Occurrence of *Salmonella* Typhimurium resistance under sublethal/repeated exposure to cauliflower infusion and Infection effects on *Caernohabditis elegans* host test organism

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Abstract

Resistant bacteria to antimicrobials are increasingly emerging in medical, food industry and livestock environments. The present research work assesses the capability of *Salmonella enterica* var Typhimurium to become adapted under the exposure to a natural cauliflower antimicrobial by-product infusion in consecutive repeated exposure cycles. *Caernohabditis elegans* was proposed as *in vivo* host-test organism to compare possible changes in the virulent pattern of the different rounds treated *S. enterica* var Typhimurium and untreated bacterial cells.

According to the obtained results, *S. enterica* var Typhimurium was able to generate resistance against a repeated exposure to cauliflower by-product infusion 5 % (w/v), increasing the resistance with the number of exposed repetitions. Meanwhile at the first exposure, cauliflower by-product infusion was effective reducing *S. enterica* var Typhimurium (≈1 $\log_{10}$ cycle), *S. enterica* var Typhimurium become resistant to this natural antimicrobial after the second and third treatment-round, and was able to grow (≈1 $\log_{10}$ cycle). In spite of the increased resistance observed for repeatedly treated bacteria, the present study reveals no changes on *C. elegans* infection effects between resistant and untreated *S. enterica* var Typhimurium, according to phenotypic parameters evaluation (lifespan duration and egg-laying).

Keywords: Natural antimicrobials, *S. enterica* var Typhimurium, resistance, *Caernohabditis elegans*, infection.

1.Introduction

Hurdle technology is a processing concept industrially well established to achieve efficient microorganisms reduction by the combination of different preservation techniques applied at slight intensive conditions maintaining natural valuable properties of foods treated products from a nutritional and sensory point of view (Pasha et al., 2014; Pina-Pérez et al., 2013; Sanz-Puig et al., 2017a). The combination of natural or chemical antimicrobials with other physical methods is one of the most commonly used practices to synergistically enhance microbial and enzymes reduction improving the safety and shelf-life of treated products (Guerrero et al., 2017; Sudhaus et al., 2012). However, nowadays, consumers are really concerned about chemical additives, many of them close related with allergies and other possible health problems (Asioli et al., 2017). Natural antimicrobials (e.g. polyphenols, glucosinolates, vegetable pigments, antimicrobial peptides) and phytochemical extracts (from soya, *Stevia Rebaudiana* Bertoni, cauliflower infusion, citrus fruits essential oils extracts) could be introduce in food formulation as an alternative to synthetic antimicrobials (Gutierrez et al., 2009; Sansano et al., 2017; Sanz-Puig et al., 2015a; Tiwari et al., 2009), additionally with other technological (thickening agents, gelling agents) and functional effects in novel food (enhanced

antioxidant potential, enhanced fiber content in food). Many bioactive compounds can be obtained from agro-industrial by-products (Fernandez-Lopez et al., 2005; Sanz-Puig et al., 2015 a,b; Viuda-Martos et al., 2007). Sanz-Puig et al. (2015b) studied different concentrations of vegetable by-product infusions (cauliflower, mandarin, orange, lemon, and okara) against *Salmonella enterica* var Typhimurium, and according to the obtained results 5 % (w/v) of cauliflower resulted the most effective bactericidal natural aqueous extract reducing close to 6 $\log_{10}$ cycles the initial *S. enterica* var Typhimurium load. Valorization studies carried out to make profit from valuable vegetable wastes from food industry is in the priority research axes of the European Union H2020 in order to support sustainable development by means of circular economy fundamentals (EC, 2017).

Microorganisms are becoming adapted and resistant to (i) veterinary antibiotics, used in animals farming, (ii) clinical antibiotics used in human medicine and (iii) antimicrobial additives used in food preservation, representing a great concern for the scientific community (WHO, 2017).

Cross-resistances along the complete food chain, and changes in virulence of pathogenic bacteria, may even emerge (Kalily et al., 2016, 2017; Kisluk et al., 2013). Zanini et al. (2014) studied the bacterial adaptive responses to antibiotics induced by sublethal concentration of citral on first- and second-generation cells of *Listeria monocytogenes* serovar 4b and *Salmonella enterica* var Typhimurium. They concluded that the presence of citral in the culture medium of *L. monocytogenes* 4b and *S. enterica* var Typhimurium increased the antibiotic susceptibility of the first generations, while an increase in antibiotic resistance was observed in the second generation of *S. enterica* var Typhimurium.

Recent *in vivo* models are being proposed to evaluate mechanisms of pathogenic infection, and bacterial genes responsible for changes in virulence. Among those, Additionally, *C. elegans* has been defined as a valid model for human pathogen infection, disease and health

promotion (Balla and Troemel, 2013; Gammon, 2017; Kurz and Ewbank, 2000). The evaluation of the *C. elegans* lifespan has been previously used as an indicator of the impact that certain molecules, e.g. antioxidants, metabolites and synthetic compounds, could have on human health and their influence in the anti-aging process (Liu et al., 2016; Rollins et al., 2017; Wang and Wink, 2016).

Silva et al. (2015) evaluated the effect of two antimicrobial substances, carvacrol and citral, on *L. monocytogenes* and *L. innocua* cells, as well as possible virulence changes in sub-lethally damaged cells, using *C. elegans* as *in vivo* test organism. According to Silva et al. (2015) results, the lifespan of *C. elegans* population fed on a lawn of *L. monocytogenes* previously treated with carvacrol was reduced in comparison with lifespan exerted by *C. elegans* population fed on a lawn of *E. coli* OP50 (as negative control), *L. monocytogenes* treated with citral, and, treated/not treated *L. innocua*.

