Microbial interaction between *Salmonella enterica* and main postharvest fungal pathogens on strawberry fruit

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Highlights

- *Botrytis cinerea* and *Rhizopus stolonifer* caused a decrease in *S. enterica* population.
- Treatments had significant reduction of *S. enterica* after 48 h(20ºC) and 14 d(4ºC).
- The main inactivation rate was obtained for *B. cinerea* at 20 ºC (0.160±0.027/h).
- Inhibitory effect caused by moulds with environmental factors affect *S. enterica*.

Abstract

The microbial interaction between *Salmonella enterica* and the main postharvest fungal pathogens of strawberries was evaluated. Inoculation of fungal suspension was done 2 (D2) and 1 (D1) day(s) before and at the same time (D0) as *S. enterica*. Fruits were stored at 20 ºC and 4 ºC. At both temperatures, *Botrytis cinerea* and *Rhizopus stolonifer* caused a decrease in *S. enterica* population. Treatments where the mould was inoculated (D2, D1 and D0) achieved a significant logarithmic reduction ($P < 0.05$) of *S. enterica* populations after 48 h (20ºC) and 14 d (4 ºC) comapared to uninoculated fungal fruits (CK). Regarding temperature, average reductions were significantly higher at 4º C (3.38 $\log_{10}$ CFU/wound) than at 20º C (1.16 $\log_{10}$ CFU/wound) ($P < 0.05$). Average reductions comprising all treatments were 1.91 and 0.41 log10 CFU/wound for *B. cinerea* and *R. stolonifer* at 20 ºC, and 3.39 and 3.37 $\log_{10}$ CFU/wound for *B. cinerea* and *R. stolonifer* at 4 ºC. A linear $\log_{10}$ model was fitted in order to predict the inactivation rate (kmax, $\log_{10}$ CFU/h) of *S. enterica*. Inactivation rates were higher at 20 ºC for D2 treatments than at 4 ºC throughout the running time. The main inactivation rate was obtained for *B. cinerea* at 20 ºC (0.160±0.027/h), which was found to have stronger inhibitory activity against *S. enterica* than *R. stolonifer*. Univariate analysis ANOVA was carried out to evaluate the effect of different external variables on the inhibition of *S. enterica*. Results found that single effects were significant ($P < 0.05$) except for the pH. The inhibitory effect caused by the action of moulds in conjunction with some environmental factors could indicate the potential interactions between strawberry fungal pathogens and *S. enterica.*

Keywords*: Botrytis cinerea; Rhizopus stolonifer*; metabiotic association; survival.

Introduction

The increase in demand for red fruits in Spain has brought with it a considerable upsurge in the production area (Granatstein et al., 2010; Das et al., 2017). Fresh berry produce industry is exposed to constant innovation, comprising raw fruits which are not subjected to any step that can eliminate postharvest pathogens (e.g. wash or heat treatment) (Abadias et al., 2008; Alegbeleye et al., 2018). Strawberries have a high content of water and carbohydrates, making it vulnerable to physical damage and microbial contamination during harvest and transportation. In fact, this fruit is exposed to microbial contamination at each stage of production: cultivation, harvest, transportation, packaging, storage and final sale (Delbeke et al., 2015). Strawberries are especially highly sensitive to deterioration by microorganisms after harvest mainly due to the appearance of rot caused by *Botrytis cinerea* and *Rhizopus stolonifer*, which results in their short post-harvest shelf-life. These moulds are necrotrophic fungi and obtain the nutrients from dead host cells killed by them, decreasing the pH values of matrix fruit (Adikaram et al., 2010; Elmer et al., 2000; Manteau et al., 2003). Tournas et al. (2006) showed that *B. cinerea* was by far the most common worldwide spoiler of strawberry contamination (77%), followed by *Rhizopus* spp. (23%). Shocking berry losses due *B. cinerea* have been reported in the past (Pitt & Hocking, 2009). On the other hand, in other regions like UK, species of *Mucor* (in particular *M. piriformis*) constitute a major cause of soft rot of strawberries and raspberries (Snowdon et al., 1990)

Strawberries are generally considered to be low-risk food in terms of pathogenic bacterial infections due to their naturally low pH (Knudsen et al., 2001). *Salmonella* spp. is one of the most common human pathogenic bacteria contaminating fresh produce world-wide. However, according to the reported outbreaks connected to fresh and frozen produce, very little information can be found on the prevalence of *Salmonella* spp. in strawberries, but everything seems to indicate that it is low (Graça et al., 2017; Macori et al., 2018; Ortiz-Solà et al., 2019). Nevertheless, berries could be contaminated by *Salmonella* spp. due to irrigation water, animals near the area or improper handling (Roth et al., 2018). Some investigations have shown that surface-inoculated *Salmonella enterica* was able to survive but was not able to grow or multiply in strawberries at different stored temperatures, potentially due to the low pH or other intrinsic

70 factors associated with fruit (Delbeke et al., 2015; Sreedharan et al., 2015). However, it has been
71 seen that other extrinsic factors like improper refrigeration during storage and preparation, poor
72 product quality, or the presence of other microorganisms could allow the growth of *Salmonella*
73 spp. For example, studies with healthy and soft rot tissues of pepper, potato and carrot inoculated
74 with *Salmonella* Typhimurium demonstrated that the population of viable cells multiplied 3- to
75 10-fold on soft rot tissues compared to healthy tissues (Gurtler et al., 2018; Wells et al., 1997).
76 Metabiotic effects occurring between other microorganisms like fungi and foodborne pathogens
77 are another topic of global concern. It has been seen that some moulds, such as *Aspergillus*
78 *fumigatus* and *Emericella nidulans*, increased the survival of *E. coli* O157: H7 in whole and cut
79 tomatoes (Bevilacqua et al., 2009). Growth of *E. coli* O157:H7 was also stimulated by the
80 colonization of *Fusarium spp*. in portions of tomato even when the contamination was not visible
81 (Bevilacqua et al., 2008). Riordan et al. (2000) observed enhanced growth of *E. coli* O157:H7 in
82 wounds on apples co-inoculated with *Glomerella cingulata* and stored at 22 ºC. This growth was
83 correlated with a rise in pH at the infected site, even though decay was not evident.
84 On the other hand, microbiota present in fruit matrix may negatively influence the growth and
85 survival of pathogenic bacteria, which are able to compete for nutrients or/and change pH of
86 macerated tissue. In apple, Conway et al. (2000) detected that *L. monocytogenes* inoculated in
87 tissue infected with *Glomerella cingulata* (increase the pH from 4.7 to 7) can grew, whereas it
88 did not survive when the fruit was infected with *Penicillium expansum* (decrease pH of fresh-cut
89 apple slices from 4.7 to 3.7). It was observed that new substrate presented by *G. cingulate* on the
90 fruit matrix was more suitable for survival and growth of *L. monocytogenes* than the substrate
91 modified by *P. expansum.* Other studies reported that no metabiotic interactions were observed
92 between several moulds causing postharvest damage and *Salmonella poona* in melon (Richards
93 and Beuchat, 2005). In strawberry, there is no work describing the metabiotic interactions
94 between the main moulds that cause rot (*Botrytis cinerea* and *Rhizopus stolonifer*) and *Salmonella*
95 *enterica*.

