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In vivo Antimicrobial Activity Assessment of a Cauliflower By-Product Extract Against *Salmonella* Typhimurium

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The main objective of this work was to study the antimicrobial effect of a cauliflower by-product infusion into an affordable *in vivo* model (*Caenorhabditis elegans*). The infusion demonstrated some protective effect on non-infected and infected worms with *Salmonella* Typhimurium as indicated by higher survival percentile values (75, 50, 25, and 5% percentiles) as compared with those from worms unexposed to the infusion. The antimicrobial effect of the infusion was evaluated on *Salmonella* intestinal colonization of infected worms (24, 48, and 96 h post-infection). At 96 h post-infection, the concentration of *Salmonella* was reduced around 2 log cycles in infected cauliflower treated group ($p < 0.05$) as compared with infected non-cauliflower group. Here we show that cauliflower by-products extend survival and have an antimicrobial effect in an *in vivo* nematode model, *C. elegans*, as a previous validation step to longer and costlier farm animal studies.

Keywords: agro-industrial waste, by-product, cauliflower, antimicrobial activity, *C. elegans*, *Salmonella* Typhimurium

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INTRODUCTION

Some natural antimicrobials by-products from plants (essential oils and plant extracts) have demonstrated their effectiveness against *Salmonella* Typhimurium *in vitro* (Sanz-Puig et al., 2015a; Mohamed et al., 2016). It has also been found that many vegetables in the *Cruciferae* family have antimicrobial properties against several microorganisms of clinical importance. The *in vitro* antibacterial effect of *Brassica oleracea* products on several foodborne pathogens was evidenced by Brandi et al. (2006) and Sanz-Puig et al. (2017), between others. Some of the antimicrobial properties of these plant extracts are associated with antioxidant compounds such as polyphenols (Sanz-Puig et al., 2015b; Marchese et al., 2016; Bakari et al., 2018). It is believed that the antibacterial activity of phenolic compounds is due to direct interference with bacterial growth or to inhibition of the production of virulence factors, resulting in attenuated pathogenesis (Ivanova et al., 2013).

Salmonellosis is one of the most recurrent food safety problems in the EU (100,000 cases reported/year, European Food Safety Authority (EFSA)., 2014). It is a zoonotic disease or infection that can be transmitted directly or indirectly between animals and humans. *Salmonella* spp. are commonly found in the intestines of healthy birds and mammals and, in food, it is most often found in eggs and raw meat from pigs, turkeys, and chickens contaminated during the slaughterhouse process. To reduce the risk of infection, it is important to prevent the disease at the farm. Reduction

of the microbial load in the intestine of animals prior to slaughter could be a control measure for salmonellosis infections transmitted by food by reducing the level of contamination of flesh at the slaughterhouse.

Before testing the effect of antimicrobials in the intestine of farm animals, which requires resources that are sometimes substantial, some *in vivo* assays could be carried out in a more affordable system. In this way, the nematode *C. elegans* can play an interesting role as a live test organism. This organism is an invertebrate hermaphrodite nematode that feeds on bacteria and lives in the soil. Owing to the conservation of many homologous biological processes with mammals, *C. elegans* has been chosen as a test organism in many studies on the virulence effect of pathogenic bacteria (Kurz and Ewbank, 2000; Jiang and Wang, 2018). In addition, aspects of aging are similar between nematodes and mammals, including humans (Wilson et al., 2006).

The use of by-product extracts or infusions obtained from by-products offers another interesting way to comply with a circular economy by reducing and revalorizing agro-industrial residuals. Considering these findings, the use of these natural extracts in animal feed could be an alternative, non-curative, control option for the pathogenic bacteria in animal intestines.

The objective of the present work was to study the effect of a by-product infusion from *B. oleracea* var. botrytis on *C. elegans*, evaluating the survival of the infected by *Salmonella* Typhimurium and non-infected nematodes and the survival of Salmonella in the worm's intestine fed with the infusion.