Understanding the behaviour of foodborne pathogens exposed to sublethal concentrations of natural antimicrobials, and the possible implications in terms of bacterial resistance generation and changes in virulence are key questions to search for alternatives to conventional preservatives in food and to assess the safety of the hurdle technology processing application (Pina-Perez et al., 2013; Sanz-Puig et al., 2017a). Evaluation of some *C. elegans* phenotypic factors (such as lifespan, reproduction patterns, and mobility) under the exposure to pathogenic human bacteria could be a preliminary indicator of risks for consumers when these natural antimicrobials are used below the bactericidal dosage for food formulation with preservative objectives (Kastbjerg et al., 2010; Schroeder et al., 2017).

The main aim of the present study is the assessment of *S. enterica* var Typhimurium behaviour under the repeatedly exposure to a cauliflower natural antimicrobial extract, subsequently evaluating the effects of infection by repeatedly treated/untreated *S. enterica* var Typhimurium on the *C. elegans in vivo* host-model organism.

2. Materials and methods

2.1 Cauliflower by-product extract

Cauliflower by-product (mainly external leaves of cauliflower plants) was provided as dehydrated residues from primary production of TRASA S.L., and was washed in sterile water, dried and homogenized using a laboratory grinder (Janke & Kunkel IKA Labortechnik) (Brandi et al., 2006).

Five per cent (w/v) of dried cauliflower by-product was selected in the previous study according to previous results of Sanz-Puig et al. (2015a). The infusion was prepared as follows: buffered peptone water (0.1% (w/v)) was boiled and then the dry by-product was added and allowed to infuse for 30 min (Sanz-Puig et al., 2016). The extracts were then centrifuged at 4,000 rpm for 15 min at 4 °C and filtered three times, using filters with a pore size of 11 and 2.5 µm to eliminate smaller particles (Whatman), and 0.45 µm (PVDF syringe filter) to sterilize.

2.2 Bacterial strain

A pure culture of *S. enterica* var Typhimurium (CECT 443) was provided freeze-dried by the Spanish Type Culture Collection. It was rehydrated with 10 mL of tryptic soy broth (TSB) (Scharlab Chemie, Barcelona, Spain). After 20 min, it was transferred to 500 mL of TSB and incubated at 37 °C with continuous shaking (Selecta Unitronic) at 200 rpm for 14 h to obtain cells in a stationary growth stage. The cells were centrifuged (Beckman Avanti J-25) twice at 4.000 rpm and at 4 °C for 15 min and then resuspended in TSB. The cells were finally resuspended in 20 mL of TSB, then dispensed in 2 mL vials with glycerol at 20% to a final concentration of 10^8 CFU/mL obtained by plate count, and finally frozen and stored at –80 °C.

130 **2.3 Evaluation of microbial resistance**

131 The initial population of *S. enterica* var Typhimurium (10^7 CFU/mL) was exposed to three
132 consecutive antimicrobial treatments. Each treatment consisted in exposure to 5 % (w/v)
133 cauliflower by-product infusion at 37 °C for 4 h with continuous shaking (200 rpm) (sub-lethal
134 treatment) (Sanz-Puig et al., 2015a). Afterwards, the sample was centrifuged to recover the
135 microbial cells. Recovered cells were grown in TSB culture overnight to achieve stationary
136 phase (10^9 CFU/mL), inoculated in cauliflower by-product extract (10^7 CFU/mL) and treated
137 again using the same conditions as described above. Before and after each treatment, the
138 antimicrobial effect against the *S. enterica* var Typhimurium population was evaluated by plate
139 count in tryptic soy agar (TSA) (Scharlab Chemie, Barcelona, Spain). The entire experiment was
140 carried out in triplicate with three different infusion batches.
141 *S.* Typhimurium treated once and *S. enterica* var Typhimurium treated three times were
142 included in the *C. elegans* infection study, because they were the most different populations
143 with regard to their resistance to the cauliflower antimicrobial exposure.
144

145 **2.4 *C. elegans* studies**

146 *C. elegans* strain N2, obtained from the College of Biological Sciences, Minnesota University,
147 USA, was used to evaluate the possible virulence changes in *S. enterica* var Typhimurium
148 populations as a consequence of repeated exposure to antimicrobial from cauliflower by-
149 product infusion. *C. elegans* was maintained in plates with Nematode Growth Medium (NGM)
150 agar and a bacterial lawn of *E. coli* OP50 (Silva et al., 2015).
151 In order to evaluate the effect of different *S. enterica* var Typhimurium subpopulations on the
152 survival of *C. elegans*, 5 repetitions of 50 synchronized young adult nematodes (250 in total),
153 distributed in 5 plates of 10 worms each, were transferred to NGM agar with a lawn of
154 untreated *S. enterica* var Typhimurium (control) (10^7 CFU/per plate). This was repeated for

155 treated *S. enterica* var Typhimurium populations (*Salmonella* treated once and treated three
156 times with cauliflower by-product extract) (10^7 CFU/per plate). The worms were maintained at
157 20 °C during their life cycle (approximately three weeks) and were examined at 48 h intervals.
158 Worms were considered dead when they did not move and did not respond to stimulation
159 (contact with a platinum worm picker).
160
161 The effect of selected *S. enterica* var Typhimurium populations on egg laying of *C. elegans* was
162 also assessed. For this purpose, 10 worms were transferred to NGM plates with a lawn of
163 untreated *S.* Typhimurium or *S.* Typhimurium treated with antimicrobial by-product extract,
164 and the number of eggs laid was counted at 48 h intervals.
165
166 **2.5 Statistical analysis of data**

