International Journal of Food Microbiology 390 (2023) 110106

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Formation of *Listeria monocytogenes* persister cells in the produce-processing environment

Xiran Li [a], Xavier F. Hospital [b], Eva Hierro [b], Manuela Fernández [b], Lina Sheng [c], Luxin Wang [a,*]

[a] Department of Food Science and Technology, University of California Davis, Davis, CA 95616, United States
[b] Department of Food Technology, Faculty of Veterinary, Complutense University of Madrid, 28040 Madrid, Spain
[c] School of Food Science and Technology, Jiangnan University, Wuxi, Jiangsu 214126, China

ARTICLE INFO

Keywords:
Fresh produce
Antimicrobial persistence
Cell density
Adherence
Foodborne pathogen
Chlorination

ABSTRACT

Persisters are a subpopulation of growth-arrested cells that possess transient tolerance to lethal doses of antibiotics and can revert to an active state under the right conditions. Persister cells are considered as a public health concern. This study examined the formation of persisters by *Listeria monocytogenes* (LM) in an environment simulating a processing plant for leafy green production. Three LM strains isolated from California produce-processing plants and packinghouses with the strongest adherence abilities were used for this study. The impact of the cells' physiological status, density, and nutrient availability on the formation of persisters was also determined. Gentamicin at a dose of 100 mg/L was used for the isolation and screening of LM persisters. Results showed that the physiological status differences brought by culture preparation methods (plate-grown vs. broth-grown) did not impact persister formation ($P > 0.05$). Instead, higher persister ratios were found when cell density increased ($P < 0.05$). The formation of LM persister cells under simulated packinghouse conditions was tested by artificially inoculating stainless steel coupons with LM suspending in media with decreasing nutrient levels: brain heart infusion broth (1366 mg/L O_2), produce-washing water with various organic loads (1332 mg/L O_2 and 652 mg/L O_2, respectively), as well as sterile Milli-Q water. LM survived in all suspensions at 4 °C with 85 % relative humidity for 7 days, with strain 483 producing the most persister cells (4.36 ± 0.294 Log CFU/coupon) on average. Although persister cell levels of LM 480 and 485 were reasonably steady in nutrient-rich media (i.e., BHI and HCOD), they declined in nutrient-poor media (i.e., LCOD and sterile Milli-Q water) over time. Persister populations decreased along with total viable cells, demonstrating the impact of available nutrients on the formation of persisters. The chlorine sensitivity of LM persister cells was evaluated and compared with regular LM cells. Results showed that, despite their increased tolerance to the antibiotic gentamicin, LM persisters were more susceptible to chlorine treatments (100 mg/L for 2 min) than regular cells.

1. Introduction

Consumer demand for and consumption of fresh fruit and vegetables have both increased considerably over past decades, as a result of the promotion of healthier lifestyles (Kenner, 2020). To facilitate its national and international distribution, fresh produce goes through processing plants or packinghouses before entering commerce. Although control measurements have been implemented to reduce the microbial risks associated with fresh and fresh-cut produce, spoilage and pathogenic microorganisms can survive or be reintroduced into the product through cross-contamination (DiCaprio et al., 2015; Foong-Cunningham et al., 2012). Fresh produce, which undergoes minimum preparation and is frequently consumed raw, has therefore, been implicated in an increasing number of foodborne outbreaks in the past decade (CDC, 2020).

Listeria monocytogenes (LM) is a Gram-positive, facultative intracellular foodborne pathogen that can cause listeriosis in humans (Schlech, 2000). Most listeriosis cases have been caused by the consumption of contaminated food (Posfay-Barbe and Wald, 2009). The high mortality rate (20–30 %) associated with listeriosis has made LM one of the deadliest foodborne threats (Bintsis, 2017). Although LM is ubiquitous in the environment and can enter the food supply chain at different points, several listeriosis-associated outbreaks and recalls have been linked to fresh produce processing plants (CDC, 2015a, 2015b, 2022;

* Corresponding author.
E-mail address: lxwang@ucdavis.edu (L. Wang).

https://doi.org/10.1016/j.ijfoodmicro.2023.110106
Received 20 October 2022; Received in revised form 26 December 2022; Accepted 19 January 2023
Available online 26 January 2023

Chen et al., 2016; FDA, 2019a, 2019b; Gaul et al., 2012). Cleaning and sanitation plans have been implemented by the food industry with the goal of controlling LM contamination during food manufacturing and preparation. However, intensive research has shown that the adverse stresses present in the postharvest environment, such as oxidative stress, limited nutrients, wide range of pH, and the presence of antimicrobials may prime bacteria including LM to enter the persister state (Hong et al., 2012; Thao et al., 2019; Wu et al., 2017).

Persisters are a subpopulation of growth-arrested cells that process transient tolerance to lethal doses of antimicrobials in an otherwise susceptible clonal population (Balaban et al., 2019; Fisher et al., 2017; Gollan et al., 2019). It has been shown to be one stress response status for many bacteria (Cohen et al., 2013; Kint et al., 2012). The biphasic killing curve is one iconic phenomenon associated with the identification and selection of persister cells (Balaban et al., 2019; Van den Bergh et al., 2017). Biphasic killing curves are often formed/observed when an antibiotic-sensitive, isogenic population containing persister cells is exposed to increased antibiotic concentration or a fixed concentration for a prolonged period of time (Kint et al., 2012; Van den Bergh et al., 2017). When looking at the dynamic changes of the whole population (or the shape of a biphasic curve), sensitive cells that make up the majority of the population at the beginning are quickly eliminated when first exposed to antibiotics. The population then gradually becomes dominated by persisters, and the killing rate decreases and approaches a plateau (Van den Bergh et al., 2017). Unlike resistant mutants, the persister state is a non-inheritable phenotypic feature (Van den Bergh et al., 2017). Transferring and regrowing persister cells in media without antibiotics leads to the formation of new generations that are as susceptible to antibiotics as their parental population (Balaban et al., 2019; Keren et al., 2004). Persister cells were originally thought to be produced stochastically (Balaban et al., 2004); however, increasing numbers of recent studies have shown that frequent environmental changes and the selective pressure posed by the food production environment may also induce persister formation (Fisher et al., 2017; Kussell et al., 2005; Nguyen et al., 2011; Van den Bergh et al., 2017). For example, Martins et al. (2021) showed that environmental pressures such as the presence of copper and zinc as well as high temperatures could induce the formation of persister cells by the plant pathogen *Xanthomonas citri*. Thao et al. (2019) proved that the formation of shiga-toxin-producing *Escherichia coli* (STEC) persisters could be enhanced when inoculating and incubating in spinach lysate or surface field water, where the bacteria exhibited slow growth rates.

Given persister cells' tolerance to antibiotic treatments and environmental stresses and their involvement in the relapse of bacterial infection, the presence of persister cells in the fresh produce production environment may pose significant food safety concerns (Cohen et al., 2013; Liu et al., 2020). However, whether LM persisting in a produce-processing environment can lead to the formation of LM persister cells remains largely uninvestigated. The two studies that are available tested the formation of LM persisters with in vitro models (Knudsen et al., 2013; Narimisa et al., 2020). Therefore, the first objective of this study is to evaluate if LM strains can enter the persister stage under the conditions of a simulated leafy-green processing plant environment. Secondly, it has been well accepted that culture preparation methods (plate-grown vs. broth-grown cultures) as well as cell density contribute to the bacteria fitness and their adaptive abilities to environmental stresses (Cabral et al., 2018; Ghosh et al., 2018; Goswami et al., 2017; Harris and Wang, 2012; Keller et al., 2012; Schenk et al., 2022). Based on these previous observations, the second objective of this study is to find out if culture preparation methods and cell density determine the ratios of persister cells formed in a given environment. The third objective of this study is to investigate the sanitizer sensitivity of persister cells by assessing the efficiency of chlorination for the control of LM persister cells.

2. Materials and methods

2.1. Bacterial strains and the preparation of inocula

Thirty-four LM strains used in this study were isolated from produce-processing plants and packinghouses in California and kindly provided by Dr. Trevor Suslow at the University of California Davis. Every frozen (−80 °C) stock culture was activated by streaking onto trypticase soy agar (TSA; Difco, Sparks, MD, USA) and incubated at 37 °C overnight. On the next day, one colony from each plate was transferred into 10 mL of brain heart infusion broth (BHI, Difco) and incubated at 37 °C overnight. One loopful (10 μL) of the overnight culture was then transferred into 10 mL of fresh BHI and incubated at 37 °C for an additional 24 h (broth-grown culture). To prepare plate-grown culture, fresh overnight broth culture was spread onto TSA plates (250 μL per plate, one plate per strain) and incubated for 24 h at 37 °C. On the next day, 5 mL of 0.85 % saline was pipetted onto each plate, and an L-shaped plastic cell spreader was used to loosen and scrape the lawn. *Re*-suspended plate-grown cells were pipetted into a 15 mL Falcon™ tube (Corning, Pittsburgh, PA, USA).