MATERIALS AND METHODS

Culture of Nematodes and Microbial Strains

Caenorhabditis elegans strain N2 was provided by the College of Biological Sciences, Minnesota University, USA. For optimal growth of the nematodes, Nematode Growth Medium (NGM) agar was used in medium-size plates (60 mm diameter) for general strain maintenance, and larger plates (100 mm diameter) for growing larger quantities of worms, with a bacterial lawn of *Escherichia coli* strain OP50 (*E. coli* OP50) at 20°C (Stiernagle, 2006). The study was performed with young adult *C. elegans* (L4 growth stage) that were previously synchronized.

E. coli strain OP50 was grown in Luria-Bertani broth overnight before its inoculation on NGM plates (Roth et al., 1985).

S. Typhimurium was provided by the Spanish Type Culture Collection (CECT 443). The Salmonella strain was grown in Tryptic Soy Broth (TSB) (Scharlab Chemie) and incubated for 14 h at 37°C to obtain a stock of cells.

Preparation of Nematode Growth Medium (NGM) Agar With Cauliflower By-Product Infusion

Cauliflower infusion was made from by-product consisting of dried leaves of *B. oleracea* var. botrytis. These by-products were provided by TRASA S.L. from agro-industrial primary production.

To prepare a 3% (w/v) cauliflower infusion, 0.1% peptone water was boiled. The by-product was added when the water boiled and it was left infuse for 30 min. After that, the infusion was centrifuged at 6,000 g for 15 min at 4°C. Next, it was vacuum filtered with Whatman filter paper (90 mm diameter). Finally, NGM medium with cauliflower infusion was prepared by replacing the distilled water with the infusion that had been obtained.

The concentration of 3% (w/v) cauliflower was chosen because it was the maximum that had shown *in vitro* antimicrobial activity and allowed the growth of *E. coli* OP50.

C. elegans Survival Studies

For survival studies, uninfected nematodes were distributed on NGM agar with *E. coli* OP50 lawn plates (N) or on NGM agar with *E. coli* OP50 lawn supplemented with 3% cauliflower infusion plates (N_CA).

In the same way, infected nematodes were distributed on NGM agar with *E. coli* OP50 lawn plates (I) or NGM agar with *E. coli* OP50 lawn supplemented with 3% cauliflower by-product infusion plates (I_CA), depending on the survival study.

In all study cases (uninfected and infected nematodes), five repeats were evaluated with 5 plates each. Each plate contained 10 synchronized worms, i.e., 50 nematodes per repeat. The total number of synchronized worms used in the study was 250 for each process.

Live worms on each plate were counted every 48 h for 21 days.

Salmonella Infection

For *S.* Typhimurium infection, the nematodes were transferred to plates with NGM agar and *Salmonella* Typhimurium lawn and kept in contact with the pathogen for 5 h. After that time, they were transferred to the appropriate medium for the survival study (I or I_CA plates, for the media without or with cauliflower, respectively).

Quantification of *S.* Typhimurium Infection in the Intestine of *C. elegans*

For the quantification of *Salmonella* Typhimurium in the digestive tract of the nematode, 5 worms infected with *Salmonella* Typhimurium were lysed 24, 48, and 96 h after feeding in the various media already described (I or I_CA plates, for the media without or with cauliflower, respectively). For this purpose, the worms were placed in a plate of NGM medium and were washed twice with 10 µl of M9 medium. The five washed worms were transferred to a 1.5 ml Eppendorf containing 1 ml of M9 with 1% Triton X-100 and five glass beads. Lysis of *C. elegans* contained in the Eppendorf tubes was performed by mechanical action using a vortex. The concentration of *Salmonella* (CFU/ml) in the digestive tract of *C. elegans* was obtained from lysates, which were serially diluted, and incubated in Eosin Methylene Blue agar (EMB agar) at 37°C for 48 h. Base dilutions were made from 10 to 10^{-4} to achieve a reliable count that established the concentration of *Salmonella* sp. Presumptive colonies of lactose-negative *Salmonella* sp. were counted when bacteria showed absence of coloration in this EMB culture medium. The assay plates were prepared in duplicate.