96 The objective of this study was to determine the survival of *Salmonella enterica* on fresh
97 strawberries and its interaction with the main postharvest strawberry fungal pathogens at different
98 storage temperatures. Changes in pH caused by growth of moulds were monitored.

99 2. Material and Methods

100 2.1. Experimental design and preparation of samples

101 Fresh strawberries were obtained from a single vendor in Lleida (Catalonia, Spain) in the spring
102 of 2017 and 2018. For each experiment, 336 fruits of the same batch were divided into 2 groups
103 according to the storage temperature at 20 and 4 ºC. For each temperature, fruits were divided
104 equally in a control group (CK), which were inoculated only with a cocktail of *S. enterica* (no
105 fungal inoculation), a group with *S. enterica* and the mould inoculated on the same day (D0), a
106 group where the mould was spotted one day before *S. enterica* (D1) and a group where the mould
107 was inoculated two days (D2) before *S. enterica* (Figure 1). For the preparation of the samples,
108 42 strawberries were available in alveoli for each group. Stems of all strawberries were gently
109 removed. A wound was made on the surface with a size of 1 mm in diameter and 2 mm deep with
110 a sterile nail. The microbial population and the pH of the wound were monitored during the
111 storage time at 20 and 4 ºC. The experiment was carried out twice, in two different years.

112 2.2. Preparation of *S. enterica* cocktail

113 The strains used for the experiments were *Salmonella enterica* subsp. *enterica* (Smith) Weldin
114 serotype Agona (BAA-707), Michigan (BAA-709), Montevideo (BAA-710), Gaminara (BAA-
115 711) and Enteritidis (CECT-4300).

116 For each strain of studied *S. enterica*, a single colony from a streak in Tryptone Soy Agar (TSA;
117 Biokar Diagnostics) medium (20-24 h, 37 ± 1ºC) was inoculated in 5 mL of Tryptic Soy Broth
118 (TSB; Biokar Diagnostics) and incubated at 37 ± 1ºC for 18-24 h. Afterwards, all cultures were
119 combined in one centrifuge tube. The volume of the tube was centrifuged (Sorvall Legend XTR
120 Centrifuge, Thermo Fischer, US) at 9800×g for 10 min at 10 °C and resuspended with half of the
121 initial volume (12.5 ml) of saline solution (SS; 0,85% w/v NaCl). The inoculum was diluted to a

concentration of about 1×10^7 CFU/ml with deionized sterile water before being added to the wound. The real concentration of the inoculum was checked by plating in TSA and Xylose Lysine Desoxycholate Agar (XLD; Biokar Diagnostics) incubated at 37 ± 1 ºC for 18-24 h.

2.3. Preparation of postharvest pathogen (fungi)

The strains *Botrytis cinerea* BC03 (CECT 20973) and *Rhizopus stolonifer* RSF, belonging to the collection of Postharvest Pathology Group of IRTA (Lleida, Catalonia), were used for the experiments. They were subcultured on Potato Dextrose Agar (PDA; Biokar) and incubated at 25 ± 1 ºC. *B. cinerea* cultures were grown for a minimum of 7-15 days and *R. stolonifer* cultures for 5-7 days. Conidia were harvested with inoculating loop and conidial suspensions were adjusted to 10^4 conidia/ml for *B. cinerea* (BC) and 10^3 conidia/ml for *R. stolonifer* (RSF) in 10 ml tubes containing tween 20 (0.20% w/v). Tween 20 was added to sterile water and the conidial suspensions to ensure homogeneous distribution of the conidia. Cell suspension was determined using a haemocytometer (Thoma cell counting chamber, Marienfield-Superior, UK). Two special coverslip provided with the counting chamber were properly positioned. The cell suspension was applied to the edge of the coverslip which completely fills the chamber with the sample. The number of cells in the chamber have been determined by direct counting using a microscope and was defined as:

$$conidia/mL = \frac{(number\ of\ cells\ counted)(dilution\ factor)}{(number\ of\ large\ squares\ counted)\ (volume\ of\ 1\ large\ square)} \quad \text{Equation 1}$$

2.4. Inoculation of *S. enterica* and the fungi on strawberry tissue

For all experiments, strawberries were removed from storage before inoculation, allowed to warm for a few minutes. The surface of the fruit was marked with nail polish to locate the wound. The wound was done using a nail (1 mm wide and 2 mm deep) at approximately 10 - 12 mm distance from the mark. Before the inoculation, strawberries were homogeneously placed under UV light in a biosafety laminar cabinet (class II – type A, Telstar, Terrassa, Spain) for the disinfection. The time exhibition of UV light was 10 min per face. Wounds on strawberry were inoculated with 10 µl of the fungal suspension of BC (10^4 conidia/ml) or RSF (10^3/ml) 2, 1 and 0 days before

148 inoculation (Figure 1). Fungal inoculum on strawberries was allowed to dry for 1-2 h in a
149 biosafety laminar air cabinet (class II - type A, Telstar, Terrassa, Spain) at room temperature.
150 Then, the berries stored at 20 ºC. *S. enterica* suspension (10 µl) at a concentration of 1×10^7
151 CFU/ml in 0.85% NaCl was pipetted into the same wound in which the fungus was inoculated
152 the same day (D0), 1 (D1) or 2 days (D2) before. Control strawberries (no fungal pathogen) were
153 only inoculated with 10 µl of *S. enterica* suspension. The fruits with both fungi and bacteria or
154 only with bacteria were dried in a laminar cabinet. Strawberries were placed in a box with 42-
155 cells alveoli (21 strawberries were for monitoring the population of *S. enterica* and 21 strawberries
156 were for pH determination) and subsequently stored at 4 ºC and 20 ºC.

