4.1. Introduction

Listeria species are widespread in the environment and are known to be associated with soil and decaying matter (Fenlon, 1999). The genus Listeria is differentiated into six species: L. monocytogenes, L. ivanovii, L. innocua, L. welshimeri, L. seeligeri and L. grayi based on DNA homology, 16S rRNA homology, chemotaxonomic properties and multilocus enzyme analysis (Rocourt, 1999). Of the different species L. monocytogenes is often associated with opportunistic infections in man and animals with a propensity to cause severe illness in pregnant women, neonates, in the elderly and immunosuppressed individuals. L. ivanovii is commonly considered as an animal pathogen particularly in sheep. Other Listeria spp. viz. L. innocua, L. welshimeri, L. seeligeri, L. grayi, though generally avirulent, occasional reports of infections with some of these species have been documented.

The Listeria species recovered from the intestinal contents of P. americana viz. L. grayi, L. innocua, L. monocytogenes were tested for important virulence properties.

4.2. Materials and methods

4.2.1. Bacterial strains

L. grayi, L. innocua and L. monocytogenes strains obtained from the intestinal contents of P. americana were maintained at 4°C in TSA containing 0.6% yeast extract. Bacterial suspension equilibrated to
McFarland standard 0.5 (approximately $10^8$ CFU/mL) was used as the test inoculum.

### 4.2.2. Haemolysis

Haemolysis test was performed by the method as described by Twedt et al., (1994). Sheep blood agar plates were prepared and a grid of 5x5 cm$^2$ was marked at the bottom of each plate. The grids were labeled and typical colonies from TSA were spot inoculated into each marked square on the grid and incubated at $37^0\text{C}$ for 48hr. After incubation the plates were examined for haemolysis under transmitted light.

### 4.2.3. CAMP test

The Christie-Atkins-Munch-Peterson (CAMP) test was performed as described by Groves and Welshimer (1977). To perform the test, plates of TSA containing 5% sheep blood was used. With a sterile inoculating wire loop *Staphylococcus aureus* (NCTC 1803) was streaked in a straight line across the centre of the medium with the test isolates of *Listeria* in close proximity with the streak line of *Staphylococcus* at right angles about 1-2 mm apart. Plates were incubated at $37^0\text{C}$ for 24 hr and then observed for enhanced haemolysis by the test isolates to be designated as CAMP test positive.

### 4.2.4. Phospholipase C detection

To determine the phospholipase C production of the *Listeria* isolates, the inoculum was spot inoculated on to the centre of each of the ChromoCult® Listeria selective agar (Merck). The plates were observed for opacity around the colonies after incubation at $37^0\text{C}$ overnight.
4.2.5. hlyA and plcA gene detection

*Listeria monocytogenes* isolate from *P. americana* was screened for the presence of *hly A* and *plcA* genes coding for haemolysin and phosphatidylinositol phospholipase C (PI-PLC) respectively. The DNA isolation of the *L. monocytogenes* isolate was carried out following the methodology by Medrano, *et al.*, (1990) as described in chapter 3. Isolated genomic DNA was used as template for PCR using primers specific to virulence associated genes *hlyA* and *plcA*. The forward and reverse primers used for *hylA* and *plcA* gene detection are given in Appendix II. PCR amplification was carried out in 25µL reaction volume containing 50 ng of extracted genomic DNA, 10 picomoles of both forward and reverse primers, 2.5 units of *Taq* DNA polymerase (Sigma Aldrich, Bangalore), 10 mM of each dNTPs and 2.5 mL of 10X PCR reaction buffer (Appendix II). The PCR was performed in a Biorad Personal PCR (Biorad, USA) with the initial denaturation at 95°C for 3 min followed by 35 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s and extension for 90 s at 72°C followed by a final extension for 10 min at 72°C. The PCR products were further analyzed by electrophoresis using 1.5% (w/v) agarose gel. The amplified PCR products were gel purified and were used for sequencing. The sequenced data was analyzed by using BLAST (Basic Local Alignment Search Tool) at NCBI GenBank (www.ncbi.nlm.nih.gov).

