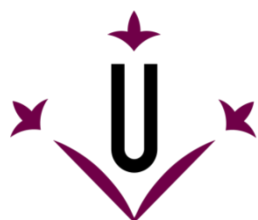


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1 **Highlights**

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

6

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

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21 **Abstract**

22 The microbial interaction between *Salmonella enterica* and the main postharvest fungal
23 pathogens of strawberries was evaluated. Inoculation of fungal suspension was done 2 (D2) and
24 1 (D1) day(s) before and at the same time (D0) as *S. enterica*. Fruits were stored at 20 ºC and
25 4 ºC. At both temperatures, *Botrytis cinerea* and *Rhizopus stolonifer* caused a decrease in *S.*
26 *enterica* population. Treatments where the mould was inoculated (D2, D1 and D0) achieved a
27 significant logarithmic reduction ($P < 0.05$) of *S. enterica* populations after 48 h (20ºC) and 14 d
28 (4 ºC) comapared to uninoculated fungal fruits (CK). Regarding temperature, average
29 reductions were significantly higher at 4º C (3.38 $\log_{10}$ CFU/wound) than at 20º C (1.16 $\log_{10}$
30 CFU/wound) ($P < 0.05$). Average reductions comprising all treatments were 1.91 and 0.41
31 log10 CFU/wound for *B. cinerea* and *R. stolonifer* at 20 ºC, and 3.39 and 3.37 $\log_{10}$ CFU/wound
32 for *B. cinerea* and *R. stolonifer* at 4 ºC. A linear $\log_{10}$ model was fitted in order to predict the
33 inactivation rate (kmax, $\log_{10}$ CFU/h) of *S. enterica*. Inactivation rates were higher at 20 ºC for
34 D2 treatments than at 4 ºC throughout the running time. The main inactivation rate was obtained
35 for *B. cinerea* at 20 ºC (0.160±0.027/h), which was found to have stronger inhibitory activity
36 against *S. enterica* than *R. stolonifer*. Univariate analysis ANOVA was carried out to evaluate
37 the effect of different external variables on the inhibition of *S. enterica*. Results found that
38 single effects were significant ($P < 0.05$) except for the pH. The inhibitory effect caused by the
39 action of moulds in conjunction with some environmental factors could indicate the potential
40 interactions between strawberry fungal pathogens and *S. enterica.*

41

42 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

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

98 interactions between the main moulds that cause rot (*Botrytis cinerea* and *Rhizopus stolonifer*)
99 and *Salmonella enterica*.
100 The objective of this study was to determine the survival of *Salmonella enterica* on fresh
101 strawberries and its interaction with the main postharvest strawberry fungal pathogens at
102 different storage temperatures. Changes in pH caused by growth of moulds were monitored.

## 103 2. Material and Methods

### 104 2.1. Experimental design and preparation of samples

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

### 117 2.2. Preparation of *S. enterica* cocktail

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

121 For each strain of studied *S. enterica*, a single colony from a streak in Tryptone Soy Agar (TSA;
122 Biokar Diagnostics) medium (20-24 h, 37 ± 1ºC) was inoculated in 5 mL of Tryptic Soy Broth
123 (TSB; Biokar Diagnostics) and incubated at 37 ± 1ºC for 18-24 h. Afterwards, all cultures were

124 combined in one centrifuge tube. The volume of the tube was centrifuged (Sorvall Legend XTR
125 Centrifuge, Thermo Fischer, US) at 9800×g for 10 min at 10 °C and resuspended with half of
126 the initial volume (12.5 ml) of saline solution (SS; 0,85% w/v NaCl). The inoculum was diluted
127 to a concentration of about $1 \times 10^7$ CFU/ml with deionized sterile water before being added to
128 the wound. The real concentration of the inoculum was checked by plating in TSA and Xylose
129 Lysine Desoxycholate Agar (XLD; Biokar Diagnostics) incubated at 37 ± 1 ºC for 18-24 h.

