Internalization capacity of *Salmonella enterica* subsp. *enterica* serovar Thompson in strawberry plants via root

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1.1. Abstract

Strawberry production represents an agriculture sector of high relevance for the Spanish economy, due to their strawberries, highly appreciated for their organoleptic characteristics and health benefits. However, the reported outbreaks in different countries by enteric pathogens associated with this product have increased the safety concerns among different stakeholders. A number of factors and pathways for introducing pathogens in the strawberry production chain has been investigated. However, information on the potential internalization of enteric pathogens in strawberries is still scarce. The present study aimed to evaluate the potential internalization of *Salmonella enterica* subsp. *enterica* serovar Thompson via root in strawberry plants under the application of successive contamination events through capillary irrigation. Strawberry plants were organized in three groups (16 plants per group), and they were submitted to different contamination scenarios; groups 1, 2 and 3 were irrigated 1, 2 and 3 times respectively, with 100 mL of water containing *S*.Thompson (8.8 log10 CFU/mL) every other day for 9 days. Fruits, calyces, and leaves were analyzed during the experiment, while roots were analyzed on the 9th day. The results showed that all calyces analyzed were negative, while one strawberry sample (group 3; day 8), four leaves samples (groups 2 and 3; day 8) and one root sample (group 3; day 9) were positive for *S*.Thompson, representing the 0.85% (95% C.I.: [0.02–4.63%]), 8.88% (95% C.I.: [2.48–21.22%]), and 2.22% (95% C.I.: [0.06–11.77%]), respectively, of the overall samples analyzed. A significant association between the increased frequency of contamination (three irrigations) and the presence of the pathogen in the samples was elucidated. Our findings, in comparison with other studies, also point out the importance of watering by drip irrigation as a more hygienic and safer agronomic practice than systems like sprinkling irrigation.

Keywords: strawberry, *Salmonella enterica* subsp. *enterica* serovar Thompson, internalization, irrigation

1.2. Introduction

Strawberry is a fruit highly appreciated for its sensory characteristics and functional properties attributed to its content of nutrients, phytochemicals and fiber (Basu et al., 2014; Battino et al., 2020; Jimenez-Garcia et al., 2013). According to the latest FAOSTAT report, Spain leads the strawberry production in the European Union with 344,679 tons and is also considered the leading exporter worldwide (Food and Agriculture Organization of the United Nations [FAO], 2020). Fresh market strawberries account for about 80% of total strawberries production, while the rest is intended for industrial transformation (Šamec et al., 2016).

Berries have been traditionally considered as a low-risk food category mainly due to their low pH and the presence of natural antimicrobial substances such as polyphenols. However, it has been recognized that there is the possibility for microbial contamination during cultivation, harvest and post-harvest operations (Sreedharan et al., 2015). Pre-harvest microbial contamination by enteric pathogens can often occur through contaminated irrigation water, agricultural soil, raw or improperly composted manure, poor agricultural hygiene practices and/or feces deposited by intruding domestic or wild animals (Iwu& Okoh, 2019). These issues may lead to unsafe products, and thus, cases and outbreaks such as the one caused by the consumption of contaminated fresh strawberries by *Escherichia coli* O157:H7 from deer feces (Laidler et al., 2013). Other outbreak investigations associated to the consumption of strawberries contaminated with animal manure or human fecal matter have also been documented (Beuchat, 2006). Likewise, contaminated irrigation water has been linked to a hepatitis A outbreak caused by consumption of strawberries (Centers for Disease Control and Prevention [CDC], 2016). On the other hand, recent outbreaks caused by viruses with unknown contamination routes in many cases (Bernard et al., 2014; European Commission [EC], 2018a, 2018b) have increased the concern about the safety of strawberries, since that these products are not always washed or disinfected (European Food Safety Authority [EFSA], 2014).

During primary production, pathogens may become internalized into plant tissues as shown in various studies. Erickson et al. (2010b) and Solomon et al. (2002) demonstrated the internalization of *E. coli* O157:H7 in lettuce leaves. Ongeng et al. (2011) also showed the internalization of *E. coli* O157:H7 together with *Salmonella* Typhimurium in cabbage leaf tissues. Zheng et al. (2013) found the internalization of *Salmonella* in stems, leaves and fruit of tomatoes.