4.2.6. Resistance to lysozyme

Lysozyme sensitivity was performed on TSA containing different concentrations of lysozyme (Hi-Media) ranging from 1mg/mL to 5 mg/mL. One loopful (0.01 mL) of overnight broth culture of each *Listeria* strain was inoculated on to the medium and the plates were incubated at 37°C for 24 hr to assess the growth.
4.2.7. Resistance to bile

One loopful (0.01 mL) of overnight broth culture of the *Listeria* strains was inoculated into TSA plates supplemented with different concentrations of Ox bile (Hi-Media) (0.5–2%). The plates were incubated at 37°C for 24 hr and growth was assessed.

4.2.8. Resistance to serum bactericidal effect

Serum sensitivity test was performed by methods as described by Keller *et al.*, (1998) with modification. 1mL of each bacterial suspension in TSB (Hi-Media) was distributed in test tubes and an equal volume of freshly prepared sera obtained from healthy human volunteers was added. Serum heated to 56°C for 30 min served as control. The tubes were incubated at 37°C for 60 min and the viability of each strain was assessed by plating on to TSA. The *Listeria* strains were considered serum resistant if they were able to grow in both fresh serum (complement active) and heat-inactivated serum (complement deprived).

4.2.9. Biofilm formation

4.2.9.1. Preparation of bacterial cultures

The *Listeria* isolates tested include 2 strains of *L. monocytogenes*, 6 strains of *L. innocua* and 25 strains of *L. grayi*. Prior to each experiment, *Listeria* strains were inoculated into 10mL of TSB and incubated at 37°C for 24 hr.

4.2.9.2. Microtiter plate assay for biofilm formation at different temperatures

The assay was performed by the method described by O'Toole and Kolter, (1998), adopted by Harvey *et al.*, (2007). The prepared cultures were vortexed for 1min and 100µL volumes of each individual culture was
transferred into the wells of three microtiter plates previously rinsed with 70% ethanol and air dried. Plates were covered with a tightly fitting lid and incubated at 4°C, 22°C, and 37°C for 72 hr. Control wells of 100µL of uninoculated growth medium are included in each plate. After incubation, the medium from each well was removed and unattached cells were removed by rinsing with 150 µL sterile distilled water three times and then dried in an inverted position at 30°C for 30 min. The biofilm in each well was stained with 150µL of 1% crystal violet solution in water and left at room temperature for 45 min. The crystal violet solution was removed, the wells were washed three times with 150 µL sterile distilled water and air dried for 30 min. 100µL of 95% ethanol was added to destain the biofilm and the concentration of crystal violet was determined by measuring the optical density (OD$_{595nm}$) using a Micro ELISA Auto Reader (Bio-Rad).

### 4.2.9.3. Influence of pH, sodium chloride and glucose on biofilm formation

The influence of pH on biofilm was studied by allowing biofilm formation as described in 4.2.9.2. in TSB media adjusted to pH values of 5.5, 7.5 and 8.5. For analyzing the influence of different concentrations of NaCl or glucose on biofilm formation of *Listeria*, TSB supplemented with either glucose (1% and 5%) or NaCl (1% and 5%) was used.

### 4.3. Statistical analysis

To study the influence of different factors affecting *Listeria* species in biofilm formation, one way ANOVA was carried out (F test) with 5% significant level.
4.4. Result

Plate: 5

Haemolysis shown by *L. monocytogenes* on blood agar

Any of the *L. grayi* or *L. innocua* isolates under study showed clearing around the colonies in blood agar. However, the two strains of *L. monocytogenes* presented a narrow zone of beta haemolysis around the colonies and were designated as haemolytic.

Plate: 6

CAMP test shown by *L. monocytogenes*

Enhanced haemolysis of the *L. monocytogenes* isolates in proximity with *Staphylococcus aureus* streak line was observed presenting a positive
CAMP test. On the other hand, no change was observed in the medium with *L. grayi* or *L. innocua* isolates.

**Plate: 7**

**Phospholipase C activity shown by *L. monocytogenes***

The present study noticed the failure of *L. grayi* and *L. innocua* strains in producing opacity around the colonies indicating the absence of phospholipase C production in these bacterial species. *L. monocytogenes*, conversely, owing to the phospholipase C production showed narrow zone of opacity around colonies.