167 An ANOVA analysis was carried out to detect significant differences (p-value ≤ 0.05) between
168 microbial resistance under the exposure to cauliflower by-product infusion, and phenotypic
169 differences in *C. elegans* infection due to different bacterial populations. Additionally, survival
170 data for *C. elegans* were analysed by using the Kaplan–Meier method, and the hazard function
171 and percentiles of estimated survival distribution were obtained. All statistical analyses were
172 performed using Statgraphics Centurion XII software (Statpoint Technologies, Inc., Warrenton,
173 VA, USA).
174
175 **3.Results**

176 Figure 1 shows the microbial inactivation and growth ($\log_{10}$ cycles) of the initial *S. enterica* var
177 Typhimurium population (10^7 CFU/mL) caused by each cauliflower by-product infusion
178 treatment round. As can be seen graphically, 1.04 $\log_{10}$ cycles of inactivation were achieved
179 after the first cauliflower by-product exposure. However, subsequently exposed cells were

resistant to the antimicrobial cauliflower effect, and even able to grow (≈1 $\log_{10}$ cycle after the third exposure).

In a second step, *C. elegans* was used as a host-model organism for infection, fed with *S. enterica* var Typhimurium: untreated (control), treated once (sensitive to cauliflower effect), and treated three times (resistant to cauliflower bactericidal effect and able to grow). Lifespan, movement, and egg-laying were phenotypic factors under study to evidence possible changes in bacterial virulence between untreated and different populations of resistant treated *S. enterica* var Typhimurium bacteria.

Figure 2 shows the percentage of surviving nematodes during their life cycle, when they were fed with (i) untreated *S. enterica* var Typhimurium, (ii) treated once, and (iii) treated three times with cauliflower by-product infusion. The *C. elegans* survival curve indicated that during their life cycle (until day 18) the number of living nematodes was higher (p-value < 0.05) for *C. elegans* fed with treated *S. enterica* var Typhimurium (one or three times) than for *C. elegans* fed with untreated *S. enterica* var Typhimurium. Owing to the life cycle of the worm, the number of nematodes decreased with time; but, survival was up to 21 days for worms fed with untreated *S. enterica* var Typhimurium, and 23 and 25 days for nematodes fed with *S. enterica* var Typhimurium treated once and three times, respectively. Nevertheless, non-significant differences were found between survival data of nematode populations fed with *S. enterica* var Typhimurium treated once and three times (p-value > 0.05).

Survival data were analysed by using a Kaplan–Meier method to obtain the hazard function for different *C. elegans* individuals fed with different *S.* Typhimurium subpopulations (see Figure 3) and percentiles (Table 1). As can be seen in the figure 3, the hazard was always higher when the nematodes were fed with untreated *S.* Typhimurium than when they were fed with treated *S. enterica* var Typhimurium. From day 14 onwards, the hazard increased more slowly

205 for *C. elegans* fed with *S. enterica* var Typhimurium treated once and three times than for *C.*
206 *elegans* fed with untreated *S. enterica* var Typhimurium.
207 Also the percentiles of estimated survival distribution were obtained. Table 1 shows
208 percentiles of estimated survival distribution for each of the *C. elegans* populations. If we focus
209 on the 5 % percentile (percentage of worms surviving for a given time), it can be seen that
210 there are significant differences (p-value < 0.05) between *C. elegans* fed with treated *S.*
211 *enterica* var Typhimurium (treated once: 19.3 days, and three times: 19.9 days) and untreated
212 *S.* Typhimurium (16.5 days).
213
214 Egg lying was also studied as a complementary test. In optimal conditions, *C. elegans* lays
215 about 300–350 eggs during its life cycle (approximately 21 days) (Labrousse et al., 2000).
216 However, when the nematodes were fed and infected with any of the subpopulations of *S.*
217 *enterica* var Typhimurium considered in this study, egg-laying was only maintained until the 5^{th}
218 day. Figure 4 shows a comparison of the number of eggs laid by *C. elegans* fed with the three
219 different *S. enterica* var Typhimurium subpopulations for two periods of time (from 0 to 2 days
220 and from 2 to 4 days). In the first period of time (0 to 2 days) significant differences (p-value <
221 0.05) were found in the number of eggs laid by nematodes fed with the three *S.* Typhimurium
222 subpopulations. Nematodes fed with untreated *S. enterica* var Typhimurium laid fewer eggs
223 than nematodes fed with *S. enterica* var Typhimurium treated once. The highest number eggs
224 laid in the first period are corresponding with nematodes fed with *S. enterica* var Typhimurium
225 treated three times. For the second time period (2 to 4 days) the number of eggs laid by the
226 worms was almost the same in all cases, and lower than the number of eggs laid in the first
227 period of time. These results are important because, despite the effect of *S. enterica* var
228 Typhimurium on the egg-laying rate during the life cycle of the nematode, it appears that *S.*
229 *enterica* var Typhimurium treated with the cauliflower by-product extract was less virulent in

relation to egg laying than the untreated *Salmonella*, thus corroborating the findings observed in the survival data and the Kaplan–Meier analysis.

Discussion

Antimicrobial resistance and adaptation is mediated by specific genes encoded for bacterial protection or generally due to spontaneous mutations in the bacterial chromosome. Once the development of resistance has occurred, the mutated gene is directly transferred to the bacteria's progeny during replication (Tiwari and Tiwari, 2011). The results of this study are revealing the capability of *S.* Typhimurium to develop microbial resistance/adaptation to cauliflower by-product infusion after being exposed to consecutive sub-lethal treatments that inactivated only part of the initial microbial population. Various authors (Di Pasqua et al., 2006; Ultee et al., 2000) have shown specific adaptation of *E. coli*, *S.* Senftenberg, *S.* Typhimurium and *Bacillus cereus* to sublethal concentrations of essential oils. Kalily et al. (2016) found linalool-resistant mutants of *S*. Senftenberg. Development of resistance to these natural antimicrobial has been associated to structural and functional changes in microbial cells, specifically in their cell membranes (Ali et al., 2018; Kalily et al., 2016; McMahon et al., 2007). Recent scientific studies have also demonstrated development of microbial resistance of *S. enterica* var Typhimurium to several antibiotics such as cephalosporins or fluoroquinolones or cross-protection against antibiotics (Kalily et al., 2017; Kariuki et al., 2015; Zanini et al., 2014). Additional studies are also demonstrated the development of resistance when *S. enterica* var Typhimurium is treated with non-essential oil natural antimicrobials (polyphenols) (Gupta and Birdi, 2017). Under other sub-lethal physical treatments, like Pulsed Electric Fields and High Hydrostatic Pressure, *S. enterica* var *Typhimurium* has also demonstrated ability to be adapted (Sanz-Puig et al., 2018)