Pathogenic internalization constitutes a risk event with serious implications for public health as pathogens remain protected within the tissues, which limits the action of disinfectant agents in fresh products (Meireles et al., 2016). Internalization of pathogens depends upon different factors such as plant species, plant age, microbial ecology, contamination routes and levels of contamination (Deering et al., 2012; Ge et al., 2014). Many internalization studies have focused on vegetables (Erickson, 2012; Hirneisen et al., 2012) and fruits such as tomatoes, melons and cucumbers (Bartz et al., 2015; Burris et al., 2020, 2021). In 2014, the Panel on Biological Hazards of EFSA published a Scientific Opinion highlighting the paucity of information on the potential of *Salmonella* or Norovirus to be internalized within berries or plants (EFSA, 2014). Since then, a few investigations have been carried out; for example, DiCaprio et al. (2015) evidenced the internalization of Norovirus surrogates via root in strawberry plants and its subsequent dissemination to leaves and fruits in a soil growth system. The study of Brassard et al. (2012) focused on the study of water as a source of contamination; however, they did not find any link between irrigation water and viral contamination in strawberries. In the same fashion, another study found a low persistence of *E. coli* in strawberries after irrigation with naturally contaminated water under field conditions (Généreux et al., 2015).

According to EFSA, *Salmonella* is the most common cause of foodborne outbreaks in the European Union (EFSA, 2019). Although most of these outbreaks have been related to eggs and egg products, foods of plant origin have also been implicated. According to the latest EU One Health Zoonoses Report, *Salmonella* caused 2 strong-evidenced foodborne outbreaks in fruits, berries and juices and other categories thereof, both associated with *S*. Enteritidis (1.1% of total) (EFSA & European Centre for Disease Prevention and Control [ECDC], 2019). Further, several notifications have alerted the presence of

this pathogen in berries through the Rapid Alert System for Food and Feed (EC, 2017, 2019, 2020). In the United States, *Salmonella* outbreaks linked to strawberries consumption were reported in 2003 and 2018 (CDC, 2018). Since *Salmonella* can be found in wastewater and survive for a long time in soil (ca. 300 days), contamination of plant products constitutes more than a plausible event (Baloda et al., 2001; Beuchat, 2006). Internalization of this pathogen via root from contaminated water has been extensively studied in different crops such as lettuce, onion and tomato (Ge et al., 2012, 2014; Hintz et al., 2010; Solomon et al., 2002). Besides, although it is expected that continuous irrigation with contaminated water increases the risk of contamination of fresh produce during field cultivation (Alegbeleye et al., 2018), published results seem to be contradictory. Lapidot and Yaron (2009) found a higher concentration of *S.* Typhimurium in parsley stems and leaves after the third irrigation. However, in the study of Jablasone et al. (2004), *S.* Enteritidis was not detected in leaves, stems or fruits of tomato plants watered every other day with contaminated water for 5 weeks.

Research on internalization ability of *Salmonella* in strawberries would support accurate estimations about the potential risk of salmonellosis by fresh strawberries consumption. The present study aims to evaluate the possible internalization of *Salmonella enterica* subsp. *enterica* serovar Thompson (*S.* Thompson) via root in strawberry plants under the application of successive contamination events through irrigation water.

1.3. Materials and methods

1.3.1. Bacterial suspension and contaminated water preparation

S. Thompson pGT-Kan mB156, a strain Green-Fluorescent-Protein (GFP) labelled, was used in the study in order to easily distinguish it from the microbiota present in the tissues. This serovar has been previously described as a clinical isolate from a patient in a cilantro-linked outbreak in California (Brandl et al., 2005). This strain was made resistant to gentamicin (15 μg/mL) to facilitate enumeration of

Salmonella on strawberry plant samples, minimizing interferences from native microbiota. The choice of such strain has been motivated because of its resistance to acidic conditions of berry fruits in preliminary studies done in strawberry juice (data not shown). Furthermore, the strain has been already used in other challenge testing studies with strawberries (Delbeke et al., 2015) as a target microorganism in vegetable matrices. *S.* Thompson has been eventually deemed as the cause of outbreaks originated by consumption of foods of plant origin, e.g. fresh cilantro (Campbell et al., 2001) or rucola lettuce (Nygård et al., 2008).