The genomic DNA isolated from *L. monocytogenes* was of good quality. PCR amplification of *hlyA* and *plcA* gene from *L. monocytogenes* resulted in the formation of products of size of 456 bp (*hlyA*) and 1484 bp (*plcA*) respectively (Plate: 8). The blast result showed 100% identity to *L. monocytogenes hlyA* and *plcA* gene (GenBank Submissions grp 4055386). The gene sequence data of *hly A* and *plcA* is shown in Appendix II.
Plate: 8
PCR amplified product of \( hlyA \) and \( plcA \) gene of \( L. \) monocytogenes

(From left to right) Lane 1: molecular marker (1kb), Lane 2: \( hlyA \) gene, Lane 3: \( plcA \) gene

Table 5
Virulence related resistance properties of \( Listeria \) isolates

<table>
<thead>
<tr>
<th>Resistance properties tested</th>
<th>Concentration (mg/mL)</th>
<th>( L. ) monocytogenes (2 No.)</th>
<th>( L. ) innocua (6 No.)</th>
<th>( L. ) grayi (25 No.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysozyme</td>
<td>1</td>
<td>2(100)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1(50)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bile</td>
<td>0.5</td>
<td>2(100)</td>
<td>6(100)</td>
<td>25(100)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2(100)</td>
<td>6(100)</td>
<td>25(100)</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>2(100)</td>
<td>6(100)</td>
<td>25(100)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2(100)</td>
<td>6(100)</td>
<td>25(100)</td>
</tr>
<tr>
<td>Growth in serum</td>
<td></td>
<td>2(100)</td>
<td>6(100)</td>
<td>25(100)</td>
</tr>
</tbody>
</table>

*percentage is given in brackets
The antibacterial activity of lysozyme on *Listeria* isolates showed the ability of the two tested strains of *L. monocytogenes* to resist lysozyme at a concentration of 1 mg/mL. At a higher concentration of 2 mg/mL, one of the isolates survived but was noticed to be killed on further increasing the concentration to 5 mg/mL. The *L. grayi* and *L. innocua* isolates, however, showed susceptibility to lysozyme at all the tested concentrations. The *Listeria* species under study were noticed to be growing in the presence of bile at different tested concentrations indicating their tolerance to bile. The ability of *Listeria* isolates irrespective of the species to grow in fresh as well as in the complement-deprived sera noticed in the study showed its resistance to the bactericidal effect of the serum.

The influence of temperature, pH and different concentrations of glucose and sodium chloride on the biofilm formation of *L. grayi*, *L. innocua* and *L. monocytogenes* is shown in Figure 4-6, Figure 7-9 and Figure 10-12 respectively. Intensity of biofilm formation shown by the *Listeria* isolates in relation to temperature, pH, different concentrations of glucose and sodium chloride is given in Table 6, 7, 8 & 9 respectively. Strains were arbitrarily designated as weak (<0.6), moderate (0.6-1.2) or strong (>1.2) biofilm formers according to the measured OD_{595} values (Harvey *et al.*, 2007).
Chapter 4

The box plot showing OD$_{595\text{nm}}$ of biofilm of *L. grayi* (25 strains) at 4°C, 22°C and 37°C

The OD of biofilm formed by *L. grayi* isolates at 4°C was noticed within the range of 0.101-0.228 (median OD 0.123) (Appendix III). A further increase in temperature to 22°C, the OD range increased to 0.217 - 0.497 (median OD 0.352). 37°C was noticed to be the optimum temperature for producing biofilm and the OD was in the range of 0.309-0.638 (median OD 0.487). The mean optical density at different temperatures were found to be statistically significant by one way ANOVA ($F = 131.668$).
4°C was not found to be supporting moderate or strong biofilm formation of *L. innocua* as obvious from the OD (0.122-0.169) with the median OD of 0.132 (Appendix III). On increasing the temperature to 22°C, however, the OD range increased (0.303-0.487) and the median value was 0.357. At 37°C, higher density biofilm formation was noticed (OD range 0.435-0.660 with the median value 0.499). The mean optical density at different temperatures were found to be statistically significant and was established by one way ANOVA test (F=131.668).
The optical density of biofilm formed by the two tested strains of *L. monocytogenes* at 4°C was noticed to be 0.257 and 0.289 respectively which is increased to 0.537 and 0.601 when temperature increased to 22°C. The OD at 37°C was still higher and were 0.813 and 0.881. The F test was conducted and found to be significant (p = 0.002) indicating that there exists a significant difference among mean OD at different temperatures.

