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Short communication

Exposure of *Listeria monocytogenes* to sublethal amounts of *Origanum vulgare* L. essential oil or carvacrol in a food-based medium does not induce direct or cross protection

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ABSTRACT

In this study, the effects of *Origanum vulgare* L. essential oil (OVEO) and carvacrol (CAR) on the growth and survival of *Listeria monocytogenes* ATCC 7644 were evaluated. The induction of direct protection against OVEO and CAR and of cross protection against various stresses (high temperature 45 °C; lactic acid, pH 5.2; NaCl 10 g/100 mL) after exposure to sublethal amounts of OVEO and CAR was also evaluated. Both OVEO and CAR decreased the cell viability of *L. monocytogenes* in meat broth over 120 min of exposure at all assayed concentrations (MIC, 1/2 MIC and 1/4 MIC). The overnight exposure of *L. monocytogenes* to sublethal amounts of OVEO or CAR did not induce direct protection or cross protection against high temperature, lactic acid or NaCl. Cells that were subcultured (24 h cycles) in meat broth containing progressively increasing amounts of the antimicrobials were able to survive exposure to up to 2×MIC (two-fold increase) of OVEO and up to the MIC (one-fold increase in MIC) of CAR, suggesting that there were only minor changes in the antimicrobial susceptibility to these substances. Overall, these data indicate that OVEO and CAR have little effect on the acquisition of direct resistance or cross resistance by *L. monocytogenes*.

1. Introduction

Listeria monocytogenes causes listeriosis, a foodborne disease that occurs predominantly in pregnant woman, the elderly and immunosuppressed individuals and can lead to miscarriages and death (Kim & Kathariou, 2009; Swaminathan & Gerner-Smidt, 2007). *L. monocytogenes* can be found in raw and processed foods such milk, dairy products, meat products, seafood and vegetables (Franklin, Cooksey, & Getty, 2004; Nguyen, Gidley, & Dykes, 2008) causing many times the recall of foods with substantial economic losses to the food industry worldwide (Gandhi & Chikindas, 2007).

Studies have found a number of isolates of *L. monocytogenes* that are resistant to one or more antimicrobial compounds or procedures applied by the food industry to control the growth and survival of microorganisms in foods (Karatzas & Bennik, 2002; Rajkovic et al., 2009). Food processing conditions resemble natural environmental stresses that bacteria may encounter, such that sublethal conditions may induce changes in the cellular physiology of bacteria, causing the bacteria to mount adaptive responses to the antimicrobial interventions used to treat foods (Hill, Cotter, Sleator, & Gahan, 2002; Shadbolt, Ross, & McMeekin, 2001).

The threat posed by this anticipated adaptive response of *L. monocytogenes* to antimicrobials compounds used in foods has prompted studies on the development of novel technologies to control the survival of this pathogen; such technologies must have broad-spectrum antimicrobial activity, be of low toxicity for consumers and involve a low risk of increasing the prevalence of microbial resistance (Nascimento, Locatelli, Freitas, & Silva, 2000; Pazhani et al., 2004). In this context, essential oils and the compounds contained therein have received the attention of researchers and industry for use as alternative antimicrobials in foods (Marino, Bersani, & Comi, 2001).

Early studies showed that *Origanum vulgare* L. essential oil (OVEO) possesses strong and broad-spectrum antimicrobial activity against spoilage-related and pathogenic food-related bacteria (D'Antuono, Galletti, & Bochinni, 2000; Oliveira, Stamford, Gomes Neto, & Souza, 2010; Souza, Barros, Conceição, Gomes Neto, & Costa, 2009). The antimicrobial property of OVEO has been shown to be related primarily to the phenolic compound carvacrol (CAR), which is often the major component of this oil (Azerêdo, Stamford, Nunes, Gomes Neto, & Souza, 2011; Seydim & Sarikus, 2006).