The strain was stocked in cryobeads and kept in vials at −80 °C. The stock culture was re-activated by introducing a cryobead into a 10-mL tube of TSB (Tryptone Soya Broth, Oxoid, UK) supplemented with 15 μg/mL of gentamicin (TSB-Gent), which was then incubated at 37 °C and throughout 24 h. Subsequently, two successive cultures were made by transferring 10 μL in TSB-Gent tubes. Finally, the TSB-Gent culture was surface plated on PCA (Plate Count Agar, Oxoid, UK) supplemented with 15 μg/mL of gentamicin (PCA-Gent), and PCA plates were maintained at 4 °C.

For the preparation of contaminated water for irrigation, from the above PCA plates, three successive transfers of the pathogen were made in TSB-Gent at 37 °C for 24 h. The last culture, prepared in 500 mL of TSB-Gent, was withdrawn after 16 h incubation, centrifuged at 3900 rpm for 10 min at 4 °C, and the pellet was washed twice in PBS (Phosphate Buffered Saline, Medicago, Sweden) at pH 7.2 and resuspended in the same volume of buffer. The suspension was added to 4500 mL of tap water to obtain a final concentration of *S.* Thompson of 8–9 $\log_{10}$ CFU/mL. Cell density was confirmed by plate count on PCA-Gent.

1.3.2. Strawberry plants conditions

In order to have enough study material for the experiment, more than 250 full-grown Strawberry plants (*Fragaria* × *ananassa* 'San Andreas' var.), of 3–4 months old (at flower and fruit stages), were acquired from a local greenhouse company in Tolox (Málaga, Spain). Every plant was supplied by this company in a 500-mL flower pot (with drainage holes) in which the soil matrix used was 'baltic peat moos'

supplemented with $CaCO_3$ as additive (to achieve a nominal pH = 5.5 and a salinity level around 1.2–1.6 g/L) and also with 2 g of slow-release encapsulated fertilizer to ensure an adequate nutrient level of the peat (ca. 170–250 mg/L of N, 80–160 mg/L of P_2O_5 and 200–280 mg/L of K_2O, among other components) for 5–6 months. Transport of the plants from Tolox to the University of Córdoba (Spain) was carried out under refrigeration conditions (~5–10 °C), and they were finally placed in a greenhouse-umbraculum. At this point, each plant/pot in the umbraculum was individually laid on a plastic tray to allow for direct watering, avoiding any contamination of the aerial parts. After allocation of plants, they were allowed to accommodate to the umbraculum environment for 15 days. After this time, experimental work started and extended around one month. The umbraculum conditions were as follows (average ± SD): temperature: 29.9 ± 4 °C; relative humidity: 26.4 ± 4%; and 14.5 daylight hours per day. These parameters were measured with a PCE-EM 882 multisensor instrument (PCE Instruments, Spain).

1.3.3. Evaluation of decontamination treatments of roots

To select the best method for the removal of *S*. Thompson from the surface of roots, they were subjected to different decontamination treatments. The roots were washed in tap water until the visible soil particles were removed, and roots were placed on filter paper to let them dry at room temperature. The roots were then inoculated by immersion in a 2 L suspension of *S*. Thompson at 8 $\log_{10}$ CFU/mL prepared in 1% w/v peptone water (PW, Oxoid, UK), for 10 min. Afterwards, they were dried for 1 h in a laminar flow cabinet and disinfected by application of different immersion treatments as follows: in 0.5% (v/v) of sodium hypochlorite for 15 and 20 min (I and II treatments); in 1% (v/v) sodium hypochlorite for 15 and 20 min (III and IV treatments); as reported by (Franz et al., 2007), immersion in 1% (w/v) of silver nitrate (Labkem, Spain) for 5 min (V treatment); and in Ethanol 80% (v/v) for 10 s followed by 1% (w/v) silver nitrate for 5 min (VI treatment). After decontamination treatments, the roots were washed twice in distilled water. In the case of those treated with sodium hypochlorite, the residual chlorine was finally neutralized with 0.05 M sodium thiosulfate (Sigma-Aldrich, USA). The chlorine solutions prepared from

commercial bleach were adjusted to pH 6.8 with 35% HCl (Panreac, Spain). Likewise, the free chlorine was measured through a multiparameter analyzer device (Hanna HI 83214).