130 **2.3. Preparation of postharvest pathogen (fungi)**

131 The strains *Botrytis cinerea* BC03 (CECT 20973) and *Rhizopus stolonifer* RSF, belonging to the
132 collection of Postharvest Pathology Group of IRTA (Lleida, Catalonia), were used for the
133 experiments. They were subcultured on Potato Dextrose Agar (PDA; Biokar) and incubated at
134 25 ± 1 ºC. *B. cinerea* cultures were grown for a minimum of 7-15 days and *R. stolonifer* cultures
135 for 5-7 days. Conidia were harvested with inoculating loop and conidial suspensions were
136 adjusted to $10^4$ conidia/ml for *B. cinerea* (BC) and $10^3$ conidia/ml for *R. stolonifer* (RSF) in 10
137 ml tubes containing tween 20 (0.20% w/v). Tween 20 was added to sterile water and the
138 conidial suspensions to ensure homogeneous distribution of the conidia. Cell suspension was
139 determined using a haemocytometer (Thoma cell counting chamber, Marienfield-Superior, UK).
140 Two special coverslip provided with the counting chamber were properly positioned. The cell
141 suspension was applied to the edge of the coverslip which completely fills the chamber with the
142 sample. The number of cells in the chamber have been determined by direct counting using a
143 microscope and was defined as:

144 $$conidia/mL = \frac{(number\ of\ cells\ counted)(dilution\ factor)}{(number\ of\ large\ squares\ counted)\ (volume\ of\ 1\ large\ square)} \quad \text{Equation 1}$$

145 **2.4. Inoculation of *S. enterica* and the fungi on strawberry tissue**

146 For all experiments, strawberries were removed from storage before inoculation, allowed to
147 warm for a few minutes. The surface of the fruit was marked with nail polish to locate the
148 wound. The wound was done using a nail (1 mm wide and 2 mm deep) at approximately 10 - 12
149 mm distance from the mark. Before the inoculation, strawberries were homogeneously placed

150 under UV light in a biosafety laminar cabinet (class II – type A, Telstar, Terrassa, Spain) for the
151 disinfection. The time exhibition of UV light was 10 min per face. Wounds on strawberry were
152 inoculated with 10 µl of the fungal suspension of BC ($10^4$ conidia/ml) or RSF ($10^3$/ml) 2, 1 and
153 0 days before inoculation (Figure 1). Fungal inoculum on strawberries was allowed to dry for 1-
154 2 h in a biosafety laminar air cabinet (class II - type A, Telstar, Terrassa, Spain) at room
155 temperature. Then, the berries stored at 20 ºC. *S. enterica* suspension (10 µl) at a concentration
156 of $1\times10^7$ CFU/ml in 0.85% NaCl was pipetted into the same wound in which the fungus was
157 inoculated the same day (D0), 1 (D1) or 2 days (D2) before. Control strawberries (no fungal
158 pathogen) were only inoculated with 10 µl of *S. enterica* suspension. The fruits with both fungi
159 and bacteria or only with bacteria were dried in a laminar cabinet. Strawberries were placed in a
160 box with 42-cells alveoli (21 strawberries were for monitoring the population of *S. enterica* and
161 21 strawberries were for pH determination) and subsequently stored at 4 ºC and 20 ºC.

162 **2.5. *S. enterica* determination analysis**

163 Inoculated strawberries were analysed for populations/presence of *S. enterica* within 2 h of
164 inoculation (0 day) and at 8, 24, 30 and 48 h for samples stored at 20ºC. For the strawberries
165 stored at 4ºC, *S. enterica* population were examined after 2, 6, 9, 12 and 14 days. For each
166 condition (fungal strain, temperature, inoculation time), three strawberries were analysed
167 individually (n=3). To recover *S. enterica* from strawberries, a small and equal portion of fruit
168 that contained the entire wound was taken out with cork borer and placed in a sterile stomacher
169 bag (BagPage®, Interscience, France) with 5 mL of Buffered Peptone Water (BPW, Biokar
170 Diagnostics). Afterwards, it was homogenized in a stomacher blender (Stomacher Minimix®,
171 Mathias S.L.) for 2 min at normal speed (9 strokes/sec). BPW was used for a better resuscitation
172 of injured cells and reduction of the lag phase to obtain higher recovery rates at shorter
173 incubation times (Jasson et al., 2009). Ten-fold dilutions of the homogenates were made with
174 saline peptone (SP) (0,85% w/v NaCl; 0,1% w/v Peptone) tubes and they were plated in the
175 selective media XLD (Biokar Diagnostics). The plates were incubated at 37 ºC for 18 - 24h.
176 Microbial population was expressed in $\log_{10}$ CFU/wound. Bags containing the homogenates