Despite the fact that essential oils (and their compounds) are potential antimicrobials that can be used in foods, there is a lack of reports about the development of direct protection and/or cross

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protection by *L. monocytogenes* when exposed to these compounds at sublethal concentrations. This study assessed the ability of OVEO and the major component thereof, CAR, to inhibit the growth and survival of *L. monocytogenes* ATCC 7644 and evaluated the development of direct protection and cross protection when this strain was exposed to sublethal concentrations of these substances in a meat-based medium.

2. Material and methods

2.1. Essential oil and carvacrol

OVEO (batch OREORG01; density at 20 °C, 0.90; refractive index at 20 °C, 1.47), as obtained by steam distillation, was purchased from Aromalândia Ind. Com. Ltda. (Minas Gerais, Brazil). CAR was purchased from Sigma Aldrich (Sigma, France). Solutions of OVEO and CAR were prepared in nutrient broth (Himedia, India) in a range of concentrations (160–0.075 μL/mL) using bacteriological agar (0.15 g/100 mL) as a stabilizing agent (Bennis, Chami, Chami, Bouchikhi, & Remmal, 2004).

2.2. Test microorganism

Listeria monocytogenes ATCC 7644 was obtained from the Collection of Reference Microorganisms, National Institute of Quality Control in Health (FIOCRUZ, Rio de Janeiro, Brazil). A stock culture was kept on nutrient agar (Himedia, India) under refrigeration (7 ± 1 °C). Unless otherwise stated, all assays used inocula obtained from stationary-phase cultures. To obtain stationary-phase cultures, *L. monocytogenes* was first grown overnight on Brain Heart Infusion agar (Himedia, India) at 37 °C. Then, liquid cultures were prepared by inoculating 100 mL of Brain Heart Infusion broth with two bacterial colonies from the overnight plates, and incubated overnight at 35 °C. After incubation, the cells were harvested from the growth medium by centrifugation at 10,000 × *g* for 12 min at 4 °C, washed twice with phosphate buffered saline (PBS, pH 7.4) and suspended in PBS. Suspensions were adjusted so that the optical density at 620 nm (OD_{620}) of a 1:100 dilution was approximately 0.3, which corresponded to approximately 10 log of cfu/mL colony forming units per milliliter (cfu/mL) (Azerêdo, Figueiredo, Souza, & Stamford, 2012). Suspensions were serially diluted in PBS (10^{-1}–10^{-3}) to provide a viable cell count of approximately 7 log of cfu/mL.

2.3. Preparation of meat broth

Time-kill assays and assays to determine the development of direct protection and bacterial cross protection were performed using a meat-based broth as a substrate for bacterial cultivation. Bovine meat steaks were trimmed of all external fat and cut into uniformly sized pieces (3 × 3 × 3 cm). Meat pieces were then boiled in distilled water for 30 min at 90 °C, yielding approximately 500 mL of meat broth that was then vacuum filtered using Whatman No. 1 filter paper. The filtrate was sterilized by autoclaving for 15 min (1.21 atm). Afterwards, the broth was stored at −20 °C in aliquots of 50 mL. When required, a single aliquot was thawed under refrigeration (7 ± 1 °C) and used for the assays (Oliveira et al., 2010).

2.4. Determination of the Minimum Inhibitory Concentration (MIC)

MIC values for OVEO and CAR were determined using macrodilution in broth. Four milliliters of double-strength nutrient broth (Himedia, India) were inoculated with 1 mL of bacterial culture, mixed with 5 mL of OVEO or CAR solutions and vortexed for 30 s. The assay was statically incubated for 24 h at 35 °C. The MIC was defined as the lowest concentration of essential oil or carvacrol that prevented visible bacterial growth (Nostro et al., 2001). Control flasks without the tested compounds were similarly tested.

2.5. Time-kill assay

The effect of OVEO and CAR on bacterial viability in meat broth following 120 min of exposure was evaluated using viable cell counts. Briefly, 4 mL of meat broth was inoculated with 1 mL of bacterial suspension; 5 mL of the OVEO or CAR solutions was then added to the assay and the mixture was gently shaken for 30 s using a vortex. The mixture was incubated at 35 °C, and at different time intervals (0, 15, 30, 45, 60 and 120 min), 1 mL of the suspension was serially diluted (10^{-1}–10^{-5}) in PBS, inoculated onto sterile nutrient agar and incubated for 24 h at 35 °C (Barros et al., 2009). Control flasks without the tested compounds were tested similarly. The results were expressed as the log of cfu/mL.

