Biofilm forming ability and disinfectant resistance of *Listeria* species from food and food processing units

A Thesis submitted to Goa University for the award of the degree of

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MICROBIOLOGY

by

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Statement

As required under the University ordinance, I hereby state that the present thesis for Ph.D. degree entitled "**Biofilm forming ability and disinfectant resistance of *Listeria* species from food and food processing units**" is my original contribution and that the thesis and any part of it has not been previously submitted for the award of any degree/diploma of any University or Institute. To the best of my knowledge, the present study is the first comprehensive work of its kind from this area.

The literature related to the problem investigated has been cited. Due acknowledgement have been made whenever facilitate and suggestions have been availed of.

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Certificate

Certified that the research work embodied in this thesis entitled "**Biofilm forming ability and disinfectant resistance of *Listeria* species from food and food processing units"** submitted by **Mr. Swapnil Prakash Doijad** for the award of **Doctor of Philosophy** degree in **Microbiology** at Goa University, Goa, is the original work carried out by the candidate himself under my supervision and guidance.

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Abbreviations

ALOA:	Agar Listeria according to Ottaviani and Agosti
BAM:	Bacteriological Analytical Manual
BC:	Benzalkonium Chloride
BHI:	Brain Heart infusion (broth)
BIS:	Bureau of Indian Standards
Bp:	Base pairs
CAMP:	Christie Atkins Munch Petersons test
CDC:	Center of Disease Control
CFU:	Colony Forming Unit
CHEF:	Contour Clamped Homogeneous Electrophoresis
CIP:	Clean-In-Place
CV:	Crystal violet stain
DNA:	Deoxyribo nucleic acid
dNTP:	Deoxy ribose nucleic acid
EPS:	Extracellular polymeric substances
EDTA:	Ethylene Nuceloside Tri Phosphate
EDS:	Energy dispersive spectroscopy
FAME:	Fatty acid methyl ester
FAO:	Food and Agriculture Organization
FDA:	Food and Drug Administration
FSIS:	Food safety and inspection service
GMP:	Good Manufacturing Practices
HACCP:	Hazard Analysis Critical Control Point

HDPE:	High density polyethylene
ILCC:	Indian *Listeria* Culture Collection
ISO:	International Organization of Standardization
LM:	*Listeria monocytogenes*
LMWT:	*Listeria monocytogenes* EGDe
LMΔ*srtA:*	*Listeria monocytogenes* EGDe mutant for sortase A enzyme
LLO:	Listeriolysin O
mdrL:	Multidrug resistance (efflux pump) of *Listeria* species
MATH:	Microbial adherence to hydrocarbons
MIC:	Minimum Inhibitory Concentration
MHFW:	Ministry of Health and Family Welfare
MLEE	Multi Locus Enzyme Electrophoresis
MLST	Multi Locus Enzyme Electrophoresis
MTCC	Microbial Type Culture Collection
MTWP	Microtiter well plate
PALCAM:	Polymyxin Acriflavin Lithium-chloride Aesculin Mannitol
PHAC:	Public Health Agency of Canada
PBS:	Phosphate Buffered Saline
PC-PLC:	Phosphatidylcholine Specific Phospholipase C
PCR:	Polymerase chain reaction
PVC:	Polyvinyl chloride
PFGE:	Pulsed field gel electrophoresis
PI-PLC:	Phosphatidylinositol Specific Phospholipase C
QAC:	Quaternary ammonium compound
qPCR:	Quantitative polymerase chain reaction

RNA:	Ribose nucleic acid
RTE:	Ready-To-Eat (Food)
SEM:	Scanning electron microscopy
SrtA:	Sortase A enzyme
Spp.:	Species
SS304:	Stainless steel 304 grade
TE:	Tris EDTA
TBE:	Tris–borate EDTA buffer
USDA:	United States Department of Agriculture
UVM:	University of Vermont media
WHO:	World Health Organization

Units of measurement

µg:	microgram
µm:	micrometer
g:	gravitational force
gm:	grams
h:	hour
kDa:	Kilodaltons
M:	Molar
mg:	mili grams
ml:	milliliter
mM:	mili molar
mm:	millimeter
ng:	nanogram
O.D.:	Optical density
^{o}C:	Degree celcius
Pmol:	Pico mole
rpm:	revolution per minute
µ:	micron

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1. Introduction

Foodborne diseases have a major public health impact. The epidemiology of foodborne diseases is rapidly changing as newly recognized pathogens emerge and well-recognized pathogens increase in prevalence or become associated with new food vehicles (Altekruse et al. 1997). In the 21st century, people are becoming dependent more and more on ready-to-eat packed food products. As the demands of such products have been increasing, the problems associated with such foods are also increasing. Emergence of newer microbial pathogen is one of the challenging and most hazardous factor that is affecting globally. With the increase in frequency of diseases caused by such pathogens, these pathogens become noticeable and termed as "emerging pathogens". New foodborne pathogens emerge when previously unrecognized pathogens are identified and are linked to foodborne transmission (Behravesh et al. 2012). Since last two decades, the incidences of pathogens such as *Salmonella* serotype Enteritidis, *Campylobacter jejuni, E. coli* O157:H7, *Vibrio vulnificus* and *Listeria monocytogenes* (*L. monocytogenes*) increased many fold and therefore these pathogens are being considered as emerging pathogens (Behravesh et al. 2012; Newell et al. 2010). Change in demographic characteristics, new life trends, industry and technology, shift towards global economy, microbial adaptations and breakdown in public health infrastructure selectively enrich the pathogens (Altekruse et al. 1997).

Food-borne pathogens are the leading cause of illness and death in developing countries, killing approximately 1.8 million people annually (WHO 2013a). In developed countries, food-borne pathogens are responsible for millions of cases of infectious gastrointestinal diseases each year (Iyer et al. 2013). Among the microbes, bacterial pathogens are incriminated most frequently and therefore most investigated (Newell et al. 2010).

Salmonella are generally transmitted to humans through consumption of contaminated food of animal origin, mainly meat, poultry, eggs and milk (WHO 2013b). Although food production practices have changed *Salmonella* spp. seem to evolve and exploit novel opportunities, and to develop antimicrobial resistance to currently used agents.

Entertoxigenic *Escherichia coli* (ETEC) is an important causative agent of diarrhea in individuals living in and traveling to developing countries transmitted by food or water (Lindsay et al. 2013).

Shigella is a causative agent for shigellosis. Most that are infected with *Shigella* develop diarrhea, fever, and stomach cramps starting a day or two after exposure. The diarrhea is often associated with its presence of blood in stool. Unlike other common foodborne pathogens (e.g. non-Typhi *Salmonella* and *Campylobacter*), humans (and, rarely, other primates) are the only natural hosts of *Shigella* (Nygren et al. 2012).

Campylobacter jejuni is transmitted mainly through consuming unpasteurized milk and dairy products as well as raw or undercooked meat, poultry, or shellfish (Alfaro 2013).

Listeria monocytogenes is a foodborne pathogen and causative agent of listeriosis that is responsible for several foodborne outbreaks. *L. monocytogenes* generally infects to immune-compromised individuals. Though, the incidence of listeriosis is rare, high mortality rate (20-30%), neonatal death rate (50%) and hospitalization rate (91%), the infection has been considered a serious one (Low & Donachie 1997; Swaminathan & Gerner-Smidt 2007). *L. monocytogenes* has been identified as third to *Campylobacter* and *Salmonella* infections as a food-borne

infectious agent contributing to the numbers of hospital bed days lost as well as the fourth most common cause of death (Barbuddhe et al. 2008). *Listeria* spp. are ubiquitous in nature and therefore can easily enter into food chain (Farber & Peterkin 1991; Haase et al. 2013; Schoder et al. 2013). Industrially processed and refrigerated foods revealed to be frequently linked to *L. monocytogenes* outbreaks than raw foods (Gianfranceschi et al. 2002; Nucera et al. 2010; Lomonaco et al. 2011). Though isolated in 1926 from gerbils, *L. monocytogenes* became noticeable after its first foodborne outbreak in 1981 in humans (Fleming et al. 1985). Since then, the organism has been reported from several food products, linked with outbreaks and deaths (Farber & Peterkin 1991; Kathariou 2002; Ramaswamy et al. 2007; Swaminathan & Gerner-Smidt 2007). Persistence of *L. monocytogenes* in food processing environment has been thought to be the most relevant cause of the contamination of food in the industries (Kathariou 2002). Research focused on persistence of *L. monocytogenes* in food industry revealed some of the characteristics of this pathogen such as stress tolerance, ability to grow at low temperature, ability to form biofilm and adapting capability. Biofilm formation ability and its relation to persistence of *L. monocytogenes* in food industry has been the emerging area of research being explored across the world.

Historically, Hülpers isolated bacteria from a liver necrosis in a rabbit in 1911 that were pathogenic for mice and called it *Bacillus hepatica* according to the isolation site (Hülpers 1911). In 1926, a bacteria was isolated by Murray, Webb and Swann from dead laboratory rabbits and guinea pigs exhibiting monocytosis and named it as *Bacterium monocytogenes* (Murray et al. 1926). Later, Pirie isolated this bacterium from wild gerbils with "Tiger River Disease" in South Africa and named as

Listerella hepatolytica to honor Lord Joseph Lister (Pirie 1927). Finally the organims renamed to '*Listeria*' in 1940 due to taxonomic reasons (Pirie 1940).

The genus *Listeria* belongs to the phylum *Firmicute,* the order *Bacillales,* the class *Bacilli* and the family *Listeriaceae* together with the genus *Brochotrix.* The *Listeria* are Gram-positive bacteria with low G+C content, closely related to *Bacillus, Clostridium, Enterococcus, Streptococcus,* and *Staphylococcus* (Barbuddhe et al. 2008). The genus *Listeria* has ten species including *L. monocytogenes, L. innocua, L. welshimeri, L. seeligeri, L. ivanovii, L. grayi* as well as four newly identified species that were reported in 2009 - *L. marthii* (Graves et al. 2010), *L. rocourtiae* (Leclercq et al. 2010) and in 2013 *L. weihenstephanensis* (Halter et al. 2013) and *L. fleischmanii* (Bertsch et al. 2013). While both *L. monocytogenes* and *L. ivanovii* infect vertebrate animals, *L. ivanovii* appears to be rare and predominantly causes disease in ruminants (Guillet 2010).

Based on serological reactions of listerial somatic (O-factor) and flagellar (H-factor) antigens with specific antisera, *Listeria* spp. are classified into serotypes or commonly names serovariants or serovars with *L. monocytogenes* comprising serovars – 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e and 7 (Chen & Knabel 2008). Using various genetic subtyping techniques, *L. monocytogenes* is separated into three lineages: lineage I contains serovars 1/2b, 3b, 4b, 4d, and 4e; lineage II contains serovars 1/2a, 1/2c, 3a, and 3c; and lineage III contains serovars 4a and 4c (Wagner & McLauchin 2008). A recent classification describes four lineages of *L. monocytogenes* with coincident niches : lineage I encompasses serotypes 1/2b, 3b, 4b and 3c; lineage II includes serotypes 1/2a, 1/2c, 3a, lineage III comprises serotypes 4a, 4b and 4c and lineage IV comprises 4a, 4b, 4c (Orsi et al., 2011).

Listeria spp. demonstrate considerable morphological, biochemical, and molecular resemblances and occupy similar ecological niches in the environment (Wagner & McLauchin 2008). Given their renowned ability to withstand arduous external conditions such as wide pH, temperature, and salt ranges there is no surprise that *Listeria* spp. are distributed in a diverse range of environments and have been isolated from soil, water, effluents, foods, wildlife, domestic animals as well as humans and mangrove ecosystems (Gorski 2008; Poharkar et al. 2013).

Being ubiquitously distributed in the natural environment, *Listeria* spp. invariably find their way into various food chains. Because of their ability to withstand extreme pH, temperature, and osmotic conditions, these bacteria remain largely unscathed after going through many food manufacturing processes (Liu 2008). In addition, food processing facilities can easily become contaminated by soil on worker's shoes, transportation and handling equipment, animal hides and raw plant material (Latorre et al. 2010; Jeyasekaran et al. 2011). *L. monocytogenes* gets added in the food by post-processing contamination, incubation of food at lower temperature and storage of contaminated food for longer period. Consumption of such contaminated foods have caused several outbreaks as well as sporadic cases (Lianou & Sofos 2007; Nucera et al. 2010; Cartwright et al. 2013).

Out of 10 species of *Listeria*, *L. monocytogenes* and *L. ivanovii* are pathogenic. *L. monocytogenes* is pathogenic to humans as well as to animals while *L. ivanovii* is pathogenic to animals and rarely to human (Guillet 2010). *L. monocytogenes* is a remarkable bacterium that has evolved over a long period of time during which the organism has acquired a diverse virulence factors, each with unique properties and functions. Its life cycle reflects its remarkable adaptation to

intracellular survival and multiplication in professional phagocytic and non-phagocytic cells of vertebrates and invertebrates (Barbuddhe et al. 2008).

The advent of genomics promoted an increasingly prolific identification and functional characterization of new *Listeria* virulence factors. The hemolysin gene (*hly*) was the first virulence determinant to be identified and sequenced in *Listeria* spp. Characterization of the *hly* locus led to discovery of the chromosomal virulence gene cluster at which most of the genetic determinants required for the intracellular life cycle of pathogenic *Listeria* spp. are located (Vázquez-Boland et al. 2001). The hemolysin produced by *L. monocytogenes* termed as listeriolysin O (LLO) has low optimum pH (5.5) and narrow pH range (4.5 to 6.5) (Geoffroy et al. 1987). To invade host cells, *Listeria* has two proteins, InlA and InlB, which have specific receptors on the host-cell surface, E-cadherin and Met, respectively (Bonazzi et al. 2009). Escape of *L. monocytogenes* from host cell vacuole gets mediated by phosphatidylinositol-specific phospholipase C (PI-PLC) (Leimeister-Wächter et al. 1992). The intracellular movement is facilitated by actin filament (ActA) (Tilney & Portnoy 1989). All these factors encoding genes that are necessary to invade mammalian system are organized in the 9.6 Kb gene cluster termed as "Virulence pathogenicity island 1" (LIPI-1) of *L. monocytogenes.* These genes in virulence cluster get controlled by a pleiotropic virulence regulator, PrfA (a 27-kDa protein encoded by the *prfA* gene). In addition to these virulence-associated genes and proteins, several other genes such as *iap, bsh, vip, inlJ, auto, ami,* and *bilA* are also contribute in virulence of *L. monocytogenes* (Barbuddhe et al. 2008).

The predominant mode of transmission of *L. monocytogenes* is via contaminated foods. Other routes include mother to fetus via the placenta or at birth

have been observed (Janakiraman 2008). Direct contact with diseased animals may lead to transmission to farmers and veterinarians during the delivery of domestic farm animals (CDC 2013). Nosocomial infections and person-to-person transmission (excluding vertical) are observed but rare (PHAC-ASPC 2012).

L. monocytogenes generally infects immune-compromised individuals such as pregnant women, neonates, children, elderly peoples etc. Others "At-risk" are cancer patient, dialysis patients, patient on immunosuppressive therapy and AIDS patients (Allerberger & Wagner 2010). The organism can tolerate the acidity of the stomach and pass to the intestine. *L. monocytogenes* breach endothelial and epithelial barriers of infected host. Once reached to intestinal lining, *L. monocytogenes* enters through enterocytes lining through ligand-receptor interaction (Vázquez-Boland et al. 2001) or by phagocytosis by the M cells of the Peyer's patches (Marco et al. 1997). The bacterium subsequently localize within professional phagocytes and antigen presenting cells (Lecuit et al. 2007). *In vivo* experiments show that *L. monocytogenes* rapidly disseminate from gut to mesenteric lymph node, presumably carried by dendritic cells (Pron et al. 2001). From mesenteric lymph node, *L. monocytogenes* disseminate to spleen and liver (Vázquez-Boland et al. 2001).

Listerial infections do not show any specific clinical symptoms. The infection is generally followed by initial flue like symptoms (e.g. chills, nausea, headache, vomiting and muscular and joint pain). In some cases gastroenteritis may be observed. Without appropriate antibiotic treatment, *L. monocytogenes* infection leads to septicemia, meningitis, encephalitis, abortions and death. These clinical features caused by *L. monocytogenes* infection are collectively termed as 'Listeriosis' (Low & Donachie 1997; Ramaswamy et al. 2007).

Similar to humans, in animals the infection of *L. monocytogenes* is asymptomatic. In animals, generally, domestic animals are found to be infected by *L. monocytogenes* through poor silage (Fensterbank 1984; Ryser et al. 1997). The *L. monocytogenes* infections in animals lead to encephalitis, abortion, gastroenteritis, and septicemia. Abortion is the most common form of listeriosis (Busch et al. 2001; Cabanes et al. 2008). The *L. monocytogenes* has also been found to cause mastitis. The infection of *L. monocytogenes* has also been reported to cause conjunctivitis, urethritis, endocarditis and disturbance of gait. The meningitis can be seen as circling disease (Hoelzer et al. 2012).

L. monocytogenes is generally sensitive to wide range of beta-lactam antibiotics (Temple & Nahata 2000; Morvan et al. 2010). Ampicillin, amoxicillin, tetracycline, chloramphenicol, β-lactam antibiotics, together with an aminoglycoside, trimethoprim, and sulfamethoxazole are recommended for the treatment of the listeria infection (Feng et al. 2013). Ampicillin is the drug of choice in cases of encephalitis. Ampicillin along with gentamicin is recommended for prolonged treatment regimens. *L. monocytogenes* infections are usually treated with a single antimicrobial agent and combined therapies are usually recommended for the treatment of immune-compromised patients (Ramaswamy et al. 2007).

Listeria spp. including human pathogen *L. monocytogenes* are ubiquitous in nature and therefore can easily contaminate the raw food (Farber & Peterkin 1991; Kathariou 2003). Since first major outbreak of 1981 from coleslaw (a regional salad dish in US), many researchers then explored different types of food products and isolated *L. monocytogenes* from food and food products such as milk (Koch et al. 2010; Jackson et al. 2011; D'Costa et al. 2012; Gelbícová & Karpísková 2012;

Giacometti et al. 2012), milk products (Fretz et al. 2010; Derra et al. 2013), different types of meats (Rahimi et al. 2010; Hosseinzadeh et al. 2012; Wang et al. 2012; Zhu et al. 2012; Derra et al. 2013; Lamden et al. 2013), fishes (Meloni et al. 2009; Pouillot et al. 2009; Gillespie et al. 2010; Yücel & Balci 2010; Kovačević et al. 2012; Lambertz et al. 2012; Nakamura et al. 2013) and raw vegetables (Cordano and Jacquet 2009; Aparecida de Oliveira et al. 2010; Mercanoglu et al. 2011; Ananchaipattana et al. 2012). Of these, foods those are industrially processed and refrigerated revealed to be frequently linked to *L. monocytogenes* outbreaks than raw foods (Gianfranceschi et al. 2002). Food industries deal with receiving raw food, bactericidal treatment and packaging of desired final products. The raw food material received may contain *L. monocytogenes* (Thimothe et al. 2002; Gelbícová and Karpísková 2012; Ning et al. 2013), however, the bactericidal treatments (e.g. pasteurisation, addition of preservatives, Clean-In-Place) performed to increase the shelf life of the product kills *L. monocytogenes* along with other bacteria. Interestingly, even after bactericidal treatment, *L. monocytogenes* found to contaminate the final food product (Lianou and Sofos 2007; D'Costa et al. 2012). The main reason behind contamination has been thought to be persistence of *L. monocytogenes* at post-processing environment (PHAC 2009; Beresford et al. 2001; Latorre et al. 2010). Though harsh sanitisation employed to environment, several reports showed that *L. monocytogenes* could enter through different routes such as exchange of workers from different departments, water used to clean, and commonly used equipment (ADASC 1999; Ivanek et al. 2004; Maitland et al. 2013). Once entered, depending upon the environmental conditions and capability of the organism, *L. monocytogenes* strains has been thought to persist in the environment and contaminate the food getting processed (Farber and Peterkin 1991).

Listeria spp. have been isolated from diverse environmental sources such as soil, water and vegetation (Liu 2008), which thought to act as the very first source to that lead to contamination of food chain (Nightingale et al. 2004). Carrying raw food from farm till the industrial level is one of the critical point from microbiological point of view. As depending upon the condition, nature and hygiene during food harvest, the fate of growth of *L. monocytogenes* gets decided (D'Costa et al. 2012). Raw food gets processed for antimicrobial treatments and microbial load gets reduced. The food being processed may get contaminated at food industrial environment due to workers hands, inadequately cleaned site, biofilm and water used (Manoj et al. 1991; Schönberg & Gerigk 1991). From the reports for contamination of industrially processed food by *L. monocytogenes*, it apparently looks like that food industry acting as a major source of *L. monocytogenes* than raw food products. Besides the animal originated RTE foods, leafy vegetables and fruits also may harbor *Listeria* from environment (Park et al. 2012).

Once entered in the food industry, depending upon the nutrient compatibility and availability, *Listeria* spp. may get established in the food industry. Proliferation of *Listeria* is promoted by high humidity and residues of nutrients in certain food production plants (Schönberg & Gerigk 1991). The washed residues of raw food material being processed may get dissolved in water or form the soil, which may get utilized by *L. monocytogenes* cells. Out of ten *Listeria* spp., *L. monocytogenes* has been studied significantly for its persistence in the food industry. Some *L. monocytogenes* strains have been observed to cause food plant contaminations over longer periods of time exhibiting persistence. Several processed foods such as milk, meat, fish and vegetables have been reported to have persistent *L. monocytogenes* strains (Almeida et al. 2010; Nakamura et al. 2013; Stessl et al. 2013; Vongkamjan et

al. 2013). However, not all *L. monocytogenes* strains found to cause persistent contamination, some strains are persistent and are found recurrently and others are non-persistent and only recovered sporadically (Lundén 2004).

Persistence of listerial strains in the food and food processing environment may lead to contamination of the products. *L. monocytogenes* may get colonized at different sites in the food industry (Carpentier & Cerf 2011). Colonization that occurs at food contact surface may add bacterial cells to processed food (Djordjevic et al. 2002). *Listeria* spp. those are in biofilm may detach and establish a new arena increasing bacterial colonization in the food processing plants (Valderrama & Cutter 2013). Such increased number of colonization increases chances of contamination of the food. Once established, such persistent bacteria are hard to detect and remove (Schönberg & Gerigk 1991). Also, persistent bacteria get adapted to the environment and can grow at sub-optimal conditions. Eventually, they evolve for resistance to disinfectants being employed (Aase et al. 2000; To et al. 2002;).

Several bacterial characteristic have been identified in relation to persistence of *L. monocytogenes*. The basic growth and physiological characteristics such as survival ability at harsh environment and enhanced tolerance capability increased the chances of persistence (Carpentier & Cerf 2011; Newell et al. 2010; Møretrø & Langsrud 2004). Similarly, *L. monocytogenes* have been found to possess characteristics such as ability to withstand extreme pH (4.5-9.5), growth at low temperature (upto 4^0C), low water activity (0.92 a_w) and high salt tolerance (upto 12%) which makes them suitable to survive in food industrial environment (Barbuddhe et al., 2008). These characteristics allow *L. monocytogenes* to tolerate, survive and multiply even at harsh conditions present at the food industry leading to

persistence. Beside these abilities, innate abilities such as antimicrobial and biofilm formation capability have been thought to contribute at greater extent for the persistence (Verghese et al. 2011). In a study, *comK* prophage junction fragments analysis indicated that extensive recombination occurs for the persistence at particular genomic site termed as rapid adaptation island (RAI). Genes within the RAI are re-characterized as "adaptons," as these genes may allow *L. monocytogenes* to rapidly adapt to different food processing facilities and foods (Verghese et al. 2011). Therefore, persistence of bacteria seems to be result of several different characteristics that *L. monocytogenes* strains possess.

For each individual food-processing plant, a limited number of bacterial clones may become established and persist for years. Microorganisms growing in biofilms are protected against cleaning and disinfection and are difficult to eradicate. *L. monocytogenes* may grow in biofilms that protect them against environmental stress and can be isolated from surfaces after cleaning and disinfection (Czaczyk & Myszka 2007). Biofilm formation ability gives several advantages to the cells such as in biofilm, bacterial cells are present in dense manner, the number of cells are more as compared to the planktonic cell, biofilm formed by specific bacteria get spread in nearby areas and therefore more likely to cause the persistence (Flemming & Wingender 2010). Though, biofilm has been suspected to play a significant role in persistence of many bacteria including *L. monocytogenes* (Borucki et al. 2003; Pan et al. 2006); the studies available till date for biofilm and its role in persistent of bacteria are conflicting and inconclusive. Few researchers have reported persistence cells as a good biofilm formers (Latorre et al. 2011), while some researchers did not find any relation between persistence and biofilm formation (Møretrø & Langsrud 2004; Giaouris et al. 2013). Generally, the biofilm former cells

and non-former cells may be isolated from single source simultaneously which are indistinguishable in their morphological and physiological characteristics. Such strains obtained repeatedly may not be always associated with the food industry or biofilm but may get entered continuously from single source (da Silva & De Martinis 2013). Therefore the question remains, whether biofilm has any role in persistence?

In several countries, criteria or recommendations for tolerable levels of *L. monocytogenes* in ready-to-eat (RTE) foods have been established (Gravani 1999). The USA and Italy require absence of *L. monocytogenes* in 25 g of foods (zero tolerance) while many European countries (Germany, The Netherlands and France) have a tolerance of below 100 cfu/g at the point of consumption. Canada and Denmark have a tolerance limit below 100 cfu/g for some food products, and zero tolerance for those which are supportive of growth and having extended shelf-lives. Several countries have concluded that a complete absence of *L. monocytogenes* for certain RTE foods is an unrealistic and unattainable requirement that limits trade without having a positive impact on public health and consequently might detract resources from other potentially more efficient measures against *L. monocytogenes* (Amalaradjou et al. 2009; Nørrung 2000). FDA has made it mandatory to recall the food product if *L. monocytogenes* is found in the final food products (FDA 2013).

In food industry, biofilm formation causes serious problem such as impeding the flow of heat across the surface, increase in the fluid frictional resistance at the surface, and increase in the corrosion rate at the surface leading to energy and product losses (Kumar & Anand 1998). In addition, the biofilms, including spoilage and pathogenic microflora formed on the food surfaces like that of milk, poultry, other meat surfaces and in processing environments also offer considerable problems of

cross-contamination and post-processing contamination (Chorianopoulos 2012; Ryu & Beuchat 2005; Srey et al. 2013). Such post processing contamination adds bacteria in processed food. If any pathogen is observed in final food products, the entire batch has to be recalled causing economical loss for the industry. Also, occurrence of such pathogen in marketed product or presence of pathogen even in food industrial premises have led to shut down of several food industries (FDA 2013). Therefore, biofilm formation by bacteria, particularly pathogens at food industrial premises is a matter of concern for economy as well as public health.

Disinfectants are chemicals or mixture of chemicals that have been used to eliminate undesirable microbial load from the food industry (Rutala et al. 2008). With the repeated and prolonged use of disinfectants, resistant or tolerant bacteria evolve under selective pressure. The resistance to disinfectant can be defined as the situation where bacterial cells are not killed or inhibited by a concentration of disinfectant that acts upon the majority of cells in that culture (Wessels & Ingmer 2013). In food industry, disinfectants such as quaternary ammonium compounds (QAC), iodophore, peracetic acid (PAA), etc. are used commonly, among QAC based Benzalkonium chloride (BC) is the most commonly used disinfectant in the food processing industry because of its high microbicidal power at lower concentration. Several mechanisms have been identified for the resistance or tolerance to disinfectants. The most widespread mechanism leading to decreased susceptibility to QAC is increased efflux pump activity. Although other mechanisms may be involved such as altered fatty acid composition and changes in the bacterial membrane (Wessels & Ingmer 2013). *L. monocytogenes* is one of the primary targets of disinfection in food and feed production. For these reasons, *L. monocytogenes* is the object of concerted disinfection with QACs in many food and feed industries. However, an additional and

confounding factor for combatting the pathogen is its propensity for reduced susceptibility to the QACs. This is well documented and seems primarily to be due to increased expression of its efflux pumps relative to more susceptible strains (Romanova et al. 2006; To et al. 2002; Wessels & Ingmer 2013).

The microbes residing in food processing environment should be removed or killed by washing procedures and disinfectant used. But such killing/removing does not happen in practical. Logically, either biofilm formation or resistance to disinfectant should be the reason behind survival and therefore caused persistence. In turn, this resistance to disinfectant could be due to the innate resistance capability or due to the biofilm formation ability of *L. monocytogenes*. The data available till date is not conclusive to determine whether the innate ability or the biofilm formation capability cause persistence of *L. monocytogenes*. The available literature strongly suggests that biofilm formation capability and therefore exhibited resistance must be contributing *L. monocytogenes* to persist. Therefore a research question comes – "Is it the innate capability or an attribute of biofilm that allows *Listeria* spp. to resist the disinfectant and cause persistence at food processing industry?" To solve this question we proposed the hypothesis as "It's the biofilm forming capability of *Listeria* spp. at food industrial premises, causes resistance to disinfectant and therefore lead to persistence". To solve this hypothesis, we proposed with objectives as……..

OBJECTIVES:

1) Isolation of *Listeria* spp. from food processing plants.
2) Characterization of the isolates by biochemical and *in-vitro* pathogenicity analysis
3) To investigate biofilm producing ability of *Listeria* species from the food and food processing plants.
4) To investigate the disinfectant resistance of *Listeria* species from the food and food processing plants.

Chapter 2:

Isolation and Identification of *Listeria* spp. from food processing plants

2.1 Introduction:

With the change in the life style, ready-to-eat foods are in demands. Several food products from the food processing industries have become part of daily life. With the increase in the food industries, the problems associated with the food industries are becoming evident. Natural food microflora is one of the major problems that food industry has to deal with. The innate microbial flora, if not removed properly causes deterioration of food (Adley 2006; Bhunia 2008; Quigley et al. 2013) while, if flora contains microbial pathogens, are hazardous to public health (Jackson et al. 2012; Liu et al. 2013; Neo et al. 2013). The studies performed over last three decades suggest that such pathogens enter in the food processing environment, utilise nutrients and establish themselves in a small niche (Carpentier & Cerf, 2011). Such establishment of microbes acts as microbial reservoir and contaminates the food being processed (Behravesh et al. 2011). Therefore, to control such spread of pathogens, government bodies have specified rules and regulations (FDA-BAM 2013). It is mandatory for all the food industries to confirm the food as 'pathogen-free' till it reaches to the consumer level. All food industries need to test the absence of bacterial pathogens such as *Salmonella, Shigella, E. coli, Campylobacter, Clostridium perfringens, Bacillus cereus, Vibrio, Staphylococcus aureus* and *Listeria monocytogenes* (Robach 2012; FDA-BAM 2013). In India, as per Food Safety and Standards Regulations 2011, standards for *L. monocytogenes* in industrially processed foods of animal origin have been prescribed (MHFW 2011) which demands the absence of *L. monocytogenes* in 25 gm of food samples.

Isolation and identification of bacteria give a validation for the presence of particular bacteria in the given area. Historically, it has been challenging to isolate *Listeria* spp. from food or other samples and this explains why it remained unnoticed

as a major foodborne pathogen until recently (Gasanov et al., 2005). Since first outbreak of *L. monocytogenes* in 1981, there has been a constant search for more rapid and sensitive methods for detection and isolation, particularly in the food industry. Previously, based on clinical procedures direct plating onto blood agar was performed which remained partially successful (Gasanov et al. 2005). Since then different culture methods have been introduced based on the specific growth characters and nutritional requirement of *L. monocytogenes*. Of the known ten species of *Listeria*, *L. monocytogenes* is a human pathogen and therefore detection of *L. monocytogenes* in food products is very important. *L. monocytogenes* easily enter into food chain and such contaminated food is a public health hazard as several outbreaks have been reported due to the presence of *L. monocytogenes* in food (Fleming et al. 1985; Piffaretti et al. 1989; Jacquet et al. 1995;de Valk et al. 2001; Kathariou 2003; CDC 2012; Cartwright et al. 2013). Therefore, regulatory bodies made it mandatory to screen random food samples to ensure absence of *L. monocytogenes* (FDA 2012; FSIS-USDA 2008; USDA 2013).

2.2 Review of Literature

2.2.1 Isolation of *Listeria* spp.

Out of 10 *Listeria* spp. known, *L. monocytogenes* is pathogenic to humans and therefore detection of *L. monocytogenes* in food becomes significant from public health point of view, while detection of other species of *Listeria* is significant as an indicator for the possible presence of *L. monocytogenes*. Several government bodies has made it mandatory to detect *L. monocytogenes* in industrially processed food (FDA 2012; FDA-BAM 2013; MHFW 2011). Earliest method for isolation of listeriae was cold enrichment. The isolation of *L. monocytogenes* used to carry out on blood

agar incubating plates at 4^0C till the colonies appear. However, the disadvantage of the method was it used to take several weeks to get isolated colonies (Gasanov et al. 2005). Also the method did not allow the growth of "injured" cells. Beside this, several other psychrotrophic non-pathogenic organisms were growing, making it difficult to identify *L. monocytogenes*. Since *Listeria* spp. are comparatively fastidious to grow, other common bacteria were outgrowing *L. monocytogenes* or *Listeria* spp.. This problem was addressed by addition of antibacterial such as acriflavin and nalidixic acids in the media (Welshimer 1981). Acriflavin inhibit the fungal growth as well as several Gram positive bacteria, while nalidixic acid is universal Gram negative bacterial inhibitor. Since antibacterial supplements are introduced, it has been employed till date in growth medium used to isolate *L. monocytogenes* and other *Listeria* spp.

As per most of the regulatory agencies, isolation method must be capable enough to detect one *L. monocytogenes* organism per 25 g of food (Jantzen et al. 2006). This sensitivity can only be achieved by using enrichment methods. Two methods are widely used for isolation of *Listeria* (i) Food and Drug Administration agency, Bacteriological and analytical method (FDA-BAM) and (ii) International Organization of Standardization (ISO) 11290 method (Hitchins 2001). In FDA-BAM method, the sample is enriched in the pre-enrichment broth at 30^oC for 48h. To avoid the contamination by fungal growth the broth contains cycloheximide as antifungal agent, in addition to acriflavin and nalidixic acid. Enriched broth then plated onto selective agar such as Oxford, PLACAM, MOX or LPM. The ISO-11290 method has two stage enrichment process: the food samples is first enriched in half Fraser broth for 24h, then an aliquot is transferred to full strength Fraser broth for further enrichment followed by isolation on selective agar as mentioned above. United States

Department of Agriculture (USDA) and Association of analytical Chemist added third method to recover environmental samples by using two stages University of Vermont (UVM) broth enrichment. Besides these three commonly used methods, several other methods for isolation of *Listeria* spp. from food gained acceptance for international regulatory purpose. The ISO-11290 is worldwide used and recommended for detection of *L. monocytogenes* in food samples (Jantzen et al. 2006); while in the United States FDA-BAM method is preferred. USDA method is preferred to isolate *L. monocytogenes* from food environmental samples. In India, Food Safety and Standards Authority has approved ISO-11290 method for the isolation of *Listeria* spp. from food (FSSAI, 2012). Besides these methods, several commercial direct *L. monocytogenes* detection systems such as biochemical based - API *Listeria*, Vitek System, Micro-Id *Listeria*, MicroLog system, Microbact system, Sherlock Microbial identification system; Immunoassay based – VIDAS LMO, Transia Plate *Listeria monocytogenes*; Molecular – Gene Trak and Gene Quench *Listeria*, AccuProbe*Listeria*, BAX, TaqMan *L. monocytogenes*, Gene vision etc. are in practice as per researcher's interest (Jantzen et al. 2006).

2.2.2 Differentiation of pathogenic and non-pathogenic *Listeria* spp.

Isolation methods described for *Listeria* spp. do not distinguish pathogenic and non-pathogenic strains. Taking the advantage of virulence characters that are exclusively present in pathogenic spp. of *Listeria*, several different approaches have been made to differentiate the pathogenic species from non-pathogenic. Earlier, hemolysis on 5% sheep blood agar was the key step to differentiate pathogenic spp. followed by fermentation of D-xylose and L-rhamnose to differentiate *L. monocytogenes* and *L. ivanonvii* (Rocourt et al. 1983). However, with the knowledge of exceptional strains of *L. seeligeri* causing hemolysis (Leimeister-Wächter &

Chakraborty 1989) there was a need for the more discrimination. Therefore, the ability of virulent strain to produce phosphatidylinositol-specific phospholipase C (PI-PLC) used to differentiate *L. monocytogenes* and *L. ivanovii* by incorporating PI-PLC substrate in media. The pathogenic spp. growing on such agar media degrades PI-PLC substrate showing halo formation (Notermans et al. 1991). However to confirm the virulence, mouse pathogenicity test (Kaufmann 1984) and the chick embryo test was mandatory (Steinmayer et al. 1989). To differentiate *L. monocytogenes* and *L. ivanovii* 'CAMP' test (Christie Atkins Munch Petersen test) is preferred method because of its reliability and reproducibility (McKellar 1994). PI-PLC combined with a chromogenic substrate (5-bromo-4-chloro-3-indolyl-β-D-glucopyranoside, X-gluc) for β-D-glucosidase activity in 'Agar *Listeria* according to Ottaviani and Agosti' (ALOA) enhanced the differentiation (Ottaviani et al. 1997). On ALOA, all *Listeria* spp. produce turquoise colonies and the pathogenic species appeared surrounded with a distinct precipitation zone (Reissbrodt 2004). Alanyl peptidase – an enzyme produced by *Listeria* spp. except by *L. monocytogenes* has been used in commercial kit – 'The MonocytogenesID' (Biolife) to differentiate *L. monocytogenes* and other spp. (Clark & McLaughlin 1997). In O.B.I.S. (Oxoid biochemical Identification System) suspected *L. monocytogenes* colonies get differentiated within 10 min. (Jantzen et al. 2006). Besides biochemical differentiation, molecular method such as PCR based virulence gene detection also has been used to directly discriminate pathogenic and non-pathogenic *Listeria* spp. (Rawool et al. 2007).

2.2.3 Differentiation / Identification of *Listeria* spp.

Listeria spp. identification is generally performed by few sugar fermentation (L-rhamnose, D-mannitol, D-xylose and α-D-methyl-mannoside) combined with biochemical tests (catalase and oxidase) (Barbuddhe et al. 2008; Gorski 2008; Huang

et al. 2007). These biochemical can be performed manually or by commercial kits. The best know kit is 'API kit' which has been developed and validated by FSIS-USDA (FSIS-USDA 2013). API *Listeria* (bio-Merieux) and Micro-ID (OrganonTeknika) are commercially kit based on batteries of biochemical that accurately differentiate all the *Listeria* spp. (Muñoz 2012; Nyenje[a] et al. 2012; Zhang et al. 2012; Jahan et al. 2013).

Table 2.1: Biochemical tests used to differentiate *Listeria* spp. (Barbuddhe et al. 2008; Liu, 2008, Graves et al. 2010; Leclercq et al. 2010; Bertsch et al. 2013; Halter et al. 2013;)

***Listeria* spp.**	Gram Staining	Catalase	Oxidase	Hemolysis	CAMP test *S. aureus*	CAMP test *R. equi*	PI-PLC	L-rhamanose	D-Mannitol	D-xylose	α-D-Methyl-Mannoside
L. monocytogenes	+	+	-	+	+	+	+	+	-	-	+
L. innocua	+	+	-	-	-	-	-	+	-	-	+
L. ivanovii	+	+	-	+	-	+	+	-	-	+	-
L. seeligeri	+	+	-	+	-	-	-	-	-	+	-
L. welshimeri	+	+	-	-	-	-	-	+	-	+	+
L. grayi	+	+	-	-	-	-	-	+	+	-	+
L. rocourtiae	+	+	-	-	-	-	-	+	+	+	+
L. marthii	+	+	-	-	-	-	-	-	-	-	+
L. fleischmannii	1	1	0	0	0	0	0	1	1	0	0
L. weihenstephanensis	1	1	0	0	0	0	0	1	1	1	0

2.2.4 Occurrence of *Listeria* spp. in food and food processing units

Listeria species have been reported to contaminate almost all the food that are processed industrially and contains moisture (da Silva & De Martinis 2013). The first outbreak due to *L. monocytogenes* was reported due to implicating improper

pasteurisation of milk (Fleming et al. 1985). Similar to many other bacteria, *Listeria* spp. easily utilise the nutrients from milk and easily grow. *Listeria* spp. has been widely reported to contaminate the raw milk and milk processing industries with the 0.5 to 30% of positive samples (Muyanja 2011; Derra et al. 2013; Giacometti et al. 2013). All dairy farms authorized to produce and sell raw milk in a province of Northern Italy showed 0.5% of *L. monocytogenes* (Giacometti et al. 2012) in in-line milk filters. In a study performed in Ethiopia, *Listeria* spp. were found to be prevalent in 27.5% of milk product samples of which 4.1% were *L. monocytogenes* (Derra et al. 2013). In another study, 13.6% of industrial cheese samples were found contaminated (Almeida et al. 2010). Up to 20% milk storage tanks samples were found to be contaminated by *Listeria* spp. (Waak et al. 2002). Mahmoodi et al. (2010) studied two milk processing plants from Iran, and found 3.3% and 6.7% of prevalence of *Listeria* spp. In Uganda, 30% of industrial milk samples were found to positive for *Listeria* spp. (Muyanja 2011). Cheese is the most frequently reported to be contaminated by *L. monocytogenes* and responsible for various outbreaks (Lambertz et al. 2012). In bulk milk samples, low prevalence (2.1%) of *L. monocytogenes* was reported (Navratilova et al. 2004). A similar frequency of findings of *L. monocytogenes* (0–5%) in bulk tank milk samples has been reported from countries such as Austria (1.5%) (Deutz et al. 1999), Spain (3.6%) (Gaya et al. 1996) and West Indies (1.7%) (Adesiyun & Krishnan 1995). In India also, the prevalence of *Listeria* spp. in raw milk as well as final products have been reported (Kalorey et al. 2008; D'Costa et al. 2012).

