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

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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*.

Keywords: Antimicrobial; Bayesian survival analysis; High Hydrostatic Pressure; Pulsed Electric Fields.

1. Introduction

Salmonella spp. are among of the most important foodborne pathogens, causing about 155000 salmonellosis-related deaths each year worldwide (Majowicz et al., 2010). *Salmonellosis* is a zoonotic infection transmitted to humans
5 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
10 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
15 non-thermal treatments such as High Hydrostatic Pressure (HHP) (Barbosa-Cánovas et al., 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).

20 Nevertheless, the higher consumption of fresh fruits and vegetables recorded in recent years has been associated with an increase in foodborne disease out-

breaks (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.

25 *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 offspring (more than 300). In addition, it has
30 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
35 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
40 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
45 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
50 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
55 Type Culture Collection (CECT 443). It was rehydrated with tryptic soy broth
(TSB) (Scharlab S. A., Barcelona, Spain) and incubated at 37°C with contin-
uous 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
60 with 20% of glycerol and dispensed to a final concentration of 10⁸ 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 lab-
65 oratory 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
70 to sterilize, consecutively). 30 mL of cauliflower by-product infusion 5% (w/v)
was inoculated with 1 mL *S. Typhimurium* overnight culture (10⁸ cfu/mL)
and was incubated at 37°C for five hours. The procedure was performed three
times. The *S. Typhimurium* population was recovered between each antimicro-
bial treatment by growing in TSB overnight with continuous shaking. The *S.*
75 *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
hours. The number of surviving microorganisms obtained after each treatment
permits to evaluate the microbial resistance development. Differences in resis-
80 tance 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 five minutes). This is a sublethal treatment to *S. Ty-*
85 *phimurium* 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
90 count in TSA. The TSA plates were incubated at 37°C for 24 hours. 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
95 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 μ s). Between PEF treatments, the bacteria were
100 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 hours. The resistance was evaluated by the number of survival microorganisms after
105 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

110 *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 a 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 characterized by censoring issues. In our study, the survival experiment accounted for interval and right-censored data. Most of the data were interval-censored: the lifespan of each individual was not known exactly, and the only relevant information about it was that its death occurred between two consecutive monitoring times. Nevertheless, right-censored data was used when the individual died accidentally during the process of changing it from one plate to another one.

The basic probabilistic elements to describe the stochastic behavior of time-to-event data are the survival function and the hazard function. The survival function, $S(t)$, is the probability that an individual survives longer than a given
145 instant in time, $S(t) = P(T > t)$, $t > 0$. The hazard function, $h(t)$, is a temporal rate, not a probability (Andersen et al., 2012), but it can be interpreted approximately as the chance that a living individual dies instantaneously.

The standard Cox proportional hazards (CPH) model (Cox and Oakes, 1984; Cox, 1992) was used to analyze the lifespan of *C. elegans* for each of the populations considered. This model is expressed in terms of the hazard function of the time-to-event of interest which factorizes into the product of a baseline hazard function, $h_0(t)$, and an exponential term that assesses the relative rate associated with a set of covariates $\mathbf{x}$ (expressed as the linear combination $\mathbf{x}'\boldsymbol{\beta}$) with regression coefficients $\boldsymbol{\beta}$,

$$h(t) = h_0(t) \exp\{\mathbf{x}'\boldsymbol{\beta}\}.$$

We carried out four different CPH analyses according to the type of food with which *C. elegans* was fed. Model 1 is the simplest and describes the effect
150 of *S. Typhimurium* on the *C. elegans* organism. Models 2, 3 and 4 analyze the lifespan of *C. elegans* with regard to the level of virulence of *S. Typhimurium* resulting from the application, in different doses, of antimicrobial treatments based on cauliflower by-product infusion, HHP and PEF, respectively. All the survival models were constructed with Weibull baseline hazard functions and
155 indicator covariates for the type of food population considered.

The statistical analysis of the CPH models was approached from Bayesian inference, which provides a powerful and flexible methodology to quantify all the uncertainties of interest in probabilistic terms and to combine all sources of information (Ibrahim et al., 2001). We assumed prior independence and elicited
160 wide proper prior distributions in order to give all inferential prominence to the data.