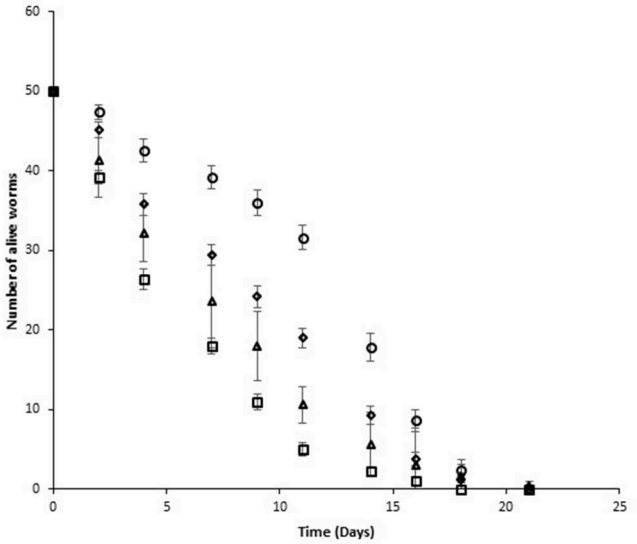

FIGURE 1 | *C. elegans* survival curves for the various nematode groups considered in the study (N: △, N_CA: ◯, I: □, I_CA: ◊). Each point represents mean ± standard deviation (error bars) of five repeat values.

This study was carried out in duplicate with 30 plates, with one synchronized nematode in each plate.

Statistical Analysis

The analysis of experimental data of microbial counts and survival curves was carried out by using Statgraphics Centurion XVI (Statpoint Technologies, Inc., USA). Kaplan Meyer analysis was performed for survival curves. The statistical significance of the microbial counts was determined by ANOVA.

RESULTS

Effect of Cauliflower By-Product Extract Infusion on Uninfected *C. elegans*

The survival curves for the various nematode groups under study until all the nematodes died are shown in **Figure 1**, **Table S1**. As can be seen, from the second day until the end of the study (21 days) the number of live nematodes at each defined length of time was significantly higher for the uninfected nematode group fed with cauliflower extract (N_CA) than for the control (N) group ($p < 0.05$). These findings in the survival curve were patent when the percentiles were analyzed (**Table 1**).

Although the number of live nematodes decreased with time until the end of the 21 days, 50 percent of the uninfected worms fed on 3% cauliflower extract (N_CA) survived for a length of time equal to 12.4 days, whereas in the control group (N) 50 percent of the worms survived for a length of time equal to 6.9 days. The difference in survival between the two groups was still maintained until the fifth percentile, with values of 16.65 ± 3.3 and 17.9 ± 2.8 days for the control (N) and cauliflower-fed (N_CA) groups, respectively. Thus, the presence of cauliflower in the feed medium appears to decrease the death rate of the nematodes at each time interval tested.

TABLE 1 | Percentiles of *C. elegans* lifespan for the various nematode groups considered in the study.

Percentile (%)	Survival time (days, standard deviation)			
	N	N_CA	I	I_CA
75	3.81 ± 1.5	7.8 ± 2.6	2.2 ± 0.6	3.6 ± 0.8
50	6.92 ± 2.0	12.4 ± 0.9	4.4 ± 1.8	8.8 ± 1.8
25	11.52 ± 2.0	15.1 ± 1.3	8.8 ± 1.9	13.0 ± 1.6
5	16.65 ± 3.3	17.9 ± 2.8	13.7 ± 1.0	17.0 ± 1.8

Values represent the survival time (days ± standard deviation) at the 75, 50, 25, and 5% percentiles in each study treatment.

Effect of Infection With *Salmonella* Typhimurium on *C. elegans*

As shown in **Figure 1**, infection produced a statistically significant ($p < 0.05$) reduction in live nematodes for a defined length of time in the I group in comparison with the uninfected N control group, both fed on standard Nematode Growth Medium. Percentile analysis (**Table 1**) indicated the difference for the 50th percentile between the uninfected group (N) (6.9 ± 2.0 days) and the infected group (I) (4.4 ± 1.8 days). This difference in lifespan between the N and I groups was also observed for the 75, 25, and 5th percentiles, where the worms were very close to the limit of their lifespan. These results indicate that *Salmonella* infection affected the survival of *C. elegans*.