Individual roots were weighed in a sterile bag and diluted with 1% buffered peptone water (Oxoid, UK) to a 1:9 ratio. The bags were then manually shaken for 1 min, and 100 µL aliquots were surface-plated in duplicate on PCA-Gent plates and incubated at 37 °C for 24 h. For recovering of injured cells, the homogenates were incubated at 37 °C for 24 h, followed by addition of TSB-Gent at double concentration. The enriched samples were subsequently plated on PCA-Gent plates and incubated at 37 °C for 24 h.

For every treatment described, three roots were used (weight average ± SD = 9.3 ± 5.6 g). For comparison purposes, baseline results, i.e. inoculated but not disinfected roots, were also tested to calculate the logarithmic reductions obtained by the decontamination treatments. Each experiment was repeated three times.

1.3.4. Irrigation plan, sample collection and preparation for analysis

The experiments were replicated three times; for each replicate the irrigation plan and sample collection is shown in Table 1.1. Plants available at fruit stage (as previously stated, more than 250 full-grown plants were ready; see section 1.3.2), were distributed in three different groups, which were placed on delimitated spaces (16 plants/group) with different contamination events conditions (Fig. 1.1). In this sense, group 1 received a single contamination event, while groups 2 and 3 underwent two and three irrigations with contaminated water, respectively. Furthermore, control plants (no contaminated) were placed in between the three groups of plants to show any potential cross-contamination event. No fertilizer was added, and each plant was irrigated, as an average, with a water supply of around 111 mL/day ≈1000 mL/9 days (to maintain the soil moisture close to field capacity). This water dose was distributed in 4–5 applications (depending on the inoculation group; see Table 1.1) in which *S.* Thompson was incorporated to the irrigation water when a contamination event was required. Irrigation was

developed by capillarity, carefully pouring water in the tray where each plant was placed. In this way, irrigation water tends to ascend from the bottom of the tray to the soil (through the drainage holes of the pots) by difference in the matric potential (Or et al., 2003). Each contamination event was carried out by using 100 mL of contaminated water (with a concentration of around 8–9 $\log_{10}$ CFU/mL of *S.* Thompson), that were added to 100–150 mL of normal irrigation water until completing the nominal irrigation dose. Strawberry samples from the three groups were collected and analyzed according to the sampling plan presented in Table 1.1 to evaluate the possible internalization and persistence of the pathogen in the fruits.

On each sampling point, at least five strawberries were collected at a red ripe stage (Fait et al., 2008; van de Poel et al., 2014) from full-grown plants; these plants also contained fruits at other ripening stages (i.e. white or light red). This variability was desirable so as to harvest red ripe fruits along the nine days of each experiment. All strawberries collected were aseptically removed from the plants using cutting scissors previously disinfected with ethanol 70% v/v (the cut was made 2 cm above the calyx). All samples were placed separately in sterile plastic bags and immediately transported to the laboratory for analysis.

Leaves and roots of each group were collected, respectively, on days 8th and 9th in every experiment. With reference to leaves, five samples consisting of 16 leaves each, were randomly taken from every group on day 8. For roots collection on day 9, 5 pots per group were also taken at random. Plants were separated from the pots, and roots were washed with tap water until visible soil was removed. They were introduced in sterilized bags and transported to the laboratory for decontamination and analysis.

Table 1.1 Irrigation plan and sample collection

Groups	**Day of experiment**								
	1	2	3	4	5	6	7	8	9
1	+	s	s-	None	s-	None	-	s	s
2	+	None	-	+	s-	None	-	s	s
3	+	None	-	+	-	None	+	s	s
Control	-	s	s-	None	s-	None	s-	s	s

+: Irrigation with water contaminated with *S*. Thompson.
-: Irrigation with uncontaminated water.
s: sampling
None: No irrigation occurred