Similar to milk industry, *L. monocytogenes* also has been found to be prevalent invariably in meat processing industry (Lambertz et al. 2012). *L. monocytogenes* has been isolated from poultry (Kosek-paszkowska et al. 2005; Cartwright et al. 2013; Zhao et al. 2013), pork (Bonardi et al. 2002; Thévenot et al.

2006; Bērziņš et al. 2010; Ochiai et al. 2010), beef (Rivera-Betancourt et al. 2004; Ochiai et al. 2010; Meyer et al. 2011; Gebretsadik et al. 2011; Dmowska et al. 2013; Hasegawa et al. 2013). In meat industry, prevalence of 65.6% for *Listeria* spp. (Zhu et al., 2012) and 29.1% for *L. monocytogenes* have been reported (Nicolas et al. 1989). A study in France showed 29.1% of meat products were contaminated by *L. monocytogenes* which were involved in the several human outbreaks (Nicolas et al. 1989). In a prevalence study of raw and cooked poultry processing environments, 46% and 29% of the samples contained *Listeria* spp. while 26% and 15% contained *L. monocytogenes*, respectively (Lawrence & Gilmour 1994). In a study performed in meat industry from China, the overall prevalence of 65.6% and 26.4% were reported for *Listeria* spp. and *L. monocytogenes* respectively (Zhu et al. 2012). A longitudinal study conducted to track listerial contamination patterns in ready-to-eat meats from meat processing plants located in three states in USA showed total 9.5% of samples to be positive for *Listeria* spp. while 6.1% samples were positive for *L. monocytogenes* (Williams et al. 2011). Raw meat market survey in Bangkok showed 15.4 % of meat samples positive for *L. monocytogenes* (Indrawattana et al. 2011). Though these raw meat products were reported positive for *L. monocytogenes*, cooked meat products did not show presence of *L. monocytogenes* (Kosek-paszkowska et al. 2005).

As compared to milk and meat, prevalence of *L. monocytogenes* in fish and fish processing environment is less. *Listeria* spp. often exist in raw fish material from water with farms and human settlement nearby (Liu 2008; Dhanashree et al. 2003; Jeyasekaran et al. 2011). A study carried out in fresh seafood samples (fish and shellfish) marketed in Zagazig city, Egypt showed 28.2% of prevalence of *L. monocytogenes* (Ahmed et al. 2013). After increase in cases of listeriosis, the survey

carried in Europe revealed 12% of fish samples were positive for *Listeria* (Lambertz et al. 2012). *L. monocytogenes* was found in 14% of both gravad and cold-smoked fish samples and in approximately 2% of hot-smoked fish samples (Lambertz et al. 2012). A study performed in Italy to investigate the sources of *L. monocytogenes* contamination in a cold smoked salmon processing environment over a period of six years (2003-2008) revealed 24% of the raw salmon samples, 14% of the semi-processed products and 12% of the final products were positive for *L. monocytogenes* (Di Ciccio et al. 2012). In an investigation of RTE meat and fish products in Vancouver, British Columbia (B.C.) *Listeria* spp. were recovered from 20% fish samples while *L. monocytogenes* was present in 5% of samples (Kovačević et al. 2012).

In case of India, *L. monocytogenes* has been reported from variety of raw as well as processed food. Dhanashree et al., (2003) have reported 5.5% of sample positive for *Listeria* spp. from food such as raw milk, meat and vegetables. The raw sea food collected from local market showed 9% occurrence of *L. monocytogenes* (Parihar et al. 2008). In recent study, Soni et al. (2013) observed 5.8% of cow milk sample positive for *L. monocytogenes.* Gawade et al. (2011) have reported 4.5% of sea food sample positive for *L. monocytogenes.* In case of food processing industrial environment, very few studies with respect to incidences of *L. monocytogenes* have performed in India. Jeyasekaran et al. (2011) have shown 4.2% sample from fish processing industry positive for *L. monocytogenes*. D'Costa et al. (2012) have shown the occurrence of *L. monocytogenes* from raw milk collection to the end product.

The literature suggests that *L. monocytogenes* is prevalent in food and food processing industry across the world. Apparently, the main reason behind the contamination seems to be the post-processing persistence of *Listeria* in food

processing environment. Such persistence could be an attribute of biofilm formation. Therefore, to determine such persistence in the food industry an attempt was made to isolate the *Listeria* spp. from the food processing plants.

2.3 Materials and methods:

2.3.1 Standards

Standard cultures of *Listeria monocytogenes* (MTCC 1143), *Staphylococcus aureus* (MTCC 1144), *Rhodococcus equi* (MTCC 1135) were obtained from Microbial Type Culture Collection Center, Institute of Microbial Technology (IMTECH), Chandigarh, India. A set of 12 *L. monocytogenes* of serotypes- 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e and 7 from Indian *Listeria* Culture Collection (ILCC), ICAR Research Complex for Goa, Goa was used as standards. Standard strains preserved in 30% glycerol at 4^0C were recovered in freshly prepared from Brain Heart Infusion (BHI) broth by growing at 37^oC for 18 h. A loopful of suspension was streaked on the PALCAM agar and plates were incubated at 37^oC for 24 h. A well isolated single colony was considered for the study.

2.3.2 Sampling

To determine the prevalence of *Listeria* spp. three milk processing plants situated approx. 250 Km distant from each other in Goa and Maharashtra, India were sampled. A total of 210 swab samples from milk processing environment including pre-pasteurization and post-pasteurization area were taken. The sampling areas were chosen which is more likely to contaminate the food. The samples were collected after the cleaning and sanitation of the food plant as per guidelines of Bureau of Indian Standards, IS 7005:1973 code of hygienic conditions for production, processing, transportation and distribution of milk. Each of the milk processing plant was visited twice for collection of samples. The plants are independent, managed by different agencies, and no plant workers get exchanged. Sterile cotton swabs from Hi-culture collection device (Hi-Media Labs, Mumbai, India) were moistened with sterile physiological saline (0.85% NaCl) at the sampling place and approx. 50 cm^2 area was

swabbed (Graham 2004). These swabs were then placed back into the collecting device. All the collecting devices were kept in icebox, transported to laboratory and processed within 24 h of collection. Details of sample locations of swabs are given in Table 2.2.

Table 2.2: Details of collection of samples in milk processing environment.

Sample source	No. of samples
Raw milk collector	27
Milk filler	33
Milk silo	25
Cheese blender	27
Product blender	24
Butter storage vessel	25
Buttermilk mixer	8
Floor	6
Drains	8
Milk can	19
Bulk milk Tanker	8
Total	**210**

2.3.3 Isolation of *Listeria* spp.

Isolation of *Listeria* spp. was attempted as per United States Department of Agriculture (USDA) method described for food environmental samples (FSIS-USDA 2013; Donnelly & Baigent 1986). The swabs were inoculated into 10 ml University of Vermont Medium-1 (UVM-1) and incubated at 30^{o}C for 24 h. Further enrichment of *Listeria* was carried by inoculating 0.1 ml of UVM-1 in 10 ml of UVM-2 containing high amount of acriflavin. Inoculated UVM-2 broth was incubated further for 24 h at 35^{o}C. A loopful of enriched broth of UVM-2 was streaked directly on PALCAM agar for selective isolation of listerial colonies. The inoculated agar plates were incubated at 37^{o}C for 48 h. A single isolated pinpoint grayish-green colonies surrounded by black zone of esculin hydrolysis were presumed as of *Listeria* spp. A well isolated single colony of presumptive listerial isolates was grown in 1 ml of BHI broth for 18 h at 37^{o}C and preserved at 4^{o}C for further study.

2.3.4 Identification

The suspected *Listeria* isolates were processed for standard biochemical described to identify the species (Barbuddhe et al. 2008). Preliminary identification was carried by analyzing isolates for their Gram character, catalase test, oxidase test and sugar fermentation (rhamnose, xylose, mannitol and a-methyl- D-mannopyranoside). Tentatively identified, *L. monocytogenes* isolates were further tested for the hemolysis on 5% sheep blood agar, CAMP test with *S. aureus* and *R. equi*, growth on ALOA agar and PI-PLC activity as per following details….

2.3.4.1 Hemolysis on Sheep Blood Agar

All the *L. monocytogenes* isolates that were confirmed using biochemical tests were analyzed for the type of hemolysis on 5% sheep blood agar (SBA) as per the

method described previously (Seeliger & Jones 1986). The isolates were streaked onto 5% SBA plates and incubated at 37^{O}C for 24 h and examined for hemolytic zones around the colonies. The characteristic β-hemolysis in the form of clear zone of hemolysis represented *L. ivanovii* while, a narrow zone of β-hemolysis was the characteristic of *L. monocytogenes*.

2.3.4.2 Christie-Atkins-Munch-Peterson (CAMP) test

All the presumptive *Listeria* isolates were tested by Christie-Atkins-Munch-Peterson (CAMP) test (McKellar 1994). Briefly, the standard strains of *Staphylococcus aureus* and *Rhodococcus equi* grown in BHI broth for 18 h/37^{0}C were streaked on sheep blood agar (SBA) plates having 7% sheep blood in a manner that these were wide apart and parallel to each other. The test cultures were streaked parallel to one another, but at right angles to and between the *S. aureus* and *R. equi* streaks. After incubation at 37^{o}C for 24-48 h, the plates were examined for hemolysis. *L. monocytogenes* hemolytic reactions were enhanced in the zone influenced by the *S. aureus* streak. The other species remained non-hemolytic.

2.3.4.3 ALOA assay

"Agar *Listeria* according to Ottaviani and Agosti" (ALOA) assay, an alternative way to assess PI-PLC activity was carried out using Chromogenic media, ALOA which helped to differentiate pathogenic *Listeria* species (Ottaviani et al. 1997). The biochemically characterized 41 *Listeria* isolates were assayed for PI-PLC activity on chromogenic ALOA (Hi-media, Mumbai, India) media. In brief, the *Listeria* isolates were grown overnight onto SBA plates at 37^{O}C. The growth of each *Listeria* isolate obtained from SBA plate was spot inoculated on ALOA plates. On this medium, all the *Listeria* species form bluish green colonies due to the presence of

a chromogenic compound X-glucosidase which detects β-glucosidase present in all *Listeria* species. Typical colonies of *L. monocytogenes* in ALOA agar are green-blue in colour, surrounded by an opaque halo.

2.4 Results and Discussion

2.4.1 Isolation of *Listeria* spp.

The presence of *Listeria* spp. has been demonstrated in a variety of food and food processing environment (Meloni et al. 2009; Atil et al. 2011; Ahmed et al. 2013). *Listeria* spp. enter into food industries by several sources such as raw food, wash water, plant workers etc. (Pagotto et al. 2006). The food industry deals with the processing of raw food to minimize the bacterial load, removal of pathogen, preparation of desirable product, increase shelf life and packaging of final products in user-friendly manner. Though the raw foods are treated for elimination of bacterial load by processes such as pasteurization, heat-treatment, addition of antimicrobials and gamma-ray treatment, chances of post-processing contamination cannot be denied. Such post-processing contamination caused by the bacteria that are persistent in food industry (Malley et al. 2013; Carpentier & Cerf 2011), while persistence have been thought to cause because of biofilm formation ability (Møretrø & Langsrud 2004). Isolation of *Listeria* spp. from samples on milk processing line was carried out. A total of 210 swabs samples were collected from areas which are more prone to come in contact with the food. A total of 41 (19.52%) presumptive *Listeria* spp. isolates were recovered by method defined by USDA (Fig. 2.1; Table 2.3). A single isolated colony from PALCAM agar plate of each sample was taken for further study. These 41 *Listeria* spp. isolates were obtained from raw milk collector (n=9), milk filling machine (n=6), pasteurized-milk silo (n=10), cheese blender machine (n=5), product (shrikhand) blender (n=5), butter storage vessel (n=2), butter milk mixer (n=3) and floor (n=1). None of the sample from drain, milk can and bulk milk tanker was found positive for *Listeria* spp. The presence of *Listeria* spp. in such environment have been reported previously (Almeida et al. 2013; Derra et al. 2013; Latorre et al.

2010). Presence of *Listeria* spp. at raw milk collector is not surprising as the raw milk itself may contain *Listeria* spp. (Kalorey et al. 2008; Le Monnier & Leclercq 2009; Schoder et al. 2011). From milking the animal at farm to raw milk collector at dairy level, several sources of *Listeria* contaminate the milk (Le Monnier & Leclercq 2009; Latorre et al. 2010). It was significant to notice the prevalence of *Listeria* spp. at post-pasteurization area. Milk filling machine, pasteurized-milk silo, cheese blender machine, product (shrikhand) blender, butter storage vessel and butter milk mixer contains direct food contact surfaces and therefore listerial contamination of food cannot be denied. Moreover, milk silo was found to contaminate with the *Listeria* spp. Milk silos are maintained at 4^{o}C, at which psychrotropic listeria gets selectively enriched. The milk stored at silo gets packed and reach directly to the consumers. From this study it can be inferred that *Listeria* species are prevalent in pre- and post-food processing industries. Entry of *Listeria* should be traced and action should be taken to decrease the incidences of *Listeria* species. Presence of such bacteria in the food processing environment suggests either inadequate cleaning or inefficacy of cleaning procedures that are currently employed.

Fig. 2.1 Typical listerial colonies on PALCAM agar (24h/35^{o}C)

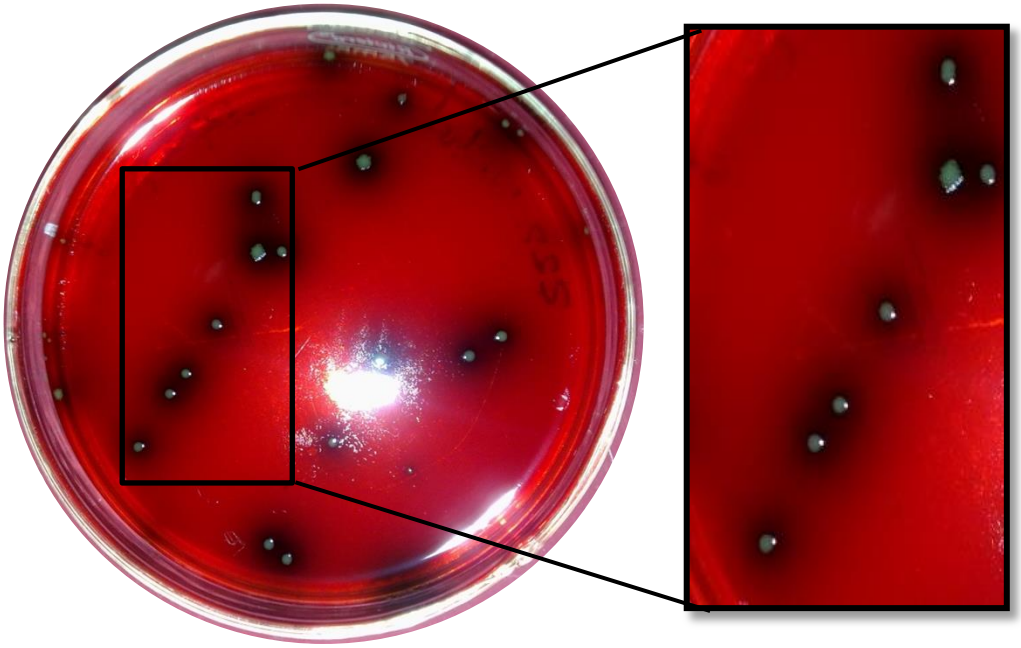


Table 2.3: *Listeria* spp. from different areas of milk processing environment.

Area of Swab	**Total No. of swabs taken**	**No. of Listerial spp.**	***L. monocytogenes***	***L. ivanovii***	***L. innocua***	***L. seeligeri***	***L. grayi***
Raw milk collector	27	9	7	1	1	0	0
Milk filing machine	33	6	0	0	6	0	0
Milk silo	25	10	4	0	4	0	2
Cheese blender machine	27	5	1	1	2	1	0
Product blender	24	5	1	0	4	0	0
Butter storage vessel	25	2	0	0	2	0	0
Butter milk mixer	8	3	3	0	0	0	0
Floor	4	1	0	0	0	0	1
Drains	8	0	0	0	0	0	0
Milk can	19	0	0	0	0	0	0
Bulk Milk tanker	8	0	0	0	0	0	0
TOTAL	**210**	**41**	**16**	**2**	**19**	**1**	**3**

2.4.2 Identification of *Listeria* spp.

The presumptive listerial isolates were further tested by a batteries of biochemical tests for species identification (Barbuddhe et al. 2008). Among 41 strains, 16 (7.61%) were confirmed as *L. monocytogenes*, 2 (0.95%) as *L. ivanovii*, 19 (9.04%) as *L. innocua*, 1 (0.48%) as *L. seeligeri* and 3 (1.43%) as *L. grayi*. Thus, 8.57% of samples from milk processing plants were found positive for presence of pathogenic *Listeria* species. Presence of *L. monocytogenes* in milk and associated environment have been reported previously (Moshtaghi & Mohamadpour 2007; Le Monnier & Leclercq 2009; Pradhan et al. 2009; Schoder et al. 2011). Various reports showed incidence of listeria from 4.1 to 19% in milk and milk processing environment (Fox et al. 2009; Almeida et al. 2013; Derra et al. 2013; Giacometti et al. 2013). The 16 *L. monocytogenes* isolates showed presence in raw milk collector (n=7), milk silo (n=4), cheese blender machine (n=1), product (shrikhand) blender (n=1) and butter milk mixer (n=3). Except raw milk collector, all mentioned sites are post-pasteurized sites where pasteurized milk or milk products come in contact. Such products are then directly packed without any bactericidal procedures, and directly reach to the consumer. Moreover, these milk products are stored at 4^0C. Being a psychrotropic nature, *L. monocytogenes* selectively gets enriched in milk and milk products stored at such a low temperature. Except milk, products such shrikhand, cheese, chakka, curd, butter-milk and butter are eaten directly by the consumers. Several processed milk products that needs to be preserve at low temperature have been reported to cause the outbreaks (MacDonald et al. 2005; Fretz et al. 2010; Johnsen et al. 2010; Koch et al. 2010; Newkirk et al. 2011; de Castro et al. 2012; Gaulin et al. 2012). Therefore, presence of *L. monocytogenes* at such product contacting areas is serious matter of concern. The contamination of a product with *L.*

monocytogenes is not acceptable in ISO 9001 and HACCP certified industries. Randomly checked food products if found positive for *L. monocytogenes*, the whole batch of the product has to be recalled. Several such recalls have been reported and thus lead to heavy losses for the food industries (FDA 2013).

Chapter 3:

Characterization of the isolates by biochemical and *in vitro* pathogenicity analysis

3.1 Introduction

Listeria species are ubiquitously distributed in the natural environment and frequently isolated from different biocenoses. Biochemical characteristics of bacteria are not always constant and can be influenced by environmental conditions attributing different characteristics (Kadner 2013). Therefore, a biochemical characteristic of a microorganism may give an idea about the recent ecological niche from which bacteria have been isolated. The identification of all listerial isolates to the species level is an important taxonomic issue (Volokhov et al. 2006). *Listeria* species isolated by using selective enrichments and selective agar, while to identify the species further biochemicals are needed. *Listeria* species have been characterized by several different ways according to the interest of study. Biochemically characterization of *Listeria* species is generally carried out for identification of species or to correlate the specific study. e.g. Phospholipase activity shows the ability of *Listeria* species to degrade host vacuole *in vitro* (Paziak-Domańska et al. 1999; Kaur et al. 2010); while pathogenic species *L. monocytogenes* and *L. ivanovii* are characterized for their virulence ability, antigenic abilities, strains subtypes etc. Such characterization gives an idea about the pathogenicity of the *L. monocytogenes* strains, its outbreak or epidemic nature. In such a way, to characterize the *Listeria* species isolates from the food processing environment further biochemical tests as well as *in vitro* tests were performed.

3.2 Review of Literature

3.2.1 Biochemical characterization

Besides biochemical test preformed to differentiate and identify *Listeria* species, biochemical tests such as methyl-red, Voges-Proskauer, carbohydrate utilization (fructose, glucose, xylitol, maltose, sucrose, galactose, lactose), motility, salt tolerance, ability to grow on MacConkeys, Enzyme activity (gelatinase, nitrate

reductase, urease) and reduction of H_2S are generally performed towards characterization of *Listeria* species as suggested in Bergey's manual of systematic bacteriology (McLauchin & Rees 2008). Also these biochemical mentioned are mandatory to differentiate novel species in *Listeria* (Graves et al. 2010; Leclercq et al. 2010; Bertsch et al. 2013; Halter et al. 2013). All *Listeria* species are positive for Methyl-red and Voges-Proskauer tests (Barbuddhe et al. 2008). *Listeria* species utilize carbohydrates such as D-fructose, D-Glucose, xylitol, maltose, lactose while not all the species of *Listeria* can utilize galactose. All the *Listeria* species can tolerate upto 12.5% of salt concentrations and show typical growth on McConkey agar. *Listeria* species cannot produce gelatinase, nitrate reductase (exception) and urease. The interesting character of *Listeria* species is its motility at 24^{o}C while immobility 37^{o}C as flagellin – a flagella protein is expressed only at 24^{o}C. *Listeria* species cannot produce hydrogen sulphide.

3.2.2 Pathogenicity testing

3.2.2.1 Hemolysis

The hemolysis character indicates the pathogenic ability of the *L. monocytogenes*. Pathogenic species of *Listeria* and few strains of non-pathogenic *L. seeligeri* exhibit weak hemolysis on 5% sheep blood agar. The hemolysin is encoded by the *hly* gene that is present within the virulence gene cluster found between *prs* and *ldh* gene in *L. monocytogenes, L. ivanovii*, and *L. seeligeri.* Such *hly* gene is absent from the genomes of the non-hemolytic *L. innocua, L. welshimeri*, and *L. grayi* species (Volokhov et al. 2006). Therefore, presence of hemolysin or *hly* gene is a strong indicator of capability to lyse the red blood cells and therefore virulence. This hemolysis ability has been employed to differentiate the pathogenic *Listeria* species from non-pathogenic (Rocourt et al. 1983). Subsequently, the method proved to be

effective in distinguishing the *L. monocytogenes* and *L. ivanovii* (Furrer et al. 1991; Churchill et al. 2006; Rawool et al. 2007; Singh et al. 2009; Le Monnier et al. 2011).

3.2.2.2 Christie Atkins Munch Petersen

The Christie Atkins Munch Petersen (CAMP) test is described for synergetic lysis of RBC in the presence of diffusible exo-substances produced by micro-organism growing adjacent to each other on the surface of blood agar (McKellar 1994). After the use of CAMP test differentiating *Staphylococcus* species, it has been used to study different pathogens including *L. monocytogenes* and *L. ivanovii*. The innate capability to lyse the RBC is weak in case of *L. monocytogenes* and *L. ivanovii.* In CAMP test, *L. monocytogenes* gives 'arrow-head' zone of lysis when grown near to *Staphylococcus aureus* while *L. ivanovii* gives 'shovel-shaped' zone of lysis near to *Rhodococcus equi* (McKellar 1994). CAMP test is a mandatory test to differentiate the *L. monocytogenes* (FDA-BAM 2013). The hemolysis and CAMP tests are crucial steps for identification of the hemolytic *L. monocytogenes*, *L. ivanovii*, and *L. seeligeri* species as well as for their differentiation from the non-hemolytic species, *L. innocua*, *L. welshimeri* and *L. grayi* (Volokhov et al. 2006).

3.2.2.3 Phosphatidylinositol-specific phospholipase C test

L. monocytogenes phosphatidylinositol-specific phospholipase C (PI-PLC) plays a critical role in escape of cell from host cell vacuoles (Wei et al. 2005). This phospholipase ability has incorporated into test by using a substrate - L-alpha-phosphatidylinositol, which get degraded because of PI-PLC-active colonies and therefore show turbid halos as a result of the release of insoluble diacylglycerol from the substrate. This activity is expressed only in the pathogenic *L. monocytogenes* (Notermans et al. 1991). The use of this assay improves the specific detection and/or

isolation of pathogenic *Listeria* species from clinical samples or food enrichment cultures.

3.2.2.4 Cell Culture assays

Cell culture assay are mainly used to determine the virulence of the pathogenic *Listeria* species (Van Langendonck et al. 1998). Since animal model studies are expensive and time consuming, several cell lines (Human Epithelial HEp-2, HeLa, and Caco-2) have utilized to evaluate adherence, invasion and virulence of *L. monocytogenes* (Jaradat & Bhunia 2003; Kushwaha & Muriana 2010). Cell culture assays have been employed to measure the cytopathogenic effects in the enterocyte-like cell line Caco-2 (Pine et al. 1991) or to form plaques in the human adenocarcinoma cell line HT-29 (Roche et al. 2001).

3.2.2.5 In vivo methods

The mouse virulence assay was one of the first methods described for *L. monocytogenes* virulence assessment (Kaufmann 1984; Liu 2006). It is capable of providing an *in vivo* measurement of all virulent determinants and is regarded as the gold standard for any newly developed tests for *L. monocytogenes* virulence (Roche et al. 2001). Differentiation of pathogenic and non-pathogenic *Listeria* species by inoculation of chick embryos through chorioallantoic (CAM) route has been described (Steinmayer et al. 1989). The pathogenic strains may cause death of embryo within 72 hours while with inoculation of non-pathogenic strains the embryo survives (Terplan & Steinmeyer 1989). The test has been reported to agree with mouse bio-assay (Notermans et al. 1991). The possibility of addressing aspects of mammalian innate immunity in invertebrates has opened a new arena for developing invertebrate models to study human infections (Mukherjee et al. 2010). The greater waxmoth, *Galleria mellonella,* has been studied as a reliable model to investigate the *Listeria*

pathogenesis (Joyce & Gahan 2010; Mukherjee et al. 2010). The *Galleria* model has been used to investigate the differences between infections caused by strains with different virulence potentials in the mouse infection model and revealed a strong correlation with virulence previously determined by the mouse model (Mukherjee et al. 2010). *G. mellonella* has been used to study brain infection and its impact on larval development as well as the activation of stress responses and neuronal repair mechanisms (Mukherjee et al. 2013).

3.2.2.6 Genotypic methods

The application of molecular techniques has facilitated the identification and characterization of major virulence-associated genes and proteins in *L. monocytogenes* (Liu, 2006). More than 80 years of listeriology have allowed the detailed description of the cycle of cellular infection and the identification of bacterial proteins implicated in each step of this process (Camejo et al. 2013). *In vitro* demonstration of LLO, PC-PLC and PI-PLC activities often provides general guidance on the pathogenic potential of *L. monocytogenes* strains, however its reliability as a virulence indicator is by no means satisfactory (Liu 2006). The genes encoding the virulence-associated proteins PI-PLC, LLO, Mpl, ActA and PC-PLC are located in a 9.6 kb virulence gene cluster (Vázquez-Boland et al. 2001). The genes encoding internalin proteins (*inlA* and *inlB*) are positioned elsewhere in the genome.

The *inlA* and *inlB* genes possess a transcription binding site similar to that recognized by PrfA and are partially regulated by PrfA. In addition to these virulence-associated genes and proteins, several other genes, such as *iap* (encoding invasion-associated protein, or Iap), are also involved in *L. monocytogenes* virulence and pathogenicity (Vázquez-Boland et al. 2001). These virulence genes can be determined by in-vitro method such as PCR (Vázquez-Boland et al. 2001). The virulent *L.*

monocytogenes strains may possess genes that are not present in avirulent strains, which serve as markers for PCR assessment of *L. monocytogenes* virulence (Liu et al. 2003). Since last two decades, with the increasing understanding of the genetic factors responsible for the virulence, several PCR methods have been standardized to detect virulence genes. Genes such as the *hly*, *inl*A, *plc*A, *act*A has been targeted to determine the virulence of pathogens (Notermans et al. 1991; Soni et al. 2013; Lomonaco et al. 2012; Churchill et al. 2006). Recently, the *lcp* gene encoding *Listeria* cellulose binding protein has been identified which has been suggested to play an important role in an attachment to vegetables and fruits (Bae et al., 2013). However, some naturally virulence-attenuated *L. monocytogenes* strains often contain mutations in their *prfA*, *hly*, *actA* and *inlA* genes, resulting in the expression of truncated or non-functional PrfA, LLO, ActA and InlA proteins (Roche et al. 2001), targeting these gene mutations as a means of determining *L. monocytogenes* virulence does not constitute a sound option in practical terms. The completion of the whole genome sequences of several *L. monocytogenes* and *L. innocua* strains (Glaser et al. 2001; Nelson et al. 2004; Kuenne et al. 2010) has facilitated the identification of novel virulence-specific genes with potential for improved determination of *L. monocytogenes* virulence and pathogenicity.

3.2.3 Detection of *Listeria* species.

L. monocytogenes and other *Listeria* species resemble closely by morphological and biochemical characteristics. Also clinical manifestations of listeriosis in animals and humans are non-specific (Vázquez-Boland et al. 2001). Therefore, rapid, specific and sensitive diagnostic tests capable of distinguishing pathogenic *Listeria* species are essential to understand the etiology of the disease. The earlier diagnostic methods for *L. monocytogenes* are largely phenotype-based that

characterize the gene products of *L. monocytogenes* through the measurement of biochemical, antigenic and bacteriophage properties (Liu 2006). The use of phenotypic tests may sometimes lead to equivocal results as these properties may vary with changing external conditions, with growth phase and with spontaneous genetic mutations (Liu 2006). Several different methods have been described to detect *Listeria* species as…..

3.2.3.1 Antibody based tests

Immunoassay methods based on antibodies specific to pathogenic *Listeria* species have been applied in detection of *Listeria* for many years. The methods are simple, sensitive, accurate and testing can be carried out directly from enrichment media without tedious sample preparation (Gasanov et al. 2005). ELISA methods with an antibody immobilized to a microtiter well for antigen capture in combination with a secondary antibody coupled to an enzyme (or another label) to detect the captured antigen, are the most widely applied methods. The VIDAS *L. monocytogenes* Xpress (LMX) test is an enzyme-linked fluorescent immunoassay designed for the specific detection of *L. monocytogenes* using a 26 h proprietary enrichment broth (Johnson et al. 2013). A capture ELISA for the identification of *L. monocytogenes* in food was standardized and validated and refined for analyzing samples of meat, seafood, dairy products, pasta and flour (Portanti et al. 2011). The method was found to be 100% specific for *Listeria* species tested, with a limit of sensitivity of 6.6×10^3 cfu/ml. Comparison of this assay against the official International Organization for Standards (ISO) method 11290-1:1996 for the isolation and identification of *L. monocytogenes* in food matrices produced a significant concordance index (Portanti et al. 2011).

Immune-capture is an elegant technique that uses magnetic beads or dip sticks coated with specific antibodies to separate *Listeria* from competing microflora and inhibitory food components (Gasanov et al. 2005). In another detection approach, paramagnetic beads coated with recombinant *Listeria* phage endolysin-derived cell wall-binding domain proteins, specific for *Listeria* species were used to detect bacteria in artificially contaminated raw milk with a sensitivity of 10^2–10^3cfu/ml (Walcher et al. 2010). Recently, a lateral-flow enzyme immune-chromatography coupled with an immune-magnetic step was developed for rapid detection of *L. monocytogenes* in food matrices (Cho & Irudayaraj 2013) and was demonstrated to be highly sensitive, specific, and rapid compared with spectroscopic methods.

3.2.3.2 Molecular tests

At low number, detection of *L. monocytogenes* in contaminated food samples pose problems (Hoffman & Wiedmann 2001). Molecular techniques are extremely accurate, sensitive and specific. Therefore, identification of *Listeria* species and *L. monocytogenes* using molecular methods is becoming increasingly popular (Gasanov et al. 2005). Datta et al. (1991) reported the first DNA probe wherein a *Hind*III-*Hind*II fragment of a presumptive haemolysin gene was used in a trial for specific detection of *L. monocytogenes*. Recently, an internalin-A probe-based genosensor for detection and differentiation of *L. monocytogenes* has been developed (Bifulco et al. 2013) which could discriminate whole DNA samples of *L. monocytogenes* strains from other non-pathogenic *Listeria* species DNA.

DNA-based methods of detection employ ways of amplifying the specific genetic signals from a few cells. Nucleic acid amplification based methods such as PCR methods have been employed to detect *L. monocytogenes* isolates (Kazmierczak et al. 2006; Liu et al. 2006; Jallewar et al. 2007; Warke et al. 2007; Mammina et al.

2009; Frece et al. 2010; Kaur et al. 2010; Ahmed et al. 2013; Park et al. 2013; Rocha et al. 2013). Multiplex PCR method makes use of multiple sets of primers to amplify a number of genes or gene fragments simultaneously (Churchill et al. 2006). A multiplex PCR assay employing four genes, the *hly*A, *plc*A, *iap* and *act*A was developed for detection of *L. monocytogenes* from clinical samples (Kaur & Malik 2007).

Real time PCR combines the DNA amplification PCR strategy and fluorescent dye. SYBR-Green chemistry is the most commonly used because of ease of use and comparative low cost (Bustin et al. 2005; Rebrikov & Trofimov 2006). SYBR green dye binds only to double stranded DNA and fluoresce. The fluorescence get captured by CCD camera and values then converted into quantities of double stranded DNA amplicon present. For *L. monocytogenes*, Real time PCR or Q-PCR has been mainly employed for detection and gene expression studies (Smith & Osborn 2009; Wong & Medrano 2005). Several qPCR methods have been standardized to detect *L. monocytogenes* in food and clinical samples (Wang & Hong 1999; Hein et al. 2001; Rodríguez-la et al. 2004; Rantsiou et al. 2008; Alessandria et al. 2010; Barbau-Piednoir et al. 2013; Wang et al. 2013). Q-PCR is also useful in understanding of the gene expressions of *L. monocytogenes* at different conditions (Dancz et al. 2002; Chatterjee et al. 2006;Werbrouck et al. 2007; Liu & Ream 2008; Bowman et al. 2008; Bae et al. 2012;). Q-PCR has also been used to detect, quantify and determine the vitality of *L. monocytogenes* in foods based on amplification of the interegenic region spacer (IGS) between the 16S and 23S genes (Rantsiou et al. 2008). Gene expression studies revealed the expression of the virulence gene at different conditions, which helped to understand the pathogenicity of *L. monocytogenes* (Rodríguez-la et al. 2004). By using real time PCR, *L. monocytogenes* can be differentiated from other

Listeria species with 98.09% of accuracy (Barbau-Piednoir et al. 2013). *In vivo* transcriptional profiling by qPCR along with mutagenesis identified new virulence factors involved in infection of *L. monocytogenes* (Camejo et al. 2009). Enrichment-free method for the detection of *L. monocytogenes* from meat samples have been developed facilitating quick detection (Ye et al. 2012). A novel method for the identification of viable *Listeria* species was developed based on reverse transcription-multiplex PCR (RT-MPCR) and restriction digestion (Rattanachaikunsopon & Phumkhachorn 2008). RT-MPCR was found to discriminate between viable and non-viable cells and to detect viable *Listeria* species in a food model.

3.2.3.3 Other detection methods

Various spectrophotometric methods like Fourier transform infrared (FT-IR), matrix-assisted laser desorption/ionisation time-of-flight mass spectroscopy (MALDI-TOF MS) have been developed to detect foodborne pathogen (Jadhav et al. 2012). MALDI-TOF MS showed promise for identification of *Listeria* species and typing and even allowed for differentiation at the level of clonal lineages among pathogenic strains of *L. monocytogenes* (Barbuddhe et al., 2008). Loop-mediated isothermal amplification (LAMP) allows a rapid amplification of nucleic acids under isothermal conditions. LAMP assay targeting the *hly* gene was developed and the amplification products were visualized by calcein and manganous ion and agarose gel electrophoresis. It has been opined that the LAMP assay can facilitate the surveillance for contamination of *L. monocytogenes* in food (Tang et al. 2011). Gold nanoparticles/horseradish peroxidase encapsulated polyelectrolyte nanocapsule was developed for signal amplification in *L. monocytogenes* detection and demonstrated that the bioconjugated nanocapsules showed 30 times greater sensitivity and a shorter

assay time (5 min) when compared to conventional ELISA using an HRP labeled antibody, for a given quantity of antibody (Oaew et al. 2012).

3.2.4 Subtyping methods

Subtyping is the process by which a bacterial species can be further separated into different subgroup or strains (Chen & Knabel 2008). A direct function of strain typing is to discriminate between different strains that belong to the same genus and/or species. Typing techniques have undergone extensive improvements and many new methods have led to significantly enhanced performance according to various criteria (Chen & Knabel 2008). Three major applications of strain typing are in taxonomy, epidemiology and phylogeny (evolutionary genetics). Taxonomy, also known as (bio)systematics, is the practice and science of classification of organisms based on their common characteristics (van Belkum et al. 2001; Chen & Knabel 2008; Liu 2006). Several genetical based ssubtyping methods have been described for *L. monocytogenes* such as serotyping (McLauchlin et al., 1998), PCR serotyping (Doumith et al., 2004), Phage typing (Capita et al., 2002), Plasmid typing (Lebrun et al., 1992), Multilocus Enzyme Electrophoresis (MEE) (Piffaretti et al., 1989), RAPD (Williams et al., 1990), Pulsed-field gel electrophoresis (PFGE) (Brosch et al., 1991), REP-PCR (Jersek et al., 1999), Hybridization-based typing (Liu et al., 2006), DNA array (Rudi et al., 2003), MLST (Salcedo et al., 2003), MVLST (Zhang et al., 2004) and Single nucleotide polymorphisms (SNPs) (Ducey et al., 2007) has been employed that distinguish *L. monocytogenes* strains isolated from clinical, food and environmental sources. Molecular subtyping of *L. monocytogenes* has been advantageous for narrowing down the strains that are clinically significant (Cheng et al., 2008; Ramaswamy et al., 2007). The major subtyping methods are described ahead.

3.2.4.1 PCR-Serotyping

Routine analysis of *L. monocytogenes* using traditional agglutination methods is limited in use because of the expense and limited availability of commercially prepared antisera. Also, intra- and inter- laboratory discrepancies arising from differences in antiserum preparation and visual determination of agglutination (Palumbo et al. 2010). *In vitro* amplification of nucleic acid is a genetic detection method for pathogen identification and diagnosis. Among several elegant approaches to nucleic acid amplification, PCR was the first and remains the most widely applied technique in both research and clinical laboratories (Liu 2006). *L. monocytogenes* strains have been differentiated into 13 serotypes of which 4b, 1/2a and 1/2b serotypes are involved in more than 98% of outbreaks (Kathariou 2002). For a rapid differentiation of these outbreaks associated *L. monocytogenes* strains, a multiplex PCR has been developed (Doumith et al. 2004) by selecting *lmo0737, lmo1118*, ORF2819 and ORF2110 as marker genes. The *prs* gene, specific for strains of the genus *Listeria*, was also included to determine the genus specificity. Each PCR product was designed for amplifying distinct fragment sizes between 370 and 906 bp (Doumith et al. 2004). The PCR based serotyping method revealed to be the easy and have been applied worldwide to differentiate 'serogroup' of *L. monocytogenes* isolates from wide variety of clinical and food samples (Chou & Wang 2006; Leite et al. 2006; Huang et al. 2007; Vasconcelos et al. 2008; Chen et al. 2010; Tamburro et al. 2010). Though the method does not give a direct identification of serotype, it gives a simple, reliable and rapid approach to shortlist the epidemic associated *L. monocytogenes* strains.

3.2.4.2 Pulsed filed gel electrophoresis typing

Pulsed field gel electrophoresis (PFGE) is a technique that separate restriction enzyme digested whole genomic DNA of microorganism. The restriction digestion of genomic DNA when separated on an agarose gel by using PFGE, a specific profile is observed for specific strain of a bacterium. Such PFGE profiles of strains are compared to determine the similarity/differences between two or more isolates. PFGE uses selected restriction enzymes to yield between 8-25 large DNA bands of 40–600 kb in size, and alternating currents to cause DNA fragments to move back and forth, resulting in a higher level of fragment resolution. Though discovered for yeasts (Schwartz & Cantor 1984), PFGE has been extensively used for subtyping of *E. coli, Salmonella, Vibrio, Campylobacter, L. monocytogenes, Shigella* and *Staphylococcus* (PulseNet 2013; CDC 2013). PFGE also has been used on other bacteria such as *Brucella* species (Ridler et al. 2005) and *Coxiella* species (van Schaik et al. 2013) to study the genetic variability as well as to differentiate the strains. In case of *L. monocytogenes*, PFGE typing has been considered as a "Gold standard technique" (Graves & Swaminathan 2001). The PFGE subtyping has been utilized in epidemiological study of *L. monocytogenes* (He et al. 2008; Félix et al. 2012; Fox et al. 2012). Also, PFGE subtyping has been employed to track the source of *L. monocytogenes* to food (Miettinen et al. 1999; Norwood & Gilmour 2001; Fugett et al. 2007; Conter 2008).

3.2.4.3 Multi-locus sequence typing

Multi-locus sequence typing (MLST) is a reference method for global epidemiology and population biology of bacteria. The application of MLST to *L. monocytogenes* effectively allows isolate comparisons across laboratories [www.pasteur.fr/mlst]. The existence of few prevalent and globally distributed clones

has been shown genotyping of 300 isolates from the 5 continents and diverse sources, some of which include previously described epidemic clones (Chenal-Francisque et al. 2011). The results obtained from MLST have been consistent with those of PFGE (Salcedo et al. 2003). The future application of this new molecular method could be a useful tool for the listeriosis surveillance systems that will allow the identification and distribution of analysis of *L. monocytogenes* clones in the environment (Salcedo et al. 2003).