In order to further understand the mechanism of resistant bacteria infection, and the possible evident changes in virulence due to this adaptation step in relation to untreated bacteria, the

256 *C. elegans in vivo* model was used. Up to date, over 40 human pathogens have been analyzed
257 in the *C. elegans* infection model (Battisti et al., 2017). The rapid growth and short generation
258 time of *C. elegans* permit extensive screens for pathogens infective potential, some of the
259 factors identified in these screens have also been shown to play roles in mammalian infections.
260 In the present study, *C. elegans* was fed with different populations, untreated *S. enterica* var
261 *Typhimurium* and cauliflower treated bacteria, once and three times, the last one highly
262 resistant to the natural cauliflower antimicrobial exposure. Firstly, the nematode intestinal
263 epithelium should recognize and respond to pathogens in a microbe-specific manner.
264 According to several previous reported studies, the accumulation of the bacteria in the
265 intestine is traduced in several pathologies in *C. elegans.* The survival of *C. elegans* is
266 dependent on the type and availability of bacteria on which to feed, among other factors
267 (Allen et al., 2015). Although some studies have indicated that the lifespan of worms fed with
268 *S. enterica* var Typhimurium decreased significantly (Aballay et al., 2000; Labrousse et al.,
269 2000), neither of those studies contemplated infecting the worms with naturally occurring
270 resistant subpopulations of *S. enterica* var Typhimurium, repeatedly exposed to natural
271 cauliflower infusion. According to results, it seems that the lifespan of worms fed with treated
272 *S. enterica* var Typhimurium was longer than the lifespan of worms fed with untreated
273 *Salmonella*. This behaviour indicating that resistant *S. enterica* var Typhimurium are not more
274 virulent for *C. elegans* than untreated type. Ahmed et al. (2016) identify the antimicrobial–
275 resistance genes in *Salmonella* Typhimurium to be mainly *blaTEM* and *floR*, detected at
276 frequencies 53 and 73 % respectively in *Salmonella* isolated from chicken meat. Wild types of
277 *Salmonella* are specifically capable to produce enterotoxins controlled by the expression of a
278 series of virulence-genes such as *stn* (Barilli et al., 2018). Antimicrobial exposure can modulate
279 the virulence of *S. enterica* var Typhimurium. In that sense, several reports are supporting the
280 capability of bacteria (*Mycobacterium, Escherichia coli, Salmonella enterica, Pseudomonas*
281 *aeruginosa*) to acquire *de novo* resistance under inadequate treatment regimes (Schroeder et

282 al., 2017). Adaptative resistance (induced genetic changes, enzymatic driven antimicrobial
283 inactivation, changes in the antimicrobial target, changes in cell permeability) is another of the
284 possible explanations for these increased survival of *S. enterica* var *typhimurium* under the
285 repetitive exposure to cauliflower infusion (Brooks and Brooks, 2014; Sanz-Puig et al., 2017b,
286 2018). In the present study, cauliflower resistant *S. enterica* var *Typhimurium* seems to be less
287 virulent for the fed nematode than untreated one.
288 The increase in the hazard from day 14 is probably related with the age of the nematodes,
289 which are already considered old at that time, and with their immunity system, which is less
290 efficient than in younger nematodes and consequently more sensitive to infection under
291 virulent subpopulations of *S. enterica* var *Typhimurium*. Thomas et al. (2004) also found that
292 the rate at which wild-type *C. elegans* was killed by the bacterial pathogens tested (*S. enterica*
293 var Typhimurium, among others) increased as nematodes aged, irrespective of the precise
294 mechanism of killing. Similar results were also found by Kurz et al. (2003) in nematodes
295 infected with *Serratia marcescens*.
296 With regard to egg laying, egg-laying events tend to be clustered in short bursts, or active
297 phases, which are separated by longer inactive phases during which eggs are retained (Hart et
298 al., 2006). Reproductive function begins on about day 5 of adulthood, and reproduction ceases
299 after 10–14 days of adulthood (Christopher and Kerry, 2013). Results of the present study
300 indicated that *S. enterica* var *Typhimurium* treated once and three times and untreated *S.*
301 *Typhimurium* modified the laying pattern of the worms. For all studied scenarios for infection,
302 egg laying stopped on the 5^{th} day after contact with the pathogen. Previous research studies
303 have indicated that live cells of *S. Typhimurium* are accumulated in the lumen of the intestine
304 of *C. elegans*, that becomes completely infected by the 5^{th} day of contact with this bacterium
305 (Aballay et al., 2000; Gardner et al., 2013; Labrousse et al., 2000). Other previous studies have
306 also registered the *C. elegans* egg-laying pattern as indicator of microbial virulence, showing
307 that under the exposure to *E. faecalis*, *C. elegans* increases the egg-in-uterus retention, being