Figure 1.1. Layout of strawberry plants in the greenhouse-umbraculum

1.3.5. Microbiological analyses

Upon arrival to the laboratory, each strawberry (weight average ± SD = 14.6 ± 6.4 g) was removed from the calyx with a sterile knife, placed in a sterile bag and weighed. Subsequently, TSB was added in a 4:1 ratio (v/w), and the content was homogenized for 90 s in a blender (Stomacher, IUL Instruments, Spain) (Pu et al., 2009). A 1-mL aliquot of the homogenate was surface plated in duplicate using PCA-Gent and incubated at 37 °C for 24 h for direct count. To recover possible injured *Salmonella* cells, the remaining homogenate suspension was enriched by the addition of TSB until reaching a ratio of 9:1 (v/w) and incubated at 37 °C for 24 h; after the first 3 h of incubation, gentamicin was added to the cultures to reach a final concentration of 15 μg/mL (Erickson et al., 2010a). After the incubation period, the enriched homogenate was cultured on both PCA-Gent and XLD Agar and subsequently incubated at 37 °C for 24 h. From each group, a number of calyces were taken, grouping them until obtaining composite samples made up of 5–7 calyces (weight average ± SD = 13.8 ± 6.0 g). Each sample was weighed in a sterile bag, then TSB was added in a ratio of 9:1 (v/w), and the content was homogenized with a blender for 1 min. Subsequently, a 1-mL aliquot was cultured in duplicate on PCA-Gent and incubated at 37 °C for 24 h for direct count. The homogenates were enriched following the above procedure.

Leaves samples (16 leaves/sample, weight average ± SD = 14.0 ± 5.9 g) were weighed in a sterile bag, and TSB broth was added in a ratio of 9:1 (v/w). The content was homogenized in a blender for 1 min. Subsequently, a 1-mL aliquot of the homogenate was cultured in duplicate on PCA-Gent and incubated at 37 °C for 24 h for direct count. The homogenate was also enriched and cultured following the above procedure to recover injured cells.

Regarding roots, these were disinfected by application of the most successful treatment according to the procedures described in section 1.3.3. After decontamination, samples were weighed (weight average ± SD = 10.6 ± 2.8 g) in a sterile bag and TSB was added at a ratio of 9:1 (v/w). To check for residual *S.* Thompson cells attached on the surface of the roots (after the decontamination process), samples were manually shaken in the same bags for 1 min, and 1-mL aliquot was cultured in duplicate on PCA-Gent;

subsequently, roots were removed from the bags. Both PCA-Gent plates and TSB bags (without roots) were incubated at 37 °C for 24 h. To investigate for internalized cells, the collected roots were introduced in sterile bags, blended for 1 min in TSB broth at a 9:1 ratio (v/w), and 1-mL aliquot was cultured in duplicate on PCA-Gent plates for direct count. TSB bags containing, on the one hand, possible cells survivors after decontamination, and on the other hand, root homogenates, were submitted to enrichment and culture according to the procedure described above. For the enrichment procedures applied, the limit of detection of the pathogen was within 0.1 CFU/g. The PCA-Gent and XLD plates were examined under UV light, and those colonies showing fluorescence were counted.

1.3.6. Statistical analysis

The internalization experiment and the root decontamination experiment were repeated three times with a different set of plants. Logarithmic reductions of *S*. Thompson on the roots' surfaces have been calculated as the difference between the initial inoculation level (or baseline) and the final concentration. A univariate analysis ANOVA with a Tukey multiple comparison posthoc tests were carried out to evaluate significant decontamination treatments. Finally, to investigate significant associations between the frequency of contamination events and the presence of *S*. Thompson in the aerial parts of the plant, the χ^2 test was used. The software SPSS v25 (Chicago, Illinois, USA) was used, with a significance level of $p < 0.05$.

1.4. Results and discussion

1.4.1. Evaluation of decontamination treatments of roots

Different investigations have been carried out to select suitable decontamination treatments of the external tissues of plants to evaluate the actual internalization of foodborne pathogens. Among the wide range of treatments are those based on the application of active compounds such as chlorine, metals

compounds such as mercury and silver, and the use of ethanol alone or in combination with other substances (Erickson, 2012; Zhang et al., 2009). In the present study, a total of six treatments based on the use of sodium hypochlorite, silver nitrate, and a combination of ethanol plus silver nitrate were evaluated for surface decontamination of *S.* Thompson in roots of strawberry plants. It was found that the use of 1% silver nitrate (alone or in combination with ethanol, treatments V and VI) achieved a reduction of *S.* Thompson in roots to undetectable levels by enumeration methods (<100 CFU/g, $p < 0.05$) as shown in Table 1.2, followed by 1% sodium hypochlorite for 20 min (treatment IV) as the second most effective treatment (>6 $\log_{10}$ CFU/g reduction). The 0.5% chlorine treatment (treatment I) for 15 min was slightly less effective than the other treatments. These results are in concordance with that reported by Franz et al. (2007) who found a more significant reduction of GFP-*Escherichia coli* O157:H7 in lettuce roots with 1% silver nitrate (10 s) compared to 1% sodium hypochlorite (5 s) plus 70% ethanol (5 s). The silver ion exerts its bactericidal action by reaction with the thiol group of proteins and interaction with DNA, affecting its replication (Matsumura et al., 2003). However, root sterilization was not achieved by the decontamination treatments (Table 1.2). This observation could be related to the roots surface structure and the fact that plants were contaminated with a high level of *S.* Thompson, i.e. 7.3 ± 0.3 $\log_{10}$ CFU/g, thus making sterilization more difficult. In this line, Zhang et al. (2009) reported the presence of *Salmonella* in lettuce roots after exposure to a combined treatment (80% ethanol for 10 s followed by immersion in 0.1% $HgCl_2$ for 10 min). Taking into account the high effectiveness of silver nitrate (>7 $\log_{10}$ CFU/g reduction), together with a very short contact time, treatment V, i.e. 1% silver nitrate for 5 min, was selected for decontamination.