2.2. Screening of the adherence ability of LM strains with the fluorescent microplate adherence assay

The adherence abilities of 34 LM strains were determined following protocols described by Kushwaha and Muriana (2009) with modifications. Briefly, 200 μL of LM overnight broth-grown cultures were 1:100,000 diluted (from ca. 9 to 4 Log CFU/mL) in BHI and transferred to a 96-well plate with a clear lid. Plates were wrapped with Parafilm™ (Bemis Company, WI, USA) to prevent evaporation and incubated at 37 °C for 24 h. After incubation, plates were washed with Tris buffer (pH 7.4, 0.05 M) for three times to remove loosely attached cells. The washing process was followed by the addition of 200 μL of fresh BHI and another cycle of incubation at 37 °C for 24 h. After the final wash, 200 μL of 5,6-carboxyfluorescein diacetate (5,6-CFDA; Invitrogen, Carlsbad, CA, USA) working solution, prepared by adding 10 μL of a 2 % 5,6-CFDA solution in dimethyl sulfoxide to 1 mL of cold Tris buffer (pH 7.4, 0.05 M), were added to each well. Plates were then incubated in the dark at ambient temperature for 15 min and washed again with the Tris buffer solution for three times. The fluorescence of each plate was measured within 5 min in the Cytation 1 imaging reader (BioTek Instruments, Winooski, VT, USA) with an excitation and detection wavelength of 485 nm and 535 nm, respectively. For each plate, negative controls without bacterial culture added were also treated, washed, and measured in the same way as described above.

2.3. Preparation of gentamicin and chlorine solutions, and simulated lettuce-washing water

Gentamicin stock solution (1 mg/mL) was prepared by diluting 10 mg/mL gentamicin (Gibco, Rockville, MD, USA) in sterile Milli-Q water. Chlorine stock solution (1000 mg/L) was made by diluting commercial Clorox (Oakland, CA, USA) with sterile Milli-Q water. The pH of the chlorine solution was adjusted to 6.5 with citric acid (Sigma-Aldrich, St. Louis, MO, USA). The active free chloride concentration of the chlorine solution was tested by using the N, N-diethyl-*p*-phenylenediamine (DPD) assay (Hach method 8021) with a Hach spectrophotometer DR 3900 (Hach, Loveland, CO, USA). Ten percent sodium thiosulphate was prepared by dissolving 10 g of sodium thiosulphate (Ward's Science, ON, Canada) in 90 mL of sterile Milli-Q water. All chemical solutions were sterilized by filtration with 0.22 μm membranes, stored at 4 °C, and used within a day.

Simulated produce-washing water (SPWW) was prepared following protocols described by Huang et al. (2020) with modifications. Romaine lettuce was bought in a local retail store. Twenty-five grams of lettuce was chopped and added into 225 mL of Milli-Q water followed by

blending at high speed in a blender (KitchenAid KFC3516IC, MI, USA). Homogenate was recovered and transferred to 50 mL conical tubes and centrifuged (Eppendorf Centrifuge 5810R, Hamburg, Germany) at 20,000 ×*g* for 10 min at room temperature to remove large food particles. The resulting supernatant was diluted in Milli-Q water and sterilized by using 0.22 μm membranes to generate SPWW with different concentrations of organic loads. The chemical oxygen demand (COD) of BHI and prepared SPWW were analyzed by using high-range COD digestion vials (Hach) following the Hach Method 8000 (Cossu et al., 2016). Briefly, samples were first digested in a DRB 200 reactor (Hach). The colorimetric analyses of the digested products were carried out by using a DR/870 colorimeter (Hach). The resulting COD contents were 1366 mg/L O_2 for BHI and 1332 and 652 mg/L O_2 for the higher organic load (HCOD) and lower organic load (LCOD), respectively.

2.4. Determination of the minimum inhibitory concentration (MIC) of gentamicin for different LM strains

The two-fold microdilution broth method was used to determine the MIC of gentamicin for 34 LM strains (Jorgensen and Turnidge, 2015). Briefly, gentamicin solutions were prepared in two-fold concentrated BHI with final concentrations ranging from 5 μg/mL to 0.04 μg/mL. For each LM strain, overnight broth-grown culture was washed with 0.85 % saline and diluted to approximately 6 Log CFU/mL in BHI. Fifty microliters of each diluted LM culture and 50 μL of each gentamicin solution were then added into each well. The final bacterial concentration in each well was ca. 5 Log CFU/mL, and the final antibiotic concentrations ranged from 0.02 to 0.25 μg/mL. Every plate included a column of positive controls in which no gentamicin was added and a column of negative controls in which no inoculum was added. After incubating at 37 °C for 24 h, the optical density (OD) was measured at 600 nm using the Cytation 1 imaging reader (BioTek Instruments). Inoculated wells with OD_{600} values that were not significantly different ($P > 0.05$) from those of negative controls were considered as having no bacterial growth. MIC was determined as the lowest concentration of gentamicin that inhibited the microbial growth (Andrews, 2001).

2.5. Determination of the gentamicin concentrations used for selecting persister cells

LM 483, 480, and 485 with the highest adherence abilities were used in this experiment. Broth-grown culture was prepared and used for this study. Gentamicin was chosen as it is a common antibiotic used for listeriosis treatment (Drevets et al., 1994). A biphasic killing curve should be observed if the population contains persister cells (Balaban et al., 2019; Van den Bergh et al., 2017). As the gentamicin concentration increases, the killing curve should reach a plateau, indicating all the susceptible cells in the population have been eliminated and the remaining cells are persisters (Kint et al., 2012). To identify the gentamicin concentration that is sufficiently high enough to select persister cells, a dose-dependent killing assay needed to be performed first. In this experiment, 1 mL of the overnight culture of each strain was washed twice with 0.85 % saline and exposed to 0, 10, 25, 50 and 100 mg/L of gentamicin in BHI for 4 h by incubating in a 37 °C heating block with the shaking speed of 650 rpm (Eppendorf ThermoMixer® C). The number of surviving cells was determined after gentamicin treatment by washing the cells with 0.85 % saline and plating onto TSA using a spiral plater (Advanced Instruments, Norwood, MA, USA). This experiment was repeated twice independently with two replicates in each trial ($n = 4$).

2.6. The effect of growth conditions and cell density on the formation of LM persister cells

Plate-grown and broth-grown LM 483, 480, and 485 were obtained as described in 2.1. The suspensions of overnight plate-grown culture contained 9.62–9.73 Log CFU/mL of cells while the concentrations of overnight broth-grown culture were 8.83–8.92 Log CFU/mL. When determining the impact of growth conditions, plate-grown culture was first diluted to approximately the same level as the broth-grown culture (8.96–9.03 Log CFU/mL) and then both cultures were exposed to 100 mg/L gentamicin (80–160 × MIC) in BHI for 4 h by holding in a 37 °C heating block with shaking speed of 650 rpm. When evaluating the impact of cell densities on the formation of persister cells, undiluted and diluted plate-growth cultures were used. The concentration of persister cells was determined after gentamicin exposure by plating onto TSA agar. Persister fractions were calculated as:

$$\textit{Persister fraction}\ (\%) = \frac{\textit{the number of CFU after gentamicin treatment}}{\textit{the number of CFU before gentamicin treatment}}$$ (Thao et al., 2019).

2.7. Confirmation of the gentamicin susceptibility of persisters

According to Balaban et al. (2019), several criteria can be used to distinguish persistence from resistance: 1. similar to the dose-dependent killing curve, a biphasic death pattern should appear in a time-dependent killing curve when a population including persisters is exposed to an antibiotic for an extended period of time at a constant dosage. 2. regrowing the persister cells in the absence of antibiotic should produce a population that is just as susceptible to the same antibiotic as the population it gets isolated from. Therefore, to confirm that the persister cells formed under 100 mg/L of gentamicin exposure were not resistant strains, exposure time-dependent studies were carried out following protocols described by Knudsen et al. (2013) and Wu et al. (2017). First, overnight plate-grown culture was exposed to 100 mg/L gentamicin in BHI by incubating in a 37 °C heating block with shaking speed of 650 rpm. The number of culturable cells was determined at 0, 1, 2, 4 and 6 h. At each sampling point, the culture was centrifuged at 8000 ×*g* for 5 min (Eppendorf centrifuge 5424R). The supernatant was discarded, and the pellet was washed twice in 0.85 % saline and plated onto TSA using a spiral plater (Advanced Instruments). Persister cells that survived the gentamicin treatment (100 mg/L for 6 h) were then revived by growing in 10 mL of BHI at 37 °C for 24 h and plated onto a TSA plate to prepare a bacterial lawn. Cells were then harvested from the plate as described in 2.1. and subjected to 100 mg/L gentamicin (re-exposure). Samples were taken again at 0, 1, 2, 4, and 6 h for enumeration. The re-exposure experiment was carried out for all three LM strains. If there is no resistance developed, the time-dependent killing curve of the original cells and the reviving cells should exhibit comparable biphasic killing patterns (Wu et al., 2017).