2.6. Induction of bacterial direct protection

The induction of direct protection in *L. monocytogenes* was performed by exposing bacteria overnight to sublethal concentrations of OVEO and CAR in meat broth as previously described (Brown, Ross, McMeekin, & Nichols, 1997; Leyer & Johnson, 1993). Meat broth (18 mL) containing the essential oil or carvacrol (final concentrations of 1/2 MIC or 1/4 MIC) was inoculated with 2 mL of bacterial suspension and shaken for 30 s using a vortex (adaptation treatment). Control broth without antimicrobials was assayed similarly (non-adaptation treatment). The assays were incubated overnight (18 h) at 35 °C, after which a 2-mL aliquot of each treatment was inoculated into fresh meat broth (18 mL) containing the OVEO or CAR (final concentration of the MIC was determined previously), shaken for 30 s using a vortex and incubated at 37 °C. Viable cells were enumerated over time (0, 30, 60, 120, 180 and 240 min) by serial dilution (10^{-1}–10^{-5}) in PBS and plating on nutrient agar for 24–48 h at 35 °C. The results were expressed as the log of cfu/mL. To determine if direct protection was induced, the viable cell counts over time of bacteria subjected to adaptation treatments were compared to the counts of non-adapted bacteria when both groups were inoculated into growth media containing the antimicrobials at their MIC values.

2.7. Induction of bacterial cross protection

The induction of cross protection in *L. monocytogenes* was performed by exposing bacteria overnight to sublethal concentrations of OVEO and CAR in meat broth followed by exposure to other environmental stressors (high temperature, low pH and NaCl) as previously described (Boziaris, Chorianopoulos, Haroutounian, & Nychas, 2011), but with minor modifications. Preliminary experiments were performed for evaluating the thermotolerance, acid tolerance and salt tolerance of the bacterial test strain. Untreated bacterial cultures were inoculated into normal meat broth and into meat broth incubated at different high temperatures (40–60 °C) or containing lactic acid (pH 4.5–6.0, at 35 °C) or NaCl (1 g–15 g/100 mL, at 35 °C) to determine the temperature, pH value and NaCl concentration that modestly inhibited the growth of the cell suspension.

After establishing the stress conditions, a 2-mL aliquot of fresh bacterial suspension was inoculated into 18 mL of meat broth containing the OVEO or CAR (final concentrations of 1/2 MIC or 1/4 MIC) and shaken for 30 s using a vortex (adaptation treatment); the same was done for control flasks without antimicrobials (non-adaptation treatment). The assays were incubated overnight (18 h) at 35 °C, after which a 2-mL aliquot of each treatment was inoculated into 18 mL of fresh meat broth acidified with lactic acid (VETEC Química Fina Ltda., Brazil) to pH of 5.2, or into fresh meat broth containing NaCl (Qeel, Brazil) at 10 g/100 mL to evaluate the induction of acid tolerance and osmotolerance, respectively. To analyze the induction of thermotolerance, a 2-mL aliquot of each treatment was inoculated into 18 mL of fresh meat broth and incubated at

45 °C. Viable cells for all assays were enumerated over time (0, 30, 60, 120, 180 and 240 min) by serial dilution (10^{-1}–10^{-5}) in PBS followed by plating on nutrient agar for 24–48 h at 35 °C. The results were expressed as the log of cfu/mL. To determine if bacterial cross protection was induced, the viable counts over time of the bacterial suspensions subjected to the adaptation treatments were compared to the counts of non-adapted bacteria following inoculation into growth media exposed to different environmental stressors.