3.2.4.4 Multi-locus virulence sequence typing

Populations of *L. monocytogenes* are genetically structured into a small number of major clonal groups, some of which have been implicated in multiple outbreaks (Chenal-Francisque et al. 2013). Since the introduction of this concept in 2004, seven ECs have been recognized, of which ECI, ECII and ECIV are serotype 4b, ECIII, ECV, ECVII are in serotype 1/2a and ECVI is in serotype 1/2b (Zhang et al., 2004; Lomonaco et al., 2013). The MVLST scheme developed by Zhang et al., (2004) has been shown to have high discriminatory power (D = 0.99), excellent epidemiological concordance (E = 1.0), stability and typiablity (Zhang et al., 2004; Chen et al., 2005; Chen et al., 2007). Also, MVLST has been successfully used to detect epidemic clones and outbreak clones (Chen et al., 2005; Chen et al., 2007; Lomonaco et al., 2013; Rocha et al., 2013). Epidemic clones are genetically related isolates implicated in geographically and temporally unrelated outbreaks that presumably of a common ancestor (Cheng et al., 2008). Given its high reproducibility and high throughput, MVLST has been suggested as first-line screening method to alleviate the PFGE workload in outbreak investigations and listeriosis surveillance (Chenal-Francisque et al. 2013). *L. monocytogenes* isolates obtained from the food

processing industries were characterized by best applicable biochemical and genetical methods.

3.3 Materials and Methods

3.3.1 Biochemical characterization

Besides the morphological characterization for the identification, all the 41 listerial isolates were characterized for carbohydrates fermentation (fructose, glucose, xylitol, maltose, sucrose, galactose, lactose), MR-VP tests, Enzyme activity (gelatinase, nitrate reductase, urease), growth on MacConkey, motility, H_2S reduction and salt tolerance as per Bergey's Manual of Systematic Bacteriology, Vol. II. (McLauchin & Rees 2008)

3.3.2 Genotypic characterization

3.3.2.1 DNA isolation

The genomic DNA of the isolates was extracted using PureLink Genomic DNA extraction kit (Invitrogen, Cat. No. K182001) as per manufacturer's instructions. The quantity and purity of the isolated DNA was determined by Nanodrop (ThermoFisher). Aliquots of 50ng of DNA was prepared and stored at -20^{o}C.

3.3.2.2 In vitro pathogenicity

In vitro pathogenicity was determined by a multiplex-PCR reaction by presence of virulence genes–*hly*A, *act*A and *plc*A among the *L. monocytogenes* isolates. Fifty microliter reaction mixtures were prepared, each containing 2 units Taq DNA Polymerase, 10x PCR buffer (50 mM TrisHCl, 10 mM KCl, 50 mM Ammonium Sulfate, 2 mM $MgCl_2$), 200 mM dNTP mix, 3mM $MgCl_2$, 2 mM each of primer (hlyA, actA and plcA) (details in Table 6) and 50 ng of DNA template. PCR was performed in Master Cycler Gradient Thermocycler (Eppendorf, Germany) having a pre-heated lid with an initial denaturation step at 94^{o}C for 5 min, 35 cycles

comprising of 94^{o}C for 30 s, 58^{o}C for 1 min 15 s, and 72^{o}C for 45 s, and one final step of 72^{o}C for 5 min in thermocycler. Samples were held at 4^{o}C until electrophoresis.

3.3.2.3 PCR based Serotyping

The serotyping of the *L. monocytogenes* isolates was carried out by using multiplex-PCR (Doumith et al. 2004). The primers used for multiplex-PCR serotyping were synthesized from Sigma Aldrich, USA. The multiplex-PCR serotyping was standardized as per the methodology described by Doumith et al. (2004). Fifty microliter reaction mixtures were prepared each containing 2 units Taq DNA Polymerase, 10x PCR Buffer (50 mM TrisHCl, 10 mM KCl, 50 mM Ammonium Sulfate, 2 mM $MgCl_2$), 300 mM dNTP mix, 2 mM $MgCl_2$, 2 mM each primers (*lmo0737*,*ORF2819, ORF2110* and *prs*) (Table:6) and 50 ng of DNA template. PCR was performed in Master Cycler Gradient Thermocycler (Eppendorf, Germany) having a pre-heated lid with an initial denaturation step at 94^{o}C for 5 min, 35 cycles consisting of 94^{o}C for 30 s, 54^{o}C for 1 min 15 s, and 72^{o}C for1 min 15 s, and one final step of 72^{o}C for 10 min in thermocycler. Samples were held at 4^{o}C until electrophoresis.

3.3.2.4 Electrophoresis

The PCR products were analyzed by agarose gel electrophoresis. Eight microliter of PCR product was separated in 1.5% agarose gel stained by ethidium bromide 0.5 µg/ml.

3.3.2.5 Pulsed Field Gel Electrophoresis

PFGE was performed according to the PulseNet standardized protocol (Graves and Swaminathan 2001). Briefly, genomic DNA was prepared by mixing 240 µl of a standardized cell suspension and 60 µl of a 10mg/ml lysozyme solution (Sigma, St. Louis, MO), followed by incubation at 37°C for 10 min. An equal volume of molten

1.2% agarose, 1% sodium dodecyl sulfate, and 0.2 mg/ml proteinase-K prepared in sterile distilled water and maintained at 55°C was added to the cell suspension, and the mixture was mixed by gently pipetting it up and down. The mixture (600 µl) was dispensed into forms of a sample reusable plug mold and allowed to cool for 5 min. The agarose plugs were transferred to tubes containing 4 ml of lysis buffer (50 mM Tris, pH 8.0, 50 mM EDTA, pH 8.0 (TE buffer), 1% sodium lauryl sarcosine, 0.15 mg/ml proteinase K), incubated for 2 h at 54°C in an orbital water bath shaker, and shaken at 200 rpm. After proteolysis, the lysis buffer solution was removed and the plugs were washed twice with 15 ml of preheated (50 to 54°C) sterile distilled water for 10 min, followed by four washes with 15 ml of preheated (50 to 54°C) TE buffer for 15 min in the orbital water bath shaker at 50 to 54°C and 200 rpm. After the final TE wash, the plugs were stored in 1.5 ml TE at 48°C until ready for restriction. Sample plugs were digested with 25 U of *Asc*I (Fermentas, MY, USA) at 37°C for 3 h or 160 to 200 U of *Apa*I (Fermentas, MY, USA) at 30°C for 5 h. Plugs were then loaded on 1% agarose gel in 0.5x TBE (45 mM Tris, 45 mM borate, 1 mM EDTA) buffer and electrophoresed on a CHEF-DR II apparatus (Bio-Rad, USA) using the following parameters: initial switch time, 4 s; final switch time, 40 s; run time, 22 h; angle, 120°; gradient, 6 V/cm; temperature, 14°C; and ramping factor, linear. Gels were stained with ethidium bromide and visualized by a UV transilluminator.

3.4 Results and Discussion

3.4.1 Biochemical characterization

Traditionally biochemical tests were performed to characterize the particular bacteria. Specific bacteria exhibit specific biochemical reactions of which few are limited to the genus or species specific. Taking the advantage, such biochemical characters have been utilized to determine the identity of the bacteria. The biochemical characters give a general guideline about physiological state of an organism (MacFaddin 1980). To determine the biochemical properties of the listerial isolates from the food processing industries sugar fermentation, MR-VP, salt tolerance, growth on MacConkeys agar, gelatinase, nitrate reductase, H_2S production, urease and motility at 24 and 37^oC were tested. All 41 listerial isolates utilized simple sugars such as fructose, glucose, xylitol, maltose, sucrose while only 9 isolates could utilize galactose showing the ability to utilize the different carbohydrates as a source of carbon (Table 3.1). Isolate were also tested for Voges-Proskauer test, Methyl red test and growth on MacConkey's agar. All the 41 isolates showed typical listerial growth on MacConkey's agar. Except four, all isolates were positive for methyl red test. None of the isolates showed the Voges-Proskauer test positive. The capacity of *Listeria* to tolerate salt stresses is of particular importance, as this pathogen is often exposed to such environments during food processing and food preservation (Gardan et al. 2003). All the listerial isolates from the food processing environment showed 10-12.5% of salt tolerance. This ability partially explains the persistence of the bacteria in the food processing environment (Duche et al. 2002). The nitrate reductase and hydrogen sulphide production ability is common among the environmental persistent bacteria (Moreno-Vivián et al. 1999; Hose et al. 2000). Though nitrate reductase is not the significant character of *Listeria* species, few exceptional strains

have been reported to utilize the nitrate (Hugas & Monfort 1997; Glass & Doyle 1989). Eight isolates showed nitrate reductase ability while thirteen isolates showed the hydrogen sulphide ability. The motility test was performed at 24^{o}C and 37^{o}C. The expected motility at 24^{o}C was observed while at 37^{o}C the motility was absent. In contrast to other bacteria, in *Listeria* species, the biosynthesis of flagella is temperature dependent and regulated by a distinctly different mechanism than other common bacteria (Lemon et al. 2007). At mammalian host physiological temperature, 37^{o}C listeria strains do not produce flagella and are non-motile (Peel et al. 1988). The flagella present in *Listeria* plays a key role in adhesion and therefore biofilm formation (Lemon et al. 2007).

Table 3.1: Biochemical characteristics of the *Listeria* species isolated from the milk processing environment

	MR	VP	Fructose	Glucose	Xylitol	Maltose	Sucrose	Galactose	Lactose	NaCl (%)	Growth on MacConkey's agar	Gelatinase	Nitrate reduction	H_2S production	Urease	37°C Motility	24°C Motility
WS3	-	-	+	+	+	+	+	-	+	10	+	-	-	-	-	-	+
WS4	+	-	+	+	+	+	+	-	+	10	+	-	-	(+)	-	-	+
WS7	+	-	+	+	+	+	+	-	+	12.5	+	-	-	-	-	-	+
WS9	+	-	+	+	+	+	+	+	+	10	+	-	-	-	-	-	+
WS10	-	-	+	+	+	+	+	-	+	10	+	-	-	-	-	-	+
WS12	+	-	+	+	+	+	+	-	+	10	+	-	-	-	-	-	+
WS44	+	-	+	+	+	+	(+)	-	+	10	+	-	+	-	-	-	+
GKS77	-	-	+	+	+	+	+	+	+	10	+	-	+	-	-	-	+
GKS85a	-	-	+	+	+	+	+	+	+	10	+	-	-	-	-	-	+
GKS85b	+	-	+	+	+	+	+	-	+	10	+	-	+	-	-	-	+
GKS86	+	-	+	+	+	+	+	-	+	10	+	-	-	(+)	-	-	+
GKS88	+	-	+	+	+	+	+	+	+	10	+	-	-	(+)	-	-	+
GKS89a1	+	-	+	+	+	+	+	+	+	12.5	+	-	-	-	-	-	+
GKS89a2	+	-	+	+	+	+	+	-	+	10	+	-	-	(+)	-	-	+
GKS89b1	+	-	+	+	+	+	+	+	+	10	+	-	-	-	-	-	+
GKS89b2	+	-	+	+	+	+	+	-	+	10	+	-	-	(+)	-	-	+
GKS90	+	-	+	+	+	+	+	-	+	10	+	-	-	(+)	-	-	+
GKS91	+	-	+	+	+	+	+	-	+	10	+	-	-	-	-	-	+
GKS93a	+	-	+	+	+	+	+	+	+	12.5	+	-	-	(+)	-	-	+
GKS93b	+	-	+	+	+	+	+	-	+	10	+	-	-	-	-	-	+
GKS96	+	-	+	+	+	+	+	-	+	10	+	-	-	(+)	-	-	+
GKS117a	+	-	+	+	+	+	+	-	+	10	+	-	-	-	-	-	+

Continued…….

	MR	VP	Fructose	Glucose	Xylitol	Maltose	Sucrose	Galactose	Lactose	NaCl (%)	Growth on MacConkey's agar	Gelatinase	Nitrate reduction	H_2S production	Urease	37°C Motility	24°C Motility
GKS117b	+	-	+	+	+	+	+	-	+	10	+	-	-	(+)	-	-	+
GKS119	+	-	+	+	+	+	+	-	+	12.5	+	-	-	-	-	-	+
GKS135	+	-	+	+	+	+	(+)	-	+	10	+	-	+	-	-	-	+
GKS136	+	-	+	+	+	+	(+)	-	+	10	+	-	-	-	-	-	+
GKS137	+	-	+	+	+	+	(+)	+	+	10	+	-	-	-	-	-	+
GaS1	+	-	+	+	+	+	+	-	+	10	+	-	+	-	-	-	+
GaS3	-	-	+	+	+	+	+	-	+	10	+	-	+	-	-	-	+
GaS5b	+	-	+	+	+	+	(+)	-	+	10	+	-	-	-	-	-	+
GaS7	+	-	+	+	+	+	+	-	+	10	+	-	-	-	-	-	+
GaS8	+	-	+	+	+	+	+	-	+	12.5	+	-	+	-	-	-	+
GaS14	+	-	+	+	+	+	+	-	+	10	+	-	+	-	-	-	+
GaS15	+	-	+	+	+	+	+	-	+	10	+	-	-	(+)	-	-	+
GaS18	+	-	+	+	+	+	(+)	+	+	12.5	+	-	-	-	-	-	+
GaS19	+	-	+	+	+	+	+	-	+	10	+	-	-	(+)	-	-	+
GaS23	+	-	+	+	+	+	+	-	+	12.5	+	-	-	(+)	-	-	+
GaS36	+	-	+	+	+	+	(+)	-	+	10	+	-	-	-	-	-	+
GaS44	+	-	+	+	+	+	+	-	+	10	+	-	-	-	-	-	+
GaS60	+	-	+	+	+	+	+	-	+	10	+	-	-	(+)	-	-	+
GaS67	+	-	+	+	+	+	+	-	+	10	+	-	-	-	-	-	+

Key: +: positive test, -: negative test

3.4.2 In-vitro pathogenicity

For a considerable period, *L. monocytogenes* has been regarded as pathogenic at the species level, with a generally accepted belief that all *L. monocytogenes* isolates are potentially virulent and capable of causing diseases. However, from the experimental data collected over the recent years, it becomes clear that *L. monocytogenes* demonstrates enormous serotype/strain variation in virulence and pathogenicity (Cheng et al. 2008). Whereas many epidemic strains are unquestionably highly infective and sometimes deadly, others (especially those from food and environmental specimens) show limited capability to establish infection and are relatively avirulent (Roche et al. 2008). Different subtypes of *L. monocytogenes* diverge in their pathogenicity for humans and/or in their ability to transmit to humans (Cheng et al. 2008). Such diversion in pathogenicity depends on the genetic constituent. Several (>40) genes has been linked with the virulence ability of the *L. monocytogenes*, however, few have been considered as main constituent - haemolysin (*hlyA*), phospholipases for disruption of intracellular phagosomal membrane (*plc*A) and actin polymerising proteins for intracellular mobility (*actA*) ([a]Kaur et al. 2007; Liu et al. 2007). Absence or mutation in such genes have been known to cause reduced virulence (Cossart et al. 1989; Gaillard et al. 1986; Jacquet et al. 2004) and presence of *hly*A, *act*A and *plc*A genes suggest the possible virulence capability in *L. monocytogenes* isolates. The screening of *L. monocytogenes* isolates from this study showed presence of *hly*A, *act*A and *plc*A genes among all the 16 isolates suggesting the potential virulence capability of the isolates (Fig. 3.1). All the virulent *L. monocytogenes* strains obtained from the clinical cases have shown presence of the *hly*A, *act*A and *plc*A genes (Roche et al. 2008; Le Monnier et al. 2011; Lomonaco et al. 2011; Lomonaco et al. 2013).

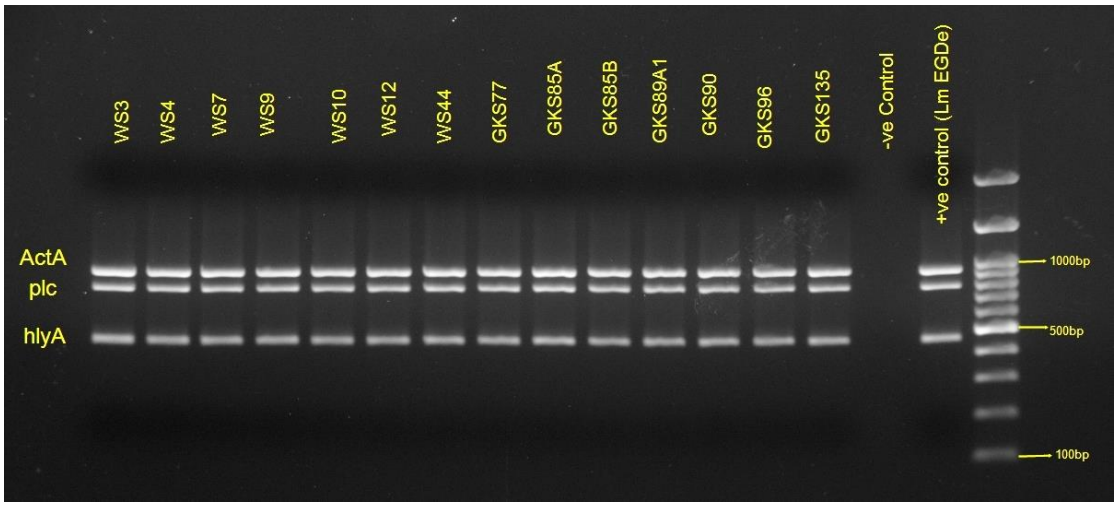


Fig. 3.1: Amplification of *act*A (965bp), *plc*A (803bp) and *hly*A (456bp) genes from the *L. monocytogenes* isolates obtained from food processing environment to determine the virulence potential.

3.4.3 Serotyping

In case of *L. monocytogenes*, as few serotypes have been linked to the foodborne outbreak, determination of serotypes of isolates from food industries is significant from the epidemiological point of view. All 16 isolates identified as *L. monocytogenes*, when subjected for PCR based serotyping revealed to be 1/2a, 1/2c, 3a and 3c serovar group (Fig. 3.2). The data is consistent with the previous report showing *L. monocytogenes* serotype 1/2a strains are frequently observed in milk and associated environment and caused several outbreaks (Waak et al. 2002; Van Kessel et al. 2004; Fretz et al. 2010; Kalorey et al. 2008; Gelbícová & Karpísková 2012).

L. monocytogenes serotype 1/2a strains along with 4b and 1/2b are responsible for more than 98% of outbreaks. Earlier, all *L. monocytogenes* isolates from milk processing environment from Brazil were found to be 1/2a serotype (Brito et al., 2008). Whereas an equal frequency of genomic types was reported for serotypes 1/2b

or 3b and 1/2a or 3a by Chambel et al. (2007) from Portugal dairy. The observation indicated the potential of milk and milk products to serve as vehicles of transmission of virulent *L. monocytogenes* strains. Distinguishing occurrence of 1/2a serotype as compared to other *L. monocytogenes* serotypes in milk and related environment suggested a close affinity/survival ability of *L. monocytogenes* 1/2a strains with/in milk.

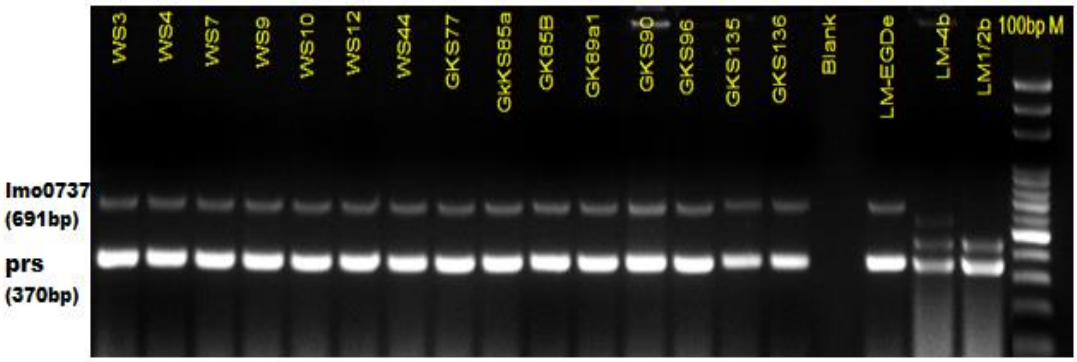


Fig. 3.2: Serotyping of the *L. monocytogenes* isolates obtained from food processing environment. (WS3-GKS136: isolates, *L. monocytogenes* EGDe as a standard for serotypes 1/2a; *L. monocytogenes*-4b: standard for serotype 4b and LM1/2b: standard for 1/2b)

3.4.4 Pulsed Field Gel Electrophoresis

Among the different serotyping methods, PFGE typing for *L. monocytogenes* has been considered as a "Gold Standard" because of its reproducibility, accuracy, discriminatory power (Simpson's index of discrimination *D*= 0.995) and epidemiological concordance (Swaminathan et al. 2001; Fugett et al. 2007; Chen & Knabel 2008). The relation of *L. monocytogenes* PFGE types from wide sources have shown the relation between the two strains that are geographically differentiated (Fugett et al. 2007). PFGE has been successfully used to study the genetic variability (Wang et al. 2012), discriminate the epidemiological or outbreak or non-outbreak

clones (Aarnisalo et al. 2003) and to trace the source of contamination (Dauphin et al. 2001). Pulsed-field gel electrophoresis have been successfully utilized to demonstrate the linkage between recalled chocolate milk and a multistate invasive listeriosis outbreak in the Unites States (Proctor et al. 1995).

PFGE typing of the listerial isolates was carried out to determine the genetic relation between the strains that have been isolated from three different industries. As recommended by PulseNet, the *L. monocytogenes* genome was digested by *Asc*I and *Apa*I restriction enzymes. The PFGE types were then analyzed by Gel Compare II software. A total of 17 pulsotypes were observed by *Asc*I enzyme. To distinguish further, pulsotypes of all the isolates were also studied by *Apa*I enzyme. Incorporation of *Apa*I enzyme added 11 more pulsotypes. Thus, combined *Asc*I and *Apa*I enzyme distinguished forty one isolate into total of 28 pulsotypes. Forty one *Listeria* species got divided into four major clusters by both the enzyme. (Fig. 3.3-3.6). Though no predominant PFGE type was observed, isolates were clustered separately according to the food industry with few exceptions. *Asc*I and *Apa*I pattern of few isolates 3, 4, 7, 9, 10, 12, 44, 135, 136 and 137 showed different pattern. *Asc*I enzyme could well discriminate the isolate according to food plant. While, except few, *Apa*I enzyme also could discriminate the isolates as per food industry. Isolates obtained from different sources showed similar PFGE pattern and got grouped together suggesting prevalence of single clone at each food industrial environment analyzed, e.g. In case of *Asc*I-pulsotypes isolates from butter milk processor and isolate from raw milk collector got grouped together or isolates from raw milk collector and isolates from milk silo got grouped together suggesting either cross contamination or single source of origin (Fig. 3.4). Except few isolates, same clustering was observed for the *Apa*I-pulsotypes, where isolates obtained from raw milk collector were grouped with isolates from

butter milk or milk silo (Fig. 3.6). Latorre et al. (2010) also observed 6 PFGE types for 15 *L. monocytogenes* isolates obtained from milking equipment, parlor, milk bulk tank, and milk filters with combined *Asc*I and *Apa*I restriction analysis.

Fig. 3.3: Pulsed field gel electrophoresis type (Pulsotypes) of listerial isolates obtained from species after restriction digestion by *Asc*I enzyme

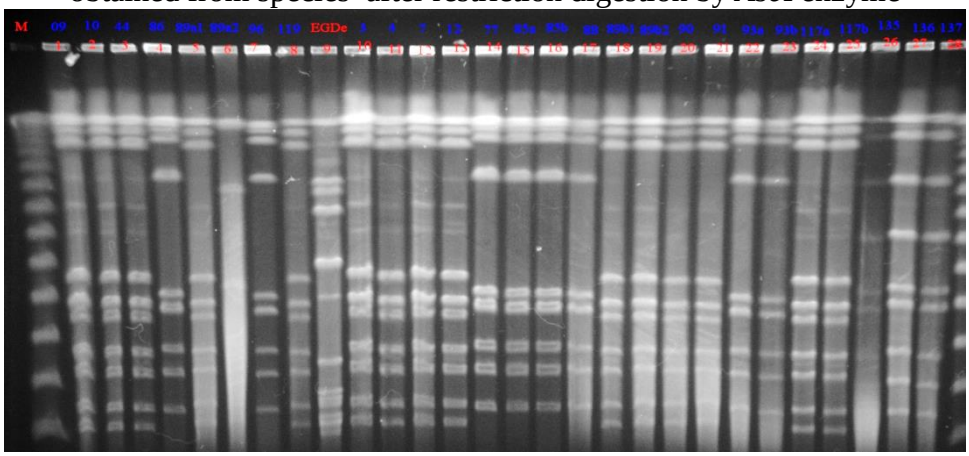
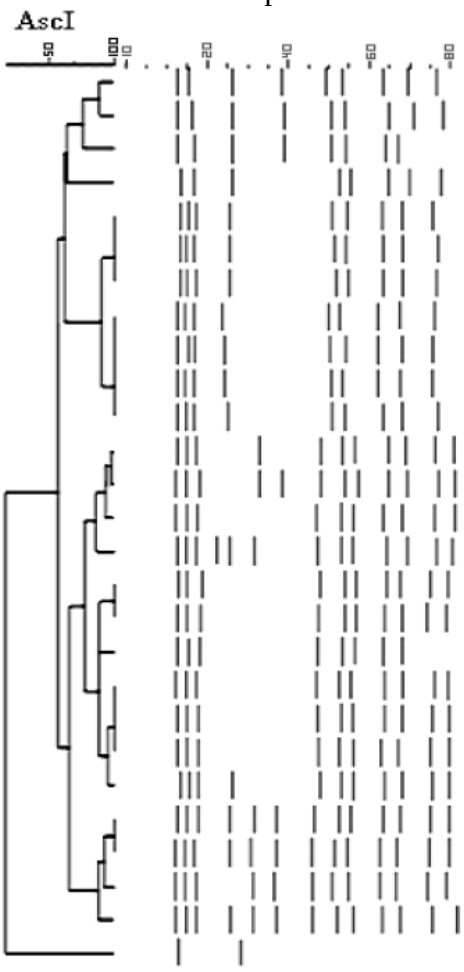


Fig.3.4: Dendrogram showing clustering of *Asc*I restriction digestion pulsotypes of *Listeria*species obtained from food processing environment



Isolate	Serotype	Source	Plant
136	*1/2a L. monocytogens*	Buttermilk processor	B
137	*1/2a L. monocytogens*	Buttermilk processor	B
135	*1/2a L. monocytogens*	Buttermilk processor	B
96	*1/2a L. monocytogens*	Raw milk collector	B
88	*L. innocua*	Product blender machine	B
119	*L. innocua*	Milk filling machine	C
93a	*L. innocua*	Butter storage vessel	B
77	*1/2a L. monocytogens*	Product blender machine	B
85a	*1/2a L. monocytogens*	Raw milk collector	B
85b	*1/2a L. monocytogens*	Raw milk collector	B
86	*1/2a L. monocytogens*	Cheese blender machine	B
117a	*L. ivanovii*	Raw milk collector	C
117b	*L. ivanovii*	Raw milk collector	C
44	*1/2a L. monocytogens*	Milk silo	A
10	*1/2a L. monocytogens*	Milk silo	A
4	*1/2a L. monocytogens*	Raw milk collector	A
93b	*1/2a L. monocytogenes*	Raw milk collector	B
90	*L. innocua*	Butter storage vessel	B
91	*L. innocua*	Cheese blender machine	B
89b1	*L. innocua*	Product blender machine	B
89b2	*L. innocua*	Product blender machine	B
89a1	*L. innocua*	Milk silo	B
12	*1/2a L. monocytogens*	Milk silo	A
7	*1/2a L. monocytogens*	Raw milk collector	A
3	*1/2a L. monocytogens*	Raw milk collector	A
9	*1/2a L. monocytogens*	Milk silo	A
89a2	*L. innocua*	Product blender machine	B

Fig. 3.5: Pulsed field gel electrophoresis type (Pulsotypes) of listerial isolates obtained from species after restriction digestion by *Apa*I enzyme

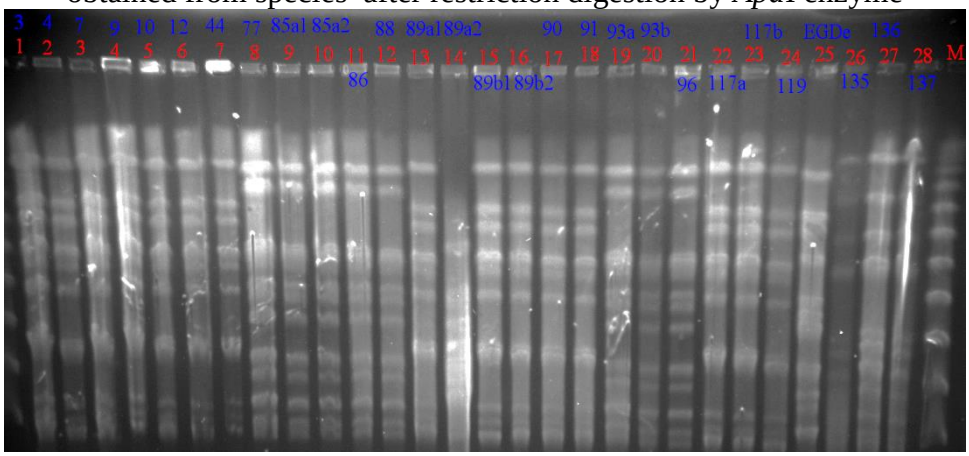
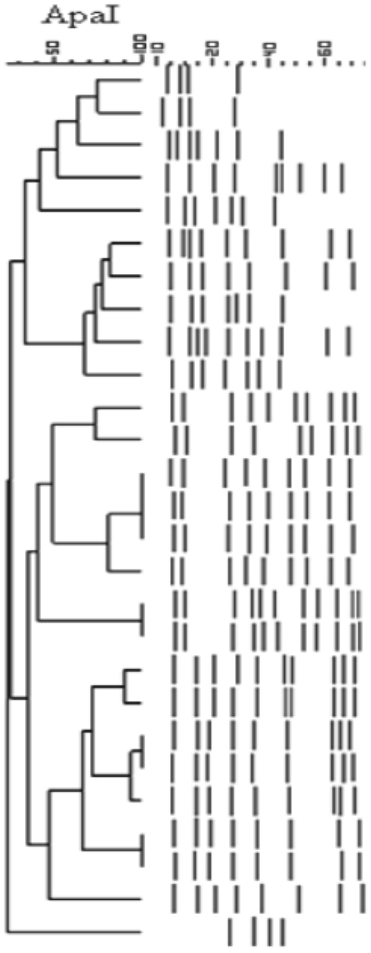


Fig. 3.6: Dendrogram showing clustering of *Apa*I restriction digestion pulsotypes of *Listeria* species obtained from food processing environment



Isolates	Serotype	Source	Plant
135	*1/2a L. monocytogenes*	Buttermilk processor	B
137	*1/2a L. monocytogenes*	Buttermilk processor	B
7	*1/2a L. monocytogenes*	Raw milk collector	A
136	*1/2a L. monocytogenes*	Buttermilk processor	B
9	*1/2a L. monocytogenes*	Milk silo	A
10	*1/2a L. monocytogenes*	Milk silo	A
4	*1/2a L. monocytogenes*	Raw milk collector	A
44	*1/2a L. monocytogenes*	Milk silo	A
3	*1/2a L. monocytogenes*	Raw milk collector	A
12	*1/2a L. monocytogenes*	Milk silo	A
88	*L. innocua*	Product blender machine	B
93a	*L. innocua*	Butter storage vessel	B
77	*1/2a L. monocytogenes*	Product blender machine	B
85b	*1/2a L. monocytogenes*	Raw milk collector	B
86	*1/2a L. monocytogenes*	Cheese blender machine	B
85a	*1/2a L. monocytogenes*	Raw milk collector	B
93b	*1/2a L. monocytogenes*	Raw milk collector	B
96	*1/2a L. monocytogenes*	Raw milk collector	B
90	*L. innocua*	Butter storage vessel	B
91	*L. innocua*	Cheese blender machine	B
89a1	*L. innocua*	Milk silo	B
89b1	*L. innocua*	Product blender machine	B
89b2	*L. innocua*	Product blender machine	B
117a	*L. ivanovii*	Raw milk collector	C
117b	*L. ivanovii*	Raw milk collector	C
119	*L. innocua*	Milk filling machine	C
89a2	*L. innocua*	Product blender machine	B

Genotyping of *L. monocytogenes* isolates can discriminate single-source clusters of food-borne infection and contribute to the identification and investigation of outbreaks (Chen & Knabel 2008). The high case fatality rate of listeriosis makes a strong case for the importance and priority of improved surveillance in India (Barbuddhe et al. 2012). *Listeria* present in raw milk could also pose a risk of contamination for a milk processing plant. The presence of *L. monocytogenes* in a processing plant could lead to post processing contamination, which also draws attention to the need to reduce the level of contamination of milk that will eventually be transported to a milk processing plant. The present study indicated the prevalence of *L. monocytogenes* in milk and milk products processing plants which may possess a potential threat to public health.

Chapter 4:

To investigate biofilm producing ability of *Listeria* species from the food and food processing plants.

4.1 Introduction

During the last decades, it has become increasingly clear that bacteria including foodborne pathogens such as *Staphylococcus aureus, Salmonella enterica, Listeria monocytogenes, Escherichia coli and Pseudomonas* spp. grow predominantly as biofilms on surfaces, in most of their habitats, rather than in planktonic mode (Giaouris et al. 2013). However, the biofilm formation of *L. monocytogenes* is of particular interest because of its association with high mortality rate, morbidity rate, hospitalization rate, increasing incidences of listeriosis outbreaks from ready-to-eat products, growth at low temperature, dual nature as a sporophyte as well as a pathogen and wide range of antimicrobial resistance (Borucki et al. 2003; Burmølle et al. 2006; Swaminathan & Gerner-Smidt 2007; Liu 2008). *L. monocytogenes* has been reported to form biofilm on meat, milk, seafood, vegetables and other food industries (Latorre et al. 2010; Meloni et al. 2012; Nakamura et al. 2013). Now it is well known that biofilm formation gives *L. monocytogenes* ability to withstand attack of antimicrobials, desiccation, low nutrients, hot water, detergents etc. (Saá Ibusquiza et al. 2011). Biofilm formation in food industries have been suspected for the persistence of *L. monocytogenes* (Carpentier & Cerf 2011). Such persistence of *L. monocytogenes* has been linked to the cross-contamination of the food product. Therefore, to control *L. monocytogenes* and its biofilm at the food industry, biofilm formation ability and its characteristics is being studied worldwide.

4.2 Literature review

4.2.1 Biofilm

The concept of biofilm can be traced back to 1920s, where many researchers showed that the bacteria can attach to glass surfaces. In 1943, Zobell reported that cells attach to the surface and form multilayers. However, the term “Biofilm” was

coined by the Characklis (1981). Biofilm is an aggregate or a layer/s of the cells, may encapsulate in self-produced (or occasionally non-self) extracellular matrix on the non-living or (rarely at living) surfaces. Biofilms have been of considerable interest in the context of food hygiene (Kumar & Anand 1998). Biofilm formation is a quorum sensing phenomenon i.e. cells when come together at a surface produce extracellular polysaccharides in three dimensional orientations, within which they live a different physico-chemical life (Garg et al. 2013). When biofilm formation is undesired and cause decomposition/ harm to the material at which they attach is generally referred as biofouling (Kumar & Anand 1998).

The biofilm formation is a dynamic process and can be divided into four different phases as- adhesion, multiplication, maturation and detachment (Flemming & Wingender 2010). Adhesion is the very first step in biofilm formation and therefore, a critical one. The adhesion capability depends upon the surface characteristics of bacterial cell surface as well as the solid surface at which they are contacting with (Garrett et al. 2008). In food industry, the bacterial cells from raw food material or from environment come in contact with the solid surfaces such as stainless steel, floor, polyvinyl chloride pipes, nylon belts etc. (Van Houdt & Michiels 2010). Depending upon the bacterial cell surface characteristics, the fate of attachment of that specific bacterium gets decided (Yang et al. 2013). If nutrients get available, the bacteria may grow and multiply establishing a niche. Eventually the bacteria grow to certain number forming a community (Ingle et al. 2011; Mann & Wozniak 2012). After certain number of cells, the bacteria sense the population and altogether show change in the physico-chemical property. This phenomenon is called as ‘Quorum sensing’ (Molloy 2013). One of the characteristics of the quorum sensing is a bacterium secreting extracellular polymeric substance (Nadell et al. 2008).

Bacteria get embedded themselves in self-produced matrix, grows adding layer over layer. As the biofilm matures, the outermost layers become loose and may get detached with the simple disturbance. Such detached cells or layer subsequently adhere to new surface and start forming a new niche (Hunt et al. 2004; Picioreanu et al. 2001). Once formed, the biofilm resists to cleaning and disinfectant procedures employed and therefore hard to remove. Areas such as grooves and ridges at the surfaces, joints of the equipment, rough floors etc. are more prone to adhesion and therefore, biofilm formation by bacterial cells (Srey et al. 2013).

4.2.2 *L. monocytogenes* biofilm in food industry

Listeria species have been found to possess the ability to adhere to solid surfaces at which they proliferate and produce extracellular polymeric substances within which they get embedded, which can be said as listerial biofilm (Djordjevic et al. 2002; Harvey et al. 2007; Carpentier & Cerf 2011; Nilsson et al. 2011). The *L. monocytogenes* has been shown to form biofilm on wide range of surfaces that are significant in medical uses and food processing premises (Chaturongkasumrit et al. 2011; [a]Bae et al. 2012; Skovager et al. 2013). However, the biofilm of *L. monocytogenes* is a major problem in food industries as it suspected to play a key role in persistence and therefore causing contamination (Møretrø & Langsrud 2004;Vestby et al. 2009; Pan et al. 2010; Carpentier & Cerf 2011). The biofilm forming *L. monocytogenes* isolates have been isolated from wide range of food processing plants such as poultry (Farber & Peterkin 1991; CDC 2009; Meloni et al. 2012; Zhao et al. 2013), fish (Manoj et al. 1991; Nørrung et al. 1999; Jeyasekaran & Karunasagar 1996; Van Coillie et al. 2004; Soumet et al. 2005; Parihar et al. 2008; Gawade et al. 2010; Nakamura et al. 2013), milk (Wong 1998; Warke et al. 2007; Latorre et al. 2010; Giacometti et al. 2012; Ning et al. 2013; Derra et al. 2013; Weiler

et al. 2013) and meat (Blackman & Frank 1996; Nørrung et al. 1999; Van Coillie et al. 2004; Gilmour et al. 2010; Wang et al. 2012). Different strains of *L. monocytogenes* found to possess different biofilm capabilities at different food processing surfaces (Borucki et al. 2003; Nilsson et al. 2011). Such biofilm producing *L. monocytogenes* strains gets protected from disinfectants, desiccation and hot water and therefore thought to be persistence in the food industry. Also, once established as a biofilm, it is very hard to remove and may act as a source of contamination. Several such biofilm producing strains recovered from food processing industry have been responsible for outbreaks (Valderrama & Cutter 2013).

4.2.3 Mechanism of biofilm formation by *L. monocytogenes*

Similar to many other bacteria, the biofilm of *L. monocytogenes* can be broadly divided into four steps as (i) adherence to surface, (ii) growth, (iii) maturation and (iv) dispersion.

(i) Adhesion

L. monocytogenes cells enter into food processing premises by different routes and get attached with the different food processing industrial surfaces. Depending upon the properties of the surfaces encountered, adherence of *L. monocytogenes* cells occur (Nyenje et al. 2012). *L. monocytogenes* cells have been reported to adhere to wide range of industrially important surfaces such as stainless steel, polystyrene, polypropylene, glass, nylon belts, ceramic-tiles, granite and marbles (Silva & Teixeira 2008). In adherence, *L. monocytogenes* cell surface properties such as hydrophobicity-hydrophilicity, electron donation-acceptance ability, overall charge on the surface, external appendages and cell surface proteins contribute to the fate of adhesion (Giaouris et al. 2013; Renier et al. 2011). The *L. monocytogenes* cell surface is negatively charged, hydrophilic, weak electron acceptor and of good electron

donating nature (Chavant et al. 2002; Szlavik et al. 2012; Skovager et al. 2013). Because of these characteristics, *L. monocytogenes* cell wall becomes adherent to wide range of surfaces. Though the bacterial cell-surface possesses hydrophobicity due to fimbriae, flagella and lipo-polysaccharide (LPS) (Bonaventura et al. 2008), hydrophobic interactions between the cell surface and the substratum may enable the cell to overcome repulsive forces resulting in adhesion of *L. monocytogenes* cells (Donlan 2002). In addition, external appendages such as flagella, pilli and cell wall proteins have been thought to entangle at abiotic surfaces leading adhesion (Vatanyoopaisarn et al. 2000; Lemon et al. 2007). Presence or absence of certain nutrients may contribute to the biofilm formation e.g. presence of L-leucine alters the fatty acid composition causing certain changes in cell wall, which has been shown to enhance the adhesion to stainless steel (Skovager et al. 2013). Beside the direct contact, it is more likely that *L. monocytogenes* cells may encounter with the resident biofilm and merge with multispecies biofilm (Giaouris et al. 2013). The adhesion of bacteria at this step occurs due to weak forces and therefore is reversible.

(ii) Growth

The adhered *L. monocytogenes* cells subsequently start multiplying and become firmly adhered. Unlike to the initial adhesion, cell in this phase are firmly adhered (Renier et al. 2011). The growth and multiplication of the cells depends upon the availability of nutrients. *L. monocytogenes* cells may obtain nutrients from the food being passed and dissolved nutrients in the wash water. As the time progresses, *L. monocytogenes* cells multiply and forms a niche (Kumar & Anand 1998). Till the growth phase, cells are exposed to environment and antimicrobials applied can reach directly to the cells causing effective microbicidal action. When the population of adhered cells reaches to certain threshold, cells synthesis and secrete small signal

molecules called autoinducers (Duan & Surette 2006). These signal molecules sensed by the bacterial population in the vicinity and induce regulation of gene expression and therefore change in microbial physiology (Garmyn et al. 2012). This phenomenon is known as quorum sensing.

