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Influence of stressing conditions caused by organic acids and salts on tolerance of *Listeria monocytogenes* to *Origanum vulgare* L. and *Rosmarinus officinalis* L. essential oils and damage in bacterial physiological functions

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ABSTRACT

This study evaluated whether the pre-exposure (24, 48 and 72 h) to sublethal conditions caused by acetic acid (AA), lactic acid (LA), sodium chloride (NaCl) or potassium chloride (KCl) could induce increased cross-tolerance to the essential oils from *Origanum vulgare* L. (OVEO) and *Rosmarinus officinalis* L. (ROEO) in different *Listeria monocytogenes* strains. Damage to membrane integrity, membrane potential, enzymatic activity and efflux activity in *L. monocytogenes* cells pre-exposed (24 h) to AA or NaCl and further treated with OVEO or ROEO (8 and 24 h) were investigated using flow cytometry (FC). Results of minimum inhibitory concentration (MIC) modulation test showed that pre-exposure to sublethal conditions caused by organic acids or salts increased cross-tolerance only to ROEO, since MIC of ROEO increased up to 4.8-fold against pre-exposed cells. Otherwise, MIC of OVEO against these pre-exposed cells was up to ten-fold lower than that observed against not pre-exposed cells, indicating no increase in cross-tolerance. Bacterial survival assays showed that ROEO only decreased the counts over time of cells not pre-exposed to organic acids or salts, while OVEO decreased similarly or more the counts of pre-exposed cells compared to not pre-exposed cells. Results of FC analysis showed that all measured functions in *L. monocytogenes* cells pre-exposed to AA or NaCl and treated with OVEO or ROEO were affected, although with different intensities. These data indicate that exposure to sublethal conditions imposed by organic acids or salts could result in a phenotype of increased cross-tolerance to ROEO but not to OVEO in *L. monocytogenes*.

1. Introduction

Listeria monocytogenes is one of the most important pathogens affecting food safety, being the causative agent of listeriosis, a foodborne disease that occurs predominantly in pregnant women, the elderly and immunosuppressed people individuals and can lead to miscarriage and death (Shen et al., 2015, 2016). Regulatory agencies have established a "zero tolerance" policy for *L. monocytogenes* in foods (Brazil, 2001; USDA-FSIS, 2014). However, listeriosis outbreaks and frequent recalls of food products, contaminated with *L. monocytogenes* have been continuously reported (Shen et al., 2015; CDC, 2017).

This fact could be associated with the ability of *L. monocytogenes* to survive and grow in relatively wide ranges of temperature, pH and water activity, besides to its facultative anaerobic metabolism (Ferrentino et al., 2015). Previous studies have shown that the exposure of *L. monocytogenes* to sublethal stressing conditions imposed by food preservation treatments may induce a greater tolerance to higher levels of the same (direct-tolerance) or other stressing agents (cross-tolerance) (Omori et al., 2017; Al-Nabulsi et al., 2015). Increase of stress tolerance could confer to *L. monocytogenes* the ability to persist under conditions found during food processing, in addition to allow this pathogen to survive in the gastrointestinal tract of hosts (NicAogáin and O'Byrne, 2016; Paudyal and Karatzas, 2016).

Researchers and industry have searched for innovative and effective strategies to control *L. monocytogenes* in the food chain (Josewin et al., 2018; Espina et al., 2013). The use of essential oils (EOs) as *anti-L. monocytogenes* agents in foods has been considered a potential control measure (Pesavento et al., 2015; Gouveia et al., 2016). Among the EOs that have demonstrated potential antimicrobial activities, those obtained from *Origanum vulgare* L. (OVEO) and *Rosmarinus officinalis* L.

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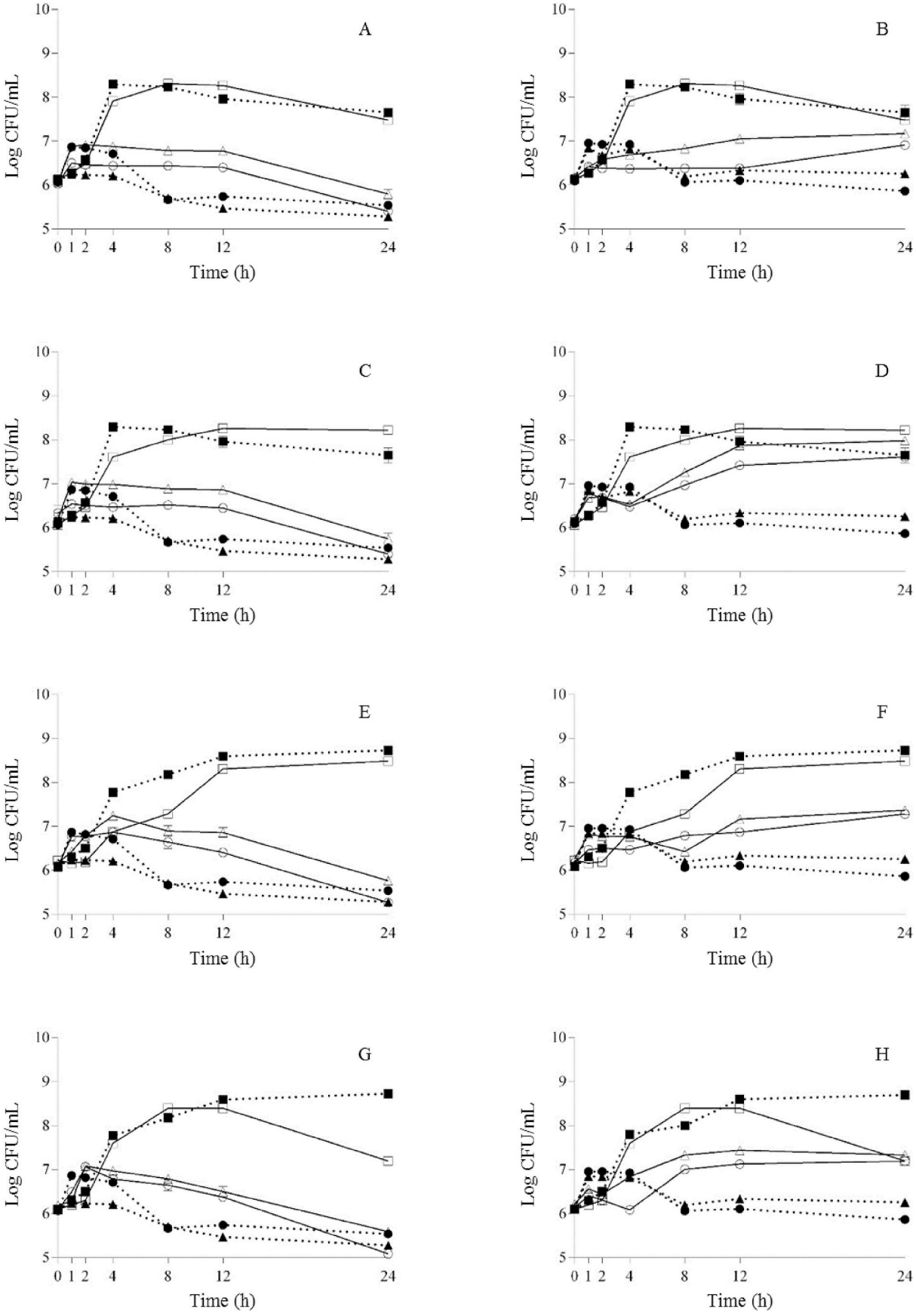

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Fig. 1. Viable counts of *Listeria monocytogenes* ATCC 7644 treated with 1/2 MIC (1.25 μL/mL) or 1/4 MIC (0.625 μL/mL) of *Origanum vulgare* L. essential oil (OVEO) (A, C, E and G) or 1/2 MIC (2.5 μL/mL) or 1/4 MIC (1.25 μL/mL) of *Rosmarinus officinalis* L. essential oil (ROEO) (B, D, F and H) after 24 h of pre-exposure (stress adaptation treatment) to NaCl (A and B), KCl (C and D), acetic acid (AA) (E and F) or lactic acid (LA) (G and H). Continuous lines: stress-adapted cells; dashed lines: non-adapted cells. (□■) Controls; (○●) 1/2 MIC of OVEO or ROEO; (Δ▲) 1/4 MIC of OVEO or ROEO; 1/2 MIC of NaCl: 45 mg/mL; 1/2 MIC of KCl: 60 mg/mL; 1/2 MIC of AA: 0.625 μL/mL; 1/2 MIC of LA: 1.25 μL/mL. Standard deviation was always ≤0.3 log CFU/mL.

(ROEO) have shown effective to inhibit *L. monocytogenes* (Azerêdo et al., 2011, 2012; Barbosa et al., 2016).

Previous studies have shown that the exposure of *L. monocytogenes* to sublethal concentrations of OVEO, ROEO and their related compounds carvacrol and 1,8-cineole, respectively, does not induce direct-tolerance to these EOs and cross-tolerance to NaCl, heat and low pH (Gomes Neto et al., 2012; Luz et al., 2012). However, the effects of the exposure of *L. monocytogenes* to sublethal stressing conditions caused by antimicrobial preservatives traditionally used in foods on the induction of increased cross-tolerance to OVEO and ROEO have not been investigated. The results of these studies could provide additional information on the antimicrobial efficacy of these EOs, which are beyond their action on bacterial cells not adapted to environmental conditions well known to induce increased tolerance responses in foodborne pathogens.