2.8. Induction of bacterial direct tolerance throughout successive habituation 24 h-cycles

The capacity of *L. monocytogenes* to develop direct tolerance to OVEO and CAR was assessed in meat broth by exposing the bacteria to increasing amounts of the antimicrobials (1/16 MIC, 1/8 MIC, 1/4 MIC, 1/2 MIC, MIC and 2×MIC) throughout successive 24-h habituation cycles to prolong the time of exposure according to the procedure described by To, Favrin, Romanova, and Griffiths (2002), but with minor modifications. Thus, 2 mL of the bacterial suspension was inoculated into 18 mL of meat broth containing the OVEO or CAR (final concentrations of 1/16 MIC), shaken for 30 s using a vortex and incubated for 24 h at 35 °C. Then, a 100-μL aliquot of the culture was serially diluted (10^{-1}–10^{-5}) in PBS and inoculated onto sterile nutrient agar to detect viable cells (35 °C for 24 h). Concurrently, a 2-mL aliquot from the broth containing antimicrobials at 1/16 MIC (and bacterial growth) was inoculated into fresh meat broth (18 mL) containing antimicrobials at the next highest concentration (1/8 MIC); this assay was incubated at 35 °C and viable cells were detected according to the conditions cited above. This procedure was repeated with increasing concentrations of the tested compounds 1/4 MIC — 2×MIC), or until no viable cells were detected. The detection limit for the viable cell count method used was 2 log of cfu/mL for all assays.

2.9. Reproductibility and statistics

All assays were performed in triplicate on three separate occasions, and the results were expressed as averages for each of the assays. Statistical analysis was performed to determine significant differences ($P<0.05$) using ANOVA followed by Tukey's test. The Sigma Stat 3.1 computer program was used.

3. Results and discussion

3.1. MIC and time-kill assays

OVEO and CAR both exhibited MIC values against *L. monocytogenes* ATCC 7644 of 0.62 μL/mL. In a previous study, Azerêdo et al. (2011) found an MIC value for OVEO of 1.25 μL/mL against this same *L. monocytogenes* strain. The differences in the observed MIC values of essential oils and their compounds are thought to be related to the test strain or isolate used, to the composition of the growth medium and/or to other intrinsic and extrinsic characteristics (Burt, 2004).

OVEO and CAR decreased the cell viability of *L. monocytogenes* ATCC 7644 at all assayed concentrations (MIC, 1/2 MIC and 1/4 MIC). The compounds decreased the bacterial cell viability after only 15 min of exposure, and no recovery in the viable count was noted for the remainder of the evaluated time interval. The exposure of *L. monocytogenes* to the antimicrobials at concentrations of 1/2 MIC, 1/4 MIC and MIC caused a significant decrease ($P<0.05$) in the viable count in comparison with the control treatment. *L. monocytogenes* grown in broth containing both tested compounds at their MICs exhibited viable counts ≤2.0 log cfu/mL after 45 min of exposure, and no recovery in the viable counts was observed over the remainder of the evaluated time interval. OVEO and CAR at 1/2 and 1/4 MIC decreased the viable count of *L. monocytogenes* to approximately 5 log cfu/mL after 120 min of exposure, revealing that these concentrations were inhibitory to the growth of the tested strain, but not lethal.

Azerêdo et al. (2011) noted that the OVEO (1.25 μL/mL) used in this study caused a decrease in viable count of *L. monocytogenes* ATCC 7644 to 2.3 log cfu/mL over 120 min of exposure in a vegetable-based broth. In the same study, the authors identified carvacrol (66.9 g/100 g), *p*-cymene (13.9 g/100 g) and γ-terpinene (7.8 g/100 g) as the main constituents of OVEO. In other study, the same OVEO (1.25 μL/mL) caused release of intracellular material in *L. monocytogenes* and marked morphological changes in the bacterial cells, including shrinkage and condensation of the cytoplasmic content and detachment of the cell wall from the plasma membrane (Azerêdo et al., 2012).