(iii) Maturation

To date, two major communication systems (luxS and agr) have been described in *L. monocytogenes* (Garmyn et al. 2012). After discovery in *Vibrio* spp, several micro-organisms including *L. monocytogenes* has shown to possess the *luxS* system (Sela et al. 2006). Signaling molecule auto inducer 2 (AI-2) hypothesized as the universal signal for the interspecies communication. In detail, along with *Pfs*, LuxS enzymes catalyze the two-step conversion of S-adenosylhomocysteine, into homocysteine and 4, 5-dihydroxy-2, 3-pentanedione, which is very unstable molecule that can subsequently rearrange into various cyclic compounds, such as furanosyl borate diester or (2R,4S)-2-methyl-2,3,3,4- tetrahydroxytetrahydro furan which is called AI-2 (Belval et al., 2006). AI-2 is found in several Gram-negative and Gram-positive bacteria including *L. monocytogenes* and is therefore considered as good candidate for an inter-species communication signal molecule. AI-2 increases biofilm formation in many bacteria, however in the case of *L. monocytogenes*, presence of the *luxS* gene found to be suppressing the biofilm formation (Belval et al. 2006; Sela et al. 2006).

The Agr based quorum sensing is one of the best-studied quorum-sensing mechanisms in *S. aureus* and other staphylococci, which is broadly conserved in low G+C% Gram positive organism (Riedel et al. 2009). In *L. monocytogenes*, the four genes (*agrB*, *agrD*, *agrC*, and *agrA*) of the *agr* locus are organized in an operon. They encode the two-component histidine kinase (AgrC), response regulator (AgrA), a precursor peptide (AgrD) and a protein (AgrB) that is involved in the processing of

AgrD into a matured auto-inducing peptide. The AgrA has been suspected to involve in the regulation of nitrogen transport, amino acids, purine and pyrimidine biosynthetic pathways and phage-related functions when cells are present in biofilm (Rieu et al. 2007; Zhu et al. 2008; Garmyn et al. 2012).

The matured *L. monocytogenes* biofilm has been reported to show various biofilm morphologies as dense three dimensional structure (Borucki et al. 2003), honeycomb like structure (Marsh et al. 2003), mushroom like or knitted chain structure (Djordjevic et al. 2002; van der Veen & Abee 2010), non-organized and aggregated structure (Renier et al. 2011). The strong biofilm forming strains show a three dimensional biofilm while weak biofilm forming strains show patches of aggregate cells formed (Borucki et al. 2003). Also, higher extracellular polysaccharide production has been reported from higher biofilm producing strains (Borucki et al. 2003; Oliveira et al. 2010). The structural differences observed in these studies could be due to the different strains and growth conditions used. Food industrial surfaces that comes in contact with food are generally covered with the food soil (Verghese et al. 2011). A biofilm study performed on the food soil coated surfaces showed octagon shaped biofilm formation (Verghese et al. 2011). *L. monocytogenes* does not produce much extracellular polymeric substances as other strong biofilm formers (*Pseudomonas* spp. or *Staphylococcus* spp.) (Habimana et al. 2009). The *L. monocytogenes* biofilm has been found to possess well differentiated channels and thought to play a role in air and water transport (Chavant et al. 2002; Nilsson et al. 2011).

(iv) Dispersion

L. monocytogenes forms the mature three dimensional biofilm adding layer of cells and polymeric substances. The cells at the base of biofilm get firmly bound

while cells that are present at the periphery of the biofilm are comparatively loose. Such loosely associated cells may get detached with the simple disturbance like flow of wash water or food (Renier et al. 2011). If detachment occurs due to the food being passed, cells get mixed with the food leading contamination. If cells get detached with wash water or some other mean, cells may get carried over to other areas of food plants. Such detached cells may colonize at new place and start synthesizing new biofilm (Valderrama & Cutter 2013). Such detached cells have been shown to cause cross-contamination (Giaouris et al. 2013).

4.2.4 Characteristics of listerial biofilm

Depending upon the *L. monocytogenes* strains the characteristics of the biofilm formation have been found to change (Lianou & Koutsoumanis 2013). Several genes have been related to contribute in biofilm formation of *L. monocytogenes*. Comparative transcriptome analysis revealed 175 genes that get upregulated in biofilm formation of *L. monocytogenes* (Luo et al. 2013). Transcriptional regulatory factor (GntR) of biofilm termed as *Listeria* biofilm regulator (*lbr*A) has been identified recently and claimed to control the biofilm formation of *L. monocytogenes* (Wassinger et al. 2013). Cell-surface proteins such as Lmo2504 and non-cell-surface proteins such as activator of virulence genes (PrfA) and autolysin amidase (Ami) have been reported to contributed in biofilm formation (Skovager et al. 2013; Lourenço et al. 2013).

Several growth factors have been studied with regards to the biofilm formation capability of *L. monocytogenes*. The **temperature** seems to greatly influence the biofilm of *L. monocytogenes*. Some strains have shown enhanced biofilm with increase in temperature (Kadam et al. 2013) while in some strains showed decreased biofilm with decrease in temperature (Lee et al. 2013). Similar to temperature, poor or

rich level of **nutrients** found to influence the biofilm variably (Kadam et al. 2013). Some stains showed higher biofilm in rich nutrient (Galvão et al. 2012) while some strains showed lower biofilm (Zhou et al. 2012). In food industry, *L. monocytogenes* is more likely to form a **mixed species biofilm** than being present in a single species biofilm. Depending upon compatibility with other species, *L. monocytogenes* survives in mixed species biofilm (Giaouris et al. 2013). Co-culture studies showed that *L. monocytogenes* form biofilm with microorganism such as *Pseudomonas putida, Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa* (Hassan et al. 2004; Habimana et al. 2009; Giaouriset al. 2013; Nostro et al. 2013). Limited data is available for behavior of *L. monocytogenes* in mixed species biofilm. As *L. monocytogenes* does not produce much extracellular polymeric substances, very little is known about its composition. The main composition of EPS has been reported as - 98% water, and the remaining 2% contains dead cell debris, DNA and proteins (Kanmani et al. 2013).

4.2.5 Relation between biofilm formation ability and serotype of *L. monocytogenes*

L. monocytogenes has 13 serotypes, which are classified into four serovar groups (Doumith et al. 2004) as serovar group I (1/2a, 1/2c, 3a, 3c), serovar group II (1/2c, 3c), serovar group III (1/2b, 3b, 4b, 4d, 4e) and serovar group IV (4b, 4d, 4e). Of these serotypes, 98% of the strains isolated from foods and patients are of serotypes 1/2a, 1/2b, and 4b (Kathariou 2003). The adherences as well as biofilm formation study with respect to these serotypes are inconclusive as different study indicates different results. The adherence capability of the serotype has been shown as 4b>1/2a>1/2c (Norwood & Gilmour 2001). Serotype 1/2b and 4b have been reported to produce more biofilm than isolates from serovar 1/2a and 1/2c (Djordjevic et al., 2002) which is exactly opposite to the observation reported by Borucki et al. (2003).

Several researchers have tried to relate the biofilm forming ability with serotypes, the data varied depending upon the isolates and no clear correlation with serogroups or lineages could be established yet (Renier et al., 2011; Da Silva & De Martinis 2013). Therefore, biofilm formation and attachment of *L. monocytogenes* seems not to be serotype specific while strain specific (Weiler et al. 2013). The pulsed field gel electrophoresis is considered as "Gold-Standard" method for *L. monocytogenes* helped to track the pathogen during outbreak cases. However, in case of biofilm formation, PFGE pulsotypes did not show any relation (Galvão et al. 2012). Therefore, serotyping or pulsotyping of *L. monocytogenes* and their relations to biofilm formation is a question.

4.2.6 Relation between biofilm formation ability and serotype of *L. monocytogenes*

The biofilm formation ability of *L. monocytogenes* and its relation with virulence potential have been studied. Borucki et al. (2003) observed the prfA gene - a positive regulator of expression of many virulence genes, may enhance the biofilm formation of *L. monocytogenes* in HTM medium. This was later confirmed by Lemon et al. (2010). In contrast a study performed by Kumar et al. (2009) found deletion of prfA gene in *L. monocytogenes* EGDe did not affect the biofilm formation when grown in tryptic soy broth. The difference observed in these studies could be due to the use of different strains and media.

4.2.7 Methods used to study the biofilm

4.2.7.1 Microtiter well plate assay

Microtiter well plate (MTWP) is the most common, easy, reliable, reproducible and highly preferred method to test the biofilm of microorganisms (Christensen et al. 1985). The method consists of 96 well microtiter plate in which

culture suspension is allowed to form the biofilm. After incubation, the biofilm formed is stained with the 0.1 % of crystal violet (CV). The CV is then destained by using alcohol and the turbidity of the destained alcohol is measured by 96 well titer plate reader giving an indirect assessment of the biofilm formation. Since developed (Christensen et al. 1985), MTWP method has been employed to study the biofilm formation of several bacteria (O'Toole 2011). For *L. monocytogenes*, the method was standardized by Djordjevic et al. (2002) which was subsequently modified by Borucki et al. (2003). With the similar principles of MTWP assay, the biofilm formation can be tested by using **glass tube or petri dish** instead of 96 well MTWP (Hassan et al. 2011; Ponnusamy et al. 2012) and the turbidity of the CV is measured by using simple spectrophotometer. However, use of glass tube or petri dish sensitivity of the biofilm assay decreases as compared to the MTWP.

4.2.7.2 Microscopy

Microscopy gives the direct evidences for the biofilm formation and therefore preferred after MTWP assay. **Light microscopy** is the simplest method to study the biofilm of microorganisms. The biofilm is allowed to form on the glass slides, stained and observed under the microscope. Light microscopy is generally used to screen the biofilm forming abilities of bacteria (Walker & Keevil 1994). However, advances in microscopy have added several features in microscopes that made the biofilm study better. Addition of fluorescent dye specifically differentiates the bacteria of interest among the mixed species biofilm, which can easily be observed under **fluorescent microscope** (van der Veen & Abee, 2011). Djordjevic et al. (2002) studied *L. monocytogenes* biofilm by microtiter plate as well as by quantitative epifluorescence microscopy and observed similar trends of biofilm formation. While, Hefford et al. (2005) observed the presence of a carbohydrate containing extracellular polymeric

matrix by labeling hydrated adherent layers with fluorescein-conjugated Concanavalin A, indicating adherent layers are biofilms. **Confocal microscopy** gives three dimensional image of the biofilm. Rieu et al. (2008) utilized time-lapse laser-scanning confocal microscopy (LSCM) to characterize the structural dynamics of *L. monocytogenes* EGD-e sessile growth and to evaluate the possible role of the *L. monocytogenes agr* system during biofilm formation. Sela et al. (2006) studied the role of the *luxS* gene in biofilm formation of *L. monocytogenes* by using confocal microscopy coupled with the gene mutation studies. The detailed biofilm structure can be observed by scanning electron microscopy (SEM). Because of high magnification power SEM gives details of the arrangement of the cells, overall biofilm structure and extracellular polysaccharides (Frank 1996; Borucki et al. 2003; Renier et al. 2011; Combrouse et al. 2013). Minute details such as external appendages, flagella, probable water and air channel formed has been observed in *L. monocytogenes* biofilm by using SEM (Chavant et al. 2002; Nilsson et al. 2011).

4.2.7.3 Extracellular polymeric substances in biofilm (EPS) studies

Detailed composition analysis of an EPS is difficult, as EPS is often a complex mixture of proteins, carbohydrates, lipids, DNA, and humic acid substances (Flemming et al. 2007). Common physical methods to study EPS include **centrifugation, ultra-sonication** and **heating. Chemical methods** include extraction of EPS with chemical agent such as ethylenediaminetetraacetic acid (EDTA), formaldehyde, NaOH and NaOH-formamide are usually used as the extractants. Commonly, the EPS in biofilm is extracted then analyzed by chromatographic or spectroscopic methods (Ting & Wong 2012). **Chromatographic (HPLC and GC)** methods are the generally used to determine carbohydrate compositions, and therefore most frequently used in characterization of the EPS (Denkhaus et al. 2007). To study

the proteins from the EPS, the extraction is carried out by thermal denaturation by tricholoroacetic acid (Denkhaus et al. 2007). A qualitative and quantitative determination of various monosaccharides, oligosaccharides and uronic acids of the carbohydrate fraction of microbial EPS have been studied by high-performance liquid chromatography (HPLC) and refractive index (RI)/UV (Meisen et al. 2008). To study the ultrastructure level details TEM has been employed (Reese & Guggenheim 2007). **A confocal laser scanning microscopy (CLSM)** has been standardized to study the three-dimensional distributions of nucleic acids, proteins, α-d-glucopyranose polysaccharides, and β-d-glucopyranose polysaccharides in a single attempt by quadruple staining (Chen et al. 2006).

4.2.7.4 Molecular techniques to study the biofilm

Molecular techniques such as polymerase chain reaction (PCR), Quantitative PCR (qPCR), whole genome analysis, mutation studies, microarrays have been employed widely to determine the biofilm formation capabilities. PCR techniques generally have been used to screen the biofilm related genes (Sela et al. 2006; [b]Lemon et al. 2010; van der Veen & Abee 2010). Metagenomic approach have been found helpful to perform a microbial survey of a biofilm (Schmeisser et al. 2003; McLean & Kakirde 2013). The expression of a particular gene at given biofilm condition have been studied by use of qPCR (Beenken et al. 2004; Beloin & Ghigo 2005; Domka et al. 2007). To determine the role of a particular gene mutation studies have been used (Sela et al. 2006; Lemon et al. 2007; Lemon et al. 2010). The microarray techniques have been employed to determine the array of genes responsible for biofilm formation (Zhu et al. 2011; Tirumalai 2013; Wassinger et al. 2013). Other molecular methods such as pulsed filed gel electrophoresis and degradient gel electrophoresis have been employed to study the bacterial genome (Galvão et al. 2012; Latorre et al. 2010).

Exploring genome sequence and their comparative genomics have found useful to determine the novel genes involved for biofilm formation (Sauer 2003).

4.3.5 Control of *L. monocytogenes* biofilm

There is high demand from food industry to overcome the biofilm problems. To eradicate the *L. monocytogenes* biofilm from food industry, several approaches have been proposed (Krysinski et al. 1992). Most of the approaches have been shown potential to reduce the *L. monocytogenes* biofilm at laboratory scale and validated for the industrial scale. However, high cost, practicability at industrial scales and complexity of the treatment is becoming limiting factors for the actual use. The efforts taken to remove/destroy the *L. monocytogenes* biofilm can be discussed as per their mode. Chemicals compounds or disinfectants are the most preferred choice because of its known bactericidal effect at planktonic phase *L. monocytogenes*. Benzalkonium chloride (BC) and sodium hypochlorite are preferred choice because of its bactericidal activity even at low concentration (Giaouris et al. 2013; Nakamura et al. 2013; Rodrigues et al. 2011). Octenidine hydrochloride is found to be useful even in the presence of food soil (Amalaradjou et al. 2009). Due to strong oxidizing capability chlorine effectively removes polysaccharides, therefore penetrates into multilayers and becomes more effective in killing the cells in biofilm. However, in presence of food soil (e.g., milk protein), the efficacy of chlorine reduces (Meyer 2003). Other compounds such as peracetic acids, octanoic acids and peroxides have been found to be effective to reduce the number of *L. monocytogenes* to some extents (Yun et al. 2012). Sanitizers used in multiple combinations have shown success to reduce the number of *L. monocytogenes* cells as compared to use of these disinfectants singularly. Anti-hydrophobic agents such as trypsin, urea, and guanidium chloride control initial adhesion by lowering the hydrophobicity (Breslow & Halfon 1992).

Similarly, addition of 0.1 mM conc. ethylene diamine tetra acetic acid (EDTA) has been found to inhibit the initial adhesion (Chang et al. 2011). However, once biofilm formed, the addition of EDTA is not that effective to remove the biofilm. Therefore, application of EDTA has potential to inhibit the biofilm formation and not to remove matured biofilm.

Plant derivative compounds such as trans-cinnamaldehyde, carvacrol, thymol and eugenol that are generally recognized safe (GRAS), have been found to inhibit the biofilm synthesis as well as destruction of formed biofilm (Upadhyay et al. 2013). Polymeric film with oil (citronellol, eugenol and linalool) showed maximum of 60% reduction in number of cells that adhere to surface (Nostro et al. 2013). Plant derived essential oil such as oregano oil, carvacrol and thyme oil possess antimicrobial activity even in biofilm (Desai et al. 2012). Mustard glucosinolates in pure or extract form found to inhibit the *L. monocytogenes* biofilm formation (Lara-Lledó et al. 2012).

Enzymes such as polysaccharides and proteases have been studied to remove the biofilm. Though, few enzymes succeeded in removing biofilms, the stability of these enzymes in food industrial environment limits its practical use. Research is being performed to modify the structure of these enzymes so as to work at harsh conditions (Meyer 2003).

Physical effects such as photo-deactivation (McKenzie et al. 2013), radiofrequency electric current (Caubet et al. 2004), electromagnetic effect, hydrodynamic shear stress (Gião & Keevil 2013) have been found to sterilize the surfaces effectively. Combination of 'power ultrasound' and ozonation have been

reported to destroy all the *L. monocytogenes* in biofilm within 60 s. (Baumann et al. 2009).

Surface topography study showed the use of microbe-repellant surface material inhibit the adhesion and therefore biofilm formation (Hsu et al. 2013). Antimicrobial peptide grafted to surface revealed reduced adhesion of bacteria or inhibition of adhered bacteria (Peyre et al. 2012). Carvacrol and cinnamaldehyde polymeric films showed inhibition of biofilm activity against *L. monocytogenes* (Nostro et al. 2012).

Competition exclusion has shown effective reduction in number of *L. monocytogenes* cells in biofilm (Woo & Ahn 2013). In presence of *Lactobacillus* spp. and *Enterococci* (Zhao et al. 2013) the growth of *L. monocytogenes* gets inhibited (Ibarreche et al. 2014). *L. monocytogenes* phages such as P100, LiMN4L, LiMN4p, LiMN17 have been found to decrease the number of *L. monocytogenes* biofilms by 2-3 log units (Ganegama et al. 2013).

The literature available worldwide confirms the prevalence of *L. monocytogenes* in food processing industries. Also, in our study, we have found the *L. monocytogenes* at food-line in food processing industry. These isolates were isolated after routine 'Clean-In-Place' procedure performed. Presence of these surviving cells must be possessing ability to overcome such harsh treatment. There is a probability that these isolates may present as a biofilm which give them protection. Till date there is no control strategy is available and therefore it has been suggested to avoid the initial adhesion than to treat the biofilm. In addition, once formed, there is no firm method that is available to remove the biofilm. To address this serious issue of biofilm at food industry, there is need to understand the abilities of such pathogens to

form biofilm. Recognizing the biofilm formation ability will enlighten the survival as well as persistent strategies of *L. monocytogenes* in food industry. Therefore, we aimed to determine the biofilm formation capability of the *L. monocytogenes*.

4.3 Materials and Methods:

4.3.1 Isolates

A total of 16 *L. monocytogenes* isolates of serotype 1/2a were obtained in this study. To avoid bias in the biofilm study *L. monocytogenes* isolates of serotype 4b and 1/2b (n= 34) from food and food processing units from Indian *Listeria* Culture Collection Centre (ILCC) were included. Five isolates out of sixteen from this study based of their different PFGE pulsotypes were chosen. Also, to compare, clinical isolates of human and animal origin (n=44) were also included. These isolates were obtained from different food and food industry across India and well characterised for their serotypes and pulsotypes. Therefore, a total of 83 isolates were selected for the further study. The details of isolates, source and serotype are given in Table 4.1.

Table 4.1: Details of the *L. monocytogenes* isolates of different serotypes (1/2a, 1/2b and 4b) from food, food industrial environment and clinical cases.

Sr. No.	Place	Original ID	Serotype	Source	ILCC ID
1	Goa	GCM45F	1/2a	Animals	ILCC005
2	Agra	H8	4b	Humans	ILCC026
3	Agra	H28	1/2b	Humans	ILCC027
4	Kolhapur	KB92F	1/2b	Humans	ILCC040
5	Kolhapur	KB41S	1/2a	Animals	ILCC041
6	Kolhapur	KB874	1/2a	Animals	ILCC041a
7	Mumbai	KB94V	4b	Animals	ILCC046
8	Izatnagar	ISA13	4b	Animals	ILCC048
9	Izatnagar	ISA25	4b	Animals	ILCC049
10	Izatnagar	ISA85	4b	Animals	ILCC050
11	Pondicherry	LP 1	4b	Humans	ILCC094a
12	Pondicherry	LP 2	1/2b	Humans	ILCC095
13	Pondicherry	LEX	1/2b	Humans	ILCC097
14	Pondicherry	L4	4b	Humans	ILCC098
15	Pondicherry	L9	1/2b	Humans	ILCC099
16	Mumbai	BS345	4b	Animals	ILCC115
17	Kolhapur	KI3	1/2b	Humans	ILCC140
18	Mumbai	W43V	4b	Humans	ILCC142
19	Goa	GCM39F	4b	Animals	ILCC144
20	Mumbai	V11F	4b	Animals	ILCC146
21	Goa	V4U	4b	Animals	ILCC147
22	Goa	22	1/2a	Meat	ILCC155
23	Goa	5	1/2a	Meat	ILCC158
24	Goa	23	1/2a	Meat	ILCC159
25	Goa	LM	4b	Meat	ILCC161
26	Goa	2	1/2a	Meat	ILCC163
27	Goa	18	1/2a	Meat	ILCC164
28	Goa	11	4b	Animals	ILCC165
29	Goa	21	1/2a	Meat	ILCC166
30	Nagpur	Cheetah	4b	Animals	ILCC172
31	Nagpur	Sambar	4b	Animals	ILCC173
32	Nagpur	Landaga	4b	Animals	ILCC174
33	Mumbai	180	4b	Animals	ILCC175
34	Mumbai	186	4b	Animals	ILCC177
35	Mumbai	193	4b	Humans	ILCC180
36	Mumbai	182	4b	Animals	ILCC181
37	Mumbai	201	4b	Animals	ILCC182
38	Nagpur	38L	4b	Animals	ILCC243
39	Nagpur	76	4b	Milk	ILCC249
40	Nagpur	S/40	4b	Milk	ILCC264

Continued…..

41	Mumbai	RW 66	1/2b	Milk	ILCC283
42	Mumbai	RW 67	1/2b	Milk	ILCC284
43	Mumbai	RW 65	1/2b	Milk	ILCC289
44	Mumbai	RW 43	1/2b	Milk	ILCC291
45	Mumbai	RW 69	1/2b	Milk	ILCC297
46	Mumbai	RW 36	1/2b	Milk	ILCC300
47	Mumbai	RW 06	1/2a	Milk	ILCC301
48	Mumbai	RW 09	1/2a	Milk	ILCC302
49	Mumbai	RW 57	1/2a	Milk	ILCC303
50	Mumbai	RW 05	1/2a	Milk	ILCC304
51	Mumbai	RW 71	1/2a	Milk	ILCC306
52	Mumbai	RW 68	1/2b	Milk	ILCC309
53	Mumbai	RW 20	1/2a	Milk	ILCC312
54	Goa	14	1/2a	Milk	ILCC317
55	Goa	39	1/2a	Milk	ILCC325
56	Goa	75	1/2a	Milk	ILCC336
57	Kolhapur	4	1/2a	Milk	ILCC373
58	Kolhapur	Ai	1/2b	Milk	ILCC395
59	Goa	Lm501	1/2a	Milk	ILCC400
60	Goa	Lm31	1/2a	Milk	ILCC405
61	Goa	Lm481	1/2b	Milk	ILCC419
62	IVRI	G123	4b	Meat	ILCC468
63	IVRI	MG109	4b	Meat	ILCC470
64	IVRI	MG102	4b	Meat	ILCC471
65	IVRI	C94F	4b	Animals	ILCC491
66	Goa	14V	4b	Animals	ILCC492
67	IVRI	BG92	4b	Animals	ILCC493
68	Nagpur	White Peacock	4b	Animals	ILCC494
69	Mumbai	196	4b	Animals	ILCC496
70	Mumbai	178	4b	Animals	ILCC498
71	Mumbai	210	4b	Animals	ILCC499
72	Kolhapur	86	1/2a	Milk	ILCC519
73	Kolhapur	88	1/2a	Milk	ILCC530
74	Kolhapur	10	1/2a	Milk	ILCC531
75	Goa	Lm5	1/2a	Milk	ILCC535
76	Goa	Lm15	1/2a	Milk	ILCC540
77	Mumbai	SSBV	4b	Humans	ILCC557
78	Mumbai	NG3V	1/2b	Humans	ILCC559
79	Kankavli	FC2	4b	Humans	ILCC562
80	Mumbai	NS9F	4b	Humans	ILCC564
81	Mumbai	NS9B	4b	Humans	ILCC567
82	Mumbai	73	4b	Meat	ILCC169
83	Mumbai	BH2U	1/2b	Humans	ILCC569

4.3.2 Assessment of biofilm formation capability:

Microtiter plate assay was done as described by Borucki et al. (2003). Overnight grown listerial culture in brain-heart infusion broth (Stepanović et al. 2004) was transferred (200 µl) into 7 wells of a column of sterile polystyrene microtiter plate (GenAxy, Cat. No. 3370). The eighth well of the column was kept as a control by putting just brain-heart infusion broth. Plates were covered with sterile lid and edges of the plate were then sealed with parafilm. The plates were incubated for 24 h at 37°C. After 24 h, the cell turbidity was measured using a microtiter plate reader (Multiscan Ascent, Thermofisher) at 595 nm. The liquid from each of the wells was removed while unattached cells were removed by rinsing three times in 250 µl of sterile water. Plates were then dried in an inverted position for 30 min. Biofilms were stained by adding 200 µl of 0.1% CV solution (in sterile water) to each well, plates were sealed by parafilm and incubated for 45 min at room temperature. Unbound dye was removed by rinsing three times in 250 µl of sterile water. The CV was solubilized by adding 210 µl of 95% ethanol and incubated at 4°C for 30 min. The contents of each well (200 µl) were then transferred to a sterile polystyrene microtiter plate, and the optical density of each well was measured at 595 nm by microplate reader. Final OD for the turbidity and crystal violet was calculated by subtracting OD of the control wells from the average OD of seven test wells.

4.3.3 Quantification of the cells in biofilm

Quantitative biofilm formation assay was performed to enumerate the listerial cells as described by Jeyasekaran and Karunasagar (2000). Six representative isolates from strong, medium and weak biofilm formers irrespective of their serotypes were taken for study. Clean grease free glass slides were placed in 100 ml screw cap bottles containing 48 ml of BHI broth and autoclaved. The medium was inoculated with 2 ml

of overnight grown isolates in BHI broth. After 24 h incubation at 37^0C, the glass slides were aseptically removed and washed in sterile phosphate buffered saline (PBS) to remove unattached cells. The cells were removed by rubbing with sterile cotton swab (Hi-Media). The swab was transferred to 10 ml PBS containing 0.1% of Tween 20, shaken vigorously and serial tenfold dilutions were plated on BHI agar. To limit variation in the data due to incomplete removal of the cells from the glass slides, multiple swabs were used for the same area and inoculated in phosphate buffered saline containing 0.01% of Tween 20. The experiment was repeated three times to minimise the error. Colony count was performed and calculated for cells in biofilm/cm^2. The correlation coefficient was calculated by statistical analysis (Sharma 2005).

4.3.4 Microscopy

4.3.4.1 Electron microscopy

Scanning electron microscopy was performed to observe the biofilm formation at different time intervals on the glass slide. Four sets were prepared with clean grease free glass slide in 100 ml screw cap glass bottle containing 48 ml of BHI broth. An overnight grown strong biofilm forming isolate (ILCC306) was inoculated (2 ml) and bottles set were incubated at 37^0C for 2, 6, 12 and 24 h with shaking at 100 rpm/min. After respected incubation time, slides were removed and washed three times with PBS in order to remove unattached cells. Slides were fixed in 2% glutaraldehyde buffer in cacodylate buffer. The samples were dehydrated by increasing ethanol concentrations (50%, 60%, 70%, 85%, 95% and 100%) by 10 min. each. Specimen was coated with gold-palladium with sputter coater and cells were observed under SEM (JEOL, Model: 5800LV, Japan).

4.3.4.2 Biofilm formation on industrial important surfaces

The biofilm formation ability of ILCC306 isolates were also tested on stainless steel (SS304), polyvinyl chloride (PVC), high density polyethylene plastic (HDPE) and ceramic tiles materials were obtained from food industry equipment manufacturer and cut in 2x4 cm coupons. The samples were prepared and processed as mentioned above (4.3.4.1).

4.3.5 Determination of factors affecting biofilm formation ability in *L. monocytogenes*

4.3.5.1 Effect of growth conditions

The influence of time, nutrient, pH and salt concentration on biofilm formation ability of *L. monocytogenes* isolates was analyzed. Five isolates each from strong, moderate and weak biofilm forming isolates were randomly selected for the study. To determine the influence for longer time period incubation, the biofilm was accessed for 24, 48, 72, 96, 120 and 144 h. To test the effect of nutrients concentration on biofilm formation, BHI broth was diluted for 5 and 10 times and isolates were tested for change in the biofilm formation capability. Biofilm at different pH was accessed from pH 4.5 to 9.5 with increment by 0.5 pH units. To determine the effect of salt, biofilm was analyzed by BHI broth with 0, 0.85, 1, 2.5, 5, 7.5, 10, 12.5 and 15% concentrations on NaCl. The biofilm formation ability was accessed as described above (4.3.1).

4.3.5.2 Detection of *luxS* gene

The *luxS* gene was screened in all 83 *L. monocytogenes* isolates by PCR amplification. Twenty five microtiter reaction mixture consisting of 50 ng of bacterial genomic DNA extract, 15 pmol of primers (lmo1288) (details in Table: 6) in 1x ReadyMix™ Taq PCR Reaction Mix (Sigma, USA; Cat. No.P4600). The reaction was performed in an Eppendorf thermal cycler (Germany). The cycling conditions

were initial denaturation 94^0C for 5.0 min followed by 30 cycles of denaturation 94^0C for 45 s, annealing at 50^0C for 30 s; and extension at 72^0C for 20 s, and final extension at 72^0C for 5.0 min. PCR products were resolved by electrophoresis in 1.5 (w/v) agarose gels and visualized under Alpha Innotech gel documentation system (USA) after ethidium bromide staining. The single amplicon of aprox. 200 bp was considered as of the *luxS* gene. The PCR products were purified by using Wizard® SV Gel and PCR Clean-Up System (Promega, Cat. No. A9281). The PCR product was sequenced and sequence was confirmed by NCBI blast search.

4.3.5.3 Expression of the *luxS* gene

Six isolates each from strong, moderate and weak biofilm formers were randomly chosen to determine if any relation present between expressions of *luxS* gene and biofilm formation capability. For the gene expression studies, RNA was extracted by RNeasy extraction kit (Qiagen, Cat. No. 74104) and subsequently treated with the RNase-Free DNase according to the manufacturer's protocol. RNA concentration was estimated using an ND-1000 UV-VIS spectrophotometer (Nanodrop Technologies). The qPCR reaction mixture was set up by using Power SYBR Green RNA to CT 1-step kit (Invitrogen, Cat. No. 4391178). The same primer used for qPCR, as used in the detection of the *luxS* gene amplifying 200 bp. Expression of LPXTG genes was normalised against the 16S rDNA gene (Primers: Table 6). The qPCR was performed using the My-iQ2 system (Bio-Rad). The real-time cycling conditions were as follows: 95°C for 3 min for the initial activation step, 40 cycles each of denaturing at 95°C for 15 s, and annealing–extension at 60°C for 15 s. To confirm that a single PCR product was amplified, melting curve analysis was performed with the following conditions: 95°C for 1 min, 55°C for 1 min and 55.0–95.0°C with a heating rate of 0.5°C per 10 s. PCR amplicons were electrophoresed on

agarose gels to confirm the predicted sizes. Relative gene expression was calculated using the comparative threshold cycle (C_T) method (Livak & Schmittgen 2001). PCR efficiency and C_T values were determined using software supplied with the My-iQ2 system.

4.3.4 FAME analysis

Six isolates, each from strong, moderate and weak biofilm forming capability were analyzed to determine the variation in fatty acid profile as described by Whittaker et al. (2003) using Fatty acid methyl esterase (FAME) analysis. In brief, by using a sterile disposable wooden stick, approximately 40–50 mg of bacterial growth from tryptose soy agar was harvested in a sterile screw cap tube. The cellular fatty acids were saponified by adding 1.0 ml of 3.75 N NaOH in aqueous methanol and heated in a boiling water bath for 30 min. For methylation of the fatty acids, 2.0 ml of 3.25 N HCl in methanol was added, and the tubes were heated at 80^0C for 10 min. The fatty acid methyl esters (FAMEs) were extracted with 1.25 ml of 1:1 hexane/methyl tert-butyl ether. The organic phase was washed with 3.0 ml of 0.3 N NaOH, separated and measured by Agilent 6850 gas chromatography system. By comparing isolates with reference standards, total fatty acids ranging from $C_{12:0}$ to $C_{20:0}$ were recognized and their relative amounts were estimated by Sherlock Microbial Identification system.

4.3.5 Role of cell surface proteins in biofilm formation

4.3.5.1 Construction of mutant for sortase A

The sortase A deletion mutant was constructed in *Listeria monocytogenes* EGDe (LMWT) (Bierne et al. 2002) with little modification. In brief, 434 bp fragment upstream and 501 bp fragment downstream to the *srt*A gene was amplified (SrtAUF-SrtAUR; SrtADF-SrtADR; Table 6). Upstream and downstream fragments were

digested by *Hind*III and ligated to make an upstream-downstream (UD) insert. The UD insert was digested with *Bam*HI and *Eco*RI and ligated into the thermo-sensitive shuttle vector pKSV7. The plasmid was then electroporated into LMWT and gene replacement was performed (Fig. 4.1) (Camilli et al. 1993; Dramsi et al. 1997). The deletion of *srtA* in the deletion mutant (LMΔ*srtA*) was confirmed by PCR (SrtAUF1, SrtAI and SrtADR1). LMΔ*srtA* was complemented with complete 669 bp of sortase A gene by using pIMK2, a *Listeria* specific overexpression plasmid (Monk et al. 2008). In brief, the promoter-less sortase A gene was amplified (SrtACF and SrtACR) and ligated downstream to a constitutive Phelp promoter at *Nco*I/*Pst*I site within pIMK2 (Fig. 4.2; 4.3). The resulting vector pIMK2*SrtA* was transformed into the Δ*srtA* mutant strain yielding a single copy integration of pIMK2*SrtA* into the EGDe chromosome (LMΔ*srtA::pIMK2srtA*). Replacement of the functional gene was then confirmed by PCR and the *srtA* transcript was confirmed by qPCR. Change in the biofilm forming capability of the LMWT, LMΔ*srtA* and complemented strain was assessed by microtiter plate assay as described in section 4.3.1. The loss of number of adhered cells was enumerated as described in section 4.3.3. Actual change in biofilm formation on polystyrene surface was observed by electron microscopy as described in section 4.3.2.1 (except the samples were observed under Nova™ Nano SEM 630).

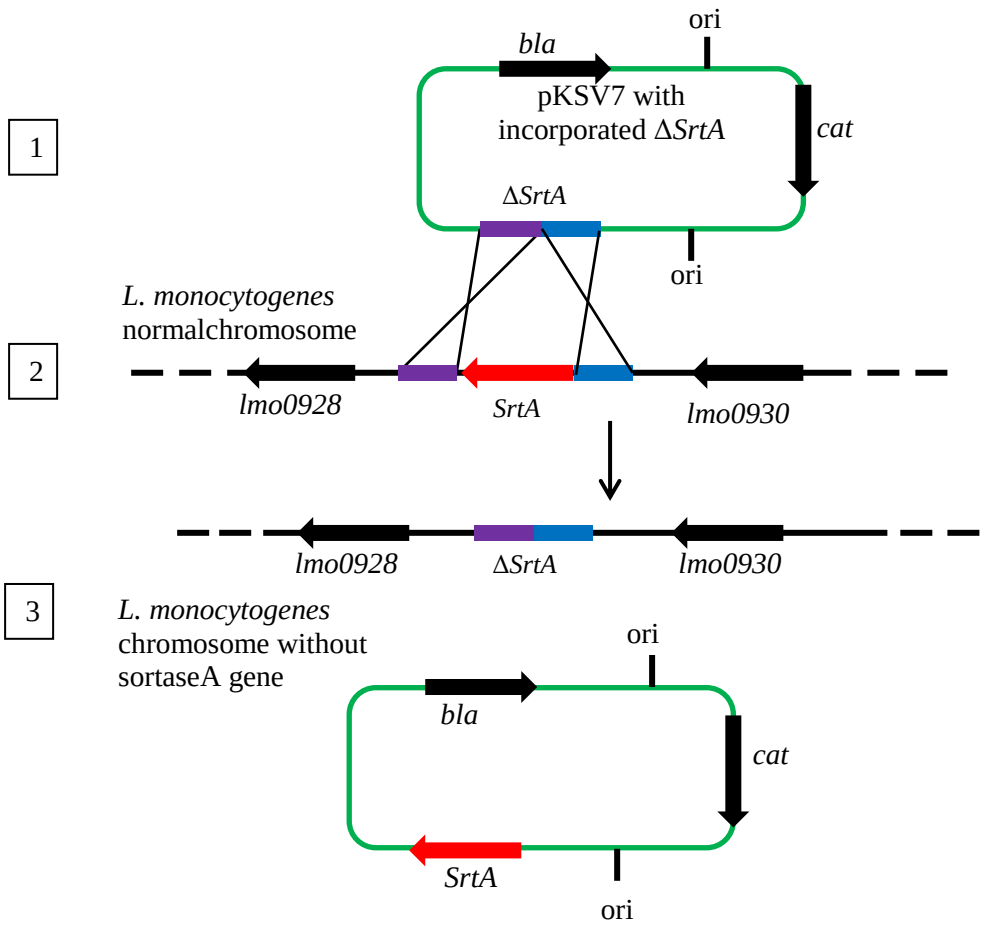


Fig. 4.1: Construction of deletion mutant for sortaseA in *L. monocytogenes* EGDe strain toward studying the role of sortase A in biofilm formation. The sortaseA gene was replaced by gene allelic exchange method by suing pKSV7 temperature sensitive shuttle vector.

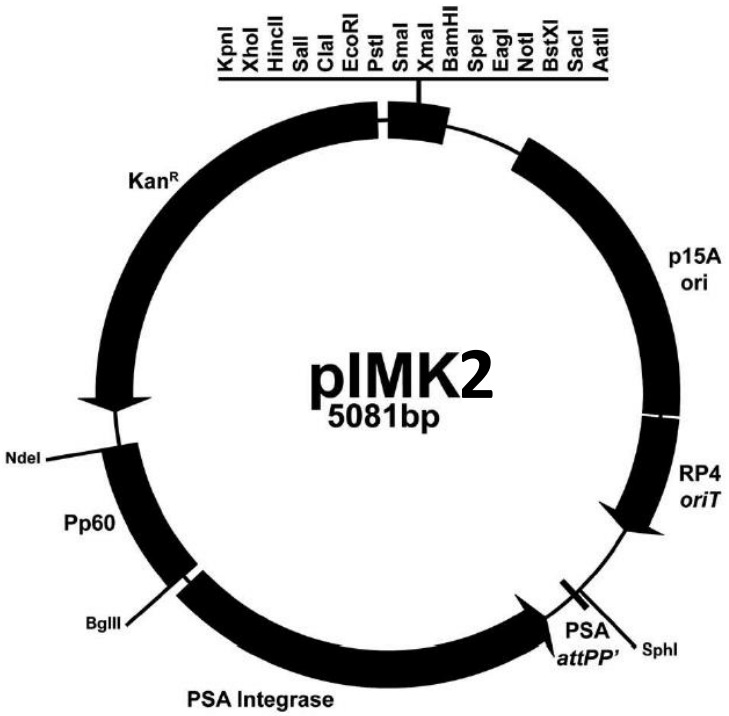


Fig. 4.2: Details of the pIMK2 plasmid used for the construction of the complement for sortaseA enzyme mutant in *L. monocytogenes* EGDe strain. The promoter less sortase A gene was placed downstream to synthetic Phelp promoter (Fig. 4.3) to construct the complement (LMΔ*srtA::pIMK2srtA*)

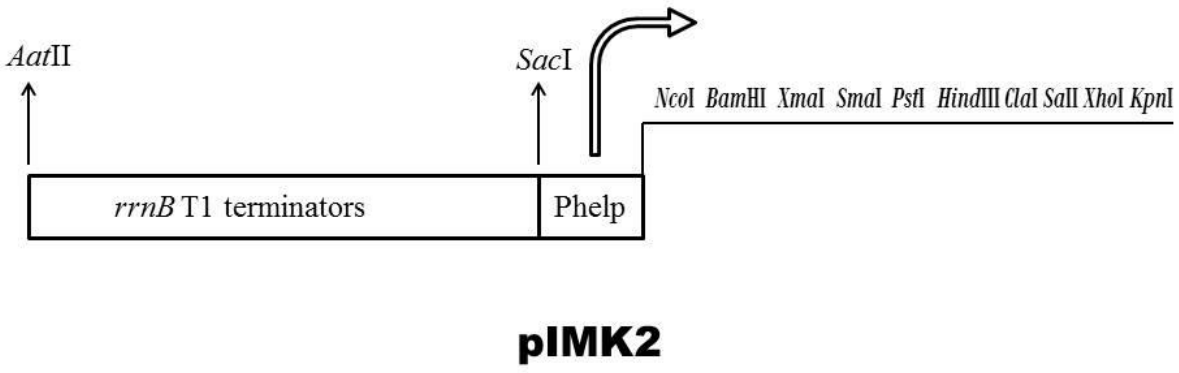


Fig. 4.3: The promoter region of pIMK2 plasmid with the synthetic Phelp promoter (Monk et al. 2008).