157 **2.5. *S. enterica* determination analysis**

158 Inoculated strawberries were analysed for populations/presence of *S. enterica* within 2 h of
159 inoculation (0 day) and at 8, 24, 30 and 48 h for samples stored at 20ºC. For the strawberries
160 stored at 4ºC, *S. enterica* population were examined after 2, 6, 9, 12 and 14 days. For each
161 condition (fungal strain, temperature, inoculation time), three strawberries were analysed
162 individually (n=3). To recover *S. enterica* from strawberries, a small and equal portion of fruit
163 that contained the entire wound was taken out with cork borer and placed in a sterile stomacher
164 bag (BagPage®, Interscience, France) with 5 mL of Buffered Peptone Water (BPW, Biokar
165 Diagnostics). Afterwards, it was homogenized in a stomacher blender (Stomacher Minimix®,
166 Mathias S.L.) for 2 min at normal speed (9 strokes/sec). BPW was used for a better resuscitation
167 of injured cells and reduction of the lag phase to obtain higher recovery rates at shorter incubation
168 times (Jasson et al., 2009). Ten-fold dilutions of the homogenates were made with saline peptone
169 (SP) (0,85% w/v NaCl; 0,1% w/v Peptone) tubes and they were plated in the selective media XLD
170 (Biokar Diagnostics). The plates were incubated at 37 ºC for 18 - 24h. Microbial population was
171 expressed in $\log_{10}$ CFU/wound. Bags containing the homogenates were also incubated at 37 ºC
172 overnight for *S. enterica* detection in case no colonies were present in plates. The limit of
173 detection was 1.40 $\log_{10}$ CFU/wound. When no colonies were counted and detection was positive,
174 an arbitrary number of half detection limit was used for calculation (1.13 $\log_{10}$ CFU/wound).

175 **2.6. pH measurement**

176 At the same time as population of *S. enterica* was determined, pH of the wound was measured
177 using a Crison pH meter (Crison GLP-21, Barcelona, Spain) equipped with a penetration probe
178 (Crison electrode 52-31, Barcelona, Spain). To avoid microbial contamination of the samples,
179 reading of the pH was carried out in different fruits that received the same treatment conditions.

180 **2.7. Data modelling**

181 The survival patterns of the different assayed treatments in the inoculated strawberries were
182 evaluated by fitting the logarithm of the number of colony-forming units per wound of sample
183 ($\log_{10}$ CFU/wound) against the storage time (h). The log-linear model (Equation 1) was fitted to
184 survival curves using the GInaFiT add-in for Excel® (Geeraerd et al., 2005).

185 $$\log N(t) = \log N_0 - k_{\max} \cdot t \qquad \text{Equation 2}$$

186 where $N(t)$ is the number of survival cells ($\log_{10}$ CFU/wound) at time t (h); N_0 corresponds to the
187 initial inoculum level ($\log_{10}$ CFU/wound); and k_{max} is defined as the specific inactivation rate (h^{-1}).
188 Model fitting was performed by using the average observed values from each data point.

189 **2.8. Statistical data analysis**

190 To gain insight into the effect of the studied factors on the survival of *S. enterica* in the inoculated
191 strawberries, a fixed effects linear model with interactions was performed. The factors considered
192 were the type of mould (*B. cinerea* and *R. stolonifer*), temperature (20 and 4 ºC), pH, storage time
193 and treatment (CK, D2, D1 and D0). A backward selection method was chosen and mean
194 estimated parameters together with goodness-of-fit indices were obtained. The latter
195 corresponded to the log likelihood (logL), Akaike Information Criterion (AIC) and Bayesian
196 Information Criterion (BIC). The model structure was defined as:

197 $$y_i = \beta_0 + \beta_1 \cdot x_{i,1} + \ldots\ldots + \beta_{p-1} \cdot x_{i,p-1} + \varepsilon_i \sim Normal(0, \sigma^2) \qquad \text{Equation 3}$$

198 Being y_i the response variable (*S. enterica* level, $\log_{10}$ CFU/wound), β_0, β_1, …β_{p-1} the unknown
199 regression parameters and σ^2 the unknown (constant) error variance. A univariate analysis
200 ANOVA with a Tukey post-hoc test was achieved to evaluate the significance of the studied
201 factors. The software R v.3.5.1 (cran.rproject.org) was used taking a value of $P < 0.05$ as a level
202 of significance.

203 To assess model predictions, the acceptable simulation zone (ASZ) approach was used, with ASZ
204 defined as ± 0.5 $\log_{10}$-units from the predicted *S. enterica* counts (Velugoti et al., 2011). To
205 determine the acceptability of the model, at least 70% of the observed $\log_{10}$ CFU/wound values
206 should be inside this zone (Oscar, 2005).

3. Results and Discussion

3.1. Survival ability of *S. enterica* in strawberries

Initial population of *S. enterica* ranged between 4.46 and 4.61 $\log_{10}$ CFU/wound on strawberries. At both temperatures studied, *S. enterica* did not grow over storage time (48 hours and 14 days at 20ºC and 4ºC, respectively) when the pathogen was inoculated alone (CK) (Figure 2-5). Previous publications reported that *Salmonella* was able to survive on fresh-cut strawberries for prolonged periods of time, but was unable to multiply (Knudsen et al., 2001). Moreover, both *B. cinerea* and *R. stolonifer* caused a decline in *S. enterica* population in comparison with uninoculated fungal fruits (CK). A multivariate ANOVA analysis was performed to find out significant differences between storage temperatures, type of mould and treatment on the $\log_{10}$ reductions of *S. enterica*. It was observed that in those treatments where moulds were inoculated (D2, D1 and D0), significant reductions were achieved in comparison to the treatment where *Salmonella* was inoculated alone ($P < 0.05$). Moreover, in those treatments where *B. cinerea* and *R. stolonifer* were inoculated on the same day (D0), the day before (D1) and two days before (D2) *S. enterica*, did not yield significant differences ($P > 0.05$) in the average reductions, ranging from 2.47 to 3.07 $\log_{10}$ CFU/wound while the uninoculated fruits presented an average reduction of 0.77 $\log_{10}$ CFU/wound.

