1 Resistance changes in *Salmonella enterica* serovar Typhimurium treated by high hydrostatic
2 pressure and pulsed electric fields and assessment of virulence changes by using
3 *Caenorhabditis elegans* as a test organism

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20

21 **Abstract**

22 The goal of this study was to evaluate the development of *Salmonella enterica* subsp
23 *enterica* serovar Typhimurium resistance against Pulsed Electric Fields (PEF) and High
24 Hydrostatic Pressure (HHP), and to study the possible virulence changes of the resistant
25 subpopulations using *Caenorhabditis elegans*. Results showed that *S.* Typhimurium
26 became resistant to both non-thermal technologies after four consecutive treatments.
27 Survival probability of the worms (*C. elegans*) increased significantly when they were
28 fed with *S*. Typhimurium treated by PEF four consecutive times in comparison with the
29 other two subpopulations, untreated and treated once.For HHP, results indicated that up
30 to percentile 50%, the survival probability of worms fed with treated microorganisms
31 (once and four times) was greater than that of worms fed with untreated ones. Also, the
32 *C. elegans* egg-laying pattern was modified and there were significant differences in the
33 number of eggs laid in the first two days among the three subpopulations studied.
34 Consequently, although *S*. Typhimurium develops microbial resistance against PEF or
35 HHP treatments, when it is applied repeatedly, its virulence against *C. elegans* appears
36 to decrease.

37

38 **Industrial relevance**

39 Among the non-thermal technologies, Pulsed Electric Fields and High Hydrostatic
40 Pressure have a great potential. Nevertheless, it is necessary to validate them from a
41 safety point of view because of the huge amount of damaged cells that can be obtained
42 if sublethal treatments are applied. These studies have industrial relevance in food
43 safety ensuring proper processing when using these non-thermal technologies.

44

Keywords

Pulsed Electric Fields (PEF); High Hydrostatic Pressure (HHP); sublethal damage; *Salmonella*; *C. elegans*

Highlights

- Consecutive sublethal treatments by PEF produce resistance in *S*. Typhimurium
- Consecutive sublethal treatments by HHP produce resistance in *S*. Typhimurium
- Damaged *S*. Typhimurium does not appear to be more virulent after being repaired
- *C. elegans* may be a good test organism to detect virulence changes in damaged cells
- Resistance generation does not imply more virulence

1. Introduction

Nowadays, consumers demand safe and healthier food products with higher quality levels. Therefore, food manufacturers and research groups have developed new technologies for food preservation with the aim of maintaining the original organoleptic and nutritional properties of foodstuffs while keeping them safe (Otunola et al., 2008).

Many non-thermal technologies have been developed, such as ionizing radiation, ultraviolet light, ozone, High Hydrostatic Pressure (HHP), and Pulsed Electric Fields (PEF) (Barba et al., 2017; Misra et al., 2017; Pan et al., 2017; Pottier et al., 2017). Among them, Pulsed Electric Field treatment is perceived as a promising cool treatment alternative to conventional thermal pasteurization for liquid products (Saldaña et al., 2014), and High Hydrostatic Pressure (HHP) is at present one of the most successful non-thermal technologies that are applied today in cool preservation of foods. Both technologies can achieve enough reductions in the microbial population of food, using lower temperatures than those traditionally used in thermal pasteurization (Rendueles et al., 2011).

However, as has been described for other mild treatments (antimicrobials, low temperature treatments), microorganisms treated by PEF or HHP could develop resistance when they receive consecutive sublethal doses (Kostyanev et al., 2015; Laxminarayan et al., 2013; Vanlint et al, 2013; Kisluk et al., 2013). This resistance can be the result of transient variations in gene expression, or be the sporadic emergence and subsequent selection of spontaneous mutants with a permanently resistance. In any case, resistant microbial populations might pose a risk to consumers, particularly if the acquired resistance becomes stable, because these subpopulations are new and their virulence is unknown (Capita et al., 2013). Consequently, it appears interesting to study the development of microbial resistance (without differentiate between stable or