2.8. Formation of LM persister cells on stainless steel (SS) coupons

SS, a corrosion-resistant metal, is preferred for use in the fresh-produce industry because of its antifouling ability (FDA, 2008). In this experiment, SS coupons and glass beads used for releasing cells from SS coupons were soaked in acetone overnight to remove debris and grease. They were then washed with commercial detergent solution (Joy professional dish soap, Joysuds, CT, USA), air dried, and autoclaved at 121 °C for 15 min before use (Ganegama Arachchi et al., 2013). To prepare the inoculum, regular plate-grown LM cultures (483, 480, and 485) were washed twice with 0.85 % saline by centrifugation and resuspended in four different solutions (BHI, HCOD, LCOD, or sterile Milli-Q water representing various nutrient levels). Two hundred and fifty milliliters of prepared inoculum were then inoculated on to each SS coupon. Persisters formed on stainless steel coupons were determined after 0, 3, 5 and 7 days of storage at 4 °C and 85 % relative humidity (RH). At each sampling point, LM cells were recovered as described by Ryu and Beuchat (2005) with modifications. Briefly, each coupon was submerged in 10 mL of BHI with 10 glass beads in a 50 mL centrifuge tube, incubated at room temperature for 30 min, and vigorously vortexed at maximum intensity for 2 min with a vortexer (Thermo Fisher, MA, USA). The total population of LM was quantified by plating and

enumeration at various times during incubation, while the persister subpopulation in each sample was determined by exposing the recovered culture to 100 mg/L of gentamicin for 4 h and then plated, incubated, and enumerated.

2.9. Chlorine sensitivity of LM persister cells

Persister cells of LM 483 were obtained as described in 2.8. To overcome the impact generated by cell density, a dead cell culture was prepared based on the procedure provided by the LIVE/DEAD BacLight Bacterial Viability Kit (Invitrogen). Cells were washed twice and resuspended in 0.85 % saline. Regular LM cell culture was prepared by 1:100 diluting the overnight culture in dead cell culture. By doing so, the levels of culturable bacteria and the levels of organic matter in both persister and regular cell cultures were at similar levels. One milliliter of LM culture was washed and resuspended in 0.85 % saline. After that, 1000 mg/L of chlorine solution at a ratio of 1:10 (*v*/v) was added to resuspended LM to obtain a final chlorine concentration of 100 mg/L. After 2 min of incubation, 1 mL of a 10 % sodium thiosulfate solution was added to the mixture to neutralize the residue chlorine (Kemp and Schenider, 2000). The number of surviving cells in each type of culture was then washed with 0.85 % saline twice and determined by plate count.

2.10. Statistical analysis

All tests described above were repeated twice independently with three replicates for each trial ($n = 6$) unless otherwise indicated. Data analyses were conducted using general linear models (GLM) from JMP (SAS, Cary, NC, USA). Mean values were compared by Tukey's test. P values of <0.05 were considered significant.

3. Results

3.1. Different adherence ability of LM strains

The microplate fluorescence adherence assay was applied to evaluate the adherence ability of 34 LM strains isolated from California fresh-produce-processing plants and packinghouses. The strains were arranged (from top to bottom) in the order of increasing fluorescence intensities (from 1099 to 16,488 RFU). The higher the fluorescence intensity, the stronger the adherence ability of the LM strain (Fig. 1). Bacterial adhesion has been proven to be directly associated with microorganisms' persistence in the food processing environment, and their chance of being transferred to food and infecting humans (Kushwaha and Muriana, 2009; Latorre et al., 2011). Studies have also demonstrated a positive relationship between bacterial adhesion and persister development (Aslan et al., 2021; Flemming et al., 2016; Manandhar et al., 2022; Uzoechi and Abu-Lail, 2020). Therefore, strains with stronger adherence are anticipated to produce more persister cells, which would be advantageous for our survival and disinfection tests. As shown in Fig. 1, LM 485, 480, and 483 exhibited the best adhesion capabilities with respective fluorescence intensities of 11,960 ± 5039, 12,685 ± 4720 and 16,488 ± 5732, thus were employed in the following experiments.

3.2. Determination of the gentamicin concentration used for selecting LM persister cells

Persisters are defined as the surviving subfractions of cells that become stabilized even when the concentration of antibiotics keeps increasing (Balaban et al., 2019; Kint et al., 2012). Thus, a gentamicin concentration needed to be determined before any further experiment to efficiently select and separate persister cells from other surviving populations. The MICs of gentamicin for each LM strain were first determined; they were 1.25 μg/mL for strains LM 480 and 483 and 0.62 μg/mL for LM 485. Biphasic killing patterns, the key characteristic showing

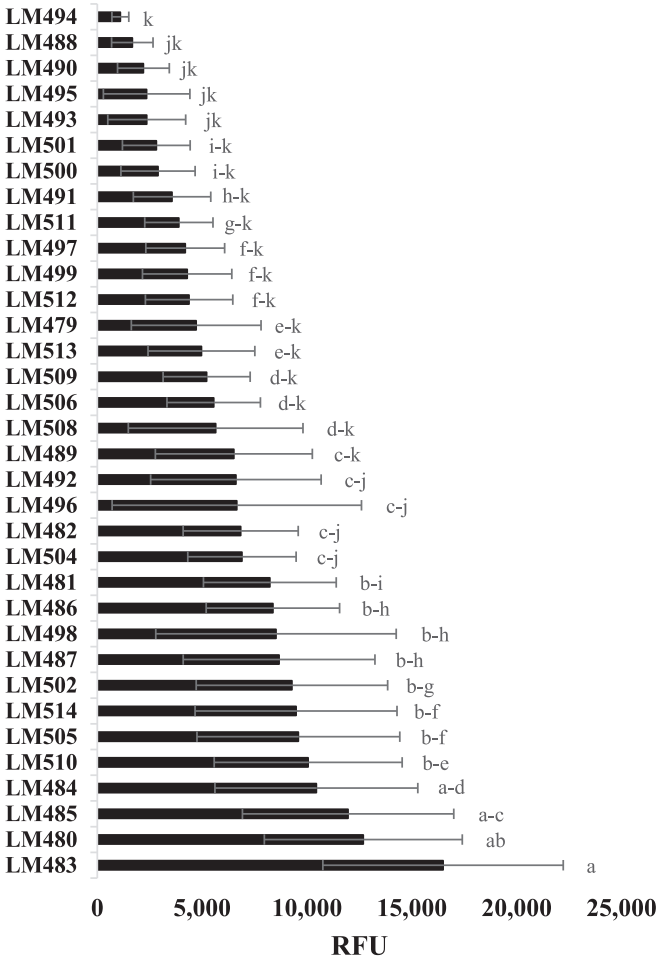

Fig. 1. Evaluation of the adherence ability of 34 *Listeria monocytogenes* (LM) strains isolated from California fresh produce processing plants and packinghouses by using the microplate fluorescence attachment assay. RFU, relative fluorescence units. Each black horizontal bar represents the mean RFU of six measurements ($n = 6$). Values with different letters are significantly different ($P < 0.05$).

the presence of persister cells within a population, were observed (Fig. 2). As shown in Fig. 2, the number of surviving cells after treatment correlated negatively with gentamicin concentrations until the drug concentration reached 50 mg/L. The number of surviving cells plateaued when the gentamicin increased from 50 to 100 mg/L ($P > 0.05$), suggesting that the remaining cells are persisters. This observation was applicable to all three LM strains. Therefore, 100 mg/L of gentamicin was used in the following experiment for the selection of LM persisters.

3.3. Impact of culture preparation and cell density on the formation of LM persisters

The impact of culture preparation methods and the inoculum sizes/population sizes on the formation of LM persisters were evaluated. Overnight broth-grown culture and plate-grown culture generated ca. 8.84–8.92 and 9.62–9.73Log CFU/mL LM cells, respectively. As shown in Fig. 3, the percentages of formed LM persister cells from the broth-grown culture were 0.022 %, 0.011 %, and 0.009 % for LM 483, 480, and 485, respectively. To match the initial cell density of the broth-grown culture, the plate-growth culture was 1:10 diluted before treating with gentamicin. The resulting persister fractions (0.003–0.004 %) were at the same level as the broth-grown culture ($P > 0.05$). However, when the plate-grown culture was held at the original cell density and exposed to the 100 mg/L gentamicin treatment, approximately

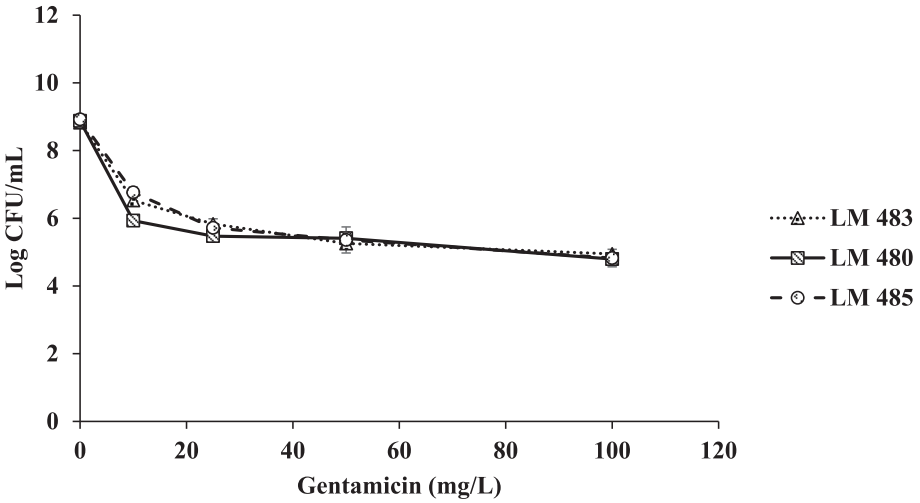

Fig. 2. Dose-dependent killing kinetics of three LM strains. Cultures were treated with different concentrations of gentamicin (0–100 mg/L) at 37 °C for 4 h and the surviving cultures were enumerated on non-selective agar ($n = 4$).

