fair, R.J., Tor, Y., 2014. Perspectives in Medicinal Chemistry Antibiotics and Bacterial Resistance in the 21st Century. Perspect. Medicin. Chem. 25–64. doi:10.4137/PMC.S14459.Received

المنارة للاستشارات
www.manaraa.com

Farber, J.M., Sanders, G.W., Johnston, M.A., 1989. A survey of various foods for the presence of Listeria species. J. Food Prot. 52, 456–458.

Fernández, L., Hancock, R.E.W., 2012. Adaptive and mutational resistance: Role of porins and efflux pumps in drug resistance. Clin. Microbiol. Rev. 25, 661–681. doi:10.1128/CMR.00043-12

Flemming, H., Wingender, J., 2010. The biofilm matrix. Nat. Rev. Microbiol. 8, 623–33. doi:10.1038/nrmicro2415

Flemming, H.C., Neu, T.R., Wozniak, D.J., 2007. The EPS matrix: The "House of Biofilm Cells." J. Bacteriol. 189, 7945–7947. doi:10.1128/JB.00858-07

Fong, J.N.C., Yildiz, F.H., 2015. Biofilm Matrix Proteins. Microbiol. Spectr. doi:10.1128/microbiolspec.MB-0004-2014

Forbes, S., Dobson, C.B., Humphreys, G.J., McBain, A.J., 2014. Transient and sustained bacterial adaptation following repeated sublethal exposure to microbicides and a novel human antimicrobial peptide. Antimicrob. Agents Chemother. 58, 5809–5817. doi:10.1128/AAC.03364-14

Gao, H., Liu, C., 2014. Biochemical and morphological alteration of Listeria monocytogenes under environmental stress caused by chloramine-T and sodium hypochlorite. Food Control 46, 455–461. doi:10.1016/j.foodcont.2014.05.016

García-Gonzalo, D., Pagán, R., 2015. Influence of Environmental Factors on Bacterial Biofilm Formation in the Food Industry: A Review. Postdoc J. 3. doi:10.14304/SURYA.JPR.V3N6.2

Gasanov, U., Hughes, D., Hansbro, P.M., 2005. Methods for the isolation and identification of Listeria spp. and Listeria monocytogenes: a review. FEMS Microbiol. Rev. 29, 851–875. doi:10.1016/j.femsre.2004.12.002

Genigeorgis, C.A., Dutulescu, D.A.N., Garayzabal, J.F., 1989. Prevalence of Listeria spp . in Poultry Meat at the Supermarket and Slaughterhouse Level 52, 618–624.

Giotis, E.S., Blair, I.S., McDowell, D.A., 2007. Morphological changes in Listeria monocytogenes subjected to sublethal alkaline stress. Int. J. Food Microbiol. 120, 250–258. doi:10.1016/j.ijfoodmicro.2007.08.036

Gottlieb, S.L., Newbern, E.C., Griffin, P.M., Graves, L.M., Hoekstra, R.M., Baker, N.L., Hunter, S.B., Holt, K.G., Ramsey, F., Head, M., Levine, P., Johnson, G., Schoonmaker-Bopp, D., Reddy, V., Kornstein, L., Gerwel, M., Nsubuga, J., Edwards, L., Stonecipher, S., Hurd, S., Austin, D., Jefferson, M.A., Young, S.D., Hise, K., Chernak, E.D., Sobel, J., 2006. Multistate outbreak of Listeriosis linked to turkey deli meat and subsequent changes in US regulatory policy. Clin. Infect. Dis. 42, 29–36. doi:10.1086/498113

المنارة للاستشارات
www.manaraa.com

Gray, M.J., Wholey, W., Jakob, U., 2014. NIH Public Access 141–160. doi:10.1146/annurev-micro-102912-142520.Bacterial

Greenberg, J.T., Monach, P., Chou, J.H., Josephy, P.D., Demple, B., 1990. Positive control of a global antioxidant defense regulon activated by superoxide-generating agents in Escherichia coli. Proc. Natl. Acad. Sci. U. S. A. 87, 6181–6185. doi:10.1073/pnas.87.16.6181

Hammoudeh, D.I., Zhao, Y., White, S.W., Lee, R.E., 2013. Replacing sulfa drugs with novel DHPS inhibitors. Future Med. Chem. doi:10.4155/fmc.13.97

How, Z.T., Linge, K.L., Busetti, F., Joll, C.A., 2017. Chlorination of Amino Acids: Reaction Pathways and Reaction Rates. Environ. Sci. Technol. 51, 4870–4876. doi:10.1021/acs.est.6b04440

Ioannou, C.J., Hanlon, G.W., Denyer, S.P., 2007. Action of Disinfectant Quaternary Ammonium Compounds against Staphylococcus aureus . Antimicrob. Agents Chemother. doi:10.1128/AAC.00375-06

Kelly, B.G., Vespermann, A., Bolton, D.J., 2009. Horizontal gene transfer of virulence determinants in selected bacterial foodborne pathogens. Food Chem. Toxicol. 47, 969–977. doi:10.1016/j.fct.2008.02.007

Kerr, K., Dealler, S.F., Lacey, R.W., 1988. Listeria in cook-chill food. Lancet (London, England).

Kerr, K.G., Dealler, S.F., Lacey, R.W., 1988. Materno-fetal listeriosis from cook-chill and refrigerated food. Lancet (London, England).

Knezevic, P., Petrovic, O., 2008. A colorimetric microtiter plate method for assessment of phage effect on Pseudomonas aeruginosa bio fi lm 74, 114–118. doi:10.1016/j.mimet.2008.03.005

Kolar, M., Urbanek, K., Latal, T., 2001. Antibiotic selective pressure and development of bacterial resistance. Int. J. Antimicrob. Agents 17, 357–363.

Kose, A., Yakupogullari, Y., 2015. A rapidly fatal sepsis caused by listeria monocytogenes type-4b in a patient with chronic renal failure. Jundishapur J. Microbiol. 8, e19980. doi:10.5812/jjm.19980

Kreuzer, K.N., 2013. DNA damage responses in prokaryotes: regulating gene expression, modulating growth patterns, and manipulating replication forks. Cold Spring Harb. Perspect. Biol. 5, 1–23. doi:10.1101/cshperspect.a012674

Lee, M.S., 1994. Vancomycin-Resistant Leuconostoc mesenteroides and Lactobacillus 176, 260–264.

المنارة للاستشارات
www.manaraa.com

Lee, S., Ward, T.J., Graves, L.M., Tarr, C.L., Siletzky, R.M., Kathariou, S., 2014. Population structure of listeria monocytogenes serotype 4b isolatesfrom sporadic human listeriosis cases in the United States from 2003 to 2008. Appl. Environ. Microbiol. 80, 3632–3644. doi:10.1128/AEM.00454-14

Levin, B.R., Rozen, D.E., 2006. Non-inherited antibiotic resistance. Nat. Rev. Microbiol. 4, 556–562. doi:10.1038/nrmicro1445

Liu, D., Lawrence, M.L., Gorski, L., Mandrell, R.E., Ainsworth, A.J., Austin, F.W., 2006. Listeria monocytogenes Serotype 4b Strains Belonging to Lineages I and III Possess Distinct Molecular Features 44, 214–217. doi:10.1128/JCM.44.1.214

Lukinmaa, S., Aarnisalo, K., Suihko, M.-L., Siitonen, A., 2004. Diversity of Listeria monocytogenes isolates of human and food origin studied by serotyping, automated ribotyping and pulsed-field gel electrophoresis. Clin. Microbiol. Infect. 10, 562–568. doi:10.1111/j.1469-0691.2004.00876.x

Martins, A., Hunyadi, A., Amaral, L., 2013. Mechanisms of resistance in bacteria: an evolutionary approach. Open Microbiol. J. 7, 53–8. doi:10.2174/1874285801307010053

McClain, D., Lee, W.H., 1988. Development of USDA-FSIS method for isolation of Listeria monocytogenes from raw meat and poultry. J. Assoc. Off. Anal. Chem. 71, 660–664.

Mcdonnell, G., Russell, A.D., 1999. Antiseptics and disinfectants: Activity, action, and resistance. Clin. Microbiol. Rev. 12, 147–179. doi:10.1007/s13398-014-0173-7.2

McMahon, M.A.S., Xu, J., Moore, J.E., Blair, I.S., McDowell, D.A., 2007. Environmental stress and antibiotic resistance in food-related pathogens. Appl. Environ. Microbiol. 73, 211–217. doi:10.1128/AEM.00578-06

Mead, P.S., Dunne, E.F., Graves, L., Wiedmann, M., Patrick, M., Hunter, S., Salehi, E., Mostashari, F., Craig, A., Mshar, P., Bannerman, T., Sauders, B.D., Hayes, P., Dewitt, W., Sparling, P., Griffin, P., Morse, D., Slutsker, L., Swaminathan, B., 2006. Nationwide outbreak of listeriosis due to contaminated meat. Epidemiol. Infect. 134, 744–751. doi:10.1017/S0950268805005376

Mead, P.S., Slutsker, L., Dietz, V., McCaig, L.F., Bresee, J.S., Shapiro, C., Griffin, P.M., Tauxe, R. V., 1999. Food-related illness and death in the United States. Emerg. Infect. Dis. 5, 607–625. doi:10.3201/eid0505.990502

Muenster, L., Romanova, N. a, Wolffs, P.F.G., Brovko, L.Y., Griffiths, M.W., 2006. Role of Efflux Pumps in Adaptation and Resistance of Listeria monocytogenes to Benzalkonium Chloride Role of Efflux Pumps in Adaptation and Resistance of Listeria monocytogenes to Benzalkonium Chloride 72, 3498–3503. doi:10.1128/AEM.72.5.3498

المنارة للاستشارات
www.manaraa.com

Muhterem-Uyar, M., Dalmasso, M., Bolocan, A.S., Hernandez, M., Kapetanakou, A.E., Kuchta, T., Manios, S.G., Melero, B., Minarovičová, J., Nicolau, A.I., Rovira, J., Skandamis, P.N., Jordan, K., Rodríguez-Lázaro, D., Stessl, B., Wagner, M., 2015. Environmental sampling for Listeria monocytogenes control in food processing facilities reveals three contamination scenarios. Food Control 51, 94–107. doi:10.1016/j.foodcont.2014.10.042

Munita, J.M., Arias, C.A., Unit, A.R., Santiago, A. De, 2016. HHS Public Access 4, 1–37. doi:10.1128/microbiolspec.VMBF-0016-2015.Mechanisms

Mureddu, A., Mazza, R., Fois, F., Meloni, D., Bacciu, R., Piras, F., Mazzette, R., 2014. Listeria monocytogenes persistence in ready-to-eat sausages and in processing plants. Ital. J. Food Saf. 3. doi:10.4081/ijfs.2014.1697

National Academy of Sciences, 1977. Drinking Water and Health : Volume 5, Drinking Water and Health. doi:10.1016/0160-4120(91)90308-D

Nelson, R.R.S., 1999. Intrinsically vancomycin-resistant Gram-positive organisms: Clinical relevance and implications for infection control. J. Hosp. Infect. 42, 275–282. doi:10.1053/jhin.1998.0605

Nightingale, K.K., Schukken, Y.H., Nightingale, C.R., Fortes, E.D., Ho, A.J., Her, Z., Grohn, Y.T., Mcdonough, P.L., Wiedmann, M., 2004. Ecology and Transmission of. Society 70, 4458–4467. doi:10.1128/AEM.70.8.4458

Nikolaidis, I., Favini-Stabile, S., Dessen, A., 2014. Resistance to antibiotics targeted to the bacterial cell wall. Protein Sci. doi:10.1002/pro.2414

Oliveira, N.M., Martinez-Garcia, E., Xavier, J., Durham, W.M., Kolter, R., Kim, W., Foster, K.R., 2015. Biofilm formation as a response to ecological competition. PLoS Biol. 13, 1–23. doi:10.1371/journal.pbio.1002191

Ooi, S.T., Lorber, B., 2005. Gastroenteritis due to Listeria monocytogenes. Clin. Infect. Dis. 40, 1327–1332. doi:10.1086/429324

Palková, Z., 2004. Multicellular microorganisms: laboratory versus nature. EMBO Rep. doi:10.1038/sj.embor.7400145

Pan, Y., Breidt, F., Kathariou, S., 2006a. t. Appl. Environ. Microbiol. doi:10.1128/AEM.01065-06

Pan, Y., Breidt, F., Kathariou, S., Science, F., Carolina, N., Carolina, N., 2006b. Resistance of Listeria monocytogenes Biofilms to Sanitizing Agents in a Simulated Food Processing Environment □ † 72, 7711–7717. doi:10.1128/AEM.01065-06

للاستشارات المنارة
www.manaraa.com

Pearce, D.A., Bridge, P.D., Hughes, K.A., Sattler, B., Psenner, R., Russell, N.J., 2009. Microorganisms in the atmosphere over Antarctica. FEMS Microbiol. Ecol. 69, 143–157. doi:10.1111/j.1574-6941.2009.00706.x

Peel, M., Donachie, W., Shaw, A., 1988. Temperature-dependent expression of flagella of Listeria monocytogenes studied by electron microscopy, SDS-PAGE and western blotting. J. Gen. Microbiol. 134, 2171–2178. doi:10.1099/00221287-134-8-2171

Pomposiello, P.J., Demple, B., 2001. Redox-operated genetic switches: The SoxR and OxyR transcription factors. Trends Biotechnol. 19, 109–114. doi:10.1016/S0167-7799(00)01542-0

Puga, C.H., Orgaz, B., Sanjose, C., 2016. Listeria monocytogenes Impact on Mature or Old Pseudomonas fluorescens Biofilms During Growth at 4 and 20 ◦ C 7, 1–9. doi:10.3389/fmicb.2016.00134

Sahoo, K.C., Tamhankar, A.J., Johansson, E., Lundborg, C.S., 2010. Antibiotic use, resistance development and environmental factors: a qualitative study among healthcare professionals in Orissa, India. BMC Public Health 10, 629. doi:10.1186/1471-2458-10-629

Sallum, U.W., Chen, T.T., 2008. Inducible resistance of fish bacterial pathogens to the antimicrobial peptide cecropin B. Antimicrob. Agents Chemother. 52, 3006–3012. doi:10.1128/AAC.00023-08

Scallan, E., Hoekstra, R.M., Angulo, F.J., Tauxe, R. V., Widdowson, M.A., Roy, S.L., Jones, J.L., Griffin, P.M., 2011. Foodborne illness acquired in the United States-Major pathogens. Emerg. Infect. Dis. 17, 7–15. doi:10.3201/eid1701.P11101

Scharff, R.L., 2012. Economic Burden from Health Losses Due to Foodborne Illness in the United States 75, 123–131. doi:10.4315/0362-028X.JFP-11-058

Schlech, W.F. 3rd, Lavigne, P.M., Bortolussi, R.A., Allen, A.C., Haldane, E. V, Wort, A.J., Hightower, A.W., Johnson, S.E., King, S.H., Nicholls, E.S., Broome, C. V, 1983. Epidemic listeriosis--evidence for transmission by food. N. Engl. J. Med. 308, 203–206. doi:10.1056/NEJM198301273080407

Schlech III, W.F., 2006. Epidemiology and clinical manifestations of Listeria monocytogenes infection, in: Gram-Positive Pathogens, Second Edition. American Society of Microbiology, pp. 601–608.

Schonberg, A., Gerigk, K., 1991. Listeria in effluents from the food-processing industry. Rev. Sci. Tech. 10, 787–797.

Seeliger, H.P., 1984. Modern taxonomy of the Listeria group relationship to its pathogenicity. Clin. Invest. Med. 7, 217–221.

المنارة للاستشارات

www.manaraa.com

Seier-Petersen, M.A., 2013. Development of bacterial resistance to biocides and antimicrobial agents as a consequence of biocide usage. Div. Epidemiol. Microb. Genomics Natl. Food Inst. Tech. Univ. Denmark.