83 transient adaptation) against mild control measures, and possible changes in pathogen
84 virulence.
85 One of the most important groups of foodborne pathogens contaminating raw food is
86 *Salmonella* spp. It is the most frequent cause of foodborne outbreaks (22.5%), eggs and
87 egg products being the main contributors (44.9%) (EFSA, 2016). Also, salmonellosis is
88 the second most frequent zoonotic disease in the European Union, with 94,625 cases in
89 2015. The most frequent serotypes are *Salmonella enterica* subsp *enterica* serovar
90 Enteritidis and serovar Typhimurium, with 39.5% and 20.2% of confirmed cases,
91 respectively (EFSA, 2016).
92 Therefore, it is important to evaluate the possible resistance developed by *S.*
93 Typhimurium against alternative preservation treatments because many raw materials
94 can be contaminated by this microorganism and are used in food preparation and
95 production. It is also important to know whether these sublethal treatments could induce
96 virulence changes. A novel option could be to use the nematode *Caenorhabditis elegans*
97 as a test organism. It belongs to nematode species. Its body is transparent which allows
98 observation of most of internal organs and most of the main physiological processes and
99 stressed responses of higher organisms like those that humans are preserved in *C.*
100 *elegans.* It is easy and economical to use in optimal laboratory conditions due to its
101 short lifespan and it has been successfully employed in some studies (Chai-Hoon *et al.,*
102 2010; Silva et al., 2015; Ewbank et al., 2011).
103 The aim of this study was to evaluate the microbial resistance against PEF and HHP
104 treatment developed by *Salmonella enterica* serovar Typhimurium, and to study the
105 possible virulence changes of the resistant subpopulations by using *C. elegans* as a test
106 organism.
107

108 **2. Material and methods**

109 2.1 Microbial strain

110 The freeze-dried *S.* Typhimurium was provided by the Spanish Type Culture Collection
111 (CECT 443). The pure culture was rehydrated with tryptic soy broth (TSB) (Scharlab
112 Chemie) and incubated with continuous shaking (Selecta Unitronic) for 14 h at 37 °C to
113 obtain a stock of cells. Then the cells were centrifuged (Beckman Avanti J-25) twice at
114 2450 g and at 4 °C for 15 min. After centrifugation, the cells were resuspended in TSB
115 with 20% glycerol and dispensed in 2 mL vials to a final concentration of 10^8 cfu/mL.
116 The cryovials were frozen and stored at –80 °C until needed.

117

118 2.2 PEF treatment against *S.* Typhimurium

119 On the basis of previous studies (Sanz-Puig et al., 2016), a *S.* Typhimurium overnight
120 culture (10^8 cfu/mL) coming from a defrozen cryovial was used to inoculate 500 mL
121 TSB (Scharlab Chemie). It was PEF treated at 30 kV/cm for 300 μs, with bipolar, 2.5 μs
122 width squared-pulses as an intermediate sublethal treatment that caused 2.5 log cycles
123 of cellular reduction in *S.* Typhimurium and great sublethal damage. These studies were
124 carried out in triplicate by using an OSU-4D laboratory-scale system as described in
125 Sanz-Puig et al., (2016).

126

127 2.3 HHP treatment against *S.* Typhimurium

128 As for PEF treatment, a *S.* Typhimurium overnight culture (10^8 cfu/mL) coming from a
129 defrozen cryovial was used to inoculate TSB. Based on previous studies (Sanz-Puig et
130 al., 2017) it was treated by HHP at 250 MPa for 5 minutes. These studies were carried
131 out in triplicate. HHP treatments were applied using an EPSI NV unit (Temse, Belgium)
132 as described in Pina-Pérez et al., (2007).

133

134 2.4 Evaluation of development of microbial resistance

135 To evaluate the development of *S.* Typhimurium microbial resistance against PEF or
136 HHP treatments, an initial population of this microorganism (10^8 cfu/mL) was PEF or
137 HHP treated repeatedly (4 times) as described in previous sections. After each PEF or
138 HHP treatment, 1 mL of the treated sample was incubated overnight in 500 mL TSB
139 (15h) with continuous shaking at a temperature of 37 °C. Afterwards, the microbial cells
140 were recovered by centrifugation (2450 g for 15 min), and inoculated in TSB for the
141 subsequent PEF or HHP treatment. Before and after each treatment, the concentration of
142 *S.* Typhimurium was calculated by plate count in TSA (Scharlau, Scharlab). Microbial
143 resistance experiments were done in triplicate with three replicas for each repetition.

