Effects of osmotic pressure, acid, or cold stresses on antibiotic susceptibility of Listeria monocytogenes

Anas A. Al-Nabulsi a, *, Tareq M. Osailli a, Reyad R. Shaker a, Amin N. Olaimat b, Ziad W. Jaradat c, Noor A. Zain Elabedeena, Richard A. Holley b

a Department of Nutrition and Food Technology, Jordan University of Science and Technology, Irbid, Jordan
b Department of Food Science, University of Manitoba, Winnipeg, Manitoba R3T 2N2, Canada
c Department of Biological and Genetic Engineering, Jordan University of Science and Technology, Irbid, Jordan

A R T I C L E   I N F O

Article history:
Received 6 March 2014
Received in revised form 12 July 2014
Accepted 18 July 2014
Available online 7 August 2014

Keywords:
Listeria monocytogenes
Antibiotic susceptibility
Osmotic pressure
Acid stress
Cold stress
Stress adaptation

A B S T R A C T

Prevalence of antibiotic resistance of Listeria monocytogenes isolated from a variety of foods has increased in many countries. L. monocytogenes has many physiological adaptations that enable survival under a wide range of environmental stresses. The objective of this study was to evaluate effects of osmotic (2, 4, 6, 12% NaCl), pH (6, 5.5, 5.0) and cold (4 °C) stresses on susceptibility of three isolates of L. monocytogenes towards different antibiotics. The minimal inhibitory concentrations (MICs) of tested antibiotics against unstressed (control), stressed or post-stressed L. monocytogenes isolates (an ATCC strain and a meat and dairy isolate) were determined using the broth microdilution method. Unstressed cells of L. monocytogenes were sensitive to all tested antibiotics. In general, when L. monocytogenes cells were exposed to salt, cold and pH stresses, their antibiotic resistance increased as salt concentration increased to 6 or 12%, as pH was reduced to pH 5 or as temperature was decreased to 10 °C. Results showed that both meat and dairy isolates were more resistant than the ATCC reference strain. Use of sub-lethal stresses in food preservation systems may stimulate antibiotic resistance responses in L. monocytogenes strains.

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1. Introduction

Listeria monocytogenes is a Gram-positive organism and one of 5 five species including Listeria innocua, Listeria seeligeri, Listeria welshimeri, Listeria ivanovii, and Listeria grayi which comprise this genus (Farber and Peterkin et al., 1991). Recently, two newly identified species (Listeria marthii and Listeria rocourtiae) were reported (Graves et al., 2010; Leclercq et al., 2009). L. monocytogenes is widely distributed and has been isolated from a variety of ready-to-eat and raw dairy, meat and meat products, sea foods and fresh produce (Bell and Kyriakides, 2005).

L. monocytogenes is a major concern for food producers, health regulatory officials, and consumers since it is considered one of the more virulent foodborne pathogens (McLauchlin et al. 2004). Although the incidence of listeriosis is rare compared to illness caused by other foodborne pathogens such as Escherichia coli O157:H7, Campylobacter jejuni or Salmonella spp., L. monocytogenes has been extensively studied during the past several decades because of its high (20%) case-fatality and high hospitalization (90%) rates (Lianou and Sofos, 2007). Furthermore, L. monocytogenes is a versatile organism that has the ability to survive for long periods under adverse conditions including cold storage, high NaCl concentrations and acidic pH (Rhoades et al., 2009).

Antibiotic treatment of infectious listeriosis is indicated and has proven to be successful. These treatments usually rely upon the use of β-lactam antibiotics such ampicillin or penicillin, alone or in combination with an aminoglycoside (gentamicin). However, with patients allergic to β-lactams, trimethoprim and a sulfonamide have been used as an alternative with success (Conter et al., 2009).

Even though it has been experimentally shown that L. monocytogenes strains are generally susceptible to a wide range of antibiotics, occasionally antibiotic resistant L. monocytogenes strains have been observed (Depardieu et al. 2007). The first multi-drug resistant strain of L. monocytogenes was reported in 1988 by Poyart-Salmeron et al. (1990). Since then, L. monocytogenes strains have been isolated from food, environmental and human clinical samples which have shown resistance to one or more antibiotics (Morvan et al., 2010).