Despite the available literature considering the survival of *L. monocytogenes* when challenged with chemical preservatives and physical procedures used to preserve foods, little is known about the physiological state of these cells. Flow cytometry (FC) has been considered useful technique to investigate the effects of stressing conditions or inactivation treatments against foodborne pathogens (de Sousa Guedes et al., 2017). FC allows the use of selected fluorochromes to monitor physiological functions in cells, giving reliable and rapid information on the heterogeneity of responses in different bacterial subpopulations following exposure to one or more antimicrobial treatments (Ferrario and Guerrero, 2017; Arioli et al., 2019). The use of traditional plate counting and FC, as complementary analytical methods, should provide a deeper understanding of the true efficacy of antimicrobial treatments on bacterial cell survival (Arioli et al., 2019).

This study evaluated whether the pre-exposure of three different strains of *L. monocytogenes* to stressing conditions imposed by sublethal concentrations of the food preservatives acetic acid (AA), lactic acid (LA), sodium chloride (NaCl) and potassium chloride (KCl) for different time periods could induce increased cross-tolerance to OVEO or ROEO. Damage to different physiological functions caused by the sublethal stressing conditions imposed by AA and NaCl as well as by further treatment with OVEO or ROEO were also investigated in *L. monocytogenes* cells by FC analysis.

2. Material and methods

2.1. Materials

OVEO (batch ORETU679; density at 20 °C: 0.90; refractive index at 20 °C: 1.49) and ROEO (batch ROSTUN04; density at 20 °C: 0.94; refractive index at 20 °C: 1.51) were purchased from Laszlo Aromaterapia Ind. Com. Ltda. (Minas Gerais, Brazil). This supplier extracts EOs on an industrial scale by steam distillation. Most prevalent components in tested OVEO were thymol (69.3%), followed by *p*-cymene (13.1%) and γ-terpinene (6.01%); the most prevalent components in tested ROEO were eucalyptol (1,8-cineole) (35.75%), followed by camphor (28.7%) and limonene (24.88%) (Barbosa et al., 2016). Stock emulsions of OVEO or ROEO (48, 40 and 32 μL/mL) were prepared in brain-heart infusion (BHI) broth (HiMedia, Mumbai, India) using bacteriological agar (1.5 g/L) as a stabilizing agent (de Souza et al., 2010). AA, LA, NaCl and KCl were obtained from Labsynth (São Paulo, Brazil). Stock solutions of organic acids (48, 40 and 32 μL/mL) and salts (300, 280, 260, 240, 220, 200 and 180 mg/mL) were prepared in sterile BHI broth as previously described (Tavares et al., 2015).

Standard typed strains of *Listeria monocytogenes* (ATCC 7644, ATCC, 19112 and ATCC, 19117) kindly supplied by the National Institute of Health Quality Control – Oswaldo Cruz Foundation (Rio de Janeiro, Brazil) were used as test microorganisms. An inoculum of each test strain was obtained by preparing suspensions in sterile saline solution (NaCl 0.85% w/v) from overnight cultures grown in BHI agar (HiMedia, Mumbai, India) at 30 °C. Each strain was grown in BHI broth at 30 °C for 18 h (late exponential growth phase), harvested by centrifugation (4500 rpm, 15 min, 4 °C), washed twice and resuspended in sterile saline solution to obtain standard cell suspensions with an optical density reading at 625 nm (OD_{625}) of 0.08–0.1, resulting in cell counts of 7–8 log colony forming units per milliliter - CFU/mL (Barbosa et al., 2016; McMahon et al., 2008). Each target strain was tested alone as a single inoculum.

2.2. Determination of the minimum inhibitory concentration (MIC)

MIC of OVEO, ROEO, AA, LA, NaCl and KCl against the test strains was determined using a standard microdilution technique (CLSI, 2015), with modification in inoculum size. MIC of OVEO, ROEO, AA, LA, NaCl and KCl against the test strains was determined using a standard microdilution technique (CLSI, 2015), with modification in inoculum size. Initially, 200 μL-aliquots of the different stock emulsions/solutions of the test EO, organic acid or salt were dispensed into wells (first line) of a 96-well microplate. Then, 100 μL of the total volume (200 μL) contained in the wells of the first line were transferred to the following wells and homogenized. Through geometric dilutions of the reason two, the concentrations of the tested EOs, organic acids or salts in the wells in this step were in the range of 24–0.125 μL/mL for OVEO and ROEO; 24–0.125 μL/mL for AA and LA and 150–0.7 mg/mL for NaCl and KCl. Subsequently, 100 μL of a suspension (7–8 log CFU/mL) of the test bacterial strain was added to each well. The final concentrations in the wells were in the range of 12–0.06 μL/mL for OVEO and ROEO; 12–0.06 μL/mL for AA and LA; and 75–0.35 mg/mL for NaCl and KCl. The microplate was wrapped with cling film and statically incubated at 30 °C for 24 h. Each microplate included a set of positive (BHI broth + test strain inoculum) and negative controls (non-inoculated BHI broth). MIC was considered the lowest concentration of each tested antimicrobial able to cause visual growth inhibition of the target strain.

2.3. Assays for evaluating the induction of increased direct-tolerance to organic acids and salts: MIC modulation

The induction of direct-tolerance was carried out by exposing the test strains to sublethal concentrations of AA, LA, NaCl or KCl for different time periods, followed by determination of MIC values for the same agent. For this, 2 mL of the bacterial suspension was inoculated into 18 mL of BHI broth containing a sufficient volume of the stressing agent to result in a final concentration similar to the 1/2, 1/4 or 1/8 MIC and incubated statically at 30 °C for 72 h. Every 24 h, an aliquot of each system was removed, standardized to present 7–8 Log CFU/mL and used as the inoculum for MIC determination using the procedure cited in section 2.2. Cells of each strain not pre-exposed to AA, LA, NaCl or KCl were similarly tested as controls. Occurrence of direct-tolerance induction was evaluated by comparing the MIC values observed against the cells pre-exposed to an organic acid or salt with those found against the not pre-exposed cells (control) (Tavares et al., 2015).