The posterior distribution of the parameters of each model was approximated by means of Markov chain Monte Carlo methods (MCMC) using WinBUGS

software (Lunn et al., 2000). The settings were three Markov chains of 33000
 165 values each after discarding the first 3000 values in order to reach convergence
 to the posterior distribution. The chains were thinned by storing every three
 iterations thereby reducing the autocorrelation in the sample. Trace plots of
 the simulated values of the chains appeared to overlap one another indicating
 stabilization. Convergence of the chains to the posterior distribution was as-
 170 sessed through the potential scale reduction factor and the effective number of
 independent simulation draws (Gelman and Rubin, 1992; Kass et al., 1998).

3. Results and discussion

The content of this section has been arranged in four parts according to
 the four studies carried out that compare the lifespan of *C. elegans* between
 175 treated and untreated *S. Typhimurium* (control group). All results are presented
 in terms of posterior quantities concerning median lifespan (time from which
 50% of individuals are still living and the other half have died) and survival
 probabilities in order to improve their interpretability.

3.1. *S. Typhimurium*

180 The effect of *S. Typhimurium* infection on *C. elegans* was evaluated via the
 posterior distribution of the median of the lifespan of the worms according to
 the type of feed, *S. Typhimurium* and their optimal food *E. coli* OP50, as well
 as their subsequent posterior survival probabilities.

Figure 1a shows mean and 95% credible interval for the posterior distribution
 185 of *C. elegans* median lifespan for both populations. *C. elegans* posterior mean
 lifespan in optimal conditions is around 6.39 days. This was reduced to about
 4.82 days when the nematodes were fed with *S. Typhimurium*. This value is
 very close to five days, which is the time required by *S. Typhimurium* to achieve
 complete infection of the intestinal lumen of *C. elegans*, as described in other
 190 research studies (Labrousse et al., 2000; Aballay et al., 2000). The uncertainty of
 the estimates is assessed through credible intervals for the relevant parameters;

Figure 1: (a): Posterior expected median lifespan and 95% CI of *C. elegans* fed with *E. coli* OP50 (grey) and with *S. Typhimurium* (black); and (b): posterior expected survival probabilities of *C. elegans* fed with *E. coli* OP50 (grey) and *S. Typhimurium* (black).

the 95% credible intervals (95% CI) for the effect of each food indicate that the two distributions cover completely different range of values: [5.68, 7.15] days for *E. coli* and [4.27, 5.42] for *S. Typhimurium*. This provides strong evidence
195 that worms fed with *S. Typhimurium* have a shorter lifespan than those fed in optimal conditions (*E. coli* OP50), such as it has been shown in other studies as (Labrousse et al., 2000). **A result**, *C. elegans* seems to be able to highlight the virulence effect of *S. Typhimurium*.

Figure 1b presents the posterior mean of the survival probabilities for *C. elegans* fed with each of the microbial populations considered. It is easy to see
200 they show clear differences between the two survival trajectories: probabilities associated with *C. elegans* fed with *S. Typhimurium* are always lower than the corresponding ones for *C. elegans* fed with *E. coli* OP50. In particular, survival probabilities estimated at days 2, 12, and 22 from the start of the experiment
205 are 0.84, 0.22, and 0.05 for *E. coli*, and 0.78, 0.12 and 0.01 for *S. Typhimurium*, respectively. These results support the idea that survival probabilities of *C. elegans* are always lower in all their life stages when it is infected with *S. Ty-*

phimurium.

3.2. *S. Typhimurium* with cauliflower by-product infusion

210 The next step was to evaluate virulence shifts in *S. Typhimurium* resulting from repetitive exposure to cauliflower by-product infusion. Cauliflower by-product infusion contained bioactive compounds, as a ascorbic acid, carotenoids, flavonoids and, mainly, polyphenols such as galic acid, quercetin or rutin (Sanz-Puig et al., 2015; Vieira, 2013). To accomplish this goal, *C. elegans* were fed
215 with *S. Typhimurium* exposed once and three times to cauliflower by-product infusion during their entire lifespan, and subsequently compared with *C. elegans* fed with untreated *S. Typhimurium* (control group).