Sies, H., 1997. Oxidative stress: oxidants and antioxidants. Exp. Physiol. 82, 291–295. doi:10.1113/expphysiol.1997.sp004024

Siti Zulaikha, R., Sharifah Norkhadijah, S.I., Praveena, S.M., 2015. Hazardous Ingredients in Cosmetics and Personal Care Products and Health Concern : A Review. Public Heal. Res. 5, 7–15. doi:10.5923/j.phr.20150501.02

Sivaranjani, M., Gowrishankar, S., Kamaladevi, A., Pandian, S.K., Balamurugan, K., Ravi, A.V., 2016. Morin inhibits biofilm production and reduces the virulence of Listeria monocytogenes ??? An in vitro and in vivo approach. Int. J. Food Microbiol. 237, 73–82. doi:10.1016/j.ijfoodmicro.2016.08.021

Spratt, B.G., 1994. Resistance to antibiotics mediated by target alterations. Science 264, 388–393.

Taormina, P.J., Beuchat, L.R., 2001. Survival and Heat Resistance of Listeria monocytogenes after Exposure to Alkali and Chlorine. Appl. Environ. Microbiol. 67, 2555–2563. doi:10.1128/AEM.67.6.2555-2563.2001

To, M.S., Favrin, S., Romanova, N., Griffiths, M.W., 2002. Postadaptational resistance to benzalkonium chloride and subsequent physicochemical modifications of Listeria monocytogenes. Appl. Environ. Microbiol. 68, 5258–5264. doi:10.1128/AEM.68.11.5258-5264.2002

Tóth, M.E., Vígh, L., Sántha, M., 2014. Alcohol stress, membranes, and chaperones. Cell Stress Chaperones. doi:10.1007/s12192-013-0472-5

Tretyakova, N.Y., Groehler, A., Ji, S., 2015. DNA-Protein Cross-links: Formation, Structural Identities, and Biological Outcomes. Acc. Chem. Res. doi:10.1021/acs.accounts.5b00056

Upadhyay, A., Upadhyaya, I., Kollanoor-Johny, A., Venkitanarayanan, K., 2013. Antibiofilm effect of plant derived antimicrobials on Listeria monocytogenes. Food Microbiol. 36, 79–89. doi:10.1016/j.fm.2013.04.010

Valderrama, W.B., 2012. EFFECT OF ENVIRONMENTAL FACTORS ON BIOFILM FORMATION BY Listeria monocytogenes 275.

Valderrama, W.B., Cutter, C.N., 2013. An ecological perspective of Listeria monocytogenes biofilms in food processing facilities. Crit. Rev. Food Sci. Nutr. 53, 801–817. doi:10.1080/10408398.2011.561378

المنارة للاستشارات
www.manaraa.com

Vannuffel, P., Cocito, C., 1996. Mechanism of action of streptogramins and macrolides. Drugs 51 Suppl 1, 20–30.

Vasseur, C., Baverel, L., Hébraud, M., Labadie, J., 1999. Effect of osmotic, alkaline, acid or thermal stresses on the growth and inhibition of Listeria monocytogenes. J. Appl. Microbiol. 86, 469–476. doi:10.1046/j.1365-2672.1999.00686.x

Vázquez-Boland, J.A., Kuhn, M., Berche, P., Chakraborty, T., Domínguez-Bernal, G., Goebel, W., González-Zorn, B., Wehland, J., Kreft, J., 2001. Listeria Pathogenesis and Molecular Virulence Determinants. Clin. Microbiol. Rev. doi:10.1128/CMR.14.3.584-640.2001

Vellarikkal, S., Singh, A., Singh, P., 2013. Draft genome sequence of a multidrug-resistant clinical isolate of Mycobacterium tuberculosis belonging to a novel spoligotype. GenomeA 1, 6–7. doi:10.1371/journal.pone.00

Verraes, C., Van Boxstael, S., Van Meervenne, E., Van Coillie, E., Butaye, P., Catry, B., de Schaetzen, M.A., Van Huffel, X., Imberechts, H., Dierick, K., Daube, G., Saegerman, C., De Block, J., Dewulf, J., Herman, L., 2013. Antimicrobial resistance in the food chain: A review. Int. J. Environ. Res. Public Health 10, 2643–2669. doi:10.3390/ijerph10072643

Von Wintersdorff, C.J.H., Penders, J., Van Niekerk, J.M., Mills, N.D., Majumder, S., Van Alphen, L.B., Savelkoul, P.H.M., Wolffs, P.F.G., 2016. Dissemination of antimicrobial resistance in microbial ecosystems through horizontal gene transfer. Front. Microbiol. 7, 1–10. doi:10.3389/fmicb.2016.00173

Vu, B., Chen, M., Crawford, R.J., Ivanova, E.P., 2009. Bacterial extracellular polysaccharides involved in biofilm formation. Molecules 14, 2535–2554. doi:10.3390/molecules14072535

Wainwright, M., Wickramasinghe, N.C., Narlikar, J. V., Rajaratnam, P., 2003. Microorganisms cultured from stratospheric air samples obtained at 41 km. FEMS Microbiol. Lett. 218, 161–165. doi:10.1016/S0378-1097(02)01138-2

Wales, A., Davies, R., 2015. Co-Selection of Resistance to Antibiotics, Biocides and Heavy Metals, and Its Relevance to Foodborne Pathogens. Antibiotics 4, 567–604. doi:10.3390/antibiotics4040567

Webber, M.A., Whitehead, R.N., Mount, M., Loman, N.J., Pallen, M.J., Piddock, L.J. V, 2015. Parallel evolutionary pathways to antibiotic resistance selected by biocide exposure. J. Antimicrob. Chemother. 70, 2241–2248. doi:10.1093/jac/dkv109

Wenger, J.D., Swaminathan, B., Hayes, P.S., Green, S.S., Pratt, M., Pinner, R.W., Schuchat, A., Broome, C. V, 1990. Listeria-Monocytogenes Contamination of Turkey Franks - Evaluation of a Production Facility. J. Food Prot. 53, 1015–1019.

المنارة للاستشارات

www.manaraa.com

WHO, 1988. Foodborne listeriosis. WHO Working Group. Bull. World Health Organ. 66, 421–428.

WHO, n.d. 2 . Chemistry of Disinfectants and Disinfectant By-Products. EHC 216 Disinfect. Disinfect. By- Prod. 27–109.

Wiuff, C., Zappala, R.M., Regoes, R.R., Garner, K.N., Levin, B.R., 2005. Phenotypic Tolerance : Antibiotic Enrichment of Noninherited Resistance in Bacterial Populations Phenotypic Tolerance : Antibiotic Enrichment of Noninherited Resistance in Bacterial Populations. Antimicrob. Agents Chemother. 49, 1483. doi:10.1128/AAC.49.4.1483

Wu, S., Wu, Q., Zhang, J., Chen, M., Yan, Z., Hu, H., 2015. Listeria monocytogenes prevalence and characteristics in retail raw foods in China. PLoS One 10, 1–16. doi:10.1371/journal.pone.0136682

Young, C., Holder, R.C., Dubois, L., Reid, S.D., 2016. Streptococcus pyogenes Biofilm, in: Ferretti, J.J., Stevens, D.L., Fischetti, V.A. (Eds.), . Oklahoma City (OK).

Zhang, C., Zhang, M., Ju, J., Wise, J., Terry, P.M., Olson, M., Kachman, S.D., Wiedmann, M., Samadpour, M., Benson, A.K., 2003. Genome Diversi cation in Phylogenetic Lineages I and II of. Society 185, 5573–5584. doi:10.1128/JB.185.18.5573

Zhu, Q., Gooneratne, R., Hussain, M., 2017. Listeria monocytogenes in Fresh Produce: Outbreaks, Prevalence and Contamination Levels. Foods 6, 21. doi:10.3390/foods6030021

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CHAPTER III

INFLUENCE OF CHLORINE INDUCED SUB-LETHAL OXIDATIVE STRESS ON BIOFILM FORMATION IN *LISTERIA MONOCYTOGENES*

Abstract

The objective of this study was to determine whether sub-inhibitory concentrations of sodium hypochlorite could influence the biofilm forming ability of *Listeria monocytogenes* strains V7, Scott A, FSL-N1-227, FSL F6-154 and ATCC 19116. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of cells exposed or not exposed to chlorine were determined by the macro dilution method. *L monocytogenes* cells were adapted to increasing (20 ppm/day) sub-inhibitory concentrations (SIC) of total chlorine from 250 ppm to 350 ppm and finally on day 6 the chlorine concentration was increased by 25 ppm to obtain a final concentration of 375 ppm in tryptic soy broth (TSB). The biofilm forming ability of chlorine adapted and control cells were determined by the crystal violet assay in 96 well microtiter polystyrene plates at 37°C for 48 hours or 22°C for 120 h. The experiments at 37°C and 22°C were replicated five and three times, respectively. The MIC and MBC values of all five strains were 500 ppm and 600 ppm, respectively. The chlorine induced oxidative stress significantly ($p < 0.10$) reduced biofilm forming ability of all the five strains on polystyrene surface as compared to control cells. Further, a higher anti-biofilm effect of chlorine was observed at 330 (2/3 MIC) and 375 (3/4 MIC) ppm concentration for both

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the oxidative stressed and non-stressed cells in the presence of chlorine ($p < 0.10$). Scanning electron microscopy revealed that the continuous exposure of chlorine induced morphological changes in the bacteria, which impacted the biofilm structure of *L. monocytogenes*. The findings from the study indicate that continuous exposure of sublethal chlorine reduces the biofilm forming ability of *L. monocytogenes.*

Keywords: *Listeria monocytogenes*, oxidative stress, chlorine, biofilm

Introduction

Listeria monocytogenes is a ubiquitous Gram-positive foodborne bacterial pathogen. Listeriosis caused by *L. monocytogenes* is the third leading cause of foodborne illness causing 90% of food related hospitalizations and 15.9 % of food related deaths (Scallan et al., 2011).

L. monocytogenes causes a lethal disease in immunocompromised patients (AIDS and cancer), pregnant women and elderly people (Drevets and Bronze, 2008) and mild gastroenteritis in the healthy population (Ooi and Lorber, 2005). *L. monocytogenes* has been implicated in multiple foodborne outbreaks in the United States causing more than 300,000 illnesses and 337 deaths per year since 1985 (CDC, 2016). The first foodborne listeriosis case was reported in the1980's, which led to extensive investigations (Conly and Johnston, 2008; WHO, 1988). Since then, there were listeriosis outbreaks reported from all over the U.S., associated with different ready-to-eat food products such as turkey deli meat in 2002 (Gottlieb et al., 2006), cantaloupes in 2011 (Centers for Disease Control and Prevention, 2011), soft cheeses in 2015 (Zhu et al., 2017), and ice-cream in 2016 (CHEN et al., 2016). The increase in listeriosis outbreaks could be associated with the diligent surveillance program and possible adaptation of *L. monocytogenes* to food

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processing stresses. Also, *L. monocytogenes* has a strong tendency to form biofilms on different food-contact and non-food contact surfaces which may allow it to persist in the food processing environments.

L. monocytogenes has been classified into 13 different serotypes and three lineages (*L. monocytogenes* I-III) based on serotyping and molecular typing, respectively. *S*erotypes 1/2b, 3b, 4d, 4e and 4b were placed into Lineage I, and serotypes 1/2a, 1/2c, 3a and 3c were placed into Lineage II, while serotypes 4a, 4c and some strains of serotype 4b were placed in lineage III (Nightingale et al., 2004; Zhang et al., 2003).

Serotypes 1/2a, 1/2b and 4b were found to be frequently associated with 90% of human listeriosis infections (Gasanov et al., 2005; Lukinmaa et al., 2004). Serotype 4b was found to be responsible for many of the sporadic cases of listeriosis (Lee et al., 2014).

Biofilms are living bacterial colonies firmly attached to the food-contact or non-food contact surfaces and act as a potential source of food contamination in the food processing environments (Chmielewski and Frank, 2003). The two components of biofilm matrix, extracellular polymeric substances (EPS) and proteins, confers the bacteria cells within a biofilm, resistance against commonly used disinfectants and sanitizers in the food processing plants (Bogino et al., 2013). Many environmental factors may influence the biofilm forming ability of *L. monocytogenes* such as temperature, pH, hydrodynamics of surfaces, nutrients, and physiological properties of cells (Attaran and Falsafi, 2017; García-Gonzalo and Pagán, 2015).

Sodium hypochlorite is a chlorine compound, which is commonly used as a disinfectant or sanitizer that removes pathogens. It is typically used at a concentration of

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< 50 ppm on food-contact surfaces as well as carcasses and at <200 ppm on non-food contact surfaces. Chlorine acts as an oxidizing agent and induces oxidative stress in exposed bacterial cells that survive in food processing environments. A previous study has revealed that *L. monocytogenes* biofilm cells were more resistant to commonly used disinfectants and sanitizers in the food processing plant (Pan et al., 2006a). However, there is no information on the influence of sublethal oxidative stress on *L. monocytogenes* planktonic cells and their subsequent biofilm forming ability. In this study, different strains of *L. monocytogenes* were exposed gradually to increasing concentrations of sublethal oxidative stress induced by chlorine (sodium hypochlorite) to evaluate their biofilm forming ability.

Material and Methods

Bacterial Strains and culture preparation

Bacterial strains used in the present study are listed in Table 1. All bacterial strains were stored as a stock culture in tryptic soy broth (TSB) supplemented with 25% glycerol at -80°C. The bacteriological media, Difco (Becton Dickinson, Sparks, MD) was used for all experiments in the present study. Prior to each experiment, individual bacterial strains were cultured in 10 ml of TSB at 37°C for two consecutive cycles of 24 h to remove any cold stress in working cultures. Following incubation, the obtained culture was centrifuged at 5000×*g* for 10 min at 4°C. The pellet was collected and resuspended in 10 ml of TSB (pH 7.2). Serial ten-fold dilutions were plated on duplicate tryptic soy agar (TSA) and modified oxford agar plates for enumeration and confirmation, respectively. Further, the plates were analyzed for CFU to enumerate bacterial colonies after 24 h incubation at 37°C.

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Antimicrobial

In the present study, sodium hypochlorite 5% stock solution, (Acros Organics, New Jersey, USA) was used as a source of chlorine. Prior to each experiment, total and freely available chlorine was measured using HACH (chlorine test kit) Pocket Colorimeter (HACH Company, Loveland, CO, USA) according to the manufacturer's instructions. In this study, chlorine was used for inducing oxidative stress in *L. monocytogenes* cells.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Assay

The Minimum inhibitory concentration (concentration that inhibit visible bacterial growth after overnight incubation at 37°C) was determined by macro-dilution method according to Clinical and Laboratory Standards Institute, CLSI (2015) with minor modifications. A *L. monocytogenes* cell concentration of 10^6 CFU/ml was used to measure MIC. MBC (concentration that kill all bacteria cells after 24 h incubation at 37°C) was measured by plating aliquots (100 µL) on TSA plates from wells with no visible growth i.e. MIC after 24 h at 37°C.

Sublethal oxidative stress induction protocol for *L. monocytogenes* planktonic cells:

The overnight culture of *L. monocytogenes* at a concentration of 10^9 CFU/ml was obtained after 24 h incubation at 37°C. Oxidative stress adaptation was induced by using a sublethal chlorine concentration at a *L. monocytogenes* cell concentration of ~10^7 CFU/ml for all strains. *L. monocytogenes* strains were challenged with an initial chlorine concentration of 250 ppm in 10 ml of TSB for 24 hours at 37°C in a 15 ml sterile polypropylene flat cap tube (Thermo Fisher Scientific, Fair Lawn, NJ). Subsequently 100

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µl (~10^7 CFU/ml) of a previous day culture was transferred into fresh TSB culture with an increasing chlorine concentration of 20 ppm/day until reaching a 350 ppm concentration. Finally, on the day 6, the chlorine concentration was increased by 25 ppm to obtain a final concentration of 375 ppm in TSB (Figure 3.1). With this protocol, three different sublethal oxidative stress levels were achieved: (1) 1/2 MIC (250 ppm), (2) 2/3 MIC (330 ppm) and (3) 3/4 MIC (375 ppm) on day 1, day 5 and day 7. Control or non-adapted cells were also passaged along with the chlorine pre-exposed cells but in the absence of chlorine.