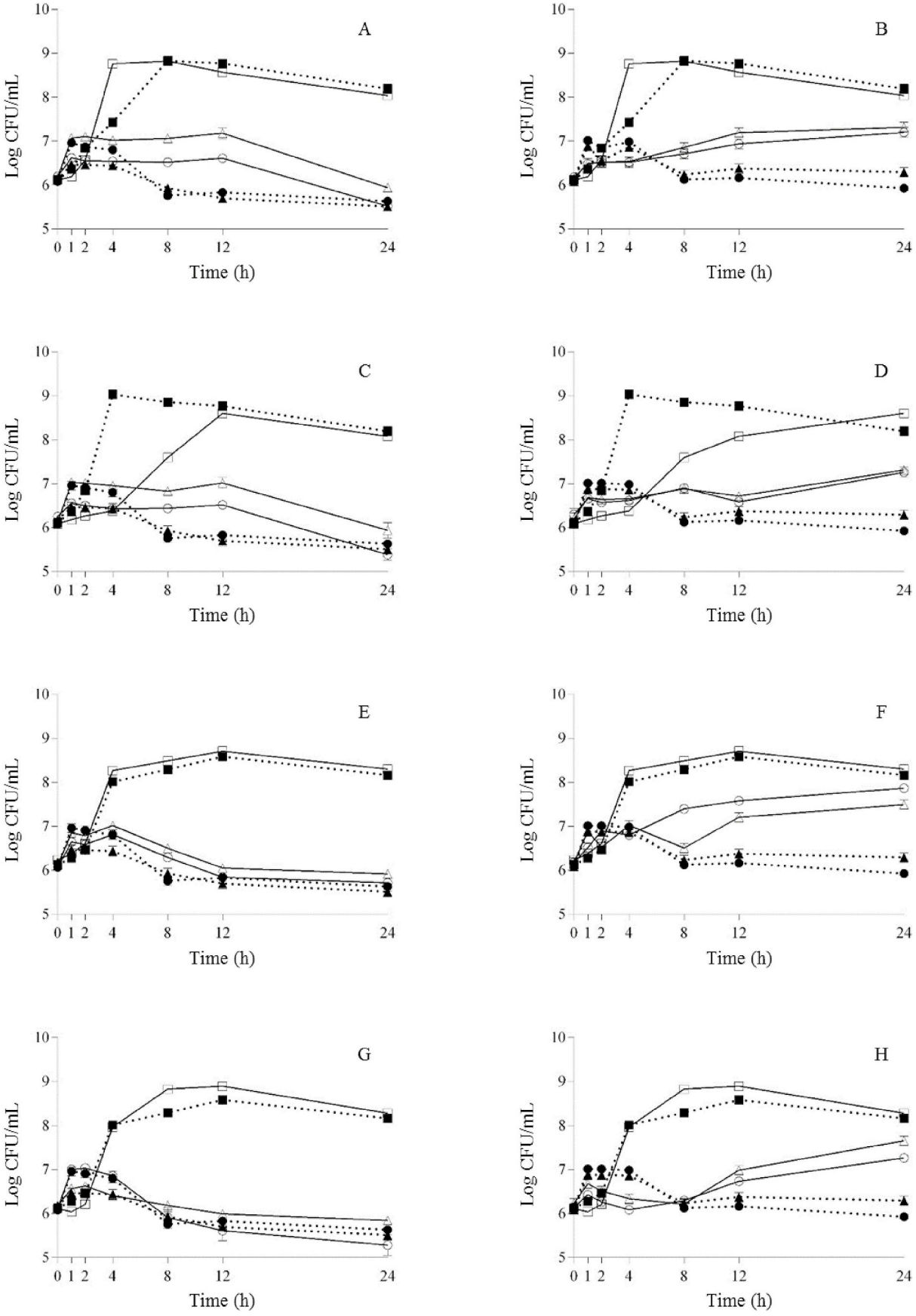

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Fig. 2. Viable counts of *Listeria monocytogenes* ATCC 19112 treated with 1/2 MIC (1.25 μL/mL) or 1/4 MIC (0.625 μL/mL) of *Origanum vulgare* L. essential oil (OVEO) (A, C, E and G) and 1/2 MIC (2.5 μL/mL) or 1/4 MIC (1.25 μL/mL) of *Rosmarinus officinalis* L. essential oil (ROEO) (B, D, F and H) after 24 h of pre-exposure (stress adaptation treatment) to NaCl (A and B), KCl (C and D), acetic acid (AA) (E and F) or lactic acid (LA) (G and H). Continuous lines: stress-adapted cells; dashed lines: non-adapted cells. (□■) Controls; (○●) 1/2 MIC of OVEO or ROEO; (Δ▲) 1/4 MIC of OVEO or ROEO; 1/2 MIC of NaCl: 45 mg/mL; 1/2 MIC of KCl: 60 mg/mL; 1/2 MIC of AA: 0.625 μL/mL; 1/2 MIC of LA: 1.25 μL/mL. Standard deviation was always ≤0.3 log CFU/mL.

2.4. Assays for evaluating the induction of increased cross-tolerance to ROEO and OVEO after pre-exposure to organic acids and salts: MIC modulation

The induction of cross-tolerance was performed by exposing the test strains to sublethal concentrations of AA, LA, NaCl or KCl for different time periods, followed by determination of the MIC values for OVEO and ROEO. For this, 2 mL of the bacterial suspension was inoculated into 18 mL of BHI broth containing a sufficient volume of AA, LA, NaCl or KCl to result in a final concentration similar to the 1/2, 1/4 or 1/8 MIC and statically incubated at 30 °C for 72 h. Every 24 h, an aliquot of each system was removed, standardized to present 7–8 log CFU/mL and used as the inoculum for MIC determination using the procedure cited in section 2.2. Cells of each strain not pre-exposed to sublethal concentrations of AA, LA, NaCl or KCl were similarly tested as controls. Induction of cross-tolerance was evaluated by comparing the MIC values of OVEO and ROEO observed against the cells pre-exposed to an organic acid or salt with those found against the not pre-exposed cells (control) (Tavares et al., 2015).

2.5. Assays for evaluating the induction of increased cross-tolerance to ROEO and OVEO after pre-exposure to organic acids and salts: bacterial survival curves

The influence of OVEO or ROEO on bacterial survival (viable counts) was determined by pre-exposing the test strains to 1/2 MIC of AA, LA, NaCl or KCl for 24, 48 and 72 h, followed by a treatment with 1/2 MIC or 1/4 MIC of OVEO or ROEO during 24 h. None of the assayed concentrations of AA, LA, NaCl and KCl used in the pre-exposure step, nor those of OVEO or ROEO used in the survival assays, were capable of causing a ≥1.5 log reduction in the viable counts of the test strains, thus characterizing a sublethal effect (data not shown) (LaPlante, 2007).

Systems were prepared by the addition of 2 mL of the bacterial inoculum into 18 mL of BHI broth containing 1/2 MIC of AA, LA, NaCl or KCl followed by incubation at 30 °C for 72 h. After 24, 48 and 72 h of pre-exposure to the stressing agent, an aliquot was removed, standardized to preset 7–8 Log CFU/mL and inoculated into fresh BHI broth containing 1/2 MIC or 1/4 MIC of OVEO or ROEO (final viable counts of 6–7 log CFU/mL). Thereafter, the viable cell count procedure was performed at different time points (i.e., 0 – just after homogenization, and after 1, 2, 4, 8, 12, and 24 h of incubation at 30 °C). For this, a 100 μL aliquot of each system was serially diluted in sterile saline solution (0.85% NaCl, w/v), and 20 μL aliquots of each dilution were subsequently inoculated onto BHI agar using the microdrop technique (Herigstad et al., 2001). The plates were incubated for 24 h at 30 °C and the results were expressed as log CFU/mL. Systems containing BHI broth without AA, LA, NaCl or KCl during the pre-exposure period were similarly tested as controls. To determine whether cross-tolerance was induced, the viable cell counts over time of strains subjected to the pre-exposure to an acid or salt were compared with the counts of the same strain not pre-exposed when both groups were inoculated into growth media with 1/2 or 1/4 MIC of OVEO or ROEO.

2.6. Assays for assessing the damage to physiological functions in bacterial cells

FC analysis was used to investigate the effects of the pre-exposure to sublethal amounts of AA or NaCl and further treatment with OVEO or ROEO on different physiological functions in *L. monocytogenes* cells as possible responses to the imposed stressing conditions. *L. monocytogenes* ATCC 7644 was selected as the test organism because it was the only tested strain able to develop direct tolerance to AA, LA, NaCl and KCl; AA and NaCl were selected to represent a stressing agent of each examined group, since AA was the only organic acid able to induce direct tolerance in all test strains and NaCl is the most common salt used as a food preservative.

L. monocytogenes ATCC 7644 was pre-exposed to 1/2 MIC of AA or NaCl for 24 h and subsequently treated with 1/2 MIC of OVEO or ROEO for 8 or 24 h at 30 °C. Before each analysis, the cells were centrifuged (7000 rpm × 15 min, 4 °C), washed twice and resuspended in PBS (pH 7.4) and stained with the following fluorochromes: propidium iodide (PI, Sigma-Aldrich, St. Louis, MO, USA) for measuring membrane integrity; bis-1,3-dibutylbarbituric acid (BOX, Molecular Probes, Invitrogen, part of Life Technologies, Eugene, OR, USA) for measuring membrane potential; ethidium bromide (EB, Sigma-Aldrich, St. Louis, MO, USA) for measuring efflux activity; 5-cyano-2,3-ditolyl tetrazolium chloride (CTC, Polysciences, Warrington, PA, USA) for measuring respiratory activity; fluorescein diacetate (FDA, ThermoFisher Scientific, Molecular Probes, F1303) for measuring enzymatic activity; and 5-cyano-2,3-di- (*p*-tolyl) tetrazolium chloride for measuring respiratory activity. The cell staining procedures were performed as described elsewhere (Silva et al., 2011; de Sousa Guedes et al., 2017). Untreated and heat-treated cells (85 °C, 30 min) were used as controls to validate the staining procedures.

FC measurements were performed using a BD Accuri C6 flow cytometer (BD Accuri C6, New Jersey, USA) with 488-nm excitation from a blue solid-state laser. Green fluorescence was collected in the FL1 channel (533 nm ± 30 nm) and red fluorescence in the FL3 channel (> 670 nm). Scatter and fluorescence signals of individual cells passing through the laser zone were collected as logarithmic signals. Fluorescence signal (pulse area measurements) was collected by FL1 (BOX and FDA) and FL3 (PI, EB and CTC) bandpass filters. Threshold level was adjusted for FSC (12,000) to eliminate noise or particles (cellular debris) that were much smaller than intact cells. Bacterial cells were gated per the FSC/SSC parameters. Sample acquisition was performed at the low flow rate setting (12 mL/min) and a total of 10,000 events were acquired for each sample. Data analysis was performed using BD Accuri C6 Software (de Sousa Guedes et al., 2017).

2.7. Reproducibility and statistics

Assays of MIC determination and bacterial survival curves were performed in triplicate in three independent experiments. MIC values are presented as modal values because the results were the same in all repetitions. Results of the bacterial survival curves are expressed as an average (± standard deviation) of the assays results and statistical analysis was performed to determine significant differences ($p \leq 0.05$) using ANOVA, followed by a post hoc Tukey test. The computational software Sigma Stat 3.5 (Jandel Scientific Software, San Jose, California) was used to perform the statistical analysis. FC analyses were performed in duplicate in two independent experiments with consistent results.