Effect of Cauliflower By-Product Extract Infusion on *C. elegans* Infected by *Salmonella* Typhimurium

When infected worms were fed on a medium containing cauliflower by-product infusion (I_CA group), differences in the survival curve were patent compared with that of the infected group fed without the infusion (I) and the control group (N) (**Figure 1**). A statistically significant ($p < 0.05$) increase in survival after defined periods of time was observed for the I_CA group, although the increase was lower than that observed in the uninfected group fed with cauliflower by-product infusion (N_CA). This increase in survival was reflected in all percentiles, as can be seen in **Table 1**. According to this table, 50 percent of the worms survived for a period of time equal to 8.8 ± 1.8 days in the I_CA group, while in the I group they survived for a length of time equal to 4.4 ± 1.8 days. Again, the cauliflower extract appears to have had a beneficial effect on *C. elegans*, extending the survival for a certain length of time. According to these results, it is important to know how *Salmonella* evolves in the worm's intestine after infection and to try to determine whether the extract could exert its antimicrobial effect inside the nematode's intestine or not, before considering its use as a control measure before animals are subjected to the slaughterhouse process.

Quantification and Evolution of *S.* Typhimurium Infection in the Intestine of *C. elegans*

The evolution of the *Salmonella* counts in the nematode's intestine expressed as log (N/N0) for the two groups, infected (I) and infected fed with cauliflower extract (I_CA), can be seen in **Figure 2**, **Table S2**.

It was observed that *Salmonella* grew until the fraction reached 1.25 log in the I group 24 h after infection, while for the I_CA Salmonella counts increased only 0.5 log cycles, being significant differences ($p \leq 0.05$) between both populations for the same period of time. This means that an increase in *Salmonella* survival in the *C. elegans* digestive system took place during the first 24 h post-infection period, irrespective of the group considered, but some control of *Salmonella* growth was exerted by the cauliflower extract that it only increased 0.5 log cycles. *Salmonella* growth decreased at 48 h after infection and remained stable until 96 h after infection in worms not fed with cauliflower extract. When worms infected with *Salmonella* were fed with cauliflower extract, death of *Salmonella* was observed at 48 and 96 h and counts were significantly lower ($p \leq 0.05$) than in nematodes infected but not fed with cauliflower extract (**Figure 2**). Cauliflower extract acted as an antimicrobial in the nematode's intestine, reducing the microbial survival fraction by about two log cycles.

According to these results, it is possible to say that the presence of cauliflower by-product infusion in NGM agar appears to control infection of the *C. elegans* digestive tract by *S.* Typhimurium.

DISCUSSION

It has been reported that the lifespan of *C. elegans* is related with genetic and environmental factors, such as temperature, food availability and composition (Blumenthal and Steward, 1997; Uno and Nishida, 2016). According to Sanz-Puig et al. (2015a) and Vieira (2013), cauliflower by-product infusion provides bioactive compounds such as polyphenols. It has been found that polyphenols contained in hydrophilic extracts of Brassica species are responsible for 80–95% of their total antioxidant capacity (Kurilich et al., 2002; Xianli et al., 2004). According to different authors (Kampkötter et al., 2008; Saul et al., 2011; Surco-Laos et al., 2011; Grünz et al., 2012; Kim et al., 2014), cauliflower polyphenols such as gallic acid, catechin, protocatechuic acid, quercetin and kaempferol prolonged the lifespan of *C. elegans* between 5.6 and 15% and produced some stress protection. However, other studies reported protection against thermal stress but no protection against acute oxidative stress (Wilson et al., 2006) or extended *C. elegans* lifespan (Chen et al., 2013).