Table 1.2 Reductions of *S.* Thompson on roots of strawberries' plants after disinfection treatments

Treatments	* Reduction (log_{10} CFU/g)	No. Positives after enrichment / No. Enriched samples
I- 0.5 chlorine, 15 min	5.74 ± 0.58^{a}	9 / 9
II- 0.5 chlorine, 20 min	5.97 ± 0.27^{ab}	9 / 9
III- 1% chlorine, 15 min	6.03 ± 0.32^{ab}	4 / 9
IV- 1% chlorine, 20 min	6.37 ± 0.19^{bc}	4 / 9
V- 1% silver nitrate, 5 min	$-^{c}$	3 / 6
VI- 80% ethanol, 10 s + 1% silver nitrate, 5 min	$-^{c}$	5 / 6

*Data represent mean log reduction (log_{10} CFU/g) of three replicates ± standard deviation, from the initial population of *S.* Thompson on roots, i.e. 7.28 ± 0.34 log_{10} CFU/g. Different superscripts letters indicate significantly different groups for the Tukey multiple comparison test ($p<0.05$). – means that results were below the quantification limit (2 log_{10} CFU/g).

1.4.2. Internalization of *S*. Thompson in strawberry plants

The present study aimed to evaluate the potential of *S.* Thompson to internalize via root into strawberry plants after the repeated application of irrigation with contaminated water for 9 days. Strawberry plants were grouped and subjected to a different number of irrigations with contaminated water at an average concentration of *S.* Thompson of 8.8 $\log_{10}$ CFU/mL. Our findings suggest that there is a low probability of occurrence of internalization events of *S.* Thompson through the root to fruits or leaves (Fig. 1.2). Therefore, it is unlikely that an eventual outbreak due to strawberry consumption could be attributed to drip irrigation with contaminated water, which is the traditional system applied in strawberry cultivars in the Andalusian region. This low likelihood could be explained by the different barriers that bacteria should overcome to, first, reach the fruit, and second, remain viable. For instance, when trying to penetrate the root, pathogen cells may become attached to the soil surface (Lapidot & Yaron, 2009); also, *Salmonella* may have to compete with natural microbiota present in the rhizosphere and in the different parts of the plant (Berg, 2007.; de Pereira et al., 2012; de Tender et al., 2016; Dias et al., 2009).

Concerning the underground part of the plant, the decontamination with 1% silver nitrate achieved the elimination of *S.* Thompson from the root surface, as confirmed after enrichment in broth, because no pathogen was detected (below detection limit). Then, internal colonization of the pathogen was checked after mashing the roots in TSB following the enumeration and detection procedures as described above. Overall, colonization was scarce after eight days from inoculation (group 1). Only in 1 out of 45 analyzed samples (2.2% of the total root samples, 95% C.I.: [0.06–11.77%]), *Salmonella* was presumptively internalized since it was found after enrichment. Supporting this finding, the low internalization of *Salmonella* may be related to its inability to degrade the materials of the plant cell wall, which means that it cannot enter directly through the root cells (Karmakar et al., 2018). In fact, research has shown that internal colonization of roots by *Salmonella* can occur mainly through wounds in the roots or through lateral roots, where an epidermis remodelling takes place (Karmakar et al., 2018; Zheng et al., 2013). In addition, it has been reported that the native microorganisms previously established in the rhizosphere

could exert an antagonistic action and induce the activation of the defence mechanisms of the plant (Berg, 2007., de Pereira et al., 2012). In line with these facts, a greater internalization of *Salmonella* has been evidenced in tomato plants grown in hydroponic media compared to those grown in soil (Guo et al., 2002; Hintz et al., 2010). Cooley et al. (2003) showed a decrease in survival of *S*. Newport and *E. coli* O157:H7 in unautoclaved soils compared to autoclaved soils, suggesting microbial competition with the soil microbiota.