308 the number of eggs retained related with the virulence of *E. faecalis* strain used in each case
309 (Gardner et al., 2013). This retention of progeny observed in the present study is
310 corresponding in time with the progressive accumulation of *S. enterica* var *Typhimurium* in the
311 lumen of the nematode. Additionally, other research studies have demonstrated that *S.*
312 Typhimurium infection can affect *C. elegans* egg laying and the eggs hatch internally, which
313 contributes significantly to killing the worms in the first days of their lifespan (Garden et al.,
314 2013; Labrousse et al., 2000). In spite of the fact that egg laying stopped on the 5^{th} day in all
315 cases, higher number of eggs was detected for *C. elegans* fed with resistant bacteria in
316 comparison with egg laying pattern of *C. elegans* fed with untreated *S. Typhimurium.*
317 In the present study, the survival data, the Kaplan–Meier analysis and the egg-laying behaviour
318 used to assess the effect of infection by resistant bacteria on *C. elegans* revealed that an
319 increase in resistance does not mean that microorganisms become more virulent for *C.*
320 *elegans*, although *S. enterica* var Typhimurium still maintains some level of virulence, as can be
321 deduced from the egg-laying study. Complex relationship between antimicrobial resistance
322 and virulence genes involved in each specific treatment process should be elucidated in order
323 to develop safest processes and products.
324
325

Conclusions

The present research work provides valuable information regarding the resistance development of *S. enterica* var Typhimurium under the repeated exposure to a natural antimicrobial obtained from the agri-food by-products. Valorization of natural compounds from vegetables, and the development of novel ingredients is feasible and demanded by industry and consumers. Potential functionalities can appear related to creation of more convenient, healthy and minimally processed products. However, associated risks should be accurately predicted. No virulence increment by the exposure to cauliflower infusion was detected in the present study using one of the most concerning foodborne pathogens using an in vivo test-organism *C. elegans*.

Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

Aballay A, Yorgey P and Ausubel FM (2000) *Salmonella Typhimurium* proliferates and establishes a persistent infection in the intestine of *Caenorhabditis elegans*. *Current Biology* 10: 1539–1542.

Allen EN, Ren J, Zhang Y and Alcedo J (2015) Sensory systems: Their impact on *C. elegans* survival. *Neuroscience* 296 : 15–25.

Ali J, Rafiq QA and Ratcliffe E (2018). Antimicrobial resistance mechanisms and potential synthetic treatments. *Future Science OA* 4(4): 1–10.

Asioli D, Aschemann-Witzel J, Caputo V, Vecchio R, Annunziata A, Næs T and Varela P (2017) *Future Science OA*Making sense of the "clean label" trends: A review of consumer food choice behavior and discussion of industry implications. *Food Research International* 99(1): 58-71.

Balla KM and Troemel ER (2013) *Caenorhabditis elegans* as a model for intracellular pathogen infection. *Cell Microbiology* 15(8): 1313–1322.

Barilli E, Bacci C, Stella Villa Z, Merialdi G, D'Incau M, Brindani F and Vismarra A (2018) Antimicrobial resistance, biofilm synthesis and virulence genes in *Salmonella* isolated from pigs bred on intensive farms. *Italian Journal of Food Safety* 3, 7(2): 1–7.

Battisti JM, Watson LA, Naung MT, Drobish AM, Voronina E and Minnick MF (2017) Analysis of the *Caenorhabditis elegans* innate immune response to *Coxiella burnetii. Innate Immunology* 23(2): 111–127.

Brandi G, Amagliani G, Schiavano GF, de Santi M and Sisti M (2006) Activity of Brassica oleracea leaf juice on foodborne pathogenic bacteria. *Journal of Food Protection* 69: 2274–2279.

Brooks BD and Brooks AE (2014) Therapeutic strategies to combat antibiotic resistance. *Advanced Drug Delivery Reviews* 30: 78, 14-27.

Christopher LP and Kerry K (2013) Age-related degeneration of the egg-laying system promotes matricidal hatching in *Caenorhabditis elegans*. *Aging Cell* 12: 544–553.

Di Pasqua R, Hoskins N, Betts G and Mauriello G (2006). Changes in membrane fatty acids composition of microbial cells induced by addiction of thymol, carvacrol, limonene, cinnamaldehyde, and eugenol in the growing media. *Journal of Agricultural and Food Chemistry* 547: 2745–2749.

European Commission (EC). 2017. Implementation of the circular economy action Plan. Available at: http://ec.europa.eu/environment/circular-economy/index_en.htm

Fernandez-Lopez J, Zhi N, Aleson-Carbonell L, Perez-Alvarez JA and Kuri V (2005). Antioxidant and antibacterial activities of natural extracts: application in beef meatballs. *Meat Science* 69: 371–380.

Gammon DB (2017) *Caenorhabditis elegans* as an Emerging Model for Virus-Host Interactions. *Journal of Virology* 1–7.

Gardner M, Rosell M, Myers EM (2013). Measuring the Effects of Bacteria on *C. elegans* Behaviour Using an Egg Retention Assay. *Journal of Visualized Experiment* 80: 1–6.

Guerrero SN, Ferrario M, Schenk M and Carrillo MG (2017). Chapter 3 - Hurdle Technology Using Ultrasound for Food Preservation. Bermudez-Aguirre D (eds) *Ultrasound: Advances for Food Processing and Preservation*. Cambridge: Elsevier Academic Press, pp: 39-99

Gupta PD and Birdi TJ (2017). Development of botanicals to combat antibiotic resistance. *Journal of Ayurveda and Integrative Medicine* 8(4): 266-275.

Gutierrez J, Barry-Ryan C and Bourke P (2009). Antimicrobial activity of plant essential oils using food model media: efficacy, synergistic potential and interactions with food components. *Food Microbiology* 26: 142–150.

Hart AC (2006). Behavior. Hart AC (eds) *The C. elegans Research Community*. Pasadena (CA): WormBook, pp: 1-67.