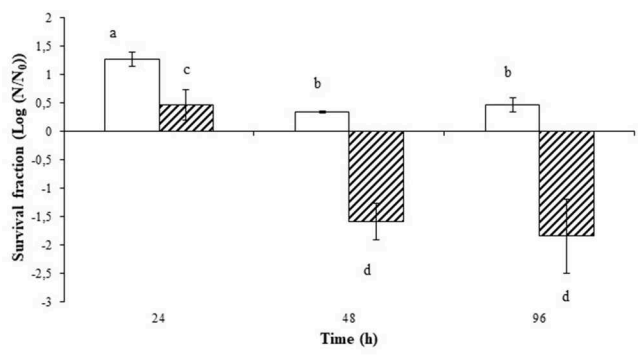

FIGURE 2 | Evolution of the microbial load of *Salmonella* in the intestine of *C. elegans* at 24, 48, and 96 h post-infection fed (stripped bar) and not fed (blank bar) with cauliflower extract. Survival of *Salmonella* Typhimurium in the intestine of *C. elegans* is expressed as survival fraction of *Salmonella* cells (CFU/mL) in the intestine of *C. elegans* (Log (N/N0)) at 24, 48, and 96 h post-infection fed and not fed with cauliflower extract. Different letters indicate significant differences in survival fractions ($p \leq 0.05$) and error bars represent standard deviation.

Studies with other compounds rich in polyphenols showed that they increased the lifespan and thermotolerance of *C. elegans* (Wilson et al., 2006; Pallauf et al., 2017). After studying the effect of an herbal mixture on *C. elegans*, Moriwaki et al. (2013) concluded that the herbal mixture prolonged its lifespan, delayed aging, and also suppressed oxidation of protein cells in the nematode. However, it appears that the longevity of the *C. elegans* mev-1 (kn1) mutant produced by gallic acid, which is present in cauliflower, was not due to its antioxidant capacity but to its antimicrobial properties (Saul et al., 2011). All these findings may help to explain why the cauliflower extract infusion clearly reflected a positive influence on the *C. elegans* lifespan of N_CA and I_CA compared with the N and I control groups.

At the same time, it has been demonstrated that *S.* Typhimurium shortened the survival of *C. elegans* (Aballay et al., 2000; Labrousse et al., 2000; Sem and Rhen, 2012). Fifty percent of L4-stage worms were alive about 9 days after being exposed to *S.* Typhimurium strain 12023 for 8 h and then returned to an *E. coli* OP50 lawn as feeding material (Labrousse et al., 2000). Aballay et al. (2000) used 1-day adult nematodes exposed to *S.* Typhimurium for their study and found that 50% of the nematodes were dead at 5.1 days when exposed to *S.* Typhimurium SL 1344 or 4.8 days when infected by *S.* Typhimurium 14028. In our study, 50% of the worms survived for 4.4 days in the case of nematodes infected by *S.* Typhimurium (CECT 443) without cauliflower extract exposure. These results are in agreement with the above-mentioned studies, although some authors have indicated that factors such as difference in culture temperature, inter-individual differences, and/or use of different Salmonella strains could affect survival of the nematode (Blumenthal and Steward, 1997). The virulence mechanism may be due to intracellular infection in the host, although Sem and Rhen (2012) described a new form of virulence that depends on the ability to induce overwhelming oxidative stress in the host through redox activity of bacterial thioredoxin 1 without intracellular invasion.

In the present work, a similar protective effect of the cauliflower extract on the infected nematodes was observed. *C. elegans* can use various strategies against *S.* Typhimurium infection (Alegado and Tan, 2008; Sem and Rhen, 2012; Curt et al., 2014). One defensive mechanism of *C. elegans* against this

pathogen is production of reactive oxygen species (ROS) while inducing an oxidative stress response that depends on DAF-16, a transcriptional factor (Chávez et al., 2007). Sem and Rhen (2012) indicated that the presence of 50 mM of ascorbic acid reduced the ROS response and significantly increased the lifespan of nematodes infected with *S.* Typhimurium by 2 days at 50% survival time, compared to the uninfected group. Vayndorf et al. (2013) noticed increased resistance to *Pseudomonas aeruginosa* in *C. elegans* pre-treated with whole apple extracts (2 days pre-treatment) as well as greater lifespan in a dose-dependent manner compared with infected non-pre-treated worms. They suggested that the pre-treatment with apple extract produced an increase in survival of up to 35.2% in infected worms, and also that the apple extract enhanced the nematode immune response.

