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Modelling of *Listeria monocytogenes* Scott A after a mild heat treatment in the presence of thymol and carvacrol: Effects on culturability and viability

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ABSTRACT

The combined effect of thymol, carvacrol and mild heat treatments against *Listeria monocytogenes* Scott A was assessed in buffered system by plate counting and flow cytometry (FCM). The susceptibility of cells increased when heat treatment was combined with terpenes. The data modelling with Bigelow and Weibull equations showed that the latter was more appropriate to describe the inactivation kinetics. After treatments, cells were no longer able to form colonies on plates; nevertheless, FCM indicated that most of the cells were damaged rather than dead. Treated cells were not able to recover the damage after 6 h. This opens the question if they could recover in a food matrix. FCM can be a helpful technique to better comprehend the physiological state of microorganisms. In the perspective of industrial applications, studies based on predictive microbiology, as well as a deeper comprehension of the action mechanisms of antimicrobials, play a key role for process optimization.

1. Introduction

Heat treatment remains one of the most important methods to achieve microbial stability in foods. However, in the last decades the trend toward the application of mild treatments asks for a deeper comprehension of the real dynamics of thermal inactivation for targeted microorganisms. In effect, as observed by some Authors (Smelt and Brul, 2014), in the traditional heat processes the margins for microbial stability are often overestimated due to a suboptimal control of the process, and can markedly affect the organoleptic profile of foods. The need to reduce food thermal treatment opens new scenarios for food microbiologists, also because it is well known that usually the thermal cell death does not follow a linear kinetic (Peleg et al., 2001). For this reason, predictive microbiology explored the potential of models alternative to the classic first order inactivation model (Bigelow, 1921; Stumbo, 1973), able to satisfactorily describe not constant death rates. Among these tools, the Weibull model (van Boekel, 2002) is extremely flexible because it can be adapted to data with increasing or decreasing death rates, as well as to linear kinetics, which are a particular case of the Weibull equation. Moreover, it is well adaptable to the description of inactivation factors different to heat, as it has been demonstrated for pulsed electric fields (San Martín et al., 2007), essential oils (Aragao et al., 2007) and non-thermal atmospheric plasma (Calvo et al., 2016).

The sub-lethally damaged cells can turn toward active cells or definitively die according to the environmental conditions during storage (Kramer and Thielmann, 2016; Rowan et al., 2015). The effectiveness of these mild thermal treatments often depends also on the application of other stabilizing factors, in the framework of "hurdle technology" (Leistner, 1994; Singh and Shalini, 2016). In the perspective of industrial applications, studies based on the use of predictive microbiology as well as a deeper comprehension of the mechanisms of action of the antimicrobial effects play a key role for optimizing the process.

The first documented foodborne outbreak caused by *Listeria monocytogenes* occurred in the beginning of '80 (Schlech et al., 2010). After this first case, the risks of food listeriosis dramatically increased and nowadays this species is considered among the most dangerous pathogens associated with the food chain (Ferrentino et al., 2015). This is also due to its ability to grow in relatively wide ranges of temperature, pH, a_w and also to its facultatively anaerobic metabolism (Välimaa et al., 2015). In this perspective, a particular attention has been posed on the study of thermal inactivation of this species. Smelt and Brul (2014) reported *D*-values ranging from about 10 min to 12 s at temperatures between 55 and 65 °C, depending on physiological state,

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treatment medium (model system *vs.* real foods) and treatment conditions (isothermal process or not). However, many other authors demonstrated that the kinetics of *L. monocytogenes* death do not follow the linear kinetics of the traditional Bigelow model. In fact, there is evidence that this kinetic presents, depending on the conditions, shoulders or tails and therefore it is often fitted with the Weibull model (Aryani et al., 2015; Buzrul and Alpas, 2007; Cava-Roda et al., 2012; Juneja et al., 2014). Further, important synergistic effects on the mortality of *L. monocytogenes* were observed when a mild thermal treatment was carried out in the presence of aroma compounds such as thymol, carvacrol, citral and (*E*)-2-hexenal (Guevara et al., 2015; Karatzas et al., 2000; Sado-Kamdem et al., 2011), vanillin (Cava-Roda et al., 2012; Char et al., 2009) or D-limonene (Maté et al., 2016). These effects were evident also when the aroma compounds were added in amount well below their minimum inhibitory concentration (MIC) or minimum bactericidal concentration (MBC). The synergic phenomena were attributed to the sum of sub-lethal damages caused by the two treatments (heat and volatile molecules), that can prevent a possible cell repair. Moreover, an increase of the antimicrobial activity of aroma compounds is due to the temperature rise, which enhances their ability to solubilize into the cell membrane, the first target of their action (Hyldgaard et al., 2012; Patrignani et al., 2015; Raybaudi-Massilia et al., 2009; Sado-Kamdem et al., 2011).