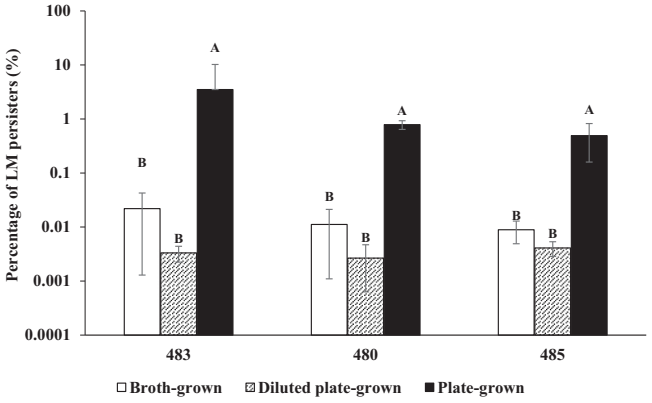

Fig. 3. The impact of growth conditions (broth-grown vs. plate-grown) and population sizes (original vs. diluted plate-grown cultures) on the formation of persisters. Strains LM 483, 480 and 485 were tested. Y-axis represents the percentages of LM persisters (%) that are calculated by dividing the number of persisters after the 4 h 100 mg/L-gentamicin exposure by the total cell counts prior to the exposure. Different capitalized letters on top of the error bars mean significantly different ($n = 6$, $P < 0.05$).

7.31–8.28 Log CFU/mL LM persisters formed. The calculated persister fractions for non-diluted, plate-grown LM 483, 480, and 485 were 3.511 %, 0.786 %, and 0.492 %, respectively, which were significantly higher than those of diluted plate-grown cultures ($P < 0.05$) (Fig. 3). Given that the undiluted plate-grown culture generated the highest ratios of persisters, plate-grown LM was used for the rest of the studies.

3.4. Confirmation of the gentamicin susceptibility of persisters

As described in the introduction section, one key feature associated with persister cells is that their tolerance to an antibiotic is not inheritable. To confirm that the LM persister cells selected by the exposure to 100 mg/L gentamicin were not actually resistant cells, the formed LM persisters were revived and re-exposed to 100 mg/L gentamicin. Fresh overnight plate-grown LM cultures were used as the control. During the 6 h exposure, subsamples were taken at hour 1, 2, 4, and 6 and plated onto TSA agar. As shown in Fig. 4, the patterns of cell inactivation by 100 mg/L gentamicin were the same for plate-grown LM and revived LM cultures. This observation was applicable to all three LM strains, proving that the selected persisters were phenotypic characters, and their progeny were as susceptible to antibiotics as the parental population.

3.5. Formation of LM persister cells on stainless steel coupons

To test if LM persisters could be formed under the simulated environment of produce-processing-plants, plate-grown LM 483, 480, and 485 were resuspended in BHI, HCOD, LCOD and sterile Milli-Q water representing different nutrient levels and inoculated onto SS coupons. The inoculated SS coupons were stored at 4 °C with 85 % RH. The total number of surviving LM cells and the formed persister cells during storage were examined at days 0, 3, 5, and 7. Figs. 5A-C show the total number of surviving LM cells recovered from SS coupons. In general, LM survived better in more nutrient-rich media such as BHI and HCOD compared to LCOD and Milli-Q water. Lower numbers ($P < 0.05$) of LM 483 and LM 480 were recovered from LCOD-inoculated and water-inoculated coupons compared to BHI- and HCOD-inoculated coupons since Day 5 (Fig. 5A and B). LM 485 was more sensitive to the nutrient level compared to other strains (Fig. 5C). Fewer LM 485 survivors were recovered in water and LCOD compared to BHI since Day 3 ($P < 0.05$). On Days 5 and 7, the surviving populations in LCOD, water, and even HCOD were smaller than that in BHI ($P < 0.05$).

Figs. 5D-F show the dynamics of LM persister cells on the SS coupon during storage. Overall, LM 483 generated a higher persister level compared to LM 480 and 485. Taking the BHI group as an example, LM 483 generated 4.59 ± 0.142 Log CFU/coupon of persister on average during the whole storage period, while LM 480 and 485 generated 3.01 ± 0.188 and 2.98 ± 0.115 Log CFU/coupon, respectively. For LM 483, both the media type (i.e., nutrient level) and storage time had limited impact on the formation of persisters; persisters formed in BHI, HCOD, LCOD, and Milli-Q water were not significantly different from each other within the same sampling day ($P > 0.05$, Fig. 5D). Similarly, the number of persister cells in each kind of medium did not vary considerably over the storage time (BHI: 4.44–4.76 Log CFU/mL; HCOD: 3.99–4.68 Log CFU/mL; LCOD: 3.82–4.53 Log CFU/mL; H_2O: 3.99–4.51 Log CFU/mL). Regardless of the medium, there was no significant difference between the number of persisters recovered from SS coupons on Day 7 and the numbers recovered on Day 0 ($P > 0.05$). For LM 480, the persister cell level in BHI (2.78–3.20 Log CFU/mL) and HCOD (2.58–3.50 Log CFU/mL) remained relatively stable during the storage period (Fig. 5E). On the contrary, the number of persister cells in LCOD and H_2O fell below the limit of detection (LOD) on Days 5 and 3, respectively (Fig. 5E). For LM 485, the persister levels in BHI, HCOD and LCOD did not change significantly throughout the storage period ($P > 0.05$; Fig. 5F). Milli-Q water did not support the formation of LM 485 persister cells, with the