**Table 6**

*Intensity of biofilm formation shown by *Listeria* isolates in relation to temperature*

<table>
<thead>
<tr>
<th><em>Listeria</em> species</th>
<th>No. of strains tested</th>
<th>4°C</th>
<th>22°C</th>
<th>37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>weak</td>
<td>moderate</td>
<td>strong</td>
<td>weak</td>
</tr>
<tr>
<td><em>L. monocytogenes</em></td>
<td>2</td>
<td>2(100)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>L. innocua</em></td>
<td>6</td>
<td>6 (100)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>L. grayi</em></td>
<td>25</td>
<td>25(100)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*percentage is given in brackets
All the *Listeria* strains, irrespective of the species formed weak biofilms at 4°C. Though *L. innocua* and *L. grayi* strains remained as weak biofilm formers at 22°C, one of the two strains of *L. monocytogenes* produced moderate biofilm at this temperature. At 37°C, 2 of the 6 tested strains of *L. innocua* and 6 of 25 strains of *L. grayi* along with 2 strains of *L. monocytogenes* became moderate biofilm formers.

**Figure. 7**

The box plot showing OD$_{595nm}$ of biofilm of *L. grayi* (25 strains) at pH 5.5, 7.5 and 8.5

At pH 5.5 the optical density of biofilm formed by *L. grayi* ranged between 0.415–0.758 (median 0.569) (Appendix III). The pH at 7.5 also seemed to be supporting biofilm production with the OD in the range of 0.363-0.727 (the median value 0.485). A decrease in optical density was noticed at pH 8.5 and it ranged between 0. 204-0.590 with the median value of 0.290. One way ANOVA was carried out and found to be significant (F=49.753).
L. innocua formed denser biofilm at pH 5.5 (OD between 0.481–0.725 and the median 0.532 (Appendix III). On increasing the pH to 7.5 the OD ranged between 0.368-0.678 (median value 0.493). At an alkaline pH of 8.5 the optical density was noticed within the range of 0.210-0.401 and the median 0.231. The difference among the mean OD was found to be significant by F test (F=15.707). Multiple comparison test conducted revealing statistically significant difference in mean OD between pH 5.5 and 8.5 and between pH 7.5 and 8.5. No significant difference was noticed between pH 5.5 and 7.5.
The OD of biofilm formed by the two \textit{L. monocytogenes} strains at pH 5.5 were 1.005 and 0.728 which became 0.613 and 0.424 respectively with a shift in pH to 8.5. The one way ANOVA carried out to indicate that the difference in mean was not significant between any pair of pH values ($F = 3.967$).

\textbf{Table 7}

\textbf{Intensity of biofilm formation shown by \textit{Listeria} isolates in relation to pH}

\begin{center}
\begin{tabular}{lrrrrrrrr}
\hline
\textit{Listeria} species & No. of strains & 5.5 & 7.5 & 8.5 & 5.5 & 7.5 & 8.5 \\
& & weak & moderate & strong & weak & moderate & strong & weak & moderate & strong \\
\hline
\textit{L. monocytogenes} & 2 & - & 2(100) & - & - & 2(100) & - & 1(50) & 1(50) & - \\
\textit{L. innocua} & 6 & 4(66.6) & 2(33.3) & - & 4(66.6) & 2(33.3) & - & 6(100) & - & - \\
\textit{L. grayi} & 25 & 15(60) & 10(40) & - & 16(64) & 9(36) & - & 25(100) & - & - \\
\hline
\end{tabular}
\end{center}

*percentage is given in brackets
No strong biofilm formation was noticed with any of the *Listeria* species under study at any of the pH tested viz. 5.5, 7.5, 8.5. However, at a pH of 5.5 and 7.5 more strains were turned out to be moderate formers. Though *L. innocua* and *L. grayi* formed weak biofilms at pH 8.5, one of the two strains of *L. monocytogenes* was found to be a moderate biofilm producer.