* Corresponding author. Tel.: +962 02 7201000; fax: +962 02 7201078.
E-mail address: anas_nabulsi@just.edu.jo (A.A. Al-Nabulsi).

http://dx.doi.org/10.1016/j.fm.2014.07.015
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During food processing bacteria may encounter a variety of conditions that may cause chemical (acids, ethanol, alkaline, chlorine and salts) or physical (heat, radiation and pressure) stresses (Yousef and Courtney, 2003). When bacteria are exposed to mild forms of these stresses, opportunity is provided for them to improve their ability to adapt and become resistant to subsequent more extreme exposures through physiological adjustment, enabling reproduction (Depardieu et al. 2007; Hill et al. 2002). Furthermore, the adaptive responses to these stresses may enhance resistance to others such as exposure to antibiotics and lead to what is termed “cross-protection” (Doyle et al. 2006; McMahon et al. 2007).

*L. monocytogenes* may develop an adaptive response during exposure to sublethal food processing conditions (Hill et al. 2002). Lou and Yousef (1997) confirmed that *L. monocytogenes* adapted to pH 4.5–5.0 had increased resistance to lethal concentrations of H₂O₂, ethanol, and acid. In addition, *L. monocytogenes* is able to transfer antibiotic resistance genes to other strains of *Listeria* spp. as well as other pathogens, such as *Staphylococcus aureus*, *Enterococcus* spp., and non-pathogenic bacteria (Courvalin, 1994; Walsh et al. 2001). Therefore, understanding the effects of stress during food processing on the antibiotic susceptibility of *L. monocytogenes* is important in developing strategies to facilitate effort to achieve clinical control over pathogens that originate from food. The objectives of the current study were to investigate the effect of osmotic, cold and acid stresses on the susceptibility of *L. monocytogenes* toward different antibiotics.

### 2. Materials and methods

#### 2.1. Preparation of bacterial cultures

Three strains of *Listeria monocytogenes* were used in the current study; *L. monocytogenes* ATCC 7644 and two *L. monocytogenes* isolates from processed meat and dairy products, respectively, in Jordan. These isolates were obtained from the Department of Nutrition and Food Technology at Jordan University of Science and Technology and were kept frozen in glycerol. They were maintained on Typtic Soy Agar (TSA, Oxoid Ltd., Basingstoke, UK) slants at 4 °C and transferred bi-weekly to maintain viability. Working cultures were prepared by growth in 10 ml Tryptic Soy Broth (TSB, Oxoid) and were kept frozen in glycerol. They were maintained on Typtic Soy Broth (TSB, Oxoid) to give a approximately 6 log10 CFU/ml in the reaction mixtures.

### 2.2. Antibiotics

The three *L. monocytogenes* isolates were challenged with 9 antibiotics (Table 1), chosen according to their mode of action and use in clinical therapy. Those that inhibit cell wall synthesis included vancomycin 98%, ampicillin 84.5% (Bio Basic Inc., Markham, ON, Canada) and penicillin G sodium salt 98% (Biochemika Int., Hangzhou, China). Those that inhibit protein synthesis included tetracycline hydrochloride 90%, gentamicin sulfate 59%, streptomycin sulfate 63% (Bio Basic Inc.), and doxycycline HCL 99.5% (Tecelo Chemicals B.V., Hertogenbosch, Netherlands). Those that inhibit nucleic acid synthesis included enrofloxacin 95.3% (Tecelo Chemicals B.V.) and ciprofloxacin 98% (Biochemika Int.) (Yanoeyama and Katsumata, 2006; Al-Nabulsi et al. 2011).