3. Results

3.1. MIC determination

MIC values of ROEO (5 μL/mL) and LA (2.5 μL/mL) were two-fold

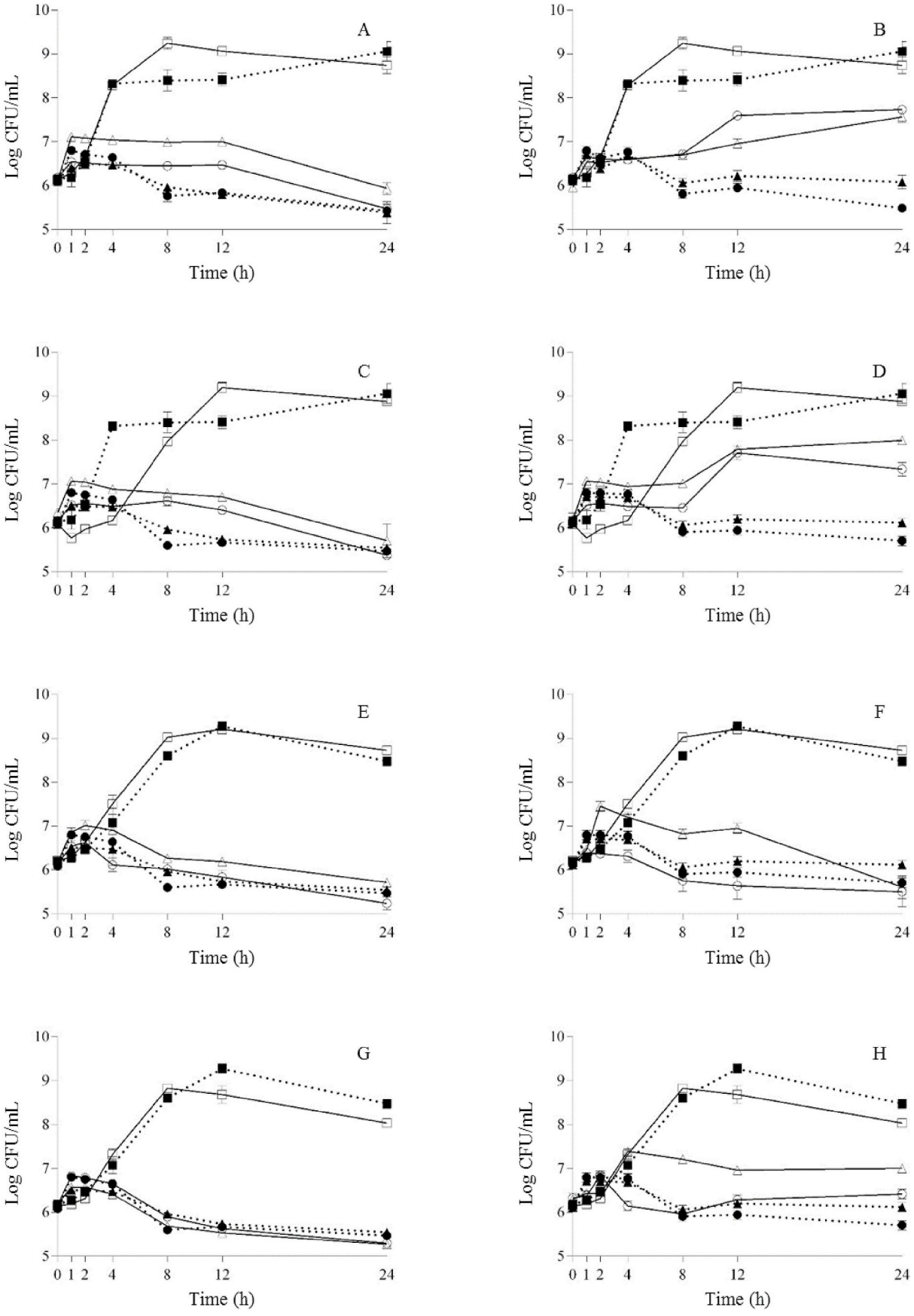

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Fig. 3. Viable counts of *Listeria monocytogenes* ATCC 19117 treated with 1/2 MIC (1.25 μL/mL) or 1/4 MIC (0.625 μL/mL) of *Origanum vulgare* L. essential oil (OVEO) (A, C, E and G) and 1/2 MIC (2.5 μL/mL) or 1/4 MIC (1.25 μL/mL) of *Rosmarinus officinalis* L. essential oil (ROEO) (B, D, F and H) after 24 h of pre-exposure (stress adaptation treatment) to NaCl (A and B), KCl (C and D), acetic acid (AA) (E and F) or lactic acid (LA) (G and H). Continuous lines: stress-adapted cells; dashed lines: non-adapted cells. (□■) Controls; (○●) 1/2 MIC of OVEO or ROEO; (Δ▲) 1/4 MIC of OVEO or ROEO; 1/2 MIC of NaCl: 45 mg/mL; 1/2 MIC of KCl: 60 mg/mL; 1/2 MIC of AA: 0.625 μL/mL; 1/2 MIC of LA: 1.25 μL/mL. Standard deviation was always ≤0.3 log CFU/mL.

higher than those of OVEO (2.5 μL/mL) and AA (1.25 μL/mL), respectively. MIC of KCl (120 mg/mL) was 1.3-fold higher than that of NaCl (90 mg/mL). There was no variation among the MIC values of ROEO, OVEO, AA, LA, NaCl or KCl against the three tested *L. monocytogenes* strains.

3.2. Induction of increased direct-tolerance to organic acids and salts: MIC modulation assays

Pre-exposure of the three *L. monocytogenes* strains to sublethal concentrations of AA (i.e., 1/8, 1/4 or 1/2 MIC) for 24, 48 or 72 h caused an increase in the original MIC values (observed against the not pre-exposed control cells) of this agent against all tested strains (Supplementary data S1). AA increased the original MIC values by 1.6 and 1.2-fold (to a MIC of 2 and 1.5 μL/mL) against *L. monocytogenes* ATCC 7644 and ATCC 19117 pre-exposed to this same agent, respectively, was observed after 24 h of pre-exposure and remained up to 72 h of pre-exposure. Original MIC value of AA against *L. monocytogenes* ATCC 19112 increased 1.2-fold (to a MIC of 1.5 μL/mL) only after 72 h of pre-exposure. An increase in original MIC value of LA was verified only against *L. monocytogenes* ATCC 7644 (1.2-fold) after 72 h of pre-exposure. Original MIC values of LA against *L. monocytogenes* ATCC 19112 and ATCC 19117 showed a decrease of 1.25 and 1.67-fold (to a MIC of 2 and 1.5 μL/mL), respectively, after pre-exposure to the sublethal concentrations (Supplementary data S1).

Original MIC of NaCl increased 1.11-fold (to a MIC of 100 mg/mL) against all the target strains, while the original MIC of KCl increased 1.17-fold (to a MIC of 140 mg/mL) against *L. monocytogenes* ATCC 7644 and 1.08-fold (to a MIC of 130 mg/mL) against *L. monocytogenes* ATCC 19112 and ATCC 19117 at the end of the 72 h pre-exposure step (Supplementary data S2).

3.3. Induction of increased cross-tolerance to ROEO and OVEO in cells pre-exposed to organic acids and salts: MIC modulation assays

MIC values of OVEO ranged from 0.25 to 1 μL/mL and from 0.25 to 0.75 μL/mL against the *L. monocytogenes* strains pre-exposed to sublethal concentrations of AA and LA (i.e., 1/2, 1/4 or 1/2 MIC), respectively, for 72 h. The greatest decrease in original MIC values of OVEO (i.e., a 10-fold reduction) was observed against *L. monocytogenes* ATCC 19117 after 48 and 72 h of pre-exposure to sublethal amounts of either AA or LA (Supplementary data S1). Similarly, the pre-exposure to sublethal concentrations of NaCl or KCl for 72 h caused reductions in original MIC values of OVEO in the range of 2 (1.25 μL/mL) to 5-fold (0.5 μL/mL). MIC of OVEO ranged from 1 to 0.63 μL/mL and from 1.5 to 0.5 μL/mL when the cells were pre-exposed to NaCl and KCl, respectively (Supplementary data S2).

The original MIC values of ROEO against all test strains increased following the pre-exposure to the sublethal amounts of AA, LA, NaCl or KCl. MIC of ROEO against *L. monocytogenes* ATCC 19112 pre-exposed to AA ranged from 6 to 10 μL/mL (increase of 1.2–2-fold); MIC of ROEO against *L. monocytogenes* ATCC 7644 and ATCC 19117 was > 24 μL/mL (an increase of > 4.8-fold). MIC of ROEO against the tested strains pre-exposed to LA varied from 8 to 12 μL/mL (an increase of 1.6–2-fold). Similar results were obtained against cells pre-exposed to either NaCl or KCl. Pre-exposure to NaCl or KCl led to MIC values of ROEO ranging from 6 to 12 μL/mL (an increase of 1.2–2.4-fold) and 6–10 μL/mL (an increase of 1.2–2.4-fold), respectively (Supplementary data S1 and S2).