On comparing the optical density values of biofilms formed without added glucose, all the tested *Listeria* strains were noticed to be forming higher density biofilms on supplementing the medium with 1% glucose. The influence of glucose on biofilm formation was more pronounced in *L. monocytogenes*. Further increase in glucose concentration to 5% had no noticeable effect in biofilm formation on any of the *Listeria* species under study. Though *Listeria* strains produced enhanced biofilm formation on 1% salt complementation of the medium, a further increase to 5% resulted in weak biofilms.

**Figure 10**
The box plot showing OD$_{595\text{nm}}$ of biofilm of *L. grayi* (25 strains) without and with 1% & 5% glucose/sodium chloride supplementation
The optical density of biofilm formed by *L. grayi* in TSB medium prior to the addition of glucose/ NaCl ranged from 0.309 - 0.638 (median value 0.477) which was found to be increased to 0.519 - 0.971 on the addition of 1% glucose (median value 0.684) (Appendix III). No noticeable difference in OD could be observed with the addition of 5% glucose. Though the strains of *L. grayi* produced enhanced biofilm formation on salt complementation (OD 0.398 – 0.681 with the median OD of 0.552), addition of 5% NaCl resulted in apparent decline in OD (0.124 - 0.298 with median 0.211). The ANOVA and LSD conducted to verify the significance in the mean OD at different glucose / NaCl concentrations and shows significant difference in their mean values (F=83.512).

**Figure 11**

The box plot showing OD$_{595nm}$ of biofilm of *L. innocua* (6 strains) without and with 1% & 5% glucose / sodium chloride supplementation

The optical density of biofilm formed by *L. innocua* with 1% glucose supplementation was within the range of 0.593- 0.875 with the median OD 0.705 (Appendix III). On addition of 5% glucose, the OD range was found to be 0.514–0.732 and the median 0.605. The supplementation of 1% NaCl in
the medium resulted in biofilm within a range of 0.501 and 0.727 (median value 0.603). When the concentration of NaCl was increased to 5%, a noticeable decrease in OD was observed in the range of 0.162–0.265 with median value 0.222.

**Figure 12**

The box plot showing OD$_{595\text{nm}}$ of biofilm of *L. monocytogenes* (2 strains) without and with 1% & 5% glucose/sodium chloride supplementation

Supplementation of medium with 1% glucose had a profound influence on biofilm formation of both *L. monocytogenes* strains. However, no noticeable difference in OD was resulted on 5% glucose supplementation from that of 1%. Though addition of 1% NaCl influenced biofilm formation, a marked decrease in the biofilm production was observed with 5% NaCl.
Table 8

Intensity of biofilm formation shown by *Listeria* isolates in relation to 1% and 5% glucose supplementation

<table>
<thead>
<tr>
<th><em>Listeria</em> species</th>
<th>No. of strains tested</th>
<th>No.&amp; percentage* of strains positive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1%</td>
</tr>
<tr>
<td><em>L. monocytogenes</em></td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td><em>L. innocua</em></td>
<td>6</td>
<td>1(16.6)</td>
</tr>
<tr>
<td><em>L. grayi</em></td>
<td>25</td>
<td>6(24)</td>
</tr>
</tbody>
</table>

*percentage is given in brackets

Moderate biofilm formation was noticed with 83.3% of *L. innocua* and 76% of *L. grayi* with 1% glucose supplementation which was 50% and 72% respectively on supplementation with 5% glucose. Both *L. monocytogenes* strains remained as moderate biofilm formers at 1% as well as 5% glucose in the medium.