4.3.5.2 Determination of LPXTG gene expression

The gene expression of the 18 representative genes encoding LPXTG proteins was studied to determine the influence of the sortase gene on expression of the LPXTG-motif proteins. RNA from LMWT, LMΔ*srtA* and complement was extracted in its planktonic phase as well in biofilm (12 h and 24 h) by using RNeasy mini kit (Qiagen, Cat No. 74524).

The RNA from planktonic cells was isolated as per manufacturer's instructions. In case of biofilm; the growth was swabbed from 7 microtiter wells and dipped in 1 ml of saline and vortexed vigorously. The suspension was subsequently analysed for isolation of RNA and treated with DNAse. The concentration of RNA was assessed by agarose gel electrophoresis and spectrophotometry. The primers used are listed in the table 6. QPCR was performed by using QuantiTect SYBR® Green RT-PCR Kit (Qiagen, Cat. No. 204243). The Q-PCR conditions were used as described in the section 4.3.5.3. Expression of LPXTG genes was normalised against the 16S rDNA gene (Primers: Table 6).

4.3.5.3 Determination of Hydrophobicity index

Hydrophobicity index of LMWT, LMΔ*srtA* and complemented strain was determined by microbial adherence to n-hexadecane (MATH) test as described by Di Bonaventura et al. (2008). In brief, an overnight (18 hour) culture (4 ml) was centrifuged at 8000 g for 5 min. Cell pellets were washed three times by using phosphate buffered saline (PBS) and re-suspended in PBS (4 ml). The O.D. of the LMΔ*srtA* and wild type *L. monocytogenes* EGDe strain were adjusted by using PBS at a constant value (A_0). Then, 1 ml of n-hexadecane (Sigma-Aldrich) was added and vortexed for 1 min. Suspensions were allowed to separate out for 15 min at room temperature. Approximately 200 µl of cell suspension was transferred to each well of

a microtiter plate and turbidity of the cell suspension was measured at 590 nm wavelength using a microtiter plate reader (BioTek, ELx800). The hydrophobicity index was calculated by the formula $100*(1-A_1/A_0)$.

4.3.6 Nanotubes

During electron microscopic studies, an interesting structure –a conduit formed between two cells were observed. The literature survey done revealed such conduit has been revealed recently, termed as 'Nanotubes' that take part in physical communication by exchanging the cytoplasmic molecule (Dubey & Ben-Yehuda 2011). As a characteristic of *L. monocytogenes* biofilm, we tried to explore this structure as a probably novel mode of communication in biofilm of *L. monocytogenes*.

4.3.6.1 Determination of nanotube in *L. monocytogenes*

Three *L. monocytogenes* strains EGDe, ATCC 08-5923 and ATCC 19115 were included in the study. EM sample preparation was performed as described by Dubey et al. (2011) with little modification for *L. monocytogenes*. Strains were tested in three different phases such as (i) on solid agar, (ii) in biofilm and (iii) in liquid medium. To observe the nanotube formed on solid medium, exponentially grown *L. monocytogenes* strains were plated on BHI agar and incubated at 37^0C for 24 h. A single colony was suspended in 1 ml of normal physiological saline (0.85% NaCl) and the suspension was filtered through 0.2 µm filter. Cells were washed by passing 0.1M sodium cacodylate buffer (pH 7.2) and then fixed with 2% glutaraldehyde in sodium cacodylate buffer (0.1M, pH 7.2) for 2 h at 25^0C. Samples on filters were treated by 1% osmium tetraoxide for 1 h at 25^0C in dark. The samples were dehydrated by increasing ethanol concentrations (50%, 60%, 70%, 85%, 95% and 100%) by 10 min. each. Specimen was coated with gold-palladium with sputter coater and cells were observed under NanoSEM (JEOL JSM 5400). In case of biofilm, cell growth was

scrapped from 24 h old biofilm on glass surface and suspended in saline, while for growth in liquid, 24 h old cultures were diluted 100 times in saline (to avoid clogging of filter) and suspension was passed through filter. Filters were processed as described above.

4.3.6.2 Determination of integrity of nanotube

To determine these tubes as an integral part of the cell and not the artefact, the approximate composition of these tubes were determined by using Energy dispersive spectroscopy (EDS). The samples were prepared as described above and the nanotube was located in the SEM field. Points from nanotube, connecting cells were focused (Fig. 2A) and EDS was performed. The EDS data obtained was collected from AZtecEnergy EDS Microanalysis software and tabulated to compare the approximate composition of the nanotube and the cell wall.

To further observe if nanotube as a part of the cell wall, 0.008% of SDS was added to BHI agar and *L. monocytogenes* cells were grown for 24 h at 37^0C. The samples were processed as described above and observed under scanning electron microscopy.

4.3.6.3 Determination of transfer of intracellular cytoplasmic molecule ability by nanotube

Presence of nanotubes raises the possibility of probable exchange of cytoplasmic material between connecting cells. We tried to determine whether there is any transfer of cytoplasmic molecules between two cells by use of calcein as described by Dubey & Ben-Yehuda (2011). Calcein is a small non-fluorescent acetoxymethylester (AM) derivative and a non-genetically encoded cytoplasmic fluorophore that is sufficiently hydrophobic to traverse cell membranes. After passage into the cytoplasm, hydrolysis of calcein by endogenous esterases gives rise to a

fluorescent hydrophilic product (623 Da) that is unable to traverse membranes and thus caged within the cytoplasm (Haugland 2005; Dubey & Ben-Yehuda 2011). Therefore, only possible calcein transfer may happen if there is a physical contact between two cells which could transfer the product as big as 623 Da. To determine the transfer, calcein treated cells were mixed with untreated cells (0.25 ml each) in 1:1 ratio, mixed by 3-4 gentle pipetting. Mixed cells were spotted on agarose gel and observed under fluorescent microscope. The microscopic field was adjusted to observe the gradient formed by transfer of fluorescent dye and time lapse microscopy was performed at the interval of 10 min. till 50 min. by using Fluoview software. The control was kept by adding 0.008% of SDS 1:1 calcein treated and untreated cells mixture. The mixture was mixed by 3-4 gentle pipetting and time lapse microscopy was performed.

4.4 Results and Discussion

4.4.1 Biofilm formation ability of *L. monocytogenes*

L. monocytogenes is a ubiquitous bacterium widely distributed in the environment that can cause a severe disease in humans (Almeida et al. 2013). *L. monocytogenes* outbreaks has been frequently linked to foods those were industrially processed and refrigerated (Gianfranceschi et al. 2002). Because of ubiquitous nature, *L. monocytogenes* has been reported in many sections of various food production plants (Latorre et al. 2010). The raw food material received may contain *L. monocytogenes* (Thimothe et al. 2002; Gelbícová and Karpísková, 2012; Ning et al. 2013), however, the bactericidal treatments (e.g. pasteurization, addition of preservatives) performed to increase the shelf life of the product kills *L. monocytogenes* along with other bacteria. Interestingly, in spite of such bactericidal treatments, *L. monocytogenes* have been reported to contaminate the final food products (Lianou and Sofos 2007; D'Costa et al. 2012). Persistence of *L. monocytogenes* at post-processing environment has been thought to be the main reason behind contamination. Though harsh sanitization employed at processing environment, several reports showed that *L. monocytogenes* could enter through different routes such as exchange of workers from different department, water used to clean, commonly used equipment (Ivanek et al., 2004; Maitland et al., 2013) and reside in the post-processing environment (Beresford et al., 2001; Latorre et al., 2010). Once entered, depending upon the environmental conditions and capability of the organism, *L. monocytogenes* strains have been thought to persist in the environment and contaminate the food getting processed (Farber and Peterkin 1991). Therefore, presence of biofilm in food production lines becomes a root cause of the contamination of ready-to-eat products with *L. monocytogenes* (Kalmokoff et al.

2001). The biofilm-forming capability of *L. monocytogenes* allows its persistence in the food processing environment, which may subsequently get added in the food unknowingly. A total of 83 listerial isolates were analysed for their biofilm formation ability using the microtiter plate assay (Fig. 4.4A). Brain heart infusion broth was preferred as compared to other broth as suggested by Stepanović et al. (2004). The biofilm was predominant at the liquid-air interface (Fig. 4.4B). Strains were designated as weak biofilm formers (WBF) (OD <0.323), moderate biofilm formers (MBF) (OD, 0.324-0.646) or strong biofilm formers (SBF) (OD >0.646) according to the measured crystal violet OD_{595}(CV-OD_{595}) values (Harvey et al., 2007). Out of 83 isolates, 53 (63.85%) were assessed as WBF, 22 (26.50%) were MBF and 8 (9.63%) were SBF.

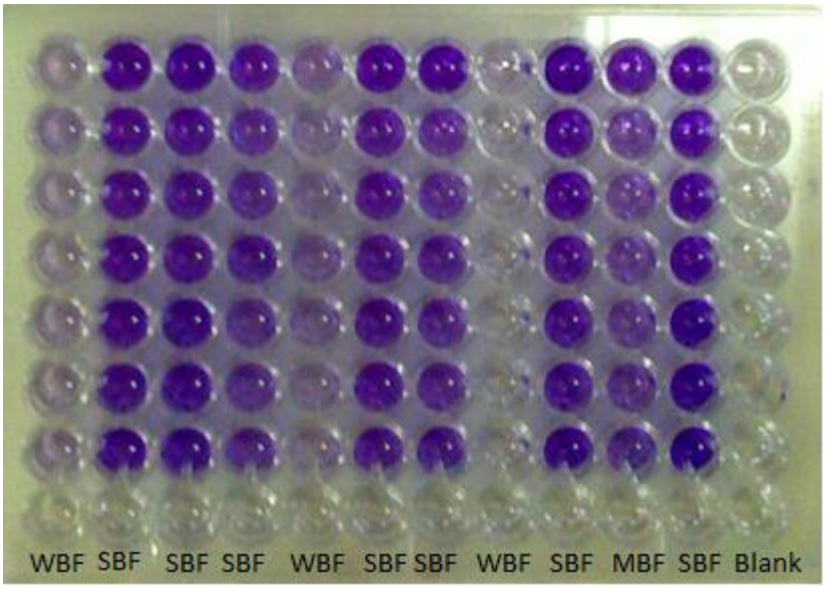


Fig. 4.4.A: 96 well microtiter plate showing destained crystal violet as a measure of biofilm.

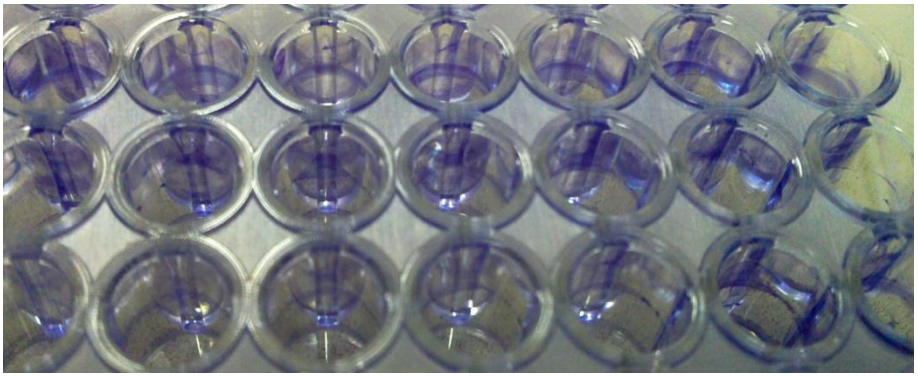


Fig. 4.4.B: The biofilm formed by *L. monocytogenes* as a ring at the air-liquid interval on the 96 well polystyrene microtiter well plate

Table 4.2: Average turbidity of the destained crystal violet (CV) (a measure of biofilm forming capability) and growth turbidity of *L. monocytogenes* isolates from different serotypes and sources. (Color indication: Red-Strong biofilm former; Yellow-moderate biofilm former and Green-weak biofilm former)

Sr. No.	Source	ILCC ID	Serotype	Average turbidity of the CV (biofilm)	Turbidity
1	Animals	ILCC005	1/2a	0.199	0.854
2	Humans	ILCC026	4b	0.085	0.712
3	Humans	ILCC027	1/2b	0.360	0.643
4	Humans	ILCC040	1/2b	0.184	0.297
5	Animals	ILCC041	1/2a	0.339	0.583
6	Animals	ILCC041a	1/2a	0.189	0.617
7	Animals	ILCC046	4b	0.291	0.657
8	Animals	ILCC048	4b	0.390	0.647
9	Animals	ILCC049	4b	0.185	0.643
10	Animals	ILCC050	4b	0.388	0.604
11	Humans	ILCC094a	4b	0.432	0.631
12	Humans	ILCC095	1/2b	0.359	0.643
13	Humans	ILCC097	1/2b	0.084	0.247
14	Humans	ILCC098	4b	0.432	0.631
15	Humans	ILCC099	1/2b	0.494	0.637
16	Animals	ILCC115	4b	0.165	0.805
17	Humans	ILCC140	1/2b	0.400	0.637
18	Humans	ILCC142	4b	0.070	0.754
19	Animals	ILCC144	4b	0.379	0.686
20	Animals	ILCC146	4b	0.198	0.666
21	Animals	ILCC147	4b	0.121	0.796
22	Meat	ILCC155	1/2a	0.223	0.725
23	Meat	ILCC158	1/2a	0.294	0.713
24	Meat	ILCC159	1/2a	0.186	0.743
25	Meat	ILCC161	4b	0.268	0.617
26	Meat	ILCC163	1/2a	0.093	0.679
27	Meat	ILCC164	1/2a	0.240	0.772
28	Animals	ILCC165	4b	0.098	0.713
29	Meat	ILCC166	1/2a	0.334	0.406
30	Animals	ILCC172	4b	0.098	0.605
31	Animals	ILCC173	4b	0.023	0.619
32	Animals	ILCC174	4b	0.159	0.661
33	Animals	ILCC175	4b	0.145	0.643
34	Animals	ILCC177	4b	0.400	0.604
35	Humans	ILCC180	4b	0.170	0.754
36	Animals	ILCC181	4b	0.268	0.686
37	Animals	ILCC182	4b	0.198	0.666
38	Animals	ILCC243	4b	0.199	0.725

Continued……..

39	Milk	ILCC249	4b	0.237	0.755
40	Milk	ILCC264	4b	0.237	0.854
41	Milk	ILCC283	1/2b	0.624	0.657
42	Milk	ILCC284	1/2b	0.395	0.701
43	Milk	ILCC289	1/2b	0.440	0.619
44	Milk	ILCC291	1/2b	0.269	0.730
45	Milk	ILCC297	1/2b	0.905	0.707
46	Milk	ILCC300	1/2b	0.269	0.755
47	Milk	ILCC301	1/2a	0.634	0.743
48	Milk	ILCC302	1/2a	0.348	0.772
49	Milk	ILCC303	1/2a	0.127	0.406
50	Milk	ILCC304	1/2a	0.229	0.679
51	Milk	ILCC306	1/2a	0.971	0.756
52	Milk	ILCC309	1/2b	0.017	0.750
53	Milk	ILCC312	1/2a	0.953	0.682
54	Milk	ILCC317	1/2a	0.187	0.657
55	Milk	ILCC325	1/2a	0.173	0.636
56	Milk	ILCC336	1/2a	0.350	0.682
57	Milk	ILCC373	1/2a	0.160	0.636
58	Milk	ILCC395	1/2b	0.905	0.632
59	Milk	ILCC400	1/2a	0.836	0.701
60	Milk	ILCC405	1/2a	0.173	0.730
61	Milk	ILCC419	1/2b	0.169	0.621
62	Meat	ILCC468	4b	0.356	0.790
63	Meat	ILCC470	4b	0.268	0.574
64	Meat	ILCC471	4b	0.268	0.617
65	Animals	ILCC491	4b	0.390	0.657
66	Animals	ILCC492	4b	0.356	0.611
67	Animals	ILCC493	4b	0.300	0.647
68	Animals	ILCC494	4b	0.121	0.796
69	Animals	ILCC496	4b	0.056	0.617
70	Animals	ILCC498	4b	0.098	0.740
71	Animals	ILCC499	4b	0.023	0.430
72	Milk	ILCC519	1/2a	0.836	0.707
73	Milk	ILCC530	1/2a	0.135	0.719
74	Milk	ILCC531	1/2a	0.174	0.750
75	Milk	ILCC535	1/2a	0.125	0.650
76	Milk	ILCC540	1/2a	0.903	0.934
77	Humans	ILCC557	4b	0.523	0.625
78	Humans	ILCC559	1/2b	0.044	0.790
79	Humans	ILCC562	4b	0.056	0.712
80	Humans	ILCC564	4b	0.523	0.625
81	Humans	ILCC567	4b	0.056	0.712
82	Meat	ILCC169	4b	0.720	0.540
83	Humans	ILCC569	1/2b	0.096	0.700

L. monocytogenes has been divided into 13 serotypes, of which only few serotypes has been strongly linked with outbreaks. Since then researchers have tried to correlate the *L. monocytogenes* serotypes in relation to many other aspects including biofilm forming capabilities. The serotype 1/2a strains were found to be good biofilm formers than serotype 4b strains (Pan et al., 2010; Soni and Nannapaneni, 2010; Nilsson et al., 2011) while, the biofilm formation capabilities of 4b and 1/2b serotypes were found to change as per growth conditions (Folsom et al., 2006). Kadam et al. (2013) reported nutrient media may influence the biofilm production level of different *L. monocytogenes* serotypes. These contradictory conclusions may not be comparable due to different experimental approaches used to evaluate attachment, sample size, and the genetic variability of the strains (Valderrama & Cutter, 2013). Therefore, after several efforts studying biofilm formation by different *L. monocytogenes* serotypes, till date evidences observed are conflicting and not specifying relation between serotypes and biofilm formation capability (Djordjevic et al. 2002; Borucki et al. 2003; Combrouse et al. 2013). Therefore, biofilm data obtained were compared according to the serotypes to determine any relation, if present.

In results, no serotype was found to be dominant for the biofilm formation. Out of 26 isolates from serotype 1/2a, 5(19.23%) were SBF, 4(15.38%) MBF and 17(65.38%) were WBF. Of 17 isolates from serotype 1/2b, 2(11.76%) were SBF, 7(41.17%) MBF and 8(47.05%) were WBF. Among the 40 isolates from serotype 4b, only a single isolate could exhibit a strong biofilm while, 11(27.5%) isolates exhibited moderate and 28(70%) showed weak biofilm formation (Table 4.2). No significant correlation was found between biofilm formation (crystal violet OD) and cell growth turbidity (correlation coefficient (CC), r =0.12). Average turbidity of growth and

crystal violet stain (indirect assessment of biofilm forming ability) of each *L. monocytogenes* isolates taken after 24 h incubation at 37^{o}C is shown in figures 4.5 (A, B & C). Specifically, isolates from serotype 1/2a showed very weak positive (r = 0.30) relation between biofilm formation while serotypes 4b and 1/2b showed weak negative (r = -0.18) and weak positive (r =0.14), respectively. We observed high variation in biofilm formation among the strains that belong to different serotypes and sources. There was no correlation observed with serotypes or growth capability. Our data is consistent with Bonaventura et al. (2008) supporting independency of strains in biofilm formation capability rather than property of serotype. Further, to determine if the biofilm formation capability gets influenced by growth, planktonic growth and biofilm forming ability was compared. As reported earlier (Pan et al., 2010) biofilm formation capability of the isolates was found to be irrespective of the growth. Consistent with earlier reports (Djordjevic et al. 2002; Borucki et al. 2003; Folsom et al. 2006; Pan et al. 2009; Soni and Nannapaneni 2010; Pan et al. 2010), biofilm formation capability of the *L. monocytogenes* are not necessarily related to their serotypes, but it is the property of the independent strain along with growth conditions. The relation between serotypes and biofilm formation that has reported could be the result of cells with different property.

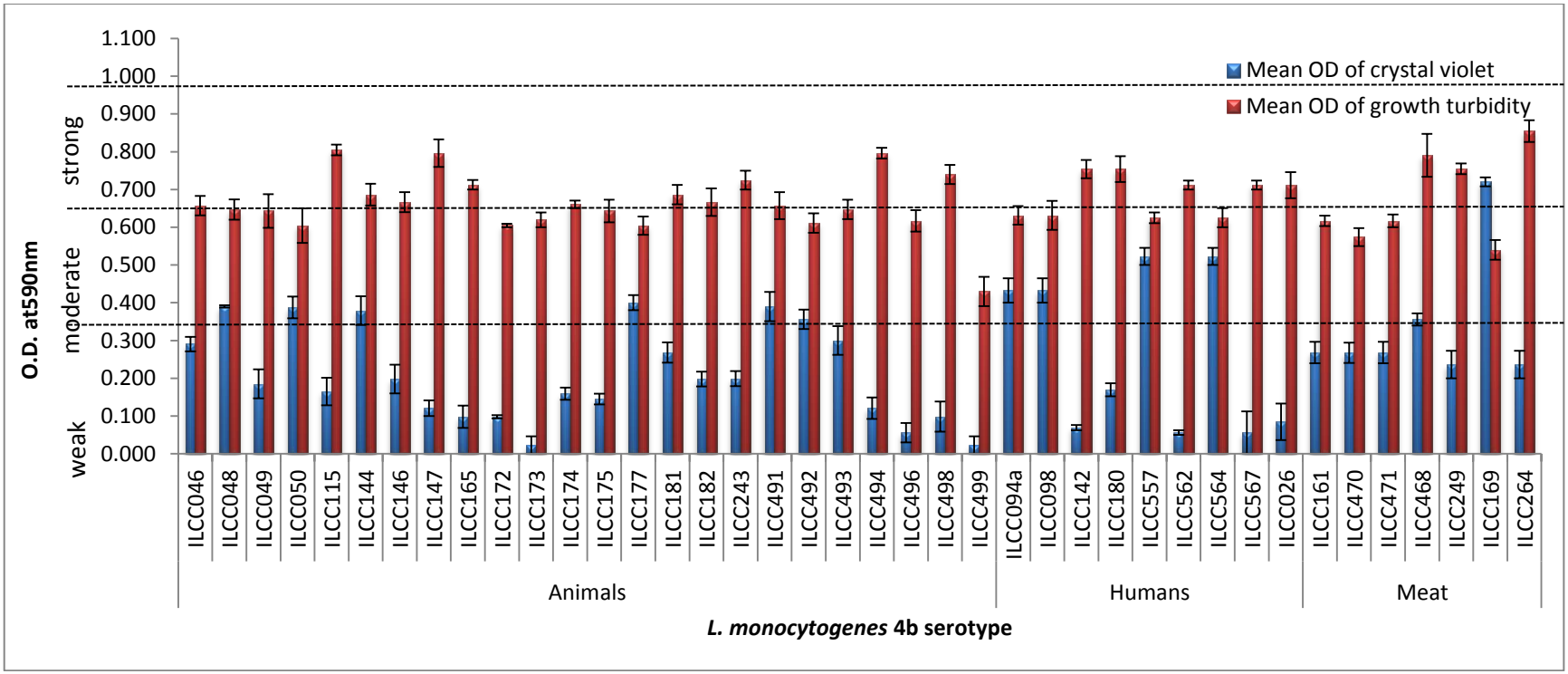


Fig. 4.5.A: showing growth turbidity (black bars) and indirect assessment of the biofilm formation (grey bars) of *L. monocytogenes* 1/2a, 1/2b and 4b isolates obtained from different sources. Standard errors are denoted by "⊥" for -ve and "┬" +ve. None of the serotype under study was predominant for biofilm formation. Also, there was no significant correlation observed between growth turbidity and biofilm formation capability of the isolates.

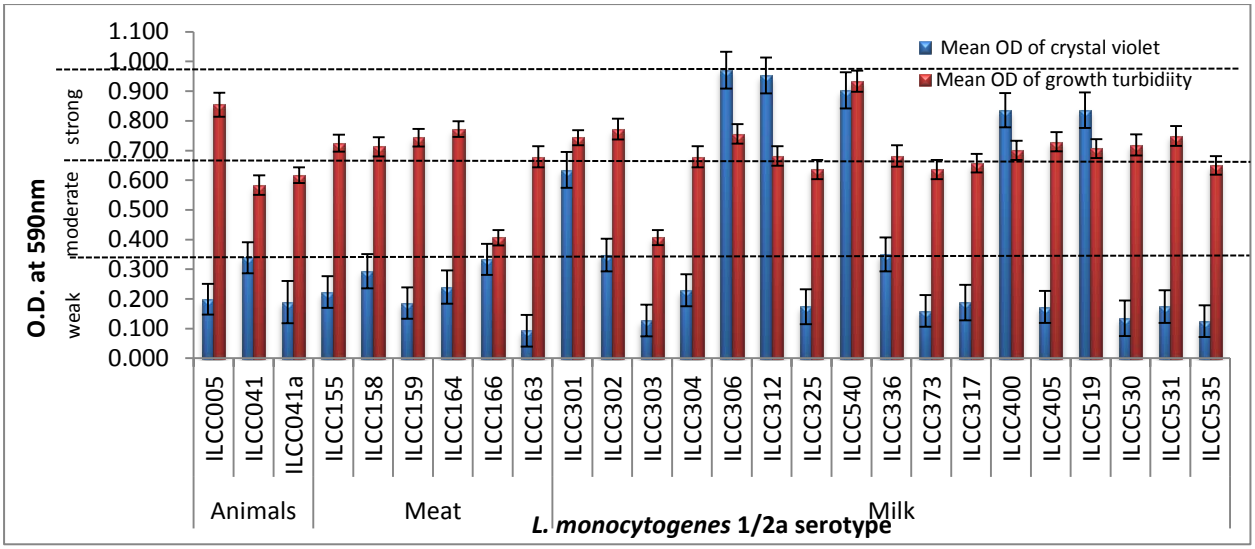


Fig. 4.5.B: showing growth turbidity (black bars) and indirect assessment of the biofilm formation (grey bars) of *L. monocytogenes* 1/2a isolates obtained from different sources

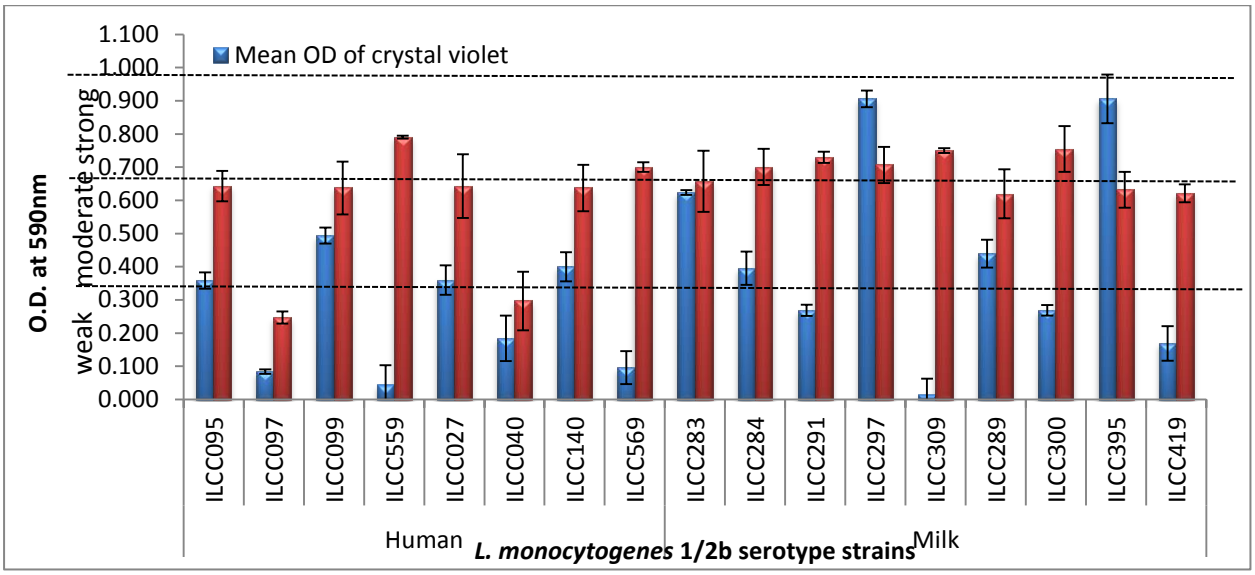


Fig. 4.5.C: showing growth turbidity (black bars) and indirect assessment of the biofilm formation (grey bars) of *L. monocytogenes* 1/2b isolates obtained from different sources

4.4.2 Quantification of *L. monocytogenes* cells in biofilm

Microtiter plate assay using crystal violet stains both cell and extracellular matrix (Pan et al. 2010). The method does not give an idea about the number of live cells contributing at the intensity of the biofilm (Kadam et al. 2013). Therefore, we enumerated the approximate cells present per square centimetre of the biofilm on the glass surface. Biofilm formation ability of the isolates was further analysed by determining the actual number of cells present in the biofilm. Six representative strains from each type (strong, medium and weak biofilm formers) of each of serogroup were considered for the study to differentiate the biofilm formation capability. Enumeration study showed average of 50×10^4 cells/cm^2 by strong biofilm formers, followed by moderate biofilm former 33×10^3 cells/cm^2 and weak biofilm former 10×10^2 cells/cm^2. Enumeration data obtained was supportive to the microtiter plate study showing the increase in the biofilm formation due to increase in the number of cells. In contrast to earlier study (Pan et al., 2010) our study revealed the intensity of the biofilm had relation to the higher number of live bacteria. However, the method used in our study could not clarify about the extracellular matrix and its role in different intensity of the biofilm.

4.4.3 Microscopic study of biofilm

To observe the actual biofilm formed, listerial biofilm was studied by scanning electron microscopy (SEM). The strongest biofilm forming strain ILCC306 was studied for its adherence, multiplication and biofilm developing capability at 37^{o}C in BHI broth. The shaking condition was incorporated so as to mimic the nutrient flow condition as occur in food industry, for ex. Milk industry. It was observed that, strain could adhere to glass surface within 2 h, showing initial attachment step (Fig. 4.6, 2h), followed by firm adherence and subsequent multiplication of cells increasing the biofilm (Fig. 4.6, 6h). Adhered cells multiplied and started forming multilayer within

6 h. By 12 h, a mature biofilm was observed (Fig. 4.6, 12 h) and cells surrounded by matrix could be seen by 24 h. Listerial cells found to be embedded in the biofilm matrix at 24 h (Fig. 4.6, 24 h). The structural composition of the *L. monocytogenes* isolate ILCC306 did not match with any of the previously reported specific biofilm structures such as dense three dimensional structure (Borucki et al. 2003), honeycomb like structure (Marsh et al. 2003), mushroom like or knitted chain structure (Djordjevic et al. 2002). The observed nature of the biofilm was not considerably organized and aggregated all over the glass material forming micro colonies as observed by Renier et al. (2011). The structural differences observed in these studies could be due to the different strains and growth conditions used.

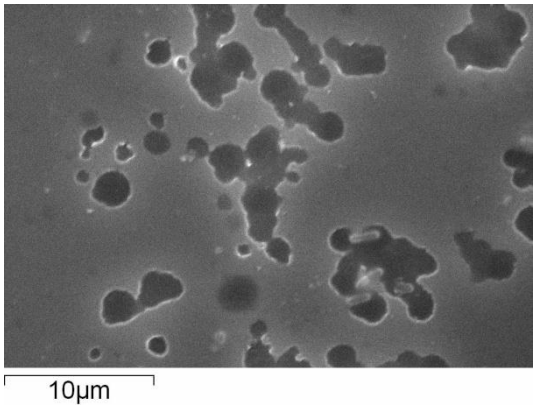
Scanning electron microscopy was also performed to observe the biofilm formed at different industrially important surfaces. Isolates were allowed to form biofilm on stainless steel (SS303), HDPE plastic, PVC pipes, ceramic tiles and glass. Biofilm formation on all the surfaces was observed after 48 h incubation. Multi-layered and mat like biofilms were observed on the PVC pipes (4.7.A). In case of tiles, the cells were found to be aggregated all over the surfaces (4.7.B). Electron microscopy revealed numbers of microscopic sutures at all the surfaces studied. Cells were aggregated in and around these sutures suggesting the probable root of adhesion. Comparatively, biofilm was more near the sutures than the plain surface area (Fig. 4.7.C). To determine the role of suture, biofilm formation was also studied on SS304 coupon with artificial sutures. Similar biofilm pattern was observed in artificial sutures (Fig. 4.7.D). Apparently, sutures protect the cells by giving shelter them from the flow of liquid (such as food or wash water). Cells situated in these sutures grow eventually and forms the strong base for the biofilm. In case of HDPE material also, biofilm rooted in the sutures could be seen in SEM (Fig. 4.7.E).

Slight change in the morphology of *L. monocytogenes* cells from very short rod to cocci were observed on all surfaces tested. Similar morphology was observed for *L. monocytogenes* on stainless steel by Somers and Wong (2004), while Wen et al. (2009) hypothesized incubation of *L. monocytogenes* for longer time push cells into 'dormant or long-term-survival phase' forming cocci morphology. The change in morphology observed could be due to exhaustion of nutrients as well as long period of incubation (48 h).

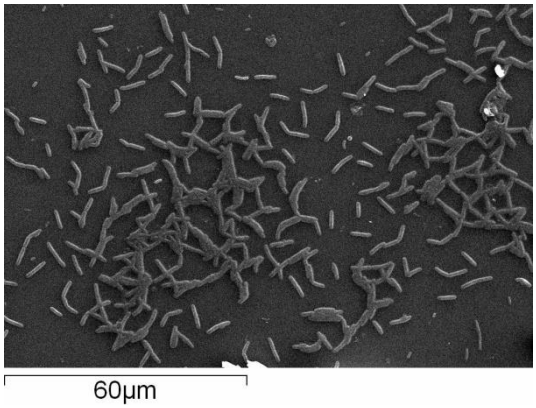
The SEM study confirms that *L. monocytogenes* can adhere and forms biofilm on different industrially important surfaces. Such biofilm formation may help *L. monocytogenes* cells to persist in the food industry and therefore a serious matter of concern.

Fig. 4.6: Scanning electron microscopy observations of *L. monocytogenes* biofilm formation at different time interval. *L. monocytogenes* strains were grown at 28°C in BHI on glass slides and observed after 2h, 6h, 12h, and 24h.

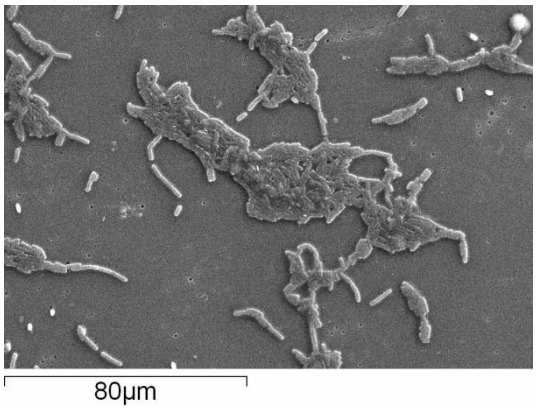
2h



6h



12h



24h

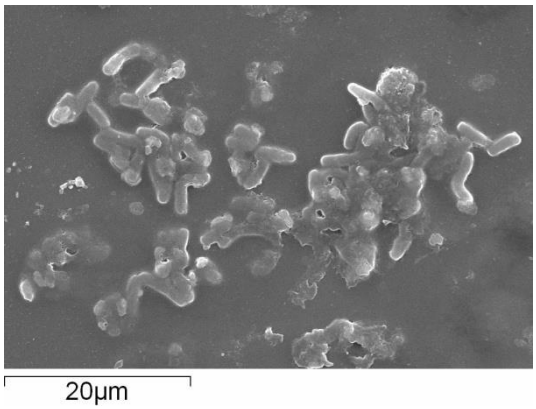


Fig 4.7: Scanning electronmicrograph of biofilm formed by *L. monocytogenes* ILCC306 on different surface important in food industries.

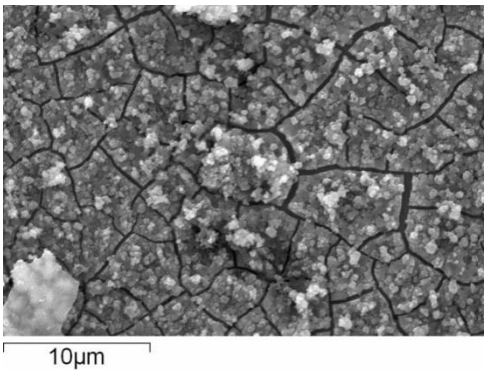


Fig. 4.7.A:*L. monocytogenes* ILCC306 on PVC pipe after 48

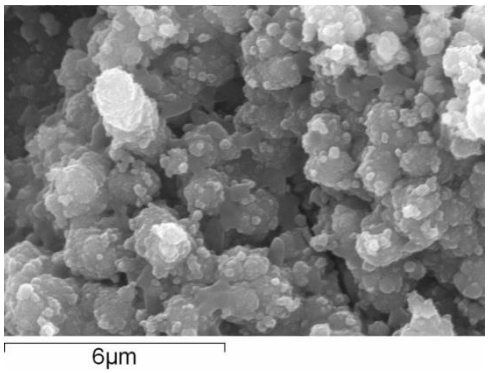


Fig. 4.7.B:*L. monocytogenes* ILCC306 on ceramic tiles after 48h

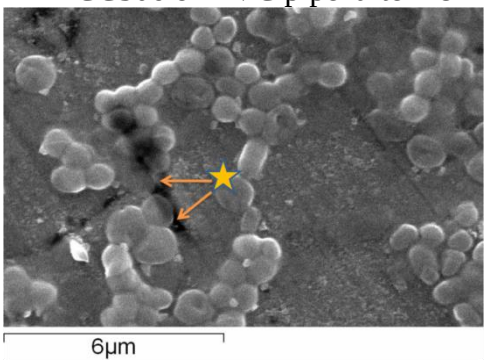


Fig. 4.7.C: *L. monocytogenes* ILCC306 on Stainless Steel (SS304) after 48h. (* Biofilm aggregates near suture)

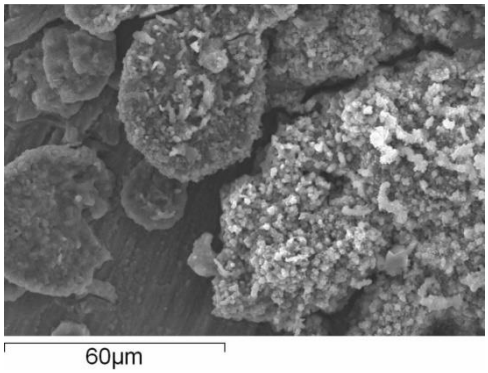


Fig. 4.7.D:*L. monocytogenes* ILCC306 on Stainless steel suture (artificially made) after 48h

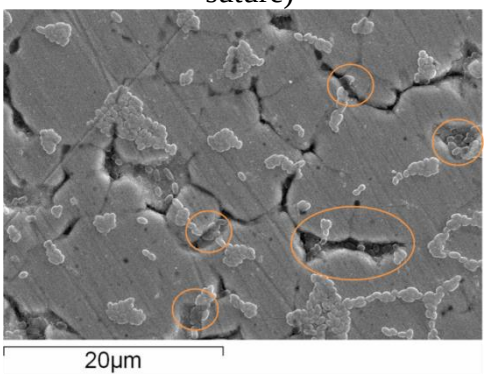


Fig. 4.7.E: *L. monocytogenes* ILCC306 on HDPE plastic after 48h (Circled area showing biofilm rooted in the sutures, Growth can be seen inside the sutures and forming aggregates toward the surfaces)

4.4.4 Influence of growth conditions

Growth time, temperature, pH, nutrient concentration and salt concentration are critical factors that must be optimum for the growth of any bacteria. These factors, if deviate from optimum, the bacterial growth rate, cell's structure and physiology may alter (Wen et al. 2009). To determine whether such factors also affect the biofilm formation capability of *L. monocytogenes*, the biofilm formation capability was accessed at various ranges of time period, temperature, pH, nutrient concentration and salt concentration.

The temperature was found to affect the biofilm formation of *L. monocytogenes*. Optimum growth temperature of *L. monocytogenes* i.e. 37^{o}C, found to be the best for biofilm formation. Decrease in temperature from optimum temperature range, decreased biofilm formation was observed up to 96 h (Fig. 4.8). The biofilm formed was increased till 96 h at all the temperature. It became approximate the same till 120 h and remained constant till the experiment terminated at 144 h. Some researchers have noted similar results (Djordjevic et al. 2002; Di Bonaventura et al. 2008; Nilsson et al. 2011; Kadam et al. 2013). Lee et al. (2013) reported the opposite effect i.e. lower the temperature, higher the biofilm formation ability. The difference in the biofilm formation ability can probably due to the use of different isolates and media in different study. The increased cell surface hydrophobicity with the increase in the temperature increased the adherence, increasing biofilm formation (Chavant et al. 2002; Di Bonaventura et al. 2008). In this study the biofilm was tested onto hydrophobic polystyrene surface may explains the increase in biofilm formation with increase in temperature. Absence of flagella have been shown to cause decrease in the biofilm formation capability (Lemon et al. 2007),

while it is known that *L. monocytogenes* do not form flagella at 37^{o}C and form at 24^{o}C; however there was not significant increase in biofilm formation capability due to presence of flagella at 24^{o}C.