Additionally, it is noticeable that an unexpected detachment of roots was observed in some samples of the present study, which could indicate a certain level of stress on these plants. Accordingly, it has been described that a high concentration of *Salmonella* gave rise to stress on the plant root (Ge et al., 2012). *Salmonella* contains a series of pathogen-associated molecular pattern molecules (PAMPs) that could activate the immune response of plants preventing the propagation of the pathogen (Amil-Ruiz et al., 2011; Deering et al., 2012). Proteins and chemicals such as jasmonate, salicylic acid, ethylene, phytoalexins and reactive oxygen species among others, are produced by plants in response to biotic stress (Alegbeleye et al., 2018; Amil-Ruiz et al., 2011). For example, Iniguez et al. (2005) found a decrease of endophytic colonization of *S. enterica* into alfalfa roots due to ethylene production.

In relation to the aerial parts, for a total of 118 fruits analyzed, all were below the quantification limit <5 CFU/g, and only one sample from group 3 was positive after enrichment in day 8 (0.8% of the total fruit samples; 95% C.I.: [0.02–4.63%]). All the calyx samples analyzed (17 samples) were negative for the pathogen. Regarding the leaves, out of 45 samples analyzed, four of them were positive after enrichment (8.9% of the total leave samples, 95% C.I.: [2.48–21.22%]), from which three corresponded to group 2 and 1 sample to group 3. The low internalization capacity of *Salmonella* in the aerial parts may be due to the fact that the exposure time used in the study (not greater than 9 days) was not sufficient to allow colonization of the pathogen together with the barriers mentioned above. In this line, other authors observed that several *Salmonella* serovars were only recovered from the inner part of the tomato stem up to 10 cm from the soil line within a week after inoculation (Zheng et al., 2013). Similarly, *Salmonella enterica* had a migration of 5 cm after 7 days of inoculation (Burris et al., 2020). Despite the low number

of contaminated samples, a very significant association ($p < 0.001$) was observed between the increased frequency of irrigation with contaminated water and the presence of the pathogen in plants, as evidenced for group 3. In agreement with our results, Lapidot and Yaron (2009) detected transfer events of *Salmonella* Typhimurium from drip irrigation to edible parts of parsley after the third irrigation with contaminated water. Also, Généreux et al. (2015) reported a low level of contamination of strawberries by *E. coli* applying irrigation with naturally contaminated water. In that study, *E. coli* count was below the detection limit (<10 CFU/g) in all strawberries' samples analyzed (n = 256) and only 5.5% of samples were positive after enrichment. Likewise, this study highlighted the higher risk of contamination of the fruit from overhead irrigation compared to subsurface drip irrigation, and higher contamination of the product when it is cultivated in straw mulch compared to plastic mulch. These findings indicate that the fruit has a higher probability of being contaminated at the surface level than internally from the root (Généreux et al., 2015). On the contrary, other authors like Miles et al. (2009) did not find dissemination events of *Salmonella* Montevideo in low acidity product via root to stems, leaves or fruits of tomato plants, after being irrigated with 350 mL of a suspension of bacteria (7 $\log_{10}$ CFU/mL) every 14 days for 70 days. In another study, research in primary production (Johannessen et al., 2015; Macori et al., 2018) demonstrated, in line with our results, a low prevalence of enteric bacterial pathogens and faecal indicators in strawberries despite their presence in environmental samples, including irrigation water.

Intrinsic factors such as low pH or presence of compounds such as polyphenols, phenolic acids, and organic acids in strawberries can affect the survival of the bacterium (Nile & Park, 2014; Puupponen-Pimiä et al., 2005). Similarly, flavonoids and phenolic compounds present in leaves, that have an essential role in the protection of the plant against pathogens (Amil-Ruiz et al., 2011), may account for the limited recovery of *S.* Thompson from the samples. In this regard, it has been widely demonstrated the antibacterial effect of berry extracts against bacterial pathogens such as *Listeria*spp, *Staphylococcus aureus, Clostridiumperfringens, S. enterica, E. coli* and *Campylobacter* (Das et al., 2017; Puupponen-Pimiä et al., 2005).