Table 9

Intensity of biofilm formation shown by the *Listeria* isolates in relation to 1% and 5% NaCl supplementation

<table>
<thead>
<tr>
<th><em>Listeria</em> species</th>
<th>No. of strains tested</th>
<th>Intensity of biofilm formation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1%</td>
</tr>
<tr>
<td><em>L. monocytogenes</em></td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td><em>L. innocua</em></td>
<td>6</td>
<td>3(50)</td>
</tr>
<tr>
<td><em>L. grayi</em></td>
<td>25</td>
<td>17(68)</td>
</tr>
</tbody>
</table>

Though 68% of the *L. grayi* strains tested remained as weak biofilm formers, 32% has been noticed as becoming moderate biofilm producers on the addition of 1% sodium chloride. A positive influence of 1% sodium
chloride supplementation was also apparent in *L. innocua* and *L. monocytogenes* as well. However, on further increasing the NaCl concentration to 5%, all the *Listeria* spp. under study were turned out to be weak biofilm producers.

**4.5. Discussion**

Pathogenic *Listeria* spp. produce quite a number of virulence linked exoproteins enabling them to cause membrane damage as well as cytolysis. Listeriolysin O (LLO) is a major virulent factor of *L. monocytogenes* required for the survival and proliferation of *L. monocytogenes* within macrophages and nonprofessional phagocytes (Gaillard et al., 1987; Kuhn et al., 1988). Phosphatidylinositol–phospholipase C (PI-PLC) and phosphatidylcholine-phospholipase C (PC-PLC) act synergistically with LLO to achieve the optimal levels of escape from primary and secondary phagosomes to facilitate its cell to cell spread (Camilli et al., 1993; Gedde et al., 2000). Hof, (1984) observed that *L. monocytogenes* responsible for natural infection are haemolytic on blood agar and virulent in mouse whereas the non-haemolytic strains are avirulent. The haemolysis shown in blood agar by the *L. monocytogenes* isolates in the current study indicates its potential in initiating an infection in human beings. The haemolytic activity of *L. monocytogenes* may be weak in blood agar especially with low haemolysin producing strains proposing the CAMP test as a fundamental criterion in the identification of the haemolytic *Listeria* species by Rocourt, (1987). The present study noticed the production of phospholipase C in *L. monocytogenes* isolates and its absence in *L. innocua* and *L. grayi*. Raveneau et al., (1992) reported the reduced virulence of mutant strains of *L. monocytogenes* lacking phospholipase C and found them being incapable of propagating in the host tissues. Though there have been isolated reports of bacteraemia with *L.*
*innocua* and *L. grayi*, their subsequent invasion or replication in tissues has not been reported so far, possibly due to the lack of the major virulence factors such as listeriolysin or phospholipase as found in pathogenic *L. monocytogenes*.

The mouse virulence assay remains the most reliable approach for the laboratory assessment of virulence potential of *L. monocytogenes*. However, it is not practiced routinely from ethical point of view. Detection of genes coding for virulence factors has been described as an alternative and reliable approach to determine the pathogenic potential and to differentiate pathogenic strains of *L. monocytogenes* from non-pathogenic strains (Hof and Rocourt, 1992). The haemolysin and phosphatidylcholine-phospholipase C, encoded by *hlyA* and *plcA* genes have been shown to play a key role in the virulence of *Listeria monocytogenes* (Cossart and Mengaud, 1989; Mengaud *et al*., 1991). The amplification of *hlyA* and *plcA* PCR products of *L. monocytogenes* isolate obtained from *P. americana* as noticed in the current study ascertained their pathogenic potential.

Lysozyme has been suggested as an efficient preservative for controlling *L. monocytogenes* in refrigerated foods which adheres to negatively charged teichoic, lipoteichoic acids and phospholipids on the bacterial surface leading to the bacterial lysis. Hof, (1984); Boneca *et al*., (2007) demonstrated the ability of *L. monocytogenes* to modify peptidoglycan (PG) in its cell wall thereby surviving the action of host’s bacteriolytic lysozyme. In the present study lysozyme resistance was noticed in both the *L. monocytogenes* strains under study with one strain tolerating 5mg/mL suggesting limited effectiveness of this food preservative against this bacterial species. The other *Listeria* isolates tested viz. *L. innocua*, *L. grayi* were, however noticed to be susceptible to all tested concentrations of lysozyme.
Many microbes have got mechanisms to evade the attack of complement present in the serum. The thick peptidoglycan layer of Gram-positive bacteria may play a role in preventing the insertion of the membrane attack complex (MAC) to the cytoplasmic membrane thereby protecting bacteria against cell lysis (Joiner, 1982). Bacteria showing serum resistance are reported to be associated with bacteremia (Roantree and Rantz, 1960). The serum resistance of \textit{L. monocytogenes} and its pathogenesis of bacteremia is well documented. The ability of \textit{L. grayi} and \textit{L. innocua} to resist the bactericidal effects of serum as shown in the current study could be the reason for bacteremia by these species as reported by Perrin \textit{et al}., (2003); Rapose \textit{et al}., (2008); Salimnia \textit{et al}., (2010).

