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Sublethal injury and virulence changes in *Listeria monocytogenes* and *Listeria innocua* treated with antimicrobials carvacrol and citral

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ABSTRACT

The aim of this study was to evaluate the effect of two antimicrobial substances, carvacrol and citral, on *Listeria monocytogenes* and *Listeria innocua* cells, as well as possible virulence changes in injured cells, using *Caenorhabditis elegans* as a model test. The results indicated that the percentage of sublethal damage was higher in *L. monocytogenes* than in *L. innocua*. The results of the study carried out by using *C. elegans* indicated that *C. elegans* fed in a lawn of *L. monocytogenes* previously treated with carvacrol showed a loss in life span ($p \leq 0.05$) as compared with *L. monocytogenes* treated with citral, *Escherichia coli* OP50 as a negative control, and treated and untreated *L. innocua*. Egg laying was also affected: worms fed in a lawn of treated and untreated *L. monocytogenes* laid fewer eggs than those fed in a lawn of treated and untreated *L. innocua* or fed with OP50 as a negative control. Worms fed in a lawn of treated and untreated *L. innocua* also laid fewer eggs than those fed with OP50 as a negative control. A phenotype named bag of worms and an undescribed new one, "vulva inflammation", were also observed.

1. Introduction

Modifications of food product formulations and processing conditions to meet consumer demands for convenient, healthy or "preservative-free" foods may concomitantly involve reduction of food preservation hurdle intensities and subsequently lead to sublethal stressing of microorganisms. As a result, surviving damaged pathogens may have increased resistance and virulence and thus be more difficult to control; and, as a result, antimicrobial hurdles may fail (Doyle et al., 2006).

Bacteria may have various mechanisms for surviving these external environmental stresses. If a population of microorganisms is exposed to a sufficiently high concentration of an antimicrobial compound, susceptible cells will be killed. However, some cells may possess a degree of natural resistance or may acquire it later through mutation or genetic exchange and will, therefore, survive and grow (Davidson and Harrison, 2002). Sublethal injury is an important aspect that should be considered in evaluating the efficacy of any food preservation method because the presence of injured bacterial cells could be as dangerous as the presence of fully viable ones. Studies have shown that sublethally injured pathogens could proliferate to microbiologically hazardous levels when water and nutrients are plentiful (Busch and Donnelly, 1992; Ariefdjohan et al., 2004) and they may acquire new or modified characteristics (Lado and Yousef, 2002).

The Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR, 2010) reported that the induction of a selective pressure by the application of biocides that may favour the selection of less susceptible bacteria and the expression and dissemination of resistance mechanisms is not fully appreciated. Biocides that include disinfectants and food preservatives have been studied only recently. Food preservatives such as the terpenes carvacrol and citral have been registered by the European Commission for use as flavourings in foodstuffs because their use does not present a risk to the health of the consumer (Burt, 2004). In fact, natural antimicrobials are becoming popular in hurdle preservation processes, allowing the use of mild treatments that minimally penalize the sensory and nutritional properties of foods. Nonetheless, caution must be exercised in light of the potential for bacterial adaptation to these terpenes. Dubois-Brissonnet et al. (2011) observed that the tolerance to disinfectants of cells cultivated with sublethal concentrations of terpenes increased significantly for

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eugenol, thymol, carvacrol and citral, probably because of alteration of the fatty acid composition. Most of the information about changes in the virulence of pathogenic microorganisms such as *Escherichia coli* O157:H7 (Isumikawa et al., 1998), *Campylobacter jejuni*, *Campylobacter coli* (Everest et al., 1992), *Cronobacter sakazakii* (Kim and Loessner, 2008) and *Bacillus cereus* (Minnaard et al., 2004) was obtained by using *in vitro* systems such as the use of the human colon carcinoma cell line Caco-2 (Coppa et al., 2006).

Efforts have been made recently to find easier model systems to study the virulence of pathogenic microorganisms. Invertebrate organisms are increasingly recognized as important in bacterial pathogenesis studies, mainly because of their practical appeal and the absence of ethical issues. One such organism is *Caenorhabditis elegans*. Studies have demonstrated that many mammalian pathogenic bacteria and fungi can infect and cause disease in a nonvertebrate host, such as *C. elegans*. The nematode *C. elegans* has been used as a model to describe the virulence of *Listeria monocytogenes* and other pathogenic bacteria (Thomsen et al., 2006; Diard et al., 2007; Jensen et al., 2008a; Forrester et al., 2007). This invertebrate animal model requires simple, inexpensive growth conditions, i.e. it can grow on a layer of bacteria in an agar plate (Diard et al., 2007). Therefore, *C. elegans* provides the opportunity simultaneously to explore pathogen virulence and host defence, and to identify novel pathogen-encoded virulence factors and universal virulence factors that are relevant in mammalian pathogenesis and specialized human pathogens (Costi et al., 2005; Ewbank, 2002; Aballay and Ausubel, 2002).

It is well known that the ability of *Listeria* to invade nonphagocytic cells is an important pathogenic property because it provides bacteria with shelter against host defence mechanisms. The objective of the present work is to study possible virulence changes in *L. monocytogenes* and *Listeria innocua* treated with citral and carvacrol antimicrobials by using *C. elegans* as an indicator of those virulence changes. *L. innocua* was also tested because it is considered a surrogate for *L. monocytogenes* in many preservation studies, and therefore it is of great interest to make comparisons between them.