#### 2.3. Preparation of antibiotic stock solutions

Antibiotic stock solutions were prepared following the manufacturers’ recommendations. Vancomycin, ampicillin, penicillin,
2.4.3. Cold stress

Cold-stressed cultures were prepared as described by Al-Nabulsi et al. (2011). One ml of each freshly prepared L. monocytogenes cell suspension was added to 9 ml of sterile 0.1 M potassium phosphate buffer (pH 6.8) in 15 ml screw capped test tubes, mixed thoroughly for 1 min and stored 24 h at 10 °C. Then, the culture was diluted to give a final concentration of approximately 6 log_{10} CFU/ml in the reaction mixtures for antibiotic challenge.

2.5. Preparation of post-stressed L. monocytogenes cultures

To examine the effect of post-stress conditions on the susceptibility of L. monocytogenes toward tested antibiotics, cells stressed by osmotic, acid, and cold exposure were inoculated into TSB and incubated at 37 °C for 24 h. The cells were harvested by centrifugation at 4000 g for 20 min, the pellets were resuspended and diluted to give a final concentration of about 6 log_{10} CFU/ml in the reaction mixtures. Cell suspensions were challenged by antibiotic exposure as described below.

2.6. Antimicrobial assays (broth microdilution method)

The minimal inhibitory concentration (MIC), defined as the lowest concentration of antibiotic required to inhibit visible growth of the test microorganisms, was determined using the broth microdilution method according to the procedure described by Langfield et al. (2004) and Klare et al. (2005).

Ninety-six well microtiter plates (CELLSTAR®, Greiner Bio-One, Frickenhausen, Germany) with a 300 μl well capacity were used to evaluate the effects of tested antibiotics on the viability of unstressed and stressed L. monocytogenes cells. In each well of the microtiter plate a total volume of 250 μl was used. First, 100 μl of a test strain previously diluted in MHB was added to give a final inoculum of 6 log_{10} CFU/ml with an optical density (OD) at 620 nm of 0.1. Then 100 μl of MHB plus 50 μl of the test antibiotic solution were added. Control wells contained 100 μl culture, 100 μl MHB and 50 μl of sterile distilled water. After incubation at 37 °C for 24 h, the microtiter plates were shaken using a Titer plate shaker (Barnstead International, Dubuque, IA, USA) at 400 rpm for 2 min before the absorbance at 620 nm was measured using a microplate reader (Dynatech MR5000, Mount Holly NJ, USA). The lowest concentration of antibiotic at which the OD reading was ≤0.1 was considered the MIC of the tested antibiotic. To confirm the MIC, a 100 μl sample was taken at the end of incubation and viable cell numbers in visibly clear wells were assessed by plating on TSA. The MIC was confirmed if the viable cell numbers after incubation were not significantly different from the numbers at initial inoculation. Each antibiotic sensitivity trial was performed in triplicate.

2.7. Interpretation of results

The breakpoint MICs of tested antibiotics against L. monocytogenes were determined using reference values recommended by the Comité de l’Antibiogramme de la Société Française de Microbiologie (CA-SFM), France (Acar et al. 1992) (Table 1).

2.8. Statistical analysis

Data obtained were evaluated using Mann–Whitney U analysis as recommended by McMahon et al. (2007) using the Statistical Package for the Social Sciences (SPSS), version 11.0.

3. Results

3.1. The susceptibility of unstressed L. monocytogenes to antibiotics

All unstressed L. monocytogenes isolates were sensitive toward the tested antibiotics (Table 2). However, some variation in the susceptibility of unstressed isolates was noted, for example, the meat isolate was more susceptible to streptomycin than the ATCC strain or the dairy isolate. The latter was the most susceptible to tetracycline, ciprofloxacin and enrofloxacin while the ATCC strain was more susceptible to gentamicin, penicillin and vancomycin. All isolates showed the same susceptibility to ampicillin and doxycycline.