Biofilm formation by sublethal chlorine adapted and control *L. monocytogenes* planktonic cells on polystyrene microtiter plate

The development of biofilm is illustrated in figure 3.2. An inoculum of 200 µL with a final concentration 10^6 CFU/ml was used for biofilm development in 96 well microtiter plates (CELLTREAT Scientific Products, MA) for this study. Plates were incubated at 37°C for 48 h or at 22°C for 120 h.

Biofilm formation by chlorine adapted cells in the presence or absence of chlorine:

Overnight incubated chlorine induced oxidative stress adapted cells (1/2MIC, 2/3MIC and 3/4MIC) were harvested after centrifugation at 5000×g for 10 min at 4°C and the pellet was retained. The pellet was re-suspended in fresh TSB that contained the concentration of chlorine used to obtain the oxidative stress and was then inoculated into eight wells of a 96 well microtiter plate (column A1-H1, Figure 3.2). Likewise, the same chlorine adapted *L. monocytogenes* cells from fresh TSB were inoculated into the next eight wells that did not contain chlorine (column A2-H2, Figure 3.2).

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Biofilm formation by control non-adapted cells:

The control non- adapted cells were harvested after centrifugation and suspended in fresh TSB. They were inoculated into eight wells of a 96 well plates both in the presence (treated 1/2 MIC, 2/3 MIC and 3/4 MIC) and in the absence of chlorine (column A3-H3 & A4-H4, Figure 3.2). The positive control was comprised of control *L. monocytogenes* cells while the negative control was only TSB without culture.

Biofilm measurement using the crystal violet assay

The crystal violet assay with minor modifications was performed to analyze the biofilm forming ability of all five *L. monocytogenes* strains (stressed and no nstressed). After biofilm formation, culture supernatant was discarded from the microtiter plate and each well was washed with sterile distilled water (250 µl) 5 times. The plates were then air-dried at room temperature for 45 minutes. Thereafter, plates were stained using 0.41% crystal violet (250 µl/well) for 45 minutes and then, washed 5 times with sterile distilled water (250 µl/well). After allowing the plates to air dry for an additional 45 minutes, individual wells were de-stained with 95% ethyl alcohol. Biofilm formation was measured at OD_{600} nm using micro-quant microplate spectrophotometer (BioTek Instruments, Winooski, VT).

Scanning Electron Microscopy

The effect of sublethal chlorine on *L. monocytogenes* biofilm morphology and structure was studied by using scanning electron microscopy (JEOL JSM-6500F Field Emission Scanning Electron Microscope). Biofilm was developed on Nunc Thermanox polystyrene cover slips (MA, USA) at 37°C for 48 hours. Coverslips were washed three

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times with sterile saline and fixed in 1/2 strength Karnowsky's fixative (pH 7.2) overnight at 4°C. Coverslips were then washed 3 times with sterile water and post fixed in 2% buffered (0.1M sodium cacodylate) osmium tetroxide, followed by dehydration through a graded ethanol series (35, 50, (2X) 70, (2X) 95 and (4X) 100). The coverslips were later dried using a critical point dryer (Autosamdri®-931, Tousimis) and sputter-coated with platinum (20nm). Thereafter, coverslips were analyzed on a scanning electron microscope (JEOL JSM-6500F Field Emission Scanning Electron Microscope, MA, USA) to obtain micrographs.

Statistical analysis

A 2 x 3 x 2 factorial arrangement of treatments in a randomized complete block design with replication considered as block using SAS V 9.4 (SAS Institute, Cary, NC, USA) was ran. The means were separated using Fisher Protected Least significance difference. The $P < 0.10$ was considered significant.

Results

Determination of MIC and MBC of chlorine

The MIC for all five strains of *L. monocytogenes* evaluated V7, Scott A, FSL-F6-154, FSL-N1-227 and ATCC 19116 was determined to be 500 ppm by the macrodilution method. The MBC of all *L. monocytogenes* strains was determined to be 600 ppm, which was the point where no colonies grew on the TSA agar plate.

Effect of strain and temperature on *L. monocytogenes* biofilm formation

The formation of biofilm by the five different strains of *L. monocytogenes* was determined at 37°C for 48 h or at 22°C for 120 h and the results are in figure 3.3 (A&B).

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Factors such as strains and temperature influenced the inherent biofilm forming ability of *L. monocytogenes* strains. Significant inter-strain variability in *L. monocytogenes* biofilm formation was observed at 37°C for 48 h. *L. monocytogenes* V7 and Scott A produced higher ($P < 0.05$) biofilm at 37°C for 48 h in 96-well polystyrene microtiter plate with OD_{600} of 0.9-1.2 as compared to the other three strains, FSL-F6-154, ATCC 19116 and FSL-N1-227 (Figure 3.3A). Under the same experimental conditions, there was no change in biofilm formation observed among all five different *L. monocytogenes* strains at 22°C for 120 h with ~ OD_{600} 0.2-0.3 (Figure 3.3B).

Effect of sublethal chlorine on *L. monocytogenes* biofilm forming ability on polystyrene microtiter plates at two different temperatures

The sublethal chlorine induced oxidative effect on the biofilm forming ability of *L. monocytogenes* were observed by crystal violet staining (CV) at OD_{600}. The control (TSB without cells) had the lowest OD_{600} 0.14-0.16 both at 37°C and 22°C. Since, similar trends of chlorine effect were found in all five *L. monocytogenes* strains studied, results of the higher biofilm forming strains V7 and Scott A and a lower biofilm forming strain FSL-F6-154 at 37°C are discussed in detail below.

At 37°C for 48 h, both the non-treated, chlorine induced oxidative stress adapted and non-adapted *L. monocytogenes* V7 cells developed higher biofilm in TSB, whereas lesser biofilm formation was observed in TSB with chlorine treatment (Figure 3.4A). At 22°C for 120 h, no significant biofilm formation on a polystyrene surface was observed when the chlorine adapted and non-adapted *L. monocytogenes* V7 cells were grown in both the presence or absence of chlorine (Figure 3.4B).

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L. monocytogenes Scott A and FSL-F6-154 biofilm forming ability was reduced significantly in the presence of chlorine treatment at all three sublethal oxidative stress levels (1/2MIC, 2/3 MIC and 3/4 MIC) at 37°C for 48 h ($p < 0.10$). A higher reduction in biofilm formation was observed when the sublethal chlorine oxidative stress was increased to 2/3 MIC or 3/4 MIC ($p < 0.10$) as compared to 1/2 MIC. Biofilm formation was also found to be less on a polystyrene surface by both chlorine adapted and non-adapted *L. monocytogenes* in both the presence and the absence of chlorine treatment at 22°C for 120 h ($p < 0.10$) (Figure 3.5 and 3.6).

Similar finding was observed for *L. monocytogenes* FSL-N1-227 and ATCC 19116 at 37°C and 22°C where chlorine adapted cells produced less biofilm than non-chlorine adapted cells ($p < 0.10$).

The antibiofilm effect of sublethal chlorine induced oxidative stress was significant at both 37°C and 22°C. The degree of biofilm inhibition was varied depending upon the individual strain's inherent biofilm forming ability and level of sublethal chlorine oxidative stress.

Effect of sublethal oxidative effect on *L. monocytogenes* biofilm morphology and its structure

Structural variation was observed in biofilms among the strains investigated in the present study. For example, *L. monocytogenes* strains V7 and Scott A had formed thick aggregates of cells on the polystyrene surface. At higher magnification SEM micrographs revealed strains V7 (Figure 3.7A) and Scott A (Figure 3.7B) formed micro colonies and a monolayer of cells, respectively with higher extracellular polymeric substance (EPS) compounds at 37°C for 4 h. Whereas strains FSL-F6-154 (Figure 3.7C) and ATCC 19116

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(Figure 3.7D) formed sparse cell aggregates with lesser EPS production under the same conditions.

Further, the antibiofilm effect of sublethal oxidative stress was investigated on higher biofilm forming strains V7 and Scott A at all three sublethal stress levels, 1/2 MIC, 2/3 MIC and 3/4 MIC. SEM micrographs revealed that the chlorine adapted but non treated and control non-adapted *L. monocytogenes* V7 and Scott A cells developed thick aggregates of cells after exposure to TSB without chlorine, whereas loosely unbound individual cells were observed after exposure to TSB with chlorine at 37°C for 48 h (Figure 3.8 and 3.9). Also, at the higher magnifications, it was revealed that the *L. monocytogenes* cells were individually attached to the polystyrene surface with less EPS production at a higher level of sublethal oxidative stress (2/3 MIC and 3/4 MIC). Along with these changes in the biofilm structure, the sublethal oxidative stress also induced structural changes in individual cells, for example, the formation of coccoid, elongated and stalk-like morphologies were observed for both the strains.

Discussion

Biofilms are responsible for up to 80% of the microbial infections in the United States (Young et al., 2016). Since biofilms are not a part of the foodborne outbreak investigations, no direct association could be established between *L. monocytogenes* biofilms and foodborne listeriosis outbreaks (Valderrama and Cutter, 2013). However, listeriosis is responsible for 260 deaths annually (CDC, 2016) with significant economic losses each year in the United States (Scharff, 2012). Hence, controlling the *L. monocytogenes* survival and biofilm formation is critical in the food processing environments.

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Chlorine is a widely used antimicrobial compound in poultry and food processing plants for disinfection and sanitation. It acts as a non-selective oxidant and generally the cytoplasmic membrane is the key target for its biocidal action (Condon et al., 2005). At sublethal concentrations, chlorine induces oxidative stress to pathogens by forming reactive chlorine species such hypochlorous acid, hypochlorite ion etc. (RCS) (Gray et al., 2014). Previous study revealed the continuous exposure to peroxide and quaternary ammonium compounds induced biocide tolerances in *Listeria monocytogenes* biofilms on stainless steel and Teflon surfaces (Pan et al., 2006b).

Other studies have investigated the plant derived antimicrobials (PDA) antibiofilm effect in *L. monocytogenes*. For example, eugenol, carvacrol, thymol, trans-cinnamaldehyde (Upadhyay et al., 2013) and morins (Sivaranjani et al., 2016) are plant derived antimicrobials that have shown a reduction in *L. monocytogenes* biofilm production. However, the influence of chlorine induced sublethal oxidative effect on biofilm forming ability of *L. monocytogenes* planktonic cells was not investigated. It is also evident that chlorine induces formation of RCS which causes oxidative stress, but it may not be permanent or stable adaptive response. Therefore, this study was envisaged to investigate the exposure to sublethal oxidative effect induced by chlorine on *L. monocytogenes* biofilms formation ability.

Prior to investigating the sublethal oxidative effect on the biofilm forming ability of *L. monocytogenes*, the MIC of *L. monocytogenes* strains exposed to chlorine were determined to identify the sublethal chlorine concentrations that induces oxidative stress. After determining the MICs, all the five strains studied were gradually exposed to sublethal chlorine concentration beginning at 250 ppm (1/2 MIC) and increasing to 375

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ppm (3/4 MIC). The crystal violet staining assay was used to measure the biofilm forming ability of *L. monocytogenes* in polystyrene wells. Several studies have previously demonstrated the biofilm formation of *L. monocytogenes* under various environmental conditions by the crystal violet staining assay (Djordjevic et al., 2002; Knezevic and Petrovic, 2008).

L. monocytogenes serotype 4b and 1/2a of lineage I and II, respectively are clinically important and responsible for the majority of human listeriosis outbreaks (Borucki et al., 2003; Liu et al., 2006). *L. monocytogenes* serotype 1/2a strains were generally found to produce more efficient biofilm than other strains like 4b (Pan et al., 2009). Similar to these previous findings, the V7 (1/2a) strain formed higher biofilm than Scott A (4b) in our studies. However, the other three strains, FSL-F6-154 (1/2a), FSL-N1-227 (1/2a), ATCC 19116 (4C) were found to form less biofilm on polystyrene surface when compared to V7 (1/2a) or Scott A (4b). Present study showed that sublethal chlorine induced oxidative effect reduce the biofilm formation ability of *L. monocytogenes* irrespective of strain differences. The antibiofilm effect of chlorine is concentration dependent since higher reduction in *L. monocytogenes* biofilm formation was observed at higher chlorine exposure.

L. monocytogenes exhibited at least five different patterns of biofilm formation after 4 h at 37°C which includes: the development of small or large micro colonies, sparse biofilm (individual cells attachment), and numerous small micro colonies that cover nearly the same area as well developed biofilm (Begley et al., 2009; Puga et al., 2016). *Listeria monocytogenes* V7 and Scott A formed three dimensional and large macro colony biofilms, respectively, after 48 hours of incubation at 37°C. Crystal violet

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assay results showed that V7 formed large biofilms at the bottom surface of the polystyrene wells, while Scott A formed large biofilms at the air-water interphase of the polystyrene wells.

L. monocytogenes attachment to the surfaces can be influenced not only by the cell surface properties but also by the presence of surface appendages such as flagella and fimbriae. It was suggested that flagellum-mediated motility is required for the initial cell attachment during biofilm formation by overcoming any repulsive interfacial forces (Herald and Zottola 1988; O'Toole and Kolter 1998; Pratt and Kolter 1998; Kalmokoff et al. 2006). Flagella were reported to serve as an adhesive structure (Moens and Vanderleyden 1996). For example, Vatanioopaisarn et al. (2000) found that flagella per se, not motility, facilitate the early stages of attachment of *L. monocytogenes* to stainless steel. In contrast, Lemon et al. (2007) found that *L. monocytogenes* flagellum-mediated motility was critical for both adhesion and biofilm formation on abiotic surfaces.

SEM micrographs revealed that *L. monocytogenes* biofilm cells when exposed to sublethal oxidative stress were devoid of any flagella. RCS may inhibit the flagella formation in *L. monocytogenes*, which could be critical for its biofilm formation.

In conclusion, the present study found that sublethal chlorine, a GRAS antimicrobial is efficient in inhibiting *L. monocytogenes* biofilm formation on plastic surface. The chlorine antibiofilm effect is found to be associated with inherent biofilm forming ability of *L. monocytogenes* strains and levels of sublethal chlorine.

المنارة للاستشارات
www.manaraa.com

References

Attaran, B., Falsafi, T., 2017. Identifi cation of Factors Associated with Biofi lm Formation Ability in the Clinical Isolates of Helicobacter pylori. Natl. Inst. Genet. Eng. Biotechnol. 15, 58–66. doi:10.15171/ijb.1368

Begley, M., Kerr, C., Hill, C., 2009. monocytogenes 4, 2–5. doi:10.1186/1757-4749-1-11

Biofilms, M., Carolina, N., Carolina, N., 2009. Competition of Listeria monocytogenes Serotype 1 / 2a and 4b Strains in 75, 5846–5852. doi:10.1128/AEM.00816-09

Bogino, P.C., Oliva, M. de las M., Sorroche, F.G., Giordano, W., 2013. The role of bacterial biofilms and surface components in plant-bacterial associations. Int. J. Mol. Sci. 14, 15838–15859. doi:10.3390/ijms140815838

Borucki, M.K., Peppin, J.D., White, D., Loge, F., Call, D.R., 2003. Variation in biofilm formation among strains of Listeria monocytogenes. Appl. Environ. Microbiol. 69, 7336–7342. doi:10.1128/AEM.69.12.7336

Centers for Disease Control and Prevention, 2011. Information for Health Professionals and Laboratories. https://www.cdc.gov/listeria/technical.html. Accessed on July 10, 2017.

Centers for Disease Control and Prevention, 2011. Multistate outbreak of listeriosis associated with Jensen Farms cantaloupe--United States, August-September 2011. MMWR. Morb. Mortal. Wkly. Rep. 60, 1357–8. doi:mm6039a5 [pii]

CHEN, Y., BURALL, L.S., MACARISIN, D., POUILLOT, R., STRAIN, E., DE JESUS, A.J., LAASRI, A., WANG, H., ALI, L., TATAVARTHY, A., ZHANG, G., HU, L., DAY, J., KANG, J., SAHU, S., SRINIVASAN, D., KLONTZ, K., PARISH, M., EVANS, P.S., BROWN, E.W., HAMMACK, T.S., ZINK, D.L., DATTA, A.R., 2016. Prevalence and Level of Listeria monocytogenes in Ice Cream Linked to a Listeriosis Outbreak in the United States. J. Food Prot. 79, 1828–1832. doi:10.4315/0362-028X.JFP-16-208

Chmielewski, R. a N., Frank, J.F., 2003. Biofilm Formation and Control in Food Processing Facilities. Compr. Rev. Food Sci. Food Saf. 2, 22–32. doi:10.1111/j.1541-4337.2003.tb00012.x

Condon, S., Raso, J., Virto, R., Man, P., 2005. Membrane Damage and Microbial Inactivation by Chlorine in the Absence and Presence of a Chlorine-Demanding Substrate 71, 5022–5028. doi:10.1128/AEM.71.9.5022

المنارة للاستشارات
www.manaraa.com

Conly, J.M., Johnston, B.L., 2008. Listeria: A persistent food-borne pathogen. Can. J. Infect. Dis. Med. Microbiol. 19, 327–328.