Regarding the effect of temperature, the results showed an inactivation of *S. enterica* population throughout storage at both temperatures studied (20 ºC and 4 ºC). However, average reductions were significantly higher at 4º C (3.38 $\log_{10}$ CFU/wound) than at 20º C (1.16 $\log_{10}$ CFU/wound) ($P < 0.05$). Moreover, as the fungi were allowed to grow for 48 h at 20 ºC before pathogen inoculation, fruit rotting was already in the initial stages when *S. enterica* inoculation was done. The acidic pH (3.61 – 3.91), nutrients availability in strawberries wounds, and the high water activity on the surface favoured the growth of *B. cinerea* and *R. stolonifer*. This fact, together with a low storage temperature could constitute a hostile environment for the growth of *S. enterica*. Delbeke et al. (2015) reported reductions of 2 – 3 $\log_{10}$ CFU in strawberry matrix after 5 days of storage at refrigeration (4 –15 °C). However, the survival experiment stopped before

day 7 at 15 ºC, as die-off of pathogen below the lower limit of detection was achieved or spoilage occurred. In fact, higher temperatures (25 ºC) conducive for *Salmonella* survival compared to lower temperatures (4 ºC) (Sreedharan et al., 2015). These results highlight the importance of refrigeration to minimize microbial risk caused by *S. enterica* contamination maintaining at the same time fruit quality for a longer shelf life period (Cantwell et al., 2001).

Regarding the inhibitory effect of the decay-causing fungi against *S. enterica*, *B. cinerea* produced a significantly higher $\log_{10}$ reduction effect than *R. stolonifer* at 20º C storage ($P < 0.05$). On the contrary, there were not significant differences in the inhibitory effect at 4º C, though maximum observed reductions of *S. enterica* population in strawberries caused by *B. cinerea* and *R. stolonifer* at this temperature were higher than 4 $\log_{10}$ CFU/wound after 14 d storage in the D2 treatment (Figures 3 and 5). Average reductions including all treatments (D2, D1, D0 and CK) were 1.91 and 0.41 $\log_{10}$ CFU/wound for *B. cinerea* and *R. stolonifer* at 20 ºC, and 3.39 and 3.37 $\log_{10}$ CFU/wound for *B. cinerea* and *R. stolonifer* at 4 ºC.

On the other hand, no significant relationship was found between the pH of strawberries during storage (average value of 3.71 for *B. cinerea* and 3.75 for *R. stolonifer*) and the reduction of *S. enterica* (data not shown). Values of pH did not change substantially throughout the storage period, regardless the applied treatment (D2, D1, D0 and CK). Cibelli et al. (2008) which clearly demonstrated with a model system (a laboratory medium added with tomato juice) that the increase of the pH approximately 1 to 1.3 of medium due to the metabolic activity of *Fusarium oxysporum* significantly enhanced the survival of *Salmonella* spp. It is reported that some postharvest fungal pathogens yield to an increase in pH, thus favouring the survival and growth of enteropathogenic bacteria in contaminated fruits and vegetables. Wade et al. (2003), reported that *Geotrichum candidum* secretes ammonia under inductive environmental conditions in fresh tomatoes and increases pH of tissues to values as high as 7.5. Storage of wound tomatoes at 15 ºC for 10 days resulted in a significant increase in population of 7.6 $\log_{10}$ CFU of *S. enterica*/g of 2-g sample of co-infected pulp tissue. On the contrary, the metabiotic interaction in our study demonstrated that the survival of the pathogen decreased pronounceably without any substantial

change in pH of strawberry's matrix, thus suggesting that metabiotic effect could be due to some metabolites different from alkalinizing or acidifying compounds. Moreover, moulds have a greater proteolytic activity and carbohydrate degradation when the postharvest pathogen is already grown. Consequently, competition of nutrients, carbohydrates and amino-acids of the fruit matrix may be critical for the bacterial growth.

3.2. Predictive modelling of *S. enterica* cocktail in strawberries during storage

Primary inactivation models were fitted to the observed $\log_{10}$ reductions of *S. enterica* at the studied conditions in strawberries. Among the models tested, $\log_{10}$ linear reductions were estimated through the calculation of the specific inactivation rate (1/h). The kinetic parameters are represented in Table 1. Log_{10} linear models overall presented an acceptable goodness of fit having R^2 values > 0.9 at most conditions tested, apart from some fittings at 20 ºC where microbial variability was much higher. However, modelling fitting was performed to proceed to a comparison between inactivation rates at different temperatures, treatments and decay-causing fungi against *S. enterica* in strawberries. It can be seen that inactivation rates were higher at 20 ºC for D2 treatments when compared to those obtained at 4 ºC. The highest inactivation rate was obtained for *B. cinerea* at 20º C (0.160±0.027/h), which was found to have stronger inhibitory activity against *S. enterica* than *R. stolonifer*. Likewise, inactivation rates obtained for D1 treatments were also higher than those calculated for CK and D0 treatments for *B. cinerea* at 20 ºC, while for *R. stolonifer*, an increased inactivation rate was found for the D0 treatment. In this later case, inactivation was more probably attributed to the microbial variability found at 20 ºC which impeded obtaining a reliable estimation of the inactivation rate. Results obtained at 4 ºC did not show such differences but in the case of *B. cinerea* there was a 32% reduction in the inactivation rate between D1 and D0 treatments, while no differences were obtained for *R. stolonifer*. However, when comparing inactivation rates at 4 ºC between D2 and D1 treatments, inactivation was similar for *B. cinerea* while for *R. stolonifer*, inactivation rate was reduced to half. Considering these results, it seems that the inhibitory action of *B. cinerea* against *S. enterica*

287 at 4 ºC is mainly exerted 24 h before inoculation, while for *R. stolonifer* the highest inhibition is
288 produced 24 – 48 h before inoculation.