In the present study, the presence of cauliflower extract extended survival by 4 days in infected nematodes at 50% survival time. This result could indicate that the presence of the cauliflower extract would produce the same effect as vitamin C in reducing the ROS response, or maybe an enhancement of the nematode immune response. It is necessary to take into account the fact that polyphenols are one of the main groups of bioactive components in cruciferous vegetables (Soengas et al., 2011). They have been related to antioxidant properties in *C. elegans*, inducing resistance to oxidative stress and delaying aging (Wu et al., 2002; Cañuelo et al., 2012).

In recent years, *Salmonella* infection has reached dramatic levels and has become a challenge in the food sector. The use of antibiotics worldwide has resulted in an increase in antibiotic resistance; consequently, new antimicrobials are necessary to avoid resistance problems with antibiotics used in human therapy. Researchers have focused efforts on the therapeutic activities of natural products from plants. There are many plant essential oils and other products from plants that can be used to control bacteria, including *Salmonella* (Bajpai et al., 2012). Essential oils added to feed or water have been used to control fecal excretion of *Salmonella*, with promising results (Borsoi et al., 2011). The authors just cited, used live animals for their trials. However, there are no studies in which cauliflower extract has been used to control *Salmonella* in the intestine of living animals. The use of live animals for trials can be expensive, so to test the effect of natural antimicrobials from plants in the intestine of infected living beings such as the *C. elegans* nematode, can be useful as a living test organism before performing field tests. The results of the present study indicate that *Salmonella* can infect the intestine of *C. elegans*. Aballay et al. (2000) reported *S.* Typhimurium and *E. coli* bacteria counts (log CFU/worm) in *C. elegans* intestine during a period of 5 days. At day 1, intestinal *Salmonella* grew by ∼1.5 log cycles. The pathogenic bacteria counts almost doubled on the second day, and they seemed to stabilize on the third day, when a 3.5 log cycle growth was achieved. In agreement with those findings, in the present work, infected worms showed around a 1.25 log increase in *Salmonella* cells at day 1; however, in contrast with the results of Aballay et al. (2000), the *Salmonella* cells decreased by ∼1 log cycle 48 h after infection, with a non-significant increase 96 h after pathogen exposure. The differences encountered in the two studies for untreated infected nematodes at 48 h and 96 h post-infection may have been due to the different *S.* Typhimurium strains used in the two studies or other factors.

Marsh et al. (2011) indicated approximate counts of 10^4 CFU of *S.* Typhimurium strain L1019 (a GFP-expressing derivative of SL1344) in every L4-stage *C. elegans* (CFU/worm) at day 1 post-infection. This datum is in accordance with the mean *S.* Typhimurium cell counts (2.97 × 104 CFU/worm) found in the present study. However, although Marsh et al. (2011) showed a rise in pathogen concentration to $\sim 10^7$ CFU/worm at day 2, our results demonstrated a mean CFU/worm decrease to 1.71 × 10^3 CFU/worm. Interestingly, both studies show a similar concentration of *Salmonella* cells around 10^3-10^4 CFU/worm at day 4. Consequently, from the results presented, it seems that *C. elegans* can be used to test the efficacy of cauliflower extract in controlling *Salmonella* in infected nematodes.

CONCLUSIONS

In conclusion it can be said that cauliflower extract exerts a protective action against aging in uninfected worms, probably owing to an antioxidant activity of the compounds in the extract. This protective effect was also shown in *Salmonella*-infected worms. Results at the 2nd and 4th days post-infection showed a positive effect of cauliflower treatment in lifespan extension related with a nearly 2 log cycle reduction in *Salmonella* cells at 48 h and even at 96 h after infection with the pathogen. Therefore, an interesting feature demonstrated in this study is the effect of cauliflower extract as feed material in controlling Salmonella in the worm intestine by reducing the microbial load after 96 h. Nevertheless, more information is needed on possible pathogen resistance to this natural antimicrobial in relation to time.

In order to optimize the incorporation of this vegetable extract with antimicrobial properties, these findings should be corroborated and completed with experimental studies in farm animals, adapting it to the necessities of each animal species. The use of agro-industrial waste to obtain the vegetable extracts contributes to sustainable agriculture development as well as a possible economic revalorization of these residues.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

DR: conceptualization and experimental design. AM: writing. GG-C: experimental analysis. DI-P: experimental and data analysis. CP-P: paper supervision.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2020.00008/full#supplementary-material

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