In the intestine, in addition to the exposure to various stress conditions associated with volatile fatty acids, variations in pH, low oxygen, competition with normal flora and elevated osmolarity, the encounter of bacteria with the bile represents a major challenge. Though the extreme concentration of bile may reach up to 2.0% during the first hour of digestion (Gotcheva \textit{et al}., 2002), its average concentration being around 0.3%. The ability of pathogens to tolerate bile is likely to be a prerequisite for the survival and colonization of bacteria in the intestine (Havenaar \textit{et al}., 1992). The bile affects the viability of bacteria by destroying their cell membranes thereby affecting the cell permeability and viability (Succi \textit{et al}., 2005). Bile resistance of certain bacteria is associated with a specific enzyme activity of bile salt hydrolase (BSH) which is prominent in organisms isolated from the intestines or faeces of animals (Tanaka \textit{et al}., 1999). It acts by hydrolyzing the conjugated bile and thus reducing its lethal effect (Du Toit \textit{et al}., 1998). Watson \textit{et al}., (2008) described the presence of a novel bile exclusion system (BilE) in \textit{L. monocytogenes} causing the active exclusion of bile from the cell resembling the
activity of multi drug efflux pumps. Listeria is a human food-borne pathogen and its primary means of infection is through consumption of contaminated food (Schlech et al., 1983; Fenlon et al., 1996; Nightingale et al., 2004; Maklona et al., 2010). The resistance to bile as demonstrated in the Listeria species under study may be considered as a virulent mechanism enabling the bacteria to survive the toxic effect of bile during its passage through the digestive tract. Begley et al., (2009) noticed an enhanced biofilm formation of L. monocytogenes on exposure to bile and proposed their enhanced survival and colonization in the intestinal tract as well as in the gallbladder.

The ability of L. monocytogenes to colonize on food processing environments and subsequently causing contamination of food products has been demonstrated in several studies. The influence of surface materials, temperature, availability of nutrients, pH, salt, sugar and the presence of other bacteria in the environment have been described to be influencing the development of biofilm by L. monocytogenes (Moretro and Langsrud, 2004). The optimum temperature for biofilm formation by the Listeria species noticed in the current study was 37°C. However, it has to be highlighted that even at this temperature the Listeria species including L. monocytogenes failed to produce biofilm which could be graded as strong, an observation in consensus with Sasahara and Zottola (1993). On comparison with 4°C, thicker biofilm was noticed to be produced 22°C by all the Listeria species which might be due to the positive influence of flagellation and motility in Listeria at 22°C. Piette and Idziak, (1991) shared a similar view in this regard suggesting the indirect role of the flagellum in adhesion by increasing the number of cells reaching the surface in a given time. The pronounced disparity in the biofilm forming potential at different temperatures shown by this bacterial species could be related to the hydrophilic-hydrophobic transition of the cells in
biofilm (Smoot and Pierson, 1998). van Loosdrechi et al., (1987) attributed hydrophobicity at high temperature as the cause for the increased adherence and observed significant differences in the hydrophobicity of the cells in relation to temperature. Chavant et al., (2002) noticed increased hydrophily in *L. monocytogenes* at low temperature making the cell colonization difficult on a hydrophobic surface or even impossible. However, Briandet et al., (1999) noticed the biofilm formation in *L. monocytogenes* at low temperature and suggest the induction of extracellular cold shock protein as the reason for the biofilm formation at low temperature. Increased attachment of cells at high temperature, however, according to Chae and Schraft, (2000); Chae et al., (2006) is possibly due to the production of heat stress proteins associated with the cell surface. Biofilms are efficient and highly effective strategies for bacterial survival. The optimum biofilm formation at 37°C of *L. monocytogenes* noticed in the study points to the possibility of this bacterial species forming biofilm on indwelling medical devices allowing their survival in hostile immune and antimicrobial environments. The ability of *L. monocytogenes* to form biofilm, though weak, at lower temperatures of 4°C or 22°C demonstrate the ability of this emerging pathogen to adapt and establish in a wide range of environmental conditions.