289 **3.3. Evaluation of the metabiotic interaction between *S. enterica* cocktail and causing decay**
290 **fungi in strawberries**

291 To evaluate the effect of the metabiotic interaction between *S. enterica* and the decay-causing
292 fungi in strawberries, a fixed effects linear model including interactions was performed.
293 Significant differences were assessed through an ANOVA analysis ($P < 0.05$) together with a
294 Tukey post-hoc test. The statistical model was able to predict the concentration of *S. enterica* as
295 a function of the studied factors ($R^2 = 0.894$; F- value = 37.47; residual std. error = 0.463). The
296 goodness of fit indices AIC, log lik and BIC were estimated as 131.32; -47.66 and 175.91,
297 respectively. Estimations of single effects and interactions are presented in Table 2. As expected,
298 time-dependent variables were found as significant ($P < 0.05$), together with D2 treatments
299 together with the interaction between D1 treatment and mould. To evaluate model predictions,
300 the percentage of $\log_{10}$ values falling within the ASZ were calculated. Predictions vs observations
301 are represented in Figure 6. It was obtained that 78.41% of the values fell inside the ASZ which
302 indicated that the fixed effect linear model provided reasonable predictions of *S. enterica* counts
303 in stored strawberries at the different assayed conditions.

304 Results from the ANOVA analysis found that single effects were significant ($P < 0.05$) apart from
305 pH (Table 3). It is generally accepted that resistance to acidity of *Salmonella* varies between
306 serovarieties and even between strains of the same serovar (Arvizu-Medrano et al., 2005; Berk et
307 al., 2005; Yuk & Schneider, 2006). A limitation of our study relies on the difficulty to quantify
308 the acid sensitivity of each *S. enterica* strain since a cocktail inoculation was performed. Indeed,
309 a combination of environmental factors prior to the storage of strawberries under certain
310 conditions could have influenced the survival of *S. enterica*. In fact, the interaction between mould
311 and pH was significant at 99% level, which explains that the inhibitory effect was attributed to
312 the inoculated mould rather than to the acidic pH of strawberries.

313 The hypothesis of the existence of a metabiotic association between the fungal and foodborne
314 pathogens that favoured growth of the later ones, would have increased the risk, in particular
315 during the period in which the mould is present and there are no rot symptoms. However, the
316 inhibitory effect caused by the action of epiphytic moulds in conjunction with some
317 environmental factors such as temperature and pH, as well as storage time, against *S. enterica* in
318 strawberries was shown. These results could indicate that the potential interactions between
319 strawberry fungal pathogens and food-borne human pathogens do not favour the later ones.

320 **4. Conlcusion**

321 *B. cinerea* and *R. stolonifer* were able to create an unfavourable microenvironment within or
322 adjacent to wound on strawberry surface that would disfavour survival and growth of *S. enterica.*
323 Treatments with mould-inoculated (D2, D1 and D0) reported significant reductions of *S. enterica*
324 compared with uninoculated fungal fruits (CK). Results reported that single effects of
325 environmental factors were significant ($p < 0.05$) except for pH. The data hereby reported
326 confirmed that *S. enterica* survival was not correlated to an increase/decrease of the pH that
327 remained unchanged throughout the running time. However, though refrigeration increased
328 *Salmonella* reductions, absence of this pathogen is not guaranteed since the survival ability of
329 *Salmonella* was also shown. Implementation of good manufacturing practices during primary
330 production, harvesting, industrial transformation and consumption seem to be crucial to avoid
331 *Salmonella* contamination and to maintain the microbiological safety of strawberries.
332 Accordingly, more consideration should be given to microbial interaction between fungal
333 pathogens of strawberry and *S. enterica*, which should be studied and assessed properly the
334 possible cause of the pathogen decay in front of fungi.

Acknowledgements

The authors are grateful to the Spanish Government (Ministerio de Economía y Competitividad, research project AGL2016-78086-R) for its financial support and to the CERCA Programme of 'Generalitat de Catalunya'. J. Ortiz thanks the University of Lleida (UdL) for PhD grant.

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445

446 **Table 1** – Microbial kinetic parameters (mean ± s.d.) inactivation rate (k_{max}, h^{-1}) and initial
447 concentration (*log* N_0, $\log_{10}$ CFU/wound) estimated by the log linear models of *S. enterica* in
448 strawberries inoculated with *B. cinerea* and *R. stolonifer* during storage at 20 and 4ºC at the
449 different studied treatments. CK: control without inoculation of postharvest pathogen, D2, D1 and
450 D0: strawberries inoculated with mould suspension 2 days, 1 day before and at the same time as
451 the *S. enterica* cocktail. MSE = Mean squared error; R^2 = determination coefficient.

Condition	Treatment	MSE	R^2	k_{max} (h^{-1})	Log N_0 (CFU/wound)
***Salmonella-Botrytis* 20 ºC**	CK	0.026	0.713	0.027±0.010	4.546±0.119
	D2	0.200	0.920	0.160±0.027	4.909±0.329
	D1	0.267	0.644	0.073±0.032	4.901±0.380
	D0	-*	-	-	-
***Salmonella Botrytis* 4 ºC**	CK	0.065	0.939	0.016±0.002	4.568±0.181
	D2	0.358	0.927	0.033±0.005	4.454±0.424
	D1	0.161	0.964	0.032±0.003	4.491±0.284
	D0	0.202	0.909	0.022±0.003	4.359±0.319
***Salmonella-Rhizopus* 20 ºC**	CK	-*	-	-	-
	D2	0.455	0.696	0.108±0.041	4.937±0.496
	D1	-*	-	-	-
	D0	0.088	0.850	0.074±0.018	4.687±0.218
***Salmonella-Rhizopus* 4 ºC**	CK	0.032	0.893	0.008±0.001	4.399±0.127
	D2	0.075	0.958	0.022±0.003	4.200±0.202
	D1	0.009	0.984	0.011±0.001	4.565±0.066
	D0	0.032	0.953	0.012±0.001	4.346±0.127

452 *Observed values could not be fitted.

453

454 **Table 2** – Estimated values and significance level (P<0.05) of the fixed effects linear model with
455 interactions for the calculation of the survival of *S. enterica* in strawberries during storage.
456 Treatments D2, D1 and D0: strawberries inoculated with mould suspension 2 days, 1 day before
457 and at the same time as the *S. enterica* cocktail.