144

145 2.5 *C. elegans* studies

146 *C. elegans* was provided by the College of Biological Sciences, Minnesota University,
147 USA. Its optimal conditions for growth in the laboratory are plates of Nematode Growth
148 Medium (NGM) agar, with a bacterial lawn of *E. coli* OP50 at 20 °C (Stiernagle, 2006).
149 To evaluate the virulence changes of the selected *S.* Typhimurium subpopulations, the
150 microbial lawn of *E. coli* OP50 was replaced by a lawn of *S.* Typhimurium as a control,
151 and *S.* Typhimurium treated once and four times by PEF or HHP to obtain resistant
152 subpopulations. The *C. elegans* behavior was monitored, focusing on its survival and
153 eggs laid when fed with the various *S.* Typhimurium subpopulations.
154 Survival studies were carried out with 250 nematodes, distributed in 25 plates (5
155 repetitions of 5 plates) with 10 synchronized nematodes in each plate, which were fed
156 with untreated *S.* Typhimurium and with *S.* Typhimurium treated once and four times
157 by PEF or HHP. At regular intervals of 48 hours all plates were examined with a

binocular microscope (COMECTA S.A.), and the number of live worms was counted. A worm was considered dead when it did not move and did not respond to stimulation. For egg laying studies, three sets of 25 plates with one nematode per plate were used. The worms on the three sets of 25 plates were fed with a lawn of one of the three selected *S.* Typhimurium subpopulations. The eggs laid were analyzed at regular intervals of 48 hours by counting the number of eggs that each worm laid.

2.6 Statistical analysis

Statgraphics Centurion XII software (StatPoint Technologies, Inc., Warrenton, VA, USA) was used to analyze results obtained by plate count for the development of *S.* Typhimurium resistance against PEF or HHP treatments, and the results obtained for *C. elegans* were analyzed by calculating the average and standard deviation. A Kaplan-Meier analysis was carried out to obtain the survival probability and hazard function for *C. elegans*, In addition, ANOVA, Friedman and Kruskal-Wallis analyses were carried out where necessary to evaluate significant differences (p-value <0.01) in *C. elegans* response when fed with different *S.* Typhimurium subpopulations.

3. Results and discussion

3.1 Development of *S.* Typhimurium resistance against PEF or HHP treatments

After consecutive treatment by PEF or HHP, resistance of *S.* Typhimurium was evaluated, focusing on the number of surviving microorganisms after each consecutive treatment. The results for PEF treatments are shown in Figure 1 (a) as the average of three independent PEF treatments with three replica for each treatment. The first treatment produced 2.91 log cycles of inactivation, the second and the third treatment produced lower inactivation levels, 1.23 and 0.57 log cycles, respectively, and the

183 fourth treatment caused 0.73 log cycles of inactivation, slightly greater than the third
184 treatment but without significant differences (p-value < 0.05) between them. These
185 results indicate a possible generation of resistance against PEF until the third
186 consecutive treatment, at which point the resistance stabilized, as can be deduced from
187 the results for the fourth treatment.
188 Other studies (Sagarzazu et al. 2013) have indicated that successive PEF treatments
189 with intermittent outgrowth of survivors selected mutants with increased resistance in
190 comparison to the non-stressed parental strain. Sagarzazu et al. (2010) and Arroyo et al.
191 (2010) studied transient resistant development immediately after sublethal stress
192 exposure/adaption because of the operation of stress responses. In the present work,
193 repetitive sublethal treatments achieved an increased resistance, which may be due to
194 permanent or transitory changes.
195 Figure 1 (b) shows the inactivation (log cycles) caused in *S.* Typhimurium after
196 consecutive HHP treatments of 250 MPa for 5 minutes. As can be seen in Figure 1 (b), a
197 reduction of 2.62 log cycles was achieved after the first treatment, the second HHP
198 treatment caused 1.8 log cycles of microbial reduction, the third treatment produced
199 0.76 log cycles of bacterial reduction, and, finally, with the fourth treatment only 0.67
200 log cycles of *S.* Typhimurium reduction was achieved. According to the results
201 obtained, it appears that *S.* Typhimurium also developed microbial resistance to HHP
202 treatment when it was applied consecutively (four times). These results are in agreement
203 with results obtained with some strains of *E. coli* by Vanlint et al. (2012). Buzrul (2014)
204 and Fioretto et al. (2005) also suggested that HHP treatments applied consecutively
205 without a recovery step to food products could increase microbial resistance to pressure
206 and temperature.

The results obtained in this study, using these two preservation technologies applied at sublethal doses, appears to indicate that a microbial resistance can be developed. Further studies should be carried out to clarify if this resistance is temporal or transient, as indicated by Gayán et al (2016). At the same time, for further studies it should be taken into account that each system (microorganism, technology, food product) is different, allowing different behavior and heterogeneity of stressed foodborne pathogens in relation to the different preservation technologies.