Djordjevic, D., Wiedmann, M., Mclandsborough, L.A., 2002. Microtiter Plate Assay for Assessment of Listeria monocytogenes Biofilm Formation 68, 2950–2958. doi:10.1128/AEM.68.6.2950

Drevets, D.A., Bronze, M.S., 2008. Listeria monocytogenes: Epidemiology, human disease, and mechanisms of brain invasion. FEMS Immunol. Med. Microbiol. 53, 151–165. doi:10.1111/j.1574-695X.2008.00404.x

García-Gonzalo, D., Pagán, R., 2015. Influence of Environmental Factors on Bacterial Biofilm Formation in the Food Industry: A Review. Postdoc J. 3. doi:10.14304/SURYA.JPR.V3N6.2

Gasanov, U., Hughes, D., Hansbro, P.M., 2005. Methods for the isolation and identification of Listeria spp. and Listeria monocytogenes: a review. FEMS Microbiol. Rev. 29, 851–875. doi:10.1016/j.femsre.2004.12.002

Gottlieb, S.L., Newbern, E.C., Griffin, P.M., Graves, L.M., Hoekstra, R.M., Baker, N.L., Hunter, S.B., Holt, K.G., Ramsey, F., Head, M., Levine, P., Johnson, G., Schoonmaker-Bopp, D., Reddy, V., Kornstein, L., Gerwel, M., Nsubuga, J., Edwards, L., Stonecipher, S., Hurd, S., Austin, D., Jefferson, M.A., Young, S.D., Hise, K., Chernak, E.D., Sobel, J., 2006. Multistate outbreak of Listeriosis linked to turkey deli meat and subsequent changes in US regulatory policy. Clin. Infect. Dis. 42, 29–36. doi:10.1086/498113

Gray, M.J., Wholey, W., Jakob, U., 2014. NIH Public Access 141–160. doi:10.1146/annurev-micro-102912-142520.Bacterial

Knezevic, P., Petrovic, O., 2008. A colorimetric microtiter plate method for assessment of phage effect on Pseudomonas aeruginosa bio fi lm 74, 114–118. doi:10.1016/j.mimet.2008.03.005

Lee, S., Ward, T.J., Graves, L.M., Tarr, C.L., Siletzky, R.M., Kathariou, S., 2014. Population structure of listeria monocytogenes serotype 4b isolatesfrom sporadic human listeriosis cases in the United States from 2003 to 2008. Appl. Environ. Microbiol. 80, 3632–3644. doi:10.1128/AEM.00454-14

Liu, D., Lawrence, M.L., Gorski, L., Mandrell, R.E., Ainsworth, A.J., Austin, F.W., 2006. Listeria monocytogenes Serotype 4b Strains Belonging to Lineages I and III Possess Distinct Molecular Features 44, 214–217. doi:10.1128/JCM.44.1.214

Lukinmaa, S., Aarnisalo, K., Suihko, M.-L., Siitonen, A., 2004. Diversity of Listeria monocytogenes isolates of human and food origin studied by serotyping, automated ribotyping and pulsed-field gel electrophoresis. Clin. Microbiol. Infect. 10, 562–568. doi:10.1111/j.1469-0691.2004.00876.x

المنارة للاستشارات
www.manaraa.com

Nightingale, K.K., Schukken, Y.H., Nightingale, C.R., Fortes, E.D., Ho, A.J., Her, Z., Grohn, Y.T., Mcdonough, P.L., Wiedmann, M., 2004. Ecology and Transmission of. Society 70, 4458–4467. doi:10.1128/AEM.70.8.4458

Ooi, S.T., Lorber, B., 2005. Gastroenteritis due to Listeria monocytogenes. Clin. Infect. Dis. 40, 1327–1332. doi:10.1086/429324

Pan, Y., Breidt, F., Kathariou, S., 2006. t. Appl. Environ. Microbiol. doi:10.1128/AEM.01065-06

Puga, C.H., Orgaz, B., Sanjose, C., 2016. Listeria monocytogenes Impact on Mature or Old Pseudomonas fluorescens Biofilms During Growth at 4 and 20 ◦ C 7, 1–9. doi:10.3389/fmicb.2016.00134

Scallan, E., Hoekstra, R.M., Angulo, F.J., Tauxe, R. V., Widdowson, M.A., Roy, S.L., Jones, J.L., Griffin, P.M., 2011. Foodborne illness acquired in the United States-Major pathogens. Emerg. Infect. Dis. 17, 7–15. doi:10.3201/eid1701.P11101

Scharff, R.L., 2012. Economic Burden from Health Losses Due to Foodborne Illness in the United States 75, 123–131. doi:10.4315/0362-028X.JFP-11-058

Sivaranjani, M., Gowrishankar, S., Kamaladevi, A., Pandian, S.K., Balamurugan, K., Ravi, A.V., 2016. Morin inhibits biofilm production and reduces the virulence of Listeria monocytogenes ??? An in vitro and in vivo approach. Int. J. Food Microbiol. 237, 73–82. doi:10.1016/j.ijfoodmicro.2016.08.021

Upadhyay, A., Upadhyaya, I., Kollanoor-Johny, A., Venkitanarayanan, K., 2013. Antibiofilm effect of plant derived antimicrobials on Listeria monocytogenes. Food Microbiol. 36, 79–89. doi:10.1016/j.fm.2013.04.010

Valderrama, W.B., Cutter, C.N., 2013. An ecological perspective of Listeria monocytogenes biofilms in food processing facilities. Crit. Rev. Food Sci. Nutr. 53, 801–817. doi:10.1080/10408398.2011.561378

WHO, 1988. Foodborne listeriosis. WHO Working Group. Bull. World Health Organ. 66, 421–428.

Young, C., Holder, R.C., Dubois, L., Reid, S.D., 2016. Streptococcus pyogenes Biofilm, in: Ferretti, J.J., Stevens, D.L., Fischetti, V.A. (Eds.), . Oklahoma City (OK).

Zhang, C., Zhang, M., Ju, J., Wise, J., Terry, P.M., Olson, M., Kachman, S.D., Wiedmann, M., Samadpour, M., Benson, A.K., 2003. Genome Diversi cation in Phylogenetic Lineages I and II of. Society 185, 5573–5584. doi:10.1128/JB.185.18.5573

للاستشارات المنارة

www.manaraa.com

Zhu, Q., Gooneratne, R., Hussain, M., 2017. Listeria monocytogenes in Fresh Produce: Outbreaks, Prevalence and Contamination Levels. Foods 6, 21. doi:10.3390/foods6030021

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Table 3.1 List of *Listeria monocytogenes* strains and their sources used in the present study

No.	*L. monocytogenes* Strains	Lineage	Serovar	First reported outbreak
1.	*L. monocytogenes* FSL F6-154	II	1/2a	Food epidemic (sliced turkey) (2000)
2.	*L. monocytogenes* FSL N1-227	I	1/2a	Food, epidemic (US 1998-99)
3.	*L. monocytogenes* ATCC 19116	III	4c	Poultry, UW
4.	*L. monocytogenes* Scott A	I	4b	Human epidemic (Mass., 1983)
5.	*L. monocytogenes* V 7	II	1/2a	Raw milk, FDA

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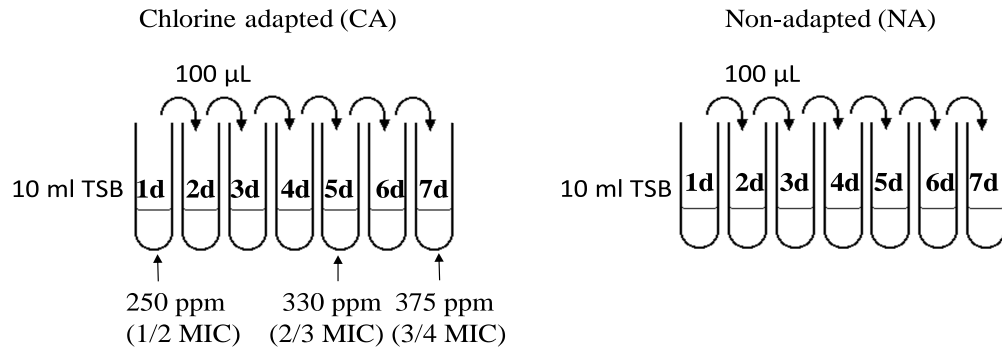

Figure 3.1 Sequence of steps for chlorine induced sublethal oxidative stress adaptation in *L. monocytogenes* planktonic cells.

Chlorine induced sublethal oxidative stress adaptation in *L. monocytogenes* planktonic cells by gradual exposure to increasing concentrations of chlorine from 250 to 375 ppm in 7 day

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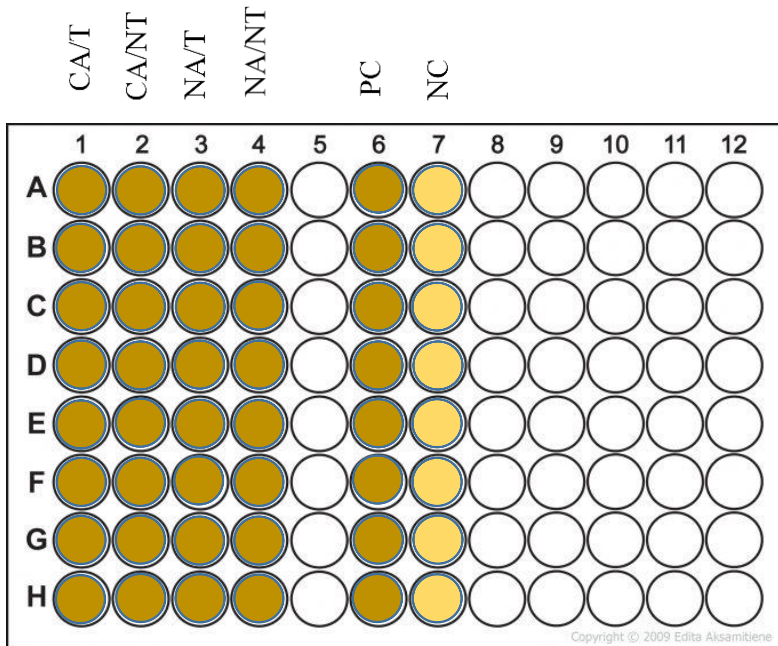

Figure 3.2 Experimental layout containing four treatments for biofilm formation by chlorine adapted and non-adapted *L. monocytogenes* cells with or without different concentrations of chlorine treatments.

Chlorine adapted and chlorine treated (CA/T), chlorine adapted and chlorine non-treated (CA/NT), Non adapted and treated NA/T and Non adapted and non-chlorine treated NA/NT

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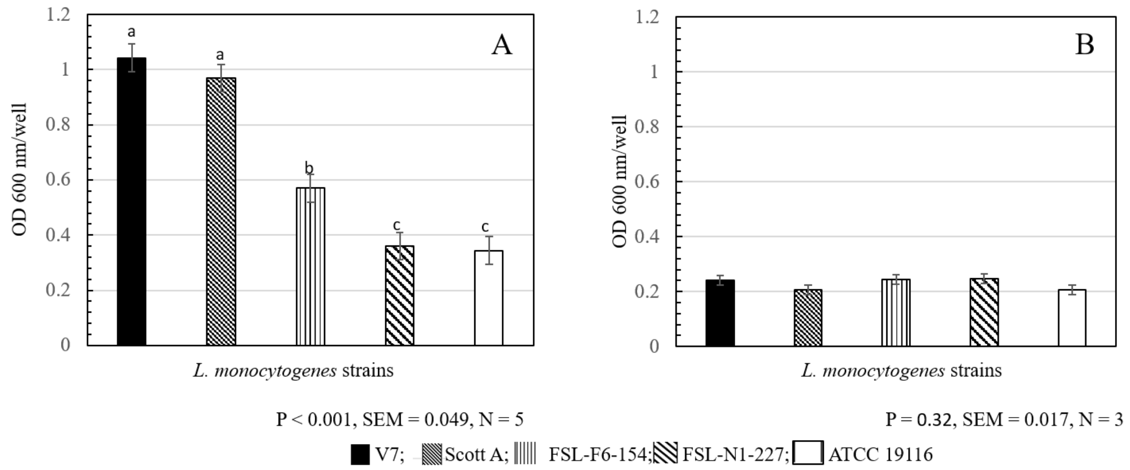

Figure 3.3 Biofilm formation by five strains of *L. monocytogenes* strains at two temperatures: (A) 37°C for 48 h; and (B) 22°C for 120 h.

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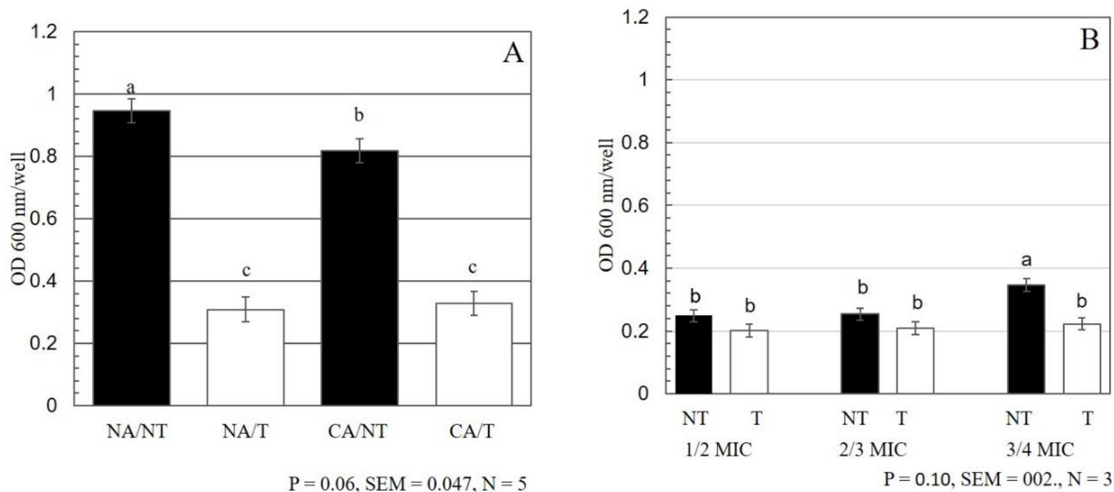

Figure 3.4 Effect of sublethal chlorine induced oxidative stress on biofilm forming ability of *L. monocytogenes* V7 at two temperatures: (A) at 37°C and (B) at 22°C for 48 and 120 h, respectively.