In spite of the wide literature regarding the cell count reduction in the presence of combined treatments measured with traditional plate counting methods, little information is still available on the physiological state of *L. monocytogenes* cells as a consequence of such treatments. In her paper, Davey (2011) stressed the possible failure of these traditional methods to evidence cryptobiotic, dormant, moribund and latent cells, which can however have other measurable activities or restore their cell integrity and restart to grow during the storage of food. Among the tools available to explore the physiological state of viable but not culturable cells, flow cytometry (FCM) plays an important role. The use of selected probes able to monitor several cellular parameters (*i.e.* metabolic activity, membrane permeability and energization, DNA/RNA content, etc.) allows to get a deeper knowledge of the metabolic characteristics of microbial subpopulations as a consequence of one or more antimicrobial treatments (Comas-Riu and Rius, 2009; Davey, 2011; Díaz et al., 2010). For this reason, FCM is nowadays considered a powerful technique to investigate the effect of chemico-physical parameters as well as of inactivation treatments on micro-organisms. Many studies indeed have used this approach to assess the efficacy of traditional (thermal) or innovative treatments (pressure, electric fields, pulsed light) on foodborne pathogens (Ferrentino et al., 2015; Fröhling and Schlüter, 2015; Kramer and Thielmann, 2016; Muñoz et al., 2009). Moreover, also the effect of variables such as extracellular pH (Hansen et al., 2016) or the presence of chemicals such as antibiotics (Saint-Ruf et al., 2016) peracetic acid and ozonated water (Fröhling and Schlüter, 2015) on bacterial viability and metabolism has been investigated by FCM.

In this context, this work was aimed to evaluate the physiological state of *L. monocytogenes* after mild thermal treatments combined or not with antimicrobial terpenes (thymol and carvacrol). The cell viability was monitored by plate counting and flow cytometry (FCM), staining the cells with i) SYBR-Green I and propidium iodide (PI) simultaneously, that allowed pointing out an intermediate state where cells show fluorescence with both probes (Díaz et al., 2010); ii) PI, an impermeant DNA stain able to enter into the cells having significant membrane damage (Díaz et al., 2010; Guyot et al., 2015); iii) $DiBAC_4$ as probe able to enter into the cells with depolarized membrane (Guyot et al., 2015). The results of the two analytical tools were compared to have a deeper insight on the real effectiveness of the treatments on cells viability and to better comprehend the risk due to the ability of cells hardly but sub-lethally damaged by heat and terpenes to recover and potentially grow during food storage.

2. Material and methods

2.1. Bacterial strains and culture conditions

The strain used in this study was *Listeria monocytogenes* Scott A belonging to the collection of the Department of Agricultural and Food Sciences (University of Bologna). The strain was maintained as freeze-dried stocks at −80 °C and, before the experiments, cultured twice in Brain Heart Infusion (BHI; Oxoid, UK) for 24 h at 37 °C.

2.2. MIC and MBC determination

For MIC and MBC determination, the method described by Sado-Kamdem et al. (2011) was used, with an inoculum level of about 6 log cfu/ml. Thymol and carvacrol (Sigma-Aldrich, St. Louis, MO) were dissolved in ethanol and the concentrations tested ranged between 0 and 500 mg/l. The MIC was defined as the lowest concentration of the compound preventing visible growth in the well after 24 h of incubation. The MBC was defined as the lowest concentration of the compound that caused the death of the inoculated cells and hence no growth after 24 h of incubation at 37 °C of a 10-µl spot plated onto BHI agar. On the basis of the results obtained, the following thermal treatments were performed using sub-inhibiting concentration, namely 50 mg/l of each terpene, alone or in combination.

2.3. Effect of aroma compounds on Listeria monocytogenes viability and injury

Cells grown until early stationary phase (24 h) at 37 °C were re-suspended in sterile sodium phosphate buffer (PBS, pH 7.4) at a cell load of approx. 6 log cell/ml in the presence of different concentrations of thymol or carvacrol: 0, 100 (half of MIC), 250 (about MIC value) and 500 (higher than MIC value) mg/l. Samples were stored at room temperature (24 ± 2 °C) and collected after 15, 30, 60, 120, 180 and 240 min and analysed both by plate counting onto BHI agar (incubation time: 48 h at 37 °C) and FCM (see section 2.6). As aroma compounds were dissolved in ethanol, a control with the same concentration of ethanol (0.5% v/v in the final medium) but without terpenes was also considered. All the data are the mean of three repetitions.

2.4. Thermal treatments in the presence of terpenes and evaluation of cells recovery

Firstly, thermal death curves were performed at 50 and 55 °C in isothermal conditions using a water bath LAUDA Ecoline (LAUDA-Brinkmann, LP., Delran, New Jersey, US). Cells of L. *monocytogenes* Scott A grown until early stationary phase (24 h) at 37 °C were re-suspended in sterile PBS, pre-heated at proper temperature (50 or 55 °C), at a cell load of approx. 6 log cfu/ml. Aroma compounds (50 mg/l of the two terpenes alone or in combination) were added just before inoculum. This procedure allowed a negligible decrease of the temperature after inoculum (0.5 °C for 2–3 s before returning to the target temperature); thus, the treatment was considered isothermal. Samples were periodically collected (about every 30–60 s in the presence of terpenes and every 2.5–5 min in the controls) and survivors were evaluated by plate counting onto BHI agar. The plates were incubated at 37 °C for 48–72 h before colonies were counted to allow injured cells to form visible colonies. The experiment was done in triplicate, and the data were merged to increase the number of raw data points and minimize the weight of outliers during the modelling process. On the basis of death curves, cells were treated at 50 and 55 °C for 30 min in the same experimental conditions and analysed by FCM to evaluate the damages induced by the treatments. Moreover, treated cells were incubated for 6 h in the presence or not of glucose (0.2% w/v) to assess the ability to recover the damage, both by plate counting and FCM analysis.