3.2 Evaluation of changes in *S.* Typhimurium virulence using *C. elegans*

Some authors have indicated that the application of specific treatments, given at sublethal intensity, could produce subpopulations of microbial cells that are not inactivated, remaining damaged, and that may finally recover and grow, becoming mutant cells with unknown virulence (Zimmermann et al., 1974; Garcia et al., 2005; Soliva-Fortuny et al., 2009; Puértolas et al., 2012).

In this study, the possibility that these mild treatments may also induce microbial virulence changes was studied by using *C. elegans* as a test organism. The nematode was fed with untreated *S.* Typhimurium and with *S.* Typhimurium treated once and four times by PEF or HHP, and the surviving worms were counted at regular time intervals of 48 hours during 21 days for each replication (5 replications). Survivor data were analysed using the Kaplan-Meier test, which provides the survival probability (figure 2). As can be seen in figure 2 (a), it seems that *C. elegans* fed with *S.* Typhimurium treated four times by PEF has a greater survival probability during its life than the worms fed with the other *S.* Typhimurium subpopulations. Nematodes fed with untreated and once-treated *S.* Typhimurium had a very close survival probability.

231 The Friedman test was used to evaluate the differences between the three
232 subpopulations at 90% significance level. No significant differences (p-value ≥ 0.01)
233 were obtained between nematodes fed with untreated *S*. Typhimurium and with *S*.
234 Typhimurium treated once by PEF. However, there were significant differences (p-
235 value < 0.05) between *C. elegans* fed with *S*. Typhimurium treated four times by PEF
236 and the other two subpopulations.
237 The Kaplan-Meier analysis also provides information about the percentage of worms
238 surviving at a specific time. Table 1 shows the estimated survival days for 20%
239 percentile of nematodes. These data confirm that there were not significant differences
240 between nematodes fed with untreated and treated once *S.* Typhimurium and, in
241 contrast, there were significant differences between nematodes fed with *S.*
242 Typhimurium treated four times with PEF and the other subpopulations. As a matter of
243 fact, in the percentile 20%, nematodes fed with untreated and treated once *S.*
244 Typhimurium subpopulations achieved this percentile at day 10 and 11, respectively,
245 whereas the nematodes fed with *S.* Typhimurium treated four times achieved this
246 percentile at 13,3 days.
247 As for the effect of HHP on virulence changes, figure 2 (b) shows the Kaplan-Meyer
248 analysis for survival data when nematodes were fed with the different *S.* Typhimurium
249 subpopulations, untreated, treated once, and treated four consecutive times with HHP.
250 As can be seen in this figure, the survival probability decreased during the life of the
251 nematodes, and almost all the nematodes were dead at 18.6 days. The nematodes fed
252 with *S.* Typhimurium treated by HHP showed a greater survival probability in the first
253 time intervals of their life cycle than the nematodes fed with untreated *S.* Typhimurium
254 (p-value ≤ 0.01). Nevertheless, the Friedman test analysis revealed that there were no

255 significant differences (p-value > 0.01) in survival data between nematodes fed with
256 different *S*. Typhimurium subpopulations at the end of their life cycle.
257 On the same way, Table 2 shows the percentiles for lifespan of *C. elegans* fed with *S*.
258 Typhimurium untreated and treated by HHP once and four times. As can be seen, the
259 50% of nematodes was died at day 4th or 6th depending on if they were fed with
260 untreated or treated *S*. Typhimurium, respectively. This confirms that the survival
261 probability of nematodes was greater in the first time intervals (up to 50%) when they
262 were fed with treated *S*. Typhimurium. This difference disappears when survival worm
263 population is lower than 25%.
264 Some authors have reported that *C. elegans* dies earlier when it is infected by *S.*
265 Typhimurium than in optimal conditions because persistent *S.* Typhimurium infection
266 colonizes the intestinal lumen and the bacterial cells increase whereas the intestinal cells
267 decrease (Aballay et al., 2000; Labrousse et al., 2000; Aballay et al., 2002). Aballay et
268 al. (2000) reported that 50% of the nematodes died in the first four days of *S.*
269 Typhimurium infection. These results are in agreement with those obtained in the
270 present work for untreated *S.* Typhimurium. When the nematodes become older, they
271 start losing their intestinal immunity and pathogen cells are accumulated, causing a
272 reduction in their lifespan (Portal-Celhay et al., 2012). Also, the nematode's pharynx is
273 a neuromuscular pump that controls the amount of bacteria that reaches the intestine,
274 and when the nematode ages, the pharynx loses its capacity, and the number of
275 microbial cells that reach the intestinal lumen can be higher, contributing to an increase
276 in the death risk (Avery, 1993).
277 If we compare the effects of PEF and HHP on *S.* Typhimurium, it seems clear that in
278 both cases there is an increase in resistance as the microorganism is subjected to
279 repeated treatments, but the effect of the PEF- or HHP-treated subpopulations on the