Kalily E, Hollander A, Korin B, Cymerman I and Yaron S (2016) Mechanisms of resistance to linalool in *Salmonella* Senftenberg and their role in survival on basil. *Environmental Microbiology* 18(11): 3673–3688.

Kalily E, Hollander A, Korin B, Cymerman I and Yaron S (2017) *Salmonella* Senftenberg adaptation to linalool and its association with antibiotic resistance and environmental persistence. *Applied and Environmental Microbiology* 1–17.

Kariuki S, Gordon MA, Feasey N and Parry CM (2015) Antimicrobial resistance and management of invasive *Salmonella* disease. *Vaccine* 33(3): 21–29.

Kastbjerg VG, Larsen MH, Gram L and Ingmer H (2010) Influence of Sublethal Concentrations of Common Disinfectants on Expression of Virulence Genes in *Listeria monocytogenes*. *Applied and Environmental Microbiology* 76(1): 303-309.

Kisluk G, Kalily E and Yaron S (2013) Resistance to essential oils affects survival of *Salmonella* enterica serovars in growing and harvested basil. *Environmental Microbiology* 15: 2787–2798.

Kurz CL, Chauvet S, Andres E, Aurouze M, Vallet I, Michel GP, Uh M, Celli J, Filloux A, De Bentzmann S, Steinmetz I, Hoffmann JA, Finlay BB, Gorvel JP, Ferrandon D and Ewbank JJ

(2003) Virulence factors of the human opportunistic pathogen *Serratia marcescens* identified by *in vivo* screening. *EMBO Journal 22*: 1451–1460.

Kurz CL and Ewbank JJ (2000) *Caenorhabditis elegans* for the study of host–pathogen interactions. *Trends in Microbiology* 8(3): 142-144.

Labrousse A, Chauvet S, Couillault C, Kurz CL and Ewbank JJ (2000) *Caenorhabditis elegans* is a model host for *Salmonella* Typhimurium. *Current Biology* 10: 1543–1545.

Liu H, Guo M, Xue T, Guan J, Luo L and Zhuang Z (2016) Screening lifespan-extending drugs in *Caenorhabditis elegans* via label propagation on drug-protein networks. *BMC Systems Biology series* 10(4): 1-11.

McMahon MAS, Xu J, Moore JE, Blair IS and McDowell DA (2007) Environmental Stress and Antibiotic Resistance in Food-Related Pathogens. *Applied and Environmental Microbiology* 73(1): 211–217.

Pasha I, Saeed F, Sultan MT, Khan MR and Rohi M (2014) Recent developments in minimal processing: a tool to retain nutritional quality of food. *Critical Reviews in Food Science and Nutrition* 54: 340–351.

Pina-Pérez MC, Martínez-López A and Rodrigo D (2013) Cocoa powder as a natural ingredient revealing an enhancing effect to inactivate *Cronobacter sakazakii* cells treated by Pulsed Electric Fields in infant milk formula. *Food Control 32*(1): 87-92.

Rollins JA, Howard AC, Dobbins SK, Washburn EH and Rogers AN (2017) Assessing Health Span in Caenorhabditis elegans: Lessons From Short-Lived Mutants*. Journal of Gerontology: A Biological Sciences and Medical Sciences* 1 72(4): 473-480.

Sansano S, Rivas A, Pina-Pérez MC, Martinez A and Rodrigo D (2017) Stevia rebaudiana Bertoni effect on the hemolytic potential of *Listeria monocytogenes*. *International Journal of Food Microbiology 5(*250): 7-11.

Sanz-Puig M, Pina-Pérez MC, Criado MN, Rodrigo D and Martínez-López A (2015a). Antimicrobial potential of cauliflower, broccoli, and okara byproducts against foodborne bacteria. *Foodborne Pathogens and Diseases* 12(1): 39-46.

Sanz-Puig M, Pina-Pérez MC, Rodrigo D and Martínez-López A (2015b) Antimicrobial activity of cauliflower (Brassica oleracea var. Botrytis) by-product against *Listeria monocytogenes*. *Food Control* 50: 435–440.

Sanz-Puig M, Santos-Carvalho L, Cunha LM, Pina-Pérez MC, Martínez A and Rodrigo D (2016) Effect of pulsed electric fields (PEF) combined with natural antimicrobial by-products against *S.* Typhimurium. *Innovative Food Science and Emerging Technologies* 37: 322–328.