3.2. The susceptibility of stressed and post-stressed L. monocytogenes cells to antibiotics

3.2.1. Effect of osmotic stress on L. monocytogenes antibiotic susceptibility

Stressed L. monocytogenes isolates at NaCl concentrations up to 12% showed increased resistance to the tested antibiotics. Osmotic stress at 2, 4, 6, and 12% NaCl for 24 h reduced the susceptibility of L. monocytogenes isolates when challenged with ampicillin, tetracycline, doxycycline and vancomycin. With increases in NaCl concentrations, bacterial antibiotic resistance increased to the extent that susceptible isolates became moderately resistant or resistant. Meat and dairy isolates previously exposed to 4, 6, and 12% NaCl were resistant to streptomycin and ciprofloxacin, although the meat isolate previously exposed to 4% was moderately resistant to ciprofloxacin. In addition, after 12% NaCl exposure only the dairy isolate was resistant to gentamycin and enrofloxacin. The development of antibiotic resistance by meat and dairy isolates during NaCl exposure was greater than that shown by the ATCC reference strain. Further, this resistance increased or remained constant after removal of the stress (Table 2).

3.2.2. Effect of acid stress on L. monocytogenes antibiotic susceptibility

It was notable that acid stress at pH 5.5 to 6.0 for 30 min tended to increase the resistance of L. monocytogenes isolates to most antibiotics, although with some isolate–antibiotic pairs, resistance development was not as pronounced as it was with osmotic stress. When L. monocytogenes cells were exposed to pH 5.0, all isolates were found to be moderately resistant to penicillin, however, the ATCC strain and the dairy isolate became resistant to streptomycin while the meat isolate became only moderately resistant. In contrast, the meat isolate became resistant to ciprofloxacin while the other two isolates became moderately resistant. The antibiotic resistance of post-acid-stressed L. monocytogenes isolates increased or remained the same as the acid-stressed cells (Table 3).

3.2.3. Effect of cold stress on L. monocytogenes antibiotic susceptibility

Cold stress (10 °C for 24 h) consistently increased the resistance of L. monocytogenes toward the tested antibiotics, although there were differences in the extent of the increases which were both strain and antibiotic dependent. Changes noted did not appear to occur more extensively with one specific strain or antibiotic; however, the meat and dairy strains tended to show enhanced resistance more often than the ATCC strain. Cold stress also increased the antibiotic resistance of L. monocytogenes isolates even when the stress was removed (Table 4).
### Table 2

Changes in MIC of antibiotics against osmotically-stressed or post-osmotically-stressed *L. monocytogenes* strains.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Salt %</th>
<th>MIC of Antibiotic (g/ml) against osmotically-stressed or post-osmotically-stressed <em>L. monocytogenes</em></th>
<th>Stress</th>
<th>Post</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC</td>
<td>0%</td>
<td>8 (S) 0.125 (S) 0.06 (S) 0.25 (S) 0.06 (S) 0.125 (S) 0.5 (S) 0.25 (S)</td>
<td>0.25 (S)</td>
<td>0.125 (S)</td>
</tr>
<tr>
<td></td>
<td>2%</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4%</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12%</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Meat</td>
<td>0%</td>
<td>4 (S) 1 (S) 0.125 (S) 0.125 (S) 0.25 (S) 0.06 (S) 0.25 (S) 0.5 (I) 0.125 (S)</td>
<td>0.06 (S)</td>
<td>0.125 (S)</td>
</tr>
<tr>
<td></td>
<td>2%</td>
<td>0</td>
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<tr>
<td></td>
<td>4%</td>
<td>0</td>
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<tr>
<td></td>
<td>12%</td>
<td>0</td>
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</tbody>
</table>

#### 4. Discussion

Historically, most *L. monocytogenes* strains isolated from clinical, food and environmental samples have been found susceptible to antibiotics active against Gram-positive bacteria; however, antibiotic resistant isolates of *L. monocytogenes* have been observed over the last two decades (Zhang et al., 2007; White et al., 2002). In the current study, the three *L. monocytogenes* isolates examined were initially sensitive toward the antibiotics tested, but when they were exposed to osmotic, acid or cold stresses, their antibiotic resistance increased. If this represents a broader phenomenon, it may in part explain the emergence of antibiotic resistance among *L. monocytogenes* isolates from food products.

Environmental stresses such as osmotic, acid and cold shock-induced mutation in bacterial cells has been reported to be associated with the development of antibiotic resistance (Foster, 2000; Jolivet-Gougeon et al. 2000). *L. monocytogenes* is osmotically tolerant and can grow at > 10% NaCl (McClure et al. 1989). The organism is able to adapt to elevated osmolarity by accumulating compatible solutes or osmolytes (Ko and Smith, 1999). In the present study, bacterial resistance toward ampicillin, tetracycline, doxycycline and vancomycin was consistently increased in response to increased osmotic stress from higher salt concentrations. With the other antibiotics, increased salt also increased bacterial resistance, but responses varied according to NaCl concentration. For example, isolates stressed at 12% NaCl generally showed increased antibiotic MICs greater than 4-fold, except for that of streptomycin against the ATCC strain which increased slightly less (2.1–4-fold). These results were similar to those of Alonso-Hernando et al. (2009) who found that the resistance of *L. monocytogenes* strains to various antibiotics increased after exposure to acidified sodium chloride. In contrast, Faezi-Ghasemi and Kazemi (2015) reported that *L. monocytogenes* cells osmotically-stressed at 7% NaCl had decreased resistance to selected antibiotics including tetracycline, rifampicin, gentamycin, penicillin, ampicillin, trimethoprim-sulfamethoxazole and chloramphenicol. However, in that work *L. monocytogenes* PTCC 1297 (serotype 4a) was exposed to 7% NaCl for only 1 h before antibiotic challenge compared to the 24 h used in the present work. In further support of the present observations, McMahon et al. (2007) found that > 4.5% NaCl increased the antibiotic resistance of *E. coli*, *Salmonella* Typhimurium and *S. aureus*. In addition, Ganjian et al. (2012) found that NaCl concentrations up to 35% increased the antibiotic resistance of *S. aureus* cells. It is also important to note that the exposure of *L. monocytogenes* to NaCl stress can lead to cross-protection against other stresses such as heat and acid (Skandamis et al., 2008).

In the presence of mild concentrations of weak acid preservatives, organisms have been shown to adapt by regulation of outer cell membrane protein synthesis (Beales, 2004), and by making changes in their cell membrane fatty acid composition to alter membrane permeability and fluidity (Diakogiannis et al., 2013). In the present study, acid stress consistently increased bacterial resistance toward ampicillin, tetracycline, doxycycline, gentamycin, vancomycin and enrofloxacin. It also increased resistance toward the remaining 3 antibiotics (streptomycin, penicillin, ciprofloxacin), but the extent of the increase varied with the bacterial strain. Nonetheless, increased antibiotic resistance was observed as the pH decreased. The MIC values of most (5–7) of the 9 antibiotics tested were increased when *L. monocytogenes* isolates were exposed to acid stress at pH 5.0. Similarly, Alonso-Hernando et al. (2009) found that *L. monocytogenes* cells exposed to citric acid were more resistant to a number of antibiotics than were unexposed cells. Al-Nabulsi et al. (2011) found that acid-stressed *Cronobacter sakazakii* cells were more resistant toward...
tetracycline, fleroxacin, amoxicillin, ampicillin, vancomycin and enrofloxacin. McMahon et al. (2007) also found that *E. coli*, *Salmonella* Typhimurium and *S. aureus* stressed at pH 4.0 to 5.0 were more resistant to antibiotics than unstressed cells. Results from a number of studies showed that exposure of *L. monocytogenes* to acid stress increased its resistance to osmotic, ethanol, and oxidative stresses (Lou and Yousef, 1997; Phan-Thanh et al., 2000; Faleiro et al., 2003). In contrast with the present findings, Faezighasemi and Kazemi (2015) reported that *L. monocytogenes* exposed to pH 5.0 was more susceptible to tetracycline, rifampicin, gentamycin, penicillin, ampicillin, trimethoprim-sulfamethoxazole and chloramphenicol. Since the lengths of acid stress exposure in the two studies were similar (<1 h), differences may have occurred because the *L. monocytogenes* cells used by Faezi-Ghasemi and Kazemi (2015) were in the exponential rather than the stationary phase as used in the present study.

Storage temperature is one of the most important parameters regulating the activities of microorganisms in food systems. *L. monocytogenes* is a psychrotrophic microorganism which has the capacity to grow at temperatures as low as 4 °C. In the current study, cold stress consistently increased resistance of tested strains toward tetracycline, doxycycline, vancomycin and enrofloxacin, and increased the resistance of the meat and dairy isolates, in particular, toward the other antibiotics. In other work with *C. sakazakii*, it was similarly found that exposure of cells to 4 °C for 4 h increased bacterial resistance to kanamycin, neomycin, tetracycline, fleroxacin, amoxicillin, ampicillin, vancomycin and enrofloxacin (Al-Nabulsi et al., 2011). However, McMahon et al. (2007) found that cold stress had the opposite effect; it reduced the antibiotic resistance of *E. coli*, *S. Typhimurium* and *S. aureus*.

Nonetheless, alternative sigma (σ) factors can produce dramatic changes in bacterial gene expression after exposure to environmental stress. The genome of *L. monocytogenes* encodes four alternative σ factors including σ^B^, σ^C^, σ^H^ and σ^A^ (Glaser et al., 2001); however, σ^B^ which is encoded by sigB plays the major role in the survival of *L. monocytogenes* under stressful conditions (Gray et al., 2006). Sigma factor B was found to regulate >170 dependent genes in *L. monocytogenes* including gbu, betL and opuc during osmotic stress; gadD1, gadD2, gadD3, gadT1 and gadT2 during acid stress, and betL, ltrC, fri and opuc during cold stress (Raengpradub et al., 2008). These genes encode cold shock proteins and compatible solutes (betaine and carnitine) that enable the bacterium to be more resistant to environmental and nutritional stresses (O’Byrne and Karatzas, 2008).

The increase in antibiotic resistance observed in the present work may be related to one or more of the following mechanisms: reduction of cell wall antibiotic binding sites, amplification of genes responsible for efflux pump synthesis and operation, and induction of stress shock proteins (McMahon et al., 2007). Bacteria can respond to adverse environmental challenges involving osmotic,
acid and cold stress by down-regulation of penicillin binding proteins in the cell wall, can enhance their ability to reduce cytoplasmic concentrations of antibiotics by enhanced efflux pump operation and can improve survival by synthesis of chaperone proteins to maintain protein functionality during stress or antibiotic challenge (Bremer and Krämer, 2000; Rahmati-Bahram and Magee, 1997).

It was observed in the present work that L. monocytogenes continued to show higher levels of antibiotic resistance after removal of each of the three types of stress. Similar results were reported by McMahon et al., 2007 who found that NaCl or acid-stressed E. coli and S. aureus cells were more resistant (<4 times) to the antibiotics tested than control cells. On the other hand, post-NaCl-stressed or post-acid-stressed S. Typhimurium cells were similar or less resistant than unstressed cells. Since retention of enhanced antibiotic resistance was also observed with the three strains of L. monocytogenes used in the present study for > 1 d (at least 10 generations) after removal of the stress, it is possible that mild stress may contribute to antibiotic resistance, at least temporarily or for longer sustained periods.

5. Conclusions

L. monocytogenes is exposed to a variety of environmental stresses during food processing and appears able to adapt to many of these stresses. It was found that cold, acid and osmotic stresses increased the resistance of this pathogen to 9 currently used antibiotics. Increases in antibiotic resistance observed were maintained for at least a day after the stress was removed. It was significant that cold (10 °C), acid (pH 5) and osmotically (12% NaCl)-stressed cells were more resistant to streptomycin, gentamycin, ampicillin, penicillin, ciprofloxacin and enrofloxacin. Exposure to milder acid or NaCl stress elicited levels of antibiotic resistance that were lower. Results suggest that the increased use of bacteriostatic (sub-lethal) stress, rather than bactericidal treatments in food preservation systems has the potential to increase the antibiotic resistance among L. monocytogenes if present.

Acknowledgment

This project was financially supported by the Deanship of Research at Jordan University of Science and Technology (grant #162/2011), Irbid, Jordan.

References


