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S. Typhimurium virulence changes caused by exposure to different non-thermal preservation treatments using *C. elegans*

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ABSTRACT

The aims of this research study were: (i) to postulate *Caenorhabditis elegans* (*C. elegans*) as a useful organism to describe infection by *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium), and (ii) to evaluate changes in virulence of *S.* Typhimurium when subjected repetitively to different antimicrobial treatments. Specifically, cauliflower by-product infusion, High Hydrostatic Pressure (HHP), and Pulsed Electric Fields (PEF).

This study was carried out by feeding *C. elegans* with different microbial populations: *E. coli* OP50 (optimal conditions), untreated *S.* Typhimurium, *S.* Typhimurium treated once and three times with cauliflower by-product infusion, *S.* Typhimurium treated once and four times with HHP and *S.* Typhimurium treated once and four times with PEF.

Bayesian survival analysis was applied to estimate *C. elegans* lifespan when fed with the different microbial populations considered. Results showed that *C. elegans* is a useful organism to describe infection by *S.* Typhimurium because its lifespan was reduced when it was infected. In addition, the application of antimicrobial treatments repetitively generated different responses: when cauliflower by-product infusion and PEF treatment were applied repetitively the virulence of *S.* Typhimurium was lower than when the treatment was applied once. In contrast, when HHP treatment was applied repetitively, the virulence of *S.* Typhimurium was higher than when it was applied once. Nevertheless, in all the populations analyzed treated *S.* Typhimurium had lower virulence than untreated *S.* Typhimurium.

1. Introduction

Salmonella spp. are among of the most important foodborne pathogens, causing about 155,000 salmonellosis-related deaths each year worldwide (Majowicz et al., 2010). *Salmonellosis* is a zoonotic infection transmitted to humans through the fecal–oral route, the primary source of transmission being infected eggs and egg-derived products, meat, poultry, milk and milk-derived products, fruits and vegetables (Gómez-Aldapa et al., 2012). *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) and *S.* Enteritidis are currently the most usual serotypes related to salmonellosis outbreaks (20.2 and 39.5% of the reported cases, respectively) (EFSA, 2015).

Different kinds of preservation treatments have been developed to reduce or eliminate these pathogens in food products, including the addition of bioactive substances from nature or from agroindustrial by-products with antimicrobial effect (O'Shea et al., 2012; Viuda-Martos et al., 2008) or the application of non-thermal treatments such as High Hydrostatic Pressure (HHP) (Barbosa-Cánovas and Juliano, 2008; Rendueles et al., 2011) and Pulsed Electric Fields (PEF) (Mosqueda-Melgar et al., 2012; Saldaña et al., 2014). However, such treatments can have important drawbacks because their repeated use could generate serious antimicrobial resistance problems (Kisluk et al., 2013; Vanlint, 2013).

Nevertheless, the higher consumption of fresh fruits and vegetables recorded in recent years has been associated with an increase in foodborne disease outbreaks (Olaimat and Holley, 2012). This is a very important issue that requires much more research work on infection by foodborne pathogens and procedures to combat them, particularly *Salmonella* spp.

Caenorhabditis elegans (*C. elegans*) is a very useful experimental organism. It belongs to a nematode species that inhabits soils around the world (Hope, 1999). Its maintenance in the laboratory is easy and cheap because it has a short lifespan, approximately 21 days, with a hermaphroditic life cycle of around three days and a high quantity of

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offspring (more than 300). In addition, it has a transparent body, which allows observation of all of its organs, and it is fed with *Escherichia coli* cells as they are eaten by the worms in optimal laboratory conditions (Leung et al., 2008). Moreover, the main physiological processes and stressed responses of higher organisms such as humans are retained in *C. elegans*. For all of these reasons, *C. elegans* is a useful organism for analyzing the effects of foodborne pathogens in host organisms and studying their virulence factors.

The aim of this paper is twofold: (i) to describe the effect of *S.* Typhimurium on *C. elegans* organisms, and (ii) to evaluate the development of microbial resistance of *S.* Typhimurium to various antimicrobial treatments applied consecutively, as well as to assess the virulence of the changes observed. For both objectives, the lifespan of the nematodes was analyzed statistically by means of Survival Analysis, specifically Cox Proportional Hazards models (Cox and Oakes, 1984; Cox, 1992; Ibrahim et al., 2001). The first study was performed to assess the effect of *S.* Typhimurium on the host organism. The remaining three studies were conducted to quantify microbial resistance and virulence changes as a consequence of three different preservation treatments: cauliflower by-product infusion, HHP and PEF. Bayesian inference was used to estimate all models thus providing a suitable framework to deal with problems with multiple comparisons and many uncertainties. WinBUGS software (Lunn et al., 2000) was used to simulate the Markov chain whose stationary distribution is the relevant posterior distribution of the parameters of interest from which all relevant inferences are derived.

2. Material and methods

2.1. Bacterial strain

A freeze-dried pure culture of *S.* Typhimurium was provided by the Spanish Type Culture Collection (CECT 443). It was rehydrated with tryptic soy broth (TSB) (Scharlab S. A., Barcelona, Spain) and incubated at 37 ° C with continuous shaking (Model 6001173, Selecta Unitronic, Barcelona, Spain) for 14 h. Cells were later centrifuged (Beckman Avanti J-25, USA) twice at 2450 g and 4 ° C for 15 min and re-suspended in TSB. Finally, cells were transferred in TSB with 20% of glycerol and dispensed to a final concentration of 10^8 cfu/mL in 2 mL vials, which were frozen and stored at −80 ° C.