Figure 2a shows the posterior expected *C. elegans* median lifespan and 95% CI for each of the two cauliflower by-product infusion treatments (**Inf1** and **Inf3**
220 for one and three doses, respectively) as well as for the control group (**St**). The estimated median lifespan values for *C. elegans* with treatments **Inf1** and **Inf3** are similar, 7.14 and 6.85 days respectively. This is not the case for treatment **St**, with an estimated value of 4.96 days, which is, as expected, very similar to the one obtained in the previous model. Not only are the punctual estimates
225 for treated and untreated *S. Typhimurium* very different but also so are their respective 95% CI values. Specifically, neither of the **Inf1** ([6.37, 7.90]) and **Inf3** ([6.20, 7.70]) credible intervals overlaps with the one corresponding to **St** ([4.43, 5.48]. Hence, when *S. Typhimurium* is exposed to cauliflower by-product infusion repetitively the lifespan increases, so *S. Typhimurium* becomes less
230 virulent than usual, with lower infection effects. This is justified because the posterior probability that the **Inf1** median lifespan is higher than the one for **Inf3** is 0.631, a value that supports a conclusion of identical virulence.

Figure 2b presents the estimated survival curves for *C. elegans* fed with microbial populations **Inf1**, **Inf3**, and **St**. It is hard to distinguish between
235 survival prospects related the two cauliflower treatments. In fact, their respective estimated survival probabilities at 2, 12 and 22 days are 0.87, 0.26, 0.06 (**Inf1**), and 0.86, 0.25, 0.05 (**Inf3**). With regard to the control group, the sur-

Figure 2: (a): Posterior expected median lifespan and 95% CI of *C. elegans* fed with untreated *S. Typhimurium* (in black and represented as **St** on the horizontal axis), *S. Typhimurium* treated once with cauliflower by-product infusion (**Inf1** in gray) and *S. Typhimurium* treated three times with cauliflower by-product infusion (**Inf3** in black dashed); and (b): posterior expected survival probabilities of *C. elegans* fed with **St** (black), **Inf1** (grey), and **Inf3** (black dashed).

vival trend is lower in relation to both cauliflower treatments, with estimated survival probabilities of 0.80, 0.12, and 0.01 at days 2, 12 and 22, respectively. Therefore, when *S. Typhimurium* is treated with cauliflower by-product infusion its virulence decreases and its infection causes lower effects on *C. elegans* than the ones for control *S. Typhimurium*, thus increasing its survival probabilities throughout its lifespan. However, there are not great differences between **Inf1** and **Inf3**. It may therefore be concluded that the application of this antimicrobial treatment repetitively does not seem to have consequences that affect the virulence of *S. Typhimurium* .

3.3. *S. Typhimurium* with HHP

Virulence changes in the *S. Typhimurium* microbial population after the application of HHP preservation treatment are now discussed. *C. elegans* were fed with *S. Typhimurium* treated once and four times with HHP and with untreated *S. Typhimurium*, which was also considered as the control group in this case.

Figure 3a displays the posterior expectation and 95% CI of the posterior distribution of *C. elegans* median lifespan for both HHP treatments (**HHP1** and **HHP4** for frequencies one and four, respectively), and the control group (**St**). *C. elegans* has an estimated median lifespan of 6.44 and 5.81 days in the case of **Hhp1** and **HHP4**, respectively, and 5.15 days for **St**. The 95% CI related to **St** does not overlap the corresponding one for **Hhp1** ([4.61, 5.68] and [5.81, 7.07], respectively). This is not the case for the comparison between **St** and **HHP4** where a clear overlap can be seen ([5.24, 6.38] is the 95% CI for **HHP4**). Nevertheless, there is a high probability, 0.97, associated with a higher effect of **HHP4** with regard to **St**. In the case of the two HHP treatments the overlap is obvious. Direct comparison between them indicates that **Hhp4** increases the virulence of bacteria with regard to **HHP1** with a probability of 0.92.

We can conclude that the application of HHP antimicrobial treatment against *S. Typhimurium* made it less virulent against *C. elegans* than in the case of the control group. However, when HHP treatment was applied repetitively (four

Figure 3: (a): Posterior expected median lifespan and 95% CI of *C. elegans* fed with untreated *S. Typhimurium* (in black and represented as St on the horizontal axis), *S. Typhimurium* treated once with HHP (HHP1 in grey) and *S. Typhimurium* treated four times with HHP (HHP4 in black dashed) ; and (b): posterior expected survival probabilities of *C. elegans* fed with St (black), HHP1 (grey), and HHP4 (black dashed).

times) the virulence of *S. Typhimurium* increased in comparison with *S. Typhimurium* treated once, although it did not achieve the virulence of untreated *S. Typhimurium*. Although, as far as we concern, there are very few scientific studies about it, the microbial changes produced for recover the cell damage after each treatment could be the responsible of the development of a major microbial virulence. In fact, studies like [Van Boeijen et al. \(2013\)](#) carried out with *Listeria monocytogenes* high pressure-resistant variants concluded that some variants showed a reduction in microbial virulence and others retained full virulence in a mouse model. The full virulence [phenotype](#) showed in the mouse model could be due to the reversion of part of the microbial population to normally cells. These results could open the door to new research studies about the effect of successive applications of HHP treatment on the virulence of *S. Typhimurium* and the subsequent comparison with results for untreated *S. Typhimurium*.