280 nematode is somewhat different for the two technologies, probably indicating different
281 mechanisms of cell damage. These differences in the survival data of nematodes
282 exposed to different subpopulations of *Salmonella* are more patent in the case of PEF-
283 treated *Salmonella* than in the subpopulations treated by HHP.
284 The higher survival probability of the nematodes fed with bacteria treated four times
285 appears to indicate that there is no direct relationship between an increase in resistance
286 and an increase in virulence. This is a very relevant conclusion for the food preservation
287 industry because it is an important factor to bear in mind with regard to the safety of
288 PEF or HHP technology.
289 Possible virulence changes were also tested by considering the number of eggs laid by
290 *C. elegans* as a complementary test. The number of eggs laid by *C. elegans* fed with *S.*
291 Typhimurium untreated and treated once and four times by PEF (figure 3 (a)) or HHP
292 (figure 3 (b)) was monitored every 48 hours. *C. elegans* lays eggs throughout its
293 lifespan when it grows in optimal conditions, although the number of eggs laid is higher
294 in the first stages and decreases during its life cycle. However, according to the results
295 of the present study, when the nematodes are infected by pathogenic bacteria the egg-
296 laying pattern may alter and they may lay a greater number of eggs during the first days
297 of the total life cycle and then stop laying eggs after the 5th day (figure 3). As can be
298 seen in the figure, the nematodes laid a greater quantity of eggs in the first time interval
299 (0–2 days) than in the second time interval (2–4 days). There were significant
300 differences in the number of eggs laid by *C. elegans* infected with the three
301 subpopulations in the first time interval (0–2 days) ($p \leq 0.05$), whereas there were no
302 significant differences between the three subpopulations in the second time interval (2–
303 4 days). This behaviour was similar for the *S.* Typhimurium subpopulations treated by
304 the two technologies, PEF and HHP. These results might indicate that PEF or HHP

305 technology modifies the pathogenic mechanisms of *S.* Typhimurium, impacting on the
306 stress mechanism of *C. elegans* when it is exposed to treated bacteria. In these cases, the
307 nematodes may feel threatened by the unknown bacterial population generated by these
308 treatments. The stress mechanisms correspond to the r-strategy, in which the nematodes
309 increase their reproductive rate in a short period of time (Hodgkin et al., 1991;
310 Schulenburg et al., 2004). This strategy enables them to protect themselves against
311 pathogenic bacteria and to ensure the continuity of their offspring before they die.

312 It appears that there was a relationship between the egg laying and the hazard function
313 of the nematodes. During the first time interval, the number of eggs laid was high
314 whereas the risk was low. In a research study carried out by Aballay et al. (2000, 2002),
315 it was suggested that *S.* Typhimurium could affect the egg-laying pattern of nematodes,
316 and that the nematodes exposed to this bacterium laid a greater quantity of eggs and
317 then, once they had left offspring, they died as a result of intestinal infection.

318 After the first four days, *C. elegans* stopped laying eggs and its survival probability
319 decreased quickly when it was fed with untreated *S.* Typhimurium or with *S.*
320 Typhimurium treated once by PEF. In contrast, *C. elegans* fed with *S.* Typhimurium
321 treated four times by PEF or HHP maintained its survival probability after the fourth
322 day. This might be related to the decrease in pathogenicity observed in *S.* Typhimurium
323 treated once or four times by PEF or HHP.

324 This is the first time that a study has indicated that infection with *S.* Typhimurium
325 treated by PEF or HHP caused nematodes to lay a greater number of eggs, although
326 they stopped laying eggs at the same time as nematodes infected with untreated *S.*
327 Typhimurium. This may be explained by some research studies that show that *C.*
328 *elegans* retains eggs in its uterus when environmental conditions are harmful until the
329 conditions become optimal again (Gardner et al., 2013).