2.2. Cauliflower by-product infusion as a natural antimicrobial

Dehydrated cauliflower by-product (from leaves of cauliflower plants) was provided from primary production of TRASA S.L. It was washed in the laboratory to eliminate contaminants, and then dried, crushed, and homogenized (Brandi et al., 2006). Cauliflower by-product 5% (w/v) infusion was obtained by boiling it in buffered peptone water (0.1% (w/v)) for 30 min. The infusion was later centrifuged at 2450 g for 15 min at 4 ° C and filtered three times (Whatman filter 11 μm pore size, Whatman filter 2.5 μm and 0.45 μm PVDF syringe filter to sterilize, consecutively). 30 mL of cauliflower by-product infusion 5% (w/v) was inoculated with 1 mL *S.* Typhimurium overnight culture (10^8 cfu/mL) and was incubated at 37 ° C for 5 h. The procedure was performed three times. The *S.* Typhimurium population was recovered between each antimicrobial treatment by growing in TSB overnight with continuous shaking. The *S.* Typhimurium concentration was evaluated before and after each antimicrobial treatment by serial dilution in buffered peptone water (0.1% (w/v)) and plate count in TSA (Scharlau, Scharlab). The plates were incubated at 37 ° C for 24 h. The number of surviving microorganisms obtained after each treatment permits to evaluate the microbial resistance development. Differences in resistance to infusion were observed between subpopulation exposed once and three times to the antimicrobial (one log reduction and one log growth, respectively).

2.3. HHP treatment

S. Typhimurium with an initial concentration of 10^8 cfu/mL was treated with HHP (250 MPa — 5 min). This is a sublethal treatment to *S.* Typhimurium that causes, mainly, cellular damage (Sanz-Puig et al., 2017). Between HHP treatments, the bacteria were grown in TSB and continuously shaken at 37 ° C overnight to recover the damaged cells. The procedure was performed four times. Before and after HHP treatments, the microbial population was evaluated by serial dilution in buffered peptone water (0.1 % (w/v)) and plate count in TSA. The TSA plates were incubated at 37 ° C for 24 h. The resistance was determined in terms of number of survival microorganisms after each HHP consecutive treatment. As in the case of cauliflower infusion, differences in resistance were observed between subpopulations (2.5 and 0.7 log cycles of microbial reduction for *S.* Typhimurium exposed once and four times to HHP treatment). HHP treatments were applied using EPSI NV equipment (Temse, Belgium) (Pérez et al., 2007a).

2.4. PEF treatment

The initial *S.* Typhimurium population (10^8 cfu/mL) was subjected to PEF treatment (30 kV/cm — 300 μs). Between PEF treatments, the bacteria were grown in TSB overnight with continuous shaking at 37 ° C. The procedure was performed four times. Before and after each PEF treatment, the *S.* Typhimurium concentration was evaluated by plate count in TSA and serial dilution in buffered peptone water 0.1% (w/v) and the plates were incubated at 37 ° C for 24 h. The resistance was evaluated by the number of survival microorganisms after each consecutive PEF treatment. The first and the fourth treatment caused, respectively, 2.9 and 0.70 log cycles of microbial reduction. An OSU-4D batch-scale continuous PEF system, designed at Ohio State University, was used to treat the samples (Pérez et al., 2007b).

2.5. C. elegans studies

C. elegans strain N2 was provided by the College of Biological Sciences, Minnesota University, USA. It was maintained in optimal conditions: plates with Nematode Growth Medium (NGM) agar and a bacterial lawn of *E. coli* OP50 (Stiernagle, 2006). The microbial concentration in the lawn of NGM agar was checked in each treatment, measuring its absorbance and it was similar in all plates. *C. elegans* was used to evaluate the effect of *S.* Typhimurium on host organisms as well as possible changes of virulence in *S.* Typhimurium populations as result of repetitive exposure to various antimicrobial treatments: cauliflower by-product infusion, HHP and PEF. For this purpose, 250 synchronized young adult nematodes, distributed in 25 plates of 10 worms each, were transferred to NGM agar with a lawn of each of the *S.* Typhimurium populations under study: *S.* Typhimurium, *S.* Typhimurium treated once and three times with cauliflower by-product infusion, *S.* Typhimurium treated once and four times with HHP, and *S.* Typhimurium treated once and four times with PEF. The worms were maintained at 20 ° C throughout all their lifespan (approximately three weeks), examined with a binocular microscope (COMECTA S.A.) at 48 hour intervals, and then transferred to new plates. They were considered dead when they did not move or respond to stimulation. All the experiments included a negative control with *C. elegans* in NGM plates with an *E. coli* OP50 lawn.

2.6. Modeling strategy

The lifespan of *C. elegans* was analyzed with regard to the different *S.* Typhimurium populations considered. Lifespan was defined for each *C. elegans* individual as the time (in days) from the start of the experiment until it was dead. The design of the study focused on time-to-event data which are typically analyzed in the framework of Survival Analysis (Klein and Moeschberger, 2003). Statistical survival modeling is mainly