	Estimate	**Std. Error**	**t value**	**Pr(>|t|)**
(Intercept)	8.577	2.154	3.981	<0.001**
Mould (*R. stolonifer*)	-6.389	2.785	-2.294	0.025*
Temperature (4ºC)	-0.181	0.172	-1.052	0.296
Treatment (D2)	-0.514	0.233	-2.204	0.031*
Treatment (D1)	-0.390	0.235	-1.657	0.102
Treatment (D0)	-0.202	0.234	-0.862	0.392
Time	-0.023	0.005	-4.817	<0.001**
pH	-0.979	0.572	-1.711	0.091
Mould (*R. stolonifer*) x Treatment (D2)	0.300	0.299	1.003	0.319
Mould (*R. stolonifer*) x Treatment (D1)	0.779	0.294	2.647	0.010**
Mould (*R. stolonifer*) x Treatment (D0)	0.012	0.284	0.043	0.966
Mould (*R. stolonifer*) x Time	0.006	0.001	5.861	<0.001**
Mould (*R. stolonifer*) x pH	1.621	0.720	2.251	0.027*
Temperature (4ºC) x Time	0.015	0.005	3.055	0.003**
Treatment (D2) x Time	-0.007	0.001	-5.610	<0.001**
Treatment (D1) x Time	-0.004	0.001	-3.485	0.001**
Treatment (D0) x Time	-0.002	0.001	-1.856	0.068

458 *Significant factors at 95% confidence level
459 **Significant factors at 99% confidence level

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466 **Table 3** – Significance of the factors Mould, Temperature, Treatment, Time and pH on the
467 survival of *S. enterica* in strawberries during storage obtained by the ANOVA analysis of the
468 fixed effects model with interactions.

	Df	Sum. Sq.	Mean Sq.	F value	Pr(>F)
Mould	1	5.519	5.519	25.746	< 0.001**
Temperature	1	29.903	29.903	139.499	< 0.001**
Treatment	3	14.445	4.815	22.4625	< 0.001**
Time	1	58.392	58.392	272.398	< 0.001**
pH	1	0.257	0.257	1.1967	0.278
Mould x Treatment	3	1.654	0.551	2.572	0.061
Mould x Time	1	6.706	6.706	31.2838	< 0.001**
Mould x pH	1	2.496	2.496	11.6419	0.001**
Temperature x Time	1	1.846	1.846	8.6121	0.004**
Treatment x Time	3	7.289	2.43	11.335	< 0.001**
Residuals	71	15.22	0.214		

469 **Significant factors at 99% confidence level

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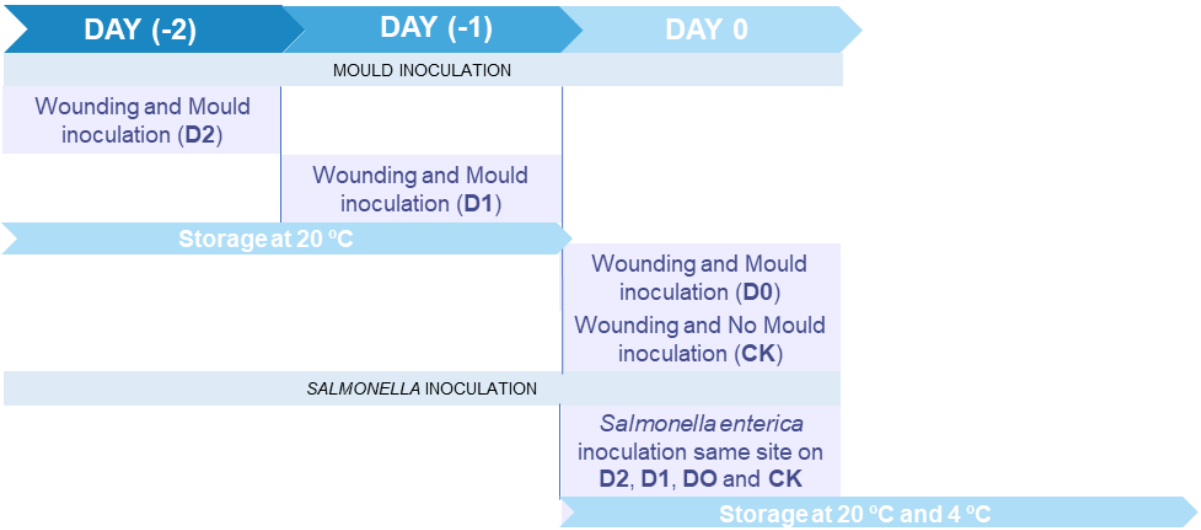
Figure 1 - Chronological representation of experimental design. D2: mould was inoculated two days before *Salmonella*; D1: mould was inoculated one day before *Salmonella*; D0: mould was inoculated at the same day as *Salmonella*.



475 **Figure 2.** Observed values and estimations provided by the log_{10} linear models for the survival of
476 *S. enterica* in strawberries inoculated with *B. cinerea* at 20 ºC. CK: control without inoculation
477 of postharvest pathogen, D2, D1 and D0: strawberries inoculated with mould's suspension 2 days,
478 1 day before and at the same time as the *Salmonella* cocktail. *Observed values of D0 could not
479 be fitted.