330

331 ## 4. Conclusions

332 The results obtained in this study could indicate that *S*. Typhimurium develops
333 microbial resistance against PEF or HHP treatment when it is applied repeatedly at
334 sublethal intensity, but, in contrast, its virulence against *C. elegans* appears to decreases.
335 This behaviour could vary among different bacteria, so individualized studies are
336 needed, depending on the pathogen in question.

337 Despite of sublethal treatments with non-thermal technologies applied to food
338 preservation are able to inactivate part of the *S.* Typhimurium population they could
339 also generate sublethal damaged cells that should be monitored to avoid the
340 development of microbial resistance and future emerging risks. With regard to the effect
341 of subpopulations coming from a mixture of sublethally damaged cells and healthy cells
342 of *S*. Typhimurium on the survival behaviour of *C. elegans*, it appears that there were
343 some differences between PEF- and HHP-treated *S*. Typhimurium that could indicate
344 different damage or repair mechanisms.

345 Future work should be carried out to investigate the molecular mechanisms underlying
346 the observed resistance and changes of virulence of S. *Typhimurium* and how these
347 changes could be transferred when the microorganism is stressed in real food matrices.

348

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479 **Figure captions**

480 **Figure 1. a)** Inactivation of *S.* Typhimurium (Log (S)) after the application of
481 consecutive PEF treatments (30 kV/cm - 300 µs). Error bars represent the deviation
482 (SD) of three independent repetitions, each one with three replica. **b)** Inactivation of *S.*
483 Typhimurium (Log (S)) after the application of consecutive HHP treatments (250 MPa
484 – 5 min). Error bars represent the deviation (SD) of three independent repetitions, each
485 one with three replica.

486 **Figure 2. a)** Survival probability of worms fed with untreated *S.* Typhimurium and *S.*
487 Typhimurium treated by PEF once and four times. **b)** Survival probability of worms fed
488 with untreated *S.* Typhimurium and *S.* Typhimurium treated by HHP once and four
489 times.

490 **Figure 3. a)** Eggs laid by worms fed with untreated *S.* Typhimurium (S2, S4) and *S.*
491 Typhimurium treated once (SPEF12, SPEF14) and four times (SPEF42, SPEF44) by
492 PEF in the first two time intervals. **b)** Eggs laid by worms fed with untreated *S.*
493 Typhimurium (S2, S4) and *S.* Typhimurium treated (SHHP12, SHHP14) and four times
494 (SHHP42, SHHP44) HHP in the first two time intervals.

495 **Table 1**: Percentiles for *C. elegans* lifespan when fed with the different *S.* Typhimurium

496 PEF treated populations.

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Microorganism	Percentil at 20% (days)
S. Typhimurium untreated	10.13 ±1.12
S. Typhimurium PEF treated once	11.36 ±1.97
S. Typhimurium PEF treated four times	13.31 ±0.84

502 **Table 2.** Percentiles for lifespan (days) of *C. elegans* fed with untreated *S.*
503 Typhimurium and *S.* Typhimurium treated once and four times by HHP.

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Percentil %	*S*. Typhimurium	*S*. Typhimurium 1	*S*. Typhimurium 4
75.0	1.6 ± 0.2	3.1 ± 0.4	2.8 ± 0.6
50.0	4.6 ± 0.9	6.3 ± 0.5	6.3 ± 0.4
25.0	9.2 ± 1.1	11.0 ± 1.0	9.6 ± 0.7
10.0	12.9 ± 4.8	14.3 ± 1.8	12.4 ± 4.5
5.0	15.6 ± 4.0	15.9 ±1.8	15.0 ± 4.2
1.0	19.6 ± 31.9	18.5 ± 72.2	17.8 ± 14.6