In case of biofilm formation at stressed nutrient media, the biofilm formation was better at full strength BHI medium. When the BHI broth was diluted for 5 times and 10 times the biofilm formation was decreased accordingly (Fig. 4.9). The results obtained were consistent with the previous reports (Stepanović et al. 2004; Pan et al. 2010). Folsom et al. (2006) previously reported that different strains used possess different ability to form biofilm at different nutrient concentration. Biofilm is made up of the polymeric substance embedded in which cells are present. Apparently it looks like exhaustion of the nutrients could be the more likely reason for the decreased level of the biofilm. Also, the contradictory result observed could be due to the specific medium used in different study.

L. monocytogenes cells could form biofilm between pH 5.5-8.5. In consistent with the Borges et al. (2011) the biofilm was found to get affected as the foci of pH shift from the optimum (Fig. 4.10). The best biofilm formation was observed at pH 7 while the decrease and increase in the pH lead to decreased biofilm. The biofilm of *L. monocytogenes* got inhibited at minimum pH 5 and maximum pH 9. Similar to temperature, pH influences the overall charge on cell surface causing decrease in adhesion (Choi et al. 2013).

Concentration of salt did not affect much the biofilm formation ability of *L. monocytogenes* isolates. *L. monocytogenes* possesses innate ability to tolerate up to 10-12.5% of salt concentration. The biofilm formation was found approximately the same till 7.5% of NaCl, while it decreased in further as the salt concentration

increased. Also, the growth of *L. monocytogenes* was also found to decrease with further increase in the salt concentration (Fig. 4.11). Similar results were noted by (Pan et al. 2010; Hingston et al. 2013; Lee et al. 2013).

From this study it can be inferred that, *L. monocytogenes* forms the best biofilm at optimum growth conditions. While deflection of growth conditions from optimum level, lead to decrease in the biofilm formation of *L. monocytogenes*. Lower temperature, nutrient stress and pH 5, possess potential to minimize the *L. monocytogenes* biofilm in the food industrial environment.

Fig. 4.8: Effect of temperature and incubation time on biofilm formation of *L. monocytogenes*

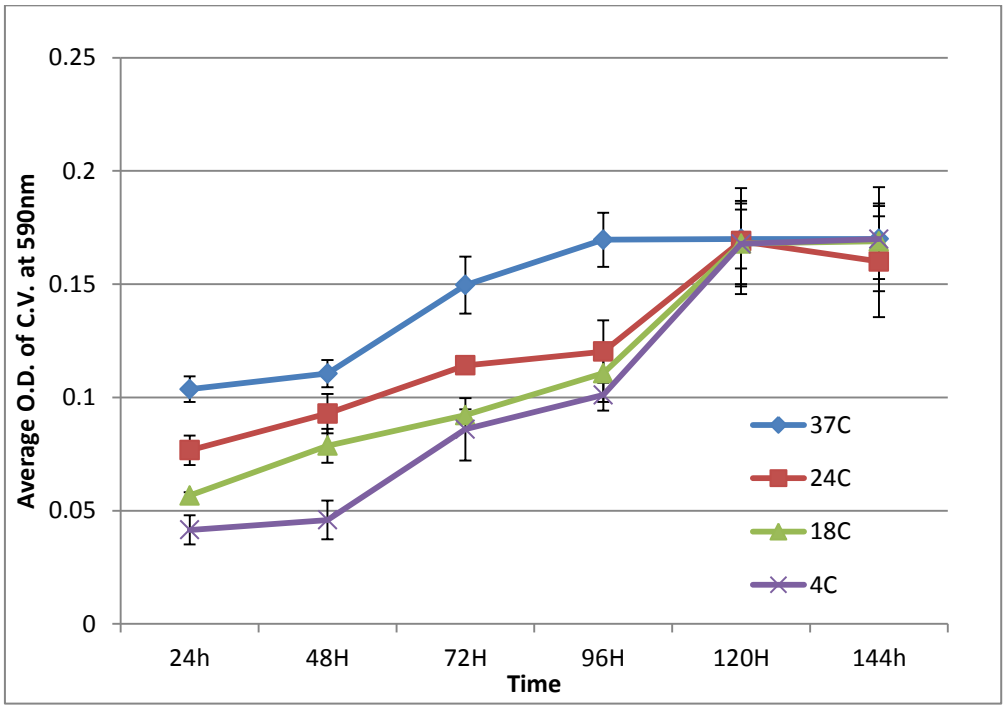


Fig 4.9: Biofilm formation of *L. monocytogenes* at nutrient stress

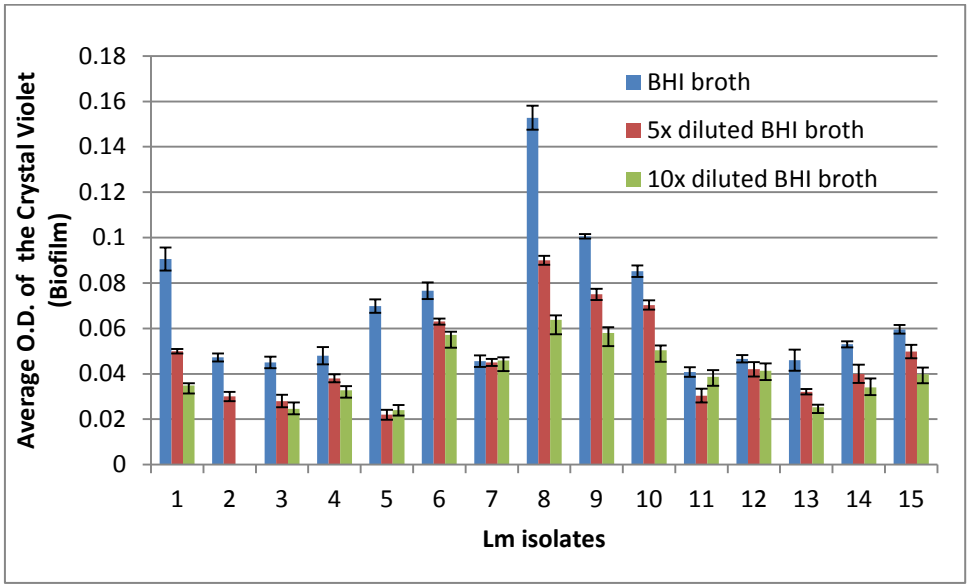


Fig. 4.10: Effect of pH on the biofilm formation of *L. monocytogenes*

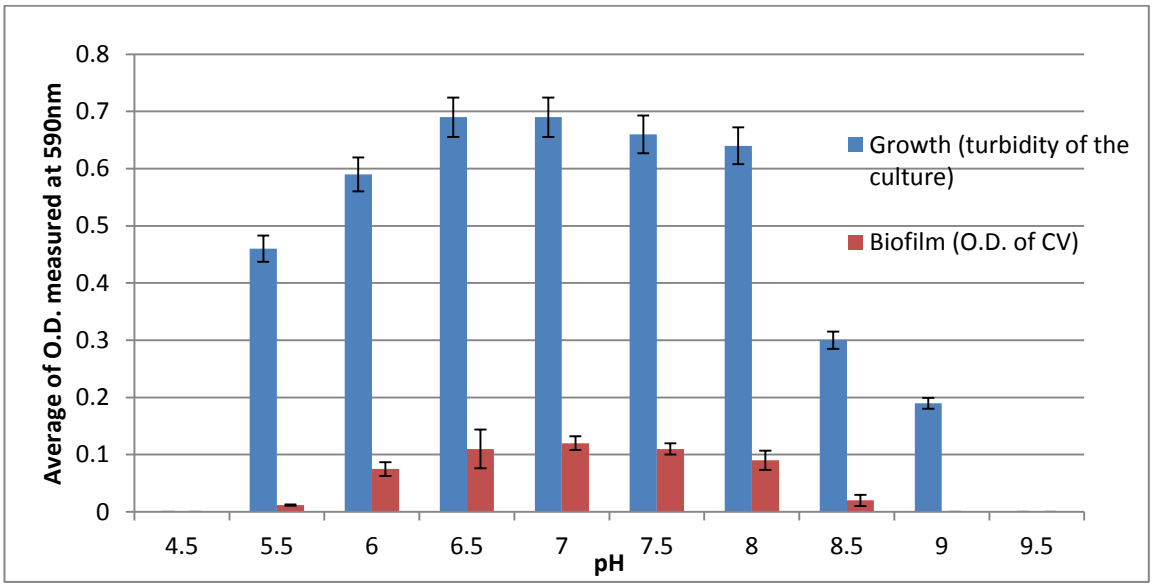
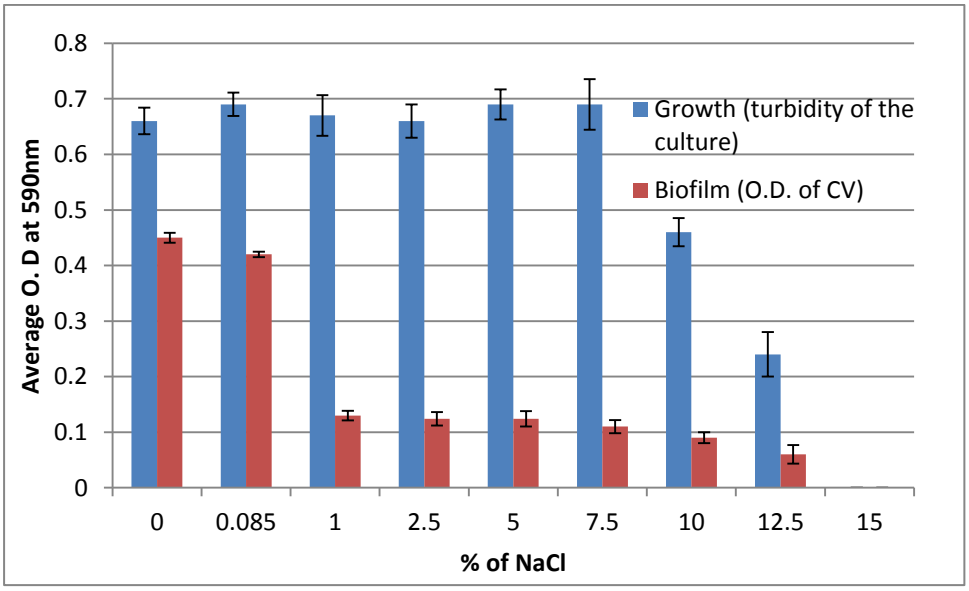


Fig. 4.11: Effect of concentration of salt (NaCl) on biofilm formation of *L. monocytogenes*



4.4.5 Role of the *luxS* gene in quorum sensing

To control the biofilm, it is necessary to understand the factor/s that causes the biofilm formation. Quorum sensing has been shown to plays an important role in biofilm formation among many bacterial cells. Signaling molecule auto inducer 2 (AI-2) hypothesized as the universal signal for the interspecies communication. A potential ortholog of *V. harveyi luxS* have been identified in *Listeria* spp. (lmo1288). Lmo1288 was found to be a functional *luxS* ortholog involved in AI-2 synthesis and interruption of *luxS* gene have been found to lose AI-2 signal (Belval et al. 2006). Therefore study was carried out to determine the role of the *luxS* gene in biofilm formation capabilities. Out of 83 isolates tested, the *luxS* gene was detected in 80 isolates (Fig. 4.12). All the three *luxS* negative isolates were from milk, two were moderate biofilm formers and one was weak biofilm formers. The failure of amplification in these three isolate could be due to the change in few nucleotide bases at primer annealing sites. It was interesting to note that the presence of *luxS* gene in *L. monocytogenes* isolates does not correspond to different biofilm forming capability (strong, moderate and weak). Therefore, further gene expression studies were carried out to determine any relation, if present. A negative correlation was observed for the expression of *luxS* gene and biofilm formation capability. The highest expression of the *luxS* gene was observed in the weak biofilm former while the expression was comparatively lower among the strong biofilm formers (Fig. 4.13). In support with the Sela et al. (2006) and Belval et al. (2006), the gene expression study shows that *luxS* seems to play a negative role in biofilm formation of *L. monocytogenes*. Sela et al. (2006) studied the *luxS* deficient mutant of *L. monocytogenes* and revealed 19-fold denser biofilm. Also, addition of exogenous AI-2 could not restore the wild-type phenotype. Further study is necessary to understand the exact molecular mechanism

involved in negative regulation of biofilm by *luxS* gene. From this study it can be concluded that, contradictory to other bacteria, in *Listeria* spp. *luxS* system negatively regulate the biofilm.

Fig. 4.12: A representative image of 1.5% agarose gel showing PCR amplicon of *luxS* gene (201bp) among the strong, moderate and weak biofilm forming isolates. strong biofilm former (Lane:1-4), moderate biofilm formers (Lane:5-8) and weak biofilm former (Lane:9-12), Lane 13 –Blank, Lane 14 –Positive control and Lane 15- M: 100bp ladder.

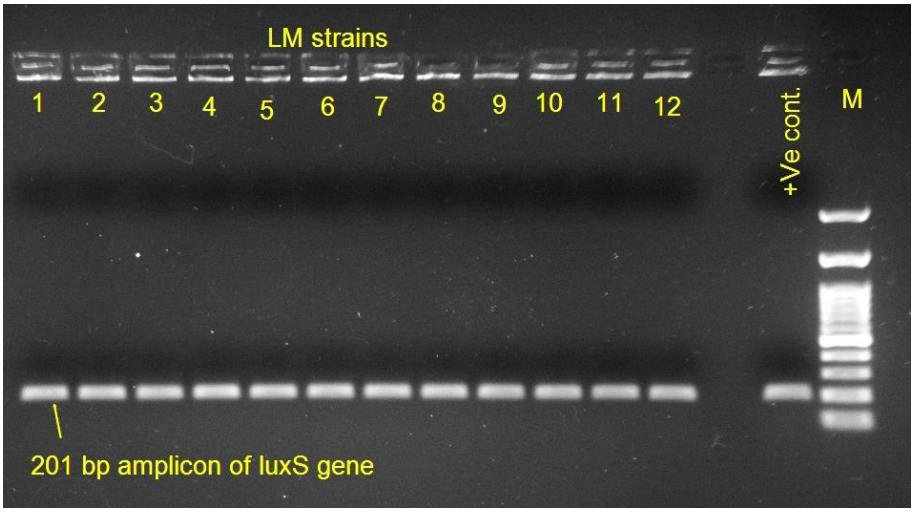
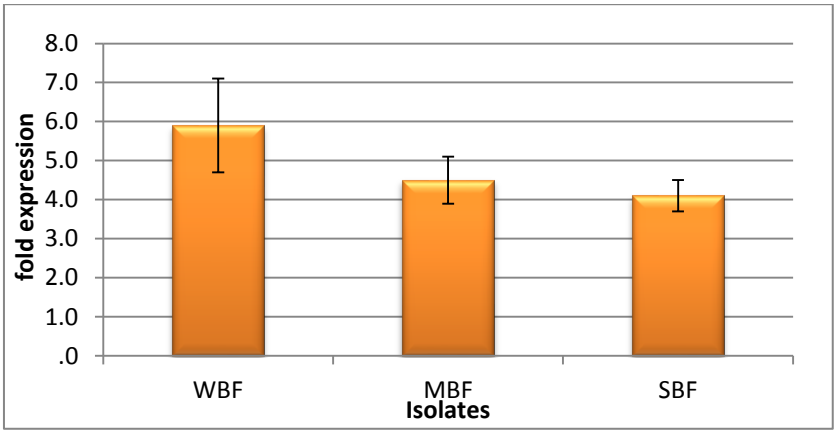


Fig. 4.13: Fold expression of the *luxS* gene in the strong modertate and weak biofilm formers (WBF: weak biofilm former; MBF: moderate biofilm former and SBF: strong biofilm former)



4.4.6 FAME analysis

It is well known that biofilm formation process greatly influenced by many factors. Many researchers have tried to relate total fatty acids composition in relation to the biofilm forming capabilities of the *L. monocytogenes*. These studies showed that the amount and type of fatty acids played differential role influencing biofilm forming properties of bacteria (Davies and Marques 2009; Chao et al. 2010; Perez et al. 2012; Pedrido et al. 2013). Fatty acids in *L. monocytogenes* cells have been studied for their role in adaptation to cold temperature (Chihib et al. 2003). However, the role of fatty acid in case of biofilm formation of *L. monocytogenes* is not adequate. We made an attempt to determine whether there is any difference present in total fatty acid profiles of the strong, moderate and weak biofilm forming isolates. Total fatty acid profiles of strong, moderate and weak biofilm forming strains were determined by using the MIDI enabled automated gas chromatographic instrument to observe its relation for biofilm formation capability. By comparing sample strains with reference standards, cell surface fatty acids ranging from 12:0 dodecanoic fatty acids to 20:0 eicosanoic fatty acids were recognized and their relative amounts were estimated. The predominant fatty acids present in all *L. monocytogenes* strains were anteiso-15:0 and anteiso-17:0 fatty acids (Table 4.3). The percentage of iso-tetradecanoic, iso and anteiso pentadeconic acid, iso and anteiso hexadecanoic acid, iso and anteiso ocatdecanoic acid was comparatively more in strong biofilm former followed by moderate and then weak biofilm former strains. Both anteiso and iso dodeacanoic acids were absent in the strong biofilm former. Analysis revealed that certain fatty such as iso-$C_{14:0}$, anteiso-$C_{15:0}$ and iso-$C_{16:0}$ acids possess a good correlation with biofilm forming capability of the *L. monocytogenes* isolates. Recently, such fatty acids have suggested to have a role in adhesion characteristics of *L. monocytogenes*

(Skovager et al. 2013). In addition, Gianotti et al. (2008) found higher amount of $C_{16:0}$ and $C_{18:0}$ in a fatty acid profile of adhered cell as compared to planktonic *L. monocytogenes* cells. The predominant fatty acids observed among strong, moderate and weak biofilm forming isolates were iso-$C_{15:0,}$ anteiso-$C_{15:0}$ and anteiso-$C_{17:0}$ fatty acids, which are also the characteristic profile of *L. monocytogenes* (Annous et al., 1997). The quantity and composition of fatty acid such as anteiso-$C_{17:0}$ and anteiso-$C_{15:0}$ seems to get easily altered toward adaptation of *L. monocytogenes* in different conditions. Such changes caused by environmental condition tend to change the quantity of anteiso-$C_{17:0}$ and anteiso-$C_{15:0}$ adjusting the bacteria to new environment (Püttmann et al. 1993; Chihib et al. 2003). Increase in the fatty acid content of the isolates was found to increase their hydrophobicity in *Pseudomonas aeruginosa* (Chao et al., 2010) and *S. aureus* (Mirani et al., 2013) promoting the bacteria to adhere to surfaces. The presence of the increasing amount of total fatty acids (iso-$C_{14:0}$, anteiso-$C_{15:0}$ and iso-$C_{16:0}$) as per intensity of the biofilm suggest that fatty acids play significant role influencing biofilm formation capabilities of *L. monocytogenes*. To determine the specificity and exact function of fatty acid toward biofilm formation need to be studied in further detail.

Table 4.3: showing the fatty acid profile of the strong moderate and weak biofilm forming isolates as analyzed by the FAME analysis

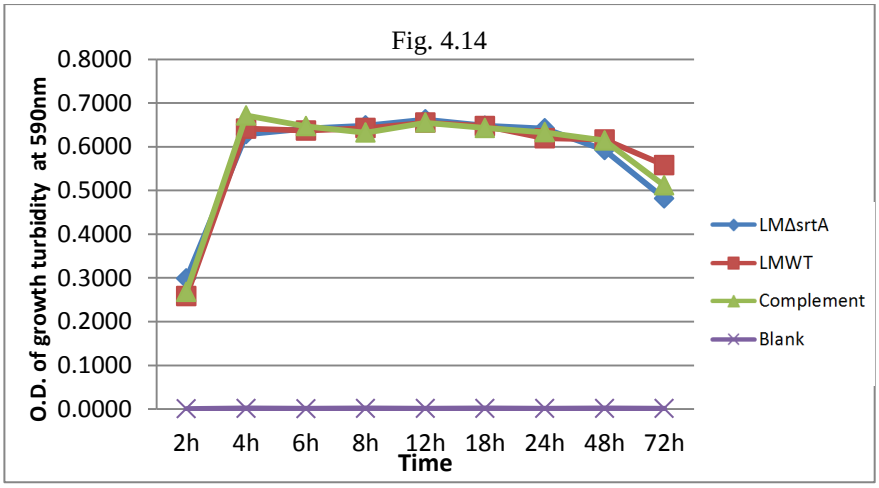
	Weak	**Moderate**	**Strong**
	Average % ± SD	**Average % ± SD**	**Average % ± SD**
Iso-$C_{12:0}$	0.22 ± 0.01	0.51± 0.09	0
Anteiso-$C_{12:0}$	0.53 ± 0.48	0.57 ± 0.12	0
Iso-$C_{13:0}$	0	0	0
Anteiso-$C_{13:0}$	0.22 ± 0.02	0.66± 0.16	0.15 ± 0.03
Iso-$C_{14:0}$	0.33 ± 0.05	0.46 ± 0.06	0.52 ± 0.03
Anteiso-$C_{14:0}$	0.42 ± 0.21	1.04 ± 0.26	0.29 ± 0.06
Iso-$C_{15:0}$	5.76 ± 1.74	7.93 ± 0.53	7.79 ± 0.87
Anteso-$C_{15:0}$	34.29 ± 2.63	37.18 ± 1.46	41.58 ± 1.55
Iso-$C_{16:0}$	1.82 ± 0.68	2.51 ± 0.12	2.93 ± 0.36
Anteiso-$C_{16:0}$	3.14 ± 1.78	3.43 ± 1.05	2.43 ± 0.39
Iso-$C_{17:0}$	3.79 ± 2.08	4.78 ± 0.20	4.37 ± 0.81
Anteiso-$C_{17:0}$	36.5 ± 2.74	42.4 ± 1.84	38.63 ± 1.41
18:1 w9c	1.66 ± 2.53	1.49 ± 0.66	1.11 ± 1.39
Iso-$C_{18:0}$	0.73 ± 0.74	0.59 ± 0.16	0.76 ± 1.04
Anteiso-$C_{19:0}$	0.33 ± 0.16	0.54 ± 0.01	0.23 ± 0.03
20:2 w6,9c	1.12 ± 1.86	0	1.41 ± 0.23

4.4.7 Role of cell surface proteins in the biofilm formation

4.4.7.1 Determination of change in biofilm formation

Sortase A is a transpeptidase enzyme encoded by many Gram positive bacteria that recognise and covalently link LPXTG motif proteins to the cell wall (Spirig et al. 2011). Several such sortase A recognised LPXTG motif cell-surface proteins have been shown to play a role in environment interactions, virulence, adhesion and biofilm formation in some bacteria (Cucarella et al. 2001; Jordan et al. 2008; Geoghegan et al. 2010; Liang et al. 2011). Certain characteristics of LPXTG motif proteins such as hydrophobic leucine reach repeats and polycysteine kidney disease like module makes these proteins structure an ideal configuration for adhesion (Bierne & Cossart 2007). Such adhesion characteristics of LPXTG proteins has been studied with respect to eukaryotic cells, however their adhesion potential to non-living surface is explored in very few bacteria (Lévesque et al. 2005; Guiton et al. 2009). Generally, sortase A controlled LPXTG proteins are present in minimum 1 to maximum 17 numbers in bacteria (Cabanes et al. 2002). However, the genomic data of *L. monocytogenes* revealed the highest number (41) of sortase A associated LPXTG proteins (Glaser et al. 2001). These proteins have been studied in relation to virulence and adhesion to eukaryotic cells (Bierne et al. 2002) however none has been explored in adhesion to non-living surfaces. Considering all these aspects about sortase A, we hypothesised that sortase A may play a role in adhesion and biofilm formation of *L. monocytogenes.* To study the role of sortase A, a mutant for sortase A in *L. monocytogenes* EGDe was prepared and analysed for its adhesion and biofilm capability. When the biofilm formation ability was assessed, the mutant and complemented strain did not show any significant growth defects, suggesting Sortase A must not be an essential entity for growth and multiplication in *L. monocytogenes*

EGDe (Fig. 4.14, 4.15, 4.16). Adhesion and biofilm formation were assessed using microtiter well plate assay. The data obtained were concluded for adhesion and biofilm formation capability as per the criteria defined by Guiton et al. (2009). Accordingly, adhesion capability of the LMΔ*srtA* was decreased by 28.03 times (96.41%), while biofilm formation capability was decreased by 7.3 times (84.26%) as compared to LMWT adhesion and biofilm capability. Interestingly, though the expression of *srtA* was increased by 21.42 folds in complement, no change in the biofilm formation capability in complement strain was observed (Fig 1, Chart B). The biofilm of LMWT that generally observed as a ring at air-liquid interface of the microtiter well plate by LMWT, was clearly absent in LMΔ*srtA*. Deletion of the *srtA* gene led to a significant decrease in adhesion, as well as in biofilm formation capability. Such decreased adhesion in *Streptococcus mutants* (75%) (Lévesque et al. 2005); in *Enterococcus faecalis* (40%) (Guiton et al. 2009) and in *S. sanguines* (5.6%) (Yamaguchi et al. 2006) has been reported previously. Here we report a highest decrease in adhesion (96.41%), as well as in the biofilm formation (84.26%) due to deletion of the *srtA* gene in *L. monocytogenes* EGDe. Replacement of *srtA* back into the mutant restored biofilm forming capability, confirming its' importance in biofilm formation by *L. monocytogenes.* This decreased biofilm formation in microtiter well plates was supported by the presence of low numbers of cells in biofilm. To compare the cell number contributing in biofilm, LMWT, LMΔ*srtA* and the complemented strain in biofilms were enumerated. LMWT cells were quantified as $50\pm19x10^3$ CFUs/cm^2 while deletion of sortase A gene led to decrease in cell numbers to $84\pm16x10^1$ CFUs/cm^2. Complementation of the mutant by restoring *srtA* restored cell numbers to $53\pm21x10^3$ CFUs/cm^2 in biofilm. The enumeration study supported the role of sortase A in biofilm formation.



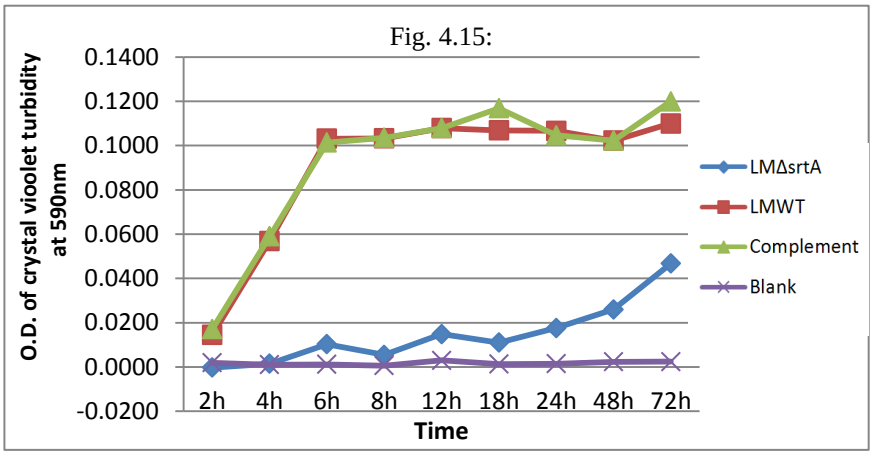


Fig 4.16

Fig. 4.14: Growth turbidity of the LMΔsrtA, WT-Control and complement strains showing there was no change in the growth rate; **Fig. 4.15**: Crystal violet turbidity as a measure of biofilm of the LMΔsrtA, LMWT-Control and complement strains. LMΔsrtA lost the ability to adhere and biofilm formation as compared to native strain WT-Control and complement strain could form biofilm; **Fig 4.16:** Microtiter well plate assay for biofilm formation of the LMΔsrtA, WT-Control and complement strains. Above images of microtiter plate showing the results of eight hour biofilm formation assay for LMΔsrtA, WT-Control, complement strains and blank. *L. monocytogenes* EGDe strain mutant for sortaseA (LMΔsrtA) could not form biofilm (no violet color of CV), while replacement of gene in mutants (complement) restored biofilm formation capability (violet color of stained cells from biofilm).

Scanning electron microscopy was performed to compare biofilm formation capabilities of the LMWT and LMΔ*srtA* on the polystyrene microtiter well plates. LMWT cells were observed in a cluster of 5-15 cells while LMΔ*srtA* were found to be present as singularly (Fig. 4.17). The average numbers of cells per microscopic field were 40-50 in case of LMWT as compared to 15-20 cells in the case of the LMΔ*srtA*. Also, a 'film' was formed by LMWT cells on polystyrene surface which was clearly absent in case of LMΔ*srtA.* The ability of *L. monocytogenes* EGDe to form clusters was lost upon deletion of sortase A gene. Scanning electron microscopy confirmed the loss of biofilm forming capability by the *srtA* mutant. Interestingly, when the LMΔ*srtA* was allowed to form biofilms, some sort of 'granular material' was observed on the polystyrene surface. Such granular materials were either very low or absent in case of LMWT. To confirm that such material was not an artefact, we repeated the experiment but noted similar observations. Mariscotti et al. (2012) reported that the lack of *srtA* resulted in a concomitant increase in the amount of protein released into the extracellular medium. In addition, sortase associated LPXTG proteins may get secreted into extracellular environments. However, further research is needed to determine whether or not the granules in question are extracellular protein aggregates.

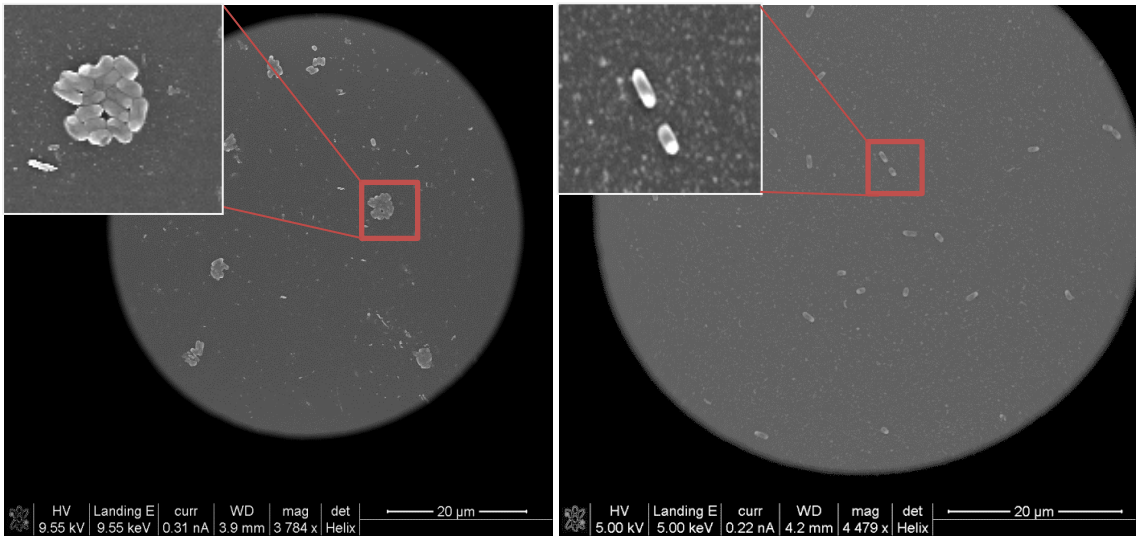


Image: A; Inset: Magnified part of image A showing cluster of cells sticking together

Image: B; Inset: Magnified part of image B showing singular cells

Fig 4.17: A typical image of electron microscopic field showing the attachment pattern of *L. monocytogenes* EGDe strain (WT) and mutant constructed for the sortaseA enzyme (LMΔsrtA) lacking LPXTG motif proteins at the cell surface. The cells were allowed to form biofilm on hydrophobic polystyrene coupons for 24 h at 37^0C. The attachment pattern of WT strains (Image A) is in cluster of 5-15 cells, while LMΔsrtA (Image B) found to adhere singularly and could not propagate into film showing the loss of biofilm capability. Also the average number of cells per microscopic field was 40-50 cells for WT while it was 15-20 in case of LMΔsrtA showing the loss of adherence capability. The 'granular' region observed at the background of LMΔsrtA (Image: A; Inset) in contrast to clean background by WT strain (Image: B, Inset).

4.4.7.2 Expression of LPXTG protein's gene

To determine if the sortase A influence the expression of LPXTG protein's gene, the respective gene expression profile of the LMWT, LMΔ*srtA* and the complemented strain was analysed in presence and in absence of sortaseA gene. In results, the expression of 18 LPXTG protein's gene was equivalent in all the three conditions tested – presence, absence or overexpression of the *srtA* gene in LMWT, LMΔ*srtA* and the complemented strain respectively (Table 4.4). The expression of the *srtA* gene and 18 LPXTG protein's genes was also analysed in biofilm (12 and 24h) (Table 4.4). The expression of the *srtA* gene was elevated by 3.91 and 1.67 times in 12h and 24h old biofilm as compared to planktonic phase. Interestingly, in 12 h biofilms, the genes encoding LPXTG proteins were significantly more upregulated than at 24 h. The LPXTG genes were found to be upregulated by 11-127 folds in case of 12h biofilm while it was 10-23 fold in 24h biofilm. In the case of LMΔ*srtA* biofilms, we could not find detectable level of RNA, probably, due to lower numbers of cells caused by loss of adherence capability. Of these 18 LPXTG proteins, four proteins that were expressed significantly higher (more than 100 fold) were peptidoglycan binding proteins (n=3) and a peptidoglycan linked protein. The 18 LPXTG protein's genes were expressed independently in LMWT, LM*ΔsrtA*, and complemented strain. There was no consistency in expression of LPXTG protein's gene in the LMWT, which was also the case for LMΔ*srtA* and complemented strain. Therefore, expression of LPXTG protein's gene does not seem to be influenced by the presence or absence or expression of the *srtA* gene. The LPXTG protein's gene were shown to be governed by factors such as *PrfA* and *SigB* gene (Cabanes et al. 2005; Sabet et al. 2008; Camejo et al. 2009; Ollinger et al. 2009; Reis et al. 2010). Our data is consistent with Mariscotti et al. (2012), who reported the absence of *srtA* affects the presence of LPXTG-motif proteins on the cell wall, however it did not necessarily

affect the expression of genes encoding LPXTG proteins. When the expression of these genes was studied in biofilm, interestingly these genes were found to be up-regulated at their initial stage (12 h biofilm), which subsequently were reduced at 24 h, suggesting a probable role in early stages of biofilm development.

Table 4.4: Expression of 18 LPXTG genes in LMΔ*srtA* and LMΔ*srtA* complemented strains, compared to LMWT as measured by Q-PCR. (* - absent in *L. innocua* –a non-pathogenic strain of *Listeria* spp.; # - detected in *L. monocytogenes* cell wall fraction in relation to sortase A; ± standard deviation)

		Fold change compared to LMWT			
Gene Identity	**Name of the gene**	**LMΔsrtA**	**Complement**	**12h Biofilm**	**24h Biofilm**
Lmo0171*	Hypothetical protein	1.22(±0.12)	1.39(±0.10)	11.39(±2.21)	10.59(±0.98)
Lmo0262*,#	Internalin G	1.82(±0.19)	0.88(±0.23)	50.21(±2.22)	13.21(±1.29)
Lmo0263*,#	Internalin H	1.32(±0.14)	1.04(±0.05)	42.22(±2.23)	11.39(±2.30)
Lmo0433*,#	Internalin A	2.51(±0.45)	1.76(±0.18)	44.01(±2.24)	17.38(±2.31)
Lmo0725	Peptidoglycan binding protein	1.08(±0.11)	1.79(±0.21)	127.11(±3.25)	12.21(±0.54)
Lmo0732	Peptidoglycan binding protein	0.46(±0.01)	1.56(±0.17)	113.77(±3.26)	11.89(±1.19)
Lmo0835*	Peptidoglycan binding protein	1.24(±0.23)	0.19(±0.08)	62.196(±4.27)	12.9(±1.85)
Lmo1115*	Hypothetical protein	0.87(±0.12)	1.03(±0.09)	34.36(±1.28)	21.33(±2.35)
Lmo1136	Hypothetical protein	1.19(±0.15)	1.29(±0.20)	54.19(±1.29)	13.98(±0.63)
Lmo1290*	Hypothetical protein	1.29(±0.41)	0.51(±0.00)	34.69(±3.30)	12.89(±0.87)
Lmo1413*,#	Peptidoglycan binding protein	1.71(±0.21)	2(±0.22)	70.03(±2.31)	21.7(±1.80)
Lmo1666*,#	Peptidoglycan linked protein	1.12(±0.30)	1.06(±1.53)	94.35(±3.32)	21.55(±2.39)
Lmo1799	Peptidoglycan linked protein	1.53(±0.12)	1.88(±0.56)	121.93(±2.33)	21.45(±2.95)
Lmo2026*	Peptidoglycan binding protein	0.98(±0.02)	0.17(±0.05)	42.1(±1.34)	23.47(±2.10)
Lmo2085*,#	Peptidoglycan binding protein	2.89(±0.15)	1.28(±0.16)	112.2(±1.35)	18.89(±1.02)
Lmo2179	Peptidoglycan binding protein	1.07(±0.09)	1.52(±0.17)	116.16(±3.36)	16.23(±2.01)
Lmo2396	Hypothetical protein	1.22(±0.13)	1.15(±0.10)	89.26(±2.37)	14.21(±0.94)
Lmo2576*	Peptidoglycan binding protein	1.07(±0.08)	0.8(±0.00)	24.19(±1.38)	18.23(±0.45)
Lmo0929	SortaseA	NA	21.42(±1.30)	3.91(±0.39)	1.67(±0.63)

4.4.7.3 Determination of Hydrophobicity index:

Microbial adherence is largely dependent upon the surface charge and hydrophobicity (Loosdrecht et al. 1990; Pagedar et al. 2010; Bujdáková et al. 2013; Guo et al. 2013). As *L. monocytogenes* does not have much biofilm forming accessories as other bacteria, the biofilm formation is thought to be one of the attribute of the hydrophobicity of the cell wall. As nineteen LPXTG proteins possess hydrophobic leucine rich repeats at the N-terminal ends which is also an active site of the proteins carrying out several interactions has been understood (Bierne & Cossart 2007), the hydrophobicity indexes of LMWT, LMΔ*srt*A and the complemented strain were determined by using n-hexadecane. LMWT showed a hydrophobicity index of 27.06 ±1.31, while for LMΔs*rtA* the hydrophobicity index was found decreased to 23.09 ±1.29. Replacement of *srtA* in LMΔs*rtA* found to restore hydrophobicity to 26.55 ±1.33, which was equivalent to LMWT. With the loss of the *srtA* gene, a loss of hydrophobicity index was observed suggesting the role of these hydrophobic proteins in overall hydrophobicity of the cell wall. The significance of *L. monocytogenes* cell surface hydrophobicity in the initial adherence and biofilm formation has been shown previously (Bonaventura et al. 2008; Takahashi et al. 2010).

In summary, the absence of sortase A gene in *L. monocytogenes* EGDe is not a critical growth factor but it significantly play role in adhesion and biofilm formation. The presence, absence or overexpression of sortase A gene does not influence the expression of LPXTG protein's gene. In addition, overexpression of sortase A gene does not affect the biofilm forming ability of *L. monocytogenes.* The LPXTG protein's seems to play an important role in an early phase of biofilm formation in *L. monocytogenes.* Sortase A associated LPXTG proteins may contribute to total cell surface hydrophobicity.

4.4.8 Nanotubes

The scanning electron microscopy studies for the biofilm formation of the *L. monocytogenes* EGDe strains showed a tubular structure that connects two cells together. Recent study by Dubey & Ben-Yehuda (2011) observed such structure in *Bacillus, Staphylococci* and *E. coli*, for first time and called it as "Nanotube". Dubey & Ben-Yehuda (2011) claimed that these tubes are novel mode of physical communication and possess ability to transfer cytoplasmic material, plasmid and genomically encoded proteins to the connecting cells. It would be valuable to know whether such nanotubes, if occur in pathogens such as *Listeria monocytogenes*.

4.4.8.1 Determination of presence of nanotubes:

To reconfirm, whether *L. monocytogenes* really forms the nanotube, *L. monocytogenes* EGDe, ATCC 08-5923 and ATCC 19115 strains were grown in different phases such as growth on solid agar, biofilm and in liquid media (BHI broth) and analysed under high resolution microscope. Interestingly, similar tubular structure connecting two neighbouring cells that described by Dubey & Ben-Yehuda (2011) were observed in growth of all the three strains (Fig. 4.18). These structures were sufficiently wide enough to differentiate from conjugation pilli or other known structure and therefore referred as "nanotube" (Dubey & Ben-Yehuda 2011). These tubes were found to be originated discretely from the cell surface and not limited to specific site of the cells. The frequency on the nanotubes was approx. 2-5 per 100 cells. The size of these tubes was 30-100 nm in width and 20-500 nm in length. Nanotubes were observed among all the three *L. monocytogenes* strains studied and there was no specific difference in size and morphology was observed. The tubes were comparable to the previously reported nanotubes (Dubey & Ben-Yehuda 2011). More than one tube originated from single cell connecting two different cells was also

observed (Fig. 4.18- D, E, F). Dubey and Yehuda et al. (2011) observed the presence of the tubes only if culture were grown in the solid agar. We observed such tubes in biofilm and the frequency of the nanotubes was approximately the same.

Such tubes have been observed before, however did not get explored much. Marsh et al. (2003), observed such structures and referred it as "channel". The SEM images published by Nillson et al. (2011) toward biofilm of *L. monocytogenes* on industrially important surfaces has such tubular structure however, author did not discussed about it. Interestingly, these tubes were found to be highly susceptible for beam of electron and used to break if the beam (5-10 Kv) concentrated for longer time (>10s).