In the current study all the three species of *Listeria* were capable of producing biofilm at pH 5.5, 7.5 and pH 8.5. Though biofilm was observed to be formed in alkaline pH conditions (pH 8.5), a noticeable increase in biofilm production occurred when the *Listeria* strains were exposed to acidic pH conditions (at pH 5.5) suggesting that formation of the mature biofilm rather more in moderate acidic growth conditions. Lee et al., (1999) noticed the optimal pH of the medium for the EPS (extracellular polymeric substance) production was between 5.5 and 6.5. Mafu et al., (1991) noticed that the
hydrophobicity of *L. monocytogenes* increased as the pH decreased and this increased hydrophobicity could be one of the reasons for the enhanced biofilm formation found at low pH as noticed in the present study.

Moderate levels of salt, sugar, and temperature commonly associated with food processing have been demonstrated to influence biofilm formation in *Listeria* on food processing environments (Møretrø and Langsrud., 2004). All the tested *Listeria* strains formed higher density biofilm when the growth medium was supplemented with glucose or NaCl (1%). The finding in the present study is in agreement with Jensen *et al.* (2007); Pan *et al*., (2010) who suggest that the addition of salt or glucose stimulated bacterial cells to produce more extra cellular matrix material. Manetti *et al*., (2010) speculated that the formation of firmly adhering biofilm on increasing concentration of glucose is because of increasing hydrophobicity of cells due to the lowering of pH. Briandet *et al*., (1999) also observed the hydrophilic – hydrophobic transition when microbial cells were cultivated in medium supplemented with glucose facilitating adhesion. The increased aggregation of *L. monocytogenes* cells leading to biofilm formation when exposed to NaCl stress has been reported by Jensen *et al.*, (2007). The transition of strong or moderate biofilm formers to weak on supplementation of the medium with high concentration of NaCl might be correlated with the down regulation of flagella as suggested by Caly *et al*., (2009). The potential for biofilm formation in the presence of moderate concentrations of glucose or NaCl indicates a possibility for the formation of biofilm on equipments used in food industry and patient care.

In the present study, all the *Listeria* spp. isolated from *P. americana* were found to be forming biofilm. Biofilms once established may function as a reservoir of microbes with increased resistance to antimicrobials and if not restricted, it may act as a source for the dissemination of bacteria and hence
becoming persistent source of contamination. Furthermore, it may lead to the development of physiologically tough and potentially virulent bacteria in the environments posing a threat to public health (Begley et al., 2009). The bacterial spp. defaecated by the cockroach are found to contain mixed bacterial flora, most of them with the potential to form biofilm. These microorganisms can form biofilms and function as a substrate for other weak biofilm formers increasing the likelihood of pathogen endurance and their further dissemination (Møretrø and Langsrud, 2004; Lehner et al., 2005; Lapidot et al., 2006). The *Listeria* species, though are not forming strong biofilm as noticed in the present study, may use a primary colonizing bacteria (Kalmokoff et al., 2001) such as *Pseudomonas* to form biofilm conglomerate on various surfaces. It has been observed that *L. monocytogenes* to form strong biofilms with *Pseudomonas fragi* (Sasahara and Zottola, 1993) or *Pseudomonas fluorescens* (Buchanan and Bagi, 1999) pointing the likelihood of natural biofilm formation with multispecies consortia in a hospital or food processing environment. Furthermore, possibilities of various interactions among bacteria in biofilm such as the co aggregation of cells (Rickard et al., 2003), conjugation (Ghigo, 2001), quorum sensing (Li and Tian, 2012) may occur leading to the formation of tougher bacterial communities resisting antimicrobial compounds such as disinfectants or antibiotics (Cowan et al., 2000) and adapting to changing environments.