2. Material and methods

2.1. Chemicals

Purified carvacrol ≥98% (5-isopropyl-2-methylphenol) and citral 95%, a mixture of *cis* and *trans* isomers (3,7-dimethyl-2,6-octadienal), were purchased from Sigma–Aldrich Company Ltd, (250-258, Cambridge Science Park, Milton Road, Cambridge, Cambridgeshire, United Kingdom).

2.2. Bacterial strain and growth conditions

The species of *L. monocytogenes* Serovar 4b (CECT 4032) and *L. innocua* Serovar 6a (CECT 910) were obtained from a pure lyophilized culture supplied by the Spanish Type Culture Collection (CECT). Glycerol stock solutions with *L. monocytogenes* Serovar 4b (CECT 4032) and *L. innocua* Serovar 6a (CECT 910) were generated. Vials containing microorganisms were obtained following the method described by Saucedo-Reyes et al. (2009). During this investigation, both stock cultures at a concentration of about 8.5×10^8 colony forming units (cfu/mL) were maintained in cryovials of 1 mL at −80 °C. In both cases, the average cell density of the vials was established by viable plate count, employing buffered peptone water (Scharlau Chemie S.A., Barcelona, Spain) for sample dilution.

E. coli OP50 is a uracil mutant used as the food source for almost all laboratory cultures of the nematode *C. elegan* (Brenner 1974) and was supplied with the worm by the College of Biological Sciences, Minnesota University, USA. The *E. coli* strain OP50 is generally thought to be innocuous to worms when grown on minimal medium (Brenner, 1974; Aballay and Ausubel, 2002).

Before each experiment, bacterial broth subcultures from stock cultures were prepared by inoculating 100 μL of *L. innocua* or *L. monocytogenes* in a sterile flask containing 10 mL of Tryptone Soya Broth (TSB; Scharlab Chemie S.A., Barcelona, Spain) incubated at 37 °C for 12 h under agitation. Then an aliquot of 100 μL was again inoculated in 10 mL of TSB, incubated at 37 °C for 12 h under agitation obtaining a suspension of 10^8 cfu/mL the cell concentration was confirmed by plate count in Tryptone Soya Agar (TSA; Scharlab Chemie S.A., Barcelona, Spain). Those cells were used in subsequent experiments.

2.3. Determination of antimicrobial activity

Bacteria were grown in TSB supplemented with different concentrations of carvacrol or citral. The terpenes were dissolved in dimethyl sulfoxide (DMSO, Sigma–Aldrich Chemie GmbH, Steinheim, Germany), as described by Firouzi et al. (1998), to give citral and carvacrol solutions of 0.100 μL/mL and 0.150 μL/mL, respectively. The concentration of each antimicrobial was chosen on the basis of previous studies of sublethal damage and growth kinetics (Belda-Galbis et al., 2011a,b; Silva-Angulo et al., 2012) and they represent up to 50% of the MIC range for carvacrol and citral, as previously described by Kim et al. (1995) using *L. monocytogenes* cultures as test organism. Briefly, the compound to be tested was added to 20 mL of TSB in sterile flasks containing 1 mL of cell suspension (10^8 cfu/mL) of an overnight culture of *L. innocua* and *L. monocytogenes* to obtain a cell concentration of 10^6 cfu/mL. After that the final antimicrobials concentrations were 0.100 and 0.150 μL/mL for citral and carvacrol respectively. Each culture was incubated under agitation for 30 h at 37 °C. The inhibitory activity of each compound tested was monitored by sampling at regular intervals of time and by a count of viable cells in TSA.

2.4. Determination of degree of injury of cells

After each interval of time, 0.1 mL of *L. innocua* and *L. monocytogenes* samples adequately diluted in sterile 0.1% (w/v) peptone water (Scharlau Chemie S.A., Barcelona, Spain) was pour-plated onto Tryptone Soya Agar (TSA; Scharlab Chemie S.A., Barcelona, Spain) supplemented with 0.6% yeast extract (TSA-YE) and onto TSA-YE selective medium with 5% (w/v) of sodium chloride added (TSA-YE-SC), in accordance with Arroyo et al. (2010).

The percentage of the number of $\log_{10}$ cycles of sublethally injured cells was estimated by the following equation (Busch and Donnelly, 1992; Dykes, 1999):

$$[1 - (\text{count on TSA} - \text{YE} - \text{SC}/\text{count on TSA} - \text{YE})]*100 \quad (1)$$

Samples recovered in selective medium and non-selective medium were incubated for 48 h at 37 °C. Then the number of colony forming units was counted by image analyser automatic counter. The error bars in the figures indicate the mean ± standard deviations from the data obtained from at least three repetitions.

2.5. C. elegans studies

To investigate possible virulence changes as a consequence of the exposure of *L. innocua* and *L. monocytogenes* to two antimicrobial substances, *L. innocua* and *L. monocytogenes* were maintained in contact with antimicrobials during the time at which the proportion of damaged cells was highest, according with the results obtained in the degree of injury section of this paper. Then the culture was recovered and washed by centrifugation in peptone