3.2. Induction of bacterial direct protection after overnight exposure to OVEO and CAR

The overnight exposure of *L. monocytogenes* to sublethal amounts of both OVEO and CAR (1/2 MIC and 1/4 MIC) did not induce direct protection (Fig. 1). The kill-curves of *L. monocytogenes* cells that were previously challenged with a sublethal amount of OVEO or CAR exhibited similar viable counts ($P>0.05$) when further cultivated in growth medium containing the same antimicrobial at the MIC. The viable counts found for the control cells (non-adapted cells) were higher ($P<0.05$) than those found for cells that have been previously treated with CAR (at 1/2 MIC and 1/4 MIC).

Although no difference ($P>0.05$) in the counts of *L. monocytogenes* treated with sublethal amounts of OVEO for adaptation or left untreated was found when the strain was exposed to the essential oil at its MIC for 240 min, the cells that were pre-adapted in broth containing 1/4 MIC or 1/2 MIC of OVEO exhibited a slight increase in the viable count between 0 and 180 min of exposure, followed by a sharp decrease in the counts at 240 min. This survival pattern was different from the linear decrease in the viable counts found for the control non-adapted cells over the same time interval. Cells submitted to pre-adaptation with CAR exhibited an initial drop in the viable counts when further exposed to the MIC, followed by the maintenance of the number of viable cells over the remainder of the evaluated time interval.

The literature regarding the assessment of tolerance development by *L. monocytogenes* when exposed to sublethal amounts of essential oils or their compounds is still limited, and most of the past studies focused on the development of direct resistance and cross resistance by *L. monocytogenes* have involved assays in which bacteria were exposed to classical chemical and physical food preservative procedures (Skandamis, Stopforth, Yoon, Kendall, & Sofos, 2009; Soni, Nannapaneni, & Tasara, 2011). Koutsoumanis, Kendall, and Sofos (2003) evaluated the acid tolerance response (ATR) of three- and five-strain mixtures of *L. monocytogenes* previously grown in Tryptic Soy Broth (TSB) containing glucose (1 g/100 mL — assumed to be acid-adapted cells) and found an enhanced ATR in adapted cultures relative to cells grown in TSB without glucose (non-acid-adapted cells). Pagán, Condón, and Sala (1997) reported an adaptive heat response (tolerance to 64–65 °C) in *L. monocytogenes* after a heat shock (48 °C) for 1–2 h. Skandamis, Yoon, Stopforth, Kendall, and Sofos (2008) reported that exposure to sublethal stress did not affect the thermotolerance of *L. monocytogenes*, whereas simultaneous exposure to multiple stresses (NaCl at 10 g/100 mL, HCl at pH 5.0, high temperature of 46 °C) simultaneously for 1.5 h in TSB resulted in increased tolerance of the bacterium to acidic environments (HCl at pH 3.5).

3.3. Induction of bacterial cross protection after overnight exposure to OVEO and CAR

In agreement with the results obtained in the assays of the induction of direct protection, the exposure of *L. monocytogenes* cells overnight to sublethal concentrations (1/4 MIC and 1/2 MIC) of OVEO or CAR did not