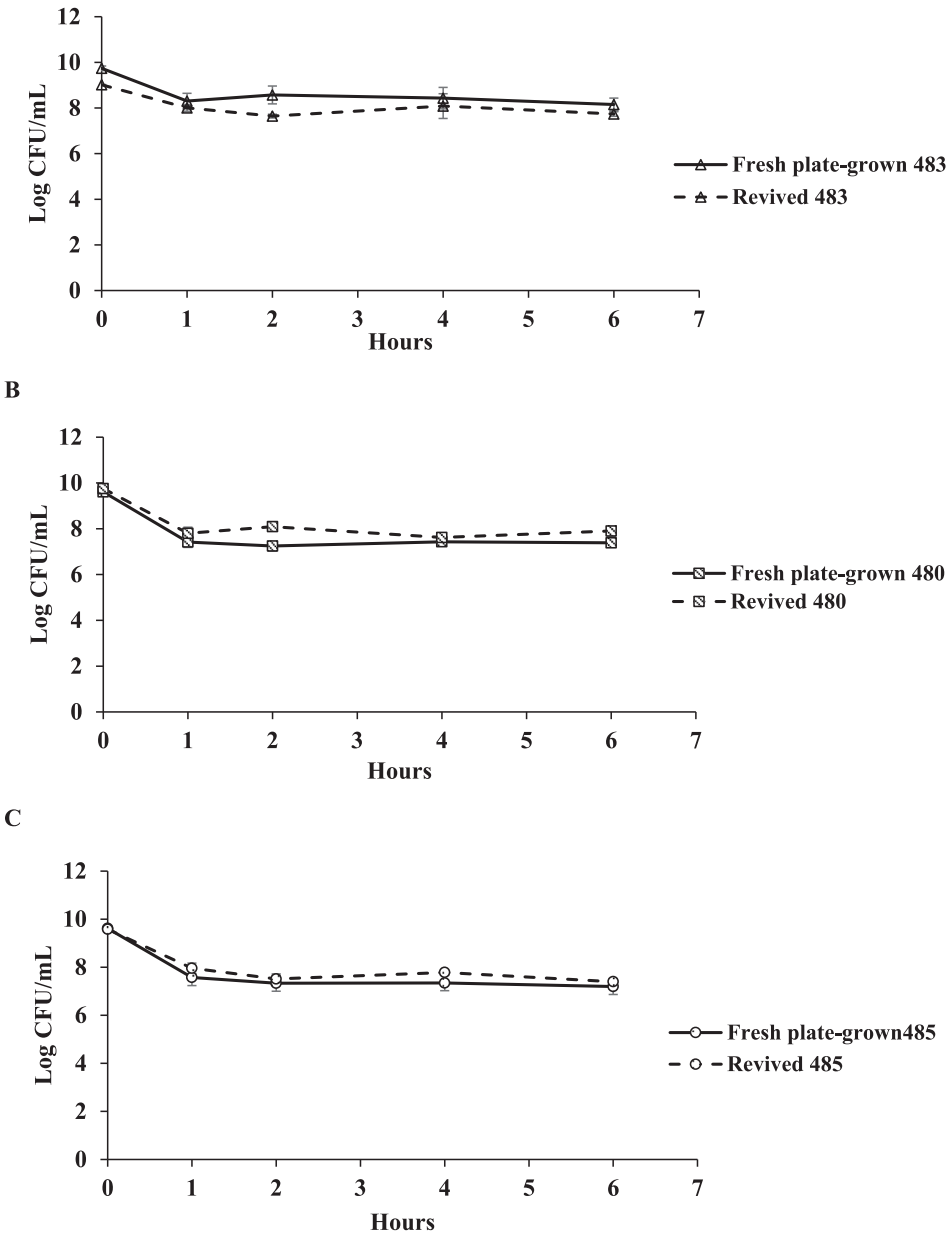

Fig. 4. The formation of persister cells by fresh plate-grown LM cultures and revived LM persisters. A, LM 483; B, LM 480, and C, LM 485. Revived LM cultures were obtained by re-growing LM persister cells in antibiotic-free media. Values presented at each sampling point represent means ± standard deviations ($n = 6$).

persister population dropping below LOD by Day 3 (Fig. 5F).

3.6. Sanitizer sensitivity of LM persister cells

Since LM 483 produced the highest percentage of persisters (Figs. 4 and 5). The regular and persister cells of LM 483 were used for the evaluation of their chlorine sensitivity. This was done by exposing regular LM and persister LM cells to 100 mg/L of chlorine for 2 min. The initial live cell concentrations of the diluted regular cells culture (plate-grown LM culture 1:100 diluted in dead cells) and the persister cell culture were on 7.84 ± 0.113 and 7.85 ± 0.578 Log CFU/mL, respectively (Fig. 6). The chlorine treatment caused a 5.65 Log CFU/mL reduction in the persister cell culture, which was significantly higher than the 3.09 Log CFU/mL reduction observed from the regular fresh plate-grown culture ($P < 0.05$; Fig. 6). This indicated that LM483 persisters were more sensitive to chlorine treatments (100 mg/L for 2 min) than their corresponding regular plate-grown cells.

4. Discussion

A persister cell is a phenotypic stage of bacteria that can be induced in response to a variety of environmental stresses, including low temperatures, limited nutrients, extreme pH, and the presence of sanitizers (Colagiorgi et al., 2016; Fisher et al., 2017; Lewis, 2007). Although these pressures are commonly found in the postharvest produce-processing environment, few studies have examined whether LM may truly develop persisters under such conditions. This study investigated the potential of LM to generate persister cells in a simulated produce- processing environment, as well as the intrinsic and extrinsic factors that may influence the persisters' formation.

The first intrinsic factor tested was the physiological status of LM cells. Previous studies have shown that growth on a solid matrix induces a distinct physiological state in *Salmonella*, which may contribute to the pathogen's increased stress resistance, such as thermal and desiccation tolerance (Keller et al., 2012; Uesugi and Harris, 2006). For example, *Salmonella* Tennessee and *Salmonella* Oranienburg survived better in

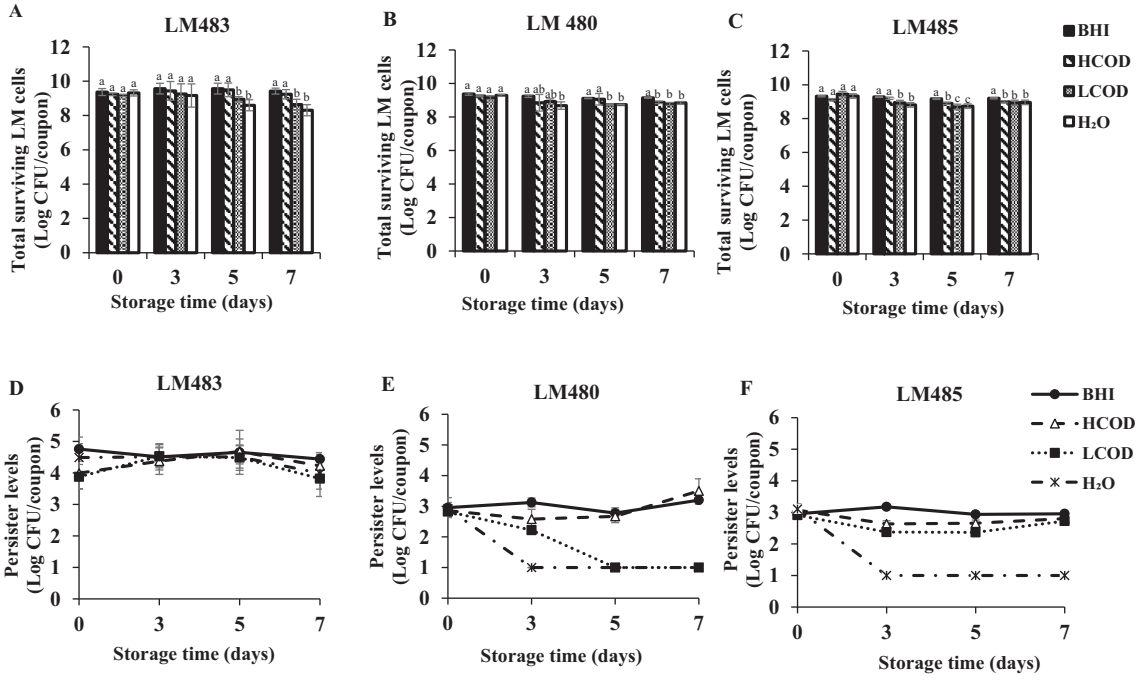

Fig. 5. The survival of LM on SS coupons and the formation and fitness of LM persisters during storage. **A-C**, the survival of LM 483 (A), LM 480 (B) and LM 485 (C) on SS coupons suspended in BHI, HCOD, LCOD, and H_2O when stored at 4 °C and 85 % RH. Different letters represent significant different among LM populations within each sampling point (n = 6, $P < 0.05$). **D—F**, Fitness of LM persisters formed by LM 483 (D), LM 480 (E) and LM 485 (F) on SS coupons during storage. **BHI:** brain heart infusion broth. **HCOD**: simulated lettuce washing water with higher organic content. **LCOD**: simulated lettuce washing water with lower organic content. **H_2O:** sterile Milli-Q water. Limit of detection (LOD) = 1 Log CFU/coupon.

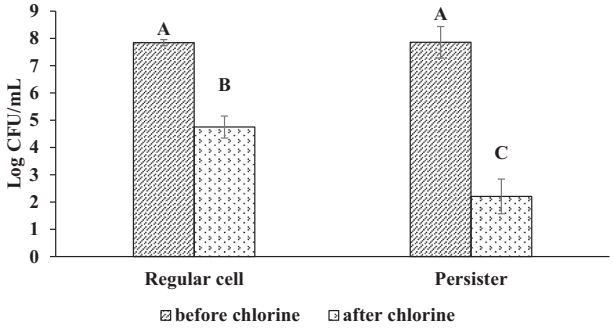

Fig. 6. Chlorine sensitivity of regular plate-grown LM 483 and LM 483 persisters. Error bars indicate the standard deviations (n = 6). Different capitalized letters on top of the bars represent significantly difference ($P < 0.05$).

peanut butter at 25 °C for the plate-grown culture (1 Log loss over 2 weeks) than the broth-grown culture (1–2 Log loss over 2 weeks) (Keller et al., 2012). Therefore, we first evaluated the physiological differences brought to LM by the form of growth media (solid agar vs. liquid broth). Based on our results, no statistically significant difference was observed in persister fractions between broth-grown and diluted plate-grown LM cultures when the inoculum sizes were the same. Similar results were reported by Knudsen et al. (2013), in which the difference between LM persister levels formed by surface-associated growth (an overnight culture was spotted onto a polycarbonate filter and incubated at 37 °C on BHI agar) and by planktonic culture was insignificant when using 100 mg/L norfloxacin to select the persister populations (Knudsen et al., 2013).