177 were also incubated at 37 ºC overnight for *S. enterica* detection in case no colonies were present
178 in plates. The limit of detection was 1.40 $\log_{10}$ CFU/wound. When no colonies were counted
179 and detection was positive, an arbitrary number of half detection limit was used for calculation
180 (1.13 $\log_{10}$ CFU/wound).

181 **2.6. pH measurement**

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

186 **2.7. Data modelling**

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

191 $$\log N(t) = \log N_0 - k_{\max} \cdot t$$ Equation 2

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

195 **2.8. Statistical data analysis**

196 To gain insight into the effect of the studied factors on the survival of *S. enterica* in the
197 inoculated strawberries, a fixed effects linear model with interactions was performed. The
198 factors considered were the type of mould (*B. cinerea* and *R. stolonifer*), temperature (20 and 4
199 ºC), pH, storage time and treatment (CK, D2, D1 and D0). A backward selection method was
200 chosen and mean estimated parameters together with goodness-of-fit indices were obtained. The

201 latter corresponded to the log likelihood (logL), Akaike Information Criterion (AIC) and
202 Bayesian Information Criterion (BIC). The model structure was defined as:

203 $$y_i = \beta_0 + \beta_1 \cdot x_{i,1} + \ldots\ldots + \beta_{p-1} \cdot x_{i,p-1} + \varepsilon_i \sim Normal(0, \sigma^2) \quad \text{Equation 3}$$

204 Being $y_i$ the response variable (*S. enterica* level, $\log_{10}$ CFU/wound), $\beta_0$, $\beta_1$, …$\beta_{p-1}$ the unknown
205 regression parameters and $\sigma^2$ the unknown (constant) error variance. A univariate analysis
206 ANOVA with a Tukey post-hoc test was achieved to evaluate the significance of the studied
207 factors. The software R v.3.5.1 (cran.rproject.org) was used taking a value of $P < 0.05$ as a level
208 of significance.

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

213 ## 3. Results and Discussion

214 ### 3.1. Survival ability of *S. enterica* in strawberries

215 Initial population of *S. enterica* ranged between 4.46 and 4.61 $\log_{10}$ CFU/wound on
216 strawberries. At both temperatures studied, *S. enterica* did not grow over storage time (48 hours
217 and 14 days at 20ºC and 4ºC, respectively) when the pathogen was inoculated alone (CK)
218 (Figure 2-5). Previous publications reported that *Salmonella* was able to survive on fresh-cut
219 strawberries for prolonged periods of time, but was unable to multiply (Knudsen et al., 2001).
220 Moreover, both *B. cinerea* and *R. stolonifer* caused a decline in *S. enterica* population in
221 comparison with uninoculated fungal fruits (CK). A multivariate ANOVA analysis was
222 performed to find out significant differences between storage temperatures, type of mould and
223 treatment on the $\log_{10}$ reductions of *S. enterica*. It was observed that in those treatments where
224 moulds were inoculated (D2, D1 and D0), significant reductions were achieved in comparison
225 to the treatment where *Salmonella* was inoculated alone ($P < 0.05$). Moreover, in those
226 treatments where *B. cinerea* and *R. stolonifer* were inoculated on the same day (D0), the day
227 before (D1) and two days before (D2) *S. enterica*, did not yield significant differences ($P >$
228 $0.05$) in the average reductions, ranging from 2.47 to 3.07 $\log_{10}$ CFU/wound while the
229 uninoculated fruits presented an average reduction of 0.77 $\log_{10}$ CFU/wound.