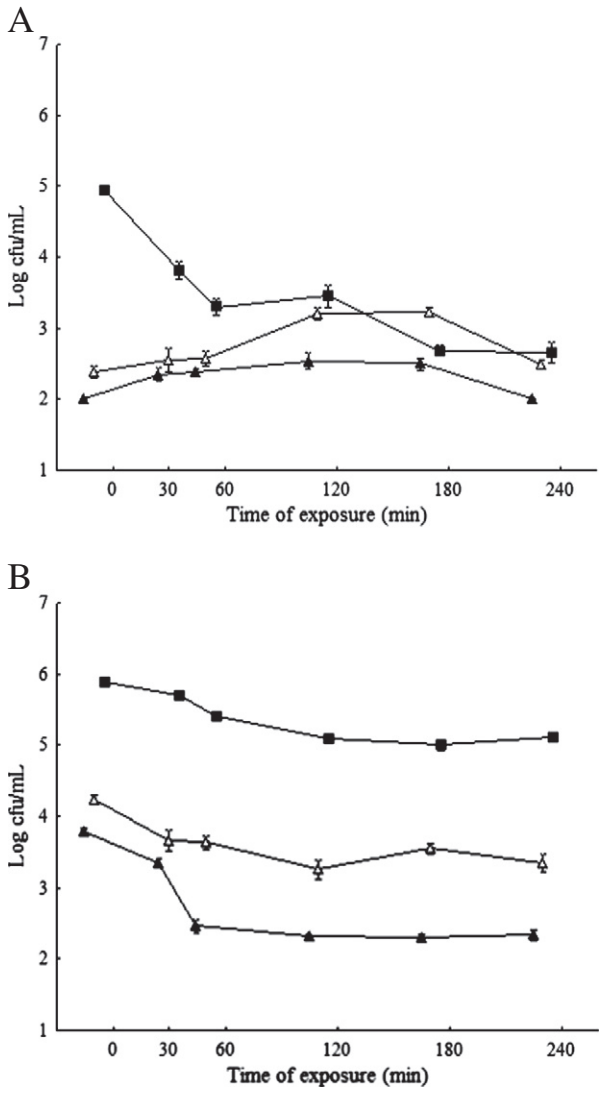

Fig. 1. Viable counts of *L. monocytogenes* ATCC 7644 in meat broth to which the *O. vulgare* L. essential oil or carvacrol (at its MIC) were added following overnight exposure at 35 °C to sublethal concentrations of *O. vulgare* L. essential oil (A) and carvacrol (B): (■) Control, non-adapted cells; (+): cells pre-adapted at 1/2 MIC — 0.3 μL/mL; (Δ): cells pre-adapted at 1/4 MIC — 0.15 μL/mL.

induce cross protection against high temperature (45 °C), lactic acid (pH 5.2) or salt (NaCl at 10 g/100 mL) (Fig. 2A–C and Fig. 3A–C). Cells of *L. monocytogenes* that were pre-adapted with the antimicrobials exhibited smaller counts ($P<0.05$) in media incubated at high temperature or containing lactic acid and NaCl relative to the cells that were not submitted to the pre-adaptation protocol (control assay) with the exception of cells that were pre-adapted with 1/2 MIC of OVEO and then challenged with lactic acid. Moreover, no difference ($P>0.05$) was found between the counts of viable *L. monocytogenes* cells submitted to pre-adaptation with OVEO or CAR at 1/2 MIC and 1/4 MIC when later exposed to the heterologous stressing agents (high temperature, low pH and NaCl).

In general, lower counts (3–4 log cfu/mL) of pre-adapted cells of *L. monocytogenes* were found when the cells were subsequently cultivated in broth containing NaCl relative to the counts observed for cells grown in broth incubated at high temperature or containing lactic acid. In the NaCl system, the viable counts of cells pre-adapted with 1/2 MIC or 1/4 MIC of OVEO or CAR were lower ($P<0.05$) than the counts for the control cells (non-adapted cells).