Fig 4.18: Intercellular nanotube formed by *L. monocytogenes* EGDe strains (selected images only); Fig: A, B –Nanotubes connecting two cells, Fig: C, D – multiple nanotubes emerging from single cells and connecting to different cells, Fig: E, F –High magnification (60,000X) images of distinguishing nanotube observed. (Red arrow indicates the nanotubes connecting two *L. monocytogenes* isolates). The width of these tubes was approx. 20-100 nm distinguishing them as a 'nanotube' as compared to conjugation tube/pilli (6-7nm width).

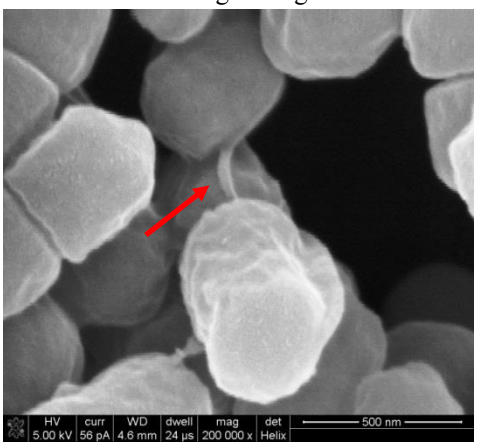


Fig: A

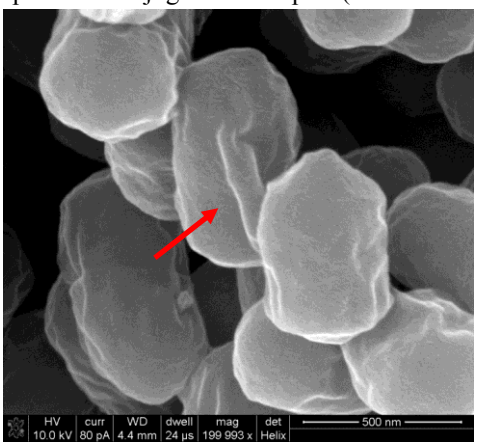


Fig: B

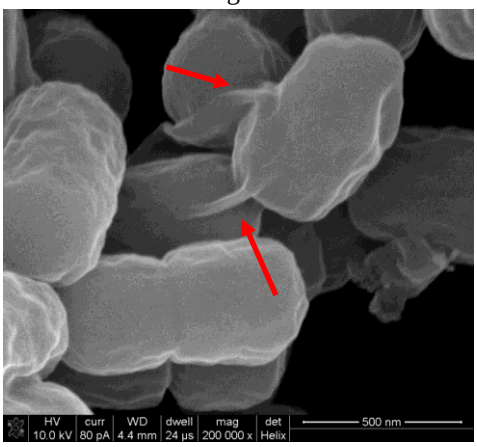


Fig: C

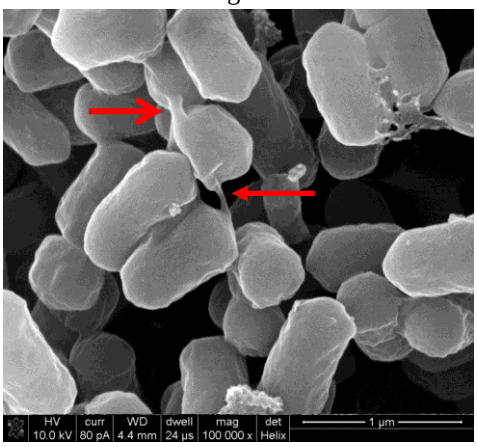


Fig: D

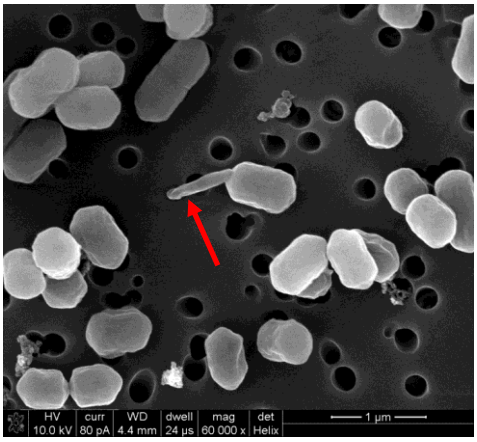


Fig: E

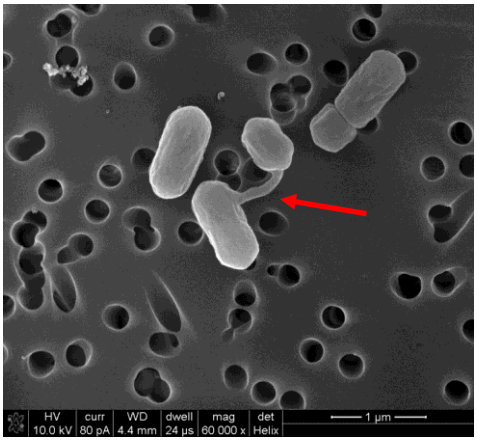


Fig: F

4.4.8.2 Determination of integrity of the tube

To furhter confirm, if these tubes are not an artefact and originated from the cells only, we determined the aproximate composition of the cellwall and nanotubes-originated from the same cells by using energy dispersive spectroscopy (EDS). The apparent concetration of the C, N, O, P and Na was determined (Fig. 4.19, 4.20). The EDS data revealed aproximately the same composition for the nanotube surface and the surface of two cells getting connected. C, N, O, P and Na elementes were present as 4.25, 3.71, 5.15, 0.75 and 0.44 units respectively in the nantoube while the concentration among the connecting cell's cell-wall was 4.28, 3.5, 4.5, 1.13 and 0.48 for one cell while 4.75, 3.93, 4.64, 0.91 and 0.31 respectivly for another cell (Table 4.5). Therfore it can be inferred that these nanotubes must be an integral part of the cells and not the artefact.

Another approach to determine the presence of such tubes as a an part of cell wall, the intgrity of the nantoubes was observed in prensce of SDS as described by Dubey and Behuda et al., (2011). Addition, of 0.008% of SDS in BHI agar inhibited the nanotube formation supporting that nanotube must be an integral part of the cellwall.

Table 4.5: The details of the spectrun taken by EDS for studying the nature and integrity of the nanotube. ('K ratio' is the ratio of the intensity from the sample and standard)

	Nanotube (Spectrum 1)					*L. monocytogenes* cell 1 (Spectrum 2)					*L. monocytogenes* cell 2 (Spectrum 3)				
Element	**Apparent Concentraton**	k Ratio	Wt%	Wt% Sigma	Atomic %	**Apparent Concentration**	k Ratio	Wt%	Wt% Sigma	Atomic %	**Apparent Concentration**	k Ratio	Wt%	Wt% Sigma	Atomic %
C	**4.25**	0.0425	43.1	0.84	56.2	**4.28**	0.04	46.4	0.55	62.7	**4.75**	0.0375	48.2	0.6	64.4
N	**3.71**	0.0066	13.9	0.67	15.6	**3.5**	0	9.84	0.6	11.4	**3.93**	0.0034	9.39	0.66	10.8
O	**5.15**	0.0173	25.2	0.51	24.7	**4.5**	0.02	21.4	0.37	21.7	**4.64**	0.0122	21	0.4	21
P	**0.75**	0.0042	1.9	0.2	0.96	**1.13**	0.01	2.85	0.21	1.5	**0.91**	0.0051	2.75	0.22	1.42
Na	**0.44**	0.0019	1.22	0.08	0.83	**0.48**	0	1.28	0.08	0.9	**0.31**	0.0013	1.01	0.08	0.7

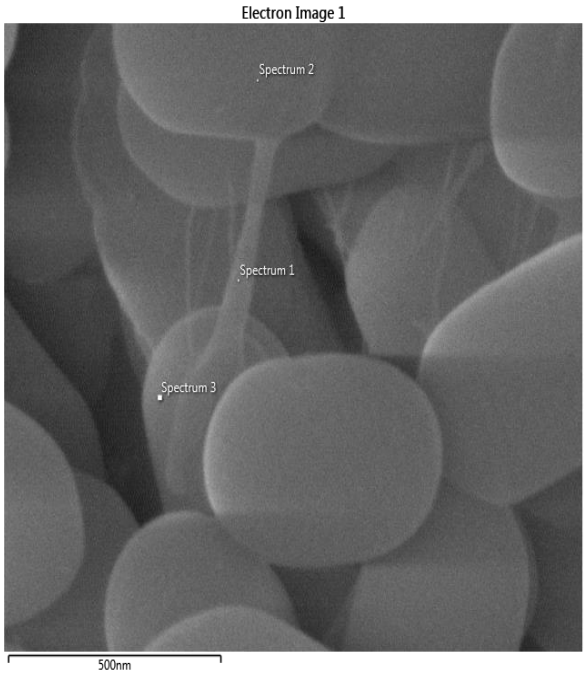


Fig: 4.17

Fig. 4.19: A scanning electron micrograph showing the nanotube between two *L. monocytogenes* cells. Energy dipsersive spectroscopy (EDS) analysis was performed at the locations as shown in the figure - nanotube (spectrum 1), *L. monocytogenes* cell 1 (sepctrum 2) and LMcell (spectrum 3).

Fig. 4.20: A, B and C: Cropped images of SEM-EDS X-ray spectrum for the - nanotube (spectrum 1), *L. monocytogenes* cell 1 (spectrum 2) and *L. monocytogenes* cell (spectrum 3) respectively.

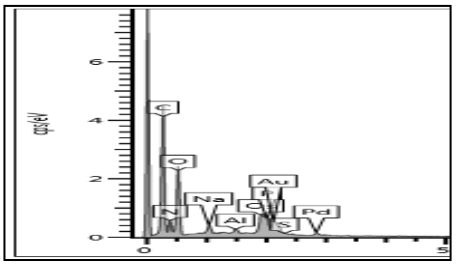


Fig: A (Spectrum 1)

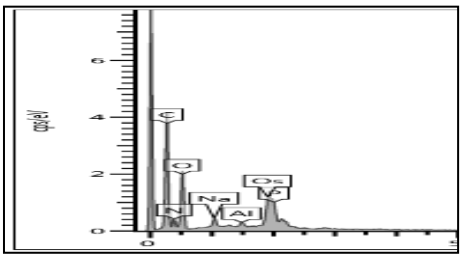


Fig: B (Spectrum 2)

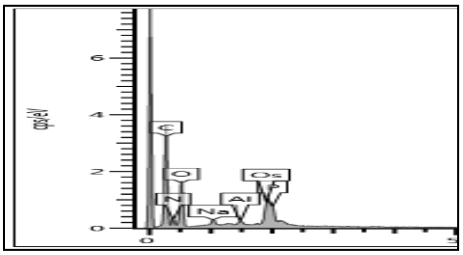


Fig: C (Spectrum 3)

4.4.8.3 Determination of the transfer of cytoplasmic molecules through nanotubes

The study was carried out to determine if the nanotubes take part in exchanging the cytoplasmic material by time lapse fluorescent microscopy. The experiment was carried out by observing transfer of calcein between calcein-treated cells (CTC) and untreated cells (CUC). CTC exhibits fluorescent color as compared to native (untreated) cells. The CTC and CUC were mixed together and the microscopic fields were adjusted to closely place fluorescent and non-fluorescent cells. As the time progressed transfer of fluorescence (i.e. calcein) was observed from CTC to CUC. The maximum transfer was observed up to 40 min. and further cells were observed to be saturated (Fig. 4.21). Transfer of calcein from one cell to other cell suggests that there must be a physical contact that could transfer this big (623 Da) molecule. Other known physical connections such as conjugation pilli cannot transfer such a big molecule. However, if the dimensions of the nanotubes are considered, calcein transfer seems possible.

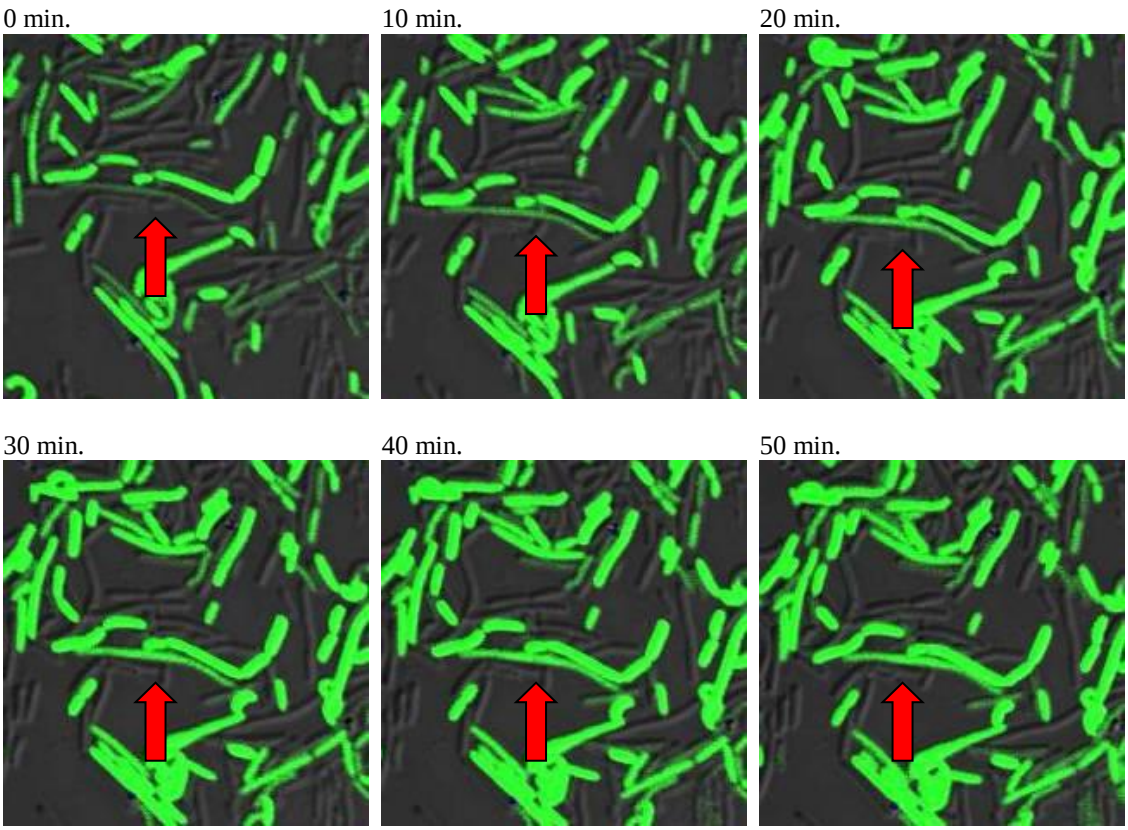
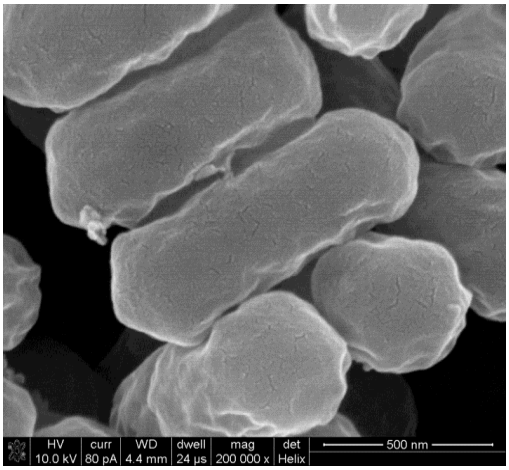


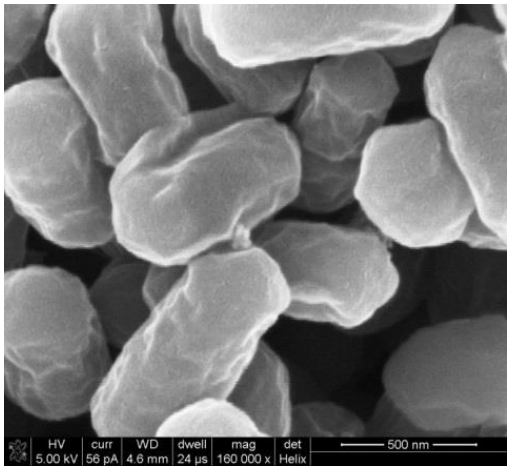
Fig 4.21: Transfer of calcein dye between two *L. monocytogenes* cells**.** Time lapse fluorescence microscopic images showing transfer of calcein between calcein-treated cells (fluorescent rods) and non-treated cells (non-fluorescent rods). Maximum transfer of calcein was observed upto 30 min. (red arrow indicated one of the pair in which calcein was being transferred)

It was interesting to note the immediate transfer of calcein between two cells. Despite the low division rate (<100 min) in *Bacillus subtilis* for duplication, Dubey and Ben-Yehuda (2011) observed the transfer of contents within 10 min. (lag phase) raising the question whether native nanotubes get involved with the new bacterium? Our electron microscopic study gives probable clues for nanotube formation within short period of time (Fig. 4.22). From the images observed, two hypotheses can be proposed for nanotube formation. Nanotube could be formed between two cells as (i) de-novo or (ii) by merging already formed tubes. The de-novo synthesis can be formed when two cells come in close proximity (Fig. 4.22 A-C). Some 'protruding' was observed among the cells whose cell walls were touching to each other (Fig. 4.22 A). Such protruding developed then may get extended (Fig. 4.22 B) which then subsequently grows and develop into a tube (Fig. 4.22 C). On the other hand, the tubes that formed previously but got detached or broken (Fig. 4.22 D) may come in contact with the cells and merge (Fig. 4.22 E, F). However, further detailed study is necessary to prove these hypotheses.

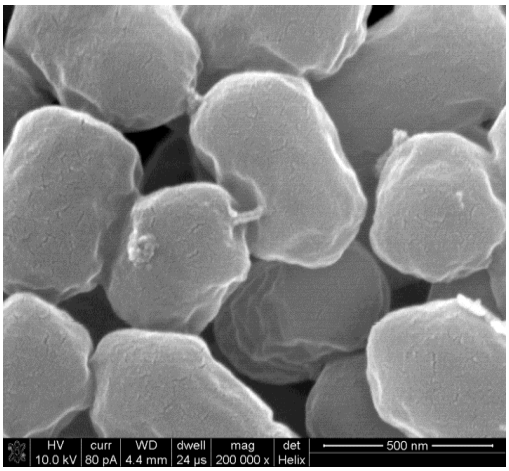
Fig. 4.22: Scanning electron microscopic image showing the probable nanotube formation steps. Fig. A, B and C shows the probable de-novo synthesis of the tubes when the cell wall of two closely associated cells comes in contact with each other. Fig. D, E and F shows the merging of already formed tubes.



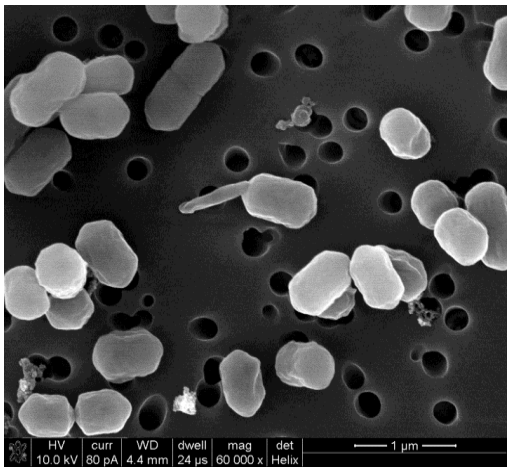
4.A



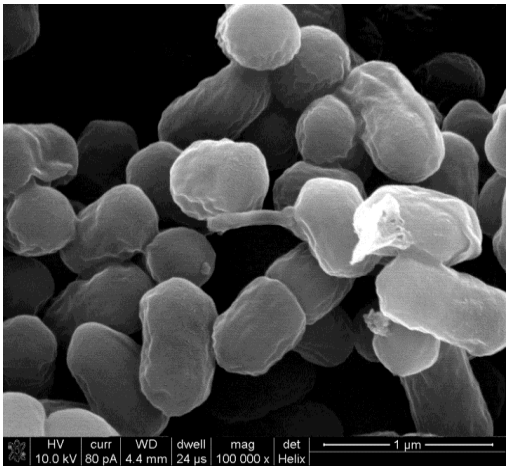
4.B



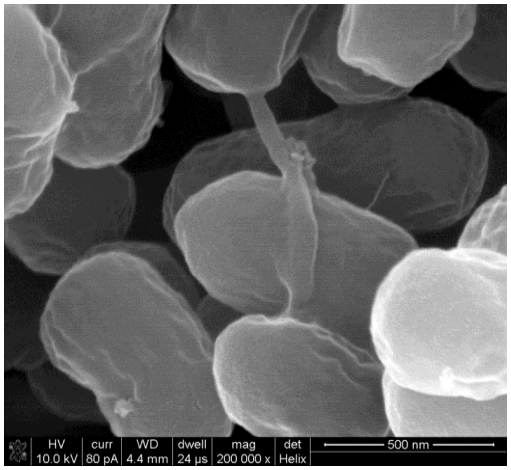
4.C



4.D



4.E



4.F

In summary, *L. monocytogenes* possesses the ability to form "nanotubes". After the discovery of the Dubey & Ben-Yehuda in 2011, this is the first report that supports the nanotube formation and its role in probable communication among the bacterial cells. Apparently, these tubes seem to be utilised by cells to transfer or exchange the cytoplasmic information between two cells. Formation of nanotubes gives a novel way to look at the bacterial communications and may give hopes to unsolved communication questions. Such nanotubes formed by intracellular pathogens such as *L. monocytogenes* raise many possibilities that may be occurring within the eukaryotic host cells. Such interaction with the formation of tube and delivering of toxin to eukaryotic host cells has been shown in the *Vibrio* spp. (Basler et al. 2012). However, further detailed study in necessary to determine the intercellular tube formation with different bacterial genus, cause of formation/induction and exact function of nanotubes.

Chapter 5:

To investigate the disinfectant resistance of *Listeria* species from the food and food processing plants

5.1 Introduction

With the understanding of microbial pathogens, the control measures to restrict the spread of these pathogens have also advanced. Antibiotics are being used to control infections, while disinfectants have been employed to remove or minimize the microbial load in the food industrial premises. However, with the time, bacteria also have evolved with the mechanisms for antimicrobial tolerance and resistance capabilities (Wessels & Ingmer 2013). Bacterial load in the raw food and food industrial premises is a major problem as normal microflora may deteriorate the food quality while prevalence of pathogens may contaminate the food and therefore harmful to public health. Therefore, it becomes mandatory to kill or minimize the microbial flora from food and food industrial premises. Several food industries employ commercially available disinfectants to kill the microbes that are prevalent at the food industry. However, it is evident that despite the application of disinfectants not all the microorganism get removed (McDonnell & Russell 1999; Bridier et al. 2011). The main reason behind failure of 100% killing/removal of microbes is the impediment for disinfectant to reach to microbes or capability of microbes to overcome the action of disinfectants (Russell 1999). Such conditions, so called "resistance" protect microbes and therefore such microbes may persist for longer period of time (Carpentier & Cerf 2011).

The listerial strains entered in the food processing premises may possess innate capability to resist the disinfectant or they may get adapted to the disinfectant if these disinfectants are employed at sub-lethal conditions (To et al. 2002). The *L. monocytogenes* strains that not possess any innate resistance, may possess good biofilm forming ability which is more likely to protect the cells despite lacking innate resistance capability (Nilsson et al. 2011; Bae et al. 2012; Nicholas et al. 2013).

Therefore, the cells that possess the innate ability or the cells that possess the good biofilm forming ability or possessing both abilities are logically more likely to persist in food industry. These abilities anyhow, result in the persistence of the bacteria in food industry. Several studies performed that suggest the persistence occurs solely either due to the disinfectant resistance or due to the biofilm formation ability (To et al. 2002; Nakamura et al. 2013); while theoretically and logically, a good biofilm forming strains will exhibit the resistance irrespective of the strains innate ability to resist the antimicrobials. Therefore, toward understanding exact cause for the persistence, we aimed to understand the role of innate resistance and resistance exhibited due to biofilm forming ability that may likely to cause the persistence.

5.2 Review of Literature

5.2.1 Resistance of *L. monocytogenes* to disinfectants

The very first case of *L. monocytogenes* as a resistant to antimicrobials was reported in the 1990 by Poyart-Salmeron et al., (1990). Since then resistance of *L. monocytogenes* has been reported to several antimicrobials including disinfectants (Njagi et al. 2004; Mullapudi et al. 2008; Rakic-Martinez et al. 2011; Ratani et al. 2012). Till date, disinfectants such as benzalkonium chloride, peracetic acid, chlorine based compounds, peroxides, iodophor, chloramine-T, phosphoric acid, formaldehyde have been employed to kill *L. monocytogenes* and other bacterial load that are prevalent in the food industry (Cordier et al. 1989; Best et al. 1990; Somers & Wong 2004; Nakamura et al. 2013). Using disinfectants at recommended concentrations, *L. monocytogenes* should be completely inactivated, however factors such as food debris, biofilm formation, acquired resistance characters, inadequate cleaning and faulty disinfection procedures or dosage failure can significantly reduce the efficiency of disinfectants (Best et al. 1990; Müller et al. 2013). Of many disinfectants,

quaternary ammonium based compound, benzalkonium chloride (BC) is widely used to kill the bacteria including *L. monocytogenes* because of its high bactericidal activity at low concentration (Soumet et al. 2005; Walton et al. 2008; van der Veen & Abee 2011). Moreover, repeated exposure to sub-lethal concentration of disinfectant has shown to induce tolerance in *L. monocytogenes* (To et al. 2002).

Worldwide studies suggested that *L. monocytogenes* from food processing plants possessed innate ability to resist BC (Romanova et al. 2006; Mullapudi et al. 2008; Ratani et al. 2012; Nakamura et al. 2013). Disinfectant resistant isolates have been obtained from turkey processing plant (Mullapudi et al. 2008), dairy products (Popowska et al. 2006), cold smoked fish (Soumet et al. 2005) and vegetables (Popowska et al. 2006). Certain major outbreaks (1998-99 from hotdog, 2001-terkey daily meat) associated *L. monocytogenes* strains also have been found to possess the disinfectant resistance capability (Nelson et al. 2004; Elhanafi et al. 2010; Gilmour et al. 2010).

5.2.2 Mechanism of resistance

At least four mechanisms have been described which can be combined to explain resistance shown by *L. monocytogenes*: (i) the exopolymeric matrix forming a physical barrier that limits the diffusion of sanitizers within the biofilm, (ii) the resistance mechanisms (e.g. detoxifying membrane transporters), which can even be encoded on a plasmid and can be horizontally transferred among biofilm cells, (iii) the differentiation of bacterial cells into different physiological states (e.g. dormant cells) less susceptible/ receptive to treatments, and (iv) the modification of the microenvironment (e.g. local acidic pH) rendering a particular sanitizer less efficient (Giaouris et al. 2013).

L. monocytogenes has been found to possess innate as well as acquired ability to extrude toxic ions which also include ionic disinfectants such as BC. The general stress or toxin removal mechanism seems to play a role in disinfectant resistance in *L. monocytogenes* increasing level of tolerance (Moorman et al. 2005). Different Gram positive bacteria have shown to possess different types of efflux pump. In case of *S. aureus, MdeA, MgrA, NorB and Tet38* while in case of *B. subtilis Erb*AB and *Pub*E have shown to exclude broad range of biocides from cell. In case of *L. monocytogenes* two types of efflux pumps have been defined – multi drug resistance (mdrL) and *Listeria* drug efflux (lde) (Godreuil et al. 2003). However, multidrug resistance (mdrL) efflux pump have been found to involve majorly to extrude toxic ions in case of *L. monocytogenes* (Romanova et al. 2006; To et al. 2002). MdrL efflux pump is a general mechanism for extrusion of toxic ions such as BC, ethidium bomide and heavy metals (Mullapudi et al. 2008). Also, the mdrL efflux pump has been shown to play a role in adaptation to BC in *L. monocytogenes* (Romanova et al. 2006). Exposure to BC or certain antibiotics or heavy metals sensitize the efflux pump and once sensitized, the efflux pump cross protect cells by removing the ions that get sensed as a toxic (Rakic-Martinez et al. 2011). Another general mechanisms exhibited by *L. monocytogenes* is change in cell wall fatty acids content. With the exposure of disinfectant at sub-lethal concentration, certain changes occurs in fatty acid content of cells (To et al. 2002; Walton et al. 2008; Bisbiroulas et al. 2011). Despite these general mechanisms, biofilm of *L. monocytogenes* may play a major role exhibiting resistance to disinfectant (Chavant et al. 2004; Somers & Wong 2004; Nakamura et al. 2013). In some cases, food-soil may shelter *L. monocytogenes* from reaching disinfectant causing "pseudo-resistance" (Ibusquiza et al. 2011). Such resistance formed by the bacteria in biofilm may not be the resistance due to change in the

bacterial characteristics and therefore has been suggested to consider as "phenotypic adaptation" (Meyer 2003). Plasmid-borne disinfectant resistance cassette (*bcrABC*) has been identified in *L. monocytogenes* and shown to be transferred through the horizontal gene transfer by conjugation (Katharios-Lanwermeyer et al. 2012). Recently, Müller et al. (2013) reported the chromosome based Tn*1688* transposon as a responsible factor for the increased tolerance of the BC. Heat shock proteins may contribute for developing resistance against disinfectants (van der Veen & Abee 2010). Overall *L. monocytogenes* cell possesses general as well as specified mechanism to extrude the BC. Apparently, such mechanism helps *L. monocytogenes* cells to survive in the food industry despite the antimicrobial efforts taken.

5.2.3 Clean-In-Place

Clean-In-Place (CIP) is a widely used unit-operation in the food industries (Grab & Benett 2001). It is done solely by circulating chemical solutions and water rinses by mechanical means onto and over surfaces that are to be cleaned (Alvarez et al. 2010; Davey et al. 2013). CIP was developed in 1950's, initially as a manual process, for hygienic cleaning of plant surfaces. It is used not only for microbiological hygiene but also, importantly, to restore heat transfer and pressure drop characteristics of plant. Today CIP is used globally as the "standard" for cleaning (Wilson 2003; Davey et al. 2013).

Clean-In-Place (CIP) can be defined as the ''cleaning of complete items of plant or pipeline circuits without dismantling or opening of the equipment and with little or no manual involvement on the part of the operator." The process involves the jetting or spraying on surfaces or circulation of cleaning solutions through the plant under conditions of increased turbulence and flow velocity (Romney, 1990). Though the procedure is not well defined, 'International Standards Organization' (ISO)

guidelines:13408-4:2005 specifies the general requirements for CIP processes applied to product contact surfaces in the manufacture of sterile health care products by aseptic processing and offers guidance on qualification, validation, operation and control (ISO 2005). A basic CIP procedure consists of hot water wash followed by alkaline, acid again alkaline and hot water wash. Many food industries incorporate disinfectant or enzyme treatment before final hot water wash (Eide et al. 2003).

The CIP procedures are mainly designed to clean the dust, food soil, planktonic microbes, other dirt particles and not to remove the biofilm (Poulsen 1999; Simões et al. 2010). However, the harshness of CIP must be having more or less effect on the biofilm. Several researchers have evaluated the effect of the CIP on biofilms. Depending upon the CIP steps employed, the effectiveness of CIP found to vary (Eide et al. 2003). Microorganisms such as *Staphyloccocus aureus*, *E. coli*, *Salmonella* spp., *Campylobacter* spp., *Pseudomonas* spp. have been shown to resist the commonly employed CIP procedures (Dufour et al. 2004; Bremer et al. 2006; Furukawa et al. 2010).

The laboratory scale study performed by addition of strong alkali/acid, use of higher temperature water (upto 95^{o}C) found to be efficient in destroying biofilm. However, use of such strong conditions has major drawbacks. After use of strong alkali/acid, it is necessary to ensure the removal of residual strong acid/alkali which needs several water washes. While use of very hot water may cause the cracks in stainless steel or it may expand of stainless steel in turn affecting the joints of pipes. Therefore, very harsh conditions are not recommended (Eide et al. 2003).

L. monocytogenes has been reported from several food industries worldwide (Taormina & Beuchat 2001; Taormina & Beuchat 2002; Adzitey & Huda 2010; da Silva & De Martinis 2013). Prevalence suggests, *L. monocytogenes* must be

possessing capability to tolerate or resist the CIP. No study is available to determine the effect of CIP on *L. monocytogenes*. To explore the actual scenario, it is necessary to understand the resistance pattern.

5.3 Materials and Methods

5.3.1 Determination of MIC

All the 83 *L. monocytogenes* isolates were screened for their minimum inhibitory concentration (MIC) towards commonly used disinfectant, benzalkonium chloride (BC) by microtiter well plate method (To et al. 2002). Overnight grown isolate (10 µl) was inoculated in 190 µl of the BHI broth in the 96 well microtiter plate containing serially increasing concentrations from 1 to 20 µg/ml of BC. Plates were incubated for 48 h at 37^0C. After incubation, microtiter plates were observed visually for the growth turbidity. The concentration at which no growth was observed after incubation was considered as MIC of the BC for that isolate. Control was prepared without adding any BC solution. Blank was prepared by adding all the components except inoculum.

5.3.2 Determination of role of efflux pump in resistance

5.3.2.1 Screening of efflux pump activity

The efflux pump activity was initially screened by determining the growth in presence of ethidium bromide (EtBr) (To et al. 2002). Isolates were spot inoculated on BHI agar plates containing 0.5 µg of EtBr per ml. The plates were incubated at 37°C for 24 h and inspected for fluorescence under UV light. Cells that showed pink fluorescence suspected to possess inactive efflux pump thus accumulate EtBr. Whereas cells that showed pale pink to white suspected to possess active efflux pump throwing off EtBr (To et al. 2002).

5.3.2.2 PCR detection of the *mdrL* gene

The efflux pump activity is mainly controlled by proton motivated multi-drug resistance (*mdrL*) gene. To determine the genetic basis of the multi-drug resistance efflux pump, *mdrL* gene was screened by PCR (Mereghetti et al. 2000). The genomic

DNA was isolated by using PureLink Genomic DNA extraction kit (Invitrogen, Cat. No. K182001). PCR was performed by using PCR master-mix (Sigma, Cat. No. P4600) with the 20 pmol of each of the two primers (lltb1 and lltb2) (details in Table: 6) and 50 ng of genomic DNA. The cycling conditions consisted of an initial denaturation step at 94°C for 120 s followed by 30 cycles comprised of denaturation at 94°C for 60 s, primer annealing at 50°C for 60 s, and extension at 72°C for 90 s and final extension at 72°C 10 min. The PCR amplicon of the 1,136 bp were considered as of *mdrL* gene. Randomly selected amplicons were purified by the Wizard® SV Gel and PCR Clean-Up System (Promega, Cat. No. A9281) and sequenced commercially (GeNei, Bangalore). The sequences were then analyzed through NCBI-blast to confirming the amplicon of *mdrL* gene from *L. monocytogenes*.

5.3.2.3 Expression of *mdrL* gene

A total of 12 *L. monocytogenes* isolates (6 sensitive and 6 resistant to BC) containing the *mdrL* gene were randomly chosen for the study. Freshly grown isolates were inoculated in 2 ml of BHI broth with BC concentration one unit lower to MIC while control was kept by growing isolates in just BHI broth. The test and control inoculated BHI broth were incubated at 37^{o}C for 12 h and processed for RNA extraction. Total RNA was extracted by RNeasy mini kit (Qiagen, Cat. No. 74104). The qPCR reactions were performed as described before (4.3.5.3) with primers mdrLq (details in Table:6).

5.3.3 Determination of disinfectant resistance by *L. monocytogenes*-biofilm

A total of 18 *L. monocytogenes* strains, 6 each from strong, moderate and weak biofilm former were randomly chosen to study the resistance formed by biofilm to BC. Freshly grown culture (10 µl) was inoculated in 190 µl of BHI broth in microtiter plate. Biofilm was allowed to form on microtiter plate for 24 h at 37^{0}C for

biofilm formation. After incubation the culture was removed by pipetting and wells were washed three times by pipetting 250 µl of PBS to remove unattached cells. Wells were treated with 250 µl of BC solution with concentrations ranging from 2 to1024 µg/ml with the contact time of 5 min. BC solution was then removed and again wells were washed three times with 250 µl of PBS to remove residuals of disinfectant. Sterile BHI broth (200 µl) was added to wells and plates were incubated for 24 h at 37^0C. Presence of turbidity was noted as growth indicating resistance to disinfectant while absence of turbidity indicating no growth thus sensitive. Control was kept without any disinfectant treatment while blank was maintained without inoculating culture. Maximum disinfectant concentration at which at which the growth turbidity was absent was considered as MIC of the disinfectant.

5.3.4 Resistance to Clean-in-place (CIP) procedure

Study was performed to determine the ability of *L. monocytogenes* biofilm to resist to CIP procedure. The CIP procedure used for study was adapted from the food industries from where isolation of *Listeria* spp. was carried out. Strong, moderate and weak biofilm forming *L. monocytogenes* strains- ILCC306, ILCC177 and ILCC161 respectively were considered for study. Two ml of freshly grown isolates were grown in 50 ml screw cap tube containing 48 ml of BHI broth and 2 X 6 cm stainless steel (SS) coupon. The sets were prepared and incubated at 37^0C for 48 h allowing to form a mature biofilm on SS coupon. Coupons were removed and treated *in-vitro* CIP procedure. The CIP was performed by series of washes. Each wash was performed sequentially by keeping coupons for desired time and temperature in the 50ml screw cap bottle containing 50 ml of specified solution with the 150 rpm rotation on shaker. The steps for washing procedure were - (1) plain water wash for 5 min., (2) hot water (55-60^0C) for 5 min.; (3) 1.2-1.5% of caustic soda for 20 min at 55-60^0C; (4) nitric

acid (0.5%); (5) then acidity was neutralized by 1.2-1.5% of NaOH for 20 min.; (6) hot water wash (55-60^{0}C) and (7) plain water wash for 5 min. each. Coupons were then thoroughly swabbed by cotton swabs. These swabs were placed in 15 ml screw cap tubes containing 10 ml of phosphate buffered saline with 0.01% tween 20. Dilutions were prepared and spread plated on PALCAM agar. The plates were incubated at 37^{0}C for 24 h. After respected incubation colony count was performed. The experiment was repeated three times and the mean results were calculated.

5.4 Results and Discussion

5.4.1 Resistance of *L. monocytogenes* to disinfectant

L. monocytogenes has been isolated from soil, silage, marine and fresh water, sewage, food processing plants, food, domestic and wild animals as well as humans and can survive in multiple habitats (Barbuddhe et al. 2012). It is well known that factors such as heavy metal ions, high salt concentration, low pH-values, low temperatures, as well as low water activity limit the bacterial growth and survival. However, due to the ability of *L. monocytogenes* to resist environmental stresses, this bacterium successfully colonizes in food processing environments. Benzalkonium chloride is a biocide belonging to the group of quaternary ammonium compounds (QACs) that is commonly used in the food industry (Ibusquiza et al. 2011). *L. monocytogenes* can be completely inactivated using disinfectants at recommended concentrations. However, many factors such as food debris, biofilm formation, inadequate cleaning and disinfection procedures can significantly reduce the efficiency of disinfectants (Pan et al. 2006; Ibusquiza et al. 2011). Between 10 to 46% of *L. monocytogenes* strains isolated from food and food processing environments can be regarded as being BC tolerant (Mullapudi et al. 2008). Continuous and over use of disinfectant has led to selective pressure on industrial microflora emerging disinfectant tolerant and resistance strains (To et al. 2002). To determine the innate ability to resist the disinfectant, all 83 *L. monocytogenes* isolates were tested against BC. The resistance criteria was used as defined previously (4 µg/ml) (To et al. 2002); accordingly, 28 (33.73%) isolates were resistant to BC (Table 5.1). The data obtained was analyzed for any correlation exists between resistance and serotypes of *L. monocytogenes*. However, no correlation was observed between disinfectant resistance and serotype or source of isolation.

Table 5.1: MIC of BC for *L. monocytogenes* isolates.

Sr. No.	Serotype	Source	ILCC ID	Benzalkonium chloride concentration
1	1/2a	Animals	ILCC005	5
2	4b	Humans	ILCC026	10
3	1/2b	Humans	ILCC027	6
4	1/2b	Humans	ILCC040	4
5	1/2a	Animals	ILCC041	4
6	1/2a	Animals	ILCC041a	4
7	4b	Animals	ILCC046	10
8	4b	Animals	ILCC048	4
9	4b	Animals	ILCC049	2
10	4b	Animals	ILCC050	6
11	4b	Humans	ILCC094a	2
12	1/2b	Humans	ILCC095	3
13	1/2b	Humans	ILCC097	2
14	4b	Humans	ILCC098	1
15	1/2b	Humans	ILCC099	2
16	4b	Animals	ILCC115	3
17	1/2b	Humans	ILCC140	2
18	4b	Humans	ILCC142	4
19	4b	Animals	ILCC144	6
20	4b	Animals	ILCC146	4
21	4b	Animals	ILCC147	1
22	1/2a	Meat	ILCC155	4
23	1/2a	Meat	ILCC158	5
24	1/2a	Meat	ILCC159	5
25	4b	Meat	ILCC161	8

Continued….

26	1/2a	Meat	ILCC163	2
27	1/2a	Meat	ILCC164	2
28	4b	Animals	ILCC165	10
29	1/2a	Meat	ILCC166	4
30	4b	Animals	ILCC172	1
31	4b	Animals	ILCC173	5
32	4b	Animals	ILCC174	5
33	4b	Animals	ILCC175	5
34	4b	Animals	ILCC177	3
35	4b	Humans	ILCC180	5
36	4b	Animals	ILCC181	10
37	4b	Animals	ILCC182	3
38	4b	Animals	ILCC243	2
39	4b	Milk	ILCC249	2
40	4b	Milk	ILCC264	3
41	1/2b	Milk	ILCC283	6
42	1/2b	Milk	ILCC284	1
43	1/2b	Milk	ILCC289	3
44	1/2b	Milk	ILCC291	5
45	1/2b	Milk	ILCC297	2
46	1/2b	Milk	ILCC300	2
47	1/2a	Milk	ILCC301	3
48	1/2a	Milk	ILCC302	3
49	1/2a	Milk	ILCC303	3
50	1/2a	Milk	ILCC304	3
51	1/2a	Milk	ILCC306	3
52	1/2b	Milk	ILCC309	6
53	1/2a	Milk	ILCC312	4
54	1/2a	Milk	ILCC317	2

Continued…..