One intriguing observation was that population size significantly impacted the ratios of persisters. When persister ratios were calculated for undiluted and diluted plate-grown LM cultures, significantly higher percentages of persisters were generated by the undiluted plate-grown cultures than by the diluted plate-grown cultures. This observation holds true for all three strains. One reason for this might be the secretion of quorum-sensing-related compounds. Studies have reported that *Pseudomonas aeruginosa* produced a greater amount of phenazine pyocyanin and acyl-homoserine lactone (the quorum-sensing-related signaling molecules) in response to increased cell density, which then significantly enhanced the number of multidrug-tolerant persisters in *P. aeruginosa* cells (Möker et al., 2010; Pesci et al., 1997). In another study conducted by Ghosh et al. (2018), the CdiA toxins utilized by the contact-dependent growth inhibition (CDI) system was associated with increased persister production in a clonal *Escherichia coli* population. Specifically, the CDI system is encoded by *cdiBAI*, in which the CdiA toxins and CdiI immunity molecule constitute a toxin-antitoxin system analog. When a population of isogenic cells with CDI systems reaches high cell density, random cell-to-cell contacts form subpopulations of cells where toxin molecules outweigh immunity molecules. In these cells, the CdiA toxin triggers the stringent response and a feedforward loop where Lon destroys the remaining CdiI protein, inducing growth arrest and the generation of persister cells. Therefore, the authors suggested that CDI systems mediate a population-density-dependent bet-hedging approach in which the proportion of non-growing cells increases only when there are a large number of cells of the same genotype (Ghosh et al., 2018). Both CDI and quorum-sensing systems have also been described in LM before (Ikryannikova et al., 2020; Riedel et al., 2009). Therefore, this could possibly explain why a greater proportion of LM persisters in our study was formed in the undiluted plate-grown culture with a higher cell density. Nonetheless, further research is needed to better explain the molecular processes behind the influence of inoculum size or cell density on the generation of persister cells.

The main extrinsic factor tested for LM-persister formation was available nutrients. LM persisters were identified on SS coupons inoculated with plate-grown LM cultures suspended in media with decreasing nutrient levels: BHI, SPWW with various organic loads (HCOD and LCOD) and Milli-Q water. Nutrient scarcity and starvation-induced

stringent response have been linked to increased persister formation (Gardner et al., 2007; Van den Bergh et al., 2017). In this study, however, reduced numbers of LM 480 and 485 persisters were found in sterile water and LCOD (i.e., lower nutrient levels) than in media with higher nutrient levels (i.e., HCOD and BHI) (Fig. 5E-F; $P < 0.05$). In concordance with our current findings, Wu et al. (2017) also found a significantly lower number of LM persisters in diluted tryptic soy broth (TSB) compared to full TSB after nisin treatment ($P < 0.05$). Thao et al. (2019) discovered that several STEC strains had considerably lower persister proportions in stationary-phase cells cultivated in spinach lysates than in rich media ($P < 0.05$). It is possible that the available nutrient directly impacted the cell densities of the bulk population, and then sequentially affected persister formation in LCOD and Milli-Q water for LM 480 and 485 during the latter days of storage. As discussed before, quorum sensing in LM may have played a role in the development of bacteria's persistence (Ghosh et al., 2018).

Chlorinated solution is currently widely used in the produce industry for controlling bacterial contamination including LM (FAO/WHO, 2009; Shen et al., 2012). In a produce processing plant, the presence of water and organic matter might consume biocides such as chlorine and reduce the concentration to sublethal levels (Carpentier and Cerf, 2011). Continuous exposure to sublethal doses of biocides/antimicrobials during cleaning and sanitizing may cause stress adaption in bacteria and co-select genes that encode resistance to the same or other biocides and antibiotics (Wales and Davies, 2015). The connection between antibiotic resistance and sanitizer resistance/tolerance has been a research topic and food safety concern. Over the past decade, mixed results have been reported about their connections. Templeton et al. (2009) exposed trimethoprim-resistant *E. coli* to 1.25 mg/L of chlorine for 15 min; the trimethoprim-resistant *E. coli* exhibited greater chlorine tolerance than the antibiotic sensitive isolate. However, the same chlorine tolerance was not observed from ampicillin-resistant isolate in the same study (Templeton et al., 2009). Similarly, Huang et al. (2011) found that antibiotic-resistant bacteria (ARB) discovered in municipal wastewater exhibited similar or higher inactivation rates than the total heterotrophic bacteria from the same source. The observed tolerance of chlorination associated with ARB (e.g., by Templeton et al., 2009) may be linked to the phenomenon commonly referred to as “cross-resistance” (Karumathil et al., 2014). The same genetic process that confers resistance to antibiotics (e.g., overexpression of the multidrug efflux pumps) may contribute to enhanced tolerance to chlorination and even other antibiotics.

As for our study, the phenotypic persisters did not show enhanced chlorine tolerance but became more sensitive to chlorination than regular cells. In support of our findings, the study conducted by Liu et al. (2022) also showed that 25 mg/L chlorine treatment for 60 min completely eradicated *Acinetobacter* persisters tolerant to kanamycin, tetracycline, and ciprofloxacin. Chlorine is regarded as a nonselective oxidant that interacts with several biological components and metabolic processes of microbes (Shang and Blatchley, 1999), while gentamicin is an aminoglycoside antibiotic that blocks protein synthesis by binding irreversibly to 30S ribosomal subunits (Ross et al., 2019). The distinct antimicrobial mechanisms between chlorine and gentamicin may be one of the reasons explaining the susceptibility of LM persisters to chlorine treatment (Van den Bergh et al., 2017). Secondly, it has been reported that the formation of persister has fitness costs. Populations with a relatively high proportion of persister cells were shown to have an increased cellular decline in resource-poor situations (Stepanyan et al., 2015). These fitness cost may also help explain the unexpected higher sensitivity of LM persister cells than regular cells to chlorine treatment. However, more studies are still needed to provide a more detailed view about the molecular mechanisms behind this phenomenon.

5. Conclusion

Persisters are transiently tolerant variants that allow populations to evade eradication by antibiotic therapy. Their tolerance to antibiotics is non-genetic and non-heritable but rather resulted from a phenotypic switch from sensitive to persistent cell type (Kint et al., 2012). This study demonstrated that LM could form persister cells under the simulated conditions of a produce-processing environment. Their formation was determined by two main intrinsic and extrinsic factors, the inoculum size, and the nutrient availability. When using gentamicin as the selective antibiotic for selecting LM persisters, the identified persisters showed higher sensitivity to chlorine treatment.

Declaration of competing interest

All Authors, Xiran Li, Xavier F. Hospital, Eva Hierro, Manuela Fernández, Lina Sheng, and Luxin Wang, certify that there is no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. There is no conflict of interest from any of the authors.

Data availability

Data will be made available on request.

Acknowledgements

The authors thank the Complutense University of Madrid and the Spanish Ministry of Science, Innovation and Universities for the mobility grants conceded to X.F. Hospital (Complutense del Amo 19/20 and José Castillejo CAS19/00257). The authors also appreciate the financial support provided by the USDA Multi-state HATCH project S-1077.

References

Andrews, J.M., 2001. Determination of minimum inhibitory concentrations. J. Antimicrob. Chemother. 48, 5–16.

Aslan, H., Petersen, M.E., De Berardinis, A., Zacho Brunhede, M., Khan, N., Vergara, A., Kallipolitis, B., Meyer, R.L., 2021. Activation of the two-component system LisRK promotes cell adhesion and high ampicillin tolerance in Listeria monocytogenes. Front. Microbiol. 12 https://doi.org/10.3389/fmicb.2021.618174.

Balaban, N.Q., Merrin, J., Chait, R., Kowalik, L., Leibler, S., 2004. Bacterial persistence as a phenotypic switch. Science 305, 1622–1625. https://doi.org/10.1126/science.1099390.

Balaban, N.Q., Helaine, S., Lewis, K., Ackermann, M., Aldridge, B., Andersson, D.I., Brynildsen, M.P., Bumann, D., Camilli, A., Collins, J.J., Dehio, C., Fortune, S., Ghigo, J.-M., Hardt, W.-D., Harms, A., Heinemann, M., Hung, D.T., Jenal, U., Levin, B.R., Michiels, J., Storz, G., Tan, M.-W., Tenson, T., Van Melderen, L., Zinkernagel, A., 2019. Definitions and guidelines for research on antibiotic persistence. Nat. Rev. Microbiol. 17, 441–448. https://doi.org/10.1038/s41579-019-0196-3.

Bintsis, T., 2017. Foodborne pathogens. AIMS Microbiol. 3, 529–563. https://doi.org/10.3934/microbiol.2017.3.529.

Cabral, D.J., Wurster, J.I., Belenky, P., 2018. Antibiotic persistence as a metabolic adaptation: stress, metabolism, the host, and new directions. Pharm. 11 https://doi.org/10.3390/ph11010014.

Carpentier, B., Cerf, O., 2011. Review-persistence of Listeria monocytogenes in food industry equipment and premises. Int. J. Food Microbiol. 145 (1), 1–8. https://doi.org/10.1016/j.ijfoodmicro.2011.01.005.

CDC, 2015. Multistate Outbreak of Listeriosis Linked to Commercially Produced, Prepackaged Caramel Apples Made From Bidart Bros. Apples (Final Update). URL. https://www.cdc.gov/listeria/outbreaks/caramel-apples-12-14/index.html.

CDC, 2015. Wholesome Soy Products, Inc. Sprouts and Investigation of Human Listeriosis Cases (Final Update). URL. https://www.cdc.gov/listeria/outbreaks/bean-sprouts-11-14/index.html.