Bars represent non-adapted/non-treated (NA/NT); non-adapted /chlorine treated (NA/T) chlorine adapted/ non-treated (CA/NT) and chlorine adapted/treated (CA/T); (B) at 22°C where bars represent non-treated (NT) and treated (T) at three different concentrations (1/2 MIC, 2/3 MIC and 3/4 MIC)

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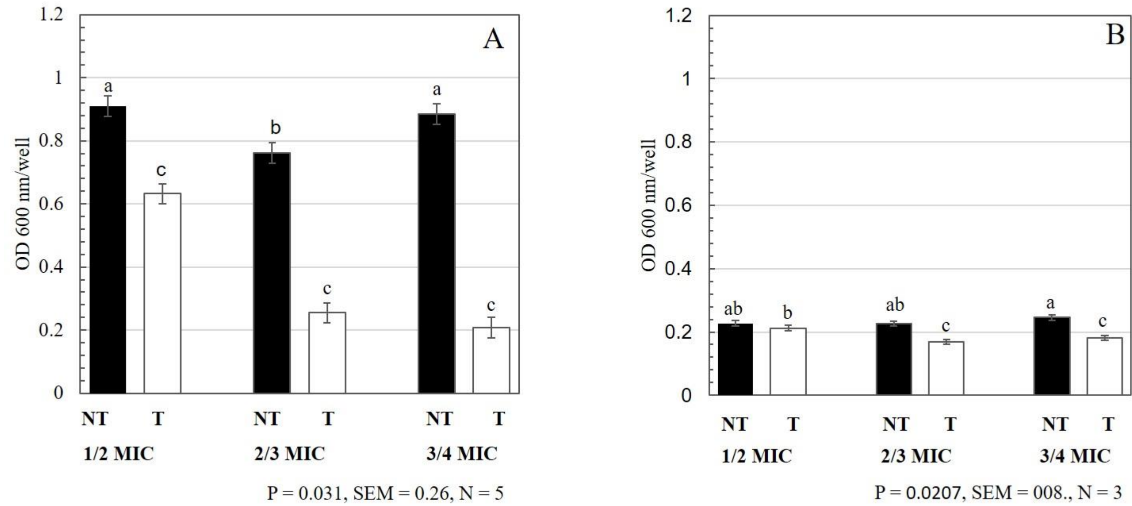

Figure 3.5 Effect of sublethal chlorine stress on biofilm forming ability of *L. monocytogenes* Scott A formation at 37°C (A) and 22°C (B) for 48 and 120 h, respectively.

Bars represented non-treated (NT) and treated (T) at different sublethal stress levels (1/2 MIC, 2/3 MIC and 3/4 MIC).

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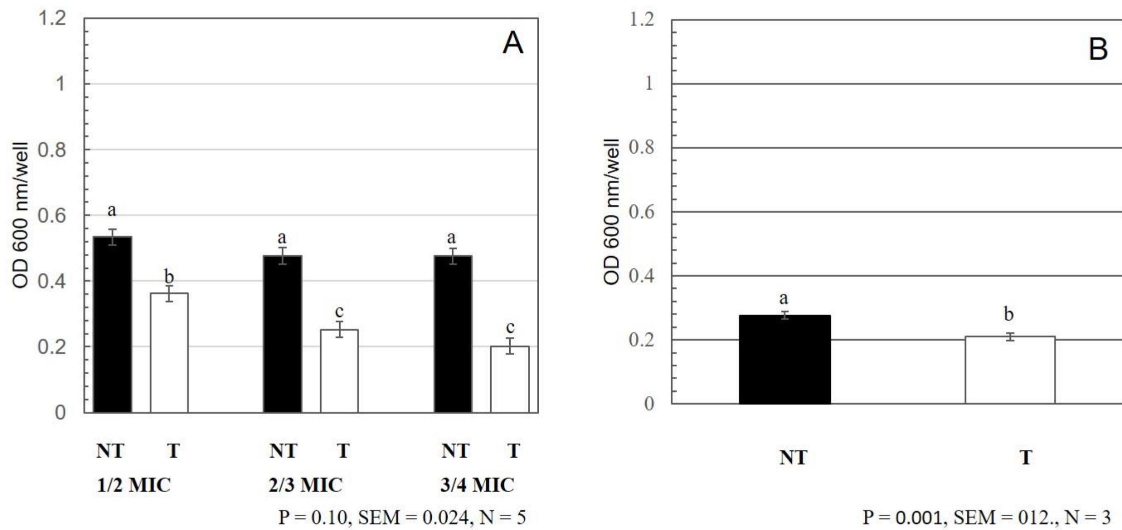

Figure 3.6 Effect of sublethal chlorine stress on biofilm forming ability of *L. monocytogenes* FSL-F6-154 formation at 37°C (A) and 22°C (B) for 48 and 120 h, respectively.

For graph (A) bars represented the chlorine treatment (T) or not (NT) interaction with different sublethal stress levels (1/2 MIC, 2/3 MIC and 3/4 MIC) and at for graph (B) main effect of chlorine treatment (T) was compared with no chlorine treatment (NT)

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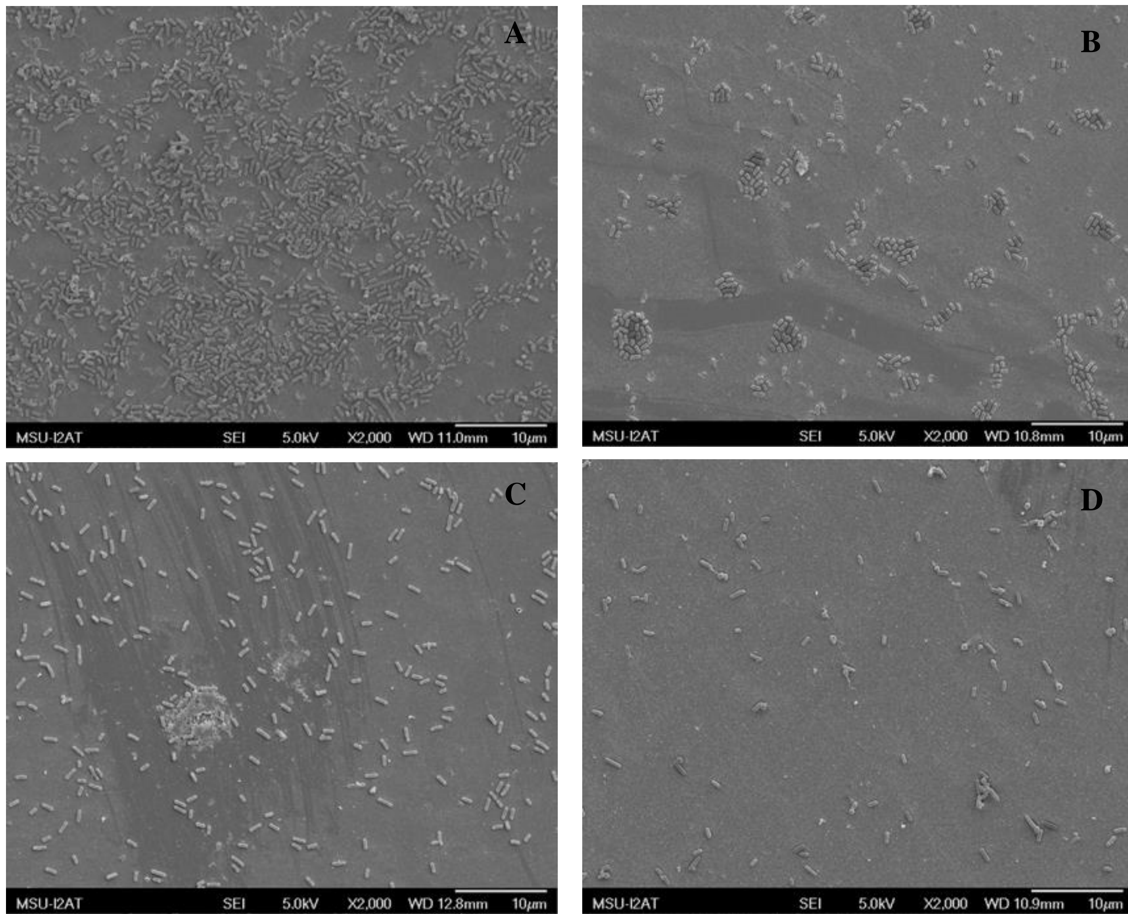

Figure 3.7 Scanning electron micrograph of biofilm formation by four strains of *L. monocytogenes* at 37°C for 24 h on polystyrene surface at 2000X: (A) Scott A; (B) V7; (C) F6-154; and (D) ATCC 19116.

Micrographs (A,B) shows Scott A and V7 forms monolayer and microcolonies like biofilms after 4 hours of attachment whereas (C,D) shows FSL-F6-154 and ATCC 19116 forms thin biofilm where cells are loosely attached

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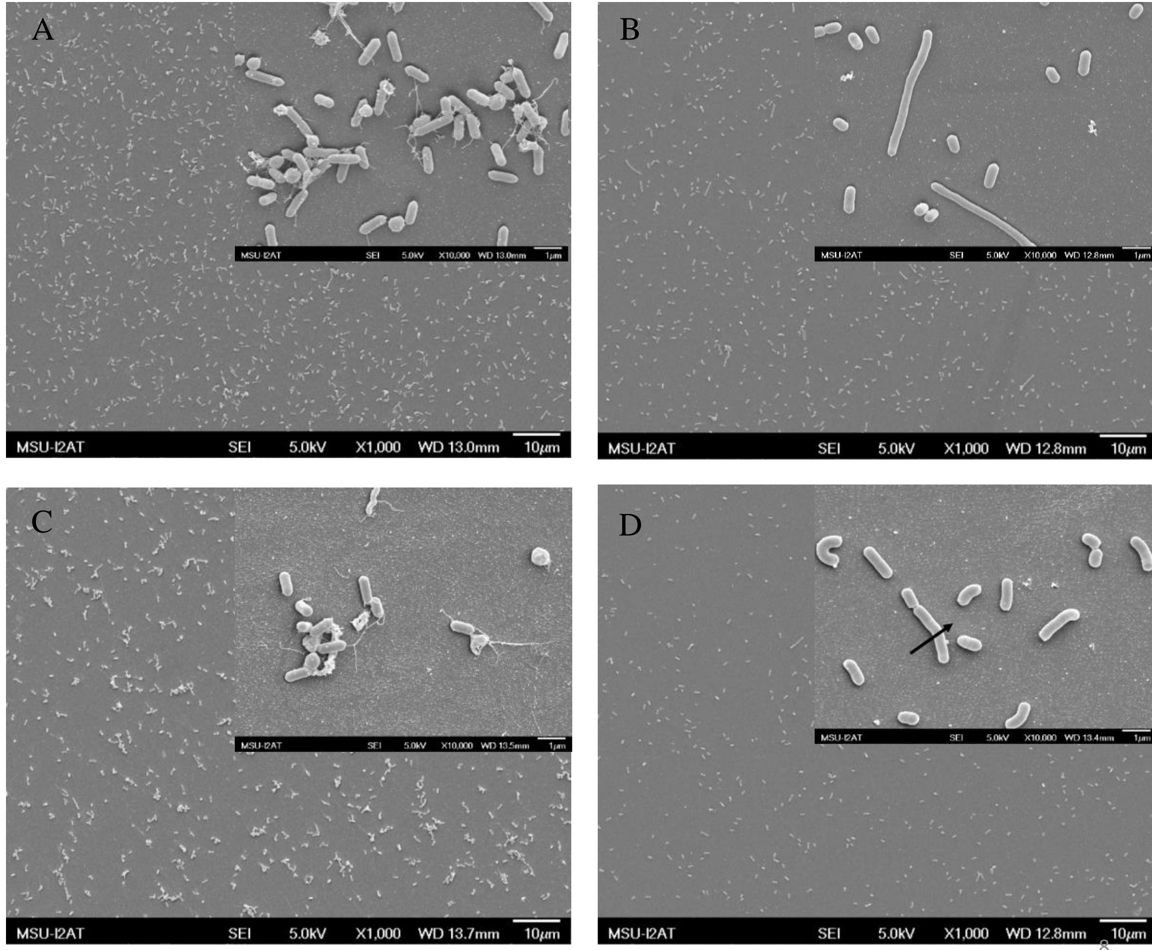

Figure 3.8 Scanning electron micrographs of chlorine induced oxidative stress adapted cells of *L. monocytogenes* Scott A biofilm formation at 37°C for 48 h at 1,000 and 10,000X.

Micrographs represent biofilm formation by (A) chlorine non adapted and non-treated, (B) chlorine adapted and non-treated, (C) Chlorine non adapted and treated, (D) Chlorine adapted and treated.

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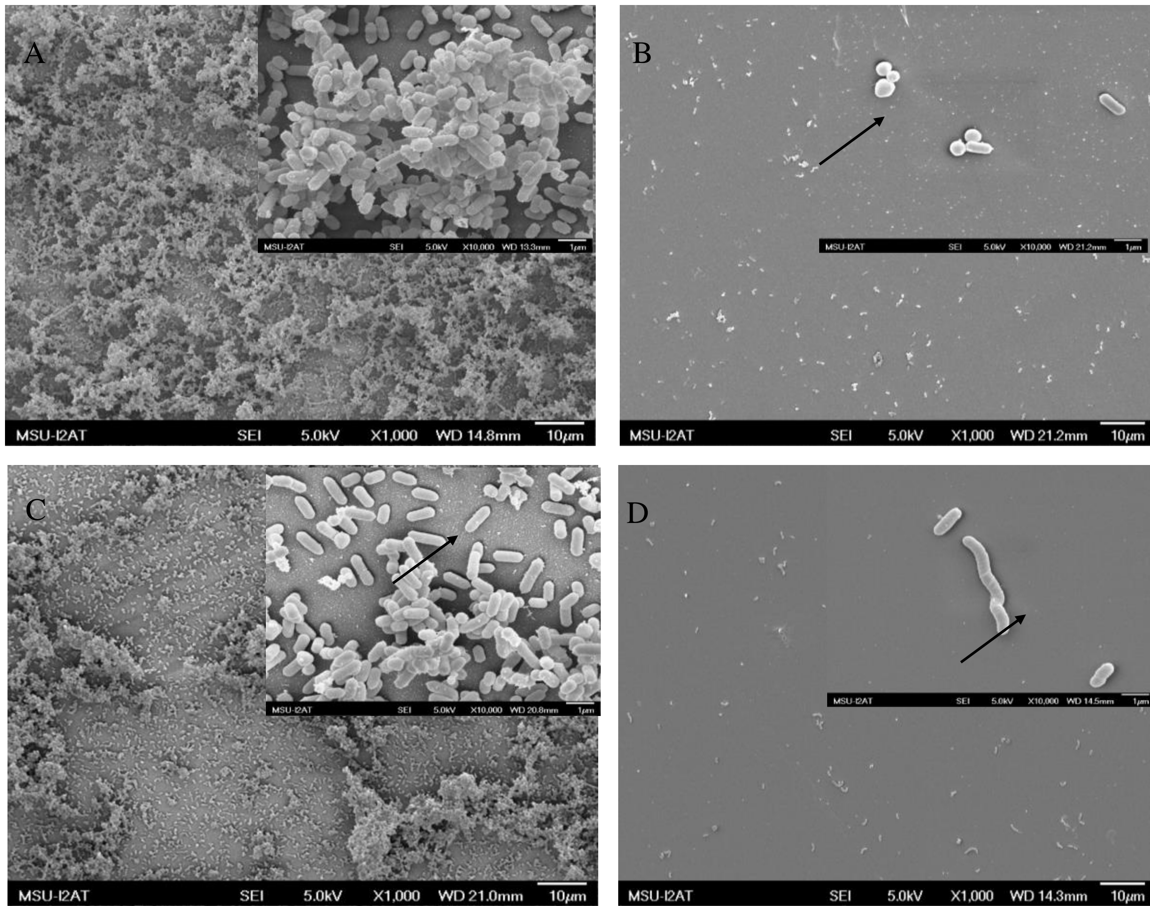

Figure 3.9 Scanning electron micrographs of chlorine induced oxidative stress adapted cells of *L. monocytogenes* V7 biofilm formation at 37°C for 48 h at 1,000 and 10,000X.

Micrographs represent biofilm formation by (A) Chlorine non adapted and non-treated, (B) Chlorine adapted and non-treated, (C) Chlorine non adapted and treated, (D) Chlorine adapted and treated.

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CHAPTER IV

LISTERIA MONOCYTOGENES RESPONSE TO SUBLETHAL CHLORINE INDUCED OXIDATIVE STRESS ON HOMOLOGOUS AND HETEROLOGOUS STRESS ADAPTATION

Abstract

When exposed to sublethal stresses, *L. monocytogenes* may induce an adaptive response to adverse stress conditions. The objective of this study was to determine the effect of sublethal oxidative stress induced by chlorine (sodium hypochlorite) against homologous and heterologous stress in five *L. monocytogenes* strains. *L. monocytogenes* cells were exposed to increasing sub-inhibitory concentrations (20 ppm/day) of total chlorine from 250 to 350 ppm and then 25 ppm on day 6 to obtain 375 ppm in tryptic soy broth (TSB). Changes in minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *L. monocytogenes* cells adapted to chlorine at 250 (1/2 MIC), 330 (2/3 MIC) and 375 (3/4 MIC) and control (non-adapted) were determined by the macro-dilution method. Chlorine-adapted *L. monocytogenes* cells were also evaluated for changes in antibiotic resistance using the Kirby-Bauer disk diffusion assay with 11 different antibiotics. Also, chlorine-adapted and non-adapted *L. monocytogenes* cells were evaluated for changes in MIC values to 8 commonly used antibiotics as per the Clinical & Laboratory Standards Institute (CLSI, 2008) guidelines. The MIC and MBC values of chlorine for *L. monocytogenes* Scott A, V7, FSL-N1-227 and FSL-F6-154

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strains when adapted to sublethal chlorine were higher (600 ppm and 700 ppm) as compared to control (500 pm and 600 ppm). The average zones of inhibition by disk-diffusion assay for chlorine-adapted *L. monocytogenes* cells was decreased by 0.5 – 2.2 mm compared to control cells against all 11 antibiotics tested. The double dilution MIC assay revealed a minor change in average MIC values for ampicillin, tetracycline, amoxicillin and ciprofloxacin. However, the average MIC values were doubled for streptomycin and gentamicin against the oxidative stress adapted *L. monocytogenes* strain V7 when compared to the control. A major change in MIC was observed for the reference strain ATCC 19116 which exhibited a significant increase in resistance to ceftriaxone. However, the changes in zones of inhibition and MIC values to all antibiotics tested for the chlorine-adapted and non-adapted (control) *L. monocytogenes* cells were still within the susceptible range. These findings indicate that the continuous exposure of *L. monocytogenes* cells to chlorine may induce changes in homologues and heterologous stress adaptation.

Key Words: Sublethal oxidative stress, sodium hypochlorite, stress adaptation, *Listeria monocytogenes*

Introduction

Listeriosis, caused by *Listeria monocytogenes*, is a fatal foodborne disease with a high hospitalization rate (>90%), and commonly causes an infection in the susceptible population which includes immunocompromised and pregnant women (Scallan et al., 2011). Though *listeriosis* has a low incidence rate (<3%) in the healthy population, it is the third leading cause of foodborne related deaths in the USA (Mead et al., 1999). This

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foodborne pathogen causes severe nervous symptoms like meningitis, meningoencephalitis and febrile gastroenteritis in the susceptible population (Vázquez-Boland et al., 2001).

L. monocytogenes is a ubiquitous Gram-positive foodborne pathogen. In the food-processing environments, *L. monocytogenes* is commonly exposed to oxidative stress induced by sanitizers and disinfectants like sodium hypochlorite (Gao and Liu, 2014; Gray et al., 2014). In the food-production environments, indiscriminate and over usage of antibiotics by veterinarians has raised concerns over the emergence of antibiotic resistance (Fair and Tor, 2014). Since the 1960s, there has been a gradual increase in the number of antibiotic resistant cases from all over the world with treatment costs exceeding $20 billion dollars annually (Fair and Tor, 2014). Other published findings show that continuous exposure of disinfectants to bacteria may induce the phenotypic resistance mechanism which is expressed in the form of changes in cell wall (McMahon et al., 2007; Wiuff et al., 2005) and membrane structures or altered activity of specific or nonspecific efflux pumps (Levin and Rozen, 2006).

Biocides are commonly used for routine cleaning, sanitation and disinfection in the food industry and hospitals (Mcdonnell and Russell, 1999; Seier-Petersen, 2013). Continuous exposure to sublethal concentrations of such biocides/antimicrobials while cleaning and sanitizing may induce stress adaptation in food borne pathogens (Capozzi et al., 2009). In response to sublethal stress, bacteria can co-select genes responsible for encoding tolerance to both the same or different biocides and antibiotics.

Published findings show that the exposure of modern food preservation processes does not inhibit the complete metabolic and/or genetic activity of bacteria while it can

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induce phenotypic and genotypic adaptations allowing bacteria to survive in food matrices (McMahon et al., 2007). Such phenotypic adaptations include changes in the permeability of the cell membrane, and increased expression of specific or nonspecific efflux pumps (Forbes et al., 2014). McMahon et al (2007) found that exposure of low pH or high NaCl can trigger the development of antibiotic resitance in subpopulations of pathogens. Apart from other factors, bile acids also have the capacity to induce oxidative stress adaptation in *L. monocytogenes*. Increased expression of bile salt hydrolase in response to bile salt exposure can aid in *L. monocytogenes* survival (Begley et al 2005).

Often, the noninvasive listeriosis cases are commonly treated with antibiotics such as ampicillin, amoxicillin, gentamicin or cephalosporin. However, a few strains of *L. monocytogenes* have been found to have intrinsic resistance against third generation cephalosporin drugs (Kose and Yakupogullari, 2015).

There is a lack of knowledge on the influence of continuously increasing doses of sublethal chlorine on *L. monocytogenes* homologous and heterologous adaptation (cross-resistance to other biocides). Therefore, 2 objectives were addressed in this study: (i) to determine the homologous stress adaptation by measuring the changes in minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) to chlorine in *L. monocytogenes* strains; (ii) to determine the cross-resistance to commonly used antibiotics by measuring changes in zones of inhibition and MIC in *L. monocytogenes* cells after exposure to chlorine.

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Material and Methods

Bacterial Strains and culture preparation

Five *L. monocytogenes* strains used in this study are listed in Table 4.1. All bacterial strains were stored as stock cultures in tryptic soy broth (TSB) supplemented with 25% glycerol at -80°C. The bacteriological media, Difco (Becton Dickinson, Sparks, MD) was used for all experiments in the present study. Prior to each experiment, individual bacterial strains were cultured in 10 ml of TSB at 37°C for two consecutive cycles of 24 h to remove any cold stress in working cultures. Following overnight incubation, obtained culture was centrifuged at 5000×*g* for 10 min at 4°C. The pellet was collected and resuspended in 10 ml of TSB (pH 7.2). Serial ten-fold dilutions were plated on duplicate tryptic soy agar (TSA) and modified oxford agar plates for enumeration and confirmation, respectively. Further, the plates were analyzed for CFU after 24 h incubation at 37°C.

Sodium hypochlorite

Sodium hypochlorite 5% stock solution (Acros Organics, New Jersey, USA) was used as the source of chlorine. Sodium hypochlorite stock solution was used to prepare the working concentrations of chlorine in Tryptic soy broth (TSB). Prior to each experiment, total and free available chlorine was measured using HACH (chlorine test kit) Pocket Colorimeter (HACH Company, Loveland, CO, USA) according to the manufacturer's instructions.

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Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Assay

The minimum inhibitory concentration (MIC) was determined by the macro-dilution method according to the Clinical and Laboratory Standards Institute, CLSI (2015) with minor modifications. The overnight cultures of *L. monocytogenes* at 10^9 CFU/ml was diluted in TSB to obtain a final concentration of 10^6 CFU/ml for the MIC assay. The total available chlorine concentration ranges of 200-800 ppm with 100 ppm increments was prepared in 10 ml volumes of TSB. An aliquot (100 µL) of *L. monocytogenes* suspension 10^6 CFU/ml was added to each tube containing working dilutions of available chlorine to observe bacterial growth by turbidity changes after 24 h of incubation at 37°C. Tubes with *L. monocytogenes* inoculum without chlorine and tubes with TSB alone were used as a positive and negative control, respectively. MIC was determined as the lowest concentration of available chlorine that inhibited the visible growth of *L. monocytogenes* after 24 h of incubation.

An aliquot of 100 µL from tubes without visible growth of *L. monocytogenes* were streaked onto duplicate tryptic soy agar (TSA) plates to determine the minimum bactericidal concentration (concentration of available chlorine that kills all *L. monocytogenes* cells after 24 h incubation at 37°C).

Chlorine induced oxidative stress adaptation in *Listeria monocytogenes*

Oxidative stress adaptation in *L. monocytogenes* was induced by exposure of bacterial cell suspensions to sublethal chlorine concentrations. An aliquot (100 µL) of a 24 h bacterial culture previously prepared, was inoculated into 10 ml of TSB with the initial 1/2 MIC (250 ppm) chlorine concentration. Subsequently 100 µl (~10^7 CFU/ml) of

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a previous day incubated culture (1/2 MIC) was transferred into fresh TSB with the increasing chlorine concentration of 20 ppm/day until 350 ppm total chlorine was obtained. Finally, on day 6, the chlorine concentration was increased by 25 ppm to obtain a final concentration of 375 ppm in TSB. Therefore, *L. monocytogenes* adapted cells from three different sublethal oxidative stress levels were obtained: (1) 1/2 MIC (250 ppm), (2) 2/3 MIC (330 ppm); and (3) 3/4 MIC (375 ppm) on days 1, 5 and 7, after 7 cycles of 24 h incubation. Control (non-adapted) cells were also passaged along with the adapted cells but in the absence of chlorine. Sublethal oxidative stress adaptation was measured by the changes in MIC and MBC for each stress level of *L. monocytogenes* culture.

Determination of antibiotic susceptibility

Antibiotic MIC determination by disk-diffusion assay

The Kirby Bauer disk-diffusion method was used to compare the zones of inhibition of *L. monocytogenes* oxidative stress adapted cells to their controls. Cells from controls and sublethal oxidative stress levels (1/2 MIC, 2/3 MIC and 3/4 MIC) were obtained and pelleted after centrifugation at 5000×g for 10 min at 4°C. Thereafter, cells were resuspended in 10 ml of fresh TSB. Colonies were isolated on fresh TSA plates after incubation at 37°C for 24 hours. McFarland standards were prepared in 0.1% peptone water from colonies obtained from both the control and oxidative stress adapted cells. Aliquots were swabbed on Mueller- Hinton agar and antibiotic discs were placed on the plate. The plates were incubated at 37°C for 24 h. The average zones of inhibition (mm) for different antibiotics were obtained for *L. monocytogenes* cells exposed to three oxidative stress levels of 1/2 MIC, 2/3 MIC and 3/4 MIC and compared to the zones of inhibition of control cells average value.

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The antibiotic discs used were: amoxicillin/clavulanic acid (AMC, 30 μg), gentamicin (GN, 10 μg), sulphamethoxazole/trimethoprim (SXT, 25 μg), streptomycin (S, 10 μg), nalidixic acid (NA, 30 μg), ciprofloxacin (CIP, 5 μg), ceftriaxone (CTX, 30 μg), ampicillin (AMP, 10 μg), vancomycin (V, 30 μg) and rifampicin (RIF, 5 μg). The zones of inhibition were measured according to the CLSI guidelines (2008).

Antibiotic MIC determination by micro-dilution assay

The stock solution of antibiotics was prepared as the manufactures recommended (Table 4.2). Working concentrations were obtained in MHB from stock solutions. The MIC was determined by serial double fold dilutions for each antibiotic, which was obtained in MHB in a 96-well round bottom sterile polystyrene microtiter plate (12 columns by 8 rows). Eight serial double dilutions were obtained in 8 columns and were duplicated in 4 rows. The MIC breakpoints recommended by the CLSI (2008) was used to observe the results as the lowest concentration of antibiotics that inhibited the visible growth as either susceptible, intermediate or resistant. The susceptible range of each antibiotic is mentioned in Table 4.2. One well of MHB with inoculum and antibiotics was used as a positive control and one well with only MHB was used as the negative control. The lowest concentration of antibiotics that inhibited the button formation and yielded no turbidity was considered the MIC. The average of MIC values from all three sublethal oxidative stress levels (1/2 MIC, 2/3 MIC and 3/4 MIC) were obtained and compared to the control cells average value.

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Determination of ultrastructure changes in the chlorine adapted cells by Transmission electron microscopy

Transmission electron microscopy (TEM) was used to investigate the ultrastructural changes in *L. monocytogenes* cells after sublethal chlorine exposure. *L. monocytogenes* cells were exposed to increasing concentrations of chlorine to the final concentration of 375 ppm beginning at 250 ppm in TSB. *L. monocytogenes* strains, Scott A, V7 and FSL-N1-227 chlorine adapted cells were harvested after exposure to a final 375 ppm chlorine concentration. Cells were harvested by centrifugation at 5,000 x g for 10 min at 4°C to remove residual chlorine and the resulting concentrated cell pellets were prepared for transmission electron microscopy (TEM) by previously described methods (Capita et al., 2014). Pellets were fixed using 1/2 strength Karnovsky fixative in 0.1M sodium cacodylate buffer (pH 7.2) overnight at 4°C. Thereafter, pellets were post fixed in 2% buffered osmium tetroxide and dehydrated through a graded ethanol series, and finally, embedded in Spurr's resin. Ultra-thin sections were prepared, collected on copper grids and stained with uranyl acetate and lead citrate for viewing on a JEOL JSM-1230 transmission electron microscope (Jeol USA, MA, USA)at 80kv.

Results

Homologous stress adaptation (chlorine to chlorine):

Homologous stress adaptation of *L. monocytogenes* to chlorine was determined by measuring the changes in MIC and MBC for sodium hypochlorite. The MIC of sodium hypochlorite for control *L. monocytogenes* was 500 ppm for all five strains tested, Scott A, V7, N1-2227, F6-154 and ATCC 19116. After exposure to the sublethal oxidative stress induced by chlorine, the average MIC of sodium hypochlorite was increased to 600

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ppm at all three sublethal stress levels, i.e., 1/2 MIC, 2/3 MIC and 3/4 MIC for the following four strains, Scott A, V7, N1-227 and F6-154. However, there was no change in MIC to sodium hypochlorite for the reference strain ATCC 19916 under these experimental conditions.

The MBC of sodium hypochlorite for *L. monocytogenes* showed similar trends of adaptive tolerance to sodium hypochlorite, where average MBC was increased to 700 ppm for strains Scott A, V7, N1-227, F6-154 from the initial 600 ppm at all sublethal stress levels (1/2 MIC, 2/3 mic and 3/4 MIC). However, no changes in MBC was observed for the reference strain ATCC 19116 (Table 4.3).

Heterologous stress adaptation (chlorine to antibiotic susceptibility):

Antibiotic susceptibility of five strains of *L. monocytogenes* was measured by the Kirby Bauer disk diffusion assay and MIC dilution assay as per CLSI (2008) guidelines. The antibiotic susceptibility patterns of the oxidative stress adapted and control cells from both the disk diffusion assay and MIC dilution method are shown in Tables 4.4 and 4.5. *L. monocytogenes* strains were screened for susceptibility to different classes of antibiotics before and after gradual exposure to the increasing concentrations of sodium hypochlorite. All five strains tested were found to have intrinsic resistance to nalidixic acid while 2 of the 5 strains tested (Scott A and N1-227) have intrinsic resistance to ceftriaxone (a third generation cephalosporin). The disk diffusion assay results showed that the sublethal oxidative stress induced by sublethal sodium hypochlorite influenced the zones of inhibition in *L. monocytogenes*. The average zone of inhibition was slightly decreased by 0.5 to 2.2 mm compared to controls for all 11 antibiotics tested. However,

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adapted cells were more susceptible to rifampicin where the zone of inhibition was increased by 0.3-0.5 mm.

The double dilution MIC assay revealed a minor change in average MIC values for ampicillin, tetracycline, amoxicillin and ciprofloxacin. However, the average MIC values were doubled for streptomycin and gentamicin against oxidative stress adapted *L. monocytogenes* strain V7 compared to control. A major change in MIC was observed for the reference strain ATCC 19116 which exhibited a significant increase in resistance to ceftriaxone (Table 4.5). A slight change in antibiotic susceptibility range was found for two more strains, Scott A and N1-227, for ceftriaxone in oxidative stress adapted cells of *L. monocytogenes*.

Changes in ultrastructure of *L. monocytogenes* in the presence of sodium hypochlorite

Sublethal chlorine induced oxidative stress caused significant morphological changes in *L. monocytogenes* strains Scott A, V7 and N1-227 which included: (1) changes in cell length, (2) changes in cell wall thickness, (3) changes in cell membrane shape; and (4) cytoplasmic changes. Oxidative stress induced cells of *L. monocytogenes* exhibited a consistent elongation of cells, presence of multiple chromosomes and membrane bleb formations which may indicate transient inhibition of cell division (figure 4.1-3).

Along with the elongated cells, outer membrane bleb formation and wavy cell envelop structures were frequently observed in oxidative stress adapted cells of *L. monocytogenes* which may be associated with the activation of a bacterial response to sublethal stresses.

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Discussion

In the present study, the influence of increasing sublethal concentrations of sodium hypochlorite was examined for changes in homologous and heterologous stress adaptation of *L. monocytogenes*. Various sanitizers and disinfectants are routinely used at >1,000 times concentrations than that of their MIC for killing foodborne pathogens in the food-processing plants. The high concentrations of sanitizers and disinfectants interacts with multiple mechanisms in bacteria cells, such as degeneration of proteins, and lipids, as well as DNA degradation (Gray et al., 2014). In other environments, bacterial cells are frequently exposed to a lower or sublethal concentrations of biocides in wastewater plants, hospitals, or in processing plant effluents. Recent findings show that the sublethal concentrations of biocides interact with a single central bacterial response and such gradual exposure of sublethal concentrations can co-select resistance to biocides as well as to antibiotics (Capita et al., 2014; Gray et al., 2014; Wales and Davies, 2015; Webber et al., 2015). Therefore, it is important to understand the role of sublethal concentrations at which biocides can select or co-select for antibiotic resistance in whole or subpopulations.

L. monocytogenes strains Scott A, V7, N1-227 and F6-154 exhibited homologous stress adaptation in TSB with an increase in the chlorine tolerance. Contrary to the present study, Kastbjerg and Gram (2012) exposed *L. monocytogenes* EGD to the disinfectants hypochlorite and Incimaxx DES (peracedic acid and hydrogen peroxide) for several hundred generations and found no changes in the minimal inhibitory concentration (MIC), whereas exposure to Triquart SUPER (quaternary ammonium compounds) caused a 2-4 fold increase in MIC.

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Chlorine reacts with organic compounds in media (TSB) and forms a reactive chlorine species (RCS) such as chloramines. The RCS then reacts with sulfur containing compounds such as amino acids (cysteine, methionine or glutathione), and primary or secondary amines, nucleotides and lipids. While the higher concentration of RCS damages the bacterial DNA, bacteria can induce adaptations when exposed to a lower or sublethal concentration. Bacteria quickly respond to RCS by the high expression of enzymes, such as catalases, peroxidases and methionine sulfoxide reductase. In response to damaged DNA induced by RCS, bacteria upregulate homologous recombination, repair and mutagenic polymerases (Gray et al., 2014) .

At sublethal concentrations chlorine and RCS species can induce the SOS response, a state of temporary stop in bacterial cell division in bacteria. During a SOS response, bacteria are in a state of mutagenesis or DNA repair and change to a rod shape with multi chromosomes containing long filaments. Bos et al., (2014) investigated the role of bud formation in antibiotic resistance propagation *E. Coli* after sublethal ciprofloxacin exposure. Asymmetrical bacterial division in the presence of sublethal ciprofloxacin stress was found to induce the bud formation, which carry resistant genes within its chromosomes. Multi chromosome condition in adapted bacteria is analogous to aneuploidy drug resistance in *candida albicans* and eukaryotic cancer cells.

The antibiotic resistance against third generation cephalosporin's in *Listeria monocytogenes* was reported from different countries. Ceftriaxone resistance in human listeriosis cases were reported from Vietnam (Chau et al., 2010), Bangladesh (Ahmed et al., 2015) and Turkey (Kose and Yakupogullari, 2015). In the present study, ceftriaxone intrinsic resistant was found in strain Scott A and N1-227, however reference strain

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ATCC 19116 showed adaptive resistance against it. Al-Nabulsi et al., (2015) revealed that antibiotic resistance in *L. monocytogenes* was increased after exposing it to 6 or 12% NaCl, reducing the pH to 5, and decreasing the temperature to 10°C. *L. monocytogenes* isolates from meat and dairy have also been found to be more resistant than its reference ATCC 19116 strain.

While the phenotypic antibiotic resistance or homologous stress adaptation may be transient (>1 d) and unstable in food, the bacterial stress response can trigger cross protection against unrelated challenges. Recently, Al-Nabulsi et al., 2015 revealed that phenotypic antibiotic resistance was retained for >1 d (at least 10 generations) after removal of the mild environmental stresses such as acidic pH, cold temperature and NaCl exposure.

Stress adapted bacterial cells change their morphology, which may help in their ability to tolerate higher concentrations of antibiotics and antimicrobials. Our present findings of *L. monocytogenes* cell elongation and cell wall roughness as a bacterial response in stress adapted cells is in agreement with previous findings (To et al., 2002). These findings suggest a major role of outer member modulations in *L. monocytogenes* adaptive responses to antimicrobials and antibiotics (Ernst et al., 2001; Sallum and Chen, 2008). Similar to previous findings (Gray et al., 2014), we have also observed that *L. monocytogenes* cells also formed multi-chromosome filaments with buds in response to chlorine stress by electron microscopy studies.

In conclusion, our findings show that the exposure to sublethal concentrations of chlorine could potentially have some impact on the cross- responses of *L. monocytogenes* to commonly used antibiotics, enabling microorganisms to adapt adverse environmental

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conditions. The changes in cell wall and membrane integrity resulting from the elongation of cells are the possible routes of *L. monocytogenes* response for its increase in tolerance to chlorine and selective antibiotics.

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References

Ahmed, D., Sadique, T., Sami, A.B., Abdullah, A., Nahid, M.A., Hossain, A., Mazumder, R., Rahaman, M.A., 2015. Listeria monocytogenes infection in a 56-year-old female cancer patient: a case report. JMM Case Reports 2, 2–5. doi:10.1099/jmmcr.0.000076

Al-Nabulsi, A.A., Osaili, T.M., Shaker, R.R., Olaimat, A.N., Jaradat, Z.W., Zain Elabedeen, N.A., Holley, R.A., 2015. Effects of osmotic pressure, acid, or cold stresses on antibiotic susceptibility of Listeria monocytogenes. Food Microbiol. 46, 154–160. doi:10.1016/j.fm.2014.07.015

Bos, J., Zhang, Q., Vyawahare, S., Rogers, E., Rosenberg, S.M., Austin, R.H., 2014. Emergence of antibiotic resistance from multinucleated bacterial filaments. doi:10.1073/pnas.1420702111

Capita, R., Riesco-Peláez, F., Alonso-Hernando, A., Alonso-Calleja, C., 2014. Exposure of Escherichia coli ATCC 12806 to sublethal concentrations of food-grade biocides influences its ability to form biofilm, resistance to antimicrobials, and ultrastructure. Appl. Environ. Microbiol. 80, 1268–1280. doi:10.1128/AEM.02283-13

Capozzi, V., Fiocco, D., Amodio, M.L., Gallone, A., Spano, G., 2009. Bacterial stressors in minimally processed food. Int. J. Mol. Sci. 10, 3076–3105. doi:10.3390/ijms10073076

Chau, T.T.H., Campbell, J.I., Schultsz, C., van Vinh Chau, N., Diep, T.S., Baker, S., Chinh, N.T., Farrar, J.J., Rogier van Doorn, H., 2010. Three adult cases of Listeria monocytogenes meningitis in Vietnam. PLoS Med. 7. doi:10.1371/journal.pmed.1000306

Ernst, R.K., Guina, T., Miller, S.I., 2001. Salmonella typhimurium outer membrane remodeling: Role in resistance to host innate immunity. Microbes Infect. 3, 1327–1334. doi:10.1016/S1286-4579(01)01494-0

Fair, R.J., Tor, Y., 2014. Perspectives in Medicinal Chemistry Antibiotics and Bacterial Resistance in the 21st Century. Perspect. Medicin. Chem. 25–64. doi:10.4137/PMC.S14459.Received

Forbes, S., Dobson, C.B., Humphreys, G.J., McBain, A.J., 2014. Transient and sustained bacterial adaptation following repeated sublethal exposure to microbicides and a novel human antimicrobial peptide. Antimicrob. Agents Chemother. 58, 5809–5817. doi:10.1128/AAC.03364-14

Gao, H., Liu, C., 2014. Biochemical and morphological alteration of Listeria monocytogenes under environmental stress caused by chloramine-T and sodium hypochlorite. Food Control 46, 455–461. doi:10.1016/j.foodcont.2014.05.016

المنارة للاستشارات
www.manaraa.com

Gray, M.J., Wholey, W., Jakob, U., 2014. NIH Public Access 141–160. doi:10.1146/annurev-micro-102912-142520.Bacterial

Kose, A., Yakupogullari, Y., 2015. A rapidly fatal sepsis caused by listeria monocytogenes type-4b in a patient with chronic renal failure. Jundishapur J. Microbiol. 8, e19980. doi:10.5812/jjm.19980

Levin, B.R., Rozen, D.E., 2006. Non-inherited antibiotic resistance. Nat. Rev. Microbiol. 4, 556–562. doi:10.1038/nrmicro1445

Mcdonnell, G., Russell, A.D., 1999. Antiseptics and disinfectants: Activity, action, and resistance. Clin. Microbiol. Rev. 12, 147–179. doi:10.1007/s13398-014-0173-7.2

McMahon, M.A.S., Xu, J., Moore, J.E., Blair, I.S., McDowell, D.A., 2007. Environmental stress and antibiotic resistance in food-related pathogens. Appl. Environ. Microbiol. 73, 211–217. doi:10.1128/AEM.00578-06

Mead, P.S., Slutsker, L., Dietz, V., McCaig, L.F., Bresee, J.S., Shapiro, C., Griffin, P.M., Tauxe, R. V., 1999. Food-related illness and death in the United States. Emerg. Infect. Dis. 5, 607–625. doi:10.3201/eid0505.990502

Sallum, U.W., Chen, T.T., 2008. Inducible resistance of fish bacterial pathogens to the antimicrobial peptide cecropin B. Antimicrob. Agents Chemother. 52, 3006–3012. doi:10.1128/AAC.00023-08

Scallan, E., Hoekstra, R.M., Angulo, F.J., Tauxe, R. V., Widdowson, M.A., Roy, S.L., Jones, J.L., Griffin, P.M., 2011. Foodborne illness acquired in the United States-Major pathogens. Emerg. Infect. Dis. 17, 7–15. doi:10.3201/eid1701.P11101

Seier-Petersen, M.A., 2013. Development of bacterial resistance to biocides and antimicrobial agents as a consequence of biocide usage. Div. Epidemiol. Microb. Genomics Natl. Food Inst. Tech. Univ. Denmark.

To, M.S., Favrin, S., Romanova, N., Griffiths, M.W., 2002. Postadaptational resistance to benzalkonium chloride and subsequent physicochemical modifications of Listeria monocytogenes. Appl. Environ. Microbiol. 68, 5258–5264. doi:10.1128/AEM.68.11.5258-5264.2002

Vázquez-Boland, J.A., Kuhn, M., Berche, P., Chakraborty, T., Domínguez-Bernal, G., Goebel, W., González-Zorn, B., Wehland, J., Kreft, J., 2001. Listeria Pathogenesis and Molecular Virulence Determinants. Clin. Microbiol. Rev. doi:10.1128/CMR.14.3.584-640.2001

Wales, A., Davies, R., 2015. Co-Selection of Resistance to Antibiotics, Biocides and Heavy Metals, and Its Relevance to Foodborne Pathogens. Antibiotics 4, 567–604. doi:10.3390/antibiotics4040567

المنارة للاستشارات

www.manaraa.com

Webber, M.A., Whitehead, R.N., Mount, M., Loman, N.J., Pallen, M.J., Piddock, L.J. V, 2015. Parallel evolutionary pathways to antibiotic resistance selected by biocide exposure. J. Antimicrob. Chemother. 70, 2241–2248. doi:10.1093/jac/dkv109

Wiuff, C., Zappala, R.M., Regoes, R.R., Garner, K.N., Levin, B.R., 2005. Phenotypic Tolerance : Antibiotic Enrichment of Noninherited Resistance in Bacterial Populations Phenotypic Tolerance : Antibiotic Enrichment of Noninherited Resistance in Bacterial Populations. Antimicrob. Agents Chemother. 49, 1483. doi:10.1128/AAC.49.4.1483

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Table 4.1 List of *Listeria monocytogenes* strains and their sources used in the present study.

No.	***L. monocytogenes*** **Strains**	**Lineage**	**Serovar**	**First reported outbreak**
1.	*L. monocytogenes* FSL F6-154	II	1/2a	Food, epidemic (sliced turkey) (2000)
2.	*L. monocytogenes* FSL N1-227	I	1/2a	Food, epidemic (US 1998-99)
3.	*L. monocytogenes* ATCC 19116	III	4c	Poultry, UW
4.	*L. monocytogenes* Scott A	I	4b	Human epidemic (Mass., 1983)
5.	*L. monocytogenes* V 7	II	1/2a	Raw milk, FDA

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Table 4.2 List of antibiotics used in the present study

No.	Antibiotic	Susceptibility Range (μg/ml)	Solvent	Diluent
1.	Ampicillin	0.0078-1	Water	MHB
2.	Amoxicillin	0.0075-0-48	Water	MHB
3.	Ceftriaxone	2-128	Water	MHB
4.	Vancomycin	0.5-32	Water	MHB
5.	Tetracycline	0.03-2	Ethanol (70%)	MHB
6.	Gentamicin	0.062-4	Water	MHB
7.	Streptomycin	4-256	Water	MHB
8.	Ciprofloxacin	0.0075-0-48	Water	MHB

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Table 4.3 Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of chlorine (ppm) for five *Listeria monocytogenes* strains control and adapted to sublethal concentrations of chlorine.

		***L. monocytogenes* strains**				
		Scott A	V7	FSL-N1-227	FSL-F6-154	ATCC 19116
MIC (ppm)	Control	500	500	500	500	500
	Adapted cells	600	600	600	600	500
MBC (ppm)	Control	600	600	600	600	600
	Adapted cells	700	700	700	700	600

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Table 4.4 Average zones of inhibition (mm) by antibiotic disk-diffusion tests for five *Listeria monocytogenes* strains after chlorine induced oxidative stress adaptation (A) at all three different stress levels (1/2 MIC, 2/3 MIC and 3/4 MIC) and control (C) cells.

Antibiotics[a]	Scott A		V7		FSL-N1- 227		FSL-F6-154		ATCC-19116	
	C	**A**	**C**	**A**	**C**	**A**	**C**	**A**	**C**	**A**
Amp	32	30.7	32	31.2	30	29	32	30.2	32	31.5
S	**20**	**17.6**	**19**	**17.6**	**18**	**17.1**	**20**	**17.7**	**20**	**18.3**
G	22	22	22	22	22	21.3	22	21.2	22	22.5
Amx	34	33.5	34	33.3	34	32.7	34	30.2	34	32
NA	R	R	R	R	R	R	R	R	R	R
ST	36	35.3	34	33.5	34	33.4	34	31.8	**34**	**31.4**
VN	22	20.5	22	21.5	22	21.2	22	21.4	22	21.6
CIP	20	19.5	22	23.4	22	21.6	20	21	21	20.6
RF	24	24.5	24	24.4	24	24.3	24	25.2	24	25.2
CFT	**R**	**R**	14	13	**R**	**R**	14	13.3	14	12.4

[a]AMP, ampicillin; S, streptomycin; GN, gentamicin; AMX, amoxicillin; NA, nalidixic acid; ST, Sulphamethaxozole and Trimethoprim; VN, Vancomycin;; CIP; ciprofloxacin; RIF' Rifampicin CFT, Ceftriaxone. Highlighted data indicate change in susceptibility relative to controls; data not highlighted indicate exposed strains with no difference in susceptibility patterns relative to controls.

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Table 4.5 Average minimum inhibitory concentrations of antibiotics for five *Listeria monocytogenes* strains after chlorine induced oxidative stress adaptation (A) at all three different stress levels (1/2 MIC, 2/3 MIC and 3/4 MIC) and control (C) cells.