230 Regarding the effect of temperature, the results showed an inactivation of *S. enterica* population
231 throughout storage at both temperatures studied (20 ºC and 4 ºC). However, average reductions
232 were significantly higher at 4º C (3.38 $\log_{10}$ CFU/wound) than at 20º C (1.16 $\log_{10}$ CFU/wound)
233 ($P < 0.05$). Moreover, as the fungi were allowed to grow for 48 h at 20 ºC before pathogen
234 inoculation, fruit rotting was already in the initial stages when *S. enterica* inoculation was done.
235 The acidic pH (3.61 – 3.91), nutrients availability in strawberries wounds, and the high water
236 activity on the surface favoured the growth of *B. cinerea* and *R. stolonifer*. This fact, together
237 with a low storage temperature could constitute a hostile environment for the growth of *S.*
238 *enterica*. Delbeke et al. (2015) reported reductions of 2 – 3 $\log_{10}$ CFU in strawberry matrix after
239 5 days of storage at refrigeration (4 –15 °C). However, the survival experiment stopped before

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

245 Regarding the inhibitory effect of the decay-causing fungi against *S. enterica*, *B. cinerea*
246 produced a significantly higher $\log_{10}$ reduction effect than *R. stolonifer* at 20º C storage ($P <$
247 $0.05$). On the contrary, there were not significant differences in the inhibitory effect at 4º C,
248 though maximum observed reductions of *S. enterica* population in strawberries caused by *B.*
249 *cinerea* and *R. stolonifer* at this temperature were higher than 4 $\log_{10}$ CFU/wound after 14 d
250 storage in the D2 treatment (Figures 3 and 5). Average reductions including all treatments (D2,
251 D1, D0 and CK) were 1.91 and 0.41 $\log_{10}$ CFU/wound for *B. cinerea* and *R. stolonifer* at 20 ºC,
252 and 3.39 and 3.37 $\log_{10}$ CFU/wound for *B. cinerea* and *R. stolonifer* at 4 ºC.

253 On the other hand, no significant relationship was found between the pH of strawberries during
254 storage (average value of 3.71 for *B. cinerea* and 3.75 for *R. stolonifer*) and the reduction of *S.*
255 *enterica* (data not shown). Values of pH did not change substantially throughout the storage
256 period, regardless the applied treatment (D2, D1, D0 and CK). Cibelli et al. (2008) which
257 clearly demonstrated with a model system (a laboratory medium added with tomato juice) that
258 the increase of the pH approximately 1 to 1.3 of medium due to the metabolic activity of
259 *Fusarium oxysporum* significantly enhanced the survival of *Salmonella* spp. It is reported that
260 some postharvest fungal pathogens yield to an increase in pH, thus favouring the survival and
261 growth of enteropathogenic bacteria in contaminated fruits and vegetables. Wade et al. (2003),
262 reported that *Geotrichum candidum* secretes ammonia under inductive environmental conditions
263 in fresh tomatoes and increases pH of tissues to values as high as 7.5. Storage of wound
264 tomatoes at 15 ºC for 10 days resulted in a significant increase in population of 7.6 $\log_{10}$ CFU of
265 *S. enterica*/g of 2-g sample of co-infected pulp tissue. On the contrary, the metabiotic
266 interaction in our study demonstrated that the survival of the pathogen decreased pronounceably

267 without any substantial change in pH of strawberry's matrix, thus suggesting that metabiotic
268 effect could be due to some metabolites different from alkalinizing or acidifying compounds.
269 Moreover, moulds have a greater proteolytic activity and carbohydrate degradation when the
270 postharvest pathogen is already grown. Consequently, competition of nutrients, carbohydrates
271 and amino-acids of the fruit matrix may be critical for the bacterial growth.

272 **3.2. Predictive modelling of *S. enterica* cocktail in strawberries during storage**

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

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

### 295 3.3. Evaluation of the metabiotic interaction between *S. enterica* cocktail and causing 296 decay fungi in strawberries

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

310 Results from the ANOVