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IDENTIFICATION OF THE MECHANISMS OF RESISTANCE AND POTENTIAL CROSS RESISTANCE OF *LISTERIA MONOCYTOGENES* UPON ADAPTATION TO DIFFERENT ANTIMICROBIALS

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ISPITIVANJE MEHANIZAMA REZISTENCIJE I MOGUĆE UNAKRSNE REZISTENCIJE *LISTERIA MONOCYTOGENES* NAKON ADAPTACIJE NA RAZLIČITA ANTIMIKROBNA SREDSTVA

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DEDICATION

I would like to dedicate this dissertation to my dear parents Zora and Ljubo with enormous respect and all my love.

Disertaciju posvećujem mojim dragim roditeljima Zori i Ljubu uz beskrajno poštovanje i svu moju ljubav.

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ABSTRACT

Ability to contaminate ready-to-eat, highly processed, cold-stored food products, relatively high fatality rate and environmental adaptations define .*Listeria monocytogenes* as a challenging foodborne pathogen of high public health significance. *Listeria*'s adaptations and prolonged persistence in food processing facilities are a key link to contamination of foods.

In recent years, bacterial multidrug resistance has been recognized as an emerging health challenge. Exposure to antimicrobials (antibiotics, disinfectants or environmentally ubiquitous heavy metals), may provide selective pressures for resistance with accompanying cross-resistance and increase in environmental fitness.

In this work I addressed some of the possible mechanisms of adaptations that may contribute to persistence of *L. monocytogenes* in the environment as well as to survival and growth in different foods. This includes adaptations to heavy metals such as cadmium, disinfectants such as benzalkonium chloride (BC), selected antibiotics (e.g. the fluoroquinolone ciprofloxacin) and other toxic compounds (e.g. tetraphenyl-phosponium, often used as an anti-carcinogenic and efflux pump substrate). In order to characterize the impact of multidrug resistance on survival and growth of *L. monocytogenes* in different foods, we tested mutants adapted to higher concentrations of antibiotic ciprofloxacin and disinfectant benzalkonium chloride. By screening of the *mariner*-based mutant librarie of *L monocytogenes* 10403S we detected tetR-like mutants with increase in the resistance to tetraphenyl-phosponium. Lastly, we tested selected *mariner*-based mutants with the change in the response to number of different stresses (cold sensitive, non-hemolytic, resistance to phage in epidemic clone II strains) deletion mutants of *wap* and region18, as well as selected food and clinical isolates.

Selection of several *L. monocytogenes* strains on either ciprofloxacin (2 μg/ml) or the BC(10 μg/ml) led to derivatives with increased MICs not only to these agents but also to several other compounds, including the antibiotic gentamicin, the dye ethidium bromide, and the chemotherapeutic drug tetraphenyl - phosphonium chloride. The spectrum of compounds

to which these derivatives exhibited reduced susceptibility was the same regardless of whether they were selected on ciprofloxacin or on BC. Strains harboring the large, cadmium and BC resistance plasmid pLM80 (identified in the epidemic clone II strain H7858) did not differ in MICs to ciprofloxacin and gentamicin from plasmid-cured strains. However, ciprofloxacin-selected derivatives of pLM80-harboring strains had higher MICs than those derived from the plasmid-cured strains. Partially restored susceptibility to the antimicrobials in the presence of the potent efflux inhibitor reserpine suggests that mutations in efflux systems are responsible for the multidrug resistance phenotype of strains selected on ciprofloxacin or BC.

Adaptive responses of bacteria to unstable environments are mediated by transcriptional regulators. A family of transcriptional regulators that is well represented and widely distributed among bacteria is the TetR family. These transcriptional repressors control genes whose products are involved in multidrug resistance, enzymes implicated in different catabolic pathways, biosynthesis of antibiotics, osmotic stress, and pathogenicity. We found a *tetR*::Tn*917* mutant with an insertion in the *tetR* repressor (next to the MDR transporter *mdrT*) to have pronounced (8-fold) increase in TPP (tetraphenylphosponium) resistance compared to the parental *L. monocytogenes* strain 10403S. Such data suggest that the observed stimulation of TPP resistance of the mutant involves increased expression of MDR transporter(s) other than *mdrT*, but also repressed by *tetR*. To test our hypothesis we screened a *mariner*-based mutant library of *L. monocytogenes* strains 10403S and F2365 for the mutants with the decrease and increase in TPP resistance.(960 mutants of each strain, 10403S and F2365, respectively, were screened). We failed to isolate mutants with decreased resistance to TPP, but 5 mutants highly resistant to TPP were isolated. One of these tetR-like mutants had insertion in tetR gene. Mutants harbored single transposon insertion and one characterized had transposon integrated in LMRG_01858 (sucrose phosphorylase).

As previously mentioned, BC resistance in *L. monocytogenes* is commonly associated with a plasmid-borne disinfectant resistance cassette (*bcrABC*).We investigated conjugative transfer of resistance to BC and to cadmium from nonpathogenic *Listeria* spp. to other nonpathogenic *Listeria*, as well as to *L. monocytogenes.* BC-resistant *L. welshimeri* and *L. innocua* harboring bcrABC, along with the cadmium resistance determinant *cadA2*, were able

to transfer resistance to other nonpathogenic listeriae as well as to *L. monocytogenes* of diverse serotypes, including strains from the latest major 2011 cantaloupe outbreak. Transfer among nonpathogenic *Listeria* spp. was noticeably higher at 25°C than at 37°C, whereas acquisition of resistance by *L. monocytogenes* was equally efficient at 25 and 37°C. When the nonpathogenic donors were resistant to both BC and cadmium, acquisition of cadmium resistance was an effective surrogate for transfer of resistance to BC, suggesting coselection between these resistance attributes.

Non-pathogenic *Listeria* spp. such as *L. innocua* and *L. welshimeri* are commonly used as an indicator of *L. monocytogenes* due to their higher prevalence and co-existence with *L. monocytogenes* in the plant ecosystem. We have analyzed the prevalence of cadmium, arsenic and bezalkonium chloride (BC) resistance among non-pathogenic *Listeria* isolates from different turkey processing plants in the United States. The aim was to elucidate the role of non-pathogenic *Listeria* spp. as resistance gene reservoirs and their gene transfer potential that may contribute to the dissemination of disinfectant resistance genes in *L. monocytogenes.* Results obtained indicate high prevalence of resistance to BC and cadmium, and of the *bcrABC* (plasmid-borne disinfectant resistance cassette), *cadA1* and *cadA2* determinants (*cadAC* efflux systems associated with cadmium resistance). Resistance to cadmium was highly prevalent, exceeding 90% regardless of species. Resistance to BC, while also highly prevalent in both species, was more common in *L. welshimeri* (83%) than *L. innocua* (73%). In contrast, arsenic resistance was relatively uncommon (<10%) in either species. Noteworthy is that Pulsed field gel electrophoresis (PFGE) profiles of isolates were largely plant-specific

Epidemiological trends in recent years suggest that *L. monocytogenes* -contaminated produce is associated with higher outbreak occurrence than previously recognized. In 2011 a multistate outbreak of listeriosis in the United States involved cantaloupe and resulted in 33 deaths and 147 illnesses. This outbreak highlighted the need for further efforts to understand the ability of *L monocytogenes* to survive and grow on cantaloupe and other produce. One of my objectives was to assess survival and growth of outbreak strains of *L. monocytogenes* on the outer surface of cantaloupe (rind) compared to inner surface (flesh) or in freshly extracted juice at various incubation temperatures. Three *L. monocytogenes* strains implicated in the

2011 multistate outbreak in cantaloupe were employed in this study. Two strains were of serotype 1 /2a and the third of serotype 1/2b. *L. monocytogenes* populations increased by approximately 10 fold following 21 days incubation at 4 or 8° C, and by approximately 100 fold following 7 days incubation at 25°C. After 24 hours at 25°C *L. monocytogenes* populations increased by approximately 10 fold. Interestingly, increases were higher on the rind than on the flesh or in the juice, with statistically significant differences after 7 days of incubation at 4°C and 72 hr at 25°C. No significant differences were noted among the three different strains. Rinsing of inoculated fragments with water prior to the incubation revealed initial decreases of *L monocytogenes* followed by subsequent growth. After the initial decrease cells grew at the same level as cells from fragments without water rinses, with exception of those incubated at the temperature of 25ºC that grew to higher levels . The results of this study suggest that *L. monocytogenes* can not only survive on the surface of cantaloupe but that it also has temperature and time-dependent potential for growth. Results also revealed that water treatment of the surface does not prevent growth of *L. monocytogenes.* These data will be valuable in design of controls to limit persistence and growth of this pathogen on cantaloupe and other produce.

Further analysis of survival and growth on the surface of cantaloupe included mutants with changes in certain phenotypes (increased resistance to the antibiotic ciprofloxacin, cold sensitivity, loss of hemolytic activity, phage susceptibility) as well as deletion mutants of wall-associated protein (*wap*) and a specific genomic region (region-18). The protein encoded by *wap* belongs to a super-family of surface-associated proteins involved in various cellular processes, including surface hydrophobicity, wall metabolism, secretion, pathogenicity, immunogenicity and cell adhesion. A genomic region (region-18) appeared to be markedly divergent in ECII strains but conserved among other serotype 4b strains. *L. monocytogenes* strains of other serotypes lack region-18, or *wap*. The results suggest no significant difference in growth between mutants and parental strains. As expected, the cold sensitive mutant of the strain F2365 failed to grow at 4ºC, whereas other mutants along with the parental strains after initial decrease grew for up to 1.3 log units over 7 days at 4C.

Milk and dairy products along with processed meats are still considered major vehicles for *L. monocytogenes* involved in food related outbreaks. Eighteen *L. monocytogenes*

strains of different serotypes involved in food-related outbreaks as well as human and animal clinical isolates were tested for growth in raw and pasteurized milk over 10 days at 8C. Included in study were also ciprofloxacin adapted mutants of two wild type strains. The goal was to assess possible difference in survival and growth between wild types and antibiotic adapted mutants, across serotypes and between two types of milk. Isolates of serotype 4b assigned to three epidemic clonal groups (ECI, ECIa and ECII) have been analyzed for difference between clonal groups as well as types of milk. Populations increased significantly over the 10 days of incubation at 8C ($p<0.0001$) Results obtained also indicate overall better growth of *L. monocytogenes* in pasteurized over raw milk. Wild type strains did not significantly differ from ciprofloxacin- adapted mutants. Comparison across serotypes showed no significant differences between serotypes or between types of milk. Significant difference, however, has been noted between clonal groups composed of serotype 4b. ECI grew significantly better in both raw ($p=0.0166$) and pasteurized ($p=0.0433$) milk compared to ECIa. Both ECI and ECIa had higher population increases then ECII with significant difference between ECI and ECII in pasteurized milk ($p=0.0358$) and ECIa and ECII in raw milk ($p=0.0026$). Interestingly, population increases of ECI in pasteurized milk were significantly higher compared to raw milk ($p=0.0001$), whereas such difference between milk types was not noticed for ECIa and ECII. Results of present study confirmed good adaptability and growth of *L. monocytogenes* in milk.

In efforts to elucidate possible sources of *L monocytogenes* resistance to different antimicrobials, in this thesis we provided evidence of gene transfer potential of non-pathogenic *Listeria* spp., their role as resistance gene reservoirs and ability to contribute, trough conjugative transfer to the dissemination of disinfectant resistance genes in *L. monocytogenes*. We also confirmed that mutations in efflux systems are responsible for the multidrug resistance phenotype of strains selected on antibiotics and disinfectants. By employing *mariner*-based transposon mutant library we screened for mutants with changes in certain phenotypes (increased resistance to the antibiotic ciprofloxacin, cold sensitivity, loss of hemolytic activity, phage susceptibility). These mutants along with mutants adapted to antimicrobials and deletion mutants of *wap* and region-18 were used to assess survival and growth of *L monocytogenes* in different foods. However, further studies are necessary in order

to better understand the distribution of epidemic clones of *L. monocytogenes*, their transmission characteristics and ability to cause foodborne disease.

KEY WORDS*: Listeria* spp., *Listeria monocytogenes*, resistance, antimicrobials, efflux system, plasmide, *tetR* repressor, conjugative transfer, cantaloupe, temperature, survival, growth, milk

DISCIPLINE: VETERINARY MEDICINE
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KRATAK SADRŽAJ

Sposobnost da kontaminira hranu pripremljenu za konzum, visoko prerađene proizvode i proizvode skladištene na niskim temperaturama kao i relativno visok stepen smrtnosti i izrazita sosobnost adaptacije na uslove okoline svrstavaju *Listeria monocytogenes* u grupu patogenih mikroorganizama prenosivih hranom koji predstavljaju jedan od najvećih izazova u oblasti javnog zdravlja. Adaptacija *L. monocytogenes* na uslove sredine i sposobnost dugotrajnog opstanka u objektima za proizvodnju i preradu hrane ključni su razlozi za kontaminaciju različitih proizvoda.

Poslednjih godina, višestruka rezistencija bakterija na različita antimikrobna sredstva nametnula se kao jedna od najznačajnijih tema u oblasti zdravstva. Izlaganje brojnim antimikrobnim sredstvima (antibiotici, dezinficijensi kao i sveprisutni teški metali) omogucava selektivni pritisak za razvoj rezistencije uz propratnu unakrsnu rezistenciju i doprinosi povećanju vitalnosti bakterija u sredini u kojoj se nalaze.

U ovom radu prikazani su neki od mogućih mehanizama adaptacije koji mogu doprineti i doprinose opstanku *L. monocytogenes* u sredini kao i sposobnost rasta ovog patogena u različitim tipovima hrane. Prikaz uključuje i mehanizme adaptacije na teške metale kao što je kadmijum, dezinficijense kao bezalkonijum hlorid (BC), odabrane antibiotike (fluorokvinolon ciprofloksacin, na primer) i druge toksične supstance (tetrafenil-fosfonijum, koji se često koristi kao anti-kancerogeno sredstvo ali i substrat efluks pumpe). U cilju karakterizacije uticaja višestruke rezistencije na preživljavanje i rast *L. monocytogenes* u razlicitim tipovima hrane, testirali smo mutante adaptirane na visoke koncentracije antibiotika ciprofloksacina i dezinficijensa bezalkonijum hlorida. Ispitivanjem kolekcije mutanata roditeljskog soja *L. monocytogenes* 10403S sa umetnutim mariner-transpozonom, registrovali smo mutante sa karakteristikama tetR mutanta sa povecanom rezistencijom na tetrafenil-fosfonijum. Konacno, testirali smo i odabrane mutante sa transpozonom koji su u toku eksperimenta iskazali promene u fenotipu nastale kao posledica odgovora organizama na razlicite uslove stresa (osetljivost na niske temperature, ne-hemoliticne sojeve, sojeve koje pripadaju epidemskom klonu II a osetljive na dejstvo faga), mutante nastale kao posledica genetskog brisanja proteina wap i genetskog regiona 18, kao i odabrane kliničke izolate i izolate iz hrane.

Selekcija nekoliko sojeva *L. monocytogenes* na ciprofloksacinu (2 μg/ml) ili BC (10 μg/ml) rezultirala je porasnom minimalnih inhibitornih koncentracija (MIC) ne samo na agense korišćene pri izolaciji već i na nekoliko drugih supstanci, ukljucujuci antibiotik gentamicin, boju etidium bromid i hemoterapeutski lek I supstrat efluks pumpe tetrafenil-fosfonijum hlorid. Spektar supstanci prema kojima su derivati ispoljili smanjenu osetljivost bio je isti bez obzira da li su originalno izolovani na ciprofloksacinu ili bezalkonijum-hloridu. Sojevi sa velikim plazmidom pLM80, na kome su smešteni geni za rezistenciju na kadmijum I BC (identifikovan u soju H7550 iz epidemskog klona II) nisu se razlikovali po MIC od derivate u kojima je plasmid uklonjen eksperimentalnom metodom. Derivati izolovani na ciprofloksacinu sa plazmidom pLM80, međutim, pokazali su veće vrednosti za MIC od onih bez plazmida. Delimicno oporavnjena osetljivost na antimikrobna sredstva u prisustvu snažnog efluks inhibitora rezerpina sugeriše da su promene u efluks sistemu odgovorne za promenu fenotipa u smeru višestruke rezistencije kod sojeva izolovanih na ciprofloksacinu ili BC.

Adaptivni odgovor bakterija na nestabilne uslove sredine često su posredovani regulatorima transkripcije. Familija TetR je jedna od najznacajnije zastupljenih I široko rasprostranjenih među regulatorima transkricije. Ovi represori kontrolišu gene čiji su proizvodi uključeni u razvoj višestruke rezistencije, enzime uključene u različite kataboličke puteve, biosintezu antibiotika, osmotski stres i patogenost. Mi smo utvrdili da tetR::Tn917 mutant sa insercijom u *tetR* represoru (smeštenom pored MDR transportera *mdrT*) ima izraženi (8-puta) porast u rezistenciji na TPP (tetraphenylphosponium) u poređenju sa roditeljskim sojem *L. monocytogenes* 10403S. Ovaj podatak sugeriše da primećena stimulacija rezistencije na TPP kod testiranog mutiranog soja uključuje i pojačanu ekspresiju drugih MDR transportera pored mdrT, koji su, međutim, regulisani represorom tetR. U cilju provere naše hipoteze testirali smo kolekciju transpozon mutanata *L. monocytogenes* sojeva 10403S i F2365 u potrazi za mutantima sa smanjenom ili povecanom rezistencijom na TPP (testirano je po 960 mutanata od svakog soja pojedinačno). Tokom eksperimena nismo uspeli da izolujemo mutante sa smanjenom rezistencijom na TPP, ali smo izolovali pet visoko rezistentnih mutanata. Utvrdili smo zatim da je jedan od pomenutih mutanata, nalik na *tetR*, sa insercijom u *tetR* genu. Mutanti su posedovali po jednu inserciju u transpozonu. Nakon karakterizacije jednog od

izolovanih mutanata utvrdili smo da je transpozon integrisan u LMRG_01858 gen (sucrose phosphorylase).

Kao što je ranjie pomenuto, rezistencija *L. monocytogenes* na BC je najčesće posledica prisustva kasete za rezistenciju (bcrABC) umetnute u plazmid. Eksperimentom u okviru ove Disertacije smo ispitivali mogućnost konjugativnog transfera rezistencije na BC i teški metal kadmijum sa ne-patogenih rezistentnih sojeva roda *Listeria* na druge, osetljive ne-patogene sojeve kao i na *L. monocytogenes*. BC-rezistentne *L. welshimeri* i *L. innocua* sa bcrABC kasetom, zajedno sa determinantom rezistencije na kadmijum cadA2, pokazale su sposobnost transfera rezistencije na druge ne-patogene sojeve I *L. monocytogenes* različitih serotipova, uključujući i sojeve izolovane tokom poslednje velike epidemije listerioze u Sjedinjenim Drzavama 2011., izazvane patogenima prisutnim u dinji. Transfer među ne-patogenim *Listeria* spp. bio je značajno viši na 25°C nego na 37°C, dok je prenos rezistencije na *L. monocytogenes* bio jednako efikasan na 25 i 37°C. Kada su ne-patogeni davaoci bili rezistentni na obadve supstance BCi kadmijum, prenos rezistencije na kadmijum se pokazao kao efikasan surogat za transfer rezistencije na BC, sugerišući koselekciju između determinati rezistencije.

Ne-patogene *Listeria* spp. kao sto su *L. innocua* i *L. welshimeri* često se uzimaju kao indikatori prisustva *L. monocytogenes* obzirom na višu prevalenciju i suživot sa *L. monocytogenes* u ekosistemu objekata za proizvodnju i preradu hrane. Jedan od predmeta interesovanja u toku izrade Disertacije bila je i analiza prevalencije rezistencije na kadmijum, arsenik i bezalkonijum hlorid među ne-patogenim sojevima iz roda Listeria izolovanih u različitim objektima za preradu živine (ćuraka) u nekoliko država u Sjedinjenim Državama. Ovu analizu smo uradili sa ciljem da rasvetlimo ulogu ne-patogenih *Listeria* spp. kao rezervoara gena rezistencije i njihov potencijal za transfer ovih gena koji može dorineti širenju rezistencije na teške metale i dezinficijense među *L. monocytogenes*. Dobijeni rezultati ukazuju na visoku prevalenciju rezistencije na BC i kadmijum, kao i značajno prisustvo *bcrABC* (kasete odgovorne za rezistenciju na BC locirane na plazmidu) i *cadA1* i *cadA2* determinanti (*cadAC* efluks sistem uključen u rezistenciju na kadmijum). Rezistencija na kadmijum je bila značajna, sa preko 90% bez obzira na vrstu. Rezistancija na BC, iako visoko zastupljena među obadve vrste, češće je registrovana medju *L. welshimeri* (83%) nego

L. innocua (73%). Na suprot ovom nalazu, rezistencija na arsenik je bila retka (<10%) kod obe vrste. Veoma je važno napomenuti da su PFGE profili u značajnoj meri bili specifični za objekat.

Epidemiološki trend poslednjih godina ukazuje da su slučajevi izazvani prisustvom *L. monocytogenes* u svežem voću i povrću mnogo češći nego što se ranije predpostavljalo. Poslednji značajan slučaj iz 2011. izazvan je prisustvom patogena u dinjama i rezultirao je sa 147 obolelih I 33 smrtna slučaja. Ova epidemija naglasila je potrebu za daljim istraživanjima sa ciljem da se bolje razume sposobnost *L. monocytogenes* da preživi i raste na dinjama i drugim sirovim proizvodima. Jedan od ciljeva u okviru ove Disertacije bio je da se utvrdi sosobnost preživljavanja i rasta sojeva izolovanih tokom poslednje epidemije listerioze 2011. i drugih sojeva povezanih sa eidemijama ili pojedinačnim slučajevima na površini dinje (kori) u poređenju sa unutrašnjom jestivom površinom (srž) ili u svežem ekstraktu pri različitim temperaturama inkubacije. Sojevi izolovani tokom epidemije 2011. pripadaju serotipu 1/2a i 1/2b i različitih su PFGE profila. Sojevi iz drugih eidemija (serotip 1/2a i 4b) su takođe testirani. Kora, srž i ekstrakt dinje su inokulisani svakim od sojeva pojedinačno tako da konačna koncentracija bakterija u matriksu iznosi 10^5cfu /fragmentu ili ml). Opstanak i rast su praćeni brojanjem kolonija tokom 21 dana inkubacije na 4 i 8°C i sedam dana inkubacije na 25°C. Dobijeni podatci su statistički obradjeni korišćenjem softvera SAS i linearnog miks efekt modela. Populacije *L. monocytogenes* uvećane su za približno 10 puta tokom 21 dana inkubacije na 4 ili 8° C i približno 100 puta nakon 7 dana inkubacije na 25°C. Nakon 24h na 25°C populacija *L. monocytogenes* uvećana je približno 10 puta. Interesantno je naglasiti da je uvećanje populacije bilo veće na kori nego u jestivom delu dinje ili u ekstraktu sa statistički značajnom razlikom (P>0.0001) zabeleženom nakon 7 dana inkubacije na 4°C i 72 h na 25°C. Sojevi izolovani tokom epidemije izazvane kontaminiranim dinjama nisu ispoljili statistički značajne razlike u rastu, dok je medju preostalim testiranim sojevima takva razlika uočena. Ispiranje fragmenata dinje u sterilnoj destilovanoj vodi pre inkubacije na 8 i 25°C rezultiralo je redukcijom populacije za približno 100 puta, ali su se patogeni mikroorganizmi oporavili i rasli na nivou patogena na netretiranim fragmentima. Rezultati ovog ispitivanja ukazuju da *L. monocytogenes* ne samo da preživljava na spoljašnoj površini dinje, već ima i potencijal da raste različitim intenzitetom direktno zavisnim od temperature i vremena inkubacije. Rezultati

sugerišu i limitiranu efikasnost tretmana sirovih proizvoda vodom obzirom da takav tretman ne zaustavlja rast *L. monocytogenes.* Dobijeni podatci mogu biti od velike važnosti u kreiranju mera kontrole u cilju limitiranog opstanka i rasta patogena na dinjama i drugim sirovim proizvodima.

U dalje analize opstanka i rasta patogena na površini dinje uvršćeni su mutanti sa izvesnim promenama u fenotipu (uvećana rezistencija na antibiotik ciprofloksacin, osetljivost na niske temperature, gubitak hemolitičke aktivnosti, osetljivost na delovanje faga) i genotipu (uklanjanje wap (protein u ćelijskom zidu) kao i specifičnog genetskog regiona – region 18). Protein kodiran wap pripada super-familiji proteina na površini bakterijske ćelije koji su uključeni u različite ćelijske procese, uključujući hidrofobnost površine, metabolizam ćelijskog zida, sekreciju, patogenost, imunogenost i ćelijsku adheziju. Genetski region (region 18) pokazao se kao značajno različit kod sojeva klonalne grupe ECII u odnosu na ostale sojeve serotipa 4b. Sojevi *L. monocytogenes* drugih serotipova ne poseduju region-18, ni wap. Rezultati eksperimenta ukazuju na nedostatak značajne razlike u rastu između roditeljskih sojeva i mutanata. Očekivano, mutant roditeljskog soja F2365, osetljiv na niske temperature nije rastao na 4ºC, dok su populacije ostalih mutanata i roditeljskih sojeva, nakon početnog pada, uvecane za 1,3 logaritamskih jedinica tokom 7 dana na 4°C.

Mleko i proizvodi od mleka uz proizvode od mesa se i dalje smatraju najznačajnijim vektorom *L. monocytogenes* odgovornu za epidemije povezane sa konzumiranjem hrane. Osamnaest sojeva *L. monocytogenes* različitog serotipa izolovanih iz hrane tokom epidemija, kao i humanih i životinjskih kliničkih izolata testirani su na rast u sirovom i pasterizovanom mleku tokom 10 dana na temperaturi od 8°C. U test su uključeni i mutant adaptirani na antibiotik ciprofloksacin uz roditeljski soj. Cilj je bio da se utvrde moguće razlike u preživljavanju i rastu između roditeljskih sojeva i mutanata, između različitih serotipova i različitih tipova mleka. Sojevi koji pripadaju serotipu 4b dodatno su analizirani kako bi se utvrdila potencijalna razlika izmedju klonalnih grupa (ECI, ECIa i ECII) u okviru navedenog serotipa. Utvrđeno je značajno ($p<0.0001$) uvećanje populacija tokom 10 dana inkubacije na 8°C. Dobijeni rezultati ukazuju i na generalno bolji rast *L. monocytogenes* u pasterizovanom u odnosu na sirovo mleko. Roditeljski soj nije se po rastu razlikovao od mutant adaptiranog na ciprofloksacin. Poređenje serotipova međusobno kao i u odnosu na tip mleka nije

rezultiralo značajnim razlikama. Statistički značajna razlika, međutim, uočena je među klonalnim grupama u okviru serotipa 4b. ECI sojevi su rasli značajno bolje kako u sirovom (p=0.0166), tako i u pasterizovanom (p=0.0433) mleku u poređenju sa klonalnom grupom ECIa. Obadve klonalne grupe, ECI i ECIa iskazale su veći porast populacija nego ECII sa značajnom razlikom između ECI i ECII u pasterizovanom mleku (p=0.0358) i ECIa i ECII u sirovom mleku (p=0.0026). Interesantno je naglasiti da je porast poulacije ECI u pasterizovanom mleku bio značajno viši u poređenju sa sirovim mlekom (p=0.0001), dok ovakva razlika nije zabeležena među klonalnim grupama ECIa i ECII. Rezultati ovog istraživanja potvrdili su dobru adaptabilnost i rast *L. monocytogenes* u mleku.
U nameri da rasvetlimo moguće uzroke rezistencije *L. monocytogenes* na različita antimikrobna sredstva, u ovoj Disertaciji smo dokazali potencijal ne-patogenih *Listeria* spp za transfer različitih gena, njihovu ulogu rezervoara gena rezistencije i sposobnost da doprinesu, putem konjugacije, širenju gena rezistencije na dezinficijense medju *L. monocytogenes*. Takodje smo potvrdili da su mutacije u efluks sistemu odgovorne za visestruku rezistenciju sojeva adaptiranih na antibiotike I dezinficijense. Korišćenjem kolekcije mutanata sa transpozonom izolovani su sojevi sa fenotipskim promenama (povećana rezistencija na antibiotik ciprofloksacin, osetljivost na niske temperature, gubitak hemoliticke sposobnosti kao I osetljivost na fage). Navedeni mutant, kao i mutant bez wap i regiona-18 korisceni su kako bi se testirala sposobnost *L. monocytogenes* da se adaptira i raste u razlicitim tipovima hrane. Dalja ispitivanja su, medjutim, neophodna kako bi se bolje razumela distribucija epidemskih klonova *L. monocytogenes*, njihove karakteristike i sposobnost da i zazovu oboljenja putem hrane.

KLJUČNE REČI

Listeria spp., *Listeria monocytogenes*, rezistencija, antimikrobna sredstva, efflux sistem, plazmid, tetR represor, konjugativni transfer, dinja, mleko, opstanak, rast

NAUČNA OBLAST: VETERINARSKA MEDICINA
UŽA NAUČNA OBLAST: HIGIJENA MLEKA
UDK BROJ: 579.62

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2.0 LITERATURE REVIEW

2.1. Antimicrobial resistance of *Listeria* spp.

Among the ten known species of the *Listeria* bacterial genus, *Listeria monocytogenes* remains the primary cause of food born infection in humans. As ubiquitous organisms, *Listeria* species are widely distributed in the environment. From the food safety viewpoint, most troubling is their persistence in food processing operations and facilities, given that some of the strains have been involved in major outbreaks of listeriosis (115). Recent epidemiologic data indicate declined incidence of listeriosis in the US, hence, *L. monocytogenes* still produce the highest mortality (16-19%) and hospitalization rate (94%) among food-borne pathogens (193). In Europe, and Canada increasing number of outbreaks and sporadic cases have been reported until 2010 (2, 55), but latest reports are encouraging and indicate a decline (3.1 % decrease in 2010 compared with 2009) (euro surveillance 2012). However, several major outbreaks that occurred recently worldwide require further prevention and food safety awareness. One of these outbreaks, a multinational 2009/2010 listeriosis outbreak caused by two different *L. monocytogenes* serotype 1/2a strains isolated from Quargel (a sour milk curd cheese) in Austria, Germany, Czech Republic, Poland and Slovakia accounted for 34 clinical cases and eight deaths (79). A 2011 major outbreak of Listeriosis associated with Jensen Farms cantaloupe-United States (32) revealed quite a few unusual and unique characteristics. Mortality rate was extremely high with 33 deaths and 147 illnesses (32). Along with being the first outbreak of Listeriosis reported to be caused by cantaloupe, *L monocytogenes* strains involved were of multiple serotypes and genotypes (122). Serotype 1/2a and 1/2 b strains from this outbreak were significantly distinct from strains of same serotype involved in previous outbreaks or sporadic cases of Listeriosis (122). Even though outbreaks attract more attention it is worthy to note that most of *L monocytogenes* infections occurred as sporadic cases and not-associated to major outbreaks (222).

In the entire *L. monocytogenes* research community, all strains are not of equal public health concern. Strains of the serotypes 1/2a, 1/2b, and 4b are especially problematic since they are most frequently associated with human illness. Of high importance are also strains that have the potential to colonize in the food processing facility environment, and to persist there as well as strains resistant to different antimicrobials.

Bacterial pathogens are often exposed to sub-lethal levels of different antimicrobial agents (i.e. disinfectants in food processing plants, antibiotics in the health environment). Such exposure is considered the main reason for the increase in *L. monocytogenes* antimicrobial resistance.

Resistance to antibiotics. Although the occurrence of antibiotic resistance in *L. monocytogenesis* is still low, its presence in food, environment, and clinical settings are well documented (38, 39, 48, 97, 153, 176, 177, 225). This pathogen is still highly susceptible to a wide range of antibiotics, including β-lactams (e.g. ampicillin) and aminoglycosides (e.g. gentamicin), which are drugs of choice for listeriosis. Emerging, however is identification of clinical isolates with enhanced resistance to fluoroquinolone ciprofloxacin which were found to also have increased resistance to EthBr and acridine orange (86). Strains selected on ciprofloxacin can have enhanced resistance not only to the antibiotic itself, but to a range of other antimicrobial compounds, including antibiotic gentamicin and the disinfectant quaternary ammonium compound benzalkonium chloride (BC) (182). Even though multi-resistant strains are not frequently isolated, evidence of their presence is available (204). Given the current wide use of antibiotics in food production and clinics, isolation of *L. monocytogenes* with reduced antibiotic susceptibility is expected to grow.

Ever since the first antibiotic-resistant strain was described in France in1988 (176), the antibiotic-resistant *L monocytogenes* have been found in foods with increasing frequency (225, 228, 230). Such resistance is frequently acquired from the commensal organisms in food or food processing environments. Another possible source are non-pathogenic *Listeria* species, primarily *L welshimeri* and *L innocua*, often more abundant in processing plants them *L monocytogenes*. A comparison of antimicrobial susceptibility between species revealed that *L. welshimeri* was resistant to more of the tested antimicrobials than the other two *Listeria*, and *L. monocytogenes* was the least resistant among the tested species (48). In response to this increase in antibiotic resistance in food borne pathogens, the European Union has banned the use of antibiotics as animal feed additives (with the exception of coccidiostats) as of January 2006 (27).

Antibiotic resistance in *L. monocytogenes* is mostly due three mobile genetic elements: self-transferable plasmids, mobilizable plasmids, and conjugative transposons (37, 40, 97, 176, 177). Efflux pumps have also been reported to be present in *Listeria* (86).

Resistance mediated by conjugation First evidence of conjugative transfer of antibiotic resistance to *L monocytogenes* involved a broad host range plasmid pIP501 in Streptococcus agalactiae (67). Few more broad-host-range plasmids with ability of conjugative transfer of resistance to *L monocytogenes* such as pAMb1 of Enterococcus faecalis and pIP823 have been reported (37, 75).

A broad host range conjugative transposon Tn916 with tetM tetracycline resistance gene, and Tn916-related transposon, Tn1545 both found in *E. faecalis* (35, 77, 177) demonstrated the transfer by conjugation to *L monocytogenes*.

Resistance mediated by efflux pumps Efflux pumps, characterized as proteins responsible for the removal of different substrates, including antibiotics from bacterial cells (both Gram positive and Gram negative) (218), may be specific to a substrate or may acquire multiple drug resistance (227). Various studies provided substantial evidence for the importance of efflux pumps in disinfectant resistance (156). Thus far, 26 Major Facilitator Super family (MFS) transporter proteins are recognized in the genome of *L. monocytogenes* EGD-e (http://www.membranetransport.org/). First efflux mechanism described in *Listeria* spp has been implicated for tetracycline resistance (36, 70). Two more efflux pumps have been characterized in *L. monocytogenes*, *MdrL* and *Lde*. *MdrL* regulating transfer of macrolide antibiotics, heavy metals and ethidium bromide and fluoroquinolones, DNA intercalating dyes acridine orange and ethidium bromide (86, 144).

Quaternary ammonium compound bezalkonium chloride (BC). The ability of *L monocytogenes* to persist in the food processing environment is highly associated with resistance to commonly used disinfectants (64, 118, 120). Different studies are suggesting variable incidence of resistance to the quaternary ammonium compound bezalkonium chloride (BC) among *L monocytogenes* isolates from foods and food processing plants. While Mullapudi et al. (154) and Soumet et al. (203) reported incidence of over 40% in turkey and fish-processing plants, respectively, Aase et al (1) suggested an incidence of 10% BC resistant *L monocytogenes* strains from fish processing plants and poultry slaughter houses.

Selective pressure has been hypothesized to contribute to the increase in *L monocytogenes* resistance as BC has been extensively used in food processing and health care environments in the US (124, 146, 151, 190). Furthermore, a number of studies have suggested cross-resistance between BC and antibiotics (182, 187, 216).

Mechanisms of resistance are various and include change in cell membrane permeability, efflux and ability to or degrade the biocide and induction of the cellular stress response.

Change in cell membrane permeability Alterations in surface teichoic acid and fatty acid are associated with increase in BC resistance of originally resistant strains upon the exposure to the sub-lethal concentrations of this disinfectant (216). Such alterations cause morphological and physicochemical changes in the cell surface and change the minimal inhibitory concentrations of BC MIC in *L monocytogenes* (138, 216). The precise nature of changes in cell surface still remains unclear.

Plasmid based BC resistance mechanisms are reported by the various studies (131, 186, 187). A plasmid-associated gene cassette encoding an efflux system (*bcrABC*) is found to be crucial for the resistance of *L monocytogenes* to BC (64). It was of importance finding the presence of the *bcrABC* cassette in strains associated to multistate outbreaks of listeriosis, in the 1998-99 hot dog outbreaks (29) and the 2000 turkey deli meat outbreak (31). Interestingly, the strain involved in 1998-99 hot dog outbreaks was the first reported strain resistant to both cadmium and BC (186). The efflux gene is harbored on a large (ca. 80 kb), pLM80 plasmid and transferred to *L monocytogenes* by conjugation. The same plasmid (pLM80) along with a three-gene cassette responsible for BC resistance harbors a cadmium resistance cassette *cadA2cadC2* (53; www.broad.mit.edu). A study by Katharios-Lanwermeyer et al (117) suggests nonpathogenic *Listeria* spp. as potential reservoirs for disinfectant and heavy metal resistance genes for other *Listeriae*, including the pathogenic species *L. monocytogenes* since both *bcrABC*, and the cadmium resistance determinant *cadA2*, were transferred to other nonpathogenic *Listeriae* as well as to *L monocytogenes.*

Chromosomal gene(s) appear to be another source of the resistance of *L monocytogenes* to BC (150, 186). Upon the adaptation of the *L monocytogenes* BC-sensitive strains to sub-lethal levels of BC increase in the transcript levels of chromosomal MFS transporters (MdrL, Lde) on MFS transporters (MdrL, Lde) (187). Their regulation and mechanisms of work, however, remain to be elucidated.

Cross resistance between disinfectant and antibiotics is important from the food safety viewpoint given that such adaptation may contribute to the persistence of the pathogen in the food processing environment. Thus, far numerous studies provided evidence for cross resistance between antibiotics, disinfectants and other compounds. Strains of *L monocytogenes* resistant to fluoroquinolones (e.g.ciprofloxacin), also expressed resistance to aminoglycoside gentamicin, dyes such as ethidium bromide, and disinfectants such as QACs (1,86, 138182, 187). Godreuil et al (86) presented evidence of MFS transporter Lde involvement in cross resistance between fluoroquinolone, ethidium bromide and acridine orange in *L monocytogenes*. Cross resistance between different disinfectants upon adaptation to QAC has been reported by Lunden et al. (138)

Resistance to heavy metals

Cadmium: Abundant presence of heavy metals, specifically cadmium and arsenic in the environment resulted in adaptation and resistance of *Listeria spp,* including *L monocytogenes,* to these toxic compounds. This lead to the high prevalence of a especially cadmium resistant *Listeria* spp in food processing environments (154, Rakic Martinez et al. unpublished data). From the food safety viewpoint it is noteworthy that several major outbreaks of listeriosis have involved cadmium-resistant strains (64, 116, 121, 156). The prevalence of the resistance to these heavy metals varies among different serotypes of *L. monocytogenes* (127, 128, 129, 147, 155).

Efflux system responsible for the, cadmium resistance in gram-positive bacteria consists of two adjacent genes (*cadA* and *cadC*) (200). The Cadmium resistance mechanism in *L monocytogenes* is diverse with four distinct cadAC energy-dependent efflux systems identified to date. The first, *cadA1*cassette is harbored on plasmids associated with a transposon (Tn5422) (121, 127, 129). Second, *cadA2* was identified in the strain H7858, implicated in the 1998–1999 hot dog outbreaks. This cassette is also harbored on plasmid (pLM80), part of a putative composite transposon. *CadA2* determinant shares this plasmid with BC – resistance genes (18, 36, 156).

The third determinant identified on the chromosome of *L monocytogenes* EGDe as a component of integrative conjugative element (ICE) (22, 83). Briers et al (19) indentified in *L. monocytogenes* Scott A novel putative cadmium resistance determinant cadA4. Presence of the determinants *cadA1* and *cadA2* alone or together seem to be most prevalent among *L monocytogenes* resistant to cadmium (155). Similar results are obtained after testing non-pathogenic *L welshimeri* and *L innocua* isolates from poultry processing plants across the US (Rakic Martinez, unpublished data).

Several studies confirmed correlation between resistance to cadmium and BC in strains harboring *cadA2* alone or with *cadA1* (155, Rakic Martinez unpublished data). Such strains also harbored BC resistant determinant *bcrABC*. All tested BC resistant strains were also cadmium resistant, however, the opposite was not true (155, Rakic Martinez unpublished data). Mullapudi et al also reported serotype specific distribution of cadA determinants (155). Resistance to cadmium in *L monocytogenes* might be acquired from non pathogenic *Listeriae* such as *L. welshimeri* and *L. innocua* harboring *bcrABC*, along with the cadmium resistant determinant *cadA2*, were able to transfer resistance to other *L.monocytogenes* of diverse serotypes (117).

Arsenic: Since none of the *Listeria* strains sequenced to date apparently harbor arsenic resistance, the responsible genes are not yet indentified.A putative arsenic resistance cassette was identified on pLI100, and harbored by *L innocua* CLIP 11262 (83, 121). Lee et al (130) recently indentified an extended arsenic resistance cassette, which includes arsR1D2R2A2B1B2 and two additional upstream genes (arsD1A1), were recently identified on the Scott A chromosome, where it is part of a 35-kb genomic Island. Generally, however, arsenic resistance determinants are highly homologous among different bacteria (162, 202).

The use of antimicrobials in both animals and humans can select for resistant bacterial populations. In food animals, antimicrobials are used for the control and treatment of bacteria associated with infectious diseases as well as for growth promotion purposes (169). Apart from the European Union (EU) ban of certain antibiotics that are used, or related to those used, in human medicine (66). An undesired consequence of antimicrobial use is the potential development of antimicrobial-resistant food borne bacterial pathogens and subsequent transmission to humans as food contaminants (62). In addition, spontaneous mutation in food borne bacteria or the spread of resistant bacteria in the absence of selective pressure may also contribute to the antimicrobial resistance burden in food (63). In fact, emerging antimicrobial resistance phenotypes have been recognized among multiple pathogens including *Listeria monocytogenes* (228).

Taken together the adaptability potential and persistence of *L monocytogenes,* along with other *Listeria* spp in the food processing environment calls for targeted interventions in order to maintain food safety at the highest possible level. Ability to propose and create such interventions require a better understanding of the mechanisms involved in *L monocytogenes* ability to colonize

food or food-processing, health care facilities, and persist and acquire resistance to antimicrobials commonly used in such environments.

2.2. Adaptation of *Listeria monocytogenes* to Ciprofloxacin or to the Disinfectant Benzalkonium Chloride Results in Reduced Susceptibility to Ciprofloxacin, Gentamicin Benzalkonium Chloride and Other Toxic Compounds

Listeria monocytogenes is a Gram-positive bacterium responsible for listeriosis, an illness that remains a leading cause of mortality and morbidity associated with foodborne infections in the United States and other industrialized nations (119, 149, 193). At risk are primarily pregnant women and their fetuses, the elderly, and those in immunocompromised states, including patients undergoing kidney dialysis or chemotherapy. Symptoms can be severe (septicemia, meningitis, stillbirths) and fatality rate is estimated at 16% (60, 163, 193).

L. monocytogenes is usually susceptible to a wide range of antibiotics, except cephalosporins and fosfomycin (102, 103, 153), and multidrug-resistant clinical isolates appear to be rare (97, 176, 180, 208). The treatment of choice for listeriosis consists of a β-lactam antibiotic (e.g. Ampicillin), alone or in combination with an aminoglycoside (e.g. gentamicin), and clinical isolates of *L. monocytogenes* generally remain susceptible to these antibiotics (102, 153). However, trends towards reduced susceptibility to tetracyclines and fluoroquinolones have been noted (153). Even though these antibiotics are not typically used for treatment of listeriosis, their extensive use in empirical therapy and treatment of other infections could create selective pressure for *L. monocytogenes* mutants with reduced susceptibility. Fluoroquinolone-resistant isolates of *L. monocytogenes* from clinical cases were found to have enhanced transcription of *lde,* encoding a drug efflux transporter of the major facilitator superfamily (MFS) (86, 153). However, limited information is available on mutants obtained upon exposure of *L. monocytogenes* to fluoroquinolones.

In addition to antibiotic-related exposures, *L. monocytogenes* is expected to be frequently subjected to selection pressures associated with the extensive use of disinfectants such as quaternary ammonium compounds in food processing plants and healthcare settings. Limited information is currently available on possible cross-resistance following exposure to antibiotics such as ciprofloxacin and disinfectants such as the quaternary ammonium compound benzalkonium chloride (BC), extensively used in the food processing industry

(146, 151). Similarly, exposure to BC may result in variants with reduced susceptibility to BC but also reduced susceptibility to antibiotics. Indeed, exposure of *L monocytogenes to* progressively increasing concentrations of this disinfectant resulted in BC-resistant mutants that also exhibited reduced susceptibility to gentamicin and kanamycin (187, 207). However, cross-resistance to fluoroquinolones (e.g. ciprofloxacin) was not reported. The potential for co-selection of antibiotic and disinfectant resistance upon exposure of *L.monocytogenes to* antimicrobial agents has important food safety and public health implications and needs to be further investigated.

In this study we determined the extent to which adaptation of *L. monocytogenes* to ciprofloxacin and to BC altered susceptibility to a panel of antimicrobial agents, including several antibiotics, BC and other toxic compounds. Outbreak-derived strains harboring a large plasmid (pLM80) and their cured derivatives were included, to assess possible impact of the plasmid on the susceptibility profiles of the adapted strains.

2.3. Identification of *L.monocytogenes* antibiotic resistance genes under control of *tetR* repressors

In their natural settings bacteria are constantly exposed to various environmental fluxes, including severe nutrient limitation, fluctuations in temperature, and changes in oxidative and osmotic tensions. In order to survive adverse environmental conditions, bacteria developed a wide range of rapid and adaptive responses. These responses are generally mediated by regulatory proteins, which modulate transcription, translation, or other events in gene expression so that the physiological responses are appropriate to the environmental changes.

Exposure to toxic compounds (e.g. quaternary ammonium disinfectants and the antibiotic ciprofloxacin) selects for mutants with multidrug resistance (MDR) (86, 182, 187). Frequent occurrence of such MDRs in food processing plants potentially leads to food contamination followed by human infection with strains that have reduced susceptibility to antibiotics; such resistant mutants in healthcare settings where disinfectants are frequently in use (hospitals, nursing homes), would result in infections with impaired response to antibiotic treatment. In our previous report, exposure of Listeria to quaternary ammonium disinfectants benzalkonium chloride (BC) or to the antibiotic ciprofloxacin (cipro) selects for mutants with multidrug

resistance (MDR) to a range of drugs, including not only BC and cipro but also toxic dyes (e.g. ethidium bromide), other antibiotics (e.g. gentamicin), and the anticancer drug tetraphenylphosphonium (TPP) (86, 182, 186). Determined reduced resistance of such mutants in the presence of the efflux inhibitor reserpine (182) suggests that increased efflux is involved in the resistance, e.g. through inactivation of repressors for MDR transporters. Recent genetic and biochemical studies have identified two *L. monocytogenes* major facilitator superfamily multidrug resistance (MDR) efflux pumps as necessary for the induction of host IFN-β involving the second messenger c-di-AMP (46, 227).

The tetracycline repressor (TetR) family transcriptional regulators constitute the third most frequently occurring transcriptional regulator family found in bacteria (164). The TetR family is named after the transcriptional regulators that control the expression of the tetR genes, whose product confers resistance to tetracycline (186). However, TetR family proteins are also involved in various other important biological processes, such as biofilm formation, biosynthesis of antibiotics, catabolic pathways, multidrug resistance, nitrogen fixation, stress responses, and the pathogenicity of Gram-negative and Gram-positive bacteria (32) Inactivation of the tetR repressor (next to the MDR transporter mdrT) in Listeria strain 10403S caused a dramatic increase in IFN-□□production by infected macrophages (46) suggesting involvement of more currently unidentified MDR transporters (besides mdrT) under control by the tetR repressor. TetR is a negative regulator of mdrT (lmo2588), a gene encoding an MDR transporter that participates in the secretion of cyclic di-AMP, which is a potent IFN-β-inducing agent. In strain 10403S inactivation of tetR resulted in the largest noted increase in IFN-□□ and in levels of c-di-AMP in infected macrophages, but deletion of the adjacent MDR transporter mdrT had no effect (46, 227). Furthermore, transcription of mdrT was not induced following treatment with TPP (46). On the other hand, we found that the tetR::Tn917 mutant had pronounced (8-fold) increase in TPP resistance. Such data suggest that the observed TPP resistance of the tetR::Tn917 mutant involve increased expression of MDR transporter(s) other than mdrT, but also repressed by tetR.

We speculate that the unidentified gene (repressed by tetR) is responsible for the observed TPP resistance of the tetR::Tn917 mutant. By screening a mariner-based mutant

library of selected L monocytogenes strains for new mutants with impaired or enhanced resistance to TPP we aim to identify this gene.

2.4. Co-selection of cadmium and benzalkonium chloride resistance in conjugative transfers from non-pathogenic *Listeria* spp. to other listeriae

Listeria spp. are Gram-positive bacteria commonly found in the environment. The single human pathogen in this genus, Listeria monocytogenes, continues to be associated with significant disease burden due to its high morbidity and mortality towards vulnerable populations such as the elderly, pregnant women and their fetuses, neonates and immunocompromised patients (54, 115, 163, 193). *L. monocytogenes* remains largely susceptible to antibiotics, with a common course for treatment being a combination of ampicillin and gentamicin. However, reported cases of listeriosis involving strains with multi-drug resistance (39, 97, 176, 180, 208) suggest the potential for enhanced resistance through horizontal gene transfer with accompanying increases in public health concerns associated with this pathogen.

Colonization of the processing plant environment by *Listeria* spp. is a major contributor to contamination of processed, ready to eat foods (120, 220). Exposure to disinfectants commonly used in food processing plants, such as the quaternary ammonium compound benzalkonium chloride (BC), may provide selective pressures for disinfectant resistance with an accompanying increase in fitness within the processing plant environment. Several studies have investigated prevalence and mechanisms of BC resistance in *L. monocytogenes* (64, 154, 186, 202); however, less is known about the behavior and response of non-pathogenic *Listeria* spp. to selection pressures in food processing plants and other environments.

The processing plant environment may present many opportunities for non-pathogenic *Listeria* spp. to interact with *L. monocytogenes*. Non-pathogenic species such as *L. innocua* and *L. welshimeri* have been found to be more common than L. monocytogenes in food processing environments and in foods, and to grow faster than L. monocytogenes in foods and other media (8, 47, 104, 140, 168, 197, 201, 221). They are also more likely than *L. monocytogenes* to exhibit antibiotic resistance and to harbor plasmids (4, 39, 48, 69, 70, 167, 184, 189). Taken together, such findings suggest the potential for horizontal gene transfer

among listeriae, with non-pathogenic strains serving as reservoirs for resistance determinants, potentially altering the fitness of the pathogenic species, *L. monocytogenes*.

In *L. monocytogenes* the efflux system encoded by *bcrABC* has been shown to confer high-level resistance to BC and other quaternary ammonium compounds (64). The *bcrABC* cassette was first identified on a large plasmid (pLM80) harbored by strains implicated in the 1998-1999 hotdog outbreak of listeriosis, and was subsequently also identified on a similar plasmid from a different strain, responsible for another multistate outbreak. The cassette on these plasmids appears to be part of a composite transposon that also includes genes conferring resistance to cadmium (64, 121,156).

Further evidence for the association between BC resistance and resistance to cadmium was obtained from characterization of *L. monocytogenes* from turkey processing plants. All BC- resistant strains were found to be also resistant to cadmium (though the reverse was not the case; ca. 30% of the cadmium-resistant isolates lacked resistance to BC) (154). This led to the speculation that BC resistance determinants (e.g. bcrABC) were acquired by plasmids that already harbored cadmium resistance determinants (154, 155).

Such findings suggest the need to characterize horizontal transfer of bcrABC in *Listeria* spp., including transfer from non-pathogenic species that may act as reservoirs. However, efforts to assess conjugative transfer of BC resistance in *Listeria* spp. can be thwarted by the frequent occurrence of spontaneous mutants exhibiting high-level resistance to BC; such mutants can be readily obtained from several strains of *L. monocytogenes* and other *Listeria* spp. (182, 207, M. Rakic-Martinez and S. Kathariou, unpublished findings). On the other hand, spontaneous mutants with high levels of resistance to cadmium are extremely rare and in fact have never been identified in our laboratory (S. Katharios-Lanwermeyer and M. Rakic-Martinez, unpublished findings). Therefore, in this study we hypothesized that BC-resistant, cadmium-resistant strains of L. innocua and L. welshimeri harbored the corresponding resistance determinants on plasmids (similarly to L. monocytogenes strains with pLM80 and related plasmids, described above), and we used cadmium resistance transfer as a surrogate for assessments of conjugative transfer of BC resistance. We examined transfer of such resistance among L. innocua and L. welshimeri as well as from these non-pathogenic species to L. monocytogenes.

2.5. Resistance gene distribution among non-pathogenenic *Listeriae* from different poultry processing plants

Listeria monocytogenes has unparalleled potential to readily demonstrate combinations of environmental and host-related adaptive attributes. From the food safety point of view, populations of Listeria within specific food processing plants are of special interest: the individual processing plant ("plant") ecosystem creates selective pressures for adaptive features relevant to environmental persistence and subsequent contamination of product. Well recognized adaptations include biofilm forming potential and tolerance to cold, disinfectants and Listeriaphage. Less clearly understood in terms of its fitness contributions, but nonetheless consistently exhibited, is Listeria's heavy metal resistance (cadmium, arsenic) (115, 118, 154, 155).

Non-pathogenic *Listeria* spp. such as *L. innocua* and *L. welshimeri* are often more abundant than L. monocytogenes in the plant ecosystem while sharing the same niche, and may thus represent excellent models for plant-specific environmental selective pressures impacting *Listeria* (104, 105, 168, 197). We recently provided evidence suggesting that non-pathogenic *Listeriae* may constitute reservoirs for resistance to the quaternary ammonium disinfectant benzalkonium chloride (BC), being able to conjugatively disseminate resistance to BC to other Listeriae, including L. monocytogenes (117). Cadmium resistance that was also exhibited by the BC-resistant non-pathogenic strains was found to be a useful marker for assessing transfer potential, and cadmium-resistant transconjugants harbored the corresponding resistance determinant (cadA1 or cadA2) (117).

Only sparse information is currently available on population structure and resistance gene distribution among non-pathogenic *Listeriae* from the processing plant environment. Such information is needed to assess gene transfer potential and to further elucidate the molecular ecology of Listeria in the context of the ecosystem of specific plants. In this study, we analyzed resistance prevalence and determinants a panel of non-pathogenic *Listeria* from different processing plants.

2.6. Survival and growth of outbreak and other strains of *Listeria monocytogenes* on cantaloupe. The facultative intracellular bacterium L. monocytogenes remains a leading

cause of severe illness and death due to foodborne disease in the United States (193). Its ubiquitous nature and persistence in the environment and equipment in food processing plants, often for years, create important food safety and public health challenges (115, 120). The disease primarily affects the elderly, pregnant women, newborns, patients undergoing chemotherapy and other immunosuppressed individuals (60, 163).

Even though several high-profile outbreaks of listeriosis have involved deli meats and soft cheeses (28, 29,82, 149), epidemiological trends suggest that L. monocytogenes - contaminated produce is associated with higher disease outcome than previously recognized. The first produce-related listeriosis outbreak involved contaminated coleslaw (cabbage); it took place in 1981 in the Maritime Provinces (Canada) and was also noteworthy in providing the first demonstration of foodborne transmission of L. monocytogenes (194). Subsequent produce-associated outbreak occurred among hospitalized patients in Boston and was epidemiologically attributed to contaminated celery, lettuce and tomato. This outbreak was followed by multistate outbreak associated with soybean sprouts (2008-09), and 2010 Texas outbreak connected to chopped celery with surprisingly high mortality (10 cases, five deaths)(123, http://www.cspinet.org/foodsafety/outbreak_report.html)].

L. monocytogenes has been isolated from numerous types of untreated produce, imported as well as domestic, including several that would be considered ready-to-eat such as cucumbers, lettuce, cabbage, salad greens, prepared salads, radishes, tomatoes, and others (10, 101). Produce can become contaminated pre-harvest as well as during the processing. In addition to their potential to be directly implicated in disease, Lm-contaminated produce can be a source of the organism for produce processing facilities and equipment. Cantaloupe has been implicated in numerous outbreaks of food-borne illnesses, primarily involving *Salmonella*. An estimated 13 out of 82 foodborne illness outbreaks associated with produce between 1996 and 2008 involved contaminated melon, resulting in 507 illnesses and 2 deaths. In recent years, numerous cases of *Salmonella* infections linked to cantaloupe had been reported including latest 2012 Multistate Outbreak of *Salmonella Typhimurium* and *Salmonella Newport* (CDC Salmonella Web Page). However, the 2011 multi state outbreak provided first evidence for cantaloupe as a vehicle for listeriosis. This outbreak had additional unusual characteristics. It had the highest mortality rate of any foodborne outbreak in the U.S. since a

listeriosis outbreak in 1998 (33). It was also one of the few common-source listeriosis outbreaks involving strains of multiple serotypes and genotypes (122). Further analysis revealed significant distinction between 1/2a and 1/2b strains involved in this outbreak and same serotypes strains associated with previously reported outbreaks and sporadic cases.

Colonization of the outer rind of melon by pathogenic microorganisms, potential for biofilm formation and efficacy of washing treatments and other interventions in pathogen reduction have been investigated (209, 210, 214, 215). Few studies provided evidence for transfer of bacteria by cutting from the outer fruit surfaces to the edible parts (211) and potential of contamination of cut melon due to improper handling and cross-contamination (214). Thus far, however, data on L. monocytogenes potential to grow on cantaloupe are limited. Ukuku at al (2002) reported survival, but no growth on fresh-cut peaces of cantaloupe at 4°C. However, increasing temperature to 8 and 20°C, respectively, led to the *L. monocytogenes* population increase of 1 log unit (211). A significant increase (6 log units) in L monocytogenes population in melon pulps stored at 10 and 20ºC, respectively, had been noted by Penteado and Leita˜o (166). Using limited strain panels, previous studies lacked reports on strain specific behavior of L monocytogenes on the surface of cantaloupe. Further more tested strains did not include isolates from 2011 cantaloupe associated listeriosis outbreak. By employing individual strains involved in food related cases and outbreaks of Listeriosis, (including strains from the 2011 cantaloupe outbreak a), the goal of the current study was to determine strain-specific differences in survival and growth of L monocytogenes on cantaloupe. Focus was also on the location-specific differences in L monocytogenes survival and growth on the outer surface, inner surface and extract of cantaloupe at various incubation temperatures as well as efficiency of water treatment in prevention of pathogen growth.

2.7. Change in phenotype and genotype as an asset for *L. monocytogenes* growth on cantaloupe. *Listeria monocytogenes* remains, globally, one of the leading food-born pathogens responsible for severe illness and death associated with infection, in spite of effective antibiotic treatments. High hospitalization rate (94%) with a mean of approx. 16% of listeriosis cases fatal cause L. monocytogenes to be a third major contributor to deaths due to food-borne deseases in the United States (163, 193).

According to the recent epidemiological data contribution of produce to human listeriosis is much higher than previously recognized (34). Various produce are accounted as a cause of multistate outbreaks including contaminated soybean sprouts in 2008-2009, chopped celery in 2010 as well as cantaloupes involved in 2011 listeriosis outbreak. Both 2010 and 2011 outbreaks had unusually high fatality rate (82). *L monocytogenes* has also been isolated from numerous types of untreated produce, including several that would be considered ready-to-eat such as cucumbers, lettuce, cabbage, salad greens, prepared salads, radishes, tomatoes, and others (10, 11, 101).

A number of special attributes (e.g. biofilm-forming potential, ability to grow in the cold, resistance to phage and disinfectants) make L monocytogenes a persistent, highly problematic contaminant of the environment and equipment in food processing plants (14, 118, 120, 154, 158). Persistent colonization of the processing plant environment is considered to be a major factor for contamination of processed, ready-to-eat foods with this pathogen, and thus contributes to the burden of human listeriosis (118, 120). A substantial amount of the information on mechanisms mediating adherence and fitness of *Listeria* on different produce has been provided (88, 89, 90, 91). However, our understanding of the basic mechanisms of adherence and fitness of *L. monocytogenes* on the surface of cantaloupe is currently minimal.

Significant number of outbreaks caused by *L. monocytogenes* had been associated with one clonal group, Epidemic clone one (115, 118). Sequencing one of the strains belonging to this group (F2365) revealed several unique genes and gene cassettes. Strain F2365 was implicated in the California outbreak of 1985 (156, 231). Interestingly, characterization of the strain involved in 1998-99, multistate outbreak of listeriosis connected to contaminated hot dogs, associated this strain with a novel epidemic clone ECII and revealed an unusual diversification in a serotype 4b specific genomic region known as “region 18” (28, 149). Interestingly, this region is flanked on one side by a large gene encoding a putative cell-wall associated protein (*wap*) and on the other side by a well-known virulence gene, inlA, encoding Internalin. In further analysis, deletion of region-18 had no effect on hemolytic ability, phage susceptibility, cell shape, or colony size, in either ECI strain F2365 or ECII strain H7550. However, it led to a defect in the utilization of certain carbon sources in ECI strain as well as enhanced death rate during post-stationary phase at 42 ºC in ECII.

In effort to better understand ability of *L. monocytogenes* to colonize and grow on cantaloupe we have employed the mariner-based transposon to construct mutant libraries of strains F2365 and H7550. Through screenings of these libraries we have isolated various mutants including non-hemolytic mutant, a mutant with altered phage susceptibility profile, and mutants in a putative biosynthesis locus that exhibited impaired growth on blood agar. Additionally, we employed deletion mutants of *wap* and genomic region 18, along with corresponding parental strains. Lastly, mutants of L. monocytogenes adapted to antibiotic ciprofloxacin described in the previous study (182) were also tested.

2.8. Assessment of *L. monocytogenes* growth in raw and pasteurised milk. Presence of *Listeria monocytogenes* in variety of foods, particularly milk and dairy products, animal products, ready-to-eat foods, fruit and vegetables has been well documented (26, 32, 52, 57, 98, 137, 152, 192, 196, 206).In USA and Canada, widespread presence and high prevalence of L monocytogenes in bulk tank raw milk samples with incidence variations from 4 - 12.6% has been reported in a number of studies (74, 108, 217)

Such a presence has raised concerns about *L. monocytogenes* survival and persistence in food processing facilities as a result of poor sanitation practices or ability of Lm to grow under abusive conditions. Persistent presence of this microorganism could lead to biofilm formation and possible cross contamination at the farm, plant or during the transport and storage whether the milk is pasteurized or not (5, 145, 159).

Ubiquitous and highly adaptable nature of *L. monocytogenes* including ability to grow in a wide range of temperatures and pH enables growth in refrigerated raw milk and animal feed at pH as low as 4.5 (45). Even dough milk and milk products depend on refrigeration for ensuring post-pasteurization microbial safety, refrigerated storage is not adequate for inhibiting the growth of *L. monocytogenes* in fluid milk as it can multiply at temeperatures as low as 4C (109, 142, 159, 175). Extended periods of refrigerated storage (up to 12 days for pasteurized milk) and improper compliance of temperature regulations during transportation and storage at retail stores (59) can allow even a few L. monocytogenes cells, weather they survived pasteurization or were introduced as post pasteurization contamination, to attain populations high enough to pose serious health risks (10^2 CFU/ ml or above) (115).

L. monocytogenes can cause neurological disease, abortion, or asymptomatic infections in cattle. Healthy, but infected animals, shed Listeria in feces and fecal contamination of pastures or vegetables has been implicated as a source of contamination for humans and ruminants. Mastitis in cows caused by *L. monocytogenes* could result with a transfer of the pathogen in milk (16, 109, 226). Further more, if bacterial concentration is high enough, *Listeria* can survive minimum HTST pasteurization, as reported by Bunning et al.(21). Consumption of such contaminated raw milk or raw milk products or ingestion of processed food cross-contaminated with pathogens present in the food processing plant environment (93) leads to human infection. Susceptible are especially certain groups of population including immunocompromised patients, pregnant woman, children and elderly. In recent years few serious outbreaks of listeriosis connected to the consumption of milk and milk products occurred worldwide. In 2005 case of contamination of cheese made from raw goat's milk occurred in Belgia (52). In Spain, two cases of pregnancy-related listeriosis associated with the Latin-style fresh cheese made from pasteurized milk in Portugal were reported by V de Castro et al. (50). Multinational 2009/2010 listeriosis outbreak caused by two different *L. monocytogenes* serotype 1/2a strains isolated from 'Quargel', a sour milk curd cheese, in Austria, Germany, the Czech Republic, Poland and Slovakia accounted for 34 clinical cases and eight deaths (79). An outbreak in California in 1985 was shown to be due to the consumption of Mexican-style fresh cheese (136). Following investigation of the outbreak suggested either mixed raw and pasteurized milk or post pasteurization contamination (199).

In the entire *L. monocytogenes* population, 13 different serotypes, only strains belonging to serotypes 4b, 1/2a and 1/2b are connected to the majority of sporadic cases and outbreaks of listeriosis. Even dough reported outbreaks were geographically and temporally distinct, involved strains were closely related (51). Based on comparative study of above mentioned outbreaks, Kathariou and al. (115, 134) defined four different epidemic clones (ECI, ECIa, ECII and ECIII). Clonal groups ECI and ECIa composed mostly of serotype 4b strains were involved in worldwide distributed major outbreaks connected to food sources (134). These two clonal groups are overrepresented among sporadic cases of listeriosis in humans and animals occurred in early 30s 40s and 50 of the last century. ECII clonal group is relatively new, observed in the connection with 1998-1999 hot-dog outbreak by Kathariou et al (134).

Employing diversity of food and clinical isolate of *L. monocytogenes* involved in older sporadic cases as well as recent major outbreaks of listeriosis we aimed to assess optimum growth rate of *L. monocytogenes* in raw and pasteurized milk over 10 days of storage at the refrigeration temperature. The objective was also to determine possible serotype and clonal associated difference in *L. monocytogenes* survival and growth in raw versus pasteurized milk stored at 8C.

3.0. TASKS AND REASEARCH

3.1. Adaptation of *Listeria monocytogenes* to Ciprofloxacin or to the Disinfectant Benzalkonium Chloride Results in Reduced Susceptibility to Ciprofloxacin, Gentamicin Benzalkonium Chloride and Other Toxic Compounds

3.1.1. Bacterial strains and growth conditions. *L. monocytogenes* strains employed in this study are listed in Table 3.1.1.

Table 3.1.1. *L. monocytogenes* strains employed in this study

Strain	Resistance[1]		Characteristics	Source (reference)
	Cd	BC		
H7550 Cd^R	+	+	Serotype 4b strain from 1998-1999 hot dog outbreak	(64)
H7550 Cd^R C_1	+	+	Ciprofloxacin-selected mutant of H7550 Cd^R	This study
H7550 Cd^R C_2	+	+	Ciprofloxacin-selected mutant of H7550 Cd^R	This study
H7550 Cd^S	-	-	Plasmid-cured derivative of H7550 Cd^R	(64)
H7550 Cd^S C_1	-	+	Ciprofloxacin-selected mutant of H7550 Cd^S	This study
H7550 Cd^S C_2	-	+	Ciprofloxacin-selected mutant of H7550 Cd^S	This study
H7550$Cd^S$$BC_1$	-	+	BC-selected mutant of H7550 Cd^S	This study
H7550$Cd^S$$BC_2$	-	+	BC-selected mutant of H7550 Cd^S	This study
SK 2802	+	-	Serotype4bstrain, sporadic case of isteriosis, USA, 2005.	CDC
SK 2802 C_1	+	+	Ciprofloxacin-selected mutant of SK 2802	This study
SK 2802 C_2	+	+	Ciprofloxacin-selected mutant of SK 2802	This study
SK 2802 BC_1	+	+	BC-selected mutant of SK 2802	This study
SK 2802 BC_2	+	+	BC-selected mutant SK 2802	This study
J0161 Cd^R	+	+	Serotype 1/2a strain from turkey deli meats outbreak, 2001	(161)
J0161 Cd^R C_1	+	+	Ciprofloxacin-selected mutant of J0161 Cd^R	This study
J0161 Cd^R C_2	+	+	Ciprofloxacin-selected mutant of J0161 Cd^R	This study
J0161 Cd^S	+	+	Plasmid-cured derivative of J0161 Cd^R	This study
J0161 Cd^S C_1	-	+	Ciprofloxacin-selected mutant of J0161 Cd^S	This study
J0161 Cd^S C_2	-	+	Ciprofloxacin-selected mutant of J0161 Cd^S	This study
J0161Cd^S BC_1	-	+	BC-selected mutant of J0161 Cd^S	This study
J0161Cd^S BC_2	-	+	BC-selected mutant of J0161 Cd^S	This study

[1] For Cd, + indicates confluent growth on isosensitest agar containing 70 µg/ml cadmium chloride anhydrous; for BC, + indicates confluent growth on media containing 10 µg/ml BC, prepared as described in Materials and Methods; - indicates absence of growth.

The serotype 4b strain H7550 was resistant to cadmium (Cd^R) and BC and was associated with the 1998-1999 hot dog-related multistate outbreak (64); it harbored pLM80 (ca. 80 kb), with genes for resistance to cadmium (*cadAC*) and to BC (*bcrABC*) (64, 156). *L. monocytogenes* J0161 was a serotype 1/2a strain associated with a listeriosis outbreak in 2001, linked to consumption of turkey deli meats (161). It is also resistant to cadmium and BC and harbors a large plasmid highly similar to pLM80 (15). *L. monocytogenes* SK2802 was from a sporadic case of listeriosis in 2005; this strain was resistant to cadmium and sodium arsenite but was susceptible to BC and lacked *bcrABC*. H7550 cadmium sensitive (Cd^S) and J0161 Cd^S were plasmid-cured derivatives of strain H7550 Cd^R and J0161 Cd^R, respectively; these derivatives were obtained following repeated passages of the bacteria at 42°C and were susceptible both to cadmium and to BC (64). Unless otherwise indicated, bacteria were grown at 37°C for 36 h on blood agar plates containing 5% sheep blood (Remel, Lenexa, KS), and long-term storage was done at - 80°C in brain heart infusion broth (BHI, Becton Dickinson and Co., Sparks, MD) with 20% glycerol (Fisher Scientific, Fairlawn, NJ).

3.1.2. Isolation of mutants selected on ciprofloxacin or BC. Bacteria were grown overnight in BHI (Becton Dickinson and Co.) at 37°C and spotted (3µl) on Mueller-Hinton agar (MHA) (Mueller Hinton broth [Becton Dickinson and Co.] with 1.2% Bacto agar [Becton Dickinson and Co.]) containing 2 µg/ml ciprofloxacin (Sigma Chemical Co., St Louis, MO). Colonies growing following 48h of incubation at 37°C were purified on the same medium. Overnight cultures of the BC-susceptible strains H7550 Cd^S, J0161 Cd^S and SK2802 were also spotted (3µl) on plates containing 10µg/ml BC (Acros, NJ) and 2% defibrinated sheep blood (Becton Dickinson and Co.) (154). Single colonies obtained following incubation at 37°C for 48h were subcultured on the same medium for purification.

3.1.3. Antimicrobial agents, susceptibility testing and MIC determinations. Antibiotics used in this study are listed in Table 3.1.2.

Table3.1.2. Antibiotics employed in this study

Antibiotic	Drug class	Concentration range (µg/ml)	MIC (µg/ml)
Ampicillin	ß-Lactam	0.125-2.0	≤ 1.0
Ciprofloxacin	Fluoroquinolone	0.5-16.0	2.0-8.0[1]
Moxifloxacin	Fluoroquinolone	5.0	ND[2]
Erythromycin	Macrolide	0.25-8.0	0.5
Gentamicin **Kanamycin** **Streptomycin**	Aminoglycoside Aminoglycoside Aminoglycoside	0.5-4.0 1.0-20.0 6.25-400.0	≤ 4.0 2.5 25.0
Rifampin	Rifamycin	0.5-4.0	0.5
Tetracycline	Tetracycline	1.0-10.0	2.5
Trimethoprim	Dihydrofolate reductase	0.5-4.0	0.5
Fosfomycin	Phosphonic acid derivative	64.0-2048.0	≥ 2048.0

[1]MIC for ciprofloxacin was 2µg/ml for all wildtype strains, except for SK 2808 (MIC, 8µg/ml).

[2]ND, not determined; Moxifloxacin susceptibility was assessed by the disk diffusion method as described in Materials and Methods.

They represented major antibiotic classes and included those used for treatment of listeriosis as well as others commonly used in human and veterinary medicine. Ampicillin, ciprofloxacin, rifampin, tetracycline, fosfomycin and trimethoprim were purchased from Sigma; gentamicin, moxifloxacin and kanamycin from Fisher Scientific; erythromycin and streptomycin from MP Biomedicals, Inc. (Solon, NJ). The isolates were tested for antibiotic susceptibility by agar dilution on MHA. The range of concentrations used for antibiotic MIC determinations are indicated in Table 2. Susceptibility to moxifloxacin was determined by the disk diffusion method using disks with 5 µg moxifloxacin (Fisher Scientific) on MHA. The diameter of the zone of inhibition was measured to the nearest whole millimeter.

Ethidium bromide (EthBr) (Fisher Scientific) resistance was determined using agar dilutions on MHA as previously described (86). Concentrations for EthBr MIC determinations were 20, 25, 50, 100, and 200µg/ml. Resistance to BC, cadmium chloride (Cd) (Sigma) and sodium arsenite (As) (Fluka, Buchs, Steinheim, Germany) was determined as described (154). The plates were incubated at 37°C for 48 h, and growth on the test plates was compared with that on the control Isosensitest agar (Oxoid, Basingstoke, England). Concentrations used for determinations of MIC values were 5, 10, 20, 35, 70 and 140µg/ml for Cd, and 5, 10, 20, 30 and 40µg/ml for BC. Tetraphenylphosphonium chloride (TPP) (Sigma) was used at 50, 100, 200, 400 and 800µM for MIC determinations. Agar dilution on MHA was used for TPP MIC determination.

The efflux inhibitor reserpine (Sigma) was used at 10µg/ml as described (86). When MICs were determined in the presence of the efflux inhibitor reserpine, MICs in the absence of reserpine were always determined as control.

3.1.4. Determination of tolerance to bile salts, triclosan and sodium dodecyl sulfate. Tolerance to bile salts was assessed by screening for growth on BHI agar plates (BHI [Becton Dickinson and Co.] with 1.2% Bacto agar [Becton Dickinson and Co.]) supplemented with 0.01-1% bile salts (mixture of sodium cholate and sodium deoxycholate; Sigma).The plates were incubated at 37°C for 48h under anaerobic conditions using BBL anaerobic jars (Becton Dickinson and Co.) and the Merck Anaerocult A Gas Pak system (Becton Dickinson and Co.) Susceptibility to triclosan (Sigma) and to sodium dodecyl sulfate (SDS, Fisher) was determined by spotting 3µl (0.125-64µg/ml for triclosan, and 100, 150, 200 and 250µg/ml for SDS) on MHA plates supplemented with 5% sheep blood (Becton Dickinson and Co.) and assessing growth following incubation at 37ºC for 24h.

3.1.5. RNA extraction and reverse-transcription PCR (RT-PCR). Expression of selected MDR genes (*mdrL, mdrM, mdrT, lde*) was assessed by using RT-PCR analysis. H7550 Cd^S and J0161 Cd^R , and their BC-selected derivatives H7550 Cd^S BC_1 and J0161 Cd^R C_1, respectively, were grown to logarithmic phase in BHI at 30ºC, RNA was extracted and RT-PCR was performed as described (64). Primers employed are listed in Table 4.1.3.

Table 3.1.3.Primers used in this study

Primer	Sequence (5' to 3')	Reference
s1	ATTACAGATGTGAGATTACGACG	(64)
s2	ACGTTTACTTGGCATAGCTAC	(64)
mdrL_ F	CTCC ACT CGT TAC ACT TCT	This study
mdrL_ R	CAG ACAAGGAAAT GAACAC	This study
mdrM _F	GTACATCAGTGAAGCGTAACG	This study
mdrM_ R	CTAGAACACAAGCGACTACAG	This study
mdrT_ F	CGGCCCGTTGATGTTAACG	This study
mdrT_ R	CATTCCGTCCAAACTAGCATC	This study
ldeF	GAA GAA GAA TTT GTA TGT TGTC	This study
ldeR	TCTCTCCATG CATTTTTCGG	This study

PCR was carried out using the Takara Ex Taq kit (Takara, Madison, WI) and a T1 thermal cycler (Biometra, Goettingen, Germany) as described (64). The housekeeping gene *spoVG* was used as internal control in the RT-PCR reactions, as described (64). RT-PCR for each gene was done in at least two independent trials.

3.2. Identification of *L monocytogenes* antibiotic resistance genes under control of *tetR* repressors

3.2.1. Bacterial strains and growth conditions

L. monocytogenes strains employed in this study are lister in Table 3.2.1.

Table3.2.1. *L. monocytogenes* strains employed in this study

Strain	Characteristics	Reference
10403S	serotype 1/2a strain streptomycin resistant derivative of a human skin lesion isolate	(61)
F2365	Serotype 4b strain cheese isolate of the California outbreak	(156)
LmDP-L5396 *tetR::Tn917*	10403S mutant with an insertion in the *tetR* repressor	(46)
MRMT1	Mutant in 10403S mariner-based mutant library with enhanced resistance to TPP	This study
MRMT2	Mutant in 10403S mariner-based mutant library with enhanced resistance to TPP	This study
MRMT3	Mutant in 10403S mariner-based mutant library with enhanced resistance to TPP	This study
MRMT4	Mutant in 10403S mariner-based mutant library with enhanced resistance to TPP	This study
MRMT5	Mutant in 10403S mariner-based mutant library with enhanced resistance to TPP	This study

Mutant libraries were constructed in the serotype 4b strain F2365 (cheese isolate of the California outbreak) (156) and in the serotype 1/2a strain 10403S (streptomycin resistant derivative of a human skin lesion isolate) (61). Bacteria were grown in brain heart infusion broth (BHI, Difco, Sparks, MD), at 37oC for 36 hours, and stored at –80oC in the presence of 20% glycerol.Following the construction of mutant libraries (see below) mutants that grew on brain heart infusion agar with 5 μg/ml erythromycin (BHI-Em) but not on BHI with10μg/ml (BHI-Km) were inoculated individually with sterile toothpicks in fresh BHI and grown at 37oC for 36 hours. Mutant libraries were stored in 96-well microtiter plates at –80oC.

3.2.2. Construction of mutant libraries

In the construction of mutant library of F2365 and 10403S, plasmid pMC38 carrying a mariner-based transposon system (TC1/mariner) (24) was used. The pMC38 plasmid DNA

was kindly provided by Dr. Marquis (Cornell University). Construction of mutant library was done as previously described (24).

3.2.3. Mutant library screening procedures

Mutant libraries were screened as described below for the impaired or enhanced resistance to tetraphenylphosphonium chloride (TPP) (Sigma). For the change resistance, 960 mutants of F2365 and 960 mutants of 10403S were screened. The mutants grown in BHI on 96-well microtiter plates were spotted on BHI supplemented with10, 20, 40, 60,100,150, 200, 400µM/ml, respectively, using a sterile 48-pin replicator. The plates were air-dried and incubated at 37oC for 2 days.

3.2.4. Southern blots and determination of transposon insertion sites

The number of transposon insertion for selected mutants were determined by Southern blots as previously described (6). Primers used to obtaine a mariner-based transposon probe are listed in Table 2. The PCR products were labeled with digoxigenin (Genius kit; Roche, Indianapolis, IN). The labeled DNA was stored at –20oC. The genomic DNA of selected mutants was isolated by using DNeasy kit (Qiagen, Valencia, CA). The genomic DNAs were digested by restriction enzyme, Hind III (New England Biolabs, Waverly, MA). X-ray film (Fuji) was exposed to the chemiluminescent light resulting from hybridization of DNA fragments labeled with the probe. Transposon insertion site was determined by sequencing DNA fragments amplified by arbitrary PCR as previously described by Cao et al. (24). The arbitrary primers are listed in Table 3.2.2.

Table 3.2.2.Primers used in this study

Primer	Sequence (5’ to 3’)	Reference
Maq205	GGT ATA GCA TAT GAATCG CAT CCG ATT GCA G	(6)
Maq254	TGT CAG ACA TAT GGG CAC ACG AAA AAC AAG T	(6)
Marq207	GGC CAC GCG TCG ACT AGT ACG TAA T	(24)
Marq208	GGC CAC GCG TCG ACT AGTAC	(24)
Marq255	CAG TAC AAT CTGCTC TGA TGC CGC ATA GTT	(24)
Marq269	CTAGAACACAAGCGACTACAG	(24)
Marq256	TAG TTA AGC CAG CCC CGA CAC CCG CCA ACA	(24)
Marq270	TGT GAA ATA CCG CAC AGA TGC GAA GGG CGA	(24)

After purification of the PCR products by QIAquick® PCR Purification Kit (Qiagen, MA), they were sequenced using Marq257 and Marq271 for the left and right end of the transposon, respectively. The sequencing of the PCR products was performed at Genomic Science Laboratory at North Carolina StateUniversity (Raleigh, NC) and at Genewiz, Inc. (South Plainfield, NJ).

3.3. Co-selection of cadmium and benzalkonium chloride resistance in conjugative transfers from non-pathogenic *Listeria* spp. to other listeriae

3.3.1. **Bacterial strains and growth conditions.** *Listeria* spp. strains employed in this study are listed in Table 3.3.1.

Table 3.3.1.Bacterial strains employed in this study

Strain	Resistance[1]	Genotype[2]			Source or reference
Donor strains		*cadA1*	*cadA2*	*bcrABC*	
L. innocua					
L0910	Cd^R	-	-	-	This study
L1214B	Cd^R	+	-	-	This study
L1306A	Cd^R	+	-	-	This study
CLIP 11262	Cd^R	-	+	-	(16)
L1221	Cd^R BC^R	-	+	+	This study
L1333a	Cd^R	-	+	-	This study
L0921	Cd^R	+	-	-	This study
L. welshimeri					
L0725	Cd^R BC^R	-	+	+	This study
L1325	Cd^R BC^R	-	+	+	This study
L0918	Cd^R BC^R	-	+	+	This study
L0926	Cd^R BC^R	-	+	+	This study
Recipient strains					
L. innocua					
L1206S	Str^R	-	-	-	This study
L. welshimeri					
L1316S	Str^R	-	-	-	This study
L0927S	Str^R	-	-	-	This study
L. monocytogenes (serotype)					
1/2a3 (1/2a)	Str^R	-	-	-	(22)
10403S (1/2a)	Str^R	-	-	-	(37)
2381L (4b)	Str^R	-	-	-	(39)
2857S (1/2a)	Str^R	-	-	-	This study
2858S (1/2b)	Str^R	-	-	-	This study

[1] Cd^R, BC^R and Str^R indicate resistance to cadmium, BC, and streptomycin, respectively, determined as described in Materials and Methods.

[2] Determined by PCR with the respective primers as described in Materials and Methods.

With the exception of the reference strain *L. innocua* CLIP 11262 (83), the non-pathogenic *Listeria* spp. strains (*L. innocua* and *L. welshimeri*) were isolated from the environment of turkey processing plants in the United States between 2003 and 2005. Isolation and characterization of *Listeria* spp. from these processing plants will be described elsewhere. Non-pathogenic *Listeria* spp. used as recipients was streptomycin-resistant derivatives of *L. welshimeri* and *L. innocua* (Table 4.3.1). Spontaneous mutants with resistance to streptomycin (MIC> 600 µg/ml) were isolated on brain heart infusion agar plates (BHIA) (BHI broth [BHI; Becton, Dickinson and Co. Sparks, MD] and 1.2% Bacto agar [Becton, Dickinson and Co.] with streptomycin sulfate (600 µg/ml; Sigma, St Louis, MO). In addition,

five *L. monocytogenes* strains were used as potential recipients. These included the streptomycin-resistant serotype 1/2a strains 1/2a3 and 10403S which have been extensively used in laboratory investigations (114, 126, 175) and the serotype 4b strain 2381L, a streptomycin-resistant derivative of a strain from the 1985 California outbreak (178). In addition, we employed strains 2857S and 2858S, streptomycin-resistant derivatives of the serotype 1/2a strain 2011L-2857 and the serotype 1/2b strain 2011L-2858, implicated in the 2011 cantaloupe outbreak (32). These streptomycin-resistant derivatives (streptomycin MIC> 600 μg/ml) were obtained as described above. Bacteria were grown in BHI or on BHIA and preserved at -80°C as described (64).

3.3.2. Cadmium and BC susceptibility and determinations of MIC. BC and cadmium susceptibility assessments were done as described (154). Strains were considered resistant to cadmium when they yielded confluent growth in the presence of 35 μg/ml cadmium chloride anhydrous (Sigma) following incubation at 37°C for 48 h. For cadmium MIC determinations, 10 μl of an overnight culture was spotted on BHIA plates with variable concentrations of cadmium chloride (2.5, 5, 10, 20, 35, 70, 140 and 200μg/ml), the plates were incubated at 25°C and observed daily for 5 days. The MIC was defined as the lowest concentration of cadmium which prevented visible growth. MIC of benzalkonium chloride (BC; Acros, New Jersey) was determined as described (31) using variable concentrations of BC (0.1, 0.5, 2.5, 5, 10, 20, 35 and 40 μg/ml) and following incubation of the plates at 37°C for 48 h.

3.3.3. Conjugations. Cultures of recipient and donor strains were grown overnight (18 h) at 37°C and mixed in a 1:10 donor to recipient ratio for conjugations. Filter matings were done as described (114). Briefly, the mixture (100 μl donor and 900 μl recipient) was centrifuged (6000 rpm, 3 min) and resuspended in 100 μl BHI which was then spotted onto sterile membrane filters (0.45μm; Millipore Corp., Bedford, MA) and incubated at the indicated temperature for 24 h. Agar matings employed the same 1:10 donor to recipient ratio; following centrifugation and resuspension in BHI (100 μl) the mixture was spotted (50 μl) on BHIA and incubated at the indicated temperature for 24 h. To isolate transconjugants, mating mixtures were rinsed off the membrane filter or, for agar matings, removed off the surface of the agar plates with a sterile glass rod and plated on double selective medium (BHIA with 600 μg/ml streptomycin and 35 μg/ml cadmium chloride), incubated at 25°C, and observed

for up to 96 h. Controls included each of the parental strains plated on the double-selective medium and incubated similarly. Conjugation frequency was determined as the ratio of the number of transconjugants over CFUs of the recipient strain at the end of the conjugation period; CFUs were determined by plating dilutions on BHIA with streptomycin (600μg/ml) and incubation at 37°C for 36 h. Experiments were done in duplicate and in at least three independent trials.

3.3.4. Polymerase chain reaction (PCR). Primers used for PCR are listed in Table 3.3.2**.**

Table 2. Primers used in this study

Gene	PCR primer sequence (5'–3')	Target gene and protein function	GenBank accession no.	Reference
cadA-Tn5422F	CAGAGCACTTTACTGACCATCAATCGTT	Tn*5422*-associated *cadA* (*cadA1*)	L28104	(32)
cadA-Tn5422R	CTTCTTCATTTAACGTTCCAGCAAAAA			
cadA-pLM80F	ACAAGTTAGATCAAAAGAGTCTTTTATT	*cadA* on pLM80 (*cadA2*)	AADR01000058	(32)
cadA-pLM80R	ATCTTCTTCATTTAGTGTTCCTGCAAAT			
cadA-EGDeF	TGGTAATTTCTTTAAGTCATCTCCCATT	*cadA* of strain EGD-e (*cadA3*)	AL591824	(32)
cadA-EGDeR	GCGATGATTGATAATGTCGATTACAAAT			
BcF	GAATGGATCCTTCAATTAGATCGAGGCACG	bcrABC	AADR01000058	(12)
BcR	GTATGAATTCGTATAATCCGGATGCTGCCC			
hlyF	CGGAGGTTCCGCAAAAGATG	*hly*		(15)
hlyR	CCTCCAGAGTGATCGATGTT			
Lw_0908_F	CTTCTCTTTCAGTAGACAGTGAAGC	*L. welshimeri*-specific sequence	NC_008555	(18)
Lw_0908_R	CCATCCTTGTTTCGCCCTTCTTC			
Li_0558_F	GGTGTCTACTTTGTAACGGCCAC	*L. innocua*-specific sequence	NC_003212.	(16)
Li_0558_R	CGGTAGCTTTTGTTAGCATCCCTG			
pLM80-0004F	CATAGCGATCATACGCATTG	LMOh7858_pLM80_0004 (hypothetical protein)	AADR01000010	This study
pLM80-0007R	AGCAGCATGTAGAAGCAGC			
pLM80-0007F	CTCAACACTAAGTCGGTACC	LMOh7858_pLM80_0007 (hypothetical protein)	AADR01000010	This study
pLM80-0007R	CCATTCAAGCTGCGAAAG			
pLM80-0008F	CCCAAGTTTTCCAGTAGCTG	LMOh7858_pLM80_0008 (conserved hypothetical protein)	AADR01000010	This study
pLM80-0008R	AGGATACACAAGCTCGGAAG			

pLM80-0010F	CTTCACTCCCACGTGTCTCA	LMOh7858_pLM80_00010 (secretion system protein)	AADR01000010	This study
pLM80-0010R	CGAGAACTGATTGCTGCTGA			
pLM80-0012F	CATCGCAATGGTATAGCC	LMOh7858_pLM80_0012 (conserved hypothetical protein)	AADR01000010	This study
pLM80-0012R	CTGGGTAAGAATTGCTG			
pLM80-0017F	GCTGTATATCACTGGTATCC	LMOh7858_pLM80_00017 (hypothetical protein)	AADR01000010	This study
pLM80-0017R	GCAAAGTTGGACCACTTGG			
pLM80-0022F	GCAACAGGTCTATCCCCAAA	LMOh7858_pLM80_00022 (TraG/TraD family protein)	AADR01000010	This study
pLM80-0022R	CCAATCGCAAGGTTTTCCTA			
pLM80-0026F	GATATGGAACATTCCACGCC	LMOh7858_pLM80_0026 (conserved hypothetical protein)	AADR01000010	This study
pLM80-0026R	GCTGGTAAGTCTACTACG			
pLM80-0031F	CAATGGCTCTACCATGATTG	LMOh7858_pLM80_0031 (conserved hypothetical protein)	AADR01000010	This study
pLM80-0031R	CCACGATAACTTGAGTGATGAG			
pLM80-0035F	GGAGAATGACTTGGCTGC	LMOh7858_pLM80_0035 (hypothetical protein)	AADR01000010	This study
pLM80-0035R	GGGCTATCAATCAGCTAC			
pLM80-0038F	GCTCCTCTAAGATCATACTGC	LMOh7858_pLM80_0038 (conserved hypothetical protein)	AADR01000010	This study
pLM80-0038R	GGATTATCTCATGCGACAC			
pLM80-0040F	GCTTCCTGATCCACCAAAAA	LMOh7858_pLM80_0040 (adenine methyltransferase, putative)	AADR01000010	This study
pLM80-0040R	ATCCAATGGTCAGGAGATCG			
pLM80-0049F	GCTTCCTGGTTAAATCCATC	LMOh7858_pLM80_0049 (pli0073)	AADR01000010	This study
pLM80-0049R	GACAACTTGCGAGATATGTC			
pLM80-0053F	CCTTACTTACCAAATGGCC	LMOh7858_pLM80_0053 (plasmid replication protein, putative)	AADR01000010	This study
pLM80-0053R	GTCATGGATAGTTCTCTCC			

.M80-0054F	GCAGGTCAAGTTGAAACTCG	LMOh7858_pLM80_0054 (DNA-dependent ATPase, SNF2 family protein)	AADR01000010	This study
.M80-0054R	GCAATCAGCTGATGATTACG			
.M80-0056F	AATCTCCTTTAGGGTTACGC	LMOh7858_pLM80_0056 (type III restriction system methylase)	AADR01000010	This study
.M80-0056R	TGATGTTCACTCACGTCAAG			
.M80-0058F	TCCAGCATCACTCGCAAAG	LMOh7858_pLM80_0058 (type III restriction system endonuclease)	AADR01000010	This study
.M80-0058R	TCAACTAGATGCCGTAGAGG			
.M80-0083F	GAAGTGTTTGCAGGAACAC	LMOh7858_pLM80_0083 (*cadA*, *cadA2* cation-transporting ATPase, E1-E2 family)	AADR01000010	This study
.M80-0083R	GAAGCTAATGGGTGTTGAG			
.M80-0090F	GTGAATGAGCTCATCCAG	LMOh7858_pLM80_0090 (ImpB/MucB/SamB family protein)	AADR01000058	This study
.M80-0090R	GTATCGACGTGATGATTACG			
.M80-0092F	CCGTTGACATCAAACC	LMOh7858_pLM80_0092 (replication-associated protein)	AADR01000058	This study
.M80-0092R	GTTGGCAAAGAAAGGC			
.M80-0093F	GGACACCGAATACATACCGA	LMOh7858_pLM80_0093 (plasmid replication protein)	AADR01000058	This study
.M80-0093R	TTTCCCATTCGCTAAAATGC			

Identification of the three different *cadA* determinants used primers *cadA*-Tn*5422*F and *cadA*-Tn*5422*R for *cadA1*, associated with Tn*5422*; *cadA*-pLM80F and *cadA*-pLM80R for *cadA2*, harbored on pLM80; *cadA*-EGDeF and *cadA*-EGDeR for *cadA3*, harbored by EGDe (155). Primers BcF and BcR were used to produce a PCR fragment containing the entire *bcrABC* cassette along with the ca. 800-nucleotide upstream intergenic region (64). PCR for *hly*, encoding the *L. monocytogenes* virulence determinant Listeriolysin O employed primers *hlyA*F and *hlyA*R (15) (Table 2). *L. welshimeri* was differentiated from *L. innocua* using primers Lw_0908_F and Lw_0908_R (*L. welshimeri*-specific) as well as primers Li_0558_F and Li_0558_R (*L. innocua*-specific) (Table 4.3.2), derived from genome sequences specific to the corresponding species (83, 99). Besides *cadA2* and *bcrABC* (harbored on pLM80), we employed a panel of several additional primer pairs to screen for other pLM80 ORFs representing diverse locations on both fragments of the plasmid (Table 4.3.2) (33). PCR was done as previously described (64, 154) using the Takara Ex Taq kit (Takara, Madison, WI).

3.4. Resistance gene distribution among non-pathogenenic *Listeriae* from different poultry processing plants

3.4.1. Bacterial strains and growth conditions. The nonpathogenic *Listeria* spp strains used in this study are listed in Tables 3.4.1a and 3.4.1b.

Table 3.4.1a. *L. innocua* isolates used in this study

L.innocua	Resistance	State	Date	Plant code	Source	cadA	bcrABC	APAI	ASCI
250a-1	Cd, BC	ND	10/12/2004	F	Enviroment	1	+		
#4MI21	Cd, BC, As	MI	12/16/2004	C	Enviroment	1	+		
244a-1	Cd, BC	ND	10/12/2004	F	Enviroment	1	+		+
282b-1	Cd, BC	NC	6/15/2004	A	Enviroment	1	+		+
#8MI21	Cd, BC, As	MI	11/29/2005	C	Enviroment	1	+		
#1MI1	Cd	MI	4/27/2004	C	Enviroment	1	+		+
297b-1	Cd, BC	ND	11/2/2004	F	Enviroment	1	+		
306a-1	Cd, BC	ND	11/2/2004	F	Enviroment	1	+		+
45-1b	Cd, BC	ND	11/10/2003	F	Enviroment	1	+		
45-1a	Cd, BC	ND	11/10/2003	F	Enviroment	1	+		+
#1MI2	Cd	MI	4/27/2004	C	Enviroment	1	-		
2#MI32	Cd, BC	MI	4/27/2004	C	Enviroment	1	+		
#4MI40	Cd, As	MI	12/16/2004	C	Food	1	-		
L1219	Cd, BC, As	VA	1/18/2005	B	Enviroment	1	+	+	+
L1306a	Cd	VA	2/28/2005	B	Enviroment	1	-	+	+
L1507	Cd, BC	VA	3/9/2005	E	Enviroment	1	+	+	+
L1814b	Cd, BC	VA	6/7/2005	E	Enviroment	1	+		
#5MI10	Cd, BC	MI	4/12/2005	C	Enviroment	1	+		
#6MI12	Cd, BC, As	MI	6/15/2005	C	Enviroment	1	+		
#7MI18	Cd, BC	MI	9/13/2005	C	Enviroment	1	+		+
#1MI11	Cd, BC	MI	4/27/2004	C	Enviroment	1	+		
LO921	Cd, BC	VA	8/19/2004	B	Enviroment	1	+	+	+
L1310	Cd, BC	VA	4/5/2005	B	Enviroment	1	+	+	+
L1312	Cd, BC	VA	4/5/2005	B	Enviroment	1	+	+	+
L1306b	Cd	VA	4/5/2005	B	Enviroment	1	-	+	+
L1201a	Cd, BC	VA	4/5/2005	B	Enviroment	1	+	+	+
L1214b	Cd, BC	VA	4/5/2005	B	Enviroment	1	+	+	+
L1206	Cd	VA	4/5/2005	B	Enviroment	1	-	+	+
L1501	Cd	VA	4/5/2005	E	Enviroment	1	-	+	+
L1307a	Cd	VA	4/5/2005	B	Enviroment	1	-	+	+
L1307b	Cd, BC	VA	4/5/2005	B	Enviroment	1	+	+	+

L1223	**Cd**	**VA**	**4/5/2005**	**B**	**Enviroment**	**1**	**-**	**+**	**+**
L1310	**Cd, BC**	**VA**	**4/5/2005**	**B**	**Enviroment**	**1**	**+**	**+**	**+**
L1333b	**Cd**	**VA**	**4/5/2005**	**B**	**Enviroment**	**1**	**-**	**+**	**+**
230a-1	**Cd, BC**	**NC**	**8/23/2004**	**A**	**Enviroment**	**1, 2**	**+**		
LO901	**Cd, BC**	**VA**	**8/19/2004**	**B**	**Enviroment**	**1, 2**	**+**	**+**	**+**
210b-2	**Cd, BC**	**NC**	**6/15/2004**	**A**	**Enviroment**	**1, 2**	**+**		
141a-1	**Cd, BC**	**NC**	**2/10/2004**	**A**	**Enviroment**	**1, 2**	**+**	**+**	
#4MI12	**Cd, BC**	**MI**	**12/16/2004**	**C**	**Enviroment**	**1, 2**	**+**		
L1816a	**Cd**	**VA**	**6/7/2005**	**E**	**Enviroment**	**1, 2**	**-**		**+**
10-1a	**Cd, BC**	**NC**	**9/24/2003**	**A**	**Enviroment**	**2**	**+**		
10-1b	**Cd, BC**	**NC**	**9/24/2003**	**A**	**Enviroment**	**2**	**+**		
104b-1	**Cd, BC**	**NC**	**12/3/2003**	**A**	**Enviroment**	**2**	**+**		
128a-1	**Cd, BC**	**NC**	**2/10/2004**	**A**	**Enviroment**	**2**	**+**		
138a-1	**Cd, BC**	**NC**	**2/10/2004**	**A**	**Enviroment**	**2**	**+**		
158a-1	**Cd, BC**	**NC**	**4/13/2004**	**A**	**Enviroment**	**2**	**+**		
176a-3	**Cd, BC**	**NC**	**4/13/2004**	**A**	**Enviroment**	**2**	**+**		
231b-3	**Cd, BC**	**NC**	**8/23/2004**	**A**	**Enviroment**	**2**	**+**		
240a-1	**Cd, BC**	**NC**	**8/23/2004**	**A**	**Enviroment**	**2**	**+**		
275a-1	**Cd, BC**	**NC**	**10/21/2004**	**A**	**Enviroment**	**2**	**+**		
283a-5	**Cd, BC**	**NC**	**10/21/2004**	**A**	**Enviroment**	**2**	**+**		
34-1a	**Cd, BC**	**NC**	**9/24/2003**	**A**	**Enviroment**	**2**	**+**	**+**	**+**
367a-2	**Cd, BC**	**NC**	**4/5/2005**	**A**	**Enviroment**	**2**	**+**		
82a-1	**Cd**	**NC**	**12/3/2003**	**A**	**Enviroment**	**2**	**-**		
L1624a-1	**Cd, BC**	**VA**	**5/18/2005**	**B**	**Food**	**2**	**+**		
L1628a-1	**Cd, BC**	**VA**	**5/18/2005**	**B**	**Enviroment**	**2**	**+**		
L1916a-1	**Cd, BC**	**VA**	**9/8/2005**	**B**	**Enviroment**	**2**	**+**		
L1930b-1	**Cd, BC**	**VA**	**9/8/2005**	**B**	**Enviroment**	**2**	**+**		
L1935a-1	**Cd, BC**	**VA**	**9/8/2005**	**B**	**Enviroment**	**2**	**+**		
L1939b-1	**Cd, BC**	**VA**	**9/8/2005**	**B**	**Enviroment**	**2**	**+**		
453a-1	**Cd, BC**	**ND**	**10/26/2005**	**F**	**Enviroment**	**2**	**+**		
L1606a-1	**Cd, BC**	**VA**	**5/18/2005**	**B**	**Enviroment**	**2**	**+**		
#2MI17	**Cd, BC**	**MI**	**7/27/2004**	**C**	**Enviroment**	**2**	**+**		
L1333a	**Cd**	**VA**	**2/28/2005**	**B**	**Enviroment**	**2**	**-**	**+**	**+**
L1712b	**Cd**	**VA**	**6/6/2005**	**D**	**Enviroment**	**2**	**-**	**+**	**+**
#7MI11	**Cd, BC**	**MI**	**9/13/2005**	**C**	**Enviroment**	**2**	**+**		

290b-1	**Cd, BC**	**ND**	**11/2/2004**	**F**	**Enviroment**	**2**	**+**		
204 b-1	**BC, As**	**NC**	**6/15/2004**	**A**	**Enviroment**	**2**	**+**		
L1921a-1	**Cd, BC**	**VA**	**9/8/2005**	**B**	**Enviroment**	**2**	**+**		
452a-1	**Cd, BC**	**ND**	**10/26/2005**	**F**	**Enviroment**	**2**	**+**		
240b-1	**Cd, BC**	**NC**	**8/23/2004**	**A**	**Enviroment**	**2**	**+**	**+**	**+**
L1221	**Cd, BC, As**	**VA**	**1/18/2005**	**B**	**Enviroment**	**2**	**+**	**+**	**+**
Table 4.4.1a. continuing									
L1724a	**Cd, BC**	**NC**	**6/6/2005**	**A**	**Enviroment**	**2**	**+**		
L0909	**Cd**	**VA**	**12/9/2004**	**B**	**Enviroment**	**2**		**+**	**+**
L0910	**Cd**	**VA**	**12/9/2004**	**B**	**Enviroment**	**2**	**-**	**+**	**+**
L0923	**Cd**	**VA**	**12/9/2004**	**B**	**Enviroment**	**2**	**-**	**+**	**+**
L0924	**Cd**	**VA**	**12/9/2004**	**B**	**Enviroment**	**2**	**-**	**+**	**+**
L0919	**Cd**	**VA**	**12/9/2004**	**B**	**Food**	**2**	**-**	**+**	**+**
232a-1	**Cd, BC**	**NC**	**8/23/2004**	**A**	**Enviroment**	**4**	**+**		
223a-5	**Cd, BC**	**NC**	**8/23/2004**	**A**	**Enviroment**	**ND**	**+**		
22-1a	**Cd**	**NC**	**9/24/2003**	**A**	**Enviroment**	**ND**	**-**		
206b-1	**Cd, BC**	**NC**	**6/15/2004**	**A**	**Enviroment**	**ND**	**+**		
174a-1	**Cd, BC**	**NC**	**4/13/2004**	**A**	**Enviroment**	**ND**	**+**		
248a-1		**ND**	**10/12/2004**	**F**	**Enviroment**	**ND**	**-**		
43-1a	**BC**	**ND**	**11/10/2003**	**F**	**Enviroment**	**ND**	**+**		
206 a-1	**Cd, BC**	**NC**	**6/15/2004**	**A**	**Enviroment**	**ND**	**+**		
172a-1	**BC**	**NC**	**4/13/2004**	**A**	**Enviroment**	**ND**	**+**		
285a-1	**BC**	**ND**	**11/2/2004**	**F**	**Enviroment**	**ND**	**+**		
L1604b-1	**-**	**VA**	**5/18/2005**	**B**	**Enviroment**	**ND**	**-**		
L1318b	**BC**	**VA**	**4/5/2005**	**B**	**Enviroment**	**ND**	**+**	**+**	**+**
LO713	**Cd**	**VA**	**5/18/2004**	**B**	**Enviroment**	**ND**	**-**		
LO907	**Cd**	**VA**	**8/19/2004**	**B**	**Enviroment**	**ND**	**-**	**+**	**+**
L1601b	**Cd, BC**	**VA**	**5/18/2005**	**B**	**Enviroment**	**ND**	**+**		
L1210a	**Cd, BC**	**VA**	**4/5/2005**	**B**	**Enviroment**	**ND**	**+**	**+**	**+**
L0913	**-**	**VA**	**12/9/2004**	**B**	**Enviroment**	**ND**	**-**	**+**	**+**
L1328a	**BC**	**VA**	**4/5/2005**	**B**	**Enviroment**	**ND**	**+**	**+**	**+**

Table 3.4.1.b *L. welshimeri* isolates used in this study

L. welshimeri	Resistance	State	Date	Plant code	Source	cadA	bcrABC	APAI	ASCI
110b-1	Cd, As	NC	2/10/2004	A	Enviroment	1	-		
117pa-1	Cd	NC	2/10/2004	A	Enviroment	1	-		
192a-2	Cd	NC	6/15/2004	A	Enviroment	1	-		
254a-1	Cd, BC	ND	10/12/2004	F	Enviroment	1	+		
290a-1	Cd, BC	ND	11/2/2004	F	Enviroment	1	+		
297a-1	Cd, BC	ND	11/2/2004	F	Enviroment	1	+		
312a-1	Cd, BC	ND	11/2/2004	F	Enviroment	1	+		
50-1a	Cd, BC	ND	11/10/2003	F	Enviroment	1	+		+
L1905a-1	Cd	VA	9/8/2005	B	Enviroment	1	-		
414a-2	Cd, BC	ND	10/13/2005	F	Enviroment	1	+		
425b-1	Cd, BC	ND	10/13/2005	F	Enviroment	1	+		
428a-1	Cd, BC	ND	10/13/2005	F	Enviroment	1	+		
475a-1	Cd, BC	ND	11/9/2005	F	Enviroment	1	+		
LO903	Cd, BC	VA	8/19/2004	B	Enviroment	1	+	+	+
139a-1	Cd, BC	NC	2/10/2004	A	Enviroment	1, 2	+	+	
143a-1	Cd	NC	2/10/2004	A	Enviroment	1, 2	-	+	
230b-2	Cd, BC	NC	8/23/2004	A	Enviroment	1, 2	+	+	
#1MI19	Cd, BC	MI	4/27/2004	C	Enviroment	1, 2	+	+	
313b-1	Cd, As	ND	11/2/2004	F	Enviroment	1, 2	-		
231a-4	Cd, BC	NC	8/23/2004	A	Enviroment	2	+		
268a-1	Cd, BC	NC	10/21/2004	A	Enviroment	2	+		
209b-1	Cd, BC	NC	6/15/2004	A	Enviroment	2	+		
102a-1	Cd, BC	NC	12/3/2003	A	Enviroment	2	+	+	+
105a-1	Cd, BC	NC	12/3/2003	A	Enviroment	2	+		
61-1b	Cd, BC	ND	11/10/2003	F	Enviroment	2	+	+	+
L1601a-1	Cd, As	VA	5/18/2005	B	Enviroment	2	-		+
L1606b-1	Cd70, BC	VA	5/18/2005	B	Enviroment	2	+		
L1625b-1	Cd, BC	VA	5/18/2005	B	Food	2	+		+
L1908a-1	Cd, BC	VA	9/8/2005	B	Enviroment	2	+		

L1916a-1	**Cd, BC**	**VA**	**9/8/2005**	**B**	**Enviroment**	**2**	**+**		
L1921b-1	**Cd, BC**	**VA**	**9/8/2005**	**B**	**Enviroment**	**2**	**+**		**+**
L1936a-1	**Cd, BC**	**VA**	**9/8/2005**	**B**	**Enviroment**	**2**	**+**		
Table 4.4.1b. continuing									
439a-1	**Cd, BC**	**ND**	**10/26/2005**	**F**	**Enviroment**	**2**	**+**	**+**	**+**
487a-2	**Cd, As, BC**	**NC**	**3/29/2006**	**A**	**Enviroment**	**2**	**+**		**+**
#104	**Cd, BC**	**NC**	**12/3/2003**	**A**	**Enviroment**	**2**	**+**		
#34	**Cd, BC**	**NC**	**9/24/2003**	**A**	**Enviroment**	**2**	**+**		
LO725	**Cd, BC**	**VA**	**5/18/2004**	**B**	**Food**	**2**	**+**	**+**	**+**
LO926	**Cd, BC**	**VA**	**12/9/2004**	**B**	**Food**	**2**	**+**	**+**	**+**
L1228a	**Cd, BC**	**VA**	**1/18/2005**	**B**	**Food**	**2**	**+**	**+**	**+**
L1625a	**Cd, BC**	**VA**	**5/18/2005**	**B**	**Food**	**2**	**+**		
L1730	**Cd, BC**	**VA**	**6/6/2005**	**D**	**Enviroment**	**2**	**+**		
#6MI19	**Cd, BC**	**MI**	**2/28/2005**	**C**	**Enviroment**	**2**	**+**		**+**
#6MI26	**Cd, BC, As**	**MI**	**6/15/2005**	**C**	**Enviroment**	**2**	**+**		**+**
L1208	**Cd, BC**	**VA**	**4/5/2005**	**B**	**Enviroment**	**2**	+	**+**	**+**
L1225	**Cd, BC**	**VA**	**4/5/2005**	**B**	**Enviroment**	**2**	+	**+**	**+**
L1228b	**Cd, BC**	**VA**	**4/5/2005**	**B**	**Food**	**2**	+	**+**	**+**
L1325	**Cd, BC**	**VA**	**4/5/2005**	**B**	**Enviroment**	**2**	+	**+**	**+**
L0914	**Cd, BC**	**VA**	**12/9/2004**	**B**	**Enviroment**	**2**	+	**+**	**+**
L0918	**Cd, BC**	**VA**	**12/9/2004**	**B**	**Food**	**2**	+	**+**	**+**
L0926	**Cd, BC**	**VA**	**12/9/2004**	**B**	**Food**	**2**	+	**+**	**+**
L0922	**Cd, BC**	**VA**	**12/9/2004**	**B**	**Enviroment**	**2**	+	**+**	**+**
202a-1	**Cd, BC**	**NC**	**6/15/2004**	**A**	**Enviroment**	**ND**	**+**	**+**	**+**
342a	**Cd, BC**	**NC**	**12/14/2004**	**A**	**Enviroment**	**ND**	**+**	**+**	**+**
361a-1	**Cd, BC**	**NC**	**4/5/2005**	**A**	**Enviroment**	**ND**	+		
405a-1	**Cd**	**ND**	**10/13/2005**	**F**	**Enviroment**	**ND**	-		
430a-1	**Cd**	**ND**	**10/26/2005**	**F**	**Enviroment**	**ND**	-		
232b-2	**BC**	**NC**	**8/23/2004**	**A**	**Enviroment**	**ND**	+		
L1203	**Cd, BC**	**VA**	**1/18/2005**	**B**	**Enviroment**	**ND**	+	**+**	**+**
L1316	**-**	**VA**	**2/28/2005**	**B**	**Enviroment**	**ND**	-	**+**	**+**

L1604a-1	**Cd,BC, As**	**VA**	**5/18/2005**	**B**	**Enviroment**	**ND**	+		
L1315	**BC**	**VA**	**2/28/2005**	**B**	**Enviroment**	**ND**	+	+	+
#138	**Cd, BC**	**NC**	**2/10/2004**	**A**	**Enviroment**	**ND**	+	+	+
210a-1	**Cd, BC**	**NC**	**6/15/2004**	**A**	**Enviroment**	**ND**	+	+	
223b-5	**Cd, BC**	**NC**	**8/23/2004**	**A**	**Enviroment**	**ND**	+	+	+
239a-3	**Cd, BC**	**NC**	**8/23/2004**	**A**	**Enviroment**	**ND**	+	+	+
L0927	**-**	**VA**	**12/9/2004**	**B**	**Enviroment**	**ND**	-	+	+

The nonpathogenic strains (n=162) were primarily contributed by four plants (A, B, C, and F): most strains were *L. innocua* and *L. welshimeri* (Table 4.4.2).

Table 3.4.2.Plant-specific distribution of the strains used in this study

Plant[1]	**Species**	
	L.welshimeri	***L. innocua***
A (n=50)	**22**	**28**
B (n=64)	**26**	**38**
C (n= 16)	**3**	**13**
D (n= 5)	**1**	**4**
E (n= 4)	**0**	**4**
F (n=25)	**14**	**11**
Total (n=162)	**66**	**96**

[1]In addition to the 162 isolates, 8 isolates (4 from plant A and 1 each from B, C, D, and F) were unidentified *Listeria* spp.

Listeriae were routinely grown in brain heart infusion broth (BHI; Difco, Sparks, MD), BHI supplemented with 1.2% agar (Difco), or Trypticase soy broth with 0.6% yeast extract (TSBYE; Becton, Dickinson and Co., Sparks, MD) at 37°C.

3.4.2. Determination of susceptibility to cadmium, sodium arsenite and BC. Resistance to cadmium, arsenic, and BC was assessed as described previously (154). Isolates were considered resistant to cadmium and arsenic if they yielded confluent growth on Iso-Sensitest

agar (ISA) (Oxoid, Hampshire, England) supplemented with 70µg/ml cadmium chloride anhydrous (Sigma, St. Louis, MO) or 500ug/ml sodium arsenite (Fluka, Steinheim, Germany), respectively, following incubation at 37°C for 48 h. BC resistance was assessed on Mueller-Hinton agar (MHA) (Mueller-Hinton broth with 1.2% Bacto agar [Becton, Dickinson and Co.]) supplemented with 10 ug/ml of benzalkonium chloride (Acros, Morris Plains, NJ) and 2% sheep blood (BBL, Sparks, MD). The plates were incubated at 37°C for 48 h. Confluent growth at 10ug/ml BC determined strains as resistant to this compound.

3.4.3. DNA extrection. DNA was extracted as described previously (64), and PCRs were performed using the ExTaq kit (Takara, Madison, WI) and a T1 thermal cycler (Biometra, Goettingen, Germany). Primers used in present study (3, 12) were purchased from Eurofin MWG Operon (Huntsville, AL)

3.4.4. PCR. Species designations for *L. welshimeri* and *L. innocua* were confirmed using primers listed in Table 3.4.3.

Table 3.4.3.Primers used in this study

Gene	PCR primer sequence (5' – 3')	Target gene and protein function	GeneBank accession no.	Ref
***CadA* – Tn5422F**	**CAGAGCACTTTACTGACCATCAATCGTT**	**Tn5422-associated *cadA*(*cadA*1)**	**L28104**	**155**
***CadA* – Tn5422R**	**CTTCTTCATTTAACGTTCCAGCAAAAA**	**Tn5422-associated *cadA*(*cadA*1)**		**155**
***CadA* – pLM80F**	**ACAAGTTAGATCAAAAGAGTCTTTTATT**	***cadA*(LMOh7858_pLM80_0083) on pLM80 (*cadA*2)**		**155**
***CadA* – pLM80R**	**ATCTTCTTCATTTAGTGTTCCTGCAAAT**	***cadA*(LMOh7858_pLM80_0083) on pLM80 (*cadA*2)**	**AADR01000058**	**155**
***CadA* - EGDeF**	**TGGTAATTTCTTTAAGTCATCTCCCATT**	***cadA*(lmo1100) in EGD-e (*cadA*3)**		**155**
***CadA* - EGDeR**	**GCGATGATTGATAATGTCGATTACAAAT**	***cadA*(lmo1100) in EGD-e (*cadA*3)**	**AL591977**	**155**
LMOSA_2330F	**GCATACGTACGAACCAGAAG**	***cadA*(LMOSA_2330) on the chromosome of Scott A (*cadA*4)**		**130**
LMOSA_2230R	**CAGTGTTTCTGCTTTTGCTCC**	***cadA*(LMOSA_2330) on the chromosome of Scott A (*cadA*4)**	**AFGI01000005.1**	**130**
BcF	**GAATGGATCCTTCAATTAGATCGAGGCACG**	**bcrABC**	**AADR01000058**	**130**
BcR	**GTATGAATTCGTATAATCCGGATGCTGCCC**	**bcrABC**		**130**
Lw_0908_F	**CTTCTCTTTCAGTAGACAGTGAAGC**	***L. welshimeri*-specific sequence**	**NC_008555**	**117**
Lw_0908_R	**CCATCCTTGTTTCGCCCTTCTTC**	***L. welshimeri*-specific sequence**	**NC_008555**	**117**
Li_0558_F	**GGTGTCTACTTTGTAACGGCCAC**	***L. innocua*-specific sequence**	**NC_003212.**	**117**
Li_0558_R	**CGGTAGCTTTTGTTAGCATCCCTG**	***L. innocua*-specific sequence**	**NC_003212.**	**117**

Detection of the four different *cadA* determinants used primers *cadA*-Tn5422F and *cadA*-Tn5422R for *cadA1*; *cadA*-pLM80F and *cadA*-pLM80R for *cadA2*; *cadA*-EGDeF and *cadA*-EGDeR for *cadA3* and primers LMOSA_2330F and LMOSA_2230R for *cadA4* (130, 155). To obtain PCR fragment with the entire bcrABC cassette along with the 800-nt upstream intergenic region we used primers BcF and BcR (64) (Table 4.4.3.).

3.4.5. Pulsed-field gel electrophoresis (PFGE). Pulsed-field gel electrophoresis (PFGE) was performed following PulseNet protocol with AscI (New England BioLabs, Ipswich, MA) and ApaI (Roche, Indianapolis, IN) (47) For normalization, analysis and matching of PFGE profiles BioNumerics (Applied Maths, Austin, TX) was employed. A dendrogram was generated from AscI and ApaI of 32 *L. innocua and* 24 *L. welshimeri* isolates with 1.5% tolerance window**.**

3.4.6. Statistical Analysis. Fisher's exact test was carried out to determine if distribution of Cd and BC resistance genes is constant across the processing plants. In case when there was evidence of plant to plant variability, pairwise comparisons among plants were made by fitting logistic regression models for each frequency variable. Significant difference ($p < 0.04$) was determined using the SAS program Version 9.1 (Cary, NC).

3.5. Survival and growth of outbreak and other strains of *Listeria monocytogenes* on cantaloupe

3.5.5. Bacterial strains and growing conditions Strains used in the study are listed in Table 3.5.1.

Table 3.5.1. *Listeria monocytogenes* strains used in this study

Strains	Genotype and features	Serotype	Source/ reference
2011L-2857	**Food, cantaloupe outbreak, 2011**	**1/2a**	**CDC**
2011L-2858	**Food, cantaloupe 2011 outbreak**	**1/2b**	**CDC**
2011L-2875	**Food, cantaloupe 2011 outbreak**	**1/2a**	**CDC**
2010L-1723	**Celery outbreak (TX) 2010**	**1/2a**	**Gaul, L.K,**
H7550	**Hot dog outbreak , 1998-1999**	**4b**	**CDC**
J0161	**Turkey deli meats outbreak, 2001**	**1/2a**	**CDC**
F8027	**Food, celery**	**4b**	**Lang, M**

Each bacterial culture was grown overnight at 37°C in brain heart infusion broth (BHI; Becton Dickinson and Co., Sparks, MD) and centrifuged at 7000×*g* (Rotor JA-14, Beckman, Palo Alto, CA, USA) for 10 min. Cells were washed twice in sterile dH_20 and re-suspended in 10ml of sterile de-ionized water.

3.5.2. Cantaloupe preparation and inoculations. For each experiment, conventionally grown cantaloupes (grown in the USA), were purchased at retail and stored at 4° C until used. Cantaloupes were tested for *Listeria* prior to inoculation. Briefly, 12.5gr of fragments containing rind (obtained as described below) were pre-enriched by re-suspending in 112.5 ml Half Frazer Broth (Oxoid LTD, Basingstoke, Hampshire, England)) and incubated at 37ºC for 48 h followed by plating 100ul on the MOX and incubation of 48h at 37ºC. Cantaloupes were cut by a flame-sterilized knife in pieces (2x2x1.0cm each), with the outer surface intact, inner surface (1cm under the rind), respectively, and the rest of flesh was extracted in to a juice using a Juice Extractor (Hamilton Beach Health Smart Juice Extractor, Southern Pines, NC, USA). Fragments were placed on sterile aluminum foil in a biosafety cabinet, pre-wetted with sterile water by spraying, left for 30 minutes to dry, followed by inoculation with different *L monocytogenes* strains, respectively.

Inoculum was prepared by diluting the washed cells in sterile water. A spot-inoculation method was used to inoculate bacteria (approx.10^5 cfu/fragment) on the rind and on the flesh of cantaloupe fragments, as previously described (147). The inoculum (100µl) was deposited as of a series of drops on the surface (rind or flesh). After air drying for 1 hour in the biosafety cabinet at room temperature the inoculated fragments were placed in polystyrene petri dishes sealed with parafilm, followed by incubation at 4, 8 and 25ºC. 10ml aliquots of freshly extracted cantaloupe juice was inoculated for a final concentration of 10^5 cfu/ml. Survival and growth were assessed at 4, 24, 168, 336 and 504 hours at 4 and 8°C; and 4, 8, 24, 72, 168 hours at 25°C. Longer incubations at 25°C were not pursued due to the visible spoilage of the product. At each time point, cantaloupe fragments (two each with inoculums on the rind and on the flesh respectively), were submerged in 10ml of sterile de-ionized water in the sterile 50ml it polypropylene conical tubes (Becton Dickinson and Company, Franklin Lakes, NJ, US) followed by vortexing at high speed for 2 minutes. Serial dilutions were plated on brain heart infusion agar (BHIA; Becton Dickinson and Co., Sparks, MD) for the

enumeration of total aerobes and on Modified Oxford Agar (MOX), (EMD Chemicals Inc., Gibbstown, NJ 08027, USA) supplemented with Modified Oxford Antimicrobic Supplement (Becton,Dickinson and Company, Sparks, MD 21152, USA) for enumeration of *L. monocytogenes*. Serial dilutions from cantaloupe juice were plated for enumeration as described above. Plates were incubated for 48 hours at 37°C and results presented as log CFU / fragment. Experiments were done in duplicate and in at least three independent trials.

3.5.3. Cantaloupe treatment with water: Fragments of cantaloupe were prepared and inoculated with *L. monocytogenes* strains F8027 and 2011L-2858, respectively, as previously described. After 1 hour two fragment of each inoculated strain were washed by submerging sterile dH_2O for 2 minutes with agitation. Fragments were then placed in polystyrene 50ml tubes with 10ml of sterile dH2O followed by 2 minutes of vortexing at high speed. Enumerations were made as described above immediately after washing and following 72 hours at 4, 8 and 25ºC. Fragments inoculated with the same strain but not washed were included as a control.

3.5.4. Statistical analysis Data were analyzed by The Statistical Analysis System (SAS Institute Inc., Cary, N.C.) using linear mixed effects model. Fixed effects of four factors were included, along with all possible interactions among them: strain (3 levels), location (3 levels) time and temperature, which had 16 combinations of levels, visible in the tables below. The experimental design was a randomized complete block with three complete replications of all 144 treatment combinations of the four factors. For each treatment and block (\trial") combination, duplicate measurements were taken, and these are considered subsamples for the purposes of the statistical analysis. Accordingly, random effects were included in the model for block and block-by-treatment interaction.

3.6. Change in phenotype and genotype as an asset for *L. monocytogenes* growth on cantaloupe

3.6.1. Bacterial strains and growing conditions. *L. monocytogenes* strains employed in this study are listed in Table 3.6.1.

Table 3.6.1: *Listeria monocytogenes* strains used in this study

Strains	Genotype and features	Source/ reference
F2365	Cheese isolate from California outbreak	6, 7
H7550	Clinical isolate from hot dog outbreak (1998-1999)	68
J0161	Serotype 1/2a strain from turkey deli meats outbreak, 2001	182
J0161C$_1$	Ciprofloxacin-selected mutant of J0161 Cd^R	31
ROA4	Transposon mutant of F2365 LMO f2365_1746 (helicase domain protein) cold sensitive	6, 7
ROA14	Transposon mutant of F2365*purA*::transposon (pMC38), EmR KmS, blood agar sensitive	6, 7
J22F	Transposon mutant of H7550-Cds,*purB*::transposon (pMC39), EmR KmS, blood agar sensitive	6, 7
J29H	Transposon mutant of H7550-Cds,*hly*::transposon (pMC39), EmR KmS, nonhemolytic	118
J46C	Transposon mutant of H7550-Cds,ORF2753::transposon (pMC39), EmR KmS, phage-susceptible at low temperature	118
F2365Δwap	F2365 with deletion of wap	42, 43
F2365Δ18R	F2365 with deletion of the gene cassette flanking by wap and inlA	42, 43
ECIIΔ18R	H7550 with deletion of the gene cassette flanking by wap and inlA	42, 43

Each bacterial culture was grown overnight at 37°C in brain heart infusion broth (BHI; Becton Dickinson and Co., Sparks, MD) and centrifuged at 7000×*g* (Rotor JA-14, Beckman, Palo Alto, CA, USA) for 10 min. The supernatant was discarded and the cell pellets were washed twice and resuspended in 10ml of sterile de-ionized water, followed by serial dilutions in sterile de-ionized water to the final inoculum concentration of 10^5cfu/fragment on the cantaloupe rind.

3.6.2. Isolation of mutants adapted on ciprofloxacin: Ciprofloxacin-adapted mutants of parental strain J0161 (serotype1/2 a strain involved in turkey deli meats outbreak, 2001) were obtained as previously described (182).

3.6.3. Mutant library constructions and genetic characterizations. The *mariner*-based transposon (24) have been employed to construct mutant libraries of strains F2365 (epidemic clone I, serotype 4b) and H7550 (epidemic clone II, serotype 4b) (1, 2, 23). All analyzed mutants harbored single transposon insertions. Through screenings of the *mariner*-based mutant libraries various mutants have been isolated. A cold-sensitive mutant, with an insertion in a gene encoding a DEAD family RNA helicase (6), and ROA14 with transposon integrated in LMOf2365_0065 (*purA*, adenylosuccinate synthetase), both mutants of strain F2365, and mutants of strain H7550- non-hemolytic mutant (J29H) harboring the transposon into ORF 0222 (*hly*, listeriolysin O) and blood sensitive mutant J22F with the transposon localized into ORF 1898 (*purB*, adenylosuccinate lyase). Non-hemolytic mutant was not able to lyse blood cells on blood agar plates (Tryptic Soy Broth supplemented with 5% of sheep blood, Remel Inc.) and blood-sensitive mutant barely grew on the tryptic soy agar with sheep blood (Remel) but grew well on other agar media, including Tryptic Soy Agar.

3.6.4. Identification of phage-susceptible mutant. One colony of strain H7550, designated J46C, was detected to be a phage susceptible on the spot assay following growth at 25°C. The phage susceptibility was confirmed individually by standard plaque assays.

3.6.5. Construction of deletion mutant of *wap* in F2365 Deletion mutants of *wap* and genomic region 18 were previously constructed in our laboratory as described (42, 43, unpublished).

3.6.6. Preparation of cantaloupe and inoculation of surface. Cantaloupe preparation and inoculation of *L. monocytogenes* on the surface were done using a spot-inoculation method described in the previous chapter.

3.6.7. Cantaloupe treatment with water: Upon the inoculation with selected mutants and parental strains, respectively, fragments of cantaloupe were treated with sterile d-H20 as described in the previous chapter. Fragments inoculated with cold-sensitive mutant (ROA4) were incubated for 504 hours at 4C. At each time point (0, 72, 168 for all strains and 336 and

504 for ROA4 at 4C) two fragments were pull out for plate count on BHI and MOX, in duplicates. Results presented are average between two trials each conducted in duplicate.

3.7. Assessment of *L. monocytogenes* growth in raw and pasteurised milk

3.7.1. Bacterial strains and growing conditions. *L. monocytogenes* strains used in this study are presented in Table 3.7.1.

Table 3.7.1.*L monocytogenes* strains employed in this study

Strain	Country/State	Source	Serotype	Class	Reference
H7550 CdR	USA	Food Hotdog Outbreak (1998-99)	4b	ECII	CDC
H7550CdRC	USA	Ciprofloxacin adapted mutant of strain H7550CdR	4b	ECII	MRM2011
J0161	USA	Food Hotdog Outbreak 2001	1/2a	ND*	CDC
J0161C	USA	Ciprofloxacin adapted mutant of strain J0161	1/2a	ND	MRM2011
2011-2857	NM	Food cantaloupe, 2011 outbreak	1/2a	ND	CDC
2011-2858	NM	Food cantaloupe, 2011 outbreak	1/2b	ND	CDC
2011-2875	NM	Food cantaloupe, 2011 outbreak	1/2a	ND	CDC
2010-1723	TX	Food Chicken salad celery	1/2a	ND	CDC
4b1	Germany	Clinical; Spinal fluid of child with meningitis	4b	ECIa	CDC
SCOTT A	Massachusetts, USA	1983 Outbreak / reference strain for dairy product studies	4b	ECIa	CDC
OLM 121	Ontario, Canada	Clinical; 1957/human	4b	ECIa	OLM
OLM 144	Brazil	Clinical; 1961/human	4b	ECIa	OLM
OLM 10	Massachusetts, USA	Clinical; 1933/child	4b	ECI	OLM
OLM 125	Ontario, Canada	Clinical; 1959/infant	4b	ECI	OLM
OLM 97	Nova Scotia, Canada	Clinical: 1954/human	4b	ECI	OLM
OLM 147	BC, Canada	Clinical; 1961/human	4b	ECI	OLM
OLM 43	Ontario, Canada	1949/cow	4b	ECI	OLM
OLM 143	Ontario, Canada	Clinical; 1961/human	4b	ECI	OLM

*ND – not determined

Set of 18 strains were chosen by the serotype and source. Six of strains were involved in food related outbreaks. 12 were clinical isolates connected to sporadic cases occurred in early 20th century. Each bacterial culture was grown overnight at 37°C in brain heart infusion broth (BHI; Becton Dickinson and Co., Sparks, MD) and centrifuged at 7000×*g* (Rotor JA-14, Beckman, Palo Alto, CA, USA) for 10 min. The supernatant was discarded and the cell pellets

were washed twice and resuspended in 10ml of sterile de-ionized water, followed by serial dilutions in sterile de-ionized water to the final concentration of 10^5cfu/ml, in raw and pasteurized milk, respectively.

3.7.2. Milk: Fresh whole raw and pasteurized milk was obtained from North Carolina State University'sDairy Plant. For each experiment, the raw and pasteurized milk were from the same batch, consisting of bulk tank milk collected from various herds in North Carolina and transported to the dairy plant. Prior to each test milk was screened and confirmed to be free of *L. monocytogenes* by enriching 25ml in 25ml of Fraser broth (Oxoid) at 37°C for 48 hrs.

3.7.3. Survival and growth determinations: Aliquots (10ml) of raw and pasteurized milk were inoculated with 100µl of each *L. monocytogenes* strain, respectively, for the final concentration of 10^5 cfu/ml. Milk was stored at 8C and samples tested each 24 hours over the 10 days of experiment. Serial dilutions were prepared in sterile deionized water and aliquots (0.1ml) plated on BHIA for the enumeration of total aerobes and MOX for *L. monocytogenes* enumeration. Plates were incubated for 48 hours at 37°C and results presented as log CFU/ml. An uninoculated control, in duplicate, at each time point, was used to determine presence of background microflora. Results present average between two independent trials each done in duplicate.

3.7.4. Statistical Analysis. Both trials were performed in duplicate, and the data presented as the standard deviations obtained for the replications. Statistical analysis was performed for multiple comparisons of the means and standard deviations for different variables. Analysis of variance (ANOVA) was carried out to determine the significant difference ($p < 0.04$) using the SAS program Version 9.1 (Cary, NC).

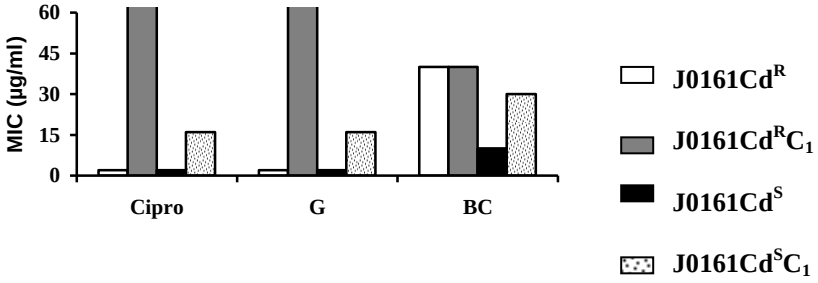
4.0. RESULTS

4.1. Adaptation of *Listeria monocytogenes* to Ciprofloxacin or to the Disinfectant Benzalkonium Chloride Results in Reduced Susceptibility to Ciprofloxacin, Gentamicin Benzalkonium Chloride and Other Toxic Compounds

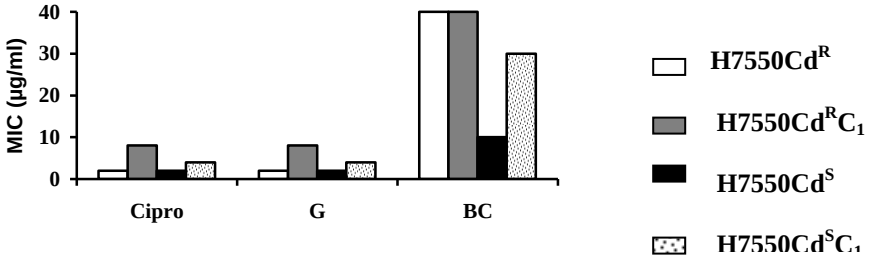
4.1.1. Presence of pLM80 does not impact MICs to antibiotics. *L. monocytogenes* H7550 Cd^R harbors the 80 kb plasmid (pLM80), which contains a cadmium resistance cassette as well as a cassette (*bcrABC*) mediating resistance to BC (4, 24). As expected, MICs to Cd and BC were markedly reduced in the cured derivatives, from 140 to 10µg/ml and from 40 to 10µg/ml, respectively. However, the cured derivatives exhibited the same MICs to the panel of antibiotics, EthBr and to TPP as the parental strains (data not shown). Similar data were obtained with the plasmid-cured derivative of the serotype 1/2a strain J0161, which harbors a pLM80-like plasmid that confers resistance to Cd and BC (15). The findings indicated that these plasmids conferred resistance to Cd and BC, but did not influence MICs to the other antimicrobial agents that were tested.

4.1.2. *L. monocytogenes* mutants selected on ciprofloxacin also exhibit reduced susceptibility to gentamicin. Colonies growing at inhibitory concentrations of ciprofloxacin were selected for further characterization, using two independent ciprofloxacin-selected mutants (C_1 and C_2) for strains H7550, J0161 and SK2802, respectively. Mutants selected on ciprofloxacin exhibited reduced susceptibility to this antibiotic, with four to 32 fold (8 to 64µg/ml) increases in MICs, depending on the strain (Fig.4.1.1A and B); independent mutants of the same strain exhibited similar increases in MIC values (data not shown).

A.



B.



C.

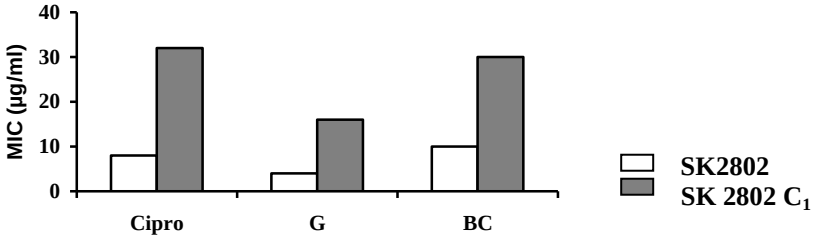


Fig.4.1.1.Mutants selected on ciprofloxacin also exhibit reduced susceptibility to gentamicin and BC. MICs for ciprofloxacin (cipro), gentamicin (G) and benzalkonium chloride (BC) were determined for: A, plasmid-harboring strain J0161 Cd^R (white columns) and its ciprofloxacin-selected mutant J0161 Cd^R C_1 (gray), as well as for the plasmid-cured strain J0161 Cd^S (black) and its ciprofloxacin-selected mutant J0161 Cd^S C_1 (stippled); B, plasmid-harboring strain H7550 Cd^R (white) and its ciprofloxacin-selected mutant H7550 Cd^R C_1 (gray), as well as for the plasmid-cured strain H7550 Cd^S (black) and its ciprofloxacin-selected mutant H7550 Cd^S C_1 (stippled); C. strain SK 2802 (white) and its ciprofloxacin-selected mutant SK 2802 C_1 (gray). MICs were determined as described in Materials and Methods.

Interestingly, even though as described above the presence of the plasmid in H7550 Cd^R and J0161 Cd^R did not impact MICs to any of the tested antibiotics, ciprofloxacin-selected mutants of the plasmid-harboring strains consistently exhibited higher MICs to ciprofloxacin than ciprofloxacin-selected mutants of the corresponding plasmid-cured strains (Fig. 4.1.1A and B). This was especially pronounced with ciprofloxacin-selected mutants of strain J0161 Cd^R, which exhibited a 32 fold increase in MIC, in contrast to the 8 fold increase in MIC observed with ciprofloxacin-selected mutants of J0161 Cd^S (Fig.4.1.1A).

Mutants selected on ciprofloxacin exhibited no change in their susceptibility to ampicillin, rifampin, streptomycin, trimethoprim, erythromycin, tetracycline, fosfomycin or kanamycin (data not shown). However, susceptibility to gentamicin was consistently reduced in all tested ciprofloxacin-selected mutants. As observed with ciprofloxacin MICs, ciprofloxacin-selected mutants of plasmid-harboring strains exhibited higher MICs to gentamicin than mutants of their plasmid-cured counterparts (Fig.4.1.1A and B). This difference was again especially noticeable with J0161 Cd^R C_1, which exhibited a 32 fold increase in MIC to gentamicin in comparison to J0161 Cd^R, whereas J0161 Cd^S C_1 exhibited 8 fold increase in MIC in comparison to J0161 Cd^S (Fig. 4.1.1A). The same trend was observed with independently isolated mutants of the same strain (data not shown). Increases in both ciprofloxacin and gentamicin MICs were also observed with ciprofloxacin-selected mutants of strain SK2802 (Fig.4.1.1C).

4.1.3. *L. monocytogenes* mutants selected for increased resistance to ciprofloxacin also exhibit markedly reduced susceptibility to BC, EthBr and TPP. As discussed above, the plasmid-cured derivatives H7550 Cd^S and J0161 Cd^S exhibited reduced tolerance to BC (MIC, 10µg/ml). However, BC susceptibility determinations of ciprofloxacin-selected mutants H7550 Cd^S C_1 and J0161 Cd^S C_1 revealed that the mutants had markedly increased MIC (30µg/ml) for BC (Fig.4.1.1A and B); identical results were obtained with H7550 Cd^S C_2 and J0161 Cd^S C_2 (data not shown). MICs were not altered in ciprofloxacin-selected mutants of the plasmid-harboring strains (Fig. 1A and B). Ciprofloxacin-selected mutants of SK 2802, which lacks resistance to BC, also exhibited increases in MIC to BC (Fig. 4.1.1C).

Resistance to ciprofloxacin was also accompanied by reduced susceptibility to EthBr and the toxic compound TPP, both of which are MDR efflux substrates. EthBr MICs increased from 20µg/ml in the parental strains to 200µg/ml in the ciprofloxacin-selected mutants, regardless of whether the strains harbored plasmids or not (data not shown). A 4 fold increase in MIC was also observed with TPP (from 100µM in the parental strains to 400µM in the ciprofloxacin-selected mutants (data not shown). However, no impact was detected on MICs of Cd or sodium arsenite (data not shown).

4.1.4. *L. monocytogenes* mutants selected for increased resistance to BC also exhibit markedly reduced susceptibility to ciprofloxacin, gentamicin, EthBr and TPP.

The plasmid-cured derivatives H7550 Cd^S and J0161 Cd^S as well as strain SK 2802 lack the BC resistance cassette *bcrABC* and exhibited relatively low tolerance to BC (MIC 10µg/ml). At least two independent mutants from each strain were isolated on media with BC and designated BC_1 and BC_2. BC-selected mutants of all strains exhibited increases in their MIC for BC (from 10 to 30µg/ml) (Fig. 4.1.2, and data not shown).

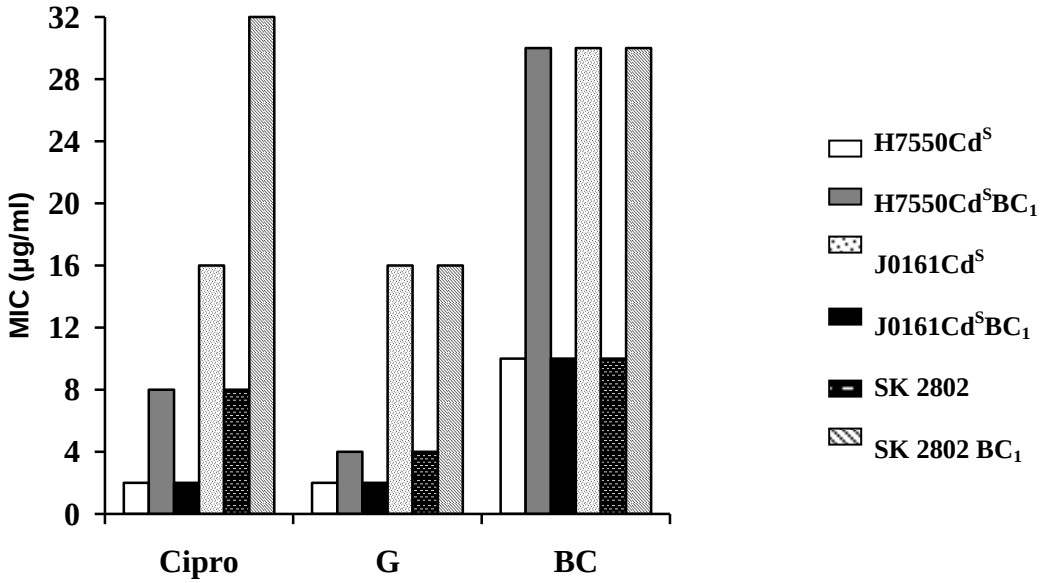


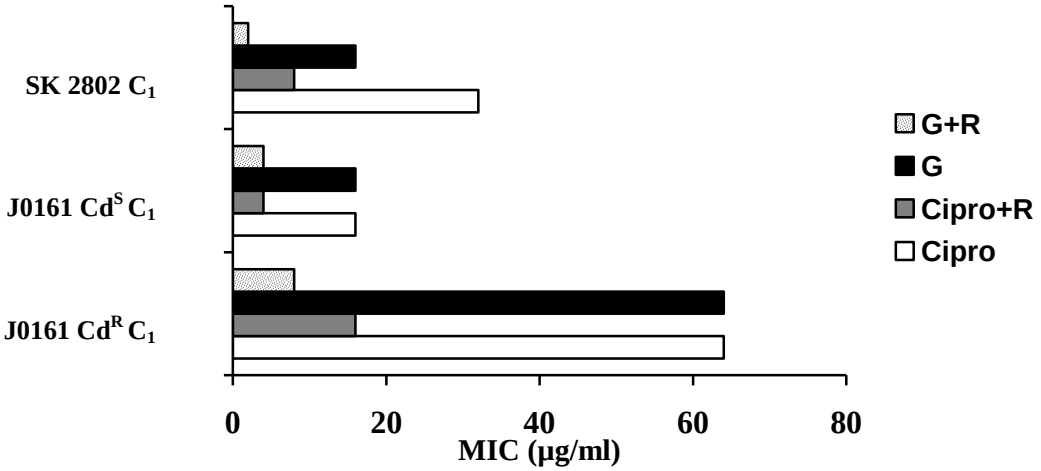
Fig. 4.1.2.Mutants selected on BC also exhibit reduced susceptibility to ciprofloxacin and gentamicin. Antimicrobial susceptibility to ciprofloxacin (cipro), gentamicin (G) and benzalkonium chloride (BC) were determined for plasmid-cured strain H7550 Cd^S (white) and its BC-selected mutant H7550 Cd^S BC_1 (gray); plasmid-cured strain J0161 Cd^S (black) and its BC-selected mutant J0161 Cd^S BC_1 (stippled); strain SK 2802 (dashed horizontal) and its BC-selected mutant SK 2802 BC_1 (diagonal). MICs were determined as described in Materials and Methods.

However, these mutants also exhibited 4 to 8 fold increase in resistance to ciprofloxacin and 2 fold to 8 fold increase in resistance to gentamicin (Fig.4.1.2). In addition, these BC-selected mutants exhibited higher tolerance to EthBr and TPP, with the increases in MICs being identical to those described above with ciprofloxacin-selected mutants (MICs increasing from 20 to 200µg/ml for EthBr and from 100 to 400µM for TPP) (data not shown). There was no impact on MICs to the other antibiotics in the panel, or to Cd and sodium arsenite (data not shown).

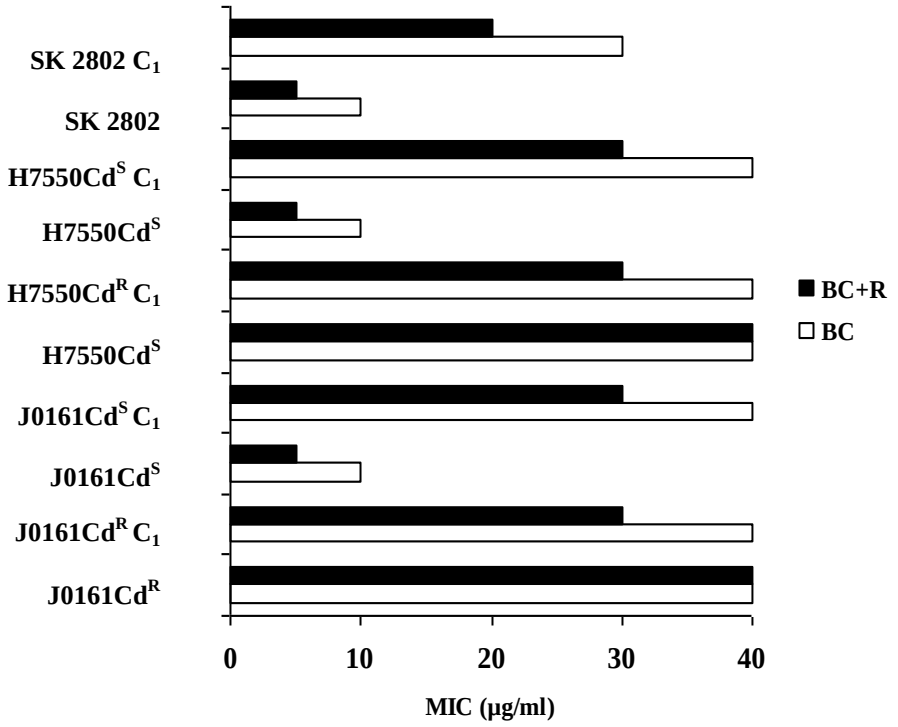
4.1.5. Antimicrobial agent MICs of ciprofloxacin-selected and BC-selected mutants of *L. monocytogenes* are reduced in the presence of the efflux pump inhibitor reserpine.

Ciprofloxacin MICs for all ciprofloxacin-selected mutants were reduced at least 4 fold in the presence of the efflux inhibitor reserpine; the extent of reduction was more pronounced in mutants with high MICs (32µg/ml or 64µg/ml), for which 8 fold MIC reductions were observed in the presence of reserpine (Fig. 4.1.3A and data not shown).

A.


SK 2802 C1
J0161 CdS C1
J0161 CdR C1
G+R
G
Cipro+R
Cipro
0
20
40
60
80
MIC (µg/ml)

B.


SK 2802 C1
SK 2802
H7550CdS C1
H7550CdS
H7550CdR C1
H7550CdS
J0161CdS C1
J0161CdS
J0161CdR C1
J0161CdR
BC+R
BC
0
10
20
30
40
MIC (µg/ml)

C.

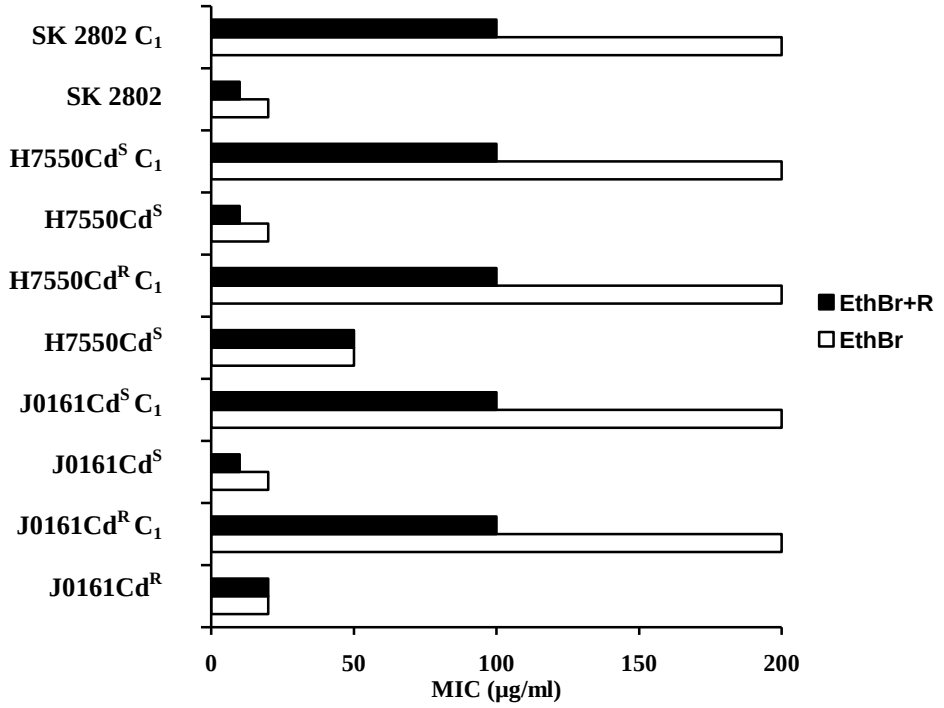
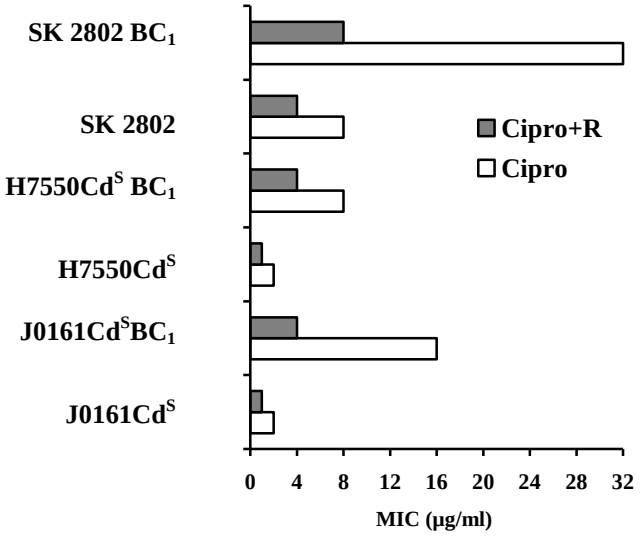


Fig. 4.1.3.Ciprofloxacin-selected mutants of *L. monocytogenes* exhibit reduced MICs to ciprofloxacin, gentamicin, benzalkonium chloride and ethidium bromide in the presence of the efflux inhibitor reserpine. A. Susceptibility to ciprofloxacin (cipro) and gentamicin (G) was determined in the presence and absence of reserpine (R). Strains are as indicated in Table 1 and in legends to Fig. 1 and Fig. 2. MICs for ciprofloxacin were determined in the absence (white) and presence (gray) of reserpine. MICs for gentamicin were also determined in the absence (black) and presence (stippled) of reserpine. B. MICs for benzalkonium chloride (BC) in the absence (white) and presence (black) of reserpine (R). C. MICs for ethidium bromide (EthBr) in the absence (white) and presence (black) of reserpine (R). MICs were determined as described in Materials and Methods.

MICs to gentamicin also declined in the presence of reserpine (e.g. from 16 to 2µg/ml in the case of SK 2802 C_1) (Fig. 4.1.3A). Ciprofloxacin and gentamicin MICs of the wildtype parental strains were also reduced (one to two-fold) in the presence of reserpine (data not shown).

In the presence of reserpine, the ciprofloxacin-selected mutants had increased susceptibility to BC (Fig. 4.1.3B), EthBr (Fig. 4.1.3C) and TPP (data not shown). MIC reductions for BC and EthBr were also noticed for the parental strains that were free of the plasmid, whereas no impact was noted in the plasmid-harboring strains H7550 Cd^R and J0161 Cd^R (Fig.4.1.3B and 3C). Mutants obtained following exposure to BC showed similar trends of reduction of MICs for ciprofloxacin, gentamicin, BC, EthBr and TPP in the presence of reserpine (Fig.4.1. 4A, B and data not shown).

A.



B.

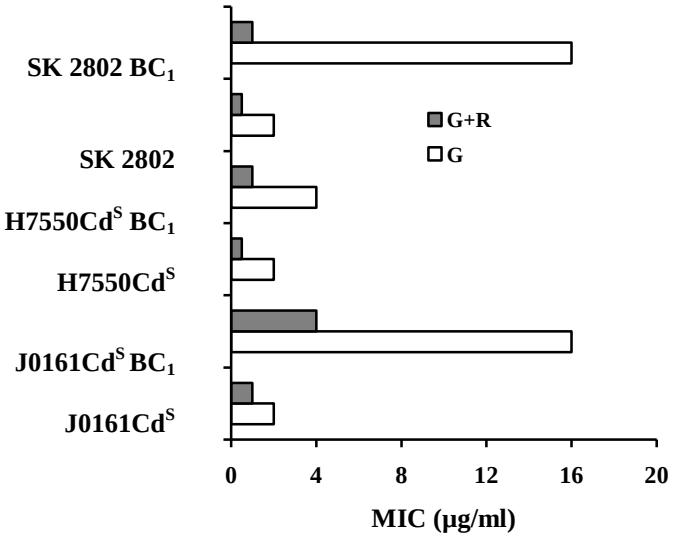


Fig.4.1.4.Benzalkonium chloride-selected mutants exhibit reduced MICs to ciprofloxacin and gentamicin in the presence of the efflux inhibitor reserpine. (A) MICs of mutants selected on BC were determined for ciprofloxacin (cipro) and gentamicin (G) (A and B, respectively). MICs were determined in the absence (white) and presence (gray) of reserpine (R). Strains are as indicated in Table 5.1.1 and in legends to Fig. 5.1.1 and Fig. 5.1.2. MICs were determined as described in Materials and Methods.

The disk diffusion method was employed to assess susceptibility of the ciprofloxacin- or BC-selected mutants to moxifloxacin, a fluoroquinolone that is not a substrate for drug efflux systems (6). None of the mutants exhibited significant changes in their susceptibility to moxifloxacin, and MICs were also not significantly affected by reserpine (Table 4.1.4 and data not shown).

Table 4.1.4.Moxifloxacin susceptibility of ciprofloxacin and BC-selected mutants of *L. monocytogenes*

Strain	Inhibition zone diameter (mm)[1]	
	Reserpine present	Reserpine absent
J0161Cd^{R}	25	26
J0161Cd^{R} C_1	25	26
J0161Cd^{S}	26	27
J0161Cd^{S} C_1	27	29
J0161Cd^{S} BC_1	26	26
H7550Cd^{R}	26	27
H7550Cd^{R} C_1	25	26
H7550Cd^{S}	25	27
H7550Cd^{S} C_1	27	28
H7550Cd^{S} BC_1	26	26

Ciprofloxacin- and BC-selected mutants of *L. monocytogenes* exhibited no change in their susceptibility to bile salts, triclosan, or SDS. MICs for bile salts, triclosan, and SDS were 0.1%, 64µg/ml, and 250µg/ml, respectively, both for the parental strains and for the mutants selected on ciprofloxacin or BC.

4.1.6. Expression of MDR transporter *lde* was enhanced in mutant obtained following exposure to BC. Expression of *lde* was enhanced in BC-selected mutant H7550 Cd^S BC_1 in comparison to the parental strain H7550 Cd^S (Fig. 4.1.5B). We failed to obtain evidence for expression of other chromosomal MFS transporters including *mdrL*, *mdrM* or *mdrT* in either the wildtype parental strains or their BC-selected and ciprofloxacin-selected derivatives (Fig. 4.1.5C and data not shown).

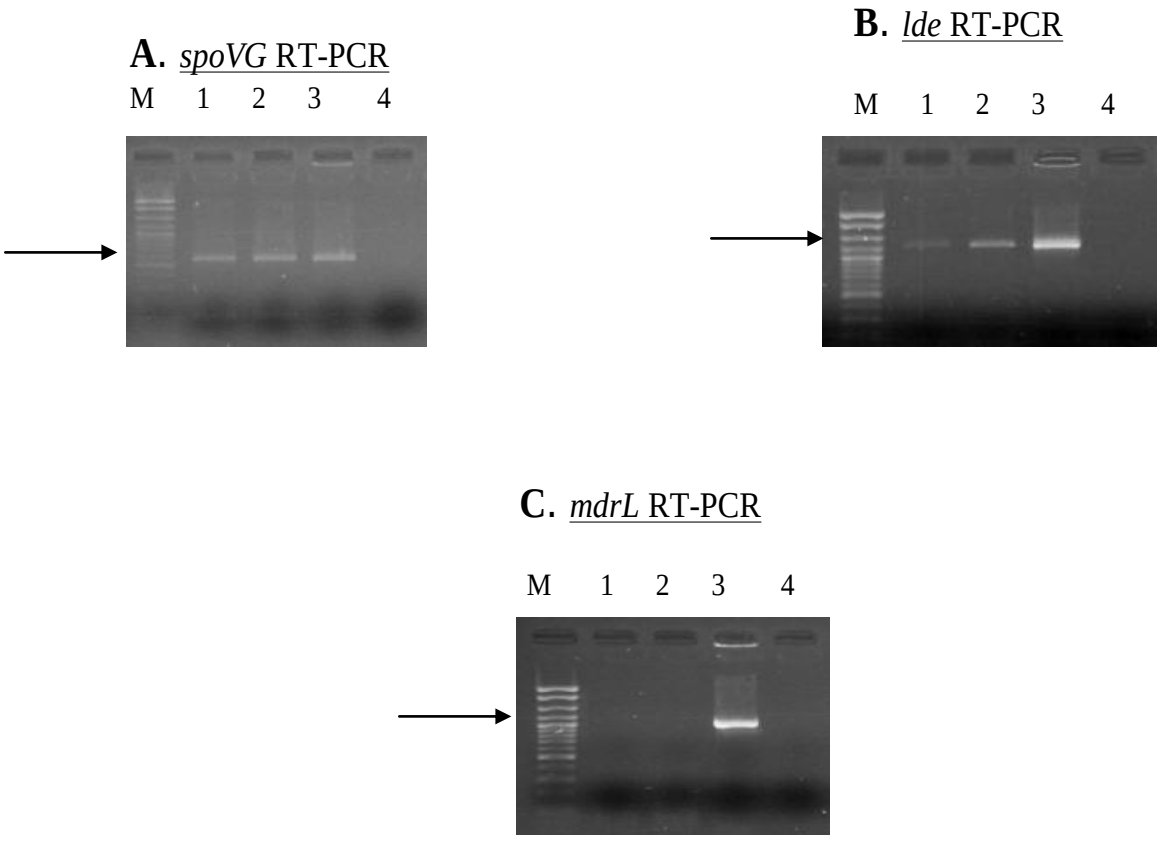


Fig.4.1.5.Benzalkonium chloride-selected mutant exhibits enhanced transcription of *lde* in comparison to parental strain. RT-PCR was employed to assess expression of the housekeeping gene *spoVG*, used as control (A), *lde* (B) and *mdrL* (C). Lanes: 1, H7550 Cd^S; 2, H7550Cd^S BC_1 ; 3, H7550 Cd^S genomic DNA; 4, H7550 Cd^S RNA (to confirm absence of genomic DNA in the RNA used for RT-PCR); M, 100 - 2,686 bp DNA molecular marker XIV (Roche). Arrows indicate the size of the expected PCR product. RT-PCR was done as described in Materials and Methods.

4.2. Identification of *L monocytogenes* antibiotic resistance genes under control of tetR repressors

4.2.1. *Listeria* MDR that may emerge in healthcare settings and food processing plants is of clear public health relevance, but its molecular basis remains uncharacterized. Previous studies focused on two genes, *mdrL* (and its repressor *ladR*) (106, 107, 144, 186, 187) and *lde* (86, 187). The transporters identified in the searches by Crimmins et al (2008) belonged to the Major Facilitator Superfamily. However, resistance of the mutants to toxic compounds known to be substrates for MDR transporters was not reported. Our analysis of these mutants (kindly provided by D. Portnoy) revealed that the *tetR*::Tn*917* mutant had pronounced increase in resistance to TPP (an anticancer drug commonly used as MDR substrate [95, 96]), and resistance was reduced in the presence of the efflux inhibitor reserpine (Fig. 1; reserpine treated cells indicated as "*tetR:* Tn917+R"). However, deletion of *mdrT* did not affect tolerance levels to TPP (Fig. 4.2.1).

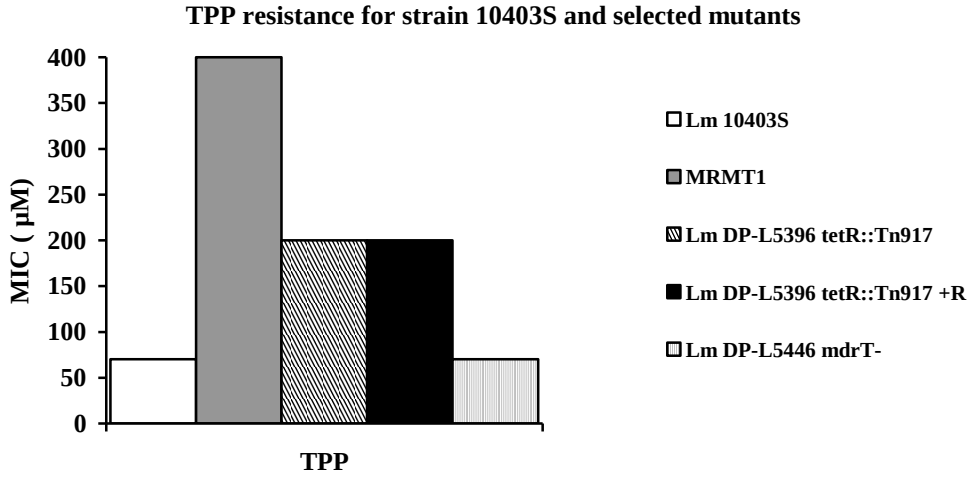


Fig.4.2.1. Resistance of *L monocytogenes* 10403S and selected mutants to TPP. *TetR::Tn917* mutant had pronounced increase in resistance to TPP (an anticancer drug commonly used as MDR substrate), and resistance was reduced in the presence of the efflux inhibitor reserpine. Mutant MRMT1 isolated from mariner-based mutant library of the strain 10403S also exhibited high resistance level

This finding was in agreement with the earlier observed failure of TPP exposure to induce expression of *mdrT* (46). Resistance of the *tetR*::Tn*917* mutant could be mediated by

increased expression of MDR transporter(s) other than *mdrT*, but also repressed by *tetR*. Screening mutant libraries of two *L. monocytogenes* strains, F2365 (serotype 4b), and 10403S (serotype 1/2a), with the *mariner*-based transposon plasmid pMC38 (Figure 4.2.2)

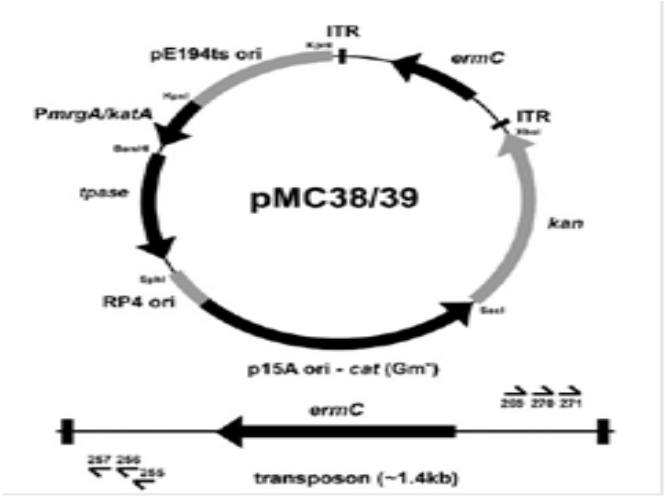


Fig. 4.2.2. Map of the mariner-based delivery vector pMc38/39 (modified from Cao et al. 2007).

(6, 24) in attempt to indentify such transporter(s) failed to isolate mutants with inpared resistance to TPP. However, five mutants of strain 10403S with significant increase in TPP resistance were indentified. Minimal inhibitory concentration (MIC) of TPP for isolated mutants was 400uM/ml, whereas parental strain 10403S had MIC of 70uM/ml and *tetR::Tn917* 200uM/ml (Fig. 4.2.3).

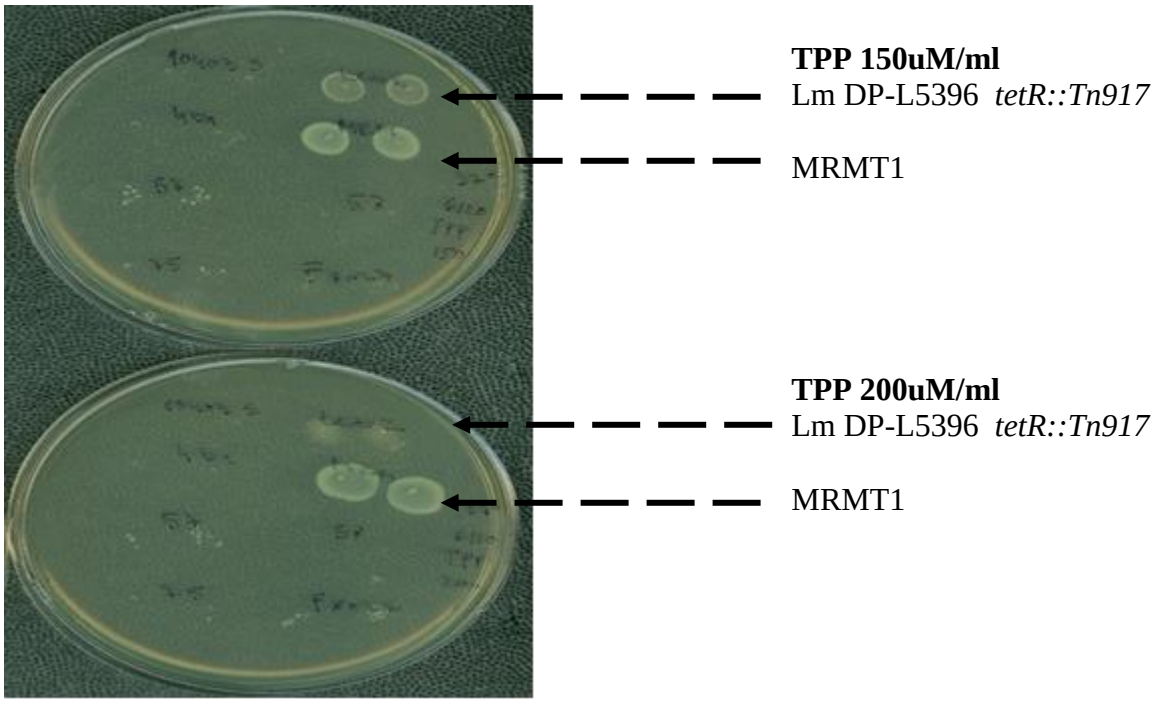


Fig. 4.2.3.MIC of TPP for LmDP-L5396 *tetR::Tn917* and mariner mutant.At the concentration of 150uM TPP both *tetR::Tn917* and mariner-based mutant grew equally, but at the concentration of 200uM/ml tetR *tetR::Tn917* failed to grow.

Further genetic characterization of mutants revealed that one of these *tetR*-like mutants had insertion in tetR gene. All mutants harbored single transposon insertion and one characterized had transposon integrated in LMRG_01858 (sucrose phosphorylase) (Fig. 5.2.4).

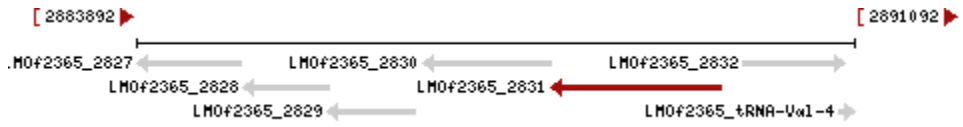


Fig. 4.2.4. LMRG_01858 (sucrose phosphorylase)

In this study we provided evidence of mutant libraries as a significant genetic tool in screening for changes in phenotype and genotype. Even though we failed to isolate mutants with inpaired resistance to TPP, *tetR*::Tn*917* mutants with pronounced (8-fold) increase in TPP resistance have been isolated. One of these mutants had insertion in *tetR* gene.

Characterization of such mutant revealed transposon integrated in LMRG_01858 (sucrose phosphorylase). Such data suggest that the observed TPP resistance of the *tetR*::Tn*917* mutant involve increased expression of MDR transporter(s) other than *mdrT*, but also repressed by *tetR*.

4.3. Co-selection of cadmium and benzalkonium chloride resistance in conjugative transfers from non-pathogenic *Listeria* spp. to other listeriae

4.3.1. Bacterial strains used in this study. Five BC-resistant strains of non-pathogenic *Listeria* spp. (four strains of *L. welshimeri* and *L. innocua* L1221) were used as potential donors of BC resistance to other non-pathogenic listeriae (*L. welshimeri* L1316S, *L. welshimeri* L0927S and *L. innocua* L1206S). All BC-resistant donor strains were also resistant to cadmium (Table 4.3.1). To further assess conjugative transfer of resistance we used as potential donors of cadmium resistance six *L. innocua* strains resistant to cadmium but susceptible to BC (Table 4.3.1).

Table 4.3.1. Bacterial strains employed in this study

Strain	Resistance[1]	Genotype[2]			Source or reference
Donor strains		*cadA1*	*cadA2*	*bcrABC*	
L. innocua					
L0910	Cd^R	-	-	-	This study
L1214B	Cd^R	+	-	-	This study
L1306A	Cd^R	+	-	-	This study
CLIP 11262	Cd^R	-	+	-	(16)
L1221	Cd^R BC^R	-	+	+	This study
L1333a	Cd^R	-	+	-	This study
L0921	Cd^R	+	-	-	This study
L. welshimeri					
L0725	Cd^R BC^R	-	+	+	This study
L1325	Cd^R BC^R	-	+	+	This study
L0918	Cd^R BC^R	-	+	+	This study
L0926	Cd^R BC^R	-	+	+	This study
Recipient strains					
L. innocua					
L1206S	Str^R	-	-	-	This study
L. welshimeri					
L1316S	Str^R	-	-	-	This study
L0927S	Str^R	-	-	-	This study
L. monocytogenes (serotype)					
1/2a3 (1/2a)	Str^R	-	-	-	(22)
10403S (1/2a)	Str^R	-	-	-	(37)
2381L (4b)	Str^R	-	-	-	(39)
2857S (1/2a)	Str^R	-	-	-	This study
2858S (1/2b)	Str^R	-	-	-	This study

[1] Cd^R, BC^R and Str^R indicate resistance to cadmium, BC, and streptomycin, respectively, determined as described in M
[2] Determined by PCR with the respective primers as described in Materials and Methods.

PCR analysis of the strains employed as donors revealed that all BC-resistant strains, regardless of species (*L. welshimeri* or *L. innocua*) harbored *bcrABC* as well as the plasmid-associated cadmium resistance determinant *cadA2*; *cadA2* was also harbored by *L. innocua* 1333a and *L. innocua* CLIP 11262, which were resistant to cadmium but susceptible to BC and lacking *bcrABC*. Three strains of *L. innocua* harbored an alternative plasmid-associated cadmium resistance determinant, *cadA1*, and one cadmium-resistant strain (*L. innocua* L0910) was found to be negative for both *cadA1* and *cadA2* (Fig.4.3.1 and Table 4.3.1).

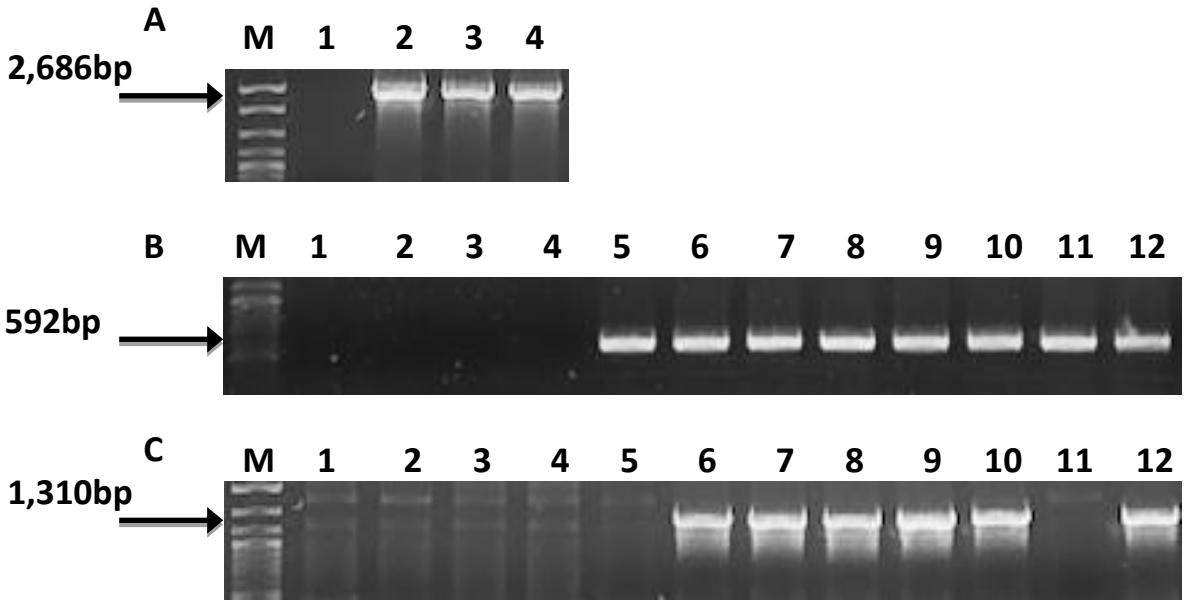


Fig.4.3.1 Presence of *cadA1, cadA2* and *bcr*ABC in nonpathogenic donor strains.

(A) PCR-based detection of *cadA1* using primers cadA-Tn5422F and cadA-Tn5422R. Lanes 1, 2, 3 and 4, *L. innocua* strains L0910, L0921, L1214B and L1306A, respectively. M, DNA molecular marker XIV (Roche, Indianapolis, IN). Arrow points to the expected *cadA1* PCR product. **(B)** PCR-based detection of *cadA2* using primers cadA-pLM90F and cadA-pLM90R. Lanes 1, 2, 3 and 4, *L. innocua* strains L0910, L0921, L1214B and L1306A, respectively; lanes 5, 10 and 11, *L. innocua* strains CLIP 11262, L1221 and L1333a, respectively; lanes 6, 7, 8 and 9, *L. welshimeri* strains L0725, L1325, L0918 and L0926, respectively. Lane 12, *L. monocytogenes* H7550, used as positive control (12). M, as in panel A. Arrow points to the expected *cadA2* PCR product. **(C)** PCR of *bcr*ABC using primers BcF and BcR. Lanes are as in panel B. With the exception of *L. innocua* strains CLIP 11262 and L1333a in lanes 5 and 11, respectively, all donors harboring *cadA2* also contained *bcr*ABC. M, as in panel A. Arrow points to the expected *bcrABC* product.

None of the strains harbored the chromosomal cadmium resistance determinant *cadA3* (data not shown). Strains used as recipients in the conjugations (*L. welshimeri* L1316S, *L. welshimeri* L0927S and *L. innocua* L1206S) were susceptible to BC and cadmium and lacked *bcrABC*, *cadA1*, *cadA2* or *cadA3* (Table 4.3.1).

4.3.2. *L. welshimeri* and *L. innocua* harboring *cadA2* can readily transfer cadmium resistance to other non-pathogenic listeriae. Results from matings performed at 25°C on filter membranes and on agar were comparable but frequency of transfer was generally higher and more consistent for the latter (data not shown). When transconjugants were obtained, transfer frequency averaged at 10^{-7} to 10^{-8} (Table 4.3.3) confirming that they were derivatives of the recipient strain and not spontaneous streptomycin-resistant mutants of the donor. Plating of the strains employed as recipients on medium with both cadmium and streptomycin failed to identify any spontaneous cadmium-resistant mutants (data not shown).

Table 4.3.3. Transfer frequency resistance in conjugations between indicated donor and recipient strains[1]

	Recipient							
	L. welshimeri		*L. innocua*	*L. monocytogenes*				
Donor	L0927S	L1316S	L1206	1/2a3	2381L	10403S	2857S	2858S
L. welshimeri								
L0725	5.8×10^{-7}	1.4×10^{-7}	$<1.0\times 10^{-9}$	1.8×10^{-7}	2.8×10^{-3}	1.8×10^{-3}	6.7×10^{-7}	1.6×10^{-7}
L1325	3.7×10^{-6}	5.6×10^{-7}	$<1.0\times 10^{-9}$	1.5×10^{-7}	2.0×10^{-3}	1.1×10^{-3}	6.7×10^{-7}	2.1×10^{-7}
L0918	2.6×10^{-6}	1.5×10^{-7}	$<1.0\times 10^{-9}$	1.5×10^{-7}	1.4×10^{-3}	7.4×10^{-9}	5.5×10^{-3}	6.1×10^{-3}
L0926	3.3×10^{-7}	1.6×10^{-7}	$<1.0\times 10^{-9}$	1.3×10^{-7}	5.5×10^{-3}	1.9×10^{-3}	4.0×10^{-7}	1.5×10^{-7}
L. innocua								
L0910	$<1.0\times 10^{-9}$	$<1.0\times 10^{-9}$	$<1.0\times 10^{-9}$	$<2.8\times 10^{-9}$	$<3.5\times 10^{-9}$	$<2.4\times 10^{-9}$	$<2.3\times 10^{-9}$	$<2.4\times 10^{-9}$
L1214B	$<1.0\times 10^{-9}$	$<1.0\times 10^{-9}$	$<1.0\times 10^{-9}$	$<3.3\times 10^{-9}$	$<4.6\times 10^{-9}$	$<2.0\times 10^{-9}$	$<1.8\times 10^{-9}$	$<2.3\times 10^{-9}$
L0921	$<1.0\times 10^{-9}$	$<1.0\times 10^{-9}$	$<1.0\times 10^{-9}$	$<3.0\times 10^{-9}$	$<3.7\times 10^{-9}$	$<2.8\times 10^{-9}$	$<1.8\times 10^{-9}$	$<16\times 10^{-9}$
L1306A	$<1.0\times 10^{-9}$	$<1.0\times 10^{-9}$	$<1.0\times 10^{-9}$	$<4.6\times 10^{-9}$	$<5.4\times 10^{-9}$	$<3.1\times 10^{-9}$	$<2.6\times 10^{-9}$	$<3.5\times 10^{-9}$
CLIP 11262	$<4.9\times 10^{-10}$	$<2.3\times 10^{-10}$	$<4.9\times 10^{-10}$	5.1×10^{-9}	7.9×10^{-9}	5.6×10^{-9}	9.7×10^{-9}	4.1×10^{-3}
L1221	6.1×10^{-3}	2.7×10^{-7}	$<4.3\times 10^{-9}$	1.5×10^{-7}	8.2×10^{-3}	1.1×10^{-3}	3.4×10^{-3}	6.6×10^{-9}
L1333a	1.3×10^{-3}	2.8×10^{-3}	$<4.1\times 10^{-9}$	6.7×10^{-3}	1.3×10^{-3}	1.6×10^{-7}	3.8×10^{-7}	3.6×10^{-7}

[1] Results are from one representative experiment and all experiments were done in at least three independent trials.

Selected putative *L. welshimeri* transconjugants obtained using *L. innocua* as donors were evaluated with PCR using species-specific primers. The putative transconjugants were positive with the *L. welshimeri*-specific primers but negative for those specific for *L. innocua* (Fig. 4.3.2 and data not shown),

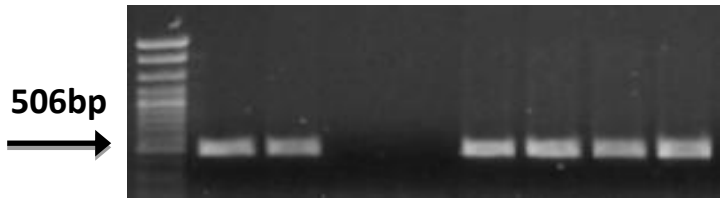


Fig.4.3.2 Identification of *L. welshimeri* transconjugants from conjugations between *L. welshimeri* as recipient and *L. innocua* as donor. PCR employed *L. welshimeri*-specific primers Lw_0908_F and Lw_0908_R. Lanes 1 and 2, *L. welshimeri* strains L0725 and L1316S , respectively; lanes 3 and 4, *L. innocua* strains L1221 and L1333a, respectively; lanes 5, 6, 7 and 8, transconjugants from conjugations of *L. welshimeri* L1316S as recipient with donors *L. innocua* 1221 (lanes 5 and 6) and *L. innocua* 1333a (lanes 7 and 8). Arrow points to the expected PCR product.

Efficiency of conjugative transfer depended markedly on the strains employed as donors as well as recipients. Few or no transconjugants were obtained from certain *L. innocua* donors (*L. innocua* strains L0910, L1214B, L1306A and L0921), regardless of the strain that was employed as potential recipient. Furthermore, one of the strains employed as recipient, *L. innocua* L1206S, failed to yield transconjugants with any of the donors (Table 4.3.3).

Based on PCR analysis of the resistance determinants, all donor strains that could efficiently transfer resistance harbored *cadA2*.With the exception of *L. innocua* strains 1333a and CLIP 11262, these strains were also resistant to BC and harbored *bcrABC* (Tables 4.3.1 and 4.3.3). In contrast, of the four other potential donors that failed to yield transconjugants three harbored *cadA1*, and one (*L. innocua* L0910) lacked *cadA1* or *cadA2* (Tables 4.3.1 and 4.3.3).

4.3.3. BC resistance determinant *bcrABC* is co-transferred with the cadmium resistance determinant *cadA2*. As mentioned above, most (five of seven) of the *cadA2*-harboring cadmium-resistant strains employed as donors also harbored the BC resistance cassette

bcrABC. PCR analysis of selected transconjugants obtained using these strains as donors revealed that, in addition to *cadA2*, they also harbored *bcrABC* (Fig. 4.3.3A and 4.3.3C).

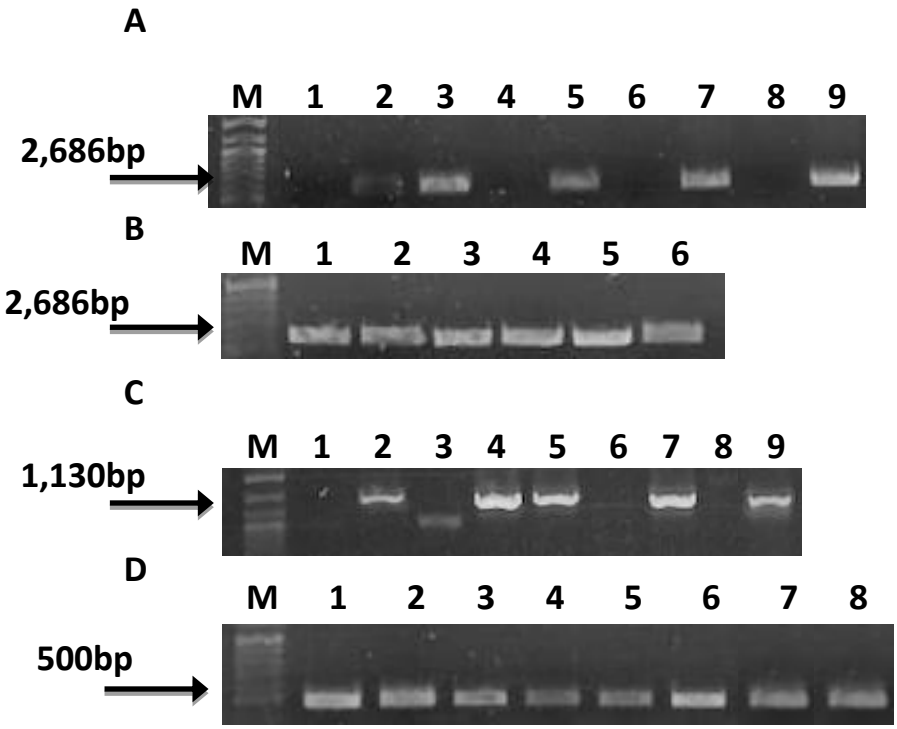


Fig.4.3.3 Presence of *cadA2* and *bcrABC* in *L.welshimeri* and *L.monocytogenes* transconjugants. (A) PCR-based detection of *cadA2* using primers cadA-pLM90F and cadA-pLM90R. Lanes 1, 4, 6 and 8, strains used as recipients (*L. welshimeri* strains L0927S and L1316S [lanes 1 and 4, respectively], *L. monocytogenes* 1/2a3 and *L. innocua* L1206 [lanes 6 and 8, respectively]); Lanes 2, 3 and 5, transconjugants from conjugations between *L. welshimeri* L0927S and *L. innocua* L1221 (lane 2), *L. welshimeri* L1316S and *L. welshimeri* L0918 (lane 3), L1316S with *L. innocua* L1221 (lane 5); lane 7, transconjugant from conjugation between *L. monocytogenes* 1/2a3 (recipient) and *L. welshimeri* L1325 (donor). Lane 9, *L. monocytogenes* H7550, used as positive control (12). M,DNA molecular marker XIV (Roche, Indianapolis, IN). Arrow points to the expected *cadA2* PCR product. **(B)** PCR-based detection of *cadA2* in transconjugants from conjugations between *L.*

monocytogenes 2381L (recipient) and *L. welshimeri* L1325 (donor). M, as in panel A. Arrow points to the expected *cadA2* PCR product. **(C)** PCR-based detection of *bcr*ABC using BcF and BcR primers. Lanes 1, 3, 6 and 8 strains used as recipients (*L. welshimeri* strains L0927S and L1316S [lanes 1 and 3, respectively, with unspecific PCR product in lane 3] and *L. monocytogenes* strains 1/2a3 and *L. innocua* L1206 [lanes 6 and 8, respectively]); Lanes 2, 4 and 5, transconjugants from conjugations between *L. welshimeri* L1316S (recipient) and *L. welshimeri* L1325 (donor); lane 7, transconjugant from conjugation between *L. monocytogenes* 1/2a3 (recipient) and *L. welshimeri* L1325 (donor); lane 9, *L. monocytogenes* H7550, used as positive control (12); M as in panel A. Arrow points to the expected *bcrABC* PCR product. Weak band in lane 3 is unspecific PCR product. **(D)** PCR-based detection of *hly* in *L. monocytogenes* transconjugants using primers hlyAF and hlyAR. Lanes 1 and 2, transconjugants from conjugations between *L. monocytogenes* 1/2a with *L. innocua* L1221 (lane 1) and *L. innocua* L1333a (lane 2); lanes 3-7, transconjugants from conjugations between *L. monocytogenes* 2381L with *L. welshimeri strains* L0725 (lane 3), L1325 (lane 4) and *L0926* (lane 5) and with *L. innocua* strains L1221 (lane 6), and L1333a (lane 7); lane 8 is *L. monocytogenes* 2381L. M, as in panel A. Arrow points to the expected *hly* PCR product.

The transconjugants exhibited elevated MICs not only for cadmium (200 μg/ml) but also for BC (35-40 μg /ml), similar to cadmium and BC MICs for the donor strains; in contrast, the strains used as recipients had MIC values of 10 μg/ml both for cadmium and for BC.

4.3.4. Non-pathogenic *Listeria* spp. strains can effectively mediate conjugative co-transfer of BC and cadmium resistance to *L. monocytogenes*. To assess the ability of non-pathogenic *Listeria* spp. to serve as donors of BC resistance to *L. monocytogenes*, we employed as potential recipients five *L. monocytogenes* strains of the three serotypes predominant in human listeriosis (1/2a, 1/2b and 4b). The strains included laboratory reference strains (10403S and 1/2a3), a streptomycin-resistant derivative of a strain from the 1985 California outbreak (2381L) as well as streptomycin-resistant derivatives of two strains from the 2011 cantaloupe outbreak (*L. monocytogenes* 2857S and 2858S of serotype 1/2a and 1/2b, respectively) (Table 4.3.1). All *L. monocytogenes* strains employed as potential recipients were susceptible to cadmium and BC (MICs 10 μg /ml for both compounds) (Table 4.3.1).

Regardless of the serotype of the *L. monocytogenes* strains used as recipients, conjugative transfer of resistance from non-pathogenic listeriae to *L. monocytogenes* generally exhibited the same dependence on donor strains that was observed with conjugations among non-pathogenic listeriae. With the exception of CLIP 11262, those *L. innocua* strains that failed to donate cadmium resistance to other non-pathogenic listeriae also failed to transfer such resistance to *L. monocytogenes* (Table 4.3.3). On the other hand, those non-pathogenic *Listeria* spp. donor strains that efficiently transferred BC and cadmium resistance in matings with other non-pathogenic listeriae were found to also transfer the resistance determinants to *L. monocytogenes*. When these strains were used as donors transconjugants were readily obtained from all *L. monocytogenes* strains in the panel, including the two strains associated with the recent cantaloupe outbreak (Table 4.3.3).

Acquisition of *cadA2* by *L. monocytogenes* was confirmed by PCR (Fig. 4.3.3A, B and data not shown). Furthermore, when the donors also harbored *bcrABC*, this cassette was detected in the cadmium-resistant transconjugants as well (Fig.4.3.3C and data not shown). Transconjugants were also tested for hemolytic activity on blood agar plates and by PCR with primers specific for *hly*, encoding the *L. monocytogenes* virulence determinant Listeriolysin O and absent from *L. innocua* or *L. welshimeri*. All tested transconjugants were hemolytic and produced the *hly* amplicon (Fig. 4.3.3D and data not shown), confirming that they were derived from the *L. monocytogenes* recipients and were not spontaneous streptomycin-resistant derivatives of the donors. As noted above with transconjugants from conjugations between non-pathogenic *Listeria* spp., the *L. monocytogenes* transconjugants exhibited elevated MICs for cadmium and BC (200 and 35-40 μg/ml, respectively), similar to those of the donor strains.

4.3.5. Temperature affects transfer of BC and cadmium resistance to non-pathogenic listeriae while transfer to *L. monocytogenes* is equally efficient at 25 and 37°C. Non-pathogenic *Listeria* spp. donor-recipient combinations that failed to yield transconjugants at 25°C were similarly negative at 37°C (data not shown). However, for the other combinations the frequency of transfer of BC and cadmium resistance among non-pathogenic listeriae was higher when conjugations were done at 25 than at 37°C. The impact of temperature was

dependent on the recipient strain, being noticeably stronger for *L. welshimeri* L0927S than for *L. welshimeri* L1316S (Fig.4.3. 4 A, B).

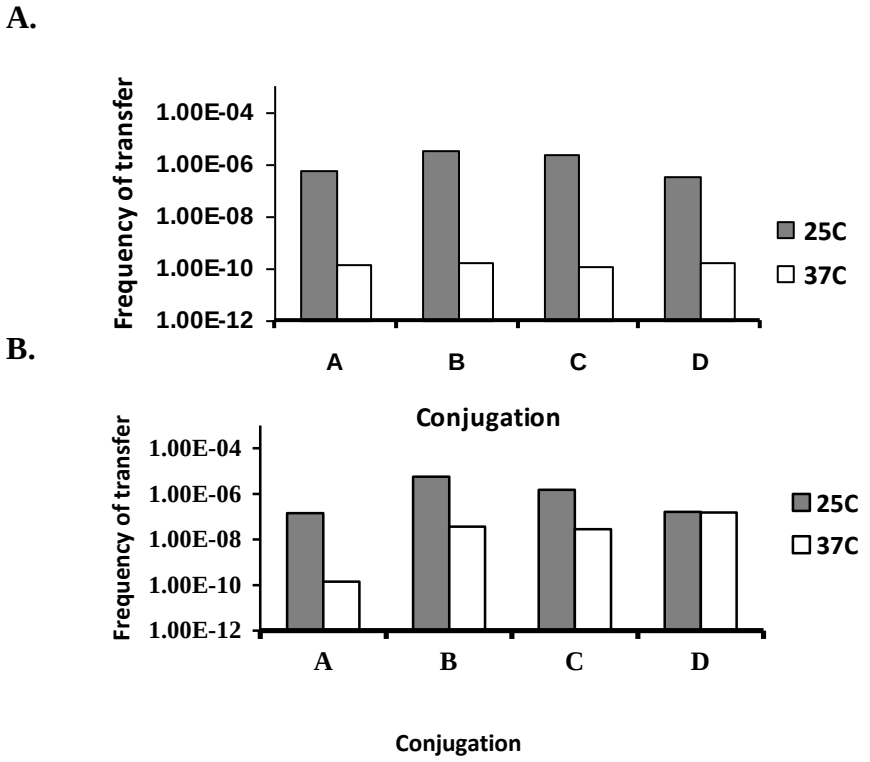


Fig. 4.3.4 Impact of conjugation temperature (25°C vs. 37°C) on frequency of transfer of cadmium resistance in conjugations among non-pathogenic *Listeria* spp. (A) A, B, C and D, transfer frequency from conjugations between *L. welshimeri* L0927S as recipient and *L. welshimeri* strains L0725, L1325, L0918 and L0926, respectively, as donors. **(B)** A, B, C and D, transfer frequency from conjugations between *L. welshimeri* L1316S as recipient and *L. welshimeri* strains L0725, L1325, L0918 and L0926, respectively, as donors. Conjugations were done on agar plates at 25°C (gray bars) or 37°C (white bars) and transfer frequency was determined as described in Materials and Methods. Results are from one representative experiment and experiments were done in at least three independent trials.

Interestingly, efficiency of transfer of resistance from these same donors to *L. monocytogenes* was not affected by temperature. Transfer was equally efficient at 25 and 37°C for all *L. monocytogenes* strains in the panel, regardless of serotype (data not shown).

4.3.6. Evidence of pLM80-like plasmids in BC and cadmium-resistant non-pathogenic donors. To determine whether the *L. innocua* and *L. welshimeri* strains employed as donors harbored plasmids similar to pLM80, these strains were tested by PCR with primers derived from a panel of ORFS at different sites of pLM80 (Table 4.3.2).

Table 4.3.2. Primers used in this study

Gene	PCR primer sequence (5'–3')	Target gene and protein function	GenBank accession no.	Reference
cadA-Tn5422F	CAGAGCACTTTACTGACCATCAATCGTT	Tn*5422*-associated *cadA* (*cadA1*)	L28104	(32)
cadA-Tn5422R	CTTCTTCATTTAACGTTCCAGCAAAAA			
cadA-pLM80F	ACAAGTTAGATCAAAAGAGTCTTTTATT	*cadA* on pLM80 (*cadA2*)	AADR01000058	(32)
cadA-pLM80R	ATCTTCTTCATTTAGTGTTCCTGCAAAT			
cadA-EGDeF	TGGTAATTTCTTTAAGTCATCTCCCATT	*cadA* of strain EGD-e (*cadA3*)	AL591824	(32)
cadA-EGDeR	GCGATGATTGATAATGTCGATTACAAAT			
BcF	GAATGGATCCTTCAATTAGATCGAGGCACG	bcrABC	AADR01000058	(12)
BcR	GTATGAATTCGTATAATCCGGATGCTGCCC			
hlyF	CGGAGGTTCCGCAAAAGATG	*hly*		(15)
hlyR	CCTCCAGAGTGATCGATGTT			
Lw_0908_F	CTTCTCTTTCAGTAGACAGTGAAGC	*L. welshimeri*-specific sequence	NC_008555	(18)
Lw_0908_R	CCATCCTTGTTTCGCCCTTCTTC			
Li_0558_F	GGTGTCTACTTTGTAACGGCCAC	*L. innocua*-specific sequence	NC_003212.	(16)
Li_0558_R	CGGTAGCTTTTGTTAGCATCCCTG			
pLM80-0004F	CATAGCGATCATACGCATTG	LMOh7858_pLM80_0004 (hypothetical protein)	AADR01000010	This study
pLM80-0007R	AGCAGCATGTAGAAGCAGC			
pLM80-0007F	CTCAACACTAAGTCGGTACC	LMOh7858_pLM80_0007 (hypothetical protein)	AADR01000010	This study
pLM80-0007R	CCATTCAAGCTGCGAAAG			
pLM80-0008F	CCCAAGTTTTCCAGTAGCTG	LMOh7858_pLM80_0008 (conserved hypothetical protein)	AADR01000010	This study
pLM80-0008R	AGGATACACAAGCTCGGAAG			

The findings suggested that strains positive for *bcrABC* as well as *cadA2* (*L. innocua* 1221 and all four *L. welshimeri* donors) harbored plasmids highly similar to pLM80, with all primer pairs in the panel yielding the expected amplicons. In contrast, strains harboring *cadA2* but

lacking *bcrABC* (*L. innocua* strains 1333a and CLIP 11262) failed to produce several of the expected amplicons (Fig. 4.3.5).

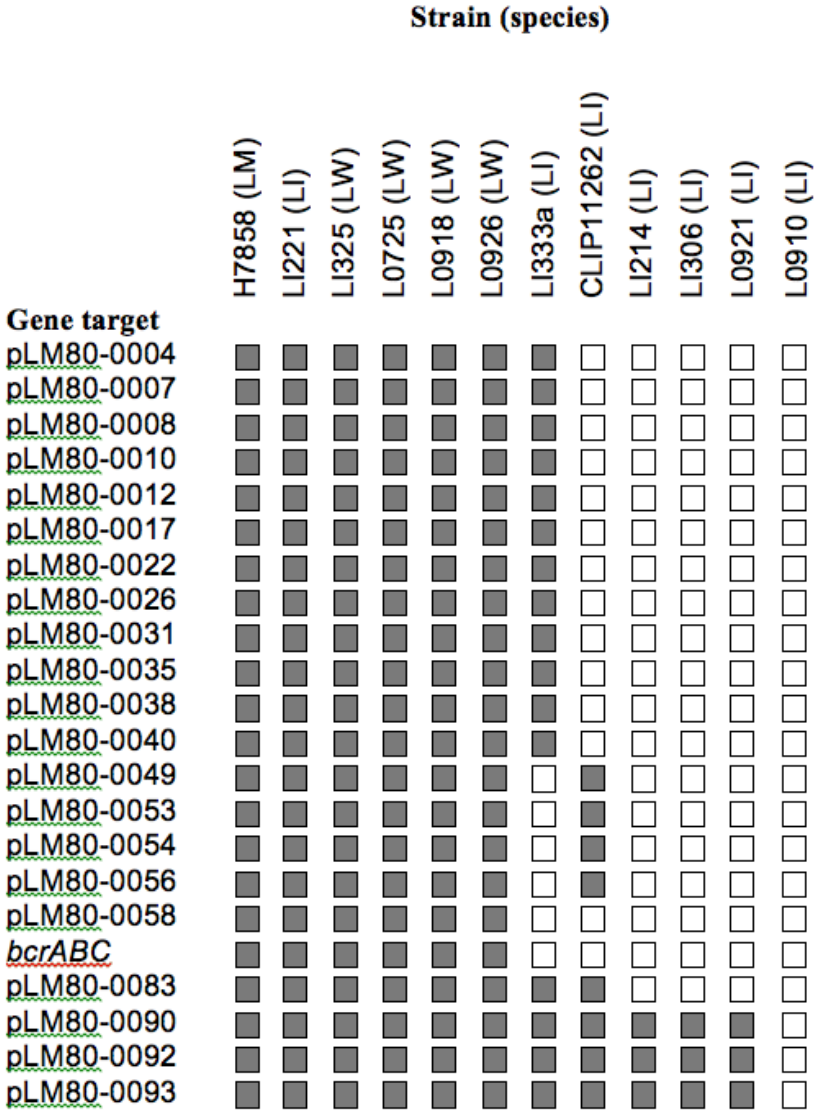


Fig. 4.3.5 Detection of pLM80 genes in non-pathogenic *Listeria* spp. used as donors. Black and white boxes indicate presence or absence, respectively, of the expected PCR product. LW, LI and LM in strain (species) designations refer to *L. welshimeri*, *L. innocua* and *L. monocytogenes*, respectively. *L. monocytogenes* H7858 harbors pLM80 (33) and is included as positive control. PCR-based detection of the indicated genes was done employing the pLM80-derived primes listed in Table 2, as described in Materials and Methods.

Strains harboring *cadA1* yielded amplicons with only three primer pairs in the panel, two of which were derived from plasmid replication-associated genes, while no amplicons were obtained using *L. innocua* L0910 (cadmium-resistant but lacking *cadA1* or *cadA2)* (Fig. 4.3.5). It is noteworthy that all donors consistently involved in high efficiency transfer (*L. welshimeri* L0725, L1225, L0918 and L0926; *L. innocua* L1333a and L1221) harbored LMOh7858_pLM80_0022, encoding a putative TraG/TraD family protein that may be involved in facilitating plasmid transfer (Fig. 4.3.5).

4.4. Resistance gene distribution among non-pathogenenic *Listeriae* from different poultry processing plants

4.4.1. Heavy metal and BC resistance in non-pathogenic *Listeria* spp. Resistance to cadmium was highly prevalent, exceeding 90% for both *L. welshimeri* and *L. innocua*. Resistance to BC, while also highly prevalent in both species, was more common in *L. welshimeri* (83%) than *L. innocua* (73%) but difference was not statistically significant. In contrast, arsenic resistance was relatively uncommon (<10%) in either species (Fig. 4.4.1).

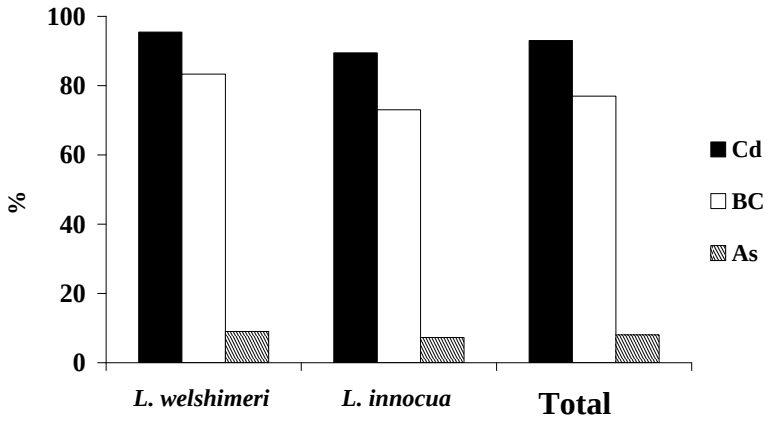


Fig. 4.4.1. Heavy metal and BC resistance in non-pathogenic *Listeriae* spp. Resistance to cadmium was highly prevalent, exceeding 90% for both *L. welshimeri* and *L. innocua.* Resistance to BC, while also highly prevalent in both species, was more common in *L. welshimeri* (83%) than *L. innocua* (73%). In contrast, arsenic resistance was relatively uncommon (<10%) in either specie

4.4.2. ***cadA*** **distribution in non-pathogenic** ***Listeria spp*****.** The majority of the isolates harbored *cadA2* (43%), followed by *cadA1* (30%). Isolates with both *cadA1* and *cadA2* were relatively uncommon (7%); only a single isolate harbored *cadA4* and *cadA3* was not detected.

A subset (12%) of cadmium-resistant isolates lacked any of the known *cadA* genes. Cadmium-susceptible isolates also lacked the known *cadA* genes (Fig. 4.4.2).

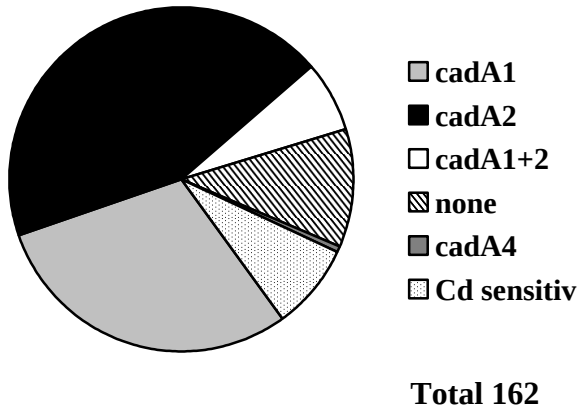


Fig. 4.4. 2. ***cadA*** **distribution in non-pathogenic** ***Listeriae*** **spp.** The majority of the isolates harbored *cadA2* (43%), followed by *cadA1* (30%). Isolates with both *cadA1* and *cadA2* were relatively uncommon (7%) and only single isolate harbored *cadA4*. A fraction (12%) of cadmium resistant isolates lacked any of the known *cadA* genes. Cadmium susceptible isolates also lacked the known *cadA* genes

4.4.3. ***cadA*** **distribution in** ***L. welshimeri*** **and** ***L. innocua:*** *cadA1* and *cadA2* were similarly common in *L. welshimeri* (A) and *L. innocua* (B). Isolates harboring both *cadA1* and *cadA2* also were similarly prevalent in the two species (6-8%). Cadmium-resistant isolates lacking known *cadA* genes were more common among *L. welshimeri* (16%) than *L. innocua* (9%) (Fig. 4.4.3)

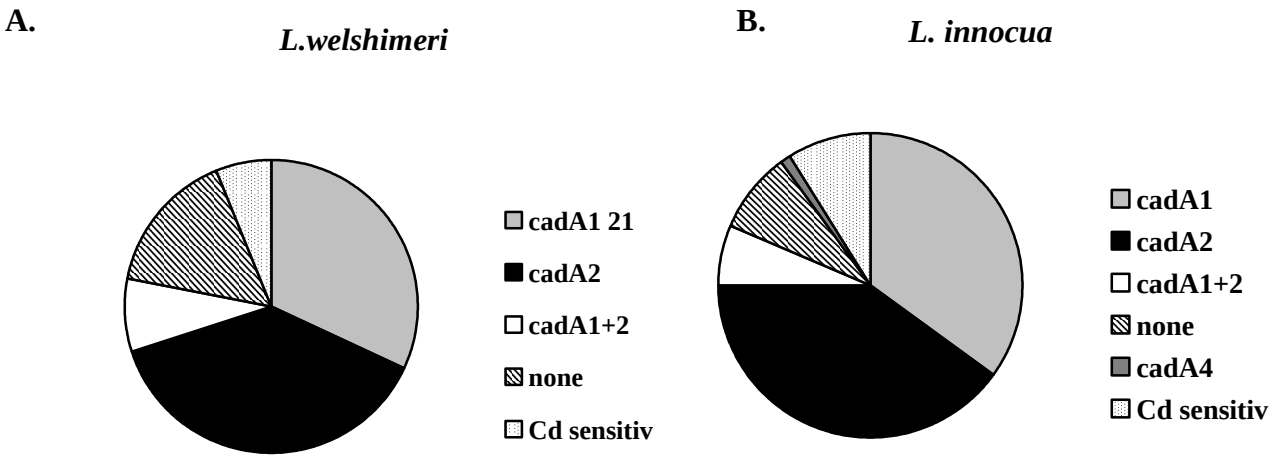


Fig. 4.4.3. *cadA* distribution in *L. welshimeri and L. innocua*. Both *cadA1* and *cadA2* were similarly common in *L. welshimeri* (A) and *L. innocua* (B). Distribution of isolates harboring both *cadA1* and *cadA2* genes seem to be slightly higher in *L. welshimeri* (8%) then *L. innocua* (6%). Cadmium-resistant isolates lacking known *cadA* genes were more common among *L. welshimeri* (16%) then *L.innocua* (9%)

4.4.4. Plant-specific prevalence of *cadA* genes in *L. welshimeri*: For *L. welshimeri*, plant-specific differences were noted in *cadA2* prevalence, which was higher in isolates from plant B (73%) than A (36%) or F (14%). Comparisons among this three plants revealed significant difference in *cadA2* prevalence between B and F (p=0.0035). In contrast, *cadA1* was more prevalent in isolates from F (64%) than A (14%) or B (8%) with difference statistically significant between plants A and F (p=0.0036) and between B and F (p=0.0003). Cadmium-resistant isolates lacking the known *cadA* genes were identified in all plants with >10 isolates and were more common in plant A (32%) than B (8%) or F (14%) with no significant difference among plants (Fig. 4.4.4).

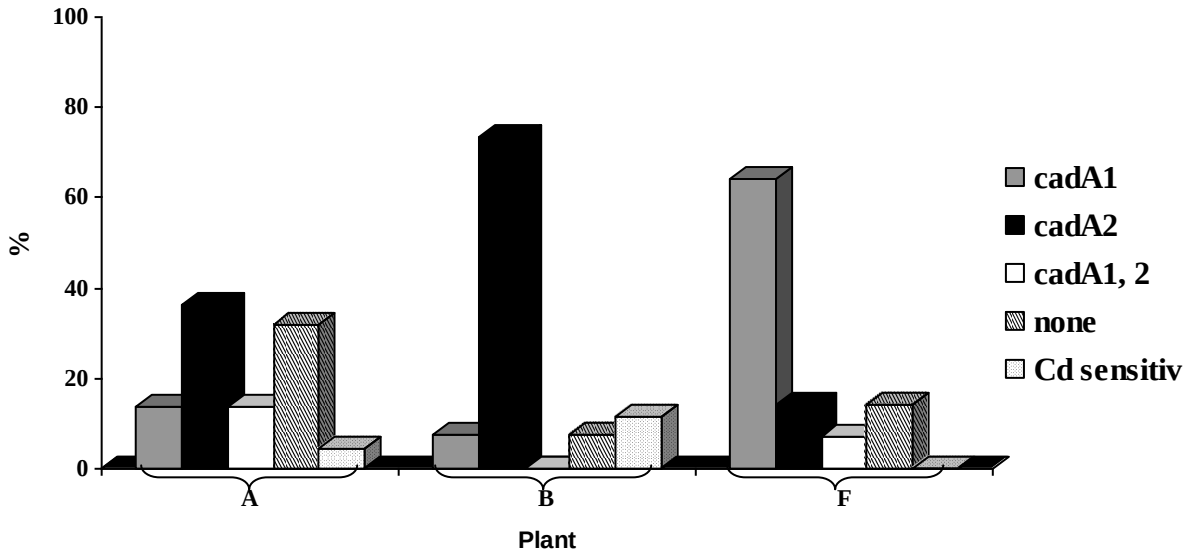


Fig. 4.4.4. Plant – specific prevalence of *cadA* genes in *L. welshimeri*: Plant-specific differences were noted in *cadA2* prevalence, which was higher in isolates from plant B (36%) or F (14%). In contrast, *cadA1* was more prevalent in isolates from plant F (64%) than A (14%) or B (8%). Cadmium- resistant isolates lacking the known *cadA* genes were identified in all plants with >10% isolates and were more common in plant A (32%) than B (8%) or F (14%)

4.4.5. Plant-specific prevalence of *cadA* genes in *L. innocua*: *cadA1* was markedly more prevalent in three plants, C (77%), F (55%) and B (37%) than in A (6%). Comparisons of *cadA1* prevalence in plant A with plants B, C and F revealed that difference was statistically significant (p=0.0102, 0.0002, 0.0049, respectively).On the other hand, *cadA2* was more prevalent in A (61%) and B (40%) than C (15%) or F (18%), with statistically significant difference between plants A and C (p=0.0129). Cadmium-resistant isolates

lacking any of the known *cadA* genes were detected in A (14%) and B (11%) with no significant difference among the plants, but not in C or F (Fig. 4.4.5).

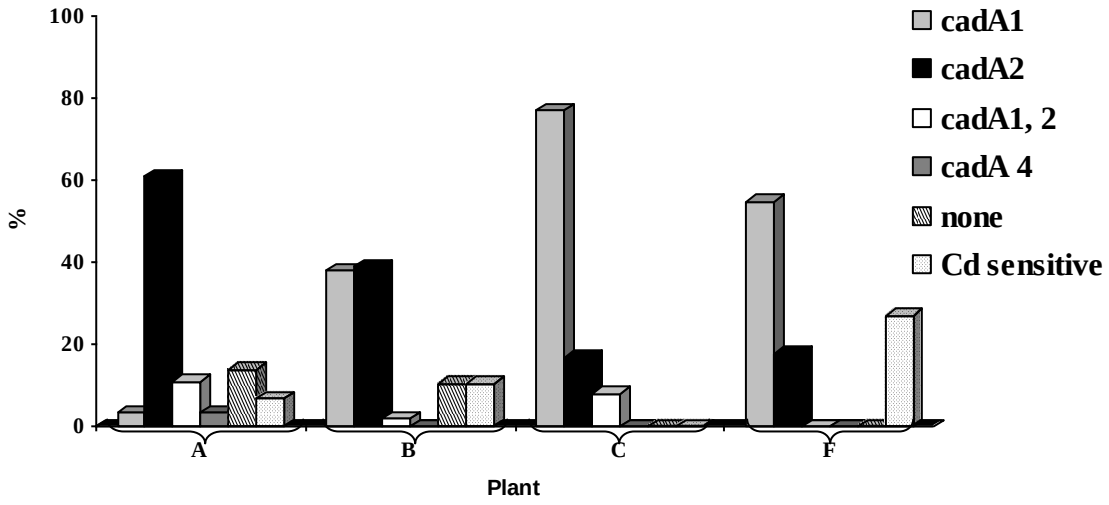


Fig. 4.4.5. Plant – specific prevalence of *cadA* genes in *L. innocua*: *cadA1* was markedly more prevalent in three plants C (77%), F (55%) and B (37%) than in A (6%). On the other hand, *cadA2* was more prevalent in A (61%) and B (40%) than C (15%) or F (18%). Cadmium-resistant isolates lacking any of the known *cadA* genes were detected in A (14%) and B (11%) but not in C or F.

4.4.6. Plant-specific comparisons between species:

Plant A: *cadA1* was relatively uncommon, regardless of species (14 and 6% of *L. welshimeri* and *L. innocua*, respectively), while *cadA2* was frequent, regardless of species (36 and 61% of *L. welshimeri* and *L. innocua*, respectively).

Plant F: *cadA1* was predominant, regardless of species (64 and 55% for *L. welshimeri* and *L. innocua*, respectively).

Plant B: *cadA1* was noticeably more common for *L. innocua* (37%) than *L. welshimeri* (8%), while the reverse was found for *cadA2* (73% of *L. welshimeri* vs. 40% of *L. innocua*).

Isolates lacking known *cadA* genes appear to be generally more common in *L. welshimeri* than *L. innocua*, primarily due to data from plants A and F, whereas distribution of Cd susceptible isolates was equal among the species**.**

4.4.7. BC resistance: Resistance to BC was highly prevalent (79-82%) among *L. welshimeri* isolates from all plants with >10 isolates (A, B, F). BC resistance prevalence was 58% in *L. innocua* from plant B, but markedly higher (85-93%) in *L. innocua* from plants A, C and F. Difference was significant between plants A and B (p=0.0052). Lower prevalence in plant B isolates was the reason for the overall lower prevalence of BC resistance in *L. innocua* (73%) than *L. welshimeri* (83%). All BC-resistant isolates harbored the *bcrABC* resistance cassette earlier found to be transferrable from non-pathogenic *Listeriae* to other *Listeria* (4). (Fig. 4.4.6)

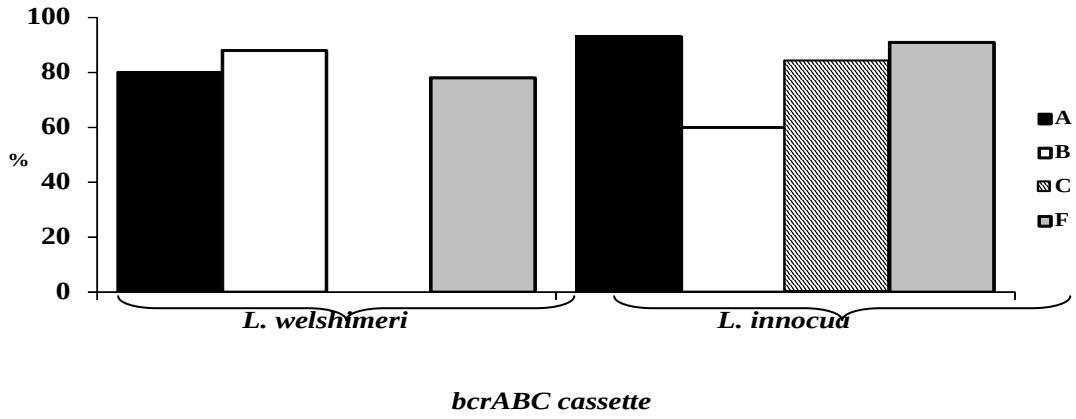
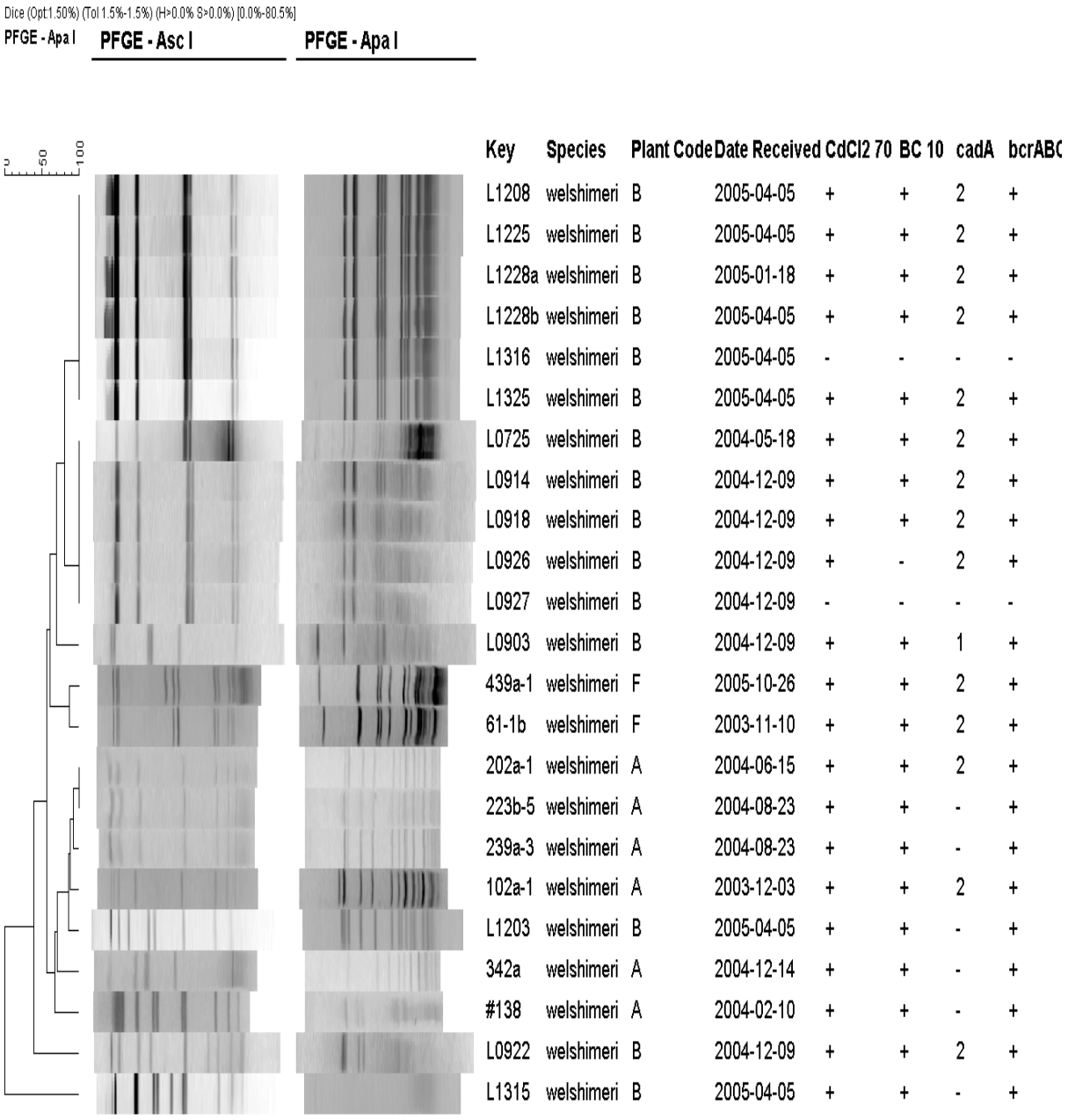


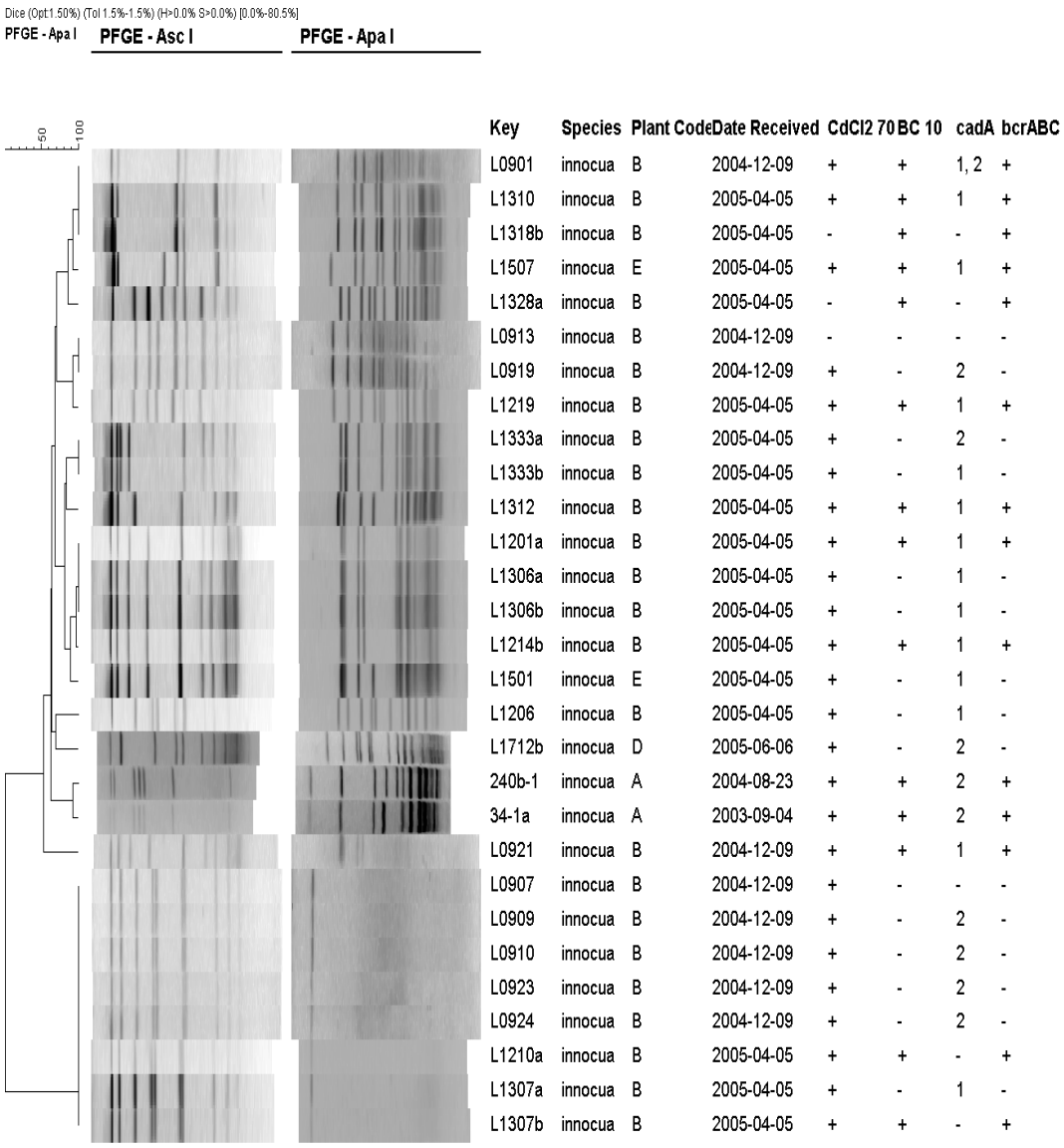
Fig. 4.4.6. Plant-specific BC resistance: Resistance to BC was highly prevalent (79-82%) among *L. welshimeri* isolates from all plants with >10 isolates (A, B, F). BC resistance prevalence was 58% in *L. innocua* from plant B, but markedly higher (85-93%) in *L. innocua* from plants A, C and F. Lower prevalence in plant B isolates was the reason for the overall lower prevalence of BC resistance in *L. innocua* (73%) than *L. welshimeri* (83%). All BC-resistant isolates harbored the *bcrABC* resistance cassette. All BC-resistant isolates were also resistant to cadmium, but the reverse was not the case. An estimated 14% *L. welshimeri* and 23% *L. innocua* isolates were resistant to cadmium but susceptible to BC. Most of these isolates harbored *cadA1* (45%), followed by *cadA2* (29%), with 16% lacking any of the known *cadA* genes.

All BC-resistant isolates were also resistant to cadmium, but the reverse was not the case. An estimated 14% *L. welshimeri* and 23% of *L. innocua* isolates were resistant to cadmium but susceptible to BC. Most of these isolates harbored *cadA1* (45%), followed by *cadA2* (29%), with 16% lacking any of the known *cadA* genes. Among isolates harboring *cadA*1 determinant highly prevalent were BC resistant ($p=0.0003$). Similarly high prevalence of BC resistance was observed among isolates harboring *cadA*2 cassette ($p<0.0001$).

4.4.8. PFGE. Clusters of isolates with indistinguishable or closely related PFGE profiles were identified both in *L. innocua* and *L. welshimeri*. Most clusters were plant-specific, and included isolates from different sites in the plant and isolation dates, suggesting strains that were persistent and disseminated in the plant. Isolates in the same cluster frequently exhibited the same resistance profiles and *cadA-bcrABC* genotype. However, several exceptions were also noted, suggesting local gene acquisition or loss events (Fig. 4.4.7).



Key	Species	Plant Code	Date Received	CdCl2 70	BC 10	cadA	bcrABC
L1208	welshimeri	B	2005-04-05	+	+	2	+
L1225	welshimeri	B	2005-04-05	+	+	2	+
L1228a	welshimeri	B	2005-01-18	+	+	2	+
L1228b	welshimeri	B	2005-04-05	+	+	2	+
L1316	welshimeri	B	2005-04-05	-	-	-	-
L1325	welshimeri	B	2005-04-05	+	+	2	+
L0725	welshimeri	B	2004-05-18	+	+	2	+
L0914	welshimeri	B	2004-12-09	+	+	2	+
L0918	welshimeri	B	2004-12-09	+	+	2	+
L0926	welshimeri	B	2004-12-09	+	-	2	+
L0927	welshimeri	B	2004-12-09	-	-	-	-
L0903	welshimeri	B	2004-12-09	+	+	1	+
439a-1	welshimeri	F	2005-10-26	+	+	2	+
61-1b	welshimeri	F	2003-11-10	+	+	2	+
202a-1	welshimeri	A	2004-06-15	+	+	2	+
223b-5	welshimeri	A	2004-08-23	+	+	-	+
239a-3	welshimeri	A	2004-08-23	+	+	-	+
102a-1	welshimeri	A	2003-12-03	+	+	2	+
L1203	welshimeri	B	2005-04-05	+	+	-	+
342a	welshimeri	A	2004-12-14	+	+	-	+
#138	welshimeri	A	2004-02-10	+	+	-	+
L0922	welshimeri	B	2004-12-09	+	+	2	+
L1315	welshimeri	B	2005-04-05	+	+	-	+



Key	Species	Plant Code	Date Received	CdCl2 70	BC 10	cadA	bcrABC
L0901	innocua	B	2004-12-09	+	+	1, 2	+
L1310	innocua	B	2005-04-05	+	+	1	+
L1318b	innocua	B	2005-04-05	-	+	-	+
L1507	innocua	E	2005-04-05	+	+	1	+
L1328a	innocua	B	2005-04-05	-	+	-	+
L0913	innocua	B	2004-12-09	-	-	-	-
L0919	innocua	B	2004-12-09	+	-	2	-
L1219	innocua	B	2005-04-05	+	+	1	+
L1333a	innocua	B	2005-04-05	+	-	2	-
L1333b	innocua	B	2005-04-05	+	-	1	-
L1312	innocua	B	2005-04-05	+	+	1	+
L1201a	innocua	B	2005-04-05	+	+	1	+
L1306a	innocua	B	2005-04-05	+	-	1	-
L1306b	innocua	B	2005-04-05	+	-	1	-
L1214b	innocua	B	2005-04-05	+	+	1	+
L1501	innocua	E	2005-04-05	+	-	1	-
L1206	innocua	B	2005-04-05	+	-	1	-
L1712b	innocua	D	2005-06-06	+	-	2	-
240b-1	innocua	A	2004-08-23	+	+	2	+
34-1a	innocua	A	2003-09-04	+	+	2	+
L0921	innocua	B	2004-12-09	+	+	1	+
L0907	innocua	B	2004-12-09	+	-	-	-
L0909	innocua	B	2004-12-09	+	-	2	-
L0910	innocua	B	2004-12-09	+	-	2	-
L0923	innocua	B	2004-12-09	+	-	2	-
L0924	innocua	B	2004-12-09	+	-	2	-
L1210a	innocua	B	2005-04-05	+	+	-	+
L1307a	innocua	B	2005-04-05	+	-	1	-
L1307b	innocua	B	2005-04-05	+	+	-	+

Fig.4.4.7. PFGE. Clusters of isolates with indistinguishable or closely related PFGE profiles were identified both in *L. welshimeri* and *L. innocua*. Most clusters were plant-specific, and included isolates from different sites in the plant and isolation dates, suggesting strains that were persistent and disseminated in the plant. Isolates in the same cluster frequently exhibited the same resistance profiles and *cadA-bcrABC* genotype. However, several exceptions were also noted, suggesting local gene acquisition or loss events

4.5. Survival and growth of outbreak and other strains of *Listeria monocytogenes* on cantaloupe

4.5.1. Growth and survival of *L. monocytogenes* on cantaloupe: *L. monocytogenes* was not isolated before inoculation from the surface of any of the whole cantaloupes used in the study. APCs on the outer surfaces were approximately 10^4cfu/g (Fig.4.5.1) while on the flesh they were approximately10^2 CFU/g (date not shown).

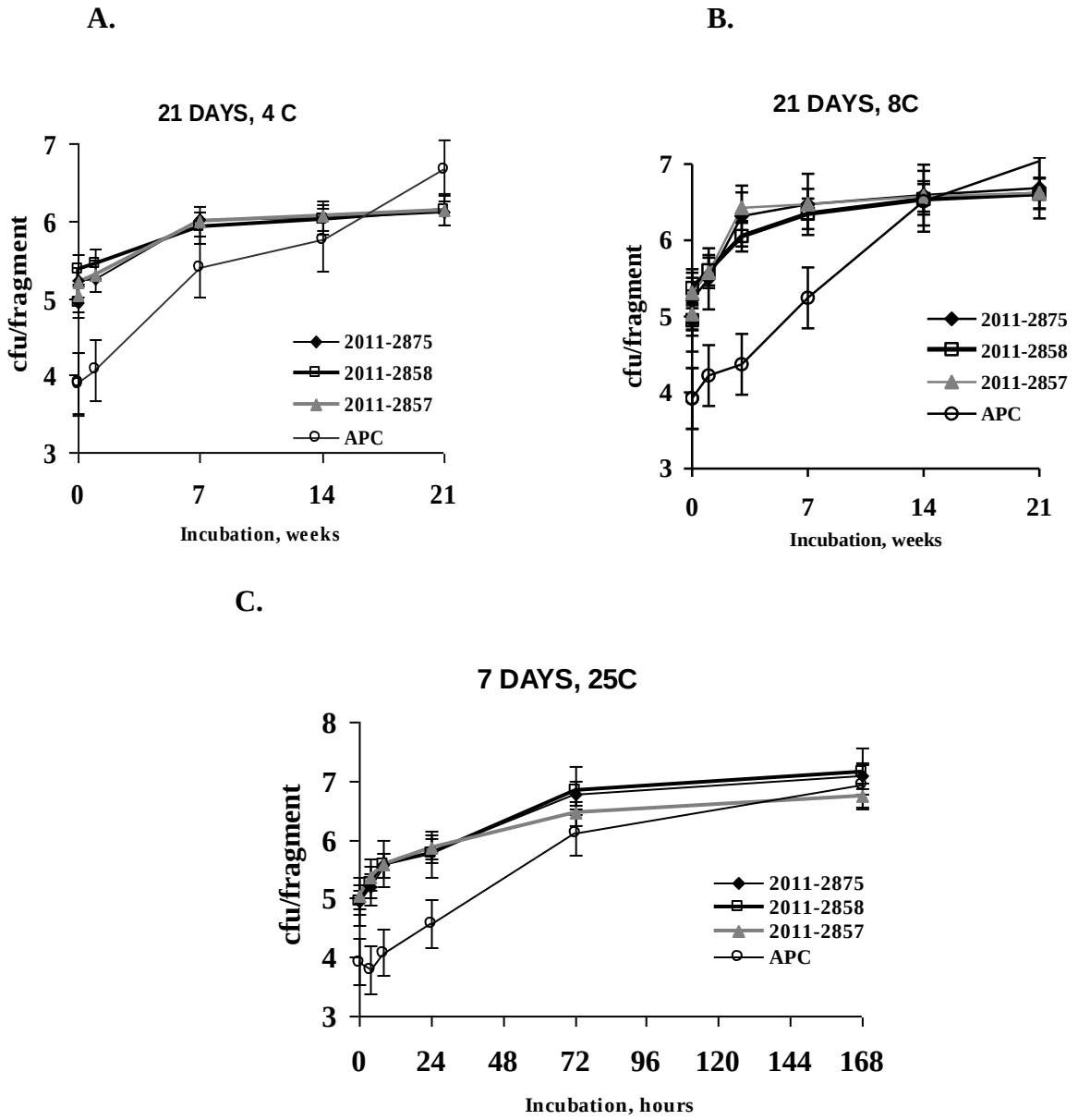


Fig.4.5.1. Growth of *L. monocytogenes* on cantaloupe rind A. *L. monocytogenes* 2011L-2875, 2011L-2858 and 2011L-2857, derived from the 2011 cantaloupe outbreak were tested for their ability to survive and grow on the cantaloupe rind during **A**, 21 days at 4ºC; **B**. **B**, 21 days at 8ºC; **C**, 7 days at 25ºC. Results represent average of three independent trials, each done in duplicate. Inoculations and enumerations were done as described in Materials and Methods.

Our results indicate that *L monocytogenes* survived and grew on cantaloupe fragments during incubation at 4 and 8ºC (monitored for up to 21 days) (Fig. 1A and 1B) and at 25ºC, monitored for up to 7 days (Fig. 1C). In all cases significant increase in the population during the incubation period was noted ($p<0.0001$). At 4ºC populations on the rind increased by an average of 1.0 to 1.2 log units during the 21 day of incubation, with no significant difference among strains (Fig. 4.5.1A). Similar trends were observed for bacteria inoculated on the flesh with slightly lower growth (0.8 log units) in the extract (data not shown).
L. monocytogenes grew significantly more on the rind then on the flesh or in the extract in certain temperature- time combinations. Specifically, after seven days at 4°C all tested strains grew more on the rind than on the flesh or in the extract ($p=0.02$) (Fig.4.5.2A and 3B).

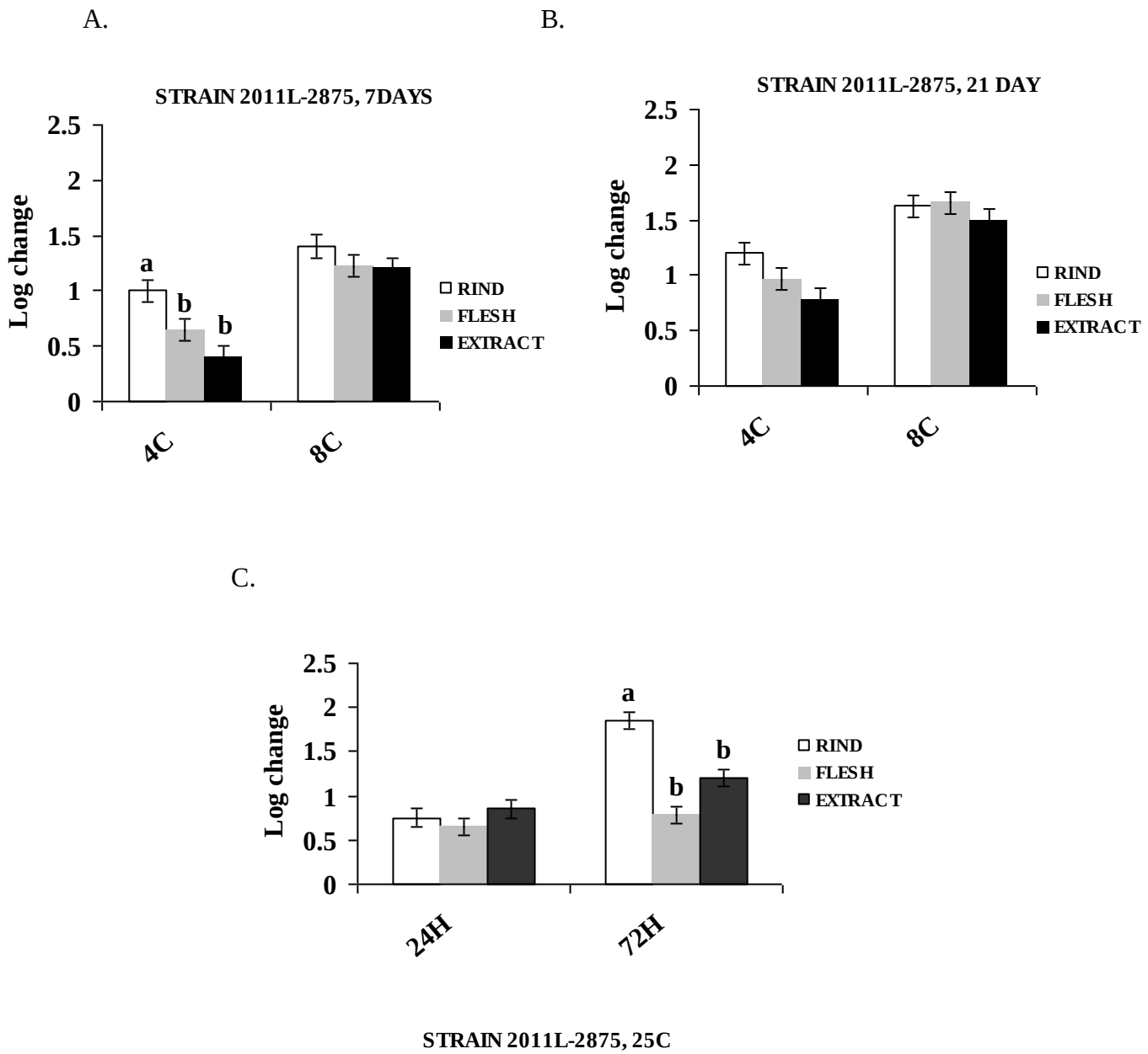


Fig. 4.5. 2. Comparative growth of *L. monocytogenes* 2011L-2875 on cantaloupe rind, flesh and extract after 24 and 72 hours at 25°C.

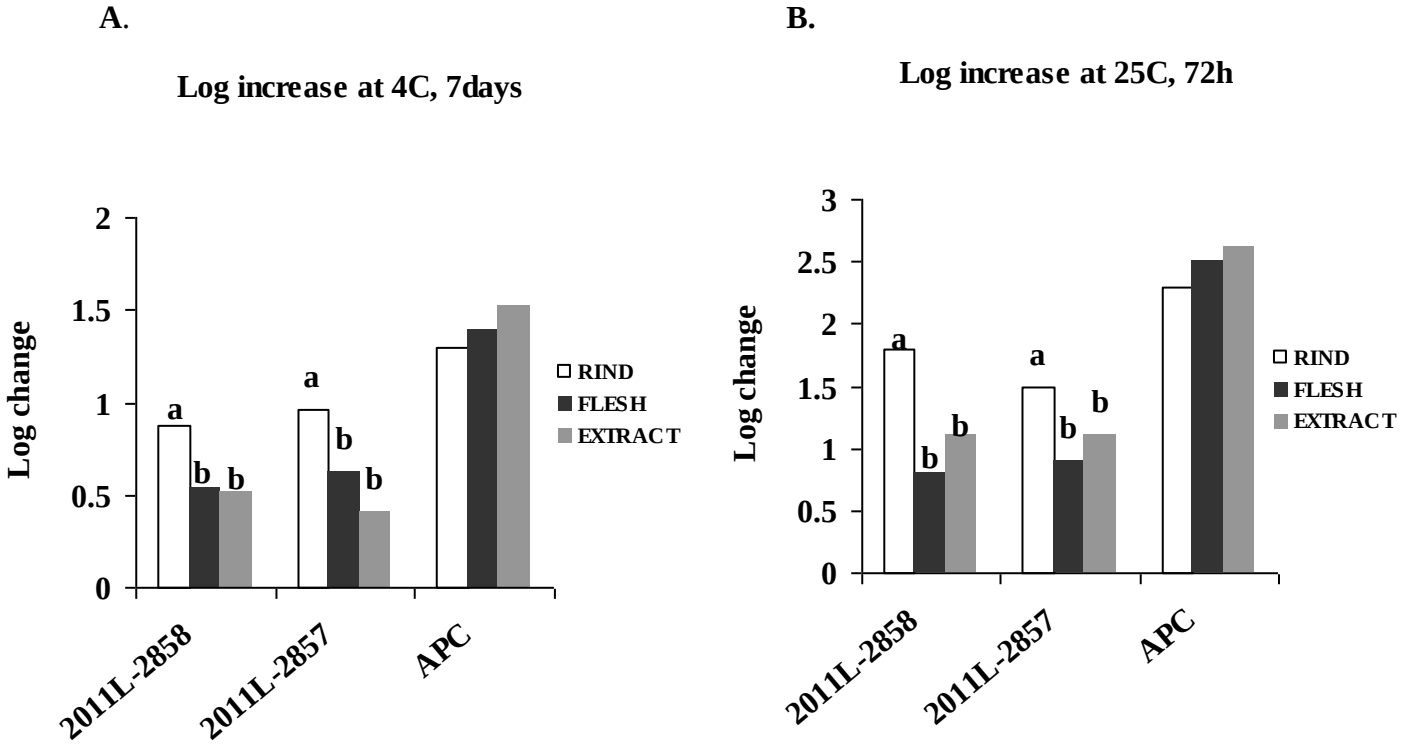


Fig. 4.5.3. Growth of *L. monocytogenes* on cantaloupe rind flesh and extract. Sample inoculations and bacterial enumerations were as described in Materials and Methods. Data indicate population increases after (A) 7 days of incubation at 4ºC and (B) 72 hours at 25ºC (B)

Increase in population (aprox. 1.7 log units) was observed at 8°C over the incubation period of 21 days with no statistically significant difference between rind, flesh and extract (Fig. 1B and 2B). Colony enumeration at 25°C showed increase in population of up to 2.1, 1.9 and 2.0 log units average, for rind flesh and extract, respectively, over the incubation period of 7 days. Noticeably more growth on the rind than on either the flesh or extract was recorded following incubation at 25°C for 72 h ($p<.0001$) (Fig. 4.5. 1C, 4.5. 2C and 4.5.3B); APCs on the *Listeria*-inoculated rind fragments increased at all three tested temperatures (up to 3 log units

over the entire incubation period) (Fig.4.5.1). Similar increases were noted on the peeled fragments and in the extract, even though initial APC levels were lower (data not shown).

4.5.2. Assessment of other *L monocytogenes* strains on cantaloupe. Testing of other *L. monocytogenes* strains of serotype 4b (H7550 and F8027) and 1/2a (2010L-1723) revealed similar location-dependent trend of growth as cantaloupe outbreak strains. Significant difference in growth ($p<.0001$) between rind, flesh and extract occurred after 7 days at 4°C as well as after 72 h at 25°C (Fig. 4.5. 4).

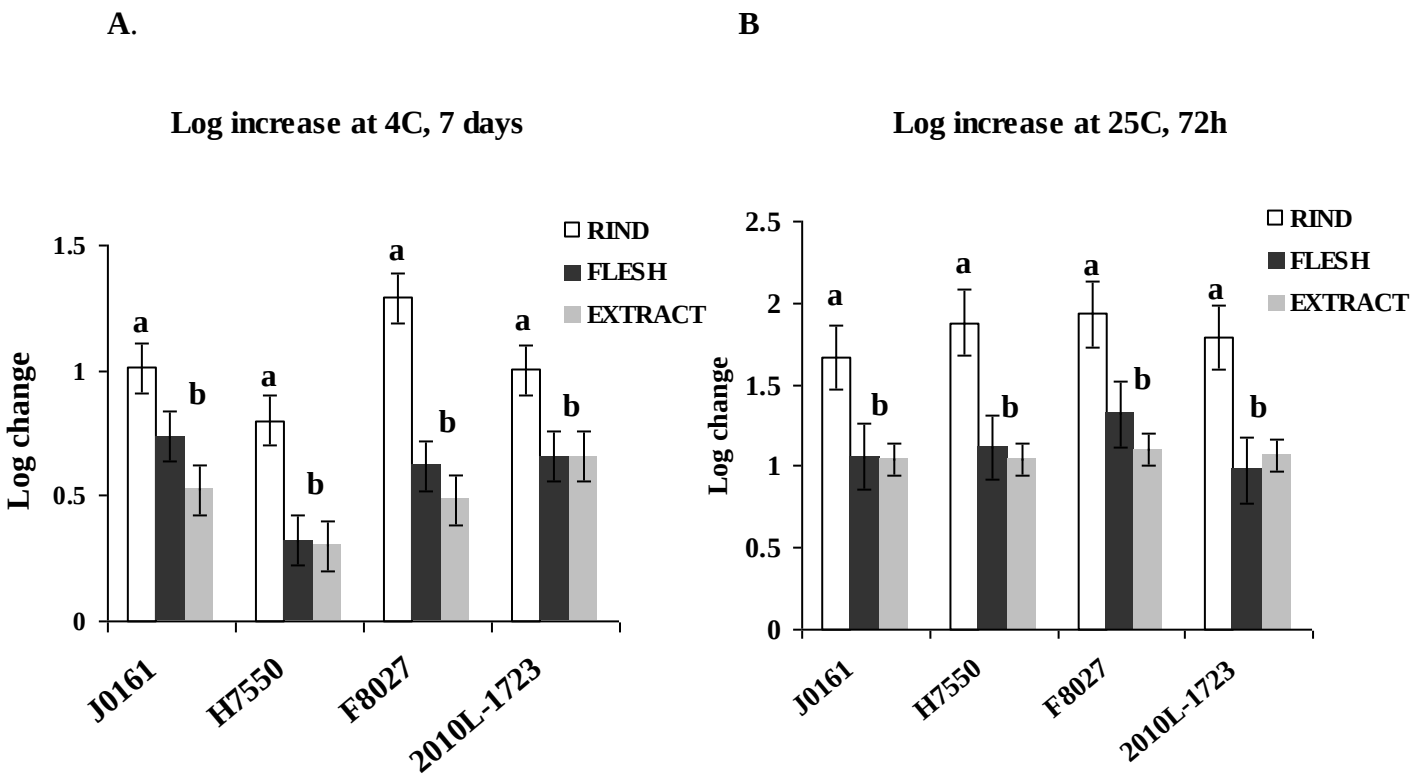


Fig. 4.5.4. Assessment of survival and growth of other *L. monocytogenes* strains on cantaloupe rind at 4 and 25°C.

Strains:

J0161 (serotype 1/2a, 2000 turkey deli meats outbreak),

2010L-1723 (serotype 1/2a, 2010 celery outbreak),

H7550 (serotype 4b, 1998-99 hot dogs outbreak) and

F8027 (serotype 4b, celery)

Unlike strains from cantaloupe outbreak, evidence of a strain effect has been noted among this panel. Strain F8027 expressed significantly higher growth ($p<.0001$) on the cantaloupe rind after 24 hours and 21 days at 4°C, respectively, compared to the strain H7550 (data not shown).

4.5.3. Growth and survival of *L. monocytogenes* on cantaloupe rind after water treatment:

Right after washings decrease in *L. monocytogenes* population on the cantaloupe rind averaged 2 log units (Fig. 4.5.5 and 4.5.6).

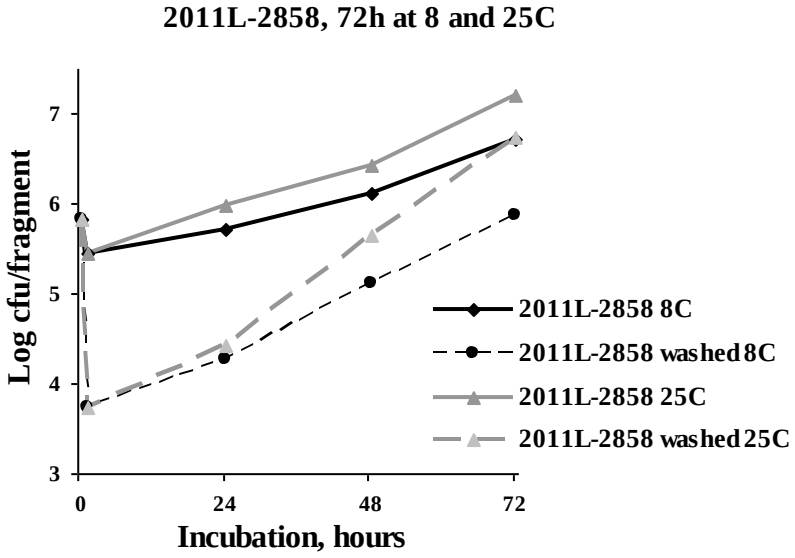


Fig.4.5.5. Growth and survival of *L monocytogenes* on the cantaloupe rind after rinsing with water: Growth and survival of *L monocytogenes* 2011L-2858 over 72 hours of incubation at 8 and 25ºC, Growth and survival of *L monocytogenes* 2011l-2858 on the fragment washed with water after inoculation, followed by incubation at 4, 8 and 25ºC. Presented results are average of two independent trials done in duplicate.

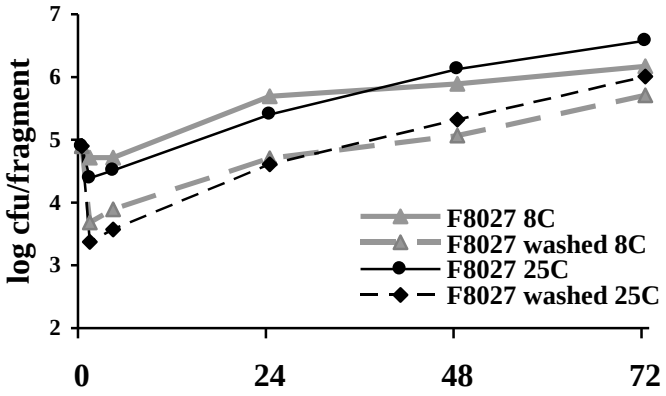
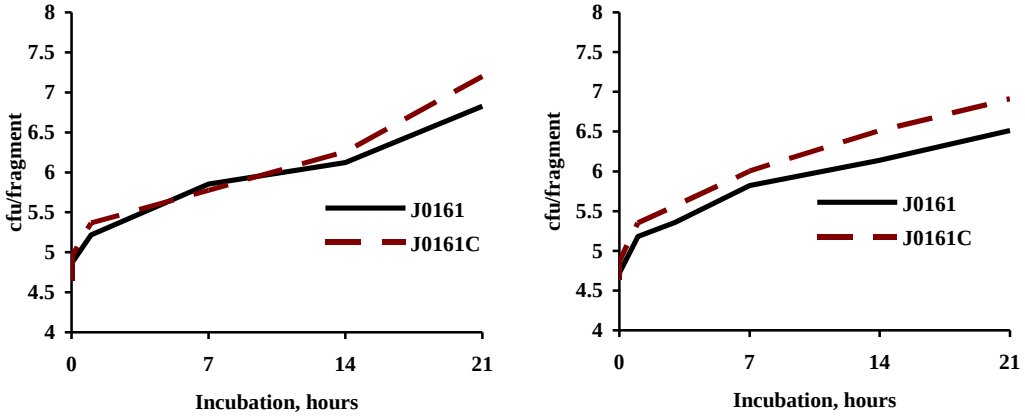


Fig. 4.5.6. Growth and survival of *L monocytogenes* on the cantaloupe rind after rinsing with water: Change in population of *L monocytogenes* F2087 over 72 hours of incubation at 8°C and 25°C before and after rinsing with water. Presented results are average of two independent trials done in duplicate.

Washed fragments were stored at 4, 8 and 25°C, respectively, over 72 hours. After 24 hours at 4°C results indicate decrease of 0.1log and 0.02units for strains 2011L-2858 and F8027, respectively. Extended storage at the same temperature leads to the recovery and increase of 0.2-0.6 after 48 and 72 hours, respectively (data not shown). Increase in population of 0.3, 0.7 and 1.7 log unites at 8ºC after 24, 48 and 72 hours, respectively, had been noted (Figure 4.5.5 and 4.5.6). At the temperature of 25ºC, population ranged from 0.7 – 3.0 log units after 24, 48 and 72h, respectively. Results obtained from the untreated cantaloupe differ with initial decrease of 0.1 log units at 4°C after 24 hours followed by slow growth and increase of 0.02 log over the 72 hours of incubation (data not shown). At the temperatures of 8 and 25ºC ranged from 0.1—1.0 and 0.5-1.3 log unites, respectively (Fig. 4.5. 5 and 4.5.6).

4.6. Change in phenotype and genotype as an asset for *L.monocytogenes* growth on cantaloupe

4.6.1. Growth and survival of *L monocytogenes* J0161 and ciprofloxacin adapted mutant on the surface of cantaloupe. *L. monocytogenes* J0161, a serotype 1/2a strain associated with a listeriosis outbreak in 2001, linked to consumption of turkey deli meats (Olsen) and mutant obtained upon the adaptation to antibiotic ciprofloxacin both expressed increase in population over the incubation period of 21 day on the cantaloupe surface. Increase ranged from 1.7 log units at 4ºC, and 3log units at 8ºC over the 21 days of incubation and 2.7log units at 25ºC over the seven days (Fig. 4.6.1A, B and C). Difference in growth between parental strain and mutant had not been observed.



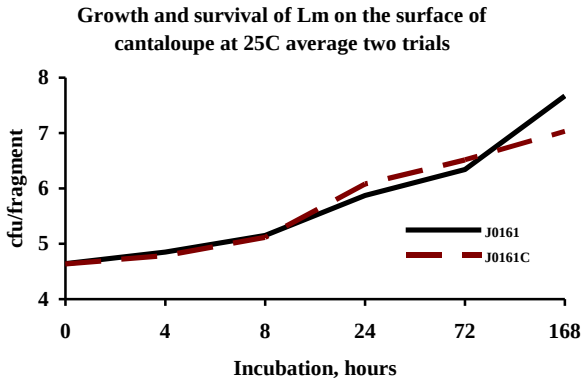


Fig. 4.6.1. Growth and survival of *L monocytogenes* J0161 and ciprofloxacin adapted mutant on the surface of cantaloupe. Growth at 4°C **(A),** 8°C **(B)** over the 3 weeks of incubation; and at 25°C **(C)** over the 7 days of incubation. Results presented are average of two trials

4.6.2. A comparison of behavior on the cantaloupe surface between *L monocytogenes* F2365 along with transposon mutant F2365*purA* and H7550Cds with mutant H7550-Cds *purB*

Strain H7550Cds and *purB* mutant and mutant, both grew at 4, 8 and 25 ºC, respectively (Fig. 4.6.2A and data not shown). Average increase in population of 2.2 log units at 8ºC had been noted with no significant difference between parental strain and mutant. Strain F2365and mutant ROA14, however, did not express significant growth in first 72 hours of incubation at 8ºC, but after this time point trough the end of the incubation period population increased 2.2 log units. There was no significant difference between parental strain and mutant. Washing of cantaloupe fragments after inoculation and before incubation at different temperatures resulted in initial 2-3 log units decrease in population for both parental strains and designated mutants (Fig. 4.6.2B and data not shown). Over the incubation period of 7 days, strains recovered and grew (3-4 log units). Final population on the treated fragments did not differ from the population of *L monocytogenes* on the untreated ones (Fig. 4.6. 2A and B).

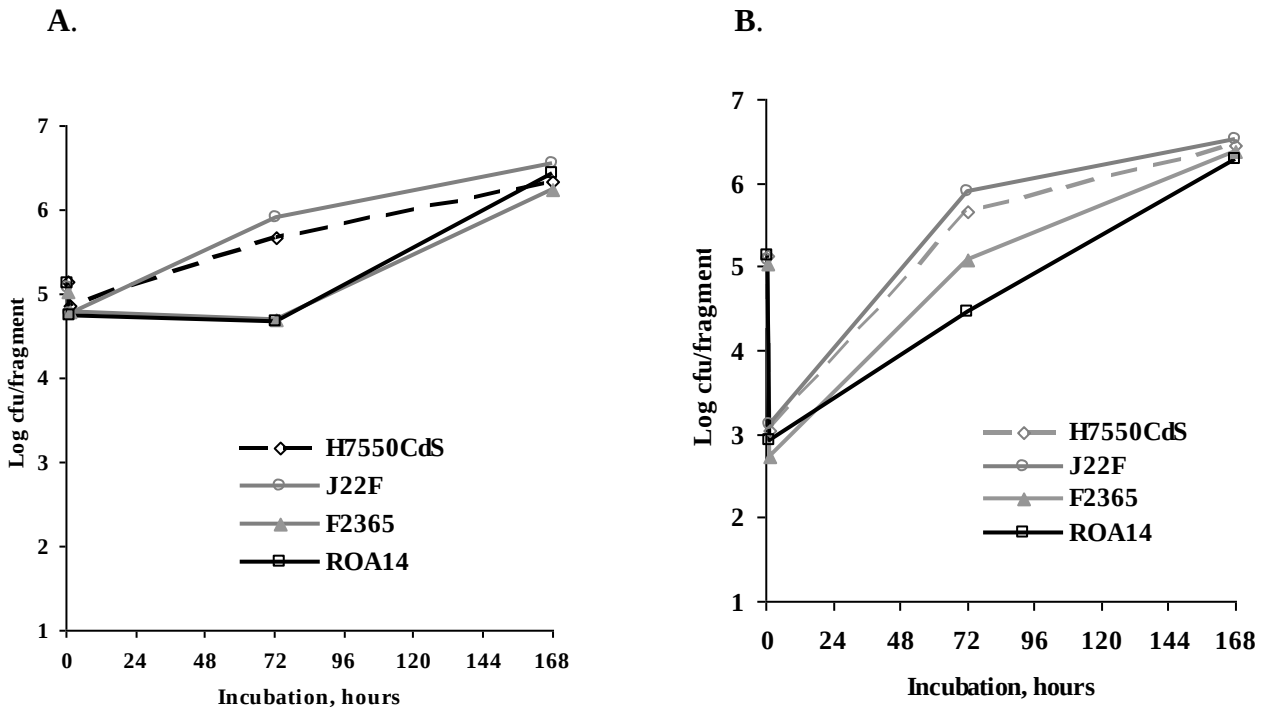


Fig. 4.6.2. A comparison of behavior on the cantaloupe surface between *L. monocytogenes* F2365 along with transposon mutant F2365*purA* and H7550Cds with mutant H7550-Cds *purB* Survival and growth at 8ºC over the seven days of incubation without (A) and with treatment with sterile d-H2O (B). Presented results are average of two independent trials done in duplicate

4.6.3. Comparison of *L monocytogenes* F2365 and cold sensitive mutant F2365 LMOf2365_1746.In first 72 hours of incubation at 4 ºC population of both, parental strain F2365 and cold sensitive mutant ROA4 decreased 1 log unit. Beyond this time point, parental F2365 grew with population increase of 3 log units at the end of the incubation period. Expectedly, mutant ROA4 did not grow. On the contrary, constant decrease in population had been noted over the incubation period of 21 days (Fig. 4.6.3A and data not shown). Treatment of the inoculated fragments with water prior the incubation at 4ºC resulted in initial decrease

of 3log units. Interestingly, in first 72 hours of incubation, both strains grew with increase of 2 logs, followed by further growth of the parental strain and stagnation of mutant ROA4 (Fig. 4.6.3B).

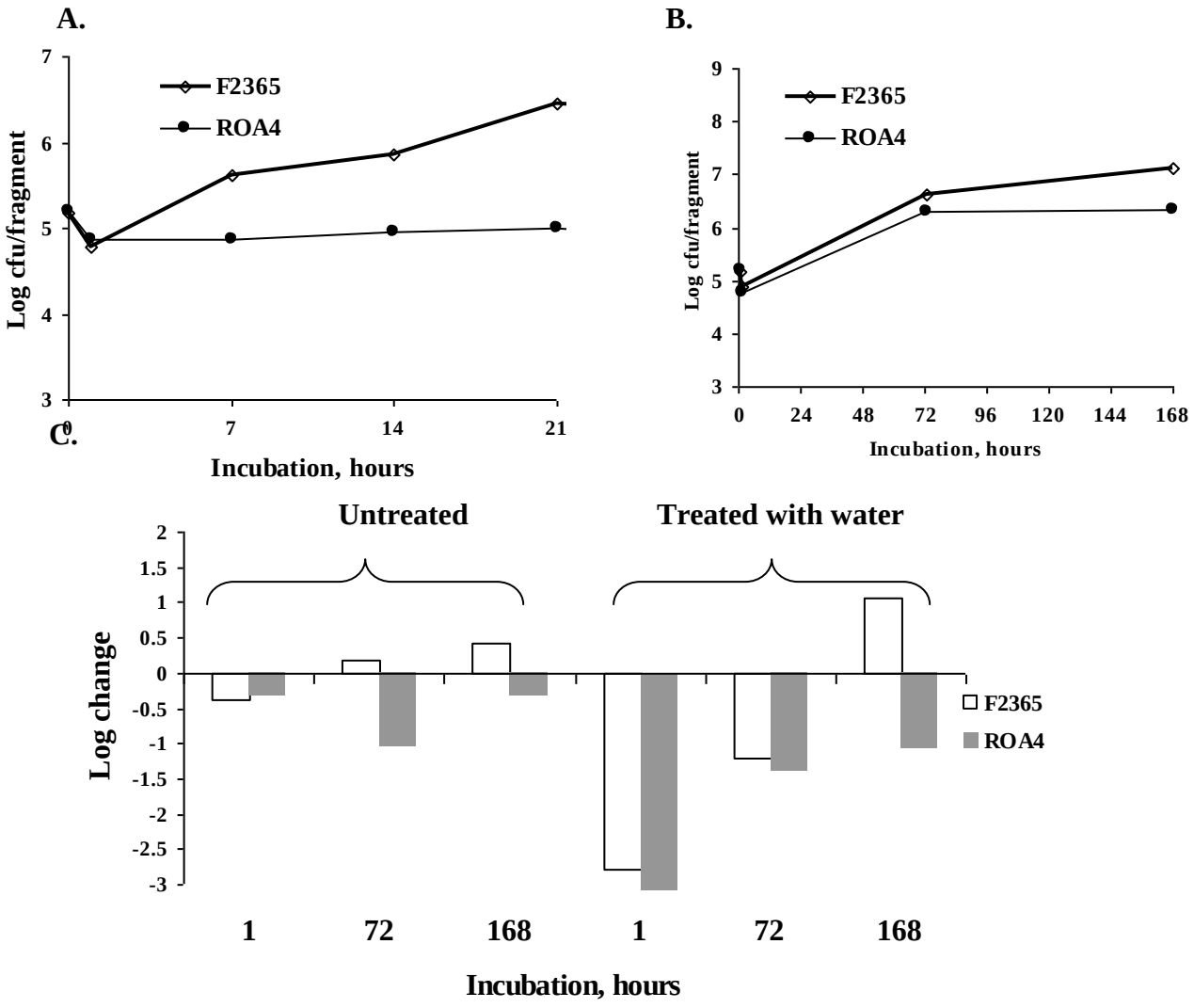


Fig. 4.6. 3.Comparison of *L. monocytogenes* F2365 and cold sensitive mutant F2365 LMOf2365_1746 Survival and growth at 4ºC over the seven days of incubation without (A) and with treatment with sterile d-H2O (B). Change in *L monocytogenes* population over the incubation period (C).

4.6.4. Deletion of the genomic region 18 in *L monocytogenes* strains F2365 and H7550 and performance on the cantaloupe surface. Within the first 72 hours of incubation at 8ºC population of *L. monocytogenes* H7550 and ECΔ18R as well as F2365 and F2365Δ18R

decreased approximately 0.4 log unit. After initial decrease strains recovered and grew 1 log unit average, over the 7 days of incubation (Fig. 4.6.4 A1). Same trend had been noted in the population of *L monocytogenes* on the washed fragments (Fig. 4.6.4 A2) with initial decrease of approximately 2.0 log units. At 25 ºC both parental strains and deletion mutants grew approximately 1.3 – 2.3 log units after 72 and 168 hours, respectively, independent of weather fragments were treated with ater or not.

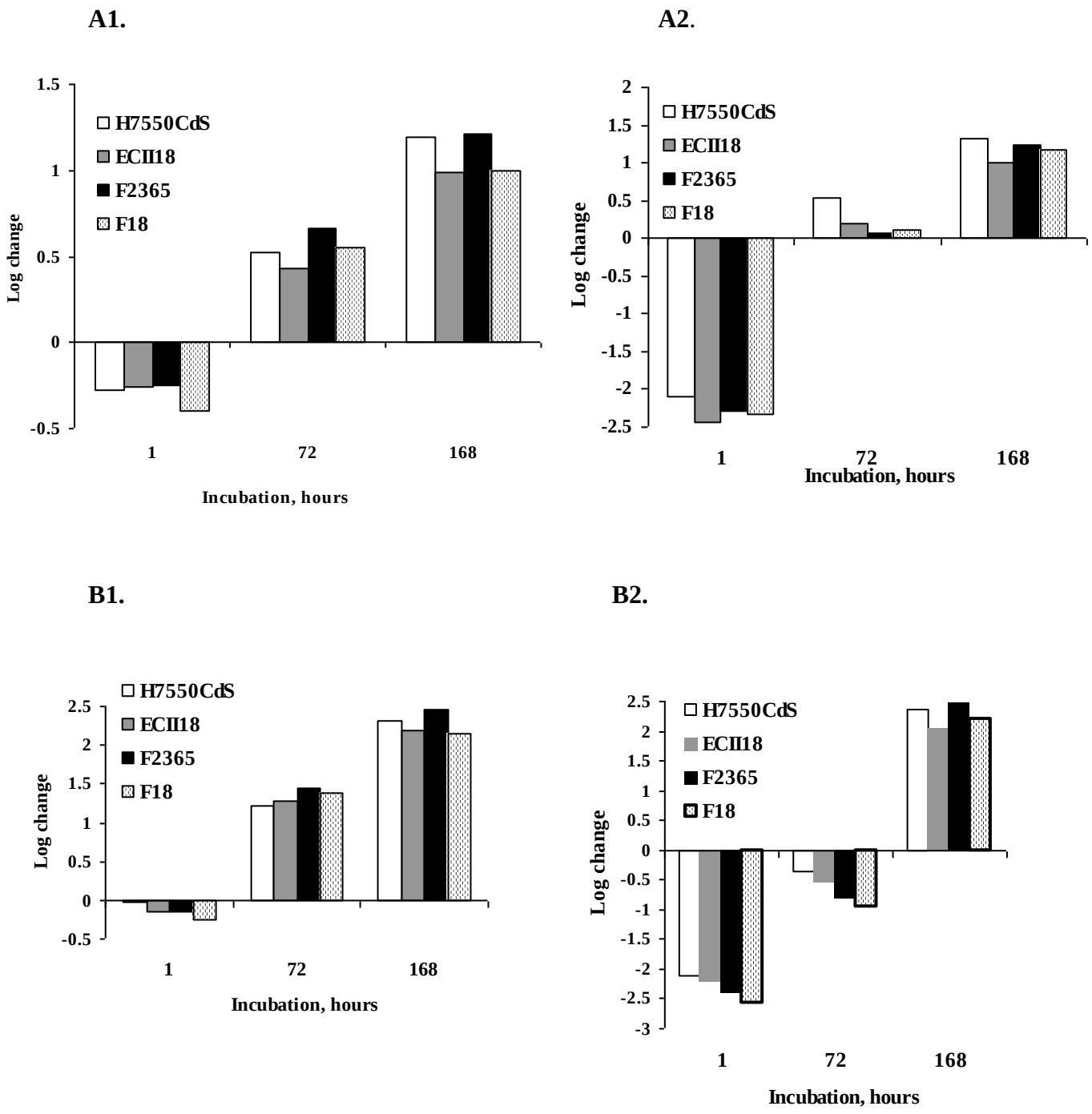


Fig. 4.6.4.Deletion of the genomic region 18 in *L monocytogenes* strains F2365 and H7550 and performance on the cantaloupe surface. Growth at 8°C before **(A1)** and after water treatment **(A2)** and 25°C before **(B1)** and after water treatment **(B2)** over the 7 days of incubation. Results presented are average of two independent trials.

Deletion of the cell-wall associated protein (*wap*) in strain F2365 did not reveal any significant difference between parental strain and mutant (data not shown).

4.7. Assessment of *L. monocytogenes* growth in raw and pasteurised milk

4.7.1. Survival and growth of *L. monocytogenes* in raw and pasteurized milk. The inoculated milk samples were monitored over a 10 days period in 24 hour intervals up to seven days and at 10^{th} day of incubation. All eighteen tested isolates were able to not only survive but also grow in both raw and pasteurized milk (Fig. 4.7.1 A and B and data not shown). *L.monocytogenes* population increased up to 3.5 log cfu/ml in raw and up to 4logcfu/ml in pasteurized milk over the 10 days of incubation at 8°C. As expected, increase in population was statistically significant over the time ($p < 0.0001$). No statistically significant difference was observed among strains. Growth appeared to be higher in pasteurized than in raw milk (Fig. 4.7. 1 A and B and data not shown). Our analysis indicate that differences in growth rate between milk types may depend on strain with most strains growing significantly better in pasteurized milk ($p < 0.0003$).

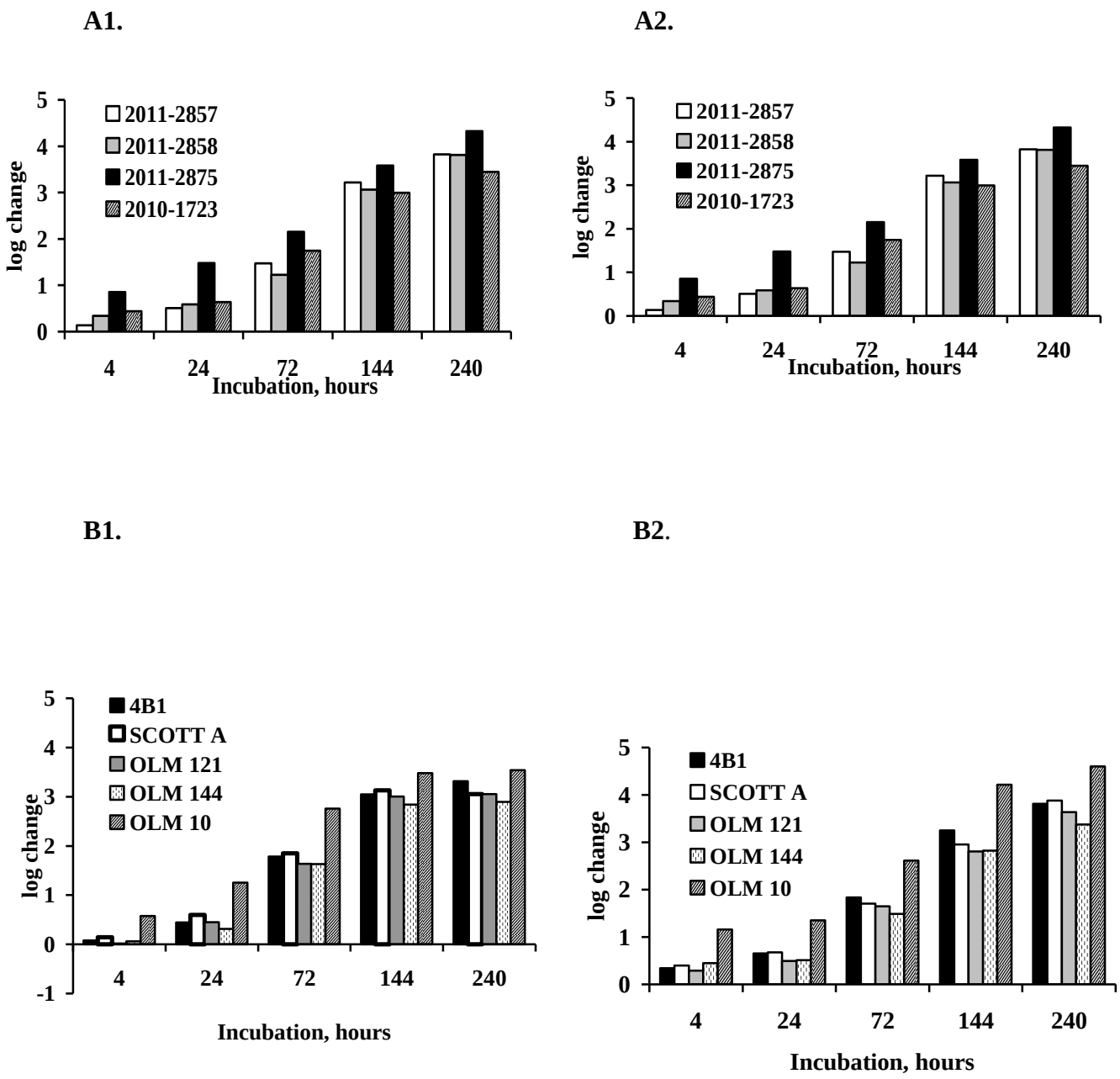


Fig. 4.7.1.Survival and growth of *L monocytogenes* in raw and pasteurized milk. A. Growth of *L monocytogenes* strains involved in 2011 listeriosis outbreak associated with cantaloupe (2011L-2857, 2011L-2858, and 2001L-2875) and 2010 celery associated outbreak (2010-1723) in raw (A1) and pasteurized (A2) milk at 8 º C over 10days;

B. Growth of *L monocytogenes* strains involved in sporadic cases of Listeriosis in the period 1930-1990 (4b1, OLM121, OLM144 and OLM10) and 1983 dairy associated outbreak (SCOTT A) in raw (B1) and pasteurized (B2) milk at 8º C over 10days ;

4.7.2. Serotype comparison in raw and pasteurized milk: Isolates were compared across serotypes (1/2a, 1/2b and 4b) with no significant difference noticed (data not showed).

4.7.3. Wild type and ciprofloxacin adapted mutants in raw and pasteurized milk: Comparison between wild type strain H7550CdR and ciprofloxacin adapted mutant H7550CdRC showed no significant difference in growth between strains or milk type (Fig.4.7. 2 A1 and A2). Strain J0161CdR, wild type, grew better in raw milk compared to ciprofloxacin adapted mutant J0161CdRC, whereas adapted mutants expressed higher population increase in pasteurized milk (Fig. 4.7.2 B1 and B2).

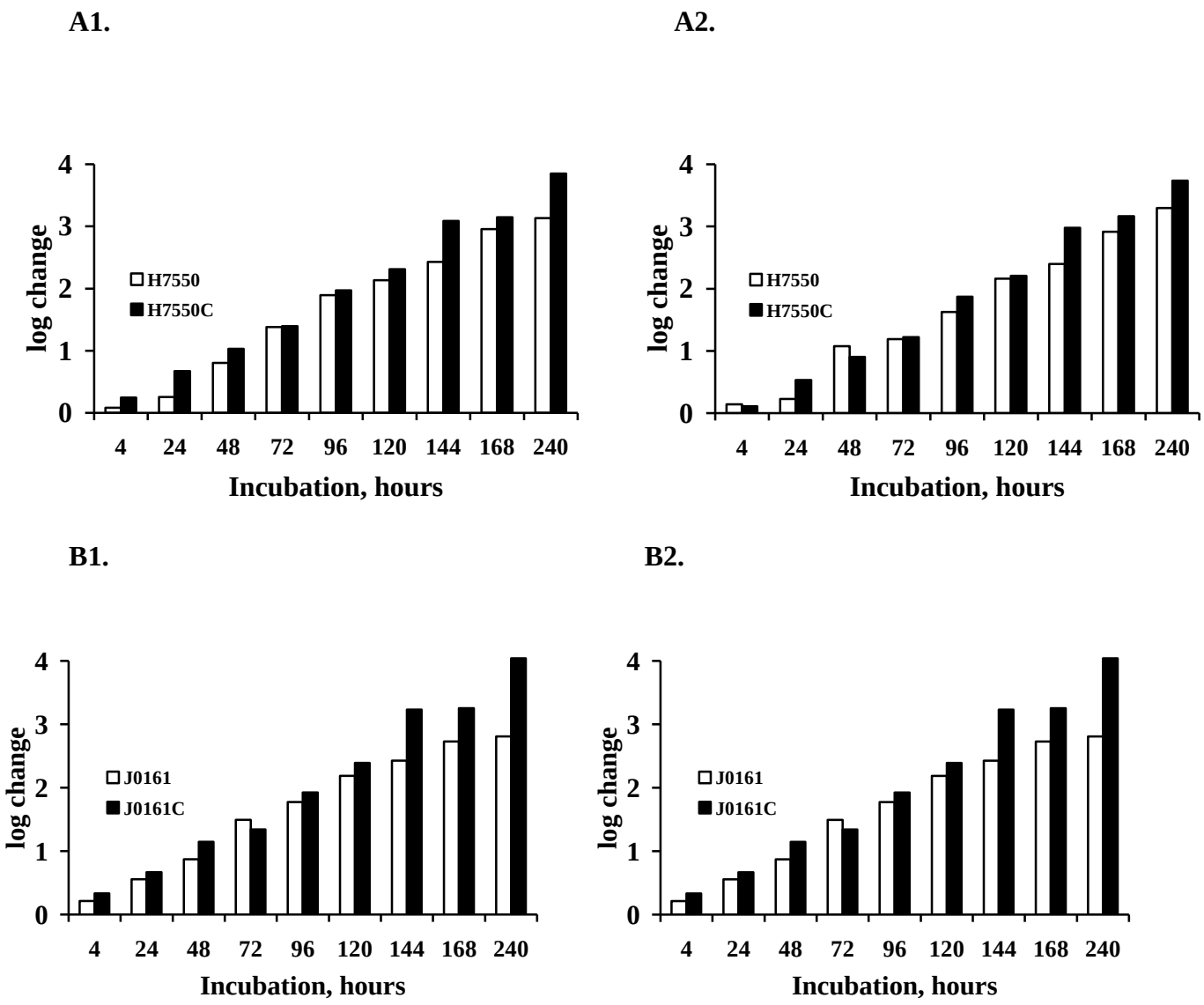


Fig. 4.7.2.Comparison of growth in raw and pasteurized milk between *L monocytogenes* parental strains and ciprofloxacin adapted mutants. A. Increase in population of *L monocytogenes* parental strain H7550 and ciprofloxacin adapted mutant in raw (A1) and pasteurized (A2) milk at 8º C over 10days; **B.** Increase in population of *L monocytogenes* parental strain J0161 and ciprofloxacin adapted mutant in raw (A1) and pasteurized (A2) milk at 8º C over 10days.

4.7.4. Difference between clonal groups: Further analysis revealed significantly better growth of isolates belonging to ECI clonal group in both pasteurized and raw milk compared to group ECIa (p=0.0433 and 0.0166, respectively)(Fig. 4.7. 3A and B) and in pasteurized milk compared to group ECII (p=0.0358) (Fig. 4.7.3.A1 and A2). ECIa associated isolates expressed significantly better growth compared to ECII group in raw milk (p=0.0026) (data not shown) with no difference in pasteurized milk. Overall ECI associated isolates grew significantly better in pasteurized then in raw milk (p=0.0001), whereas among isolates ECIa and ECII this difference was not noticed.

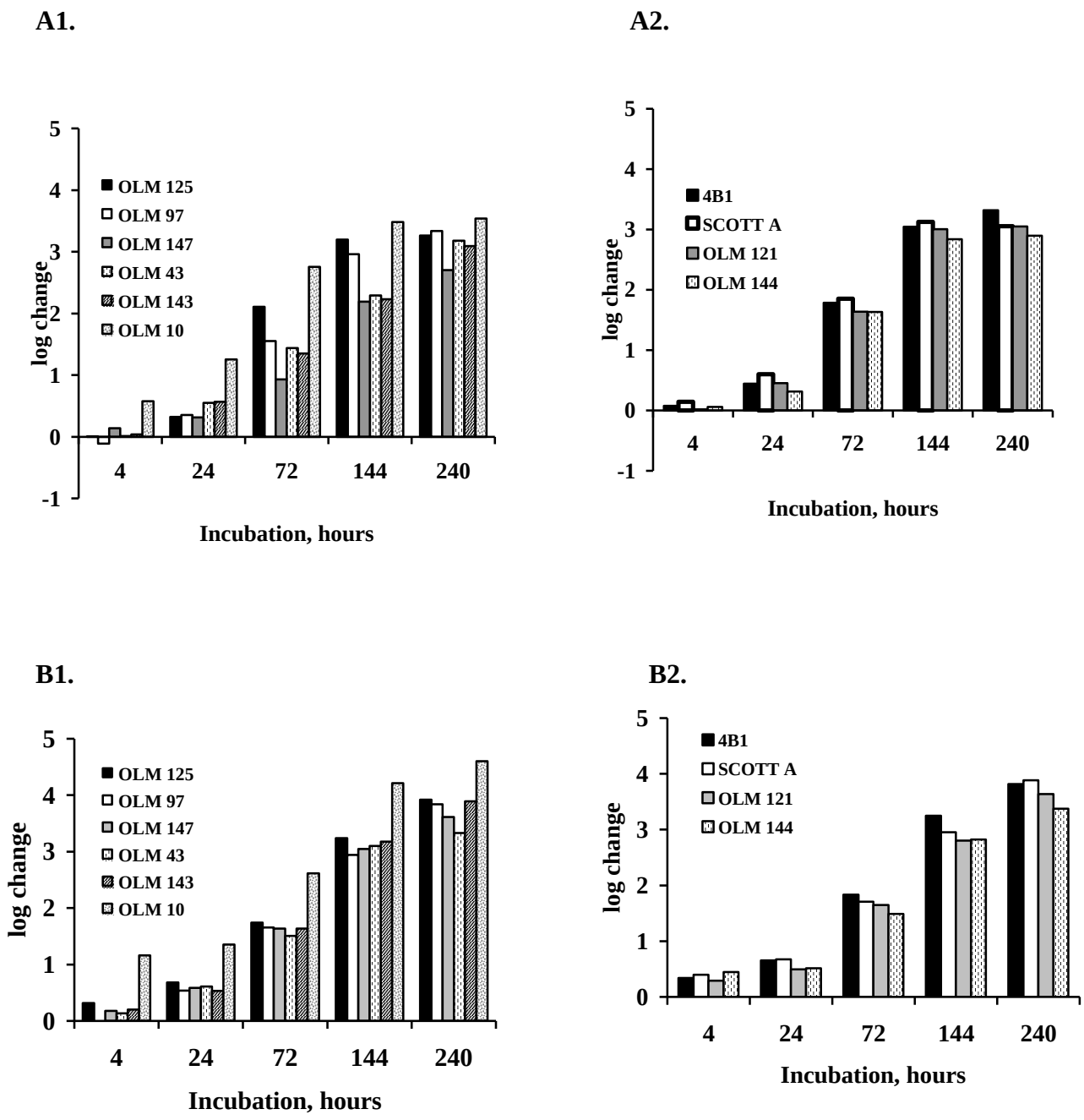


Fig.4.7.3. Comparison of growth between *L monocytogenes* strains associated with different clonal groups. Population of *L monocytogenes* strains belonging to ECI clonal group in both raw (A1) and pasteurized (B1) milk increased more compared to group ECIa in raw (A2) and pasteurized (B2) milk (p=0.0433 and 0.0166, respectively).

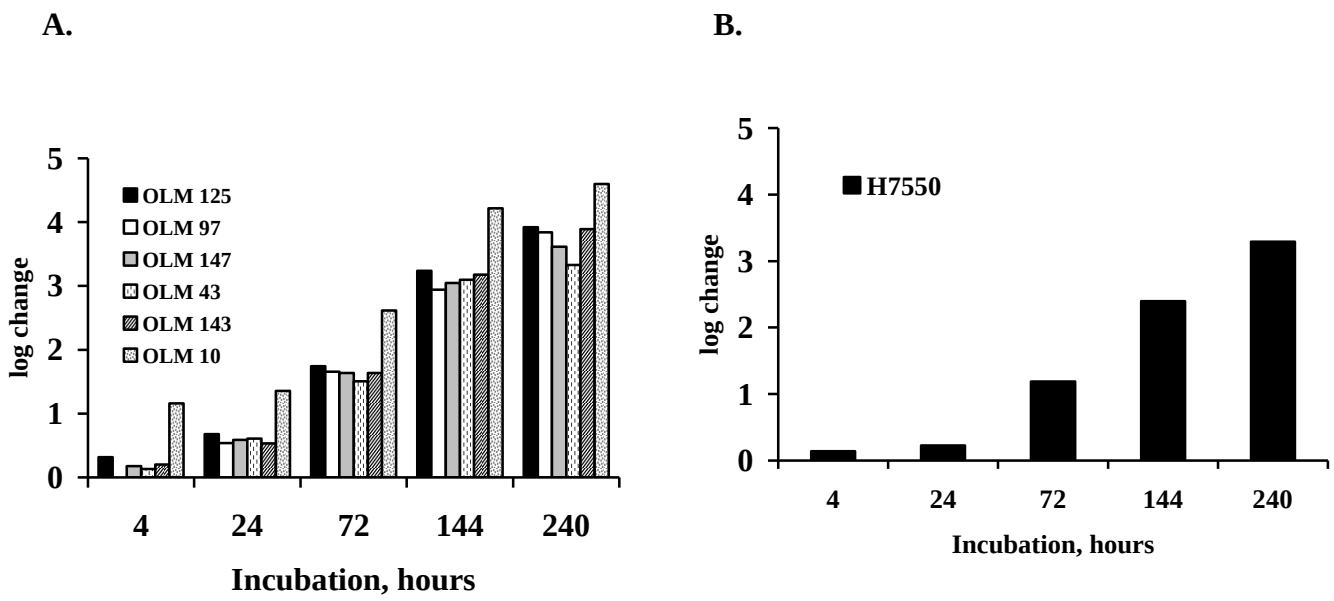


Fig.4.7.4. Comparison of growth between *L monocytogenes* strains associated with different clonal groups. Population of *L monocytogenes* strains belonging to ECI clonal group (A) increased more compared to group ECII in (B) in pasteurized milk (p=0.0358)

L. monocytogenes strains of serotype 1/2a and 1/2b involved in 2010 Texas outbreak associated with celery in chicken salad (2010-1723) and 2011 multistate outbreak connected to Jensen farm cantaloupe (2011L-2857, 2011L-2858 and 2011L-2875) survived and grew in raw and pasteurized milk. Population increase averaged from 3 log cfu/ml in raw and 4 logcfu/milk in pasteurized milk over the 10 days of incubation at 8°C (Fig. 4.7.1A1 and A2). Significant difference in growth between milk types has not been noticed. Growth of *L monocytogenes* strains associated with 2011 cantaloupe outbreak in milk both, raw and pasteurized was notably higher compared to growth of the same strains inoculated on the rind or the flash of cantaloupe, respectively (Fig. 4.7.5).

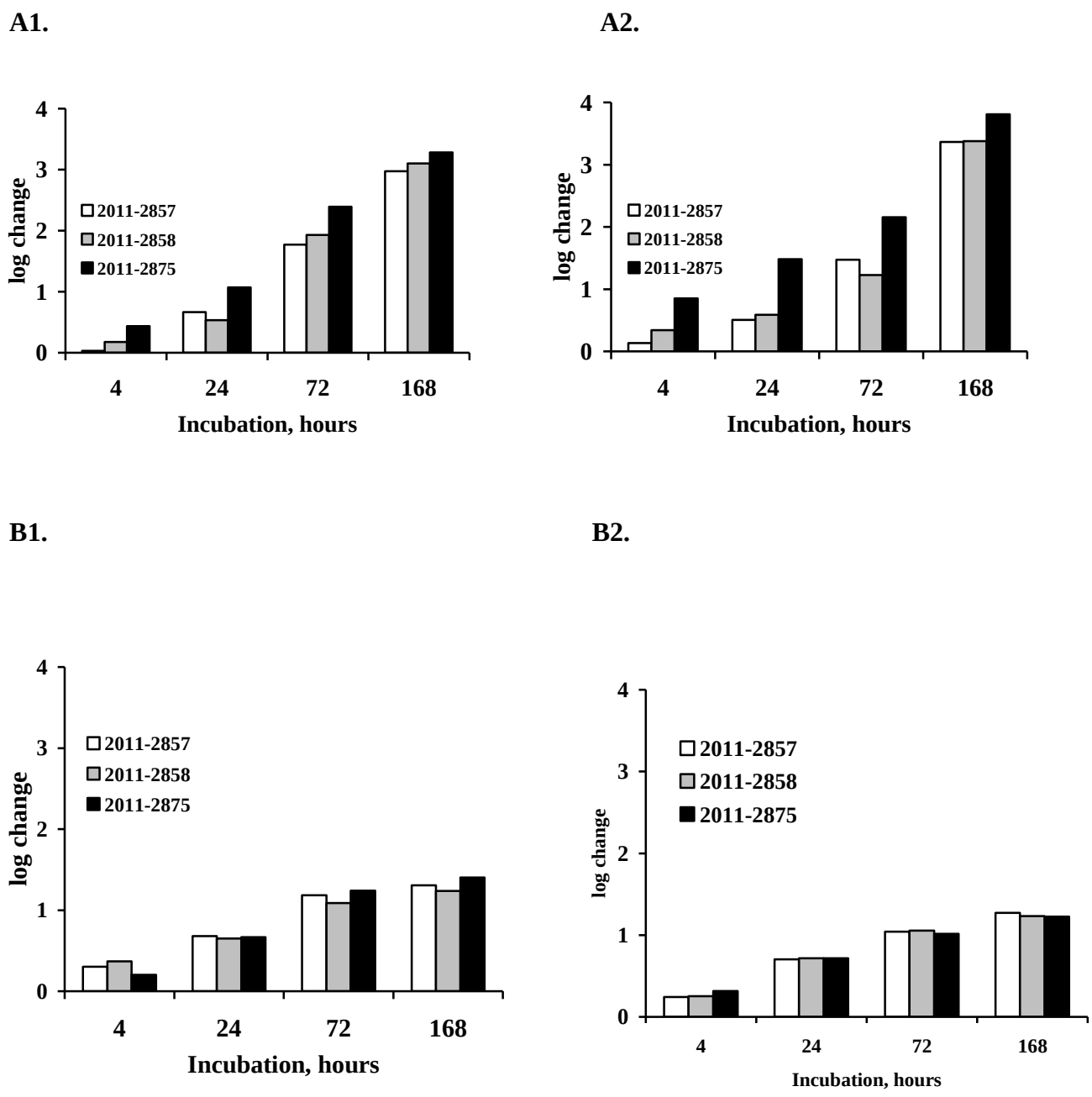


Fig. 4.7.5.Difference in *L monocytogenes* population increase in milk and on the cantaloupe surfaces. Growth of *L monocytogenes* strains (2011L-2857, 2011L-2858, and 2001L-2875) associated with 2011 cantaloupe outbreak in milk, both raw (A1) and pasteurized (A2), was notably higher compared to growth of the same strains inoculated on the rind (B1) or the flash (B2) of cantaloupe, respectively.

5.0. DISCUSSION

5.1. Adaptation of Listeria monocytogenes to Ciprofloxacin or to the Disinfectant Benzalkonium Chloride Results in Reduced Susceptibility to Ciprofloxacin, Gentamicin Benzalkonium Chloride and Other Toxic Compounds. In this study, selection of several *L. monocytogenes* strains on either ciprofloxacin or BC led to mutants with increased MICs not only to the agents employed in the selection but to several additional toxic compounds. The spectrum of compounds to which the mutants exhibited reduced susceptibility was the same regardless of whether selection was on ciprofloxacin or on BC. This multidrug resistance phenotype suggested that the mutants had increased expression of MDR efflux system(s). In accord with this notion, the ciprofloxacin and BC- selected mutants exhibited higher MICs to EthBr and TPP, which are known to serve as substrates for MDR efflux systems (95, 96). Moreover, at least 4fold reductions in MICs were observed in the presence of the potent efflux inhibitor reserpine. In contrast, the mutants did not have significant increases in MICs for the fourth generation fluoroquinolone moxifloxacin, which does not serve as a substrate for efflux systems; our finding that moxifloxacin MICs were not impacted by reserpine and were not enhanced in the mutants were similar to earlier observations with ciprofloxacin-resistant clinical isolates (86). Taken together, these data suggest that MDR efflux system mutations are likely responsible for the phenotype of the mutants in the current study. Enhanced efflux has also been implicated in multidrug resistance phenotypes of other bacteria (e.g. *Escherichia coli*, *Salmonella*, and *Pseudomonas*) following exposure to disinfectants, although other mechanisms (e.g. envelope changes) have also been implicated (18, 100, 112, 181, 205).

In previous studies, mutants of *L. monocytogenes* strains selected on BC had phenotypes partially overlapping with those of the mutants in our study. Such mutants had reduced susceptibility to BC, EthBr, gentamicin and, in several cases, kanamycin, but changes in ciprofloxacin MICs were not reported (187, 207). Additionally, these mutants exhibited increased transcription of *mdrL* (but not *lde*) and their phenotype was attributed to increased levels of the *mdrL* transporter (187). Inactivation of *mdrL* in *L. monocytogenes* LO28 resulted in increased susceptibility to macrolides, cefotaxime and certain metals (zinc, cobalt and chromium) (144). The different resistance phenotype of our mutants, which did not have

increased MICs to erythromycin (a macrolide) or kanamycin, suggests that *mdrL* is likely not involved in the multidrug resistance profile that we observed. Further support for this is provided by the fact that we failed to detect increased expression of *mdrL* in either the ciprofloxacin-selected or the BC-selected mutants. One cannot exclude the possibility that different transporters will contribute to the multidrug resistance phenotype of BC-selected mutants in different studies, depending on strain background and experimental conditions employed for the selection.

Mutants of *L. monocytogenes* selected on ciprofloxacin have not been reported. However, clinical isolates with enhanced resistance to ciprofloxacin have been identified as mentioned above, and were found to also have increased resistance to EthBr and acridine orange, as well as increased expression of the *lde* transporter (86). Insertional inactivation of *lde* resulted in reduced MICs to ciprofloxacin, EthBr and acridine orange, suggesting that this transporter mediated the observed resistance phenotype. However, susceptibility of the clinical isolates or the *lde* mutant to gentamicin or BC was not described (86). Our data showing increased expression of *lde* in the mutants also provide tentative evidence for involvement of this transporter, although involvement of additional transporters cannot be excluded. In addition to *mdrL* and *lde*, the *Listeria* genome harbors several other genes for putative MDR transporters (83, 156), some of which may contribute to the phenotype of mutants described here. Two new transporters of the major facilitator superfamily, *mdrT* and *mdrM,* were identified during a screen for genes impacting the elicitation of the innate immune response in macrophages infected with *L. monocytogenes* 10403S, and transcript levels of *mdrM* were markedly enhanced in the presence of rhodamine 6G and TPP (46). However, we failed to obtain evidence or increased expression of either *mdrT* or *mdrM* in the ciprofloxacin- or BC-selected mutants, suggesting that these transporters are likely not involved in the phenotype of the mutants. Further studies (e.g. comparative analysis of transciptome profiles) would be valuable in the effort to identify other transporters that may be expressed at higher levels in the mutants than in the parental strains, and to characterize the impact of exposure to antibiotics, disinfectant or other toxic compounds such as TPP on expression of the genes.

Strains H7550 Cd^R and J0161 Cd^R harbor large and closely related plasmids (121, 133, 156). Even though MICs to ciprofloxacin and gentamicin did not differ between the parental and

plasmid-cured strains, ciprofloxacin-selected mutants had higher MICs for either ciprofloxacin or gentamicin than mutants derived from the plasmid-cured derivatives of either strain. These findings were consistently obtained and were specific to MICs for these antibiotics; they were not observed in MICs to EthBr or TPP. Further studies are needed to determine whether the apparent impact of the plasmid is specific to pLM80 and closely related plasmids and to clarify the underlying mechanism. It is possible that a pLM80-associated efflux system for ciprofloxacin and gentamicin (but not for EthBr or TPP) was activated in the pLM80-harboring mutants, along with chromosomal transporters such as *lde*. Comparative transcriptome analyses would be valuable in assessing the validity of this hypothesis.

As has been discussed for other pathogens (100, 133), mutants such as described in the current study have a number of clinical or environmental implications. Exposure to ciprofloxacin (e.g. during empirical treatment, or in the course of treatment for other infections) may lead to *L. monocytogenes* mutants with decreased susceptibility to gentamicin, currently one of the drugs of choice for treatment of listeriosis. Such mutants may have enhanced ability to persist in the environment, as they also exhibit enhanced tolerance to disinfectants such as BC. Similarly, BC and other quaternary ammonium disinfectants are extensively used in healthcare settings as well as in the food processing industry. Mutants obtained during selection on BC can have enhanced resistance not only to BC but to a range of other antimicrobial compounds, including antibiotics such as ciprofloxacin and gentamicin.

Lastly, it is conceivable that in such mutants additional phenotypes of public health and food safety relevance may be impacted. There is increasing evidence that MDR transporters in bacterial pathogens can mediate not only resistance to antimicrobials but a number of other processes as well (95, 96, 135, 170). In the case of *L. monocytogenes*, the study by Crimmins et al. (46) has provided clear evidence that MDR transporter systems may be involved in processes of fundamental importance to host-pathogen interactions, such as elicitation of the innate immune response. Furthermore, *L. monocytogenes* virulence genes *prfA* and *inlA* were shown to be upregulated in response to sub-lethal concentrations of quaternary ammonium

compounds (113). It will be important to determine whether BC- or ciprofloxacin-selected mutants are also impacted in their virulence in cell culture or animal models, and whether attributes such as ability to persist in biofilms and persist in the environment may also be altered in such mutants.

5.2. Co-selection of cadmium and benzalkonium chloride resistance in conjugative transfers from non-pathogenic *Listeria* spp. to other listeriae. In this work we provide evidence that BC resistance mediated by *bcrABC* can be effectively transferred among certain strains of non-pathogenic *Listeria* spp. that harbored both *bcrABC* and the cadmium resistance determinant *cadA2*. We also showed that resistance could be transferred from non-pathogenic *Listeria* spp. to *L. monocytogenes* strains of all three serotypes primarily associated with human listeriosis (1/2a, 1/2b and 4b). The findings indicated that cadmium resistance transfer can be effectively used as a surrogate for transfer of resistance to BC, since transconjugants selected on cadmium were resistant to both cadmium and to BC.

Transfer of resistance was not indiscriminate: even though all tested *L. monocytogenes* strains yielded transconjugants with at least some of the non-pathogenic donors, one of the non-pathogenic strains employed as recipient failed to yield any transconjugants. Furthermore, transconjugants were produced with markedly higher frequency when non-pathogenic donors harbored *cadA2* (often while also harboring *bcrABC*) than when they harbored the alternative cadmium resistance determinant *cadA1*.

Non-pathogenic *Listeria* spp. have potential to serve as reservoirs for resistance genes and transfer them among themselves as well as to *L. monocytogenes* inhabiting the same environments, but data on such transfers remain scarce. Thus far only one study reported conjugative transfer of disinfectant resistance attributes among *Listeria* spp. (131). However, only two strains of non-pathogenic *Listeria* spp. (*L. innocua* in both cases) were tested as donors; furthermore, the donors in that study exhibited resistance to the dye ethidium bromide but not to cadmium (131). In contrast, strains employed as donors in the current study were resistant to cadmium but susceptible to ethidium bromide (M. Rakic-Martinez, unpublished findings). Thus, it is hard to compare data from this earlier study with the current findings.

In the case of non-pathogenic recipients transfer was found to be more efficient at 25 than at 37°C, suggesting that it may represent an environmental adaptation. In contrast, no difference

in transfer frequency between 25 and 37°C was noted for *L. monocytogenes*, regardless of serotype, suggesting the potential for such transfers to be taking place not only in the environment but *in vivo* as well, e.g. in the mammalian gastrointestinal tract.

In the current study conjugations were on agar or on membrane filters overlaid on solid media. Only limited information is currently available on horizontal gene transfer in listeriae in foods and other complex systems such as the gastrointestinal tract (39, 56). Further studies are needed to determine whether conjugative transfer frequency is impacted by the presence of the conjugation partners on surfaces relevant to food processing environments (e.g. stainless steel, food) and within polymicrobial biofilms.

While direct evidence of nonpathogenic *Listeria* spp. behaving as resistance gene reservoirs for *L. monocytogenes* is still lacking, co-selection and disinfectant resistance gene distribution in other pathogens perhaps illustrate analogous processes at work. For instance, Ciric et al. have shown the presence of a *Streptococcus oralis* Tn*916*-like conjugative transposon, Tn*6087 that* confers resistance to cetrimide bromide, a disinfectant in the quaternary ammonium compound (QAC) family, as well as tetracyclines, potentially providing a mechanism for co-selection of disinfectant and antibiotic resistance (44). In food-derived staphylococci, the presence of BC resistance genes linked to antibiotic resistance genes suggested the potential for similar co-selection (198). Bjorland et al. demonstrated the widespread distribution of QAC resistance genes in bovine and equine coagulase-negative staphylococci (13). The wide distribution of such genes and the potential of increased fitness through co-selection have important public health implications (191).

L. monocytogenes plasmids (e.g. pLM80) harboring both *bcrABC* and *cadA2* have been characterized in the course of genome sequencing investigations (64, 121, 156). Genome sequence data of the non-pathogenic listeriae employed as donors in the current study are currently not available. Nonetheless, when donors harbored both *bcrABC* and *cadA2* the transconjugants acquired both of these determinants even though selection was only for cadmium resistance, suggesting plasmid-associated resistance genes similar to those harbored by pLM80. Further supportive evidence for pLM80-like plasmids was provided by detection of all other tested pLM80-associated genes in these donor strains. Though these PCR-based assessments involved only a subset of pLM80-associated genes, the data provide compelling

reasons to further elucidate the sequence content of the plasmids of these *L. welshimeri* and *L. innocua* donor strains harboring *bcrABC* and *cadA2*.

Our PCR data suggest that different plasmids were harbored by strains containing *cadA1*. However, it was intriguing that such strains failed to serve as efficient donors to other listeriae. Plasmids harboring *cadA1* have been extensively described in *L. monocytogenes* (25, 121, 127, 128) and conjugative transfer of one such plasmid was demonstrated in an earlier study. A single donor-recipient strain combination was examined in that study, and non-pathogenic strains were not included (129). Further studies are needed to determine whether our findings reflect differences in the plasmids or in the types of strains employed in the conjugations.

In conclusion, we have demonstrated the potential for resistance to BC and to cadmium to be conjugatively transferred among non-pathogenic listeriae and from these strains to *L. monocytogenes* of diverse serotypes. Our findings also demonstrate co-selection of resistance to BC and cadmium when the non-pathogenic *Listeria* spp. donors were resistant to both of these agents: transconjugants selected in the presence of cadmium were also resistant to BC. Co-selection between cadmium resistance and BC resistance has not been documented before in *Listeria* or other bacteria. Future work should examine additional factors in food processing and other dynamic environments that may affect efficacy of resistance transfer, such as varied growth surfaces and the presence of mixed and single-species biofilms. Further study of conjugative dissemination of BC and cadmium resistance in *Listeria* spp. would provide the opportunity to assess impact on fitness in disinfectant-abundant environments such as food processing plants and healthcare settings. Data from such studies would be needed to characterize potential impacts of such resistance determinant acquisitions on additional attributes, including those associated with virulence.

5.3. Resistance gene distribution among non-pathogenenic *Listeriae* from different poultry processing plants.We previously reported that non-pathogenic *Listeria* spp. could serve as donors of resistance to disinfectant (BC) and to the heavy metal cadmium to other *Listeriae*, including diverse serotypes of the pathogenic species, *L. monocytogenes*. Transconjugants acquired the resistance determinants *bcrABC* (BC resistance) and *cadA1* or *cadA2* (resistance to cadmium) harbored by the donor strains (117). The current population

based analysis of non-pathogenic *Listeria* spp. from processing plants revealed high prevalence of resistance to BC and cadmium, and of the *bcrABC*, *cadA1* and *cadA2* determinants, thus providing further support for the role of non-pathogenic *Listeria* spp. as resistance gene reservoirs for pathogenic *L. monocytogenes.* The observed high prevalence of resistance may reflect responses to selective pressure stemming from exposure to disinfectant. Since all BC-resistant strains were also resistant to cadmium, cadmium resistance may have been co-selected upon exposure to BC, as observed during in vitro conjugative transfers (117). However, we also identified strains that were cadmium-resistant but BC-susceptible. Such strains may reflect recent introductions from sources outside of the processing plant, or derivatives that either have lost or have not yet acquired bcrABC and corresponding resistance to BC. Strains unable to serve as recipients were indeed identified earlier (117).

An unexpected finding was that even though *cadA1* and *cadA2* were similarly common in *L. welshimeri* and *L. innocua,* plant-specific differences in prevalence of these resistance genes were noted for each these species. For instance, in plant A *cadA2* was relatively common regardless of species, while in F *cadA1* was predominant in both species. On the other hand, in plant B prevalence of *cadA*1 and *cadA*2 differed between *L. innocua* and *L. welshimeri*. Such findings may reflect the outcome of colonization of the plant with certain strains (e.g. *cadA2*-harboring strains in plant A) and subsequent dissemination of the resistance determinant by intra- and inter-species gene transfer within the individual plant's ecosystem. This was supported by the detection of different *cadA* genes or resistance phenotypes among strains with closely related or indistinguishable PFGE profiles. PFGE profiles of *L. welshimeri* or *L. innocua* were largely plant-specific, with strain clusters suggesting the presence of certain strains disseminated and persistent in specific plants.

Non-pathogenic *Listeriae* employed in this study were isolated from the same plants as *L monocytogenes*strains described by Mullapudu et al (154, 155). This group reported high prevalence (57%) of BC- resistance among isolates of serotype 1/2a and 1/2b. Prevalence of BC resistance was markedly lower in isolates of serotype 4b (12%). All BC resistant isolated harbored *bcrABC* cassette and were also resistant to cadmium. These findings significantly correlate with current study implying similar distribution of resistance determinants among

non-pathogenic *Listeriae* and *L monocytogenes* strains of serotype 1/2a and 1/2b. Among Cd^r BC^r *L monocytogenes* strains most predominant was *cadA2* determinant either alone or together with *cadA1 (*64%), whereas 42% of non-pathogenic isolates harbored *cadA2.* In contrast, most strains resistant to cadmium but susceptible to BC (Cd^r BC^s) possessed *cadA1* (74%) *of L monocytogenes* and 60% of non-pathogenic *Listeriae).* Interestingly, plant specific distribution of Cd resistance determinants differs between *L monocytogenes* and *Listeriae* isolates. Most of the *L monocytogenes* isolates tested by Mullapudi et al. (155) originated from the plant A with predominance of *cadA*2 (~50%). In contrast, *cadA*1 was most frequent among non-pathogenic *Listeriae* isolated from the plant A (47%). Possible explanation of such a difference could be in horizontal gene transfer of cadmium resistance genes between non-pathogenic *Listeriae* and *L. monocytogenes* (117). Recent findings reported by Katharios-Lanwermeyer et al. (117) revealed that all donor strains that could efficiently transfer resistance harbored *cadA2*. Given that the BC resistance determinant *bcrABC* is co-transferred with the cadmium resistance determinant *cadA2* (117), adaptation of strains in the processing plants to BC may contribute to the higher prevalence of *cadA2*.

Findings in this study strengthen evidence that non-pathogenic *Listeria* spp. serve as reservoirs for disinfectant and cadmium resistance genes in the processing plant environment. Predominant cadmium resistance determinants vary by plant and species, possibly reflecting intra- and inter-species dissemination of the determinants within the ecosystem of individual plants.

5.4. Growth and survival of *L. monocytogenes* on cantaloupe. The results of this study provided further evidence of minimally processed fresh fruits as a good media for growth of microorganisms as previously suggested by various authors (166, 211, 212). Assessment of *L. monocytogenes* growth oh the outer surface at 4°C revealed 1.0 log increase in population with no significant difference among strains. Bacteria reached this increase within 7 day and survived at 4°C at the same level throughout the incubation period of 21 days. Our findings differ from a previous report that *L monocytogenes* survived but did not grow on fresh-cut cantaloupe pieces stored at 4°C (211). Possible reasons include differences in methodology, e.g. submerging for 10 minutes in the mixed bacterial inoculum in the earlier study (211) vs. spot inoculation of individual strains in the current one. Furthermore, in the previous study

time left for attachment of cells (2hours at room temperature) differed from current one (1 hour at room temperature). The strains employed also differed between the two studies. Similar reasons may account for the greater (approximately 10fold) increases on inoculated fragments observed at 8 and 25 º C in this study than in the earlier report (211). Population growth on the cantaloupe surface at 8º C obtained in this study is in agreement with increase in *L. innocua* population at the same temperature reported by Behrsing et al. (9).
Interestingly, in current study we observed that growth on the cantaloupe surface was higher then growth in the extract or on the cantaloupe flesh with statistically significant difference at 25°C after 72 hours ($p<0001$) and 4°C after 7 days of incubation ($p=0.0007$). This could be explained by the characteristics of rind surface that allows better attachment of the bacteria, combined with protective role of extensive netting (209, 210, 212). Lower growth in the extract could be explained by metabolism of the melon fruit. Biais et al. (12) reported strong decrease in ATP and ADP ratios and the adenylate energy charge from the periphery to the center of the fruit. From the periphery (rind) to the center of fruit there was also significant increase of ethanol concentration and other markers of hypoxia in plants which could be explanation of lower increase in *Listeria* population in the cantaloupe extract. Washing of inoculated fragments with sterile deionized water prior to the incubation resulted with initial decreases of *L monocytogenes* of approximately 2 log units followed by subsequent growth. However, washing decreases LM counts temporarily (hence consuming product immediately after washing -at least within 24h, would enhance safety), but Lm grows to the same levels as on unwashed samples within 48 h—hence, washing may not confer any benefit after 48h. In contrast to this finding Ukuku et al (215) did not observed significant reductions in *L. monocytogenes* populations after washing inoculated whole melons at day 0.
The results of this study suggest that *L. monocytogenes* can not only survive on the surface of cantaloupe but that it also has temperature and time-dependent potential for growth. Results also revealed that water treatment of the surface does not prevent growth of *L. monocytogenes.* These data will be valuable in design of controls to limit persistence and growth of this pathogen on cantaloupe and other produce.

5.5. Change in phenotype and genotype as an asset for *L. monocytogenes* growth on cantaloupe. Limited information is available on mechanisms that *L monocytogenes* employs for adherence, survival and persistence or growth on produce. Previous studies reported colonization and persistence of various pathogens such as *E. coli* O157:H7, *Salmonella* ans *L monocytogenes* on the surface of the plants (219, 224). Strain variability in interactions with plants has been noted in other foodborn pathogens (14, 118). Assessment of different *L. monocytogenes* strains revealed that one of them, F8027 associated with celery had higher potential of growth on lettuce then others . In current study, by employing various strains and selected mutants of interest we are providing evidence of *L monocytogenes* ability to colonize surface of cantaloupe, survive and grow under different conditions (various incubation temperatures, treatment with water).

Antibiotic-resistant *L. monocytogenes* have been found in foods with increasing frequency (39, 48, 228). In our previous study we isolated mutant of the *L. monocytogenes* J0161 adapted to higher concentrations of antibiotic ciprofloxacine (182). Characterization of this mutant revealed increased resistance to other antimicrobials (e.g. disinfectant bezalkonium chloride and antibiotic gentamicin). Such mutants with enhanced resistance present a burden for the food processing environment. Comparison of growth ability between parental strain J0161 and ciprofloxacin adapted mutant revealed no significant difference at any of the tested incubation temperatures (4, 8 or 25 ºC).

Using the *mariner*-based transposon (8) to construct mutant libraries presents powerful genetic tool for identification genes responsible for different changes in phenotype. Through screening of libraries of *L monocytogenes* strains F2365 and H7550 numerous mutants have been isolated in our laboratory. Analysis of such mutants revealed that they harbored single transposon insertions. Through screenings of these libraries cold-sensitive mutant with an insertion in a gene encoding a DEAD family RNA helicase have been isolated as well as nonhemolytic H7550-Cds,*hly* mutant, transposon mutant of F2365*purA*, transposon mutant of H7550-Cds,*purB* (6, 7, 118). Testing ability of these mutants to survive and grow on the surface of cantaloupe revealed no significant difference in comparison with parental strain. As expected, difference was notable between strain F2365 and cold sensitive mutant ROA4 which expressed decrease in population throughout the incubation period.

Most outbreaks of Listeriosis involved serotype 4b strains. These strains are associated with two major clonal groups. Epidemic Clone I (EC I) involved in numerous outbreaks worldwide, whereas Epidemic clone II (ECII) represents a novel epidemic clone indentified during the 1998-99 multistate outbreak in the United States. Previous studies described a genomic region (region-18) with unusual diversifications in ECII, and specific and conserved among other serotype 4b strains (68). This region was flanked on one side by a large (ca. 6.6 kb) gene encoding a putative cell wall-associated protein (*wap*) in ECII and other serotype 4b strains. On the other side, the region was flanked 119 by inlA and inlB, implicated in virulence of *L. monocytogenes* (23). *Wap* has been identified as a serotype 4b specific with possible role in sensing environmental changes (20, 81). The protein encoded by wapA belongs to a super-family of surface-associated proteins involved in various cellular processes, including surface hydrophobicity, wall metabolism, secretion, pathogenicity, immunogenicity and cell adhesion (83). In current study, deletion mutants of region 18 in *L. monocytogenes* F2365 and H7550 and *wap* in F2365 did not impact ability to survive and grow on the cantaloupe surface. After initial decrease at low temperatures (4 and 8 ºC) cells recovered and population increased trough out the incubation period.

Taken together, screening of mutant libraries and construction of different deletion mutants served as a valuable tool in the assessment of *Lmonocytogenes* survival and growth on the surface of produce. Even though growth ability of tested mutants did not differ significantly from parental strains further testing and characterization of mutants could help in better understanding of the mechanisms involved in such ability. Of great importance is finding that water treatment of inoculated surfaces does not prevent further growth of bacteria. Such finding may help in creating new strategies in food safety.

5.6. Assessment of *L. monocytogenes* growth in raw and pasteurized milk. According to our results *L. monocytogenes* grew generally better and with fewer variations in pasteurized than in raw milk at 8C. Similar findings were reported in cow milk by Northolt et al. (157) and in goat's milk by Leuchner et al (132). This is suggestive of potential impact of microbiota in raw milk, as well as inhibitory effects of antibacterial substances such as lactoperoxidase and lysozyme present in raw milk (157, 171, 229). Unlike results of these studies that indicates *L monocytogenes* population decrease over the first two days of

incubation due to the presence of antibacterial substances in raw milk, such decrease was not observed in our study. Our results are in agreement with those reported by Farber et al (71) and show increase in population over the incubation period of 10 days.

Serotype 4b has been the most common serotype connected to listeriosis outbreaks. However, serotype 4b highly represented in clinical isolates is not the most common serotype in food isolates (138). Even thou relation between serotype and virulence has not been established (115); increased virulence could be the reason for higher number of reported clinical cases of listeriosis caused by serotype 4b. Comparisons across serotypes indicate neither significant difference between serotypes nor difference in growth of strais of one serotype between two milk types. Wide geographical distribution of different *L. monocytogenes* serotypes and high adaptability to variety of environmental conditions and food composition may be explanation for the lack of difference in growth between serotypes.

Two wild type strains of *L. monocytogenes* (H7550CdR and J0161CdR) employed in this study displayed no significant difference in growth compared to their ciprofloxacin adapted mutants (H7550CdRC and J0161CdRC) in raw or pasteurized milk. This finding suggests that adaptation to antibiotics does not change growth potential of *L. monocytogenes*.

Recent epidemiological data suggest that produce and fruit contribute to listeriosis more then previously recognized. Therefore, we analyzed possible difference in *L. monocytogenes* growth in milk and on cantaloupe. For the purpose of analysis we used unpublished data on *Listeria* growth on cantaloupe, obtained in our lab. *L. monocytogenes* strains of serotype 1/2a and 1/2b involved in 2011 multistate outbreak connected to cantaloupes expressed high increase in population in both raw and pasteurized milk. Increase in population was much lower when same strains were tested on cantaloupe. These findings suggesting better nutritive composition of milk as a growth medium for *L. monocytogenes.* As reported by Donnely and Brigs (58) (milk fat content might be related to listerial lipase produced by hemolytic *L. monocytogenes* strains and this could be the reason for faster growth of such strains in milk. Explanation of results could also be in the fact that glucose concentration decrease from the surface to the center of cantaloupe fruit due to conversion of glucose to sucrose (12) and glucose tend to be one of the major substrates for growth of *Listeria* in milk (157, 188).

Eleven strains of serotype 4b assigned to three epidemic clonal groups (ECI, ECIa and ECII) were employed in this study. Epidemic clones ECIa and ECI are composed of older human and animal clinical isolates from outbreaks and sporadic cases occurred in the first half of 20th century. ECII isolate was involved in Food Hotdog Outbreak (1998-99). Clonal groups were compared for possible difference in growth in raw and pasteurized milk over the 10 days of incubation at 8C. Statistical analysis of results obtained indicate that ECI grows better in raw ($p=0.01660$) and pasteurized milk ($p=0.0433$) compared to ECIa. ECI represents widely distributed clonal group associated with multiple outbreaks in Europe and North America (194, 134). General presence across different environments indicates high adaptability of ECI, which may be one of the explanations for enhanced growth in milk compared to ECIa. Answer could also be better adaptation to inhibitory lactoperoxidase and lysozyme present in raw milk as well as unique virulence characteristics.
Compared to both ECI and ECIa lower increase in population raw and pasteurized milk, respectively, has been observed for ECII isolate. As reported by Kathariou et al (115) this clonal group was first observed in food hot dog outbreak (1998-1999). Ability of ECII to transmit among meat processing plants was discussed by various authors (41, 92). We speculate that this epidemic clone might be better adapted to meat and meat processing environment which would justify lower population increase in milk.

Demographical changes such as increase in population of elderly and immunocompromised person, as well as changes in food consumption patterns, might highly contributed to a change in epidemiological profile of human Listeriosis. Mayor food-born listeriosis outbreaks reported recently are connected to processed meats, produce or fruit. As reported by Public Health Service/Food and Drug Administration (179) less than 1% of total food-borne disease outbreaks are currently linked to milk and milk products, with a few large outbreaks occurred in last few decades (3, 15, 79). According to the same report in 1938 milk was cause of 25% of food related outbreaks. This observation and ability of old clinical isolates of *L. monocytogenes* to grow relatively high in raw and pasteurized milk reported in this study emphasized major historical role of milk as a vehicle for this pathogen. Low consumption of processed meats and still not well implemented pasteurization in first half of 20th century lead

to a speculation that raw milk was involved in most of human cases including ones presented in this study.

6.0. CONCLUSIONS:

1. Recent epidemiologic data indicate declined incidence of listeriosis in the US, hence, *L. monocytogenes* still produce the highest mortality (16-19%) and hospitalization rate (94%) among food-borne pathogens.

2. Bacterial pathogens are often exposed to sub-lethal levels of different antimicrobial agents (i.e. disinfectants in food processing plants, antibiotics in the health environment). Such exposure is considered the main reason for the increase in *L. monocytogenes* antimicrobial resistance.

3. Selection of *L monocytogenes* to antimicrobials such as antibiotic ciprofloxacin or disinfectant bezalkonium chloride (BC) led to the emergence of mutants with increased MICs not only to antimicrobial agents used in the selection process, but also a few additional toxic substances. The spectrum of agents to which they have developed resistance mutations did not differ regardless of whether the chosen selection of ciprofloxacin or BC. The change in the direction of multiresistance phenotype presented in this thesis suggests overexpress MDR efflux systems and leads to the conclusion that this system is responsible for the occurring change.

4. Screening of a *mariner*-based mutant library of selected *L monocytogenes* strains failed to isolate mutants with impaired resistance to TPP. However, *tetR*::Tn*917* mutants with pronounced (8-fold) increase in TPP resistance have been isolated. One of these mutants had insertion in *tetR* gene. Characterization of such mutant revealed transposon integrated in LMRG_01858 (sucrose phosphorylase). Such data suggest that the observed TPP resistance of the *tetR*::Tn*917* mutant involve increased expression of MDR transporter(s) other than *mdrT*, but also repressed by *tetR*.

5. Broad resistance to the tested agents confirms the hypothesis that non-pathogenic strains *Listeriae* spp could be reservoir of resistance genes for *L monocytogenes.* Resistance to disinfectant BC mediated by gene cassette *bcrABC* can be effectively transferred among certain strains of non-pathogenic *Listeria* spp. that harbored both *bcrABC* and the cadmium resistance determinant *cadA2*. Resistance could also be transferred from non-pathogenic

Listeria spp. to *L. monocytogenes* strains of all three serotypes primarily associated with human listeriosis (1/2a, 1/2b and 4b). The findings presented in this Dissertation indicated that cadmium resistance transfer can be effectively used as a surrogate for transfer of resistance to BC, since transconjugants selected on cadmium were resistant to both cadmium and to BC.

6. Analysis of non-pathogenic *Listeria* spp. from processing plants revealed high prevalence of resistance to BC and cadmium, and of the *bcrABC, cadA1* and *cadA2* determinants, providing further support for the role of non-pathogenic *Listeria* spp. as resistance gene reservoirs. The observed high prevalence of resistance may reflect responses to selective pressure stemming from exposure to disinfectant. The outcome of colonization of the plant with certain strains could result with dissemination of the resistance determinant by intra- and inter-species gene transfer within the individual plant's ecosystem. This hypothesis was supported by the detection of different *cadA* genes or resistance phenotypes among strains with closely related or indistinguishable PFGE profiles. PFGE profiles of *L. welshimeri* or *L. innocua* were largely plant-specific, with strain clusters suggesting the presence of certain strains disseminated and persistent in specific plants

7. Strains from the 2011 cantaloupe outbreak grew on cantaloupe. Generally growth was more pronounced on rind than on flesh or in cantaloupe extract. Differences were significant under certain time-temperature combinations (7 days at 4C and 72h at 25C).

•No significant difference was noted among the three tested cantaloupe outbreak strains, which differed in serotypes and PFGE profiles.

•Experiments with unrelated strains from outbreaks involving other vehicles (hot dogs, turkey deli meats, soft cheese, and celery) revealed trends similar to those with the 2011 cantaloupe outbreak strains. Growth was generally more pronounced on rind than flesh or extract.

•Growth on rind at 4C was consistently observed (ca. 1 log increase after 7 days at 4C), differing from previous reports (Ukuku 2002).

•Growth on rind and flesh at 8 and 25C, while consistently observed, was less than reported (Ukuku 2002, 2012; Brazil study). Differences in strains and inoculation method may account for the differing results.

•Washing with water decreased *L. monocytogenes* populations by ca. 2 logs but bacteria grew back, with numbers at 24h approximating those of the original inoculum.
•A cold sensitive (helicase) mutant failed to grow on cantaloupe rind at 4C, but grew at room temperature. Mutants that fail to grow on cantaloupe at room temperature

8. By employing various strains and selected mutants of interest we provided evidences of *L monocytogenes* ability to colonize surface of cantaloupe, survive and grow under different conditions (various incubation temperatures, treatment with water). Screening of mutant libraries and construction of different deletion mutants served as a valuable tool in the assessment of *L. monocytogenes* survival and growth on the surface of produce. Even though growth ability of tested mutants did not differ significantly from parental strains further testing and characterization of mutants could help in better understanding of the mechanisms involved in such ability. Of great importance is finding that water treatment of inoculated surfaces does not prevent further growth of bacteria. Such finding may help in creating new strategies in food safety.

9. Milk and dairy products along with processed meats are still considered major vehicles for *L. monocytogenes* involved in food related outbreaks. Eighteen *L. monocytogenes* strains of different serotypes involved in food-related outbreaks as well as human and animal clinical isolates were tested for growth in raw and pasteurized milk. Isolates of serotype 4b assigned to three epidemic clonal groups (ECI, ECIa and ECII) have been analyzed for such a difference between clonal groups as well as types of milk. Population expectedly, increased significantly over the 10 days of incubation at 8C ($p<0.0001$). Results obtained indicate overall better growth of *L. monocytogenes* in pasteurized over raw milk. Comparison across serotypes showed no significant differences between serotypes or individual serotype between types of milk. Significant difference, however, has been noted between clonal groups composed of serotype 4b. Results of present study confirmed good adaptability and growth of *L. monocytopgenes* in milk. However, further studies are necessary in order to understand better distribution of epidemic clones, their transmission characteristics and ability to cause foodborne disease

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BIOGRAPHY

Mira Rakić-Martinez was born in Užice, a beautiful mountain area in western Serbia. During high school Mira traveled and attended high school in Hastings, England. Upon graduation she joined the Veterinary College at the University of Belgrade where she pursued her degree in Veterinary Medicine, specifically in hygiene and technology of food of animal origin.

After receiving her degree, Mira joined PhD program at the Veterinary College under the guidance of Dr Vera Katic and during her research she focused on studying behavior of *Listeria monocytogenes* in milk and in processing plants. During this period of her life Mira enjoyed learning Spanish at the 'Instituto Cervantes' in Belgrade and more importantly she made acquaintance with her now husband Sergio.

In 2010, Mira moved to United States, continuing her research in the laboratory of Dr Sophia Kathariou at North Carolina State University. Her doctorate work at NC State has been focused on identifying the mechanisms of *Listeria monocytogenes* resistance to different antimicrobials as well as the ability of the pathogen to populate and grow in different foods.

In 2011 Mira and Sergio were blessed with the arrival of their precious little boy Gregory Alexander.

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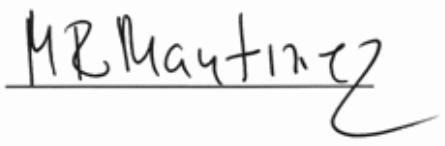

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