55	1/2a	Milk	ILCC325	2
56	1/2a	Milk	ILCC336	2
57	1/2a	Milk	ILCC373	4
58	1/2b	Milk	ILCC395	7
59	1/2a	Milk	ILCC400	10
60	1/2a	Milk	ILCC405	6
61	1/2b	Milk	ILCC419	8
62	4b	Meat	ILCC468	4
63	4b	Meat	ILCC470	3
64	4b	Meat	ILCC471	1
65	4b	Animals	ILCC491	7
66	4b	Animals	ILCC492	2
67	4b	Animals	ILCC493	8
68	4b	Animals	ILCC494	2
69	4b	Animals	ILCC496	6
70	4b	Animals	ILCC498	6
71	4b	Animals	ILCC499	2
72	1/2a	Milk	ILCC519	5
73	1/2a	Milk	ILCC530	7
74	1/2a	Milk	ILCC531	2
75	1/2a	Milk	ILCC535	2
76	1/2a	Milk	ILCC540	4
77	4b	Humans	ILCC557	4
78	1/2b	Humans	ILCC559	4
79	4b	Humans	ILCC562	2
80	4b	Humans	ILCC564	2
81	4b	Humans	ILCC567	2
82	4b	Meat	ILCC169	4
83	1/2b	Humans	ILCC569	2

5.4.2 Role of efflux pump in resistance

Out of several mechanism proposed, efflux pump activity seems to be participating majorly in resistance of the toxic ions. Efflux pumps are transport proteins involved in the extrusion of toxic substrates (including virtually all classes of clinically relevant antibiotics) from within cells into the external environment. These proteins are found in both Gram-positive and -negative bacteria as well as in eukaryotic organisms (Webber 2002). Pumps may be specific for one substrate or may transport a range of structurally dissimilar compounds (including antibiotics of multiple classes); such pumps has been also shown to associated with multiple drug resistance (Webber 2002). In case of *L. monocytogenes* two types of efflux pumps are present – multi drug resistance (mdrL) and *Listeria* drug efflux (lde) (Godreuil et al. 2003). Of these, the mdrL efflux pump is responsible for removal of wide range of toxic ions while lde is removes fluoroquinolones (Godreuil et al. 2003).

We analysed all 83 isolates for the presence of the mdrL efflux pump activity. Primary screening method based on efflux pump identified 28 BC resistant isolates showing pinkish-white colonies due to active removal of EtBr, while sensitive isolates showing orange colored colonies due to accumulation of EtBr (Fig. 5.1). To confirm the genetical constituent, BC resistant isolates were screened for the gene encoding multi-drug resistance efflux pump (mdrL) among the isolates. It was interesting to note that all 83 isolates showed presence of the *mdrL* gene irrespective of their resistance capability. Therefore further study was carried out accessing the expression of the *mdrL* genes among the BC sensitive and resistant isolates. The gene expression studies showed a positive correlation (r^2=0.907) for increase in MIC with the increase in the *mdrL* gene expression (Table 5.2). Interestingly, the *mdrL* gene expression was found higher in resistant strain than sensitive strain even in the absence of BC. It is

known that, previous exposure to the toxic ions increase the expression of the *mdrL* gene (To et al. 2002; Romanova et al. 2006). These isolates were obtained from the different food and food processing industries and the exposure of these BC resistance isolates to analogues stress cannot be denied. A study performed with clinical, food and environmental *L. monocytogenes* isolates by Mereghetti et al. (2000) observed the resistance among the food and environmental isolates only, while all the clinical isolates were sensitive to BC, reasoning the previous exposure to toxic ion stress in food industry and in environment. Increased *mdrL* gene expression among the previously laboratory adapted strains have reported by Romanova et al. (2006) et al. From this study, it can be inferred that the innate ability of *L. monocytogenes* - mdrL efflux pump is responsible for BC resistance in *L. monocytogenes*. Also, as these efflux pumps are not substrate specific (To et al. 2002), they may help cells to cross protect among the variety of ionic disinfectants in food industry causing persistence.

Fig. 5.1: *L. monocytogenes* colonies on BHI agar (24h/37^{o}C) plate with 0.5 µg/ml of Ethidium bromide (EtBr). The white colored colonies are due to actively removal of EtBr indicating active efflux pump, while pinkish colored colonies are due to the accumulation of EtBr indicating inactive efflux pump.

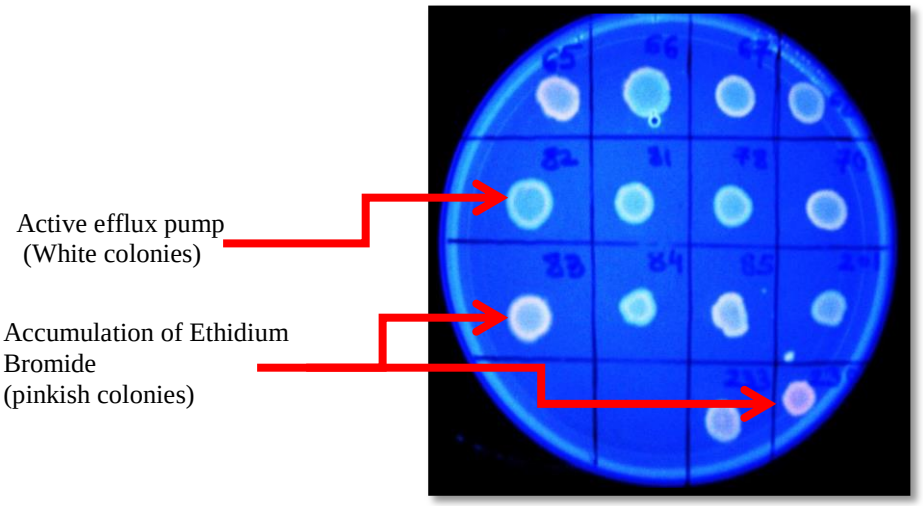


Table 5.2: Multi-drug efflux pump gene expression in presence and absence of BC in BC sensitive and resistant isolates.

Serotype	Source	ILCC ID		MIC of BC	expression of the *mdrL* gene	
					absence of BC	presence of BC
1/2a	Animals	ILCC005	**Resistant**	**5**	2.1	20.8
4b	Humans	ILCC026		**10**	2.8	28.9
1/2b	Humans	ILCC027		**6**	1.9	23.3
1/2a	Meat	ILCC158		**5**	3.1	21.1
1/2a	Meat	ILCC159		**5**	1.6	21.7
4b	Meat	ILCC161		**8**	2.4	24.9
4b	Animals	ILCC182	**Sensitive**	3	0.6	17.4
4b	Animals	ILCC243		2	1.2.	14.8
4b	Milk	ILCC249		2	1.4	18.6
1/2b	Milk	ILCC297		2	1.9	13.8
1/2b	Milk	ILCC300		2	0.8	12.8
4b	Meat	ILCC471		1	1.9	10.9

5.4.3 Resistance of *L. monocytogenes* biofilm to disinfectant

Besides the innate ability to resist the disinfectants, biofilm formation property also has been considered as a major reason for increases in tolerance or resistance (Burmolle et al. 2006; Ito et al. 2009; Hoiby et al. 2010). Toward understanding the role of biofilm as a causative agent of persistence, it is essential to understand the resistance exhibited by *L. monocytogenes* in biofilm. Therefore, the MIC of the *L. monocytogenes* isolates was tested in biofilm. In results, the disinfectant resistance pattern of isolates found elevated in biofilm as compared to planktonic phase. Also, more the biofilm formation more was the resistance to disinfectant (Fig. 5.2, 5.3 and 5.4). A good correlation (r=0.88) was observed between biofilm formation and increase in resistance. Weak biofilm formers showed 1 to 5.3 times increase in the MIC. Moderate biofilm formers showed 9.14 to 42.66 times increase in the MIC, while strong biofilm former showed 102.4 to 341.33 times increase in the MIC (Table 5.3). As compared to weak biofilm, stronger biofilm consist of multi-layers of cell (Renier et al. 2011). These layers acts as an impediment to the antimicrobials and therefore antimicrobials cannot reach to the cells that situates deep in the biofilm matrix (Bridier et al. 2011). In congruence with the electron microscopy studies (4.4.3), the resistance pattern observed in this study indicates the strong biofilm formation of *L. monocytogenes* consisting multilayers which acts as an impediment. Therefore, the biofilm formation of *L. monocytogenes* makes cells resistant to disinfectant. Higher the biofilm formation greater the resistance capability to BC. Such resistance capability due to the biofilm formation may lead to the persistence.

Table 5.3: MIC of the BC to *L. monocytogenes* isolates in planktonic and biofilm phase.

	ILCC Code	**Biofilm formation capability (CV turbidity)**	**MIC in Planktonic**	**MIC in Biofilm**
Weak Biofilm former	ILCC049	0.185	2	4
	ILCC471	0.268	1	2
	ILCC470	0.147	3	16
	ILCC530	0.128	7	16
	ILCC567	0.045	2	2
	ILCC569	0.050	2	8
Moderate Biofilm forming isolates	ILCC098	0.339	1	64
	ILCC177	0.334	3	128
	ILCC283	0.634	6	128
	ILCC336	0.348	2	32
	ILCC491	0.350	7	64
	ILCC564	0.456	2	32
Strong biofilm forming isolates	ILCC297	0.871	2	512
	ILCC306	0.953	3	1024
	ILCC312	0.803	4	512
	ILCC395	0.936	7	1024
	ILCC400	0.936	10	1024
	ILCC519	0.890	5	512

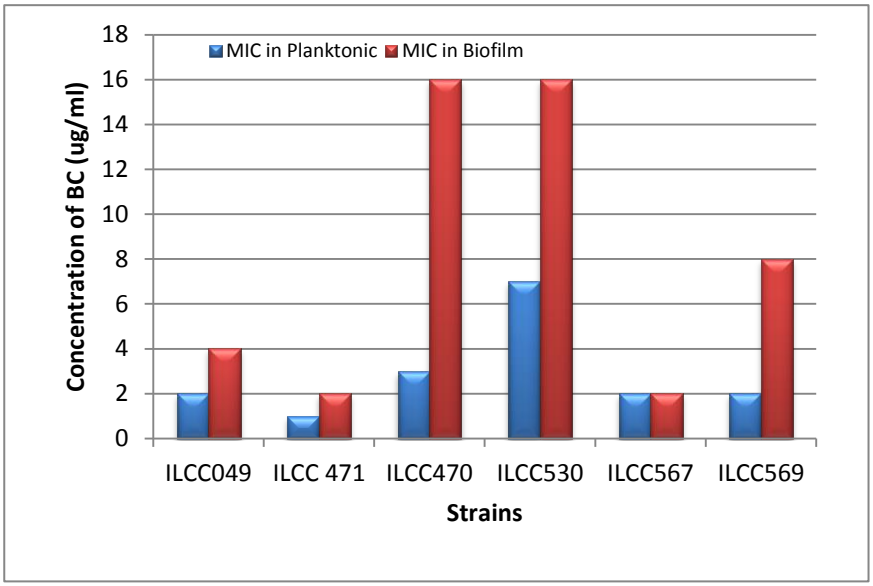


Fig. 5.2: BC resistance of weak biofilm former *L. monocytogenes* strains

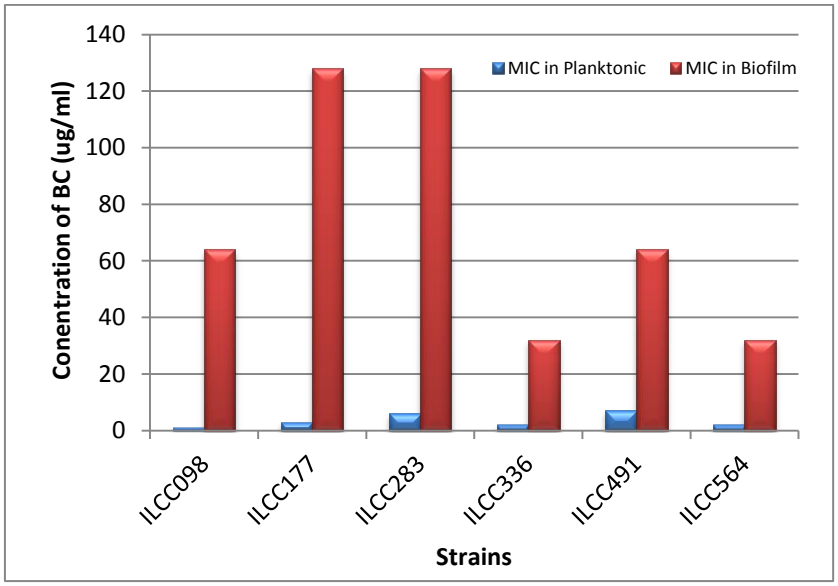


Fig. 5.3: BC resistance of moderate biofilm former *L. monocytogenes* strains

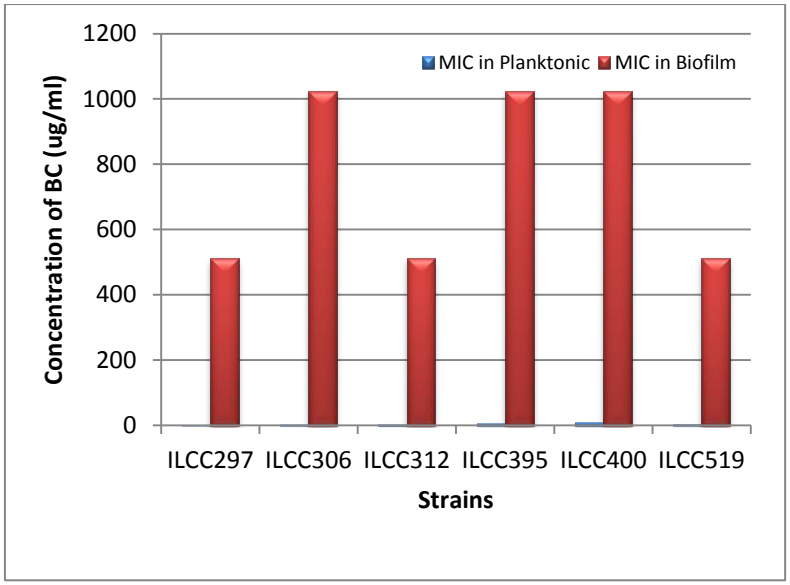


Fig. 5.4 BC resistance of strong biofilm former *L. monocytogenes* strains

5.4.4 Resistance to Clean-in-place (CIP) procedure

CIP are meant to clean the industrial equipment without dismantling it. Though there is no standard protocol defined for CIP, depending upon the nature of food products, CIP procedures are customized at food industry level. However, an ideal CIP procedure consist of six steps as hot water wash, alkali wash, acid wash, alkali wash, hot water wash and sanitization followed by cold water wash (Eide et al. 2003). The CIP is meant to clean the surface, remove food soil and wash out/kill bacterial cells (Wilson 2003; Davey et al. 2013). Though few studied have been done on commonly occurring bacteria, no study is available determining the effect of CIP on biofilm of *L. monocytogenes*. However, if *L. monocytogenes* biofilm resist or tolerate to CIP procedures, it may lead to persistence. Therefore, beside the disinfectant resistance capability, *L. monocytogenes* cells must possess the capability to tolerate CIP procedures toward persisting in the food line. To determine CIP tolerance ability, strong, moderate and weak biofilm forming isolates were tested for their resistance capability to CIP procedure.

The 48 h old biofilm of strong, moderate and weak biofilm former strains were tested for their capability to tolerate the CIP procedure. In case of weak biofilm formers no *L. monocytogenes* cell could survive while in case of moderate biofilm 30 cells/cm^2 could survive. The strong biofilm formers isolates showed the highest surviving cells (n=2600 cells/cm^2) (Fig. 5.5). The resistance pattern observed was similar to the pattern that observed for the disinfectant resistance. Similar mechanism must be responsible for the resistance. Stronger biofilm former isolates form multiple layers that prevent the acid/alkali/heat applied during CIP procedure, therefore the cells that are situated deep in the biofilm get protected. Similar observations have been made with laboratory scale CIP models with mixed bacterial culture (Bremer et

al. 2006; Forsythe & Hayes, 1998). Of the steps involved in CIP, acid and sanitization treatment are considered to be the most effective in reducing the planktonic bacterial count while they were less effective on biofilm (Anand & Singh 2013). Several reports showed the prevalence of different bacteria such as *Staphylococci, Shigella, Salmonella, Streptococci, Micrococcus, Aeromonas* and *E. coli* in commercial dairy plants after 'Clean in Place' (Sharma & Anand 2002) indicating the ineffectiveness of the CIP currently being practiced.

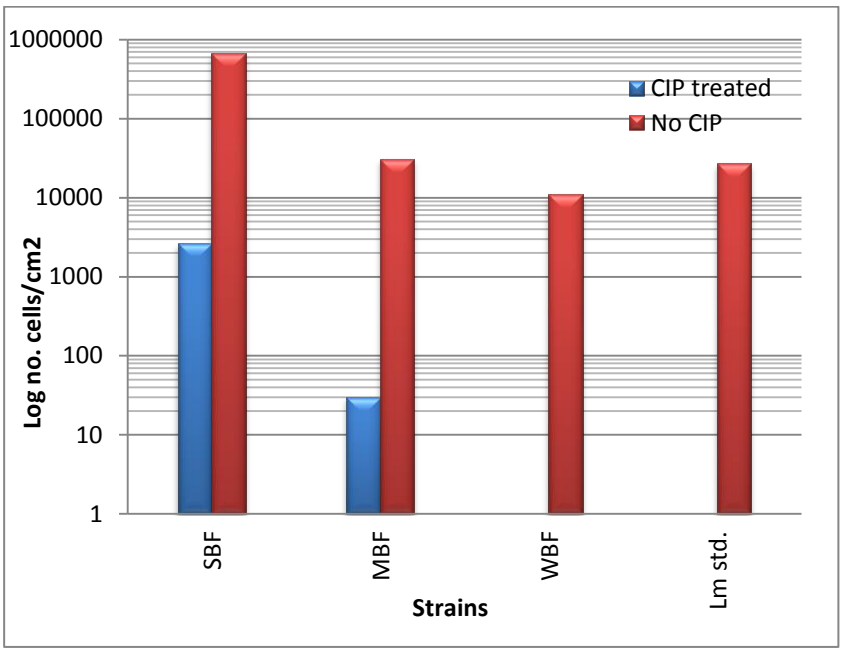


Fig. 5.5: Effect of Clean-In-Place procedure on strong, moderate and weak biofilm forming *L. monocytogenes* isolates. Keys: SBF-Strong biofilm formers, MBF-Moderate biofilm formers and WBF-Weak biofilm formers Lm std. –*L. monocytogenes* EGDe as a control.

From this study it can be concluded the *L. monocytogenes* strain that possess disinfectant resistance capability may survive through the disinfection procedure but not from the other harsh treatment such as CIP. However, strain possessing strong biofilm forming capability but not innate resistance capability may likely to persist and survive in the food industry. In such a way, biofilm forming cells are more likely to persist than the cells possessing innate resistance capability.

Need of future study

Need of future study

Listeria monocytogenes is an emerging pathogen that is showing its existence in number of food products from last three decades. *L. monocytogenes* enters in to food industry from several routes which are hard to detect and unavoidable. Once entered, *L. monocytogenes* survives at sub-optimal conditions and easily get established as a biofilm in the food industry. Such biofilm present in post-processing area contaminate the foods. With the change in life style, and advent of ready-to-eat foods, the incidence of listeriosis is increasing. Till date there is no strategy to control the biofilm and therefore has the scope for research.

The *L. monocytogenes* strains that possess good biofilm forming abilities form multilayer of cells and polymeric substances. These layers are the main impediment due to which disinfectants cannot reach to the cells that are situated deep in the biofilm, causing survival of the cells. Such survived cells grow again forming new layers, recreating the impediment. This needs to be addressed in future studies. There is a need of search for versatile enzymes which can degrade these polymeric substances. Inhibition of sortase A enzyme chemically may reduce the biofilm forming abilities of microbes. The "Bioelectric effect" in which the electricity is combined with the charged microbicidal agents, possesses potential to force charged microbicidal agents into the layers of formed biofilm.

Formation of nanotubes is new way of bacterial communication. The potential of such physical communication should be explored to understand at what level the communication can happen. Also, it would be interesting to know if the *Listeria monocytogenes* forms nanotube with other species of bacteria.

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Appendix

Table 6: Primers used in present study

Note: All the primes used in this study were synthesized from Sigma, Bangalore. (The primers were designed by PrimerQuest, IDT)

Primers used to determine the serotypes (section 3.3.2.3)		**Amplicon (Bp)**	**References**
lmo0737F	AGGGCTTCAAGGACTTACCC	691	Doumith et al., 2004
lmo0737R	ACGATTTCTGCTTGCCATTC		
lmo1118F	AGGGGTCTTAAATCCTGGAA	906	Doumith et al., 2004
lmo1118R	CGGCTTGTTCGGCATACTTA		
ORF2819F	AGCAAAATGCCAAAACTCGT	471	Doumith et al., 2004
ORF2819R	CATCACTAAAGCCTCCCATTG		
ORF2110F	AGTGGACAATTGATTGGTGAA	597	Doumith et al., 2004
ORF2110R	CATCCATCCCTTACTTTGGAC		
prsF	GCTGAAGAGATTGCGAAAGAAG	370	Doumith et al., 2004
prsR	CAAAGAAACCTTGGATTTGCGG		
Primers used to determine the *in-vitro* pathogenicity (section 3.3.2.2)			
hlyAF	5'-GCAGTTGCAAGCGCTTGGAGTGAA	456	(Rawool et al. 2007)
hlyAR	5'-GCAACGTATCCTCCAGAGTGATCG		
ActAF	5'-CAGCGACAGATAGCGAAGATT	965	This study
ActAR	5'-TGTTTCCCGGATGATTTCTAGTT		
plcAF	5'-GGAAGTCCATGATTAGTATGCCT	803	This study
plcAR	5'-CTGGAATAAGCCAATAAAGAACTCTG		
Primers used to detect the *luxS* gene and to study the gene expression (section 4.3.5.2)			
lmo1288F	GGAAATGCCAGCGCTACACTCTT	208	This study
lmo1288R	ATTGCATGCAGGAACTTCTGTCGC		This study
16s-RTF	5'-CACGCCTTTGAAAGATGGTT-3'	98	This study
16s-RTR	5'-TATGCATCGTTGCCTTGGTA-3'		This study
Primers used during construction of Sortase A deletion mutant (section 4.3.5.2)			
Name	**Sequence**	**Restriction**	**Ref:**
SrtAUF	5'-GCGCGGATCCTGACGAACAAGCCAAACAGCAA-3'	BamHI	This study
SrtAUR	5'-AAAAAGCTTAATTGTTTTCTTTAACATATG-3'	HindIII	Bierne et al., 2002
SrtADF	5'-AAAAAGCTTTAATGAGGAAAAAAAGACAGC-3'	HindIII	Bierne et al., 2002
SrtADR	5'-CCGGAATTCGAAAGTTACTTTAAACGGCC-3'	EcoRI	Bierne et al., 2002
SrtAUF1	5'-CGTACATCAAACGAATGAGGG-3'	NA	Bierne et al., 2002
SrtAI	5'-ACCTGCTAGCGGATAATTACC-3'	NA	Bierne et al., 2002
SrtADR1	5'-TTCTAAATCGCCAACTTCGGG-3'	NA	Bierne et al., 2002
SrtACF	5'-ATATCCATGGCCAATAGTTACAAGGAGGAATC-3'	NcoI	Bierne et al., 2002
SrtACR	5'-AAACTGCAGTTATTTACTAGGGAAATATTTAT-3'	PstI	This study
SrtAF	5'-ATGTTAAAGAAAACAATTGCAAT-3'	NA	This study

SrtAR	5’-TTATTTACTAGGGAAATATTTAT-3’	NA	This study
pKSV7F	5’-ATGCCTGCAGGTCGACTCTAGA-3’	NA	This study
pKSV7R	5’-ATTTCACACAGGAAACAGCTATGAC-3’	NA	This study
Primers used to study the gene expression of sortaseA controlled LPXTG protein’s gene			
Name	**Sequence**		
16s-RTF	5’-CACGCCTTTGAAAGATGGTT-3’	098	This study
16s-RTR	5’-TATGCATCGTTGCCTTGGTA-3’		This study
Lmo0171F	5’-CGTGAAAGTACCAGAAGCTAGAA-3’	105	This study
Lmo0171R	5’-TCACCAACTTCGCCAGTAATC-3’		This study
Lmo0262F	5’-GTAGAAGCGCCCGTGAATTA-3’	101	This study
Lmo0262R	5’-GGTTCAGCAGGTTCGGTTAT-3’		This study
Lmo0263F	5’-CCCGACGAAAGAAGGTTACA-3’	099	This study
Lmo0263R	5’-ATGTCCTCTGCTGGCATTT-3’		This study
Lmo0433F	5’-CGCTTCAGGCGGATAGATTAG-3’	108	This study
Lmo0433R	5’-GTGGCGTTATGTCCGTAAGT-3’		This study
Lmo0725F	5’-CGCCCATACCAAATCCCATAA-3’	109	This study
Lmo0725R	5’-ATCGTTCGCTCAGAAGGTAATG-3’		This study
Lmo0732F	5’-ATGGAGCGCAGAGGATAATTT-3’	108	This study
Lmo0732R	5’-ACACCTGCTTTCGTCGTATC-3’		This study
Lmo0835F	5’-GCGCCGTCGTAAAGAATAGA-3’	118	This study
Lmo0835R	5’-TTACTCGTCGCTGCACTATTT-3’		This study
Lmo1115F	5’-ACCAGTTGGAGCGTCATATTC-3’	090	This study
Lmo1115R	5’-GAGGGTCGCACTGATGATAAA-3’		This study
Lmo1136F	5’-ATGAAGCCAGTAGAGCCAATC-3’	101	This study
Lmo1136R	5’-GCTGGTTGTGTTGCTATTTCC-3’		This study
Lmo1290F	5’-CCACTTAAGGGCGGATAGTTATT-3’	097	This study
Lmo1290R	5’-TGCTCCAGATGGTGTTCATTTA-3’		This study
Lmo1413F	5’-GTGAGAAAGGTCCGGCTAAT-3’	106	This study
Lmo1413R	5’-CTGGAAGCTGGAGATACACTAAA-3’		This study
Lmo1666	5’-GCTGAGCCATCGTTTGTAGTA-3’	115	This study
Lmo1666	5’-ATGTGACCGCACCAGTAATC-3’		This study
Lmo1799F	5’-GCGCCTGCTAGTGAGAATAA-3’	112	This study
Lmo1799R	5’-GTTATAGCGCGGATGGAACT-3’		This study
Lmo2026F	5’-TCCAGGTATTGCACGGTAAC-3’	100	This study
Lmo2026R	5’-GCACCTTATCGACGAACTCTAA-3’		This study
Lmo2085F	5’-CCCGCTAGCTACGACAATAAG-3’	111	This study
Lmo2085R	5’-GTGGTGAAGGTAAGTGGAGAAA-3’		This study
Lmo2179F	5’-GCCAAGGATTGGTATCGTCATA-3’	100	This study
Lmo2179R	5’-GAAGGTTATGTAGGCGAGGTTAG-3’		This study
Lmo2396F	5’-GCCTCACGAAAGGAGAACTATG-3’	101	This study
Lmo2396R	5’-CCCGCCATCACGAACTAAA-3’		This study
Lmo2576F	5’-ACCATTCTCATCGGTCGTTAAA-3’	100	This study
Lmo2576R	5’-GGATAGTGAAACCGGAACTAGAC-3’		This study
srtART2F	5’-AGATGCAGTCGTTGGTTCTATC-3’		This study

srtART2R	5’-TGTTGCTCCAGCTAGCAAATTA-3’	097	This study
Primers used for detection of *mdr*L gene (section 5.3.2.2)			
Name of the primer	**Sequence**	**Amplicon size (bp)**	**Reference**
lltb1	5’-AAATGATTGCTCGTGAAGCT-3’	1136	(Mereghetti et al. 2000)
lltb2	5’-TGTAAGGTAAAATGTGCTGG-3’		
Primers used to study the gene expression of *mdrL* gene (section 5.3.2.3)			
23S rRNA	5’-GTGTCAG GTGGGCAGTTTG-3’	076	(Romanova et al. 2006)
23S rRNA	5’-CATTCTGAGGGAACCTTTGG-3’		
mdrLqF	5’-AGTGTAACGAGTGGAGCATATAAC-3’	141	This study
mdrLqR	5’-TCCCATACCAGCCAAACAAATA-3’		

Media

1. **BHI Agar**

Components	**Quantity in grams for l lt**
Brain Heart, Infusion from (Solids)	8.0
Peptic Digest of Animal Tissue	5.0
Pancreatic Digest of Casein	16.0
Sodium Chloride	5.0
Glucose	2.0
Disodium Hydrogen Phosphate	2.5
Agar	13.5

pH 7.4 ± 0.2

2. **Listeria Enrichment Medium Base (University of Vermont medium (UVM))**

Components	**Quantity in grams for l lt**
Casein enzymic hydrolysate	5.000
Proteose peptone	5.000
Beef extract	5.000
Yeast extract	5.000
Sodium chloride	20.000
Monopotassium dihydrogen phosphate	1.350
Disodium hydrogen phosphate	12.000
Esculin	1.000

Final pH (at 25°C) 7.4±0.2

Suspend 27.17 grams in 500 ml distilled water. Heat if necessary to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 50°C and aseptically add rehydrated contents of 1 vial of Listeria UVM Supplement I (FD136) for primary enrichment or 1 vial of Listeria UVM

Supplement II (FD137) for secondary enrichment. Mix well and dispense as desired. (as per Hi-Media laboratories)

3. Listeria Identification Broth Base (PALCAM)

Component	Quantity in grams for l lt
Peptic digest of animal tissue	23.000
Yeast extract	5.000
Lithium chloride	10.000
Esculin	0.800
Ammonium ferric citrate	0.500
D-Mannitol	5.000
Soya lecithin	1.000
Polysorbate 80	2.000
Phenol red	0.080

Final pH (at 25°C) 7.4±0.2

Suspend 23.69 grams in 500 ml distilled water. Heat if necessary to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 45-50°C and aseptically add sterile reconstituted contents of 1 vial of Listeria Selective Supplement (PALCAM). Mix well before dispensing. (As per HiMedia Laboratories)

4. **ALOA medium**

Component	**Quantity in grams for l lt**
Meat peptone	18.000
Casein enzymic hydrolysate	6.000
Yeast extract	10.000
Sodium pyruvate	2.000
Glucose	2.000
Magnesium glycerophosphate	1.000
Magnesium sulphate	0.500
Sodium chloride	5.000
Lithium chloride	10.000
Disodium hydrogen phosphate anhydrous	2.500
Chromogenic substrate	0.050
Agar	15.000

Final pH (at 25°C) 7.2±0.2

Suspend 36.02 grams in 460 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at15 lbs pressure (121°C) for 15 minutes. Cool to 45-50°C. Aseptically add sterile contents of 1 vial of L. mono Enrichment Supplement I and sterile rehydrated contents of L .mono Selective Supplement I, L .mono Selective Supplement II . Mix well and pour into sterile Petri plates. (As per HiMedia Laboratories)

5. **Sheep Blood agar base**

Component	Quantity in grams for l lt
Casein enzymic hydrolysate	14.000
Peptic digest of animal tissue	4.500
Yeast extract	4.500
Sodium chloride	5.000
Agar	12.500

Final pH (at 25°C) 7.3±0.2

Suspend 40.5 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 45-50°C and aseptically add 5% sterile sheep blood. Mix well and pour into sterile Petri plates.

6. **MacConkey Agar**

Component	Quantity in grams for l lt
Peptic digest of animal tissue	17.000
Proteose peptone	3.000
Lactose	10.000
Bile salts	1.500
Sodium chloride	5.000
Neutral red	0.030
Agar	15.000

Suspend 51.53 gms of medium in 1000 ml distilled water. Heat to boiling with gentle swirling to dissolve the agar completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Avoid overheating. Cool to 45 - 50°C and pour into sterile Petri plates. The surface of the medium should be dry when inoculated.

7. Tryptone Soya agar

Component	Quantity in grams for l lt
Pancreatic digest of casein	15.000
Papain digest of soyabean meal	5.000
Sodium chloride	5.000
Agar	15.000
Final pH (at 25°C) 7.3±0.2	

Buffers and Reagents

1. **TRIS stock (1M)**

Component	**Quantity**
TRIS	121.14
D/W	1000 ml

Adjust the pH to 8.0 if necessary

2. **EDTA Stock**

Component	**Quantity**
EDTA	372.24 g
D/W	1000 ml

Adjust the pH to 8.0 if necessary

3. **TE Buffer (10 mM Tris:1 mM EDTA, pH 8.0):**

Component	**Quantity**
Tris (1M, pH 8.0)	10 ml
EDTA (1M, pH 8.0)	1 ml
D/W	89 ml

Adjust the pH to 8.0 if necessary

4. **1% PFGE agarose in TE Buffer:**

Component	**Quantity**
PFGE grade agarose	1 gm
TE buffer (pH 8.0)	10 ml

5. **Preparation of phosphate-buffered saline (0.01 M; pH 7.2)**

Component	**Quantity**
Na_2HPO4 (anhydrous)	1.09 g
NaH_2PO4 (anhydrous)	0.32 g

NaCl	9.0 g
D/W	1000 ml

Mix to dissolve and adjust pH to 7.2 Store this solution at room temperature.

6. Cell lysis buffer (50mM Tris:50mM EDTA, pH 8.0 + 1% Sarcosyl)

Component	**Quantity**
Tris (1M, pH 8.0)	5 ml
EDTA (1M, pH 8.0)	5 ml
Sarcosyl	1 gm
D/W	90 ml

7. Cell Lysis/Proteinase K Buffer

Component	**Quantity**
Cell lysis buffer	5 ml
Proteinase K (20 mg/ml)	25 µl

8. SSP solution (1.2% PFGE grade agarose:1% Sodium Dodecyl Sulfate: 0.2 mg/ml proteinase K)**:**

Component	**Quantity**
PFGE grade agarose	0.12 gm
SDS	0.1 gm
Proteinase K (20 mg/ml)	100 µl
D/W	9.9 ml

9. Phosphate buffered saline

Component	**Quantity**
Sodium Chloride	8.0 gm
Disodium hydrogen phosphate	1.14 gm
Potassium chloride	0.2 gm
Potassium di hydrogen phosphate	0.2 gm
D/W	1000 ml

10. Sodium Cacodylate buffer (0.2 M)

Component	Quantity
Sodium Cacodylate Trihydrate	20.15 g
HCl (concentrated)	0.1 ml
D/W	250 ml

pH should be 7.4.

Summary

Summary: Biofilm forming ability and disinfectant resistance of *Listeria* species from food and food processing units

Listeria monocytogenes is a Gram positive, facultative intracellular foodborne pathogen and etiological agent of listeriosis. It exhibits high hospitalization rate (91%), high neonatal death rate (50%) and case fatality rate (20-30%). Although the USDA and FDA have a zero-tolerance policy for presence of *L. monocytogenes* in food, there has been reportedly increase in the incidence of listeriosis in food for the last two decades. These increased incidences is getting strongly correlated with change in lifestyle and increased consumption of ready-to-eat and packed foods. *L. monocytogenes* easily enters in food chain and contaminate food. Despite the bactericidal procedures at food industry, *L. monocytogenes* prevail in processed food. The main reason behind such contamination is occurrence of *L. monocytogenes* in post-processing area. While such occurrence is been suspected due to biofilm formation capabilities. Therefore the study was carried out to determining the role of biofilm formation in persistence of *L. monocytogenes* at food industrial premises.

Screening of 210 samples for *Listeria* spp. from three different food processing industrial premises revealed 41 samples positive for *Listeria* spp. of which 16 were *L. monocytogenes*. All the *L. monocytogenes* isolates were found to possess virulence genes and of 1/2a serotypes. PFGE revealed isolates occurring at same industry is of similar pulsotypes revealing a single source of contamination or cross-contamination occurring in food industry. The isolates were analyzed for their biofilm formation. To avoid bias of the study due to single source and serotypes, a total of 83 *L. monocytogenes* isolates from different serotype (4b, 1/2b and 1/2a) and sources (human, animal, food and environmental) from our laboratory collection (including)

were taken for study. *L. monocytogenes* strains exhibited high variation in biofilm formation capability and found to be independent of growth temperature, nutrients, serotypes and source of isolation. Electron microscopic study revealed biofilm formation by *L. monocytogenes* within 24 h on different industrially important surfaces. An interesting observation of nanotube was made during study. *L. monocytogenes* was found to form a tubular structure between two cells and suspected to function for intracellular communication. *L. monocytogenes* biofilm was analyzed for its change in MIC for quaternary ammonium compound–Benzalkonium chloride. The MIC of the isolates was found to increase as per their degree of biofilm formation. Microbial adherence to hydrocarbon (MATH) test revealed hydrophobicity as a probable reason for adherence to food industrial surface. In-situ genomic analysis showed certain proteins that are present on the cell surface gives hydrophobic characteristics (Bierne and Cossart 2007). Therefore, a mutant for sortase A (hydrophobic cell surface proteins controlling enzyme) was prepared. Deletion of sortase A gene showed a 96.41% loss in adherence, while 86.24% loss in biofilm formation concluding the major role of sortase A in biofilm of *L. monocytogenes*. The study concludes that different *L. monocytogenes* strains possess different capability to form biofilm and according to their degree of biofilm formation capability, *L. monocytogenes* resists to antimicrobial and environmental stress conditions resulting in persistence in the food industrial premises.

Publications

Publications

Research Paper:

1. **Doijad S. P.,** Vaidya V, Garg S., Kalekar S., Rodrigues J., D'Costa D., Bhosle S. N. and S. B. Barbuddhe. 2011. Isolation and characterization of *Listeria* species from raw and processed meats. *Journal of Veterinary Public Health*. 8(2):83-88.

2. **Doijad S. P.,** Garg S., Kalekar S., Rodrigues J., D'Costa D., Bhosle S. N. and S.B. Barbuddhe. 2011. Incidences and genetic variability of *Listeria* spp. from three milk processing plant environment. *Food control*. 22: 1900-1904.

3. **Doijad S. P.,** Lomonaco S., Poharkar K., Garg S., Knabel S., Barbuddhe S. and B. Jayarao. 2014. Multi-Virulence-Locus Sequence typing of 4b *Listeria monocytogenes* isolates collected from different sources in India over a 10-year period. *Foodborne pathogens and diseases.* 11(7):511-516

Book chapter:

- **Doijad S.P** and Barbuddhe S.B (2013) Diversity in the Biofilm Forming Ability of foodborne Bacterial Pathogens In: microbial diversity and its application, Ed. Barbuddhe S.B., Ramesh R., Singh N.P. New India Publishing Agency, New Delhi. pp. 91-110.

Oral presentations:

1. **Doijad S.P.,** Garg S., Kalekar S., Rodrigues J., D'Costa D., Bhosle S. and S.B. Barbuddhe. 2011. Isolation and characterization of *Listeria* species from milk processing plant. Paper presented at National Symposium on "Veterinary Public Health: New Horizon for Integrating the Animal Production, Food Safety and Human Health" organized by Mumbai Veterinary College, Mumbai during 28-29 January, 2011.

2. **Doijad S. P.,** Kumar A., Rawool D. B., Garg S., Knabel S., Barbuddhe S. B. and Bhushan Jayarao. 2013. Role of sortaseA in biofilm formation of *Listeria monocytogenes.* Paper being presented at "International symposium on Problems of Listeriosis XVIII" (ISOPOLXVIII), Goa, India, 19-22nd September 2013.

Poster presentations:

1. **Doijad S.P.,** Garg S., Poharkar K., D'Costa D., Kalekar S., Rodrigues J., Bhosle S. and S.B. Barbuddhe. 2011. Biofilm formation ability of *Listeria monocytoegenes* isolates from food processing units. Paper presented at International Conference of Microbial Diversity for Sustainable Development Chandigarh, Panjab, India.

2. **Doijad, S. P.**, Garg, S. and S. B. Barbuddhe. 2010. Heavy-metal and detergent resistance of *Listeria* species isolates from milk processing environments. Paper

presented at "International Symposium on Problems of Listeriosis"-XVII (ISOPOL XVII), Porto, Portugal, May 5-8, 2010, No. D/P 157.

3. **Doijad S. P.,** Barbuddhe S. B., Garg S., Poharkar K. V., D'Costa D., Kalorey D. R., Rawool D. B. and Chakraborty T. 2013. Biofilm formation ability and disinfectant resistance of *Listeria monocytogenes.* Paper being presented at "International symposium on Problems of Listeriosis XVIII" (ISOPOL XVIII), Goa, India, 19-22nd September 2013.

4. **Doijad S. P.,** Lomonaco S., Poharkar K., Garg S., Knabel S., Barbuddhe S. B. and Bhushan Jayarao. 2013. Multi-Virulence-Locus Sequence typing of *Listeria monocytogenes* 4b serogroup isolates of Indian Origin. Paper being presented at "International symposium on Problems of Listeriosis XVIII" (ISOPOL XVIII), Goa, India, 19-22nd September 2013.

5. **Doijad S.P.,** Knabel S., Barbuddhe S. B. and Bhushan Jayarao. 2013. Evidence of formation of nanotubes by *Listeria monocytogenes*. Paper being presented at "International symposium on Problems of Listeriosis XVIII" (ISOPOLXVIII), Goa, India, 19-22nd September 2013.

Award during PhD

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