475 Sanz-Puig M, Moreno P, Pina-Pérez MC, Rodrigo D and Martínez A (2017a) Combined effect of
476 high hydrostatic pressure (HHP) and antimicrobial from agro-industrial by-products against *S.*
477 Typhimurium. *LWT-Food Science and Technology* 77: 126-133.
478
479 Sanz-Puig M, Lázaro E, Armero C, Alvares D, Martínez A and Rodrigo D (2017b) S. Typhimurium
480 virulence changes caused by exposure to different non-thermal preservation treatments using
481 *C. elegans. International Journal of Food Microbiology* 4(262): 49-54.
482
483 Sanz-Puig M, Velázquez-Moreira A, Torres C, Guerrero-Beltrán JA, Cunha LM, Martinez A and
484 Rodrigo D (2018) Resistance changes in *Salmonella enterica* serovar Typhimurium treated by
485 High Hydrostatic Pressure and Pulsed Electric Fields and assessment of virulence changes by
486 using *Caenorhabditis elegans* as a test organism. *Innovative Food Science and Emerging*
487 *Technologies* 51: 51-56.
488
489 Schroeder M, Brooks BD and Brooks AE (2017) The Complex Relationship between Virulence
490 and Antibiotic Resistance. *Genes (Basel)* 8(1) 39: 1–23.
491
492 Silva A, Genoves S, Martorell P, Zanini SF, Rodrigo D and Martinez A (2015) Sublethal injury and
493 virulence changes in *Listeria monocytogenes* and *Listeria innocua* treated with antimicrobials
494 carvacrol and citral. *Food Microbiology* 50: 5–11.
495
496 Sudhaus N, Pina-Pérez MC, Martínez A and Klein G. (2012) Inactivation kinetics of spores of
497 Bacillus cereus strains treated by a peracetic acid-based disinfectant at different
498 concentrations and temperatures. *Foodborne Pathogens and Diseases* 9(5): 442-452.
499
500 Tiwari BK, Valdramidis VP, O'Donnell CP, Muthukumarappan K, Bourke P and Cullen PJ (2009)
501 Application of natural antimicrobials for food preservation. *Journal of Agricultural and Food*
502 *Chemistry* 57: 5987–6000.
503
504 Tiwari R and Tiwari G (2011) Use of antibiotics: from preceding to contemporary. *Scholar ' s*
505 *Research Journal* 1: 59-68.
506
507 Ultee A, Kets EP, Alberda M, Hoekstra FA and Smid EJ (2000) Adaptation of the food-borne
508 pathogen *Bacillus cereus* to carvacrol. *Archives in Microbiology* 1744: 233–238.
509
510 Viuda-Martos M, Ruiz-Navajas Y, Fernandez-Lopez J and Perez-Alvarez J (2007) Antibacterial
511 activity of lemon (Citrus lemon L.), mandarin (Citrus reticulata L.), grapefruit (*Citrus paradisi* L.)
512 and orange (*Citrus sinensis* L.) essential oils. *Journal of Food Safety* 28: 567–576.
513
514 Wang E and Wink M (2016) Chlorophyll enhances oxidative stress tolerance in *Caenorhabditis*
515 *elegans* and extends its lifespan. *Peer Journal* 4: 1-17
516
517 WHO (2017) WHO priority pathogens list for R&D of new antibiotics.
518 http://www.who.int/news-room/detail/27-02-2017-who-publishes-list-of-bacteria-for-which-
519 new-antibiotics-are-urgently-needed

Zanini S, Silva A, Rosenthal A, Rodrigo D and Martinez A (2014) Influence of the Treatment of *Listeria monocytogenes* and *Salmonella* enterica Serovar Typhimurium with citral on the efficacy of various antibiotics. *Foodborne Pathogens and Diseases* 11(4): 265–271.

544 **TABLE 1.** Percentiles for *C. elegans* when fed with the different *S.* Typhimurium sub
545 populations.

	Untreated *S.* Typhimurium	*S*. Typhimurium treated once	*S*. Typhimurium treated three times
Percentile	Time (days)	Time (days)	Time (days)
75.0	2.4	3.9	3.3
50.0	5.3	8.5	8.2
25.0	10.2	13.9	13.4

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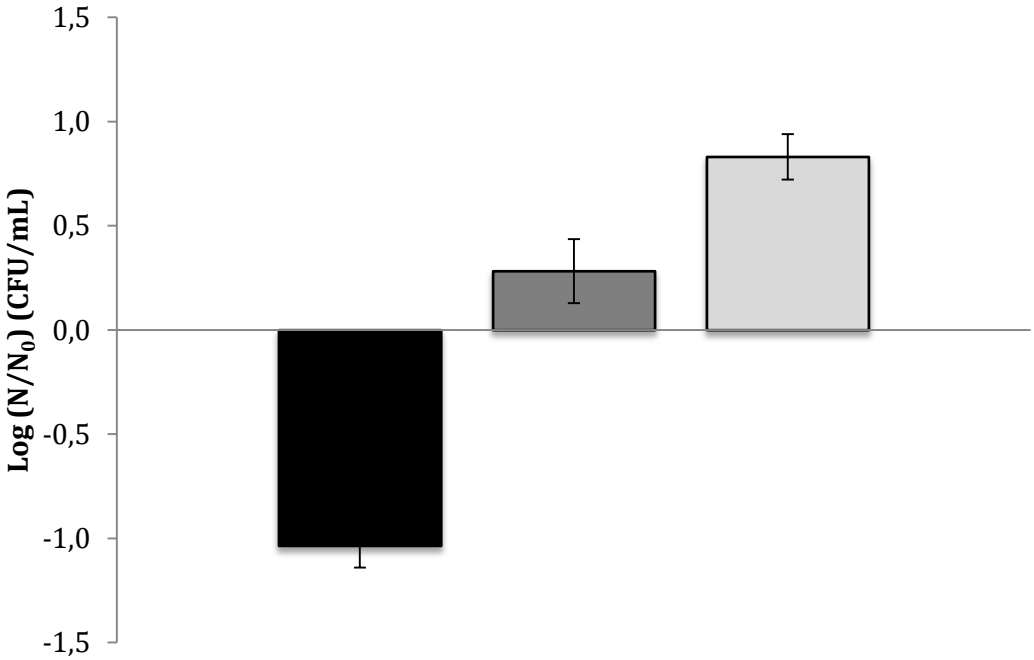


FIGURE 1. Evolution of *S*. Typhimurium resistance when subjected to repeated antimicrobial treatments with 5% cauliflower by-product infusion (Initial bacterial count 10^7 CFU/mL). Treated once, Treated two times, Treated three times