CDC, 2020. List of Selected Multistate Foodborne Outbreak Investigations [WWW Document]. URL. https://www.cdc.gov/foodsafety/outbreaks/multistate-outbreaks/outbreaks-list.html.

CDC, 2022. Listeria Outbreak Linked to Packaged Salads Produced by Fresh Express [WWW Document]. URL. https://www.cdc.gov/listeria/outbreaks/packaged-salad-12-21-b/index.html.

Chen, Y., Burall, L.S., Luo, Y., Timme, R., Melka, D., Muruvanda, T., Payne, J., Wang, C., Kastanis, G., Maounounen-Laasri, A., Jesus, A.J.De, Curry, P.E., Stones, R., K'Aluoch, O., Liu, E., Salter, M., Hammack, T.S., Evans, P.S., Parish, M., Allard, M. W., Datta, A., Strain, E.A., Brown, E.W., Dudley, E.G., 2016. Listeria monocytogenes in stone fruits linked to a multistate outbreak: enumeration of cells and whole-genome sequencing. Appl. Environ. Microbiol. 82, 7030–7040. https://doi.org/10.1128/AEM.01486-16.

Cohen, N.R., Lobritz, M.A., Collins, J.J., 2013. Microbial persistence and the road to drug resistance. Cell Host Microbe 13, 632–642. https://doi.org/10.1016/j.chom.2013.05.009.

Colagiorgi, A., Di Ciccio, P., Zanardi, E., Ghidini, S., Ianieri, A., 2016. A look inside the Listeria monocytogenes biofilms extracellular matrix. Microorganisms 4. https://doi.org/10.3390/microorganisms4030022.

Cossu, A., Ercan, D., Tikekar, R.V., Nitin, N., 2016. Antimicrobial effect of photosensitized rose Bengal on bacteria and viruses in model wash water. Food Bioprocess Technol. 9, 441–451. https://doi.org/10.1007/s11947-015-1631-8.

DiCaprio, E., Purgianto, A., Ma, Y., Hughes, J., Dai, X., Li, J., 2015. Attachment and localization of human norovirus and animal caliciviruses in fresh produce. Int. J. Food Microbiol. 211, 101–108. https://doi.org/10.1016/j.ijfoodmicro.2015.07.013.

Drevets, D.A., Canono, B.P., Leenen, P.J., Campbell, P.A., 1994. Gentamicin kills intracellular Listeria monocytogenes. Infect. Immun. 62, 2222–2228. https://doi.org/10.1128/iai.62.6.2222-2228.1994.

FAO/WHO, n.d.FAO/WHO, n.d. Benefits and risks of the use of chlorine-containing disinfectants in food production and food processing: report of a joint FAO/WHO expert meeting, Ann Arbor, MI, USA, 27-30 May 2008.

FDA, 2008. Guidance for Industry: Guide to Minimize Microbial Food Safety Hazards of Fresh-cut Fruits and Vegetables [WWW Document]. URL. https://www.fda.gov/regulatory-information/search-fda-guidance-documents/guidance-industry-guide-minimize-microbial-food-safety-hazards-fresh-cut-fruits-and-vegetables.

FDA, 2019. ac. Vandenberg, Inc. Recalls Fresh Peaches, Fresh Nectarines and Fresh Plums Because They May Be Contaminated with Listeria monocytogenes. URL. https://www.fda.gov/regulatory-information/search-fda-guidance-documents/guidance-industry-guide-minimize-microbial-food-safety-hazards-fresh-cut-fruits-and-vegetables.

FDA, 2019. Henry Avocado Recalls Whole Avocados Because of Possible Health Risk. URL. https://www.fda.gov/safety/recalls-market-withdrawals-safety-alerts/henry-avocado-recalls-whole-avocados-because-possible-health-risk.

Fisher, R.A., Gollan, B., Helaine, S., 2017. Persistent bacterial infections and persister cells. Nat. Rev. Microbiol. 15, 453–464. https://doi.org/10.1038/nrmicro.2017.42.

Flemming, H.-C., Wingender, J., Szewzyk, U., Steinberg, P., Rice, S.A., Kjelleberg, S., 2016. Biofilms: an emergent form of bacterial life. Nat. Rev. Microbiol. 14, 563–575. https://doi.org/10.1038/nrmicro.2016.94.

Foong-Cunningham, S., Verkaar, E.L.C., Swanson, K., 2012. 1 - microbial decontamination of fresh produce. In: Demirci, A., Ngadi, M.O. (Eds.), Microbial Decontamination in the Food Industry. Woodhead Publishing, pp. 3–29. https://doi.org/10.1533/9780857095756.1.3.

Ganegama Arachchi, G.J., Cridge, A.G., Dias-Wanigasekera, B.M., Cruz, C.D., McIntyre, L., Liu, R., Flint, S.H., Mutukumira, A.N., 2013. Effectiveness of phages in the decontamination of Listeria monocytogenes adhered to clean stainless steel, stainless steel coated with fish protein, and as a biofilm. J. Ind. Microbiol. Biotechnol. 40, 1105–1116. https://doi.org/10.1007/s10295-013-1313-3.

Gardner, A., West, S.A., Griffin, A.S., 2007. Is bacterial persistence a social trait? PLoS One 2, e752. https://doi.org/10.1371/journal.pone.0000752.

Gaul, L.K., Farag, N.H., Shim, T., Kingsley, M.A., Silk, B.J., Hyytia-Trees, E., 2012. Hospital-acquired listeriosis outbreak caused by contaminated diced celery—Texas, 2010. Clin. Infect. Dis. 56, 20–26. https://doi.org/10.1093/cid/cis817.

Ghosh, A., Baltekin, Ö., Wäneskog, M., Elkhalifa, D., Hammarlöf, D.L., Elf, J., Koskiniemi, S., 2018. Contact-dependent growth inhibition induces high levels of antibiotic-tolerant persister cells in clonal bacterial populations. EMBO J. 37, e98026 https://doi.org/10.15252/embj.201798026.

Gollan, B., Grabe, G., Michaux, C., Helaine, S., 2019. Bacterial persisters and infection: past, present, and progressing. Annu. Rev. Microbiol. 73, 359–385. https://doi.org/10.1146/annurev-micro-020518-115650.

Goswami, M., Bhattacharyya, P., Tribedi, P., 2017. Allee effect: the story behind the stabilization or extinction of microbial ecosystem. Arch. Microbiol. 199, 185–190. https://doi.org/10.1007/s00203-016-1323-4.

Harris, L.J., Wang, L., 2012. Influence of the Pre-harvest Environment on the Physiological State of Salmonella and Its Impact on Increased Survival Capability. Center for Produce Safety.

Hong, S.H., Wang, X., O'Connor, H.F., Benedik, M.J., Wood, T.K., 2012. Bacterial persistence increases as environmental fitness decreases. Microb. Biotechnol. 5, 509–522. https://doi.org/10.1111/j.1751-7915.2011.00327.x.

Huang, J.-J., Hu, H.-Y., Tang, F., Li, Y., Lu, S.-Q., Lu, Y., 2011. Inactivation and reactivation of antibiotic-resistant bacteria by chlorination in secondary effluents of a municipal wastewater treatment plant. Water Res. 45, 2775–2781. https://doi.org/10.1016/j.watres.2011.02.026.

Huang, K., Tian, Y., Tan, J., Salvi, D., Karwe, M., Nitin, N., 2020. Role of contaminated organic particles in cross-contamination of fresh produce during washing and sanitation. Postharvest Biol. Technol. 168, 111283 https://doi.org/10.1016/j.postharvbio.2020.111283.

Ikryannikova, L.N., Kurbatov, L.K., Gorokhovets, N.V., Zamyatnin Jr., A.A., 2020. Contact-dependent growth inhibition in bacteria: do not get too close! Int. J. Mol. Sci. 21. https://doi.org/10.3390/ijms21217990.

Jorgensen, J.H., Turnidge, J.D., 2015. Susceptibility test methods: dilution and disk diffusion methods. In: Manual of Clinical Microbiology, pp. 1253–1273. https://doi.org/10.1128/9781555817381.ch71.

Karumathil, D.P., Yin, H.B., Kollanoor-Johny, A., Venkitanarayanan, K., 2014. Effect of chlorine exposure on the survival and antibiotic gene expression of multidrug resistant Acinetobacter baumannii in water. Int. J. Environ. Res Public Health 11, 1844–1854. https://doi.org/10.3390/ijerph110201844.

Keller, S.E., Grasso, E.M., Halik, L.A., Fleischman, G.J., Chirtel, S.J., Grove, S.F., 2012. Effect of growth on the thermal resistance and survival of Salmonella Tennessee and Oranienburg in peanut butter, measured by a new thin-layer thermal death time device. J. Food Prot. 75, 1125–1130. https://doi.org/10.4315/0362-028x.Jfp-11-477.

Kemp, G.K., Schneider, K.R., 2000. Validation of thiosulfate for neutralization of acidified sodium chlorite in microbiological testing. Poult. Sci. 79, 1857–1860. https://doi.org/10.1093/ps/79.12.1857.