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507 **Figure 1**

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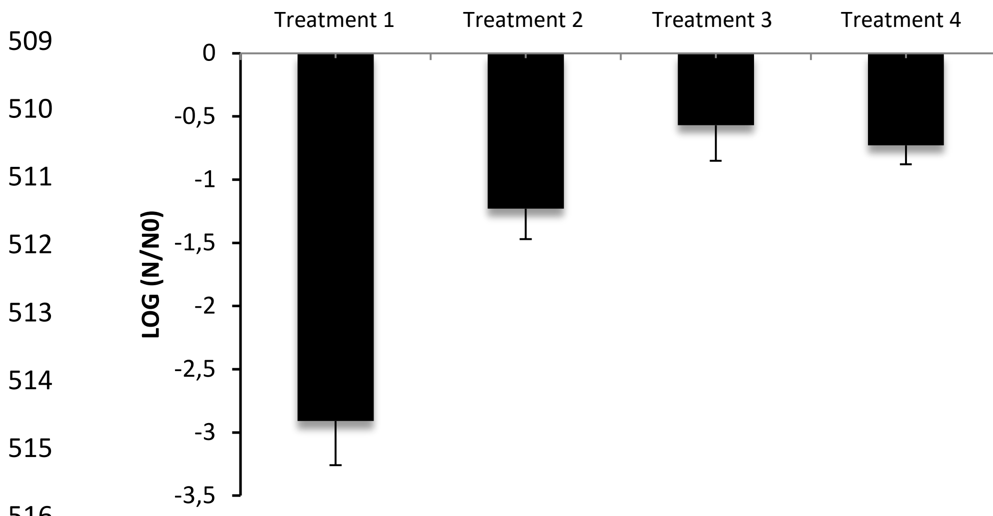

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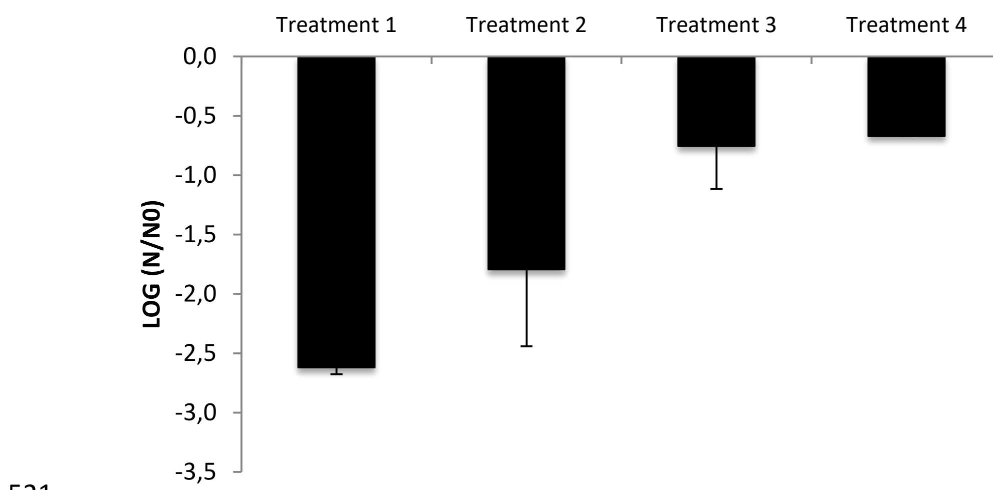
520 b)



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523 Figure 2

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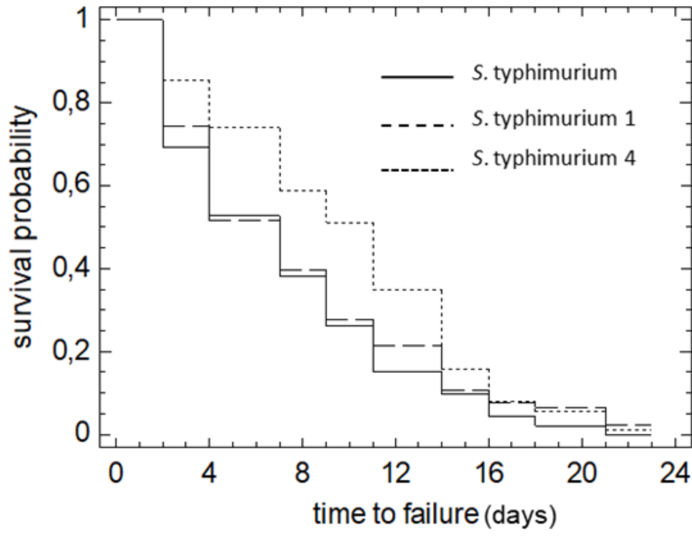