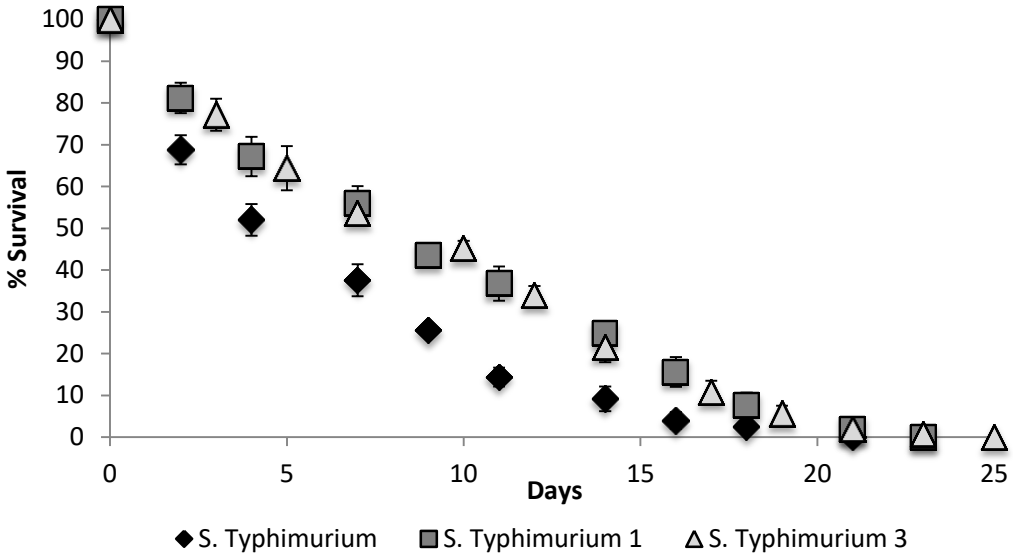
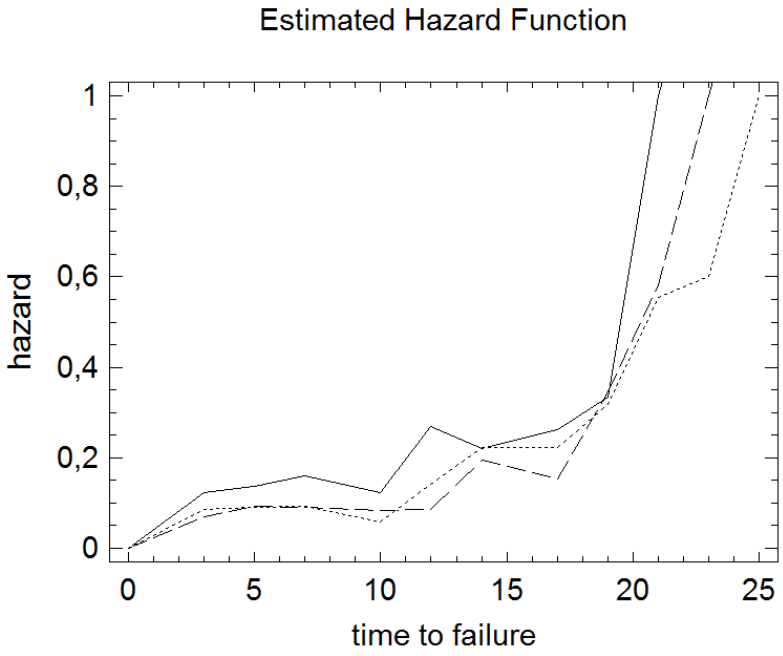


FIGURE 2. Survival function of *C. elegans* when fed with untreated *S.* Typhimurium and *S.* Typhimurium treated once and three times with cauliflower by-product extract. ◆Untreated, ■Treated once, △Treated three times.



—— *S.* Typhimurium without treatment

--- *S.* Typhimurium treated once

….. *S.* Typhimurium treated three times

FIGURE 3. Hazard function of *C. elegans* when fed with untreated *S.* Typhimurium and *S.* Typhimurium treated once and three times with cauliflower by-product extract.
—— *S.* Typhimurium, – – · *S.* Typhimurium 1, ---- *S.* Typhimurium 3.

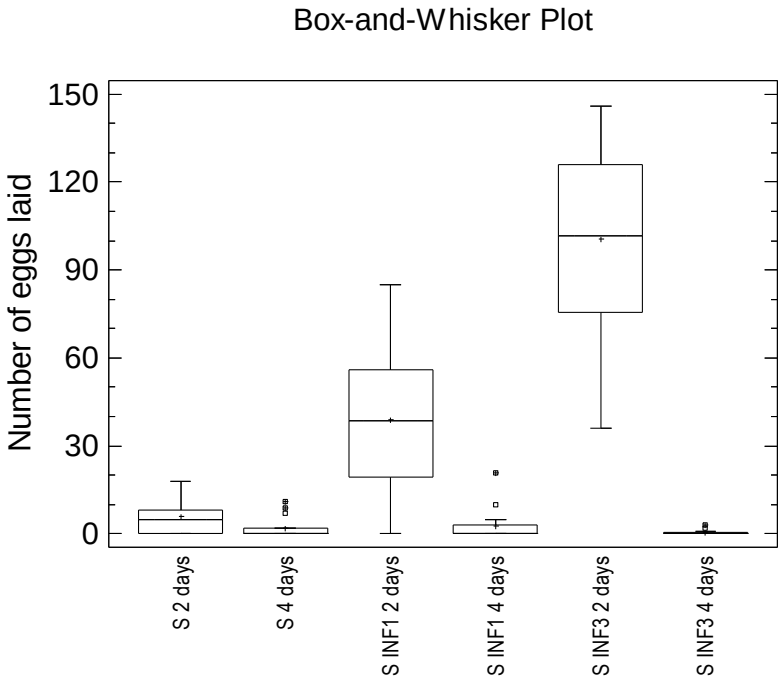


FIGURE 4. Eggs laid during first two time intervals by *C. elegans* fed with different *S.* Typhimurium populations.
S= *Salmonella* without treatment
SINFE 1= *Salmonella* treated once
SINFE 3= *Salmonella* treated three times
2 days= Eggs laid at day 2
4 days= Eggs laid at day 4