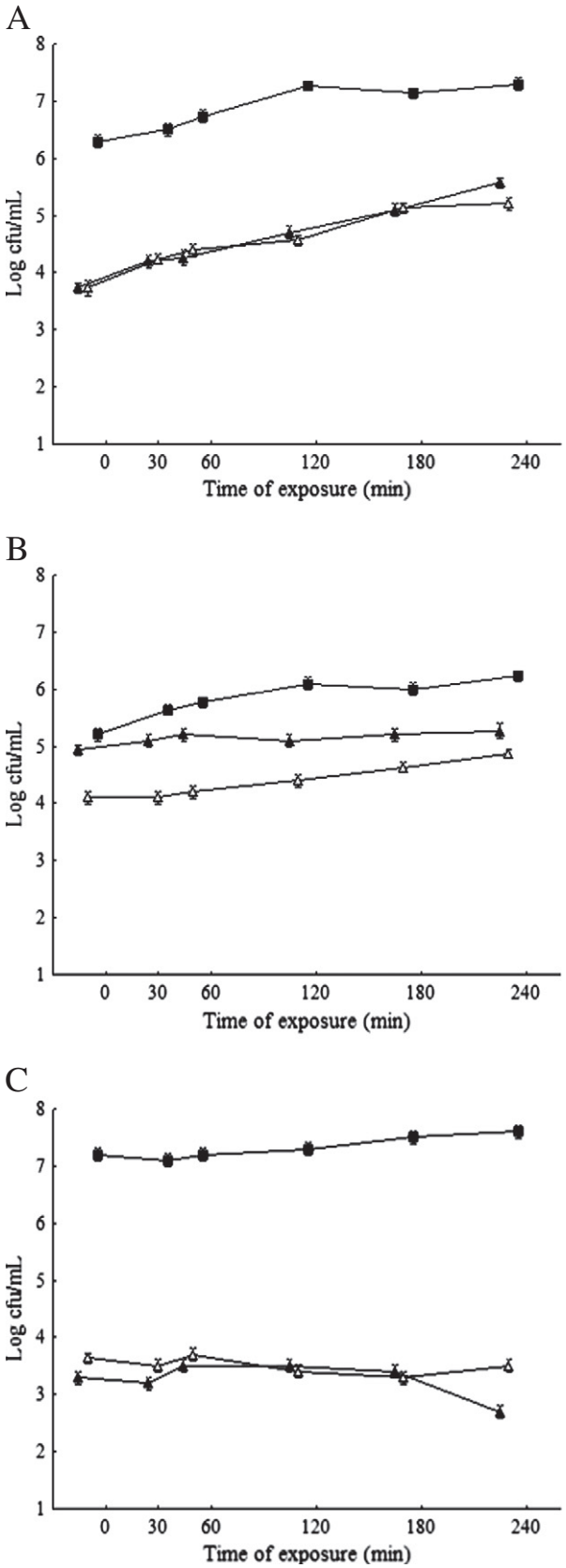

Fig. 2. Viable counts of *L. monocytogenes* ATCC 7644 grown in meat broth incubated at high temperature — 45 °C (A) or to which lactic acid — pH 5.2 (B) or NaCl — 10 g/100 ml (C) was added following overnight exposure at 35 °C to sublethal concentrations of *O. vulgare* L. essential oil. (■) Control, non-adapted cells; (+): cells pre-adapted at 1/2 MIC — 0.3 μL/mL; (Δ): cells pre-adapted at 1/4 MIC — 0.15 μL/mL.