Overall, the MIC values obtained for OVEO decreased as the duration of the pre-exposure to the sublethal stressing conditions increased. However, the MIC values obtained for ROEO increased as the duration of the pre-exposure treatment increased. The concentration of 1/2 MIC of AA, LA, NaCl and KCl was selected for use in the pre-exposure step used in bacterial survival curve assays because 1/2, 1/4 and 1/8 MIC of these agents exerted the same influence on the modulation of ROEO and OVEO MIC values.

3.4. Induction of increased cross-tolerance to ROEO and OVEO in cells pre-exposed to organic acids and salts: bacterial survival curve assays

During exposure to a sublethal concentration of OVEO (i.e., 1/2 MIC or 1/4 MIC), cells of tested *L. monocytogenes* strains pre-exposed to NaCl or KCl (Fig. 1A and C; 2A and 2C; 3A and 3C) showed an initial increase in counts up to 1 h of exposure, followed for presenting steady counts up to 12 h of exposure and a reduction in counts (ranging from 0.19 to 0.93 log CFU/mL) at the end of the measured exposure period (i.e., 24 h). In contrast to salt pre-exposed cells, cells pre-exposed to AA or LA had a reduction in their initial counts already after 4 h of treatment with the sublethal concentration of OVEO, reaching reduction levels varying from 0.2 to 1 log CFU/mL after 24 h. It is noteworthy that after 8 and 12 h, cells pre-exposed to either of the stressing agents and further treated with a sublethal concentration of OVEO showed higher counts ($p \leq 0.05$) than those not pre-exposed, with the exception of *L. monocytogenes* ATCC 19112 and ATCC 19117 pre-exposed to AA and LA (Fig. 2E and G; 3E and 3G); however, cells pre-exposed or not pre-exposed to either of the tested organic acid or salt and further treated with a sublethal concentration of OVEO showed similar counts ($p > 0.05$) after 24 h. Nevertheless, in most cases, 1/2 MIC of OVEO caused greater decreases ($p \leq 0.05$) in bacterial counts than 1/4 MIC of OVEO.

Sublethal concentrations of ROEO, in general, were not successful in inhibiting the survival of *L. monocytogenes* cells pre-exposed to AA, LA, NaCl or KCl. Cells pre-exposed to NaCl or KCl showed an increase ($p \leq 0.05$) in counts (0.75–1.8 log CFU/mL) when further treated with 1/2 MIC or 1/4 MIC of ROEO. Cells pre-exposed to AA or LA also showed an increase in counts when exposed to 1/2 MIC or 1/4 MIC of ROEO. For L. *monocytogenes* ATCC 7644 pre-exposed to AA or LA, the increase was of 1.1 log CFU/mL after 24 h of exposure to the tested concentrations of ROEO. Similarly, *L. monocytogenes* ATCC 19112 pre-exposed to AA or LA showed an increase in counts varying from 0.33 to 0.72 or 1.12 to 1.42 log CFU/mL, respectively, after a 24 h-treatment with 1/2 or 1/4 MIC of ROEO. For L. *monocytogenes* ATCC 19117 pre-exposed to LA, the increase in counts ranged from 0.1 to 0.7 log CFU/mL. In contrast, when this strain was pre-exposed to AA, a reduction of 0.57–0.64 log CFU/mL was observed after a 24 h-treatment with 1/2 or 1/4 MIC of ROEO. Overall, cells pre-exposed to either of the stressing agents and further treated with sublethal concentrations of ROEO showed higher counts ($p \leq 0.05$) than the not pre-exposed cells.

Cells not pre-exposed or only pre-exposed to the tested sublethal concentrations of organic acids or salts showed similar counts ($p > 0.05$) over the assessed time period, with an increase of up to 2.91 log CFU/mL in their initial counts after 24 h. Cells not pre-exposed to organic acids or salts and further treated with sublethal concentrations of OVEO or ROEO showed reductions in their initial counts already after 4 h, which were in the range of 0.36–0.83 and 0.23 to 0.65 log CFU/mL, respectively.

The results of the survival curves of the tested *L. monocytogenes* strains pre-exposed to AA, LA, NaCl or KCl for 48 or 72 h and further treated with OVEO and ROEO during 24 h are shown as supplementary

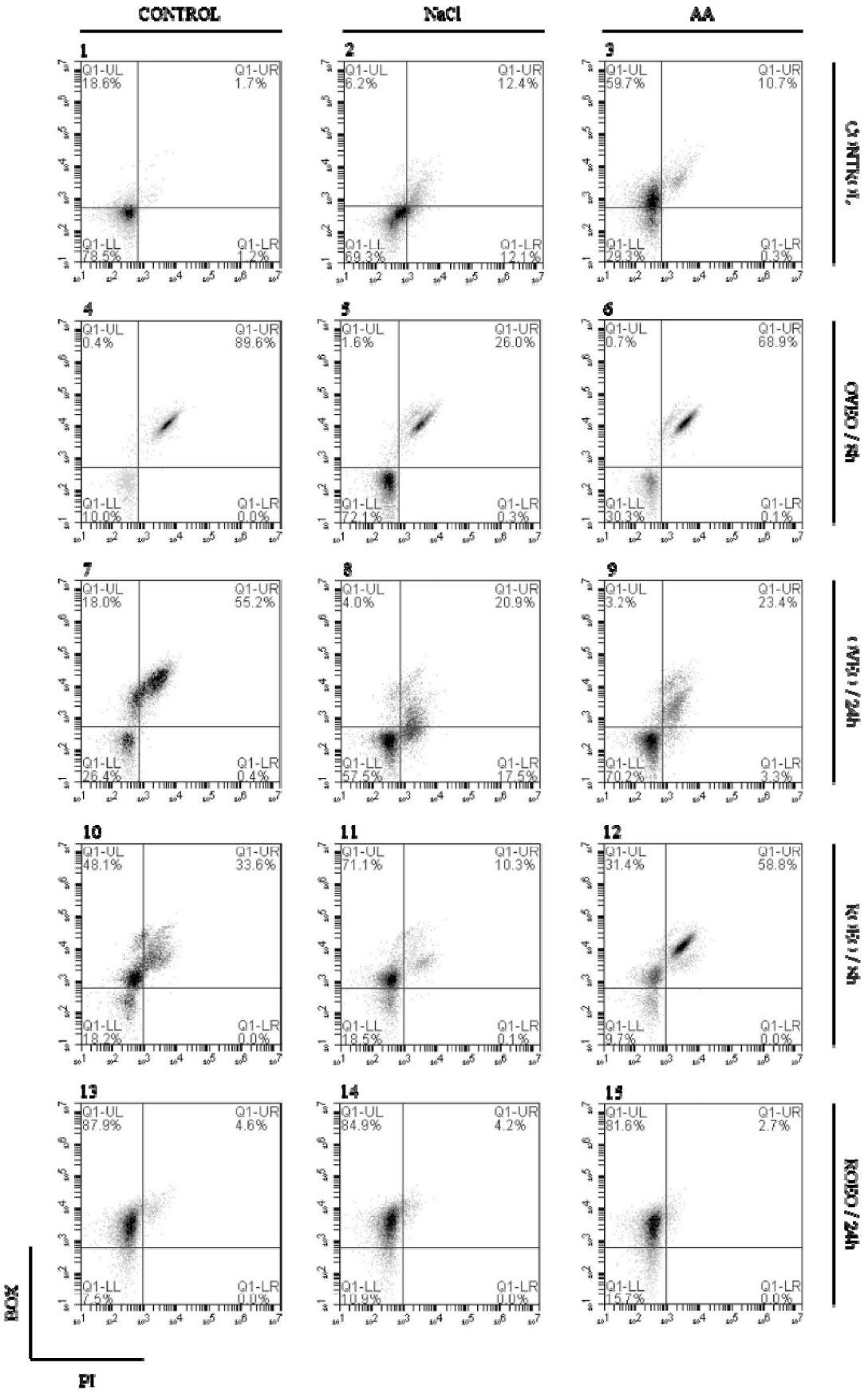

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Fig. 4. Fluorescence density plots of *Listeria monocytogenes* ATCC 7644 stained with BOX and PI after 24 h of pre-exposure to 1/2 MIC of NaCl (45 mg/mL) or acetic acid (AA, 0.625 μL/mL) and further treatment with *Origanum vulgare* L. essential oil (OVEO, 1.25 μL/mL) or *Rosmarinus officinalis* L. essential oil (ROEO, 2.5 μL/mL) for 8 or 24 h. The vertical axis indicates the fluorescence intensity of BOX; the horizontal axis indicates the fluorescence intensity of PI. The four edges indicate UL, cells with depolarized and undamaged cell membrane; LL, cells with polarized and undamaged cell membrane; UR, cells with depolarized and damaged cell membrane; and LR, cells with polarized and damaged cell membrane. The percentages of the cell population within each gate are displayed in the four corners of each plot.

data (S3–S8). The behavior of cells not pre-exposed or pre-exposed to 1/2 MIC of AA, LA, NaCl or KCl for 48 or 72 h and further treated with OVEO or ROEO was similar overall to that presented by cells pre-exposed to these stressing agents for 24 h. These findings indicate that the duration of the pre-exposure step used in this study did not influence the sensitivity/tolerance response of tested *L. monocytogenes* strains to OVEO and ROEO.