480

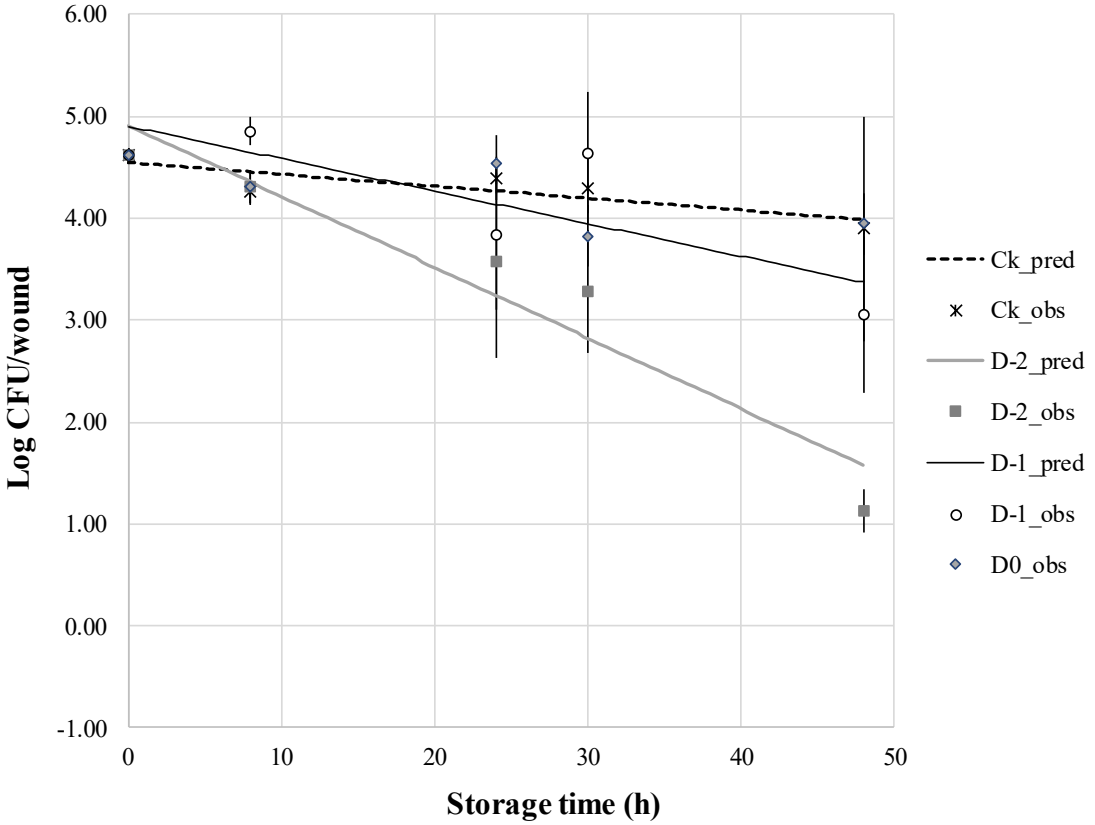


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Figure 3. Observed values and estimations provided by the log_{10} linear models for the survival of *S. enterica* in strawberries inoculated with *B. cinerea* at 4 ºC. CK: control without inoculation of postharvest pathogen, D2, D1 and D0: strawberries inoculated with mould's suspension 2 days, 1 day before and at the same time as the *Salmonella* cocktail.

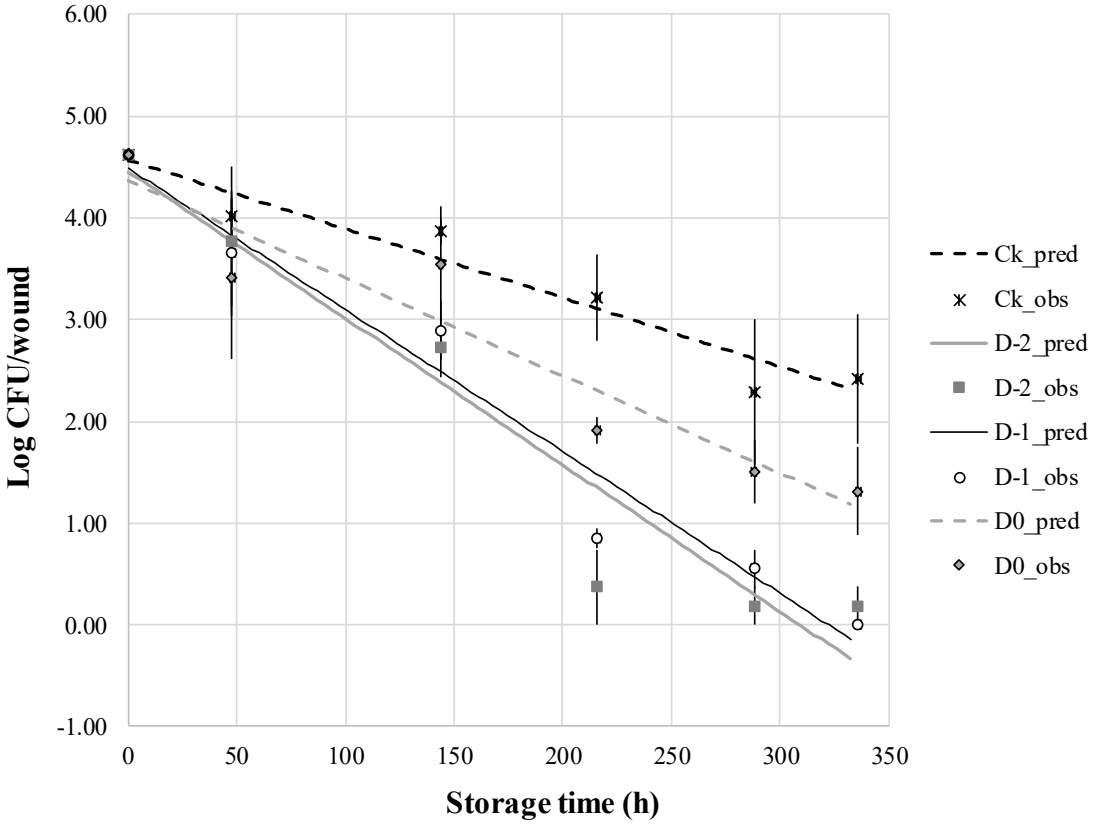

Figure 4. Observed values and estimations provided by the log_{10} linear models for the survival of *S. enterica* in strawberries inoculated with *R. stolonifer* at 20 ºC. CK: control without inoculation of postharvest pathogen, D2, D1 and D0: strawberries inoculated with mould's suspension 2 days, 1 day before and at the same time as the *Salmonella* cocktail. *Observed values of CK and D1 could not be fitted.