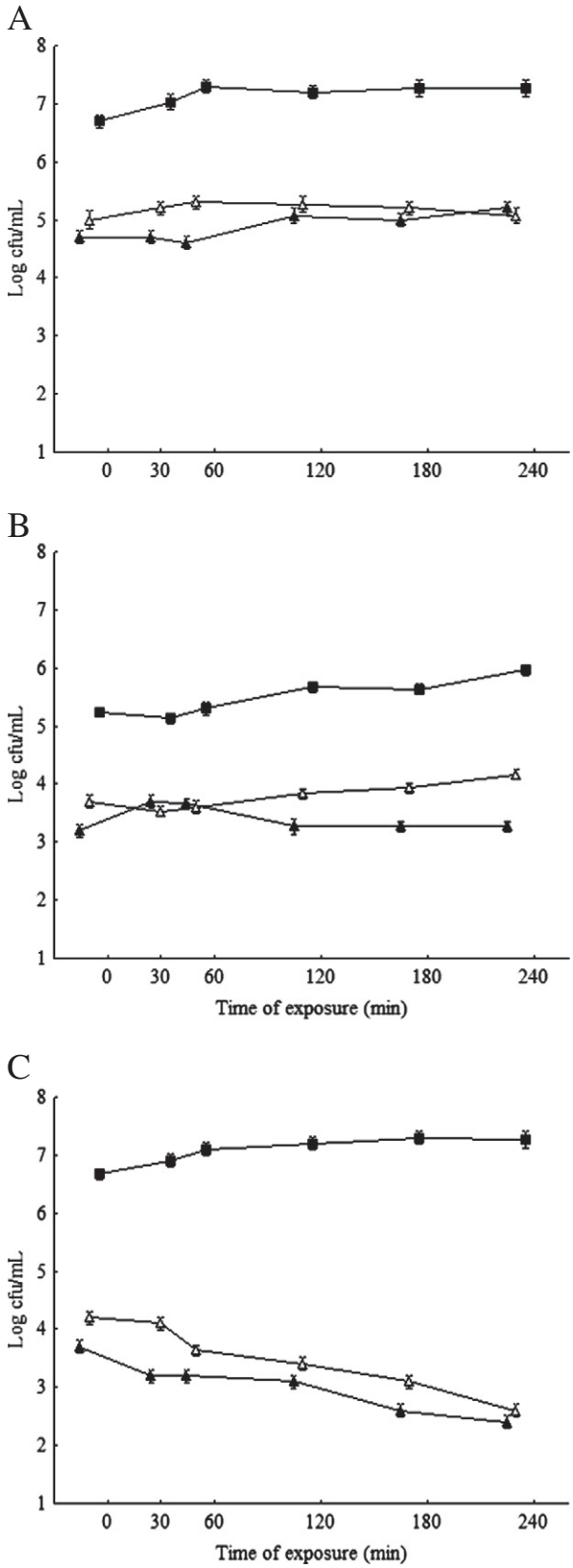

Fig. 3. Viable counts of *L. monocytogenes* ATCC 7644 grown in meat broth incubated at high temperature — 45 °C (A) or to which lactic acid — pH 5.2 (B) or NaCl — 10 g/100 ml (C) was added following overnight exposure at 35 °C to sublethal concentrations of carvacrol. (■) Control, non-adapted cells; (+): cells pre-adapted at 1/2 MIC — 0.3 μL/mL; (Δ): cells pre-adapted at 1/4 MIC — 0.15 μL/mL.

3.4. Induction of bacterial direct tolerance to OVEO and CAR throughout successive habituation 24 h-cycles

To address the possible limitations of the assays carried out with the two tested sublethal amounts of OVEO and CAR and the chosen exposure times for pre-adaptation (18 h), further experiments were performed to assess the induction of bacterial tolerance in *L. monocytogenes* ATCC 7644 when the bacteria were subcultured for 24 h cycles in meat broth containing progressively increasing amounts of OVEO or CAR (1/16 MIC – 2×MIC). The results of these assays showed that *L. monocytogenes* was able to survive (as determined by the count of viable cells) in meat broth containing up to 2×MIC (2-fold increase in MIC value) of OVEO and up to the MIC (one-fold increase in MIC value) of CAR.

The repeated exposure of *L. monocytogenes* to increasing amounts of OVEO and CAR did not induce significant changes in the bacterial susceptibility when evaluated by the standard MIC assessment criteria (Hammer, Carson, & Riley, 2012). These findings are largely in agreement with the results of previously published studies that indicated minor changes in susceptibility (2-fold increase or less in the MIC) of *Staphylococcus aureus* and *Escherichia coli* after exposure to increasing sublethal amounts of the essential oil from *Melaleuca alternifolia* and/or compounds contained therein (Hammer et al., 2012; McMahon, Blair, Moore, & McDowell, 2007); and of *Salmonella typhimurium* when exposed to sublethal amounts of OVEO and CAR (Luz et al., in press).

4. Conclusions

The results of this study reveal that *L. monocytogenes* ATCC 7644 exhibited no clear induction of direct protection or cross protection (high temperature, pH and NaCl) after an overnight exposure (one-step adaptation) to sublethal concentrations of OVEO or CAR in a meat-based medium. Moreover, the exposure of the cells to increasing sublethal amounts (multi-step adaptation) of both OVEO and CAR for a longer time resulted in no significant global effects on the acquisition of direct tolerance by *L. monocytogenes*. These findings reinforce the possible rational use of OVEO and CAR by food industry to control *L. monocytogenes* in foods regarding their efficacy to establish a fast and steady inhibitory effect, besides the low capacity to induce bacterial tolerance.

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