3.5. Damage to bacterial physiological functions

L. monocytogenes ATCC 7644 was pre-exposed to 1/2 MIC of AA or NaCl for 24 h and then treated with 1/2 MIC of OVEO or ROEO for 8 or 24 h. After each of these steps, five fluorescent probes were used (PI, BOX, FDA, EB and CTC) to measure different physiological functions in *L. monocytogenes* cells using FC. Membrane potential and integrity were evaluated by double staining cells with BOX and PI (Fig. 4). The percentages of different cell subpopulations are presented in the four edges of each plot. Control cells not pre-exposed to AA or NaCl or to OVEO or ROEO (plot 1) maintained largely polarized and undamaged membranes (78.5%). *L. monocytogenes* suspensions only exposed to a sublethal concentration of NaCl (plot 2) or AA (plot 3) showed 69.3 and 29.3% of cells with polarized and undamaged membranes, respectively; however, exposure to the sublethal concentration of AA was capable of depolarizing the membrane of 59.7% of the cells, but only 10.7% presented both depolarized and damaged cell membranes. Percentages of cells not pre-exposed to NaCl or AA and treated with OVEO (plots 4 and 7) or ROEO (plots 10 and 13) for 8 and 12 h having depolarized and damaged membranes were 89.6 and 55.2% and 33.6 and 4.6%, respectively. These values were 26 and 20.9% and 10.3 and 4.2% for cells pre-exposed to NaCl and treated with OVEO (plots 5 and 8) and ROEO (plots 11 and 14) for 8 and 24 h, respectively. Percentages of cells pre-exposed to AA and treated with OVEO (plots 6 and 9) and ROEO (plots 12 and 15) for 8 and 24 h having depolarized and damaged membranes were 68.9 and 23.4% and 58.8 and 2.7%, respectively.

Cells of L. *monocytogenes* were double stained with FDA and PI to evaluate enzymatic activity and membrane integrity (Fig. 5). Cells of L. *monocytogenes* pre-exposed or not pre-exposed to AA or NaCl and treated with OVEO for 8 h (plots 4 to 6) showed a subpopulation with damaged membranes and not detected enzymatic activity ranging from 51.3 to 76.4% of the cells. However, after a 24 h-treatment with OVEO (plots 7 to 9), the most prevalent subpopulations were formed by cells with undamaged membrane and undetected enzymatic activity (43.3–96.6%); cells pre-exposed to AA or NaCl and treated with OVEO exhibited the highest subpopulations (96.6 and 92.6%, respectively) of cells with undamaged membrane and undetected enzymatic activity (plots 8 and 9). Treatment of cells with ROEO (plots 10 to 12) for 8 h led to the formation of distinct subpopulations consisting of i) cells with detected enzymatic activity (UL and UR) and damaged (7.3–20.6%) or undamaged membrane (33.3–51%), and ii) cells with undetected enzymatic activity (LL and LR) and damaged (11.1–40%) or undamaged membrane (17.3–23.8%). Pre-exposure to AA followed by treatment with ROEO for 8 h (plot 12) led to a high percentage of cells with undetected enzymatic activity and damaged membrane (40%). After a 24 h-treatment with ROEO (plots 13 to 15), the most prevalent subpopulation consisted of cells with detected enzymatic activity and undamaged membrane (76.5–85.8%).

Efflux activity was evaluated using ethidium bromide (EB) and the results are shown in Fig. 6. EB + cells are those with a compromised efflux pump activity. Exposure to a sublethal concentrations of NaCl or AA caused damage to cell efflux pumps. Percentage of EB + cells among the control cells (plot 1) increased from 3.3 to 18.6 and 52.3% when these cells were treated only with a sublethal concentration of NaCl or AA (plots 2 and 3), respectively. Control cells treated with only OVEO or ROEO for 8 h exhibited an increase in EB + subpopulation from 3.3 to 73.5 and 38% (plots 4 and 10), respectively. Cells pre-exposed to NaCl (plot 5) had an increase of 0.8% in damaged population when treated with OVEO, whereas for cells pre-exposed to AA (plot 6) there was a decrease of 24.1% in EB + subpopulation. Similarly, cells pre-exposed to AA and NaCl and treated with ROEO showed a reduction in EB + subpopulation of 3.2 and 29.1%, respectively (plots 11 and 12). Percentage of EB + cells was increased after 24 h of treatment with OVEO or ROEO, indicating a greater disturbance of efflux pump activity (32.2–99.1%) (plots 7 to 9 and 13 to 15); the highest percentage of EB + cells was observed for cells treated with ROEO (plots 13 to 15; 86.2–99.1%).

Respiratory activity of cells was measured by staining with CTC (Fig. 7). Cells with active and inactive respiratory function were referred to as CTC + and CTC–, respectively. Exposure to only a sublethal concentration of NaCl or AA resulted in CTC– subpopulations of 84.9 and 76.4% (plots 2 and 3), representing an increase of 64.4 and 55.9%, respectively, with respect to the control group (plot 1) (20.5%) and indicating that the respiratory activity of the cells was compromised by these agents. The results obtained for cells pre-exposed to AA or NaCl and treated with OVEO (plots 5 and 6) or ROEO (plots 11 and 12) for 8 h displayed a similar response, since the percentage of CTC– subpopulation ranged from 96.2 (AA) to 98.4% (NaCl) for cells treated with OVEO and was 98.4% for cells treated with ROEO. For the control group, the percentages of CTC– cells were 80.8 and 96.8% following treatment with OVEO or ROEO (plots 4 and 10), respectively. CTC + subpopulation increased after 24 h of treatment with OVEO or ROEO (plots 7 to 9 and 13 to 15, respectively). This increase was more evident for the control group (plot 7), in which the percentage increased from 14.1 to 38.3% and from 1.6 to 40.6% for OVEO- and ROEO-treated cells, respectively. For cells pre-exposed to NaCl and treated with OVEO or ROEO (plots 8 and 14), this increase was 6.9 and 7%, respectively; for cells pre-exposed to AA, an increase of 8.5% was observed only for those treated with ROEO (plot 15), while a 0.7% reduction (plot 9) was observed for those treated with OVEO.

4. Discussion

The published literature has ordinarily reported the increase of direct-tolerance in *L. monocytogenes* cells challenged with sublethal stressing conditions imposed by traditional antimicrobials and/or physical procedures used to preserve foods (Omori et al., 2017; Al-Nabulsi et al., 2015). This behavior was confirmed in this study when all the three tested *L. monocytogenes* strains were able to increase the tolerance to AA, NaCl and KCl, and one strain was able to increase the tolerance to LA following exposure to sublethal amounts of the same antimicrobial compound.

The results obtained in assays for evaluation of increases in cross-tolerance to OVEO and ROEO by MIC modulation showed that the three