Estimated survival curves for St, HHP1 and HHP4 were based on the poste-

rior median of *C. elegans* lifespan (see Figure 3b). The survival prospects are in line with the result discussed in the previous paragraph. The lowest survival
285 probabilities are related to the control group (**St**), with estimated survival probabilities of 0.81, 0.12, and 0.01 at days 2, 12, and 22, respectively. The middle survival trend is connected with the **HHP4** group, with estimated survival probabilities of 0.86, 0.21, and 0.03 at days 2, 12 and 22, respectively, and the highest one with group **HHP1**, with probabilities of 0.84, 0.17 and 0.02 estimated at the
290 same days as before. All the survival curves look very similar.

3.4. *S. Typhimurium* with PEF

Finally, the effect of the application of PEF application on the virulence of *S. Typhimurium* against *C. elegans* was considered. *C. elegans* was fed with *S. Typhimurium* treated once and four times with PEF and with untreated *S. Typhimurium* (control group).
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There are considerable differences between the estimated median *C. elegans* lifespan values for each of the two PEF treatments (**PEF1** and **PEF4** for frequencies one and four, respectively) (see Figure 4a). The expected median lifespan related to **PEF1** is 5.14 days, quite similar to the one for the **St** group, 4.82
300 days. The 95% CI values for **PEF1** and **St** are [4.55, 5.72] and [4.27, 5.36] days, respectively and the posterior probability that the effect of **PEF1** is higher than the one for **St** is 0.20, not a value that would justify assuming differences between them. Consequently, the *S. Typhimurium* survivor population after one application of PEF shows similar virulence to the untreated population. With
305 regard to the **PEF4** median lifespan, it is noteworthy that the *S. Typhimurium* survivor population after four applications of pulsed electric fields shows the lowest virulence: the 95% CI, [5.92, 7.38] days, has the highest values and does not overlap with the 95% CI for either **St** or **PEF1**. The application of PEF treatment once seems to have no great effect on the microbial virulence of *S. Typhimurium* against *C. elegans*. In contrast, the repetitive application of PEF
310 treatment makes *S. Typhimurium* less virulent against *C. elegans*.

Posterior survival probabilities are displayed in Figure 4b. Survival probabil-

Figure 4: (a): Posterior expected median lifespan and 95% CI of *C. elegans* fed with untreated *S. Typhimurium* (in black and represented as St on the horizontal axis), *S. Typhimurium* treated once with PEF (PEF1 in grey), and *S. Typhimurium* treated four times with PEF (PEF4 in black dashed); and (b): posterior expected survival probabilities of *C. elegans* fed with St (black), PEF1 (grey), and PEF4 (black dashed).

ities corresponding to St and PEF1 are very similar with estimated probabilities of 0.78, 0.12, 0.01 and 0.80, 0.15, 0.02 at days 2, 12 and 22, respectively. Survival probabilities related to PEF4 show a different trend with respect to the other two treatments. PEF4 survival probabilities are a little higher throughout the lifespan of *C. elegans*, with particular probability values of 0.85, 0.24, 0.05 at day 2, 12 and 22, respectively.

4. Conclusions

The results of this research show that *C. elegans* can be postulated as a useful organism to describe the reduction in lifespan caused by infection by *S. Typhimurium*. In addition, the application of different antimicrobial treatments repetitively had different effects on the virulence of *S. Typhimurium*. In all cases, treated *S. Typhimurium* was less virulent than untreated *S. Typhimurium*. Nevertheless, when *S. Typhimurium* was treated repetitively with

cauliflower by-product infusion the virulence was kept low after repetitive treatment and when it was treated by PEF, its virulence was lower than when it was treated once. However, *S. Typhimurium* treated repetitively with HHP had higher virulence than *S. Typhimurium* treated once with HHP.

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