Kenner, B., 2020. U.S. Fruit Imports Grew By $8.9 Billion Over the Last Decade to Meet Rising Demand [WWW Document]. URL. https://www.ers.usda.gov/amber-waves/2020/september/us-fruit-imports-grew-by-89-billion-over-the-last-decade-to-meet-rising-demand/.

Keren, I., Shah, D., Spoering, A., Kaldalu, N., Lewis, K., 2004. Specialized persister cells and the mechanism of multidrug tolerance in Escherichia coli. J. Bacteriol. 186, 8172–8180. https://doi.org/10.1128/jb.186.24.8172-8180.2004.

Kint, C.I., Verstraeten, N., Fauvart, M., Michiels, J., 2012. New-found fundamentals of bacterial persistence. Trends Microbiol. 20, 577–585.

Knudsen, G.M., Ng, Y., Gram, L., 2013. Survival of bactericidal antibiotic treatment by a persister subpopulation of Listeria monocytogenes. Appl. Environ. Microbiol. 79, 7390–7397. https://doi.org/10.1128/AEM.02184-13.

Kushwaha, K., Muriana, P.M., 2009. Adherence characteristics of Listeria strains isolated from three ready-to-eat meat processing plants. J. Food Prot. 72, 2125–2131. https://doi.org/10.4315/0362-028x-72.10.2125.

Kussell, E., Kishony, R., Balaban, N.Q., Leibler, S., 2005. Bacterial persistence: a model of survival in changing environments. Genetics 169, 1807–1814. https://doi.org/10.1534/genetics.104.035352.

Latorre, A.A., Van Kessel, J.A., Karns, J.S., Zurakowski, M.J., Pradhan, A.K., Boor, K.J., Adolph, E., Sukhnanand, S., Schukken, Y.H., 2011. Increased in vitro adherence and on-farm persistence of predominant and persistent Listeria monocytogenes strains in the milking system. Appl. Environ. Microbiol. 77, 3676–3684. https://doi.org/10.1128/aem.02441-10.

Lewis, K., 2007. Persister cells, dormancy and infectious disease. Nat. Rev. Microbiol. 5, 48–56. https://doi.org/10.1038/nrmicro1557.

Liu, S., Brul, S., Zaat, S.A.J., 2020. Bacterial persister-cells and spores in the food chain: their potential inactivation by antimicrobial peptides (AMPs). Int. J. Mol. Sci. 21 https://doi.org/10.3390/ijms21238967.

Liu, X., Tang, R., Li, H., Wang, L., Wan, C., 2022. The physiological and ecological properties of bacterial persisters discovered from municipal sewage sludge and the potential risk. Environ. Res. 205, 112481 https://doi.org/10.1016/j.envres.2021.112481.

Manandhar, S., Singh, A., Varma, A., Pandey, S., Shrivastava, N., 2022. High level of persister frequency in clinical staphylococcal isolates. BMC Microbiol. 22, 109. https://doi.org/10.1186/s12866-022-02529-7.

Martins, P.M.M., Wood, T.K., de Souza, A.A., 2021. Persister cells form in the plant pathogen Xanthomonas citri subsp. citri under different stress conditions. Microorganisms 9. https://doi.org/10.3390/microorganisms9020384.

Möker, N., Dean, C.R., Tao, J., 2010. Pseudomonas aeruginosa increases formation of multidrug-tolerant persister cells in response to quorum-sensing signaling molecules. J. Bacteriol. 192, 1946–1955.

Narimisa, N., Sadeghi Kalani, B., Mohammadzadeh, R., Masjedian Jazi, F., 2020. Combination of antibiotics—nisin reduces the formation of persister cell in Listeria monocytogenes. Microb. Drug Resist. 27, 137–144. https://doi.org/10.1089/mdr.2020.0019.

Nguyen, D., Joshi-Datar, A., Lepine, F., Bauerle, E., Olakanmi, O., Beer, K., McKay, G., Siehnel, R., Schafhauser, J., Wang, Y., 2011. Active starvation responses mediate antibiotic tolerance in biofilms and nutrient-limited bacteria. Science 334, 982–986.

Pesci, E.C., Pearson, J.P., Seed, P.C., Iglewski, B.H., 1997. Regulation of las and rhl quorum sensing in Pseudomonas aeruginosa. J. Bacteriol. 179, 3127–3132. https://doi.org/10.1128/jb.179.10.3127-3132.1997.

Posfay-Barbe, K.M., Wald, E.R., 2009. Listeriosis. Semin. Fetal Neonatal Med. 14, 228–233. https://doi.org/10.1016/j.siny.2009.01.006.

Riedel, C.U., Monk, I.R., Casey, P.G., Waidmann, M.S., Gahan, C.G.M., Hill, C., 2009. AgrD-dependent quorum sensing affects biofilm formation, invasion, virulence and global gene expression profiles in Listeria monocytogenes. Mol. Microbiol. 71, 1177–1189. https://doi.org/10.1111/j.1365-2958.2008.06589.x.

Ross, J.D.C., Brittain, C., Cole, M., Dewsnap, C., Harding, J., Hepburn, T., Jackson, L., Keogh, M., Lawrence, T., Montgomery, A.A., Roberts, T.E., Sprange, K., Tan, W., Thandi, S., White, J., Wilson, J., Duley, L., 2019. Gentamicin compared with ceftriaxone for the treatment of gonorrhoea (G-ToG): a randomised non-inferiority trial. Lancet 393, 2511–2520. https://doi.org/10.1016/S0140-6736(18)32817-4.

Ryu, J.H., Beuchat, L.R., 2005. Biofilm formation by Escherichia coli O157:H7 on stainless steel: effect of exopolysaccharide and curli production on its resistance to chlorine. Appl. Environ. Microbiol. 71, 247–254. https://doi.org/10.1128/aem.71.1.247-254.2005.

Schenk, M.F., Zwart, M.P., Hwang, S., Ruelens, P., Severing, E., Krug, J., de Visser, J.A.G.M., 2022. Population size mediates the contribution of high-rate and large-benefit mutations to parallel evolution. Nat. Ecol. Evol. 6, 439–447. https://doi.org/10.1038/s41559-022-01669-3.

Schlech, W.F., 2000. Foodborne listeriosis. Clin. Infect. Dis. 31 (3), 770–775. https://doi.org/10.1086/314008.

Shang, C., Blatchley, E.R., 1999. Differentiation and quantification of free chlorine and inorganic chloramines in aqueous solution by MIMS. Environ. Sci. Technol. 33, 2218–2223.

Shen, C., Luo, Y., Nou, X., Bauchan, G., Zhou, B., Wang, Q., Millner, P., 2012. Enhanced inactivation of Salmonella and Pseudomonas biofilms on stainless steel by use of T-128, a fresh-produce washing aid, in chlorinated wash solutions. Appl. Environ. Microbiol. 78, 6789–6798. https://doi.org/10.1128/aem.01094-12.

Stepanyan, K., Wenseleers, T., Duéñez-Guzmán, E.A., Muratori, F., Van den Bergh, B., Verstraeten, N., De Meester, L., Verstrepen, K.J., Fauvart, M., Michiels, J., 2015.

Fitness trade-offs explain low levels of persister cells in the opportunistic pathogen Pseudomonas aeruginosa. Mol. Ecol. 24, 1572–1583.

Templeton, M.R., Oddy, F., Leung, W., Rogers, M., 2009. Chlorine and UV disinfection of ampicillin-resistant and trimethoprim-resistant Escherichia coli. Can. J. Civ. Eng. 36, 889–894. https://doi.org/10.1139/l09-040.

Thao, S., Brandl, M.T., Carter, M.Q., 2019. Enhanced formation of shiga toxin-producing Escherichia coli persister variants in environments relevant to leafy greens production. Food Microbiol. 84, 103241 https://doi.org/10.1016/j.fm.2019.103241.

Uesugi, A.R., Harris, L.J., 2006. Growth of Salmonella enteritidis phage type 30 in almond hull and shell slurries and survival in drying almond hulls. J. Food Prot. 69, 712–718. https://doi.org/10.4315/0362-028x-69.4.712.

Uzoechi, S.C., Abu-Lail, N.I., 2020. Variations in the morphology, mechanics and adhesion of persister and resister E. coli cells in response to ampicillin: AFM study. Antibiotics 9. https://doi.org/10.3390/antibiotics9050235.

Van den Bergh, B., Fauvart, M., Michiels, J., 2017. Formation, physiology, ecology, evolution and clinical importance of bacterial persisters. FEMS Microbiol. Rev. 41, 219–251. https://doi.org/10.1093/femsre/fux001.

Wales, A.D., Davies, R.H., 2015. Co-selection of resistance to antibiotics, biocides and heavy metals, and its relevance to foodborne pathogens. Antibiotics 4, 567–604. https://doi.org/10.3390/antibiotics4040567.

Wu, S., Yu, P.-L., Flint, S., 2017. Persister cell formation of Listeria monocytogenes in response to natural antimicrobial agent nisin. Food Control 77, 243–250. https://doi.org/10.1016/j.foodcont.2017.02.011.