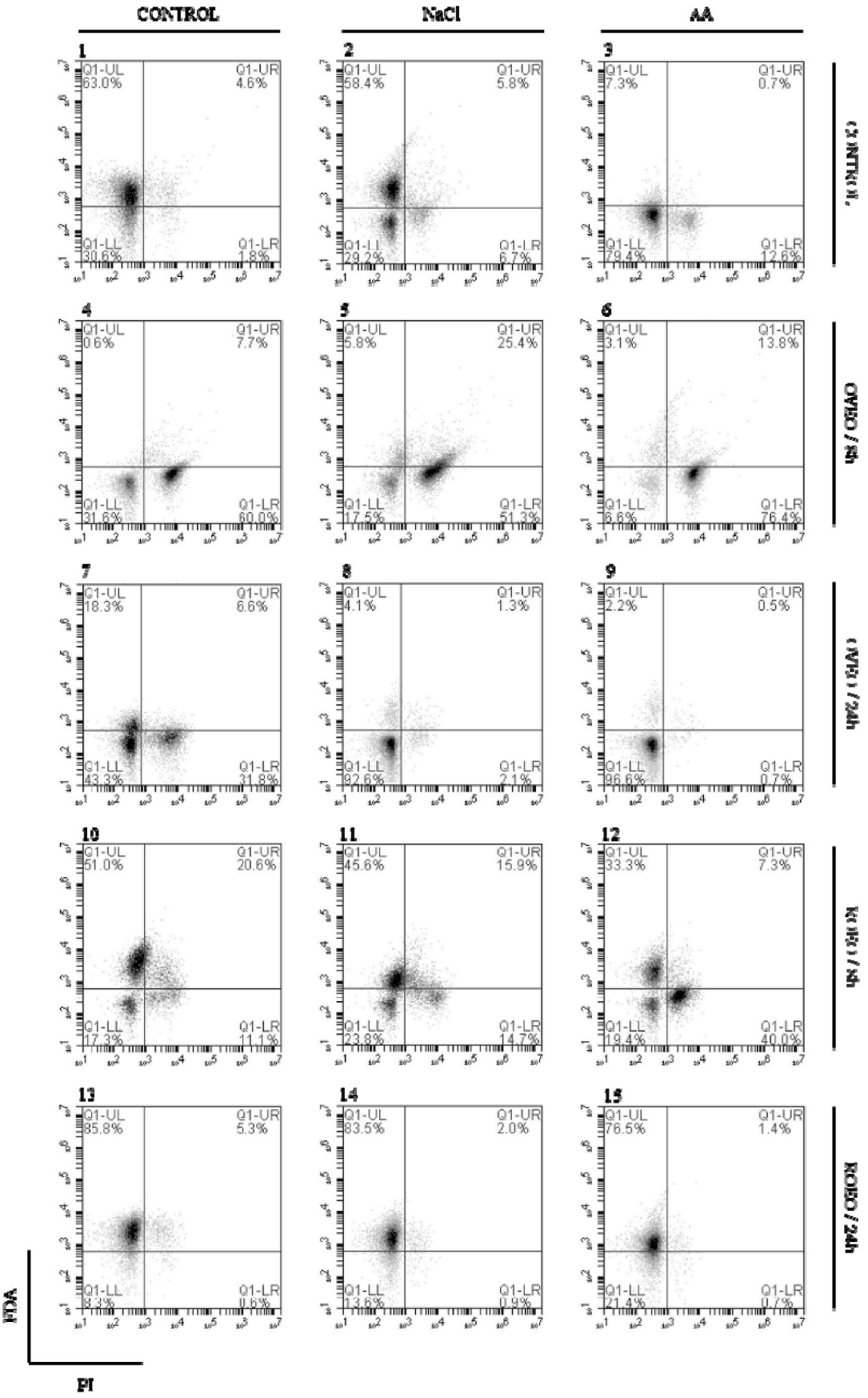

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Fig. 5. Fluorescence density plots of *Listeria monocytogenes* ATCC 7644 stained with FDA and PI after 24 h of pre-exposure to 1/2 MIC of NaCl (45 mg/mL) or acetic acid (AA, 0.625 μL/mL) and further treatment with *Origanum vulgare* L. essential oil (OVEO, 1.25 μL/mL) or *Rosmarinus officinalis* L. essential oil (ROEO, 2.5 μL/mL) for 8 or 24 h. The vertical axis indicates the fluorescence intensity of FDA; the horizontal axis indicates the fluorescence intensity of PI. The four edges indicate UL, cells with detected enzymatic activity and undamaged cell membrane; LL, cells with undetected enzymatic activity and undamaged cell membrane; UR, cells with detected enzymatic activity and a damaged cell membrane; and LR, cells with undetected enzymatic activity and a damaged cell membrane. The percentages of the cell population within each gate are displayed in the four corners of each plot.

tested *L. monocytogenes* strains pre-exposed to AA, LA, NaCl or KCl decreased their sensitivity to OVEO, as the MIC values for this EO were lower than those found against cells not pre-exposed to organic acids or salts. However, pre-exposure to AA, LA, NaCl or KCl overall decreased the sensitivity of *L. monocytogenes* to ROEO since the MIC values of this EO against cells pre-exposed to these agents increased in most cases compared with those found against the not pre-exposed cells. Although it has been reported that only increases in MIC values ≥ two-fold could confer the obvious development of bacterial direct-tolerance or cross-tolerance (Hammer et al., 2012; CLSI, 2016), any alteration in bacterial susceptibility following exposure to stressing conditions could suggest the occurrence of an adaptative response (McMahon et al., 2008).

The results observed in the bacterial survival assays were in agreement with the results of MIC modulation assays. Even at sublethal concentrations, OVEO was able to decrease the counts of *L. monocytogenes* cells pre-exposed to AA, LA, NaCl or KCl, even though the decreases in counts of salt-pre-exposed cells occurred only after 12 h. The same effect was not observed for ROEO since sublethal concentrations of this EO only decreased the counts of cells not pre-exposed to organic acids or salts. These data indicate that sublethal concentrations of AA, LA, NaCl and KCl possibly induced adaptive responses sufficient to decrease the inhibitory effects of ROEO on cell viability of the tested *L. monocytogenes* strains. In contrast, OVEO exerted similar or stronger inhibitory effects on the viable counts of *L. monocytogenes* cells pre-exposed to organic acids or salts compared to its effects on the not pre-exposed cells, indicating no increase in cross-tolerance to this EO.

The results of FC analysis showed that pre-exposure to AA or NaCl and further treatment with OVEO or ROEO led to different degrees of alteration in enzymatic, respiratory and efflux pump activities as well as in cell membrane polarization and integrity. The damage to physiological functions of *L. monocytogenes* cells induced by OVEO and ROEO observed in FC analysis did not accurately reflect the results of the bacterial survival curve assays, in which OVEO clearly induced higher reductions in counts of *L. monocytogenes* cells pre-exposed or not pre-exposed to AA or NaCl than did ROEO. In fact, the effects on the physiological functions of *L. monocytogenes* caused by OVEO and ROEO varied with the treatment duration, tested EO and measured physiological parameters, as seen in the following examples: i) damage to the cell membrane was greater when cells were treated with OVEO than when cells were treated with ROEO for 8 h; ii) the respiratory and efflux pump activities were similarly affected by OVEO and ROEO after 8 h, but efflux pump activity was more compromised by ROEO than by OVEO after 24 h; iii) the respiratory and enzymatic activities were more affected overall by OVEO than by ROEO; and iv) membrane depolarization was more affected overall by ROEO than by OVEO.

It is noteworthy that OVEO and ROEO were able to disrupt all the measured physiological functions in *L. monocytogenes* cells either not pre-exposed or pre-exposed to AA or NaCl, although at different intensities, maintaining a multitarget mechanism action toward both cell groups. Moreover, the array of damage to distinct physiological functions induced by OVEO seemed to be more effective than that of ROEO to decrease the viable counts of *L. monocytogenes* over time, even when these cells were pre-exposed to AA or NaCl. Additionally, *L. monocytogenes* cells not pre-exposed or pre-exposed to AA and NaCl and treated with ROEO were probably injured rather than dead.

Some authors have suggested that bacterial cells exposed to harsh conditions that affect different physiological functions may exist as "viable but not culturable cells" (VBNC) for prolonged durations (Robben et al., 2018). VBNC are still metabolically active but do not grow on culture media and are undetectable by traditional culture methods (Arioli et al., 2019; Paparella et al., 2008). VBNC state could possibly be associated with the results obtained in the bacterial survival assays in this study, where the viable counts of cells pre-exposed to organic acids or salts and treated with OVEO were lower than those of cells treated with ROEO. The existence of VBNC subpopulations of different sizes among *L. monocytogenes* cells treated with OVEO and ROEO should be a focus of further investigations, particularly when these cells are pre-exposed to stressing conditions that could induce increased tolerance in foodborne pathogens, using experimental models available in published literature (Robben et al., 2018; Arioli et al., 2019).

The ability of different EOs to affect bacterial cell survival has been typically associated with the chemical structure of their most prevalent constituents (Djenane et al., 2011; Carovic-Stanko et al., 2010; Nowotarska et al., 2014). OVEO tested in this study had the phenolic thymol as the most prevalent constituent (69.3%); while the tested ROEO had the cyclic ether 1,8-cineole (35.75%) and the bicyclic monoterpene ketone camphor (28.7%) as the most prevalent constituents. The high amount of thymol in the tested OVEO should be associated with the inability of the target *L. monocytogenes* strains to develop a phenotype of cross-tolerance to this EO after exposure to organic acids or salts. Thymol, even in low doses, is able to disrupt membrane structures in bacterial cells, causing increases in permeability, and impairment of enzyme systems (Arioli et al., 2019; Gill and Holley, 2006). Only one previous study evaluated the increase in cross-tolerance to *Melaleuca alternifolia* essential oil (MAEO) in six strains of *Enterococcus faecalis* pre-exposed to NaCl, finding an induction of increased tolerance to MAEO in most of the tested strains, as assessed by bacterial survival curve assays. Interestingly, MAEO has the alcohol monoterpene terpinen-4-ol (or its isomer alpha-terpinol) and not a phenolic as the active component (Lim and Hammer, 2015).

The results obtained in this study showed that the exposure to sublethal concentrations of AA, LA, NaCl and KCl increased the cross-tolerance to ROEO in three standard strains of *L. monocytogenes*, as evaluated by the MIC modulation and bacterial survival curve assays. Otherwise, the tested *L. monocytogenes* strains pre-exposed to organic acids or salts did not increase the cross-tolerance to OVEO in either the MIC modulation or bacterial survival curve assays, remaining more susceptible to this substance. OVEO and ROEO were able to cause damage to different physiological functions in *L. monocytogenes* cells not pre-exposed or pre-exposed to AA or NaCl, although the extent of these effects depended on the exposure time, tested EO and monitored physiological response. OVEO and ROEO presented a multitarget action mode on *L. monocytogenes* cells not pre-exposed and pre-exposed to AA or NaCl, although the cells treated with ROEO seemed to be injured rather than dead. These results indicate that the potential ability of *L. monocytogenes* to increase the tolerance to EOs following adaptation to sublethal stressing conditions imposed by organic acids and salts used to preserve foods could vary with the type of EO.