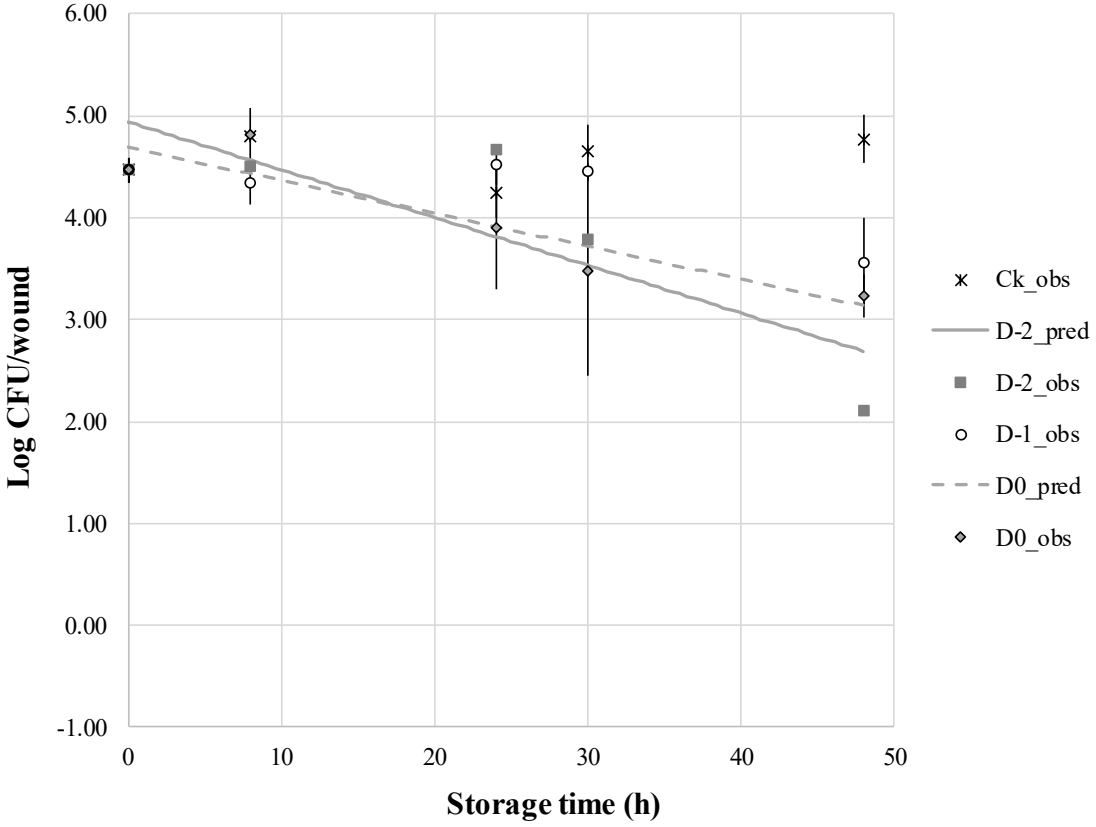

Figure 5. Observed values and estimations provided by the log_{10} linear models for the survival of *S. enterica* in strawberries inoculated with *R. stolonifer* at 4 ºC. CK: control without inoculation of postharvest pathogen, D2, D1 and D0: strawberries inoculated with mould's suspension 2 days, 1 day before and at the same time as the *Salmonella* cocktail.

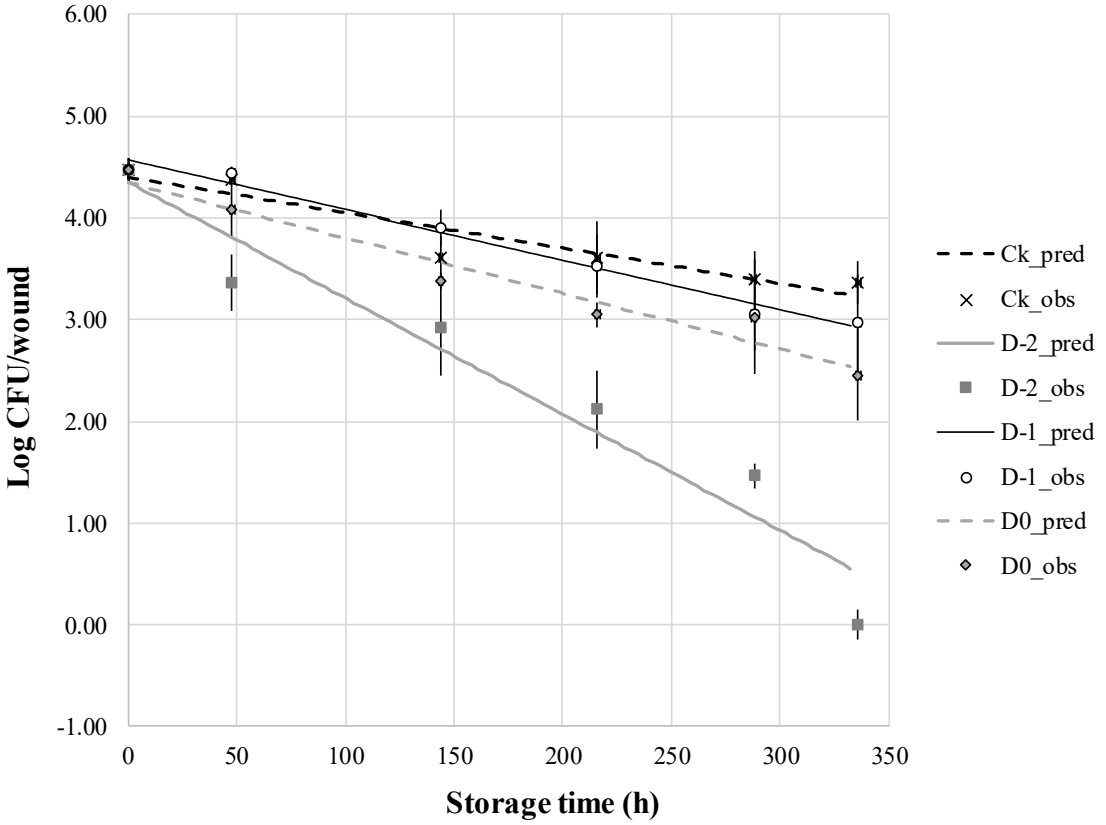

Figure 6. Graphical representation of the $\log_{10}$ counts predicted vs observed provided by the fixed effect linear model. The dashed lines define the Acceptable Simulation Zone (ASZ) of ± 0.5 $\log_{10}$ CFU/wound.