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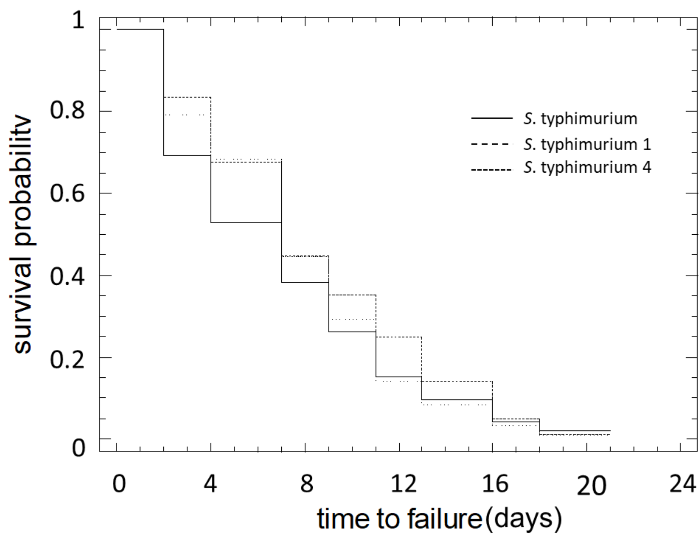

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538 Figure 3

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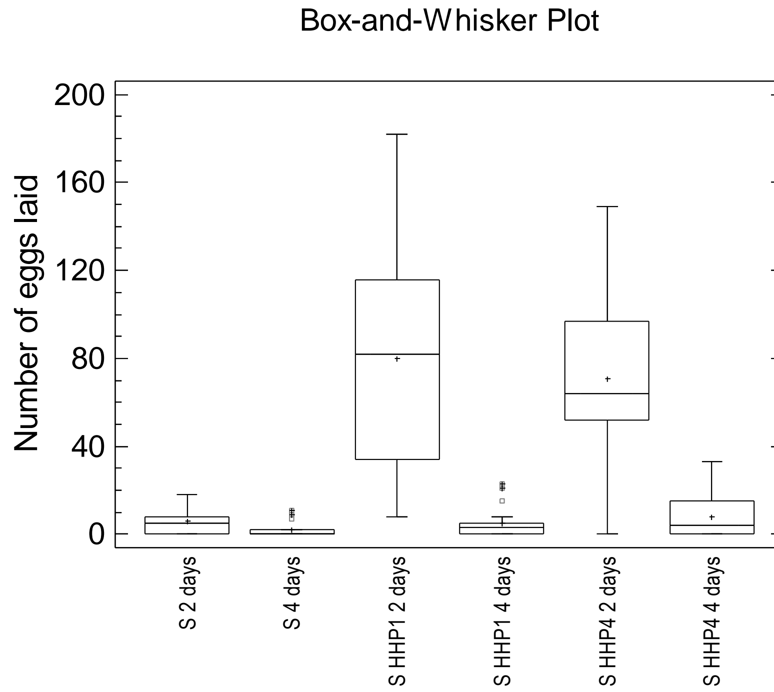

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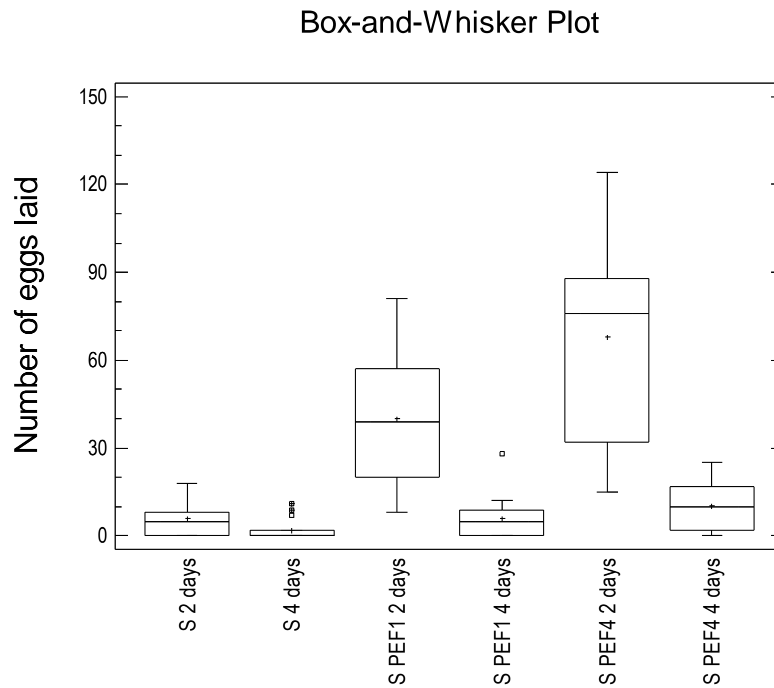

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