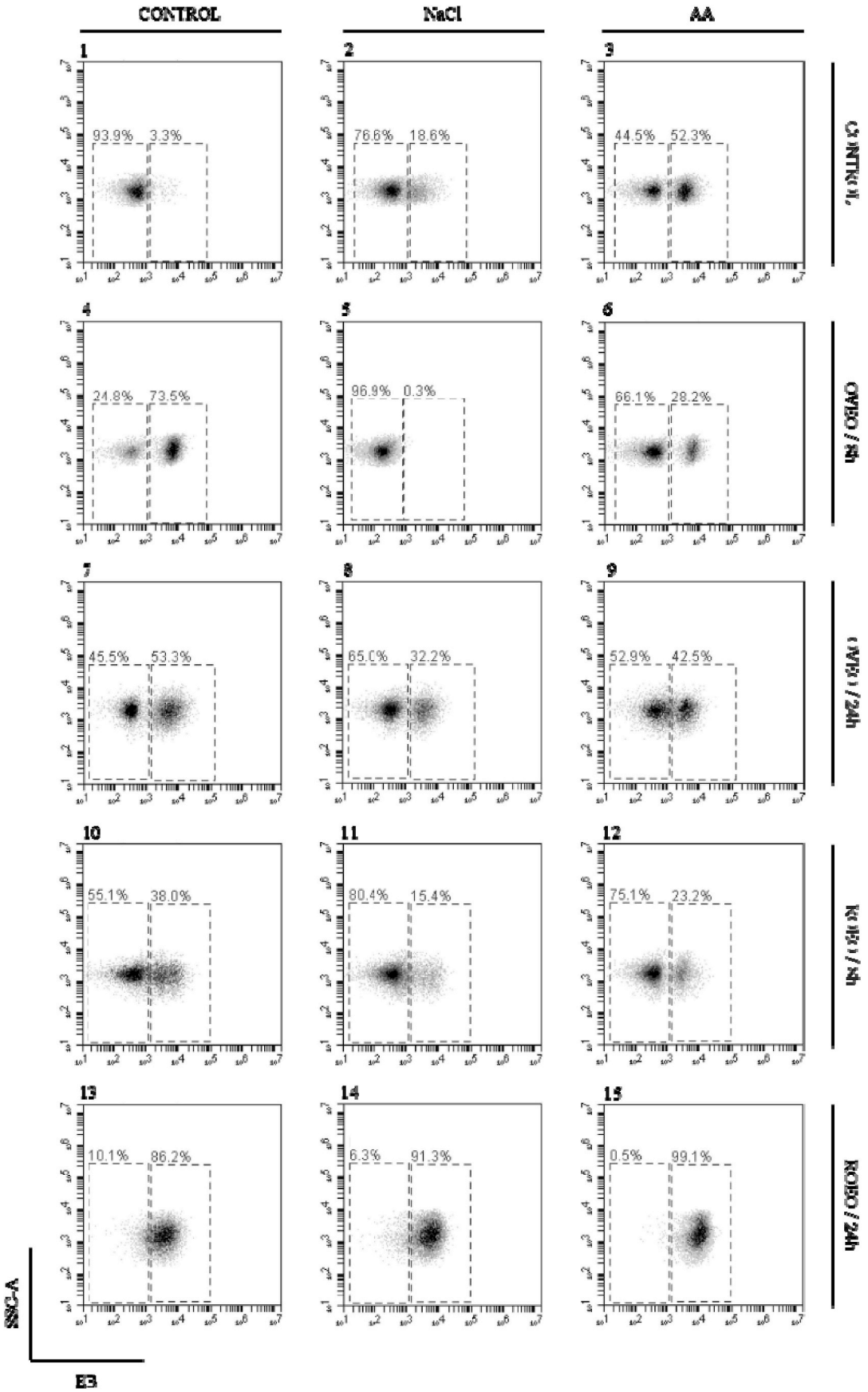

Fig. 6. Fluorescence density plots of *Listeria monocytogenes* ATCC 7644 stained with EB after 24 h of pre-exposure to 1/2 MIC of NaCl (45 mg/mL) or acetic acid (AA, 1.25 μL/mL) and further treatment with *Origanum vulgare* L. essential oil (OVEO, 1.25 μL/mL) or *Rosmarinus officinalis* L. essential oil (ROEO, 2.5 μL/mL) for 8 or 24 h. The horizontal axis indicates the fluorescence intensity of EB; the vertical axis indicates the side-scatter intensity. The unstained subpopulation (EB–) was gated in the left rectangles; the stained subpopulation (EB+) was gated in the right rectangles. The percentages of the cell population within each gate are shown in each plot.

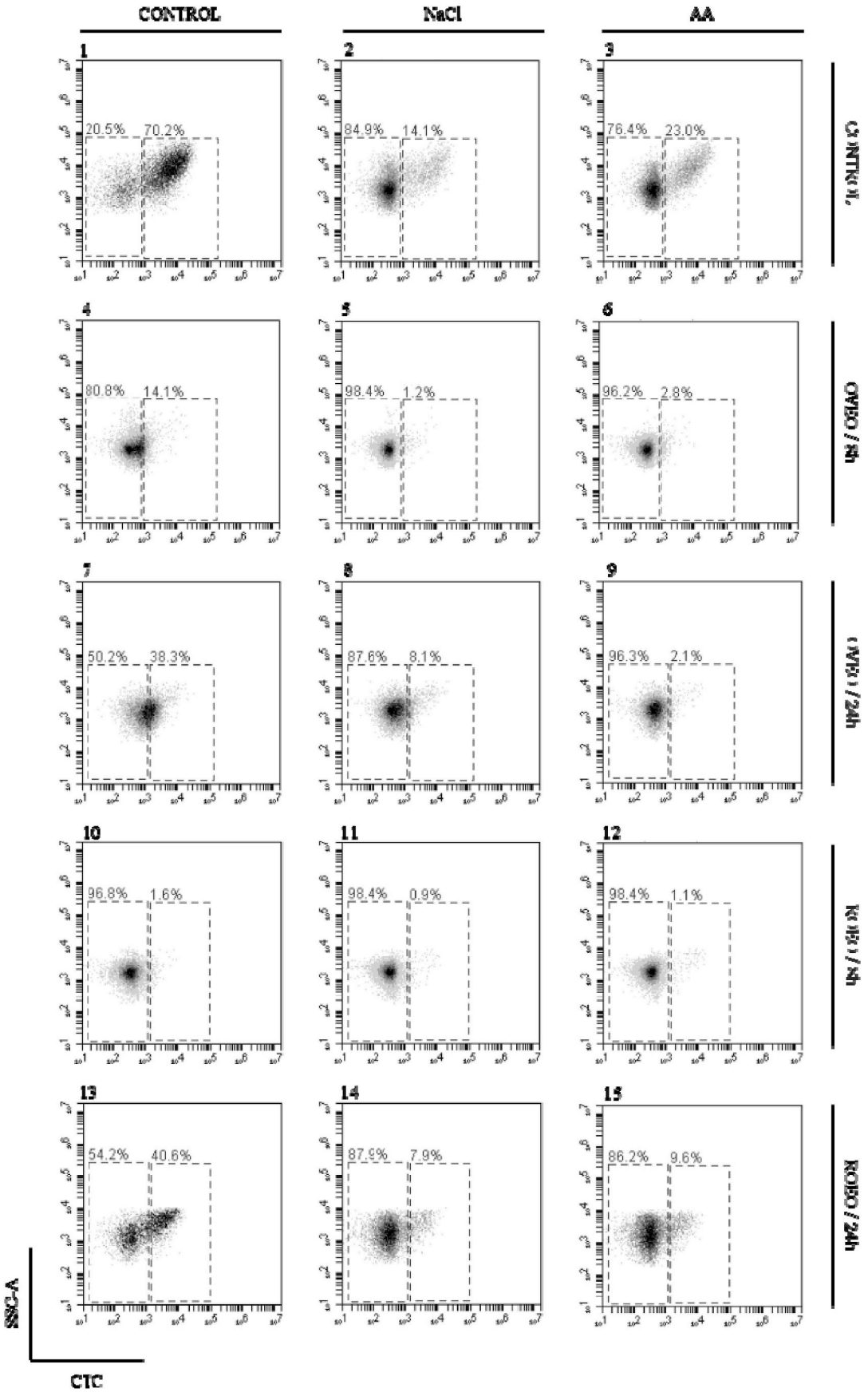

Fig. 7. Fluorescence density plots of *Listeria monocytogenes* ATCC 7644 stained with CTC after 24 h of pre-exposure to 1/2 MIC of NaCl (45 mg/mL) or acetic acid (AA, 1.25 μL/mL) and further treatment with *Origanum vulgare* L. essential oil (OVEO, 1.25 μL/mL) or *Rosmarinus officinalis* L. essential oil (ROEO, 2.5 μL/mL) 8 or 24 h. The horizontal axis indicates the fluorescence intensity of CTC; the vertical axis indicates the side-scatter intensity. The unstained subpopulation (CTC–) was gated in the left rectangles; the stained subpopulation (CTC+) was gated in the right rectangles. The percentages of the cell population within each gate are shown in each plot.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2019.103240.

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