Antibiotic[a]	Scott A		V7		FSL-N1- 227		FSL-F6-154		ATCC-19116	
	C	A	C	A	C	A	C	A	C	A
Amp	0.3/S	0.3/S	**.06/S**	**0.2/S**	0.3/S	0.3/S	0.6/S	0.6/S	**0.3/S**	**0.4/S**
T	0.3/S	0.5/S	0.3/S	0.5/S	0.3/S	0.3/S	1.1/S	1.1/S	0.5/S	0.6/S
S	3.5/S	3.5/S	**7/S**	**14/S**	15/S	15/S	**8/S**	**16/S**	**10/S**	**15/S**
GN	0.3/S	0.3/S	**0.5**/S	**1**/S	1.5/S	2/S	1.6/S	1.8/S	1.4/S	1.6/S
AMX	0.1/S	0.1/S	**0.1/S**	**0.2/S**	0.4/S	0.4/S	0.2/S	0.3/S	0.2/S	0.2/S
VN	0.5/S	0.5/S	0.5/S	0.5/S	0.5/S	0.5/S	0.5/S	0.5/S	0.5/S	0.5/S
CIP	0.2/S	0.3/S	0.3/S	0.3/S	**0.3/S**	**0.5/S**	0.4/S	0.5/S	0.4/S	0.4/S
CFT	**17/R**	**29/R**	**7.3**/S	**7.3**/S	**19/R**	**27/R**	**6.6**/S	**8**/S	**8/S**	**32/R**

[a]AMP, ampicillin; T, Tetracycline; S, streptomycin; GN, gentamicin; AMX, amoxicillin; VN, Vancomycin; CIP; ciprofloxacin; CFT, Ceftriaxone. Data are reported as S, susceptible strain; R, resistance strain. Highlighted data indicate change in susceptibility relative to controls; data not highlighted indicate exposed strains with no difference in susceptibility patterns relative to controls.

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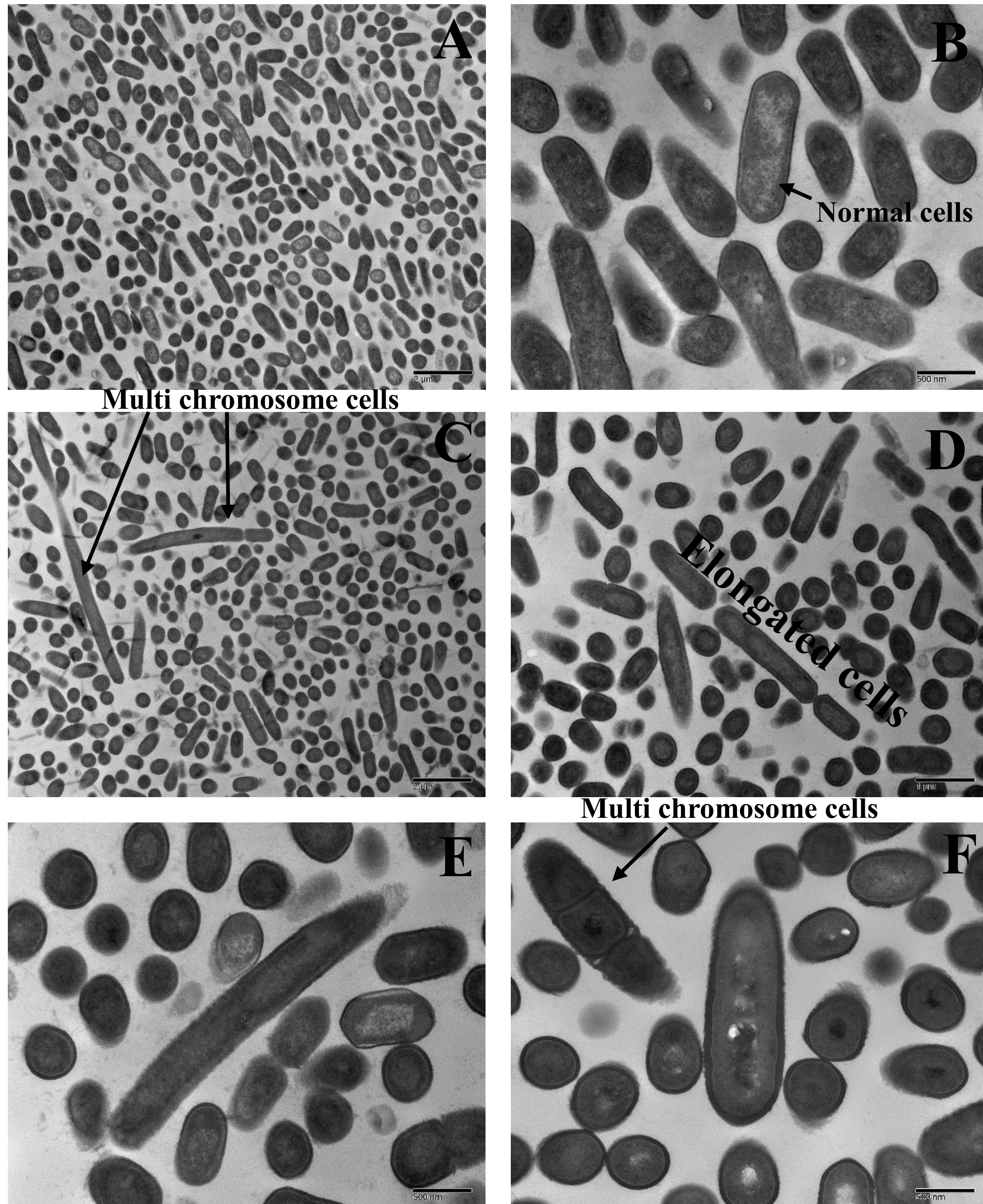

Figure 4.1 Transmission electron micrographs of *L. monocytogenes* Scott A non-adapted (A, B) and oxidative stress adapted cells (C-F) after exposing to 3/4 MIC of chlorine (375 ppm) beginning from 1/2 MIC (250 ppm).

Micrographs represent planktonic cells of (A, B) control cells at different magnifications; (C, D) elongated cells and budformation in chlorine adapted cells at different magnifications; (E, F) multi-chromosome formation in chlorine adapted cells at different magnifications.

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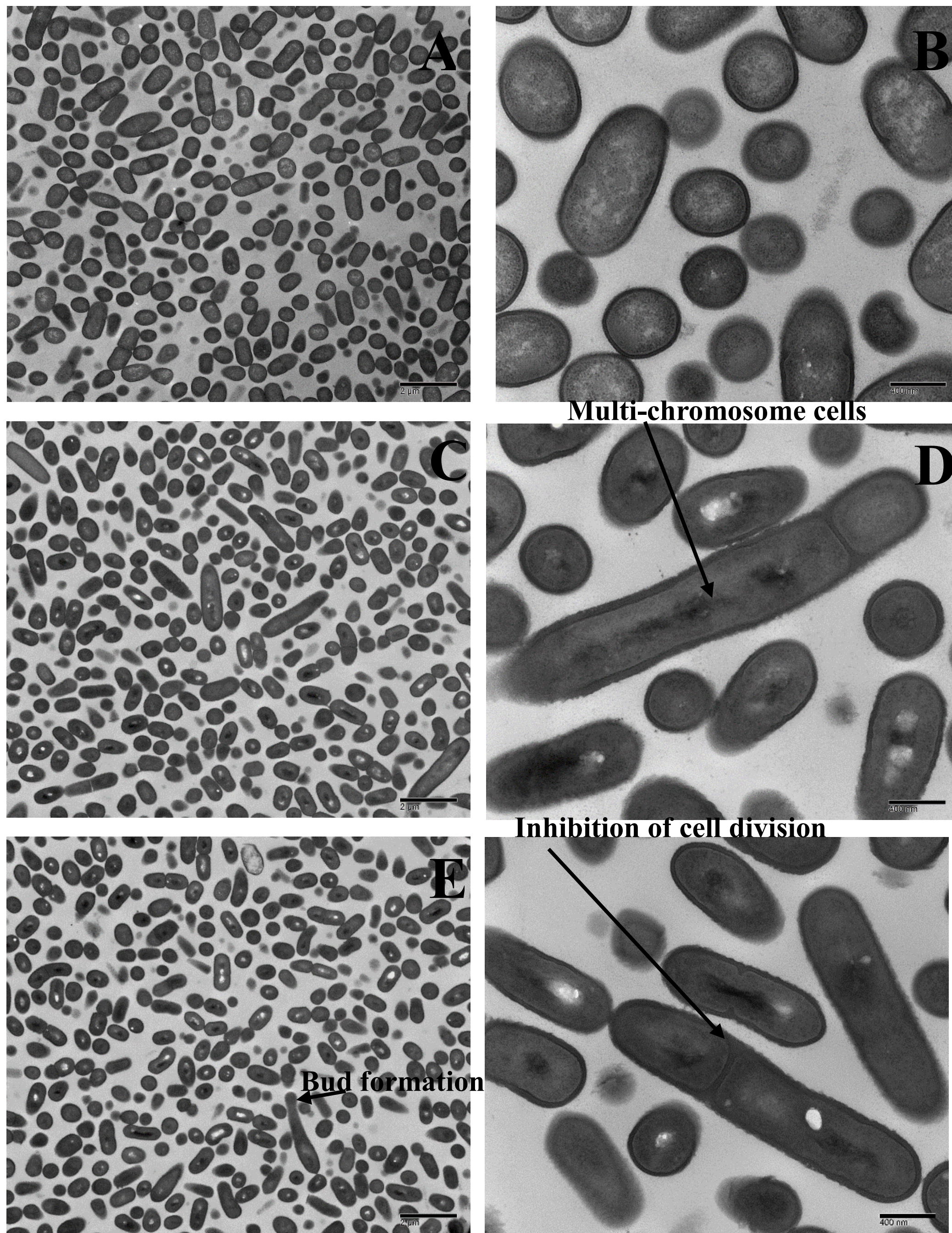

Figure 4.2 Transmission electron micrographs of *L. monocytogenes* V7 non-adapted (A-B) and oxidative stress adapted cells (C-F) after exposing to 3/4 MIC (375 ppm) of chlorine.

Micrographs represent planktonic cells of (A, B) control cells at different magnifications; (C, E) elongated cells and bleb formation in chlorine adapted cells at different magnifications; (D, F) multi-chromosome formation, and inhibition of cell division in chlorine adapted cells at different magnifications.

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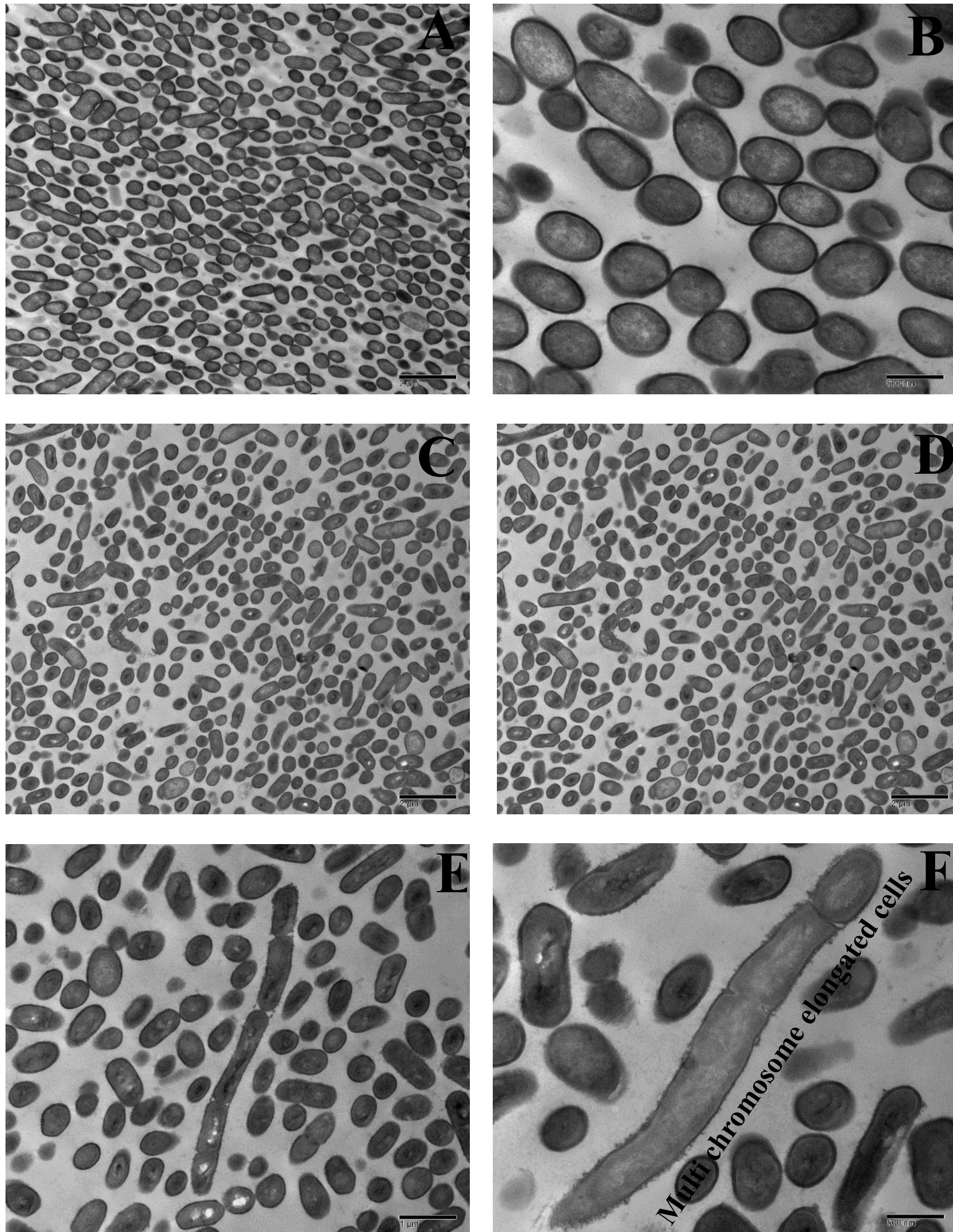

Figure 4.3 Transmission electron micrographs of *L. monocytogenes* N1-227 non-adapted (A, B) and chlorine induced oxidative adapted cells (C-F) after exposing to 3/4 MIC of chlorine (375 ppm).

Micrographs represent planktonic cells of (A, B) control cells at different magnifications; (C, D) elongated cells and bleb formation in chlorine adapted cells at different magnifications; (E, F) multi-chromosome cells and inhibition of cell division in chlorine adapted cells at different magnifications

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CHAPTER V

SUMMARY

This research determined that sublethal chlorine exposure to *L . monocytogenes* can induce physiological responses in terms of its biofilm formation ability, tolerance to chlorine as well as commonly used antibiotics. Production of less biofilm by all five *L. monocytogenes* strains on plastic surface after sublethal chlorine stress suggests that chlorine has an antibiofilm effect on *L. monocytogenes*. The antibiofilm effect of chlorine stress was found to be transient, after removal of the stress, which was concluded by *L. monocytogenes* forming more biofilm on a polystyrene surface when chlorine was not present.

The chlorine tolerance and changes in antibiotic susceptibility demonstrates that *L. monocytogenes* could adapt in adverse processing plant environments. Most of the strains tested demonstrated the development of chlorine tolerance after sublethal chlorine induced oxidative stress adaptation. A few of the *L. monocytogenes* strains have intrinsic resistance towards third generation cephalosporin's antibiotics while one strain found developed resistance to ceftriaxone after oxidative stress adaptation. The transmission electron micrographs revealed that subpopulations of *L. monocytogene* change their morphology, which may be associated with chlorine tolerance and antibiotic resistance development. There is a need for further research that explores gene expression of adapted *L. monocytogenes* to gain a better understanding of the chlorine antibiofilm effect, development of chlorine tolerance as well as antibiotic resistance.

This research emphasizes the need of proper sanitation and disinfection of food and nonfood contact surfaces. Standards procedures need to be followed to avoid any

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presence of residual chlorine on different surfaces. The result of this research demonstrates that the residuals chlorine exposure can induces physiological and morphological changes in *L. monocytogenes*, which reduces its biofilm formation but some changes can reduce susceptibility to antibiotics which may be how cells survive if they can not become a part of a biofilm for protection.

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