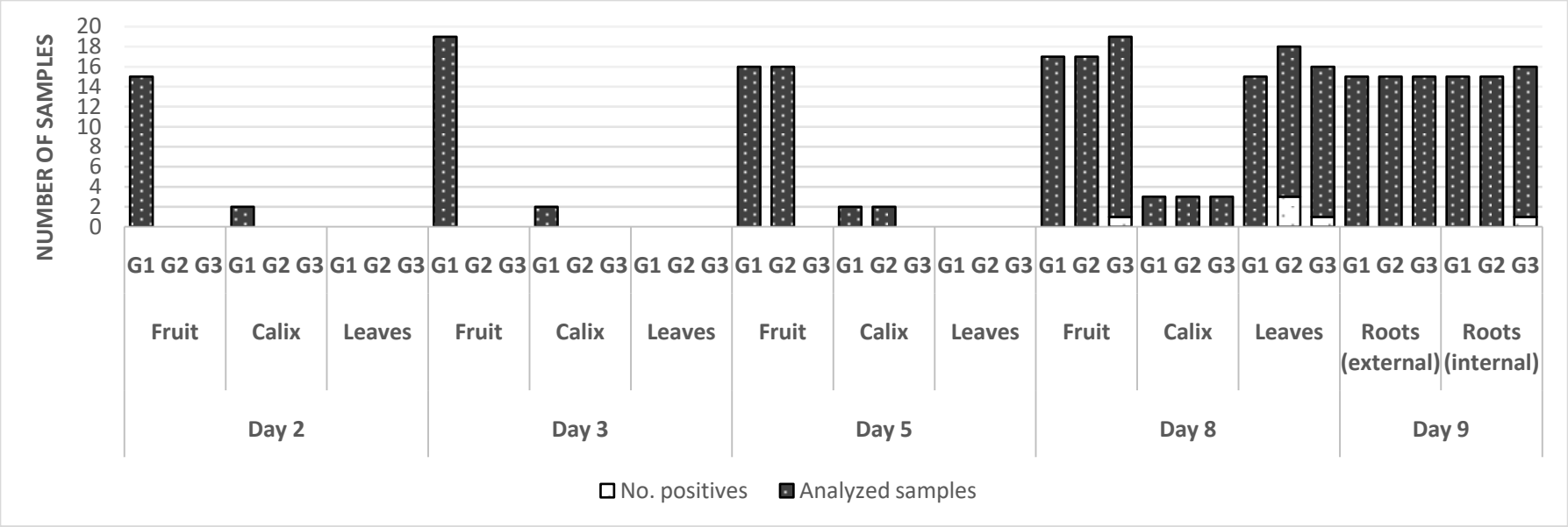

Figure 1.2. Number of samples analyzed and the presence of *S*. Thompson in different parts of the strawberry plant. G1, G2 and G3 abbreviations refer to Groups 1, 2 and 3, respectively.

1.5. Conclusion

In this study, artificial contamination of strawberry plants with an enteric bacterial pathogen, i.e. *S.* Thompson, was evaluated to study its potential internalization. It can be concluded that internalization of this pathogen via root into strawberry plants 'San Andreas' variety after frequent irrigation with contaminated water is considered a rare event; thus, other routes and pathways different from internalization should be explored as the origin of outbreaks by enteric pathogens caused by strawberries consumption. However, frequent exposure to water contaminated with a high *Salmonella* load, that could happen in waters with high proportion of fecal matter, increases the likelihood of internalization of the pathogen, reaching different parts of the strawberry plant. Our findings also point out the importance of the irrigation method to reduce or avoid any contamination event. This study shows the importance of applying drip irrigation as a more hygienic and safe agronomic practice than the overhead irrigation, even assuming that splashing events during drip irrigation may occur. In fact, other overhead agricultural practices, such as frost protection of fruits, might be a source of direct contamination with hazardous agents, so special attention should be paid to this type of practices. Future work should focus on the evaluation of other *Salmonella* serotypes, including acid-tolerant strains, plant varieties, and the effect of different factors affecting *Salmonella* survival and transfer as well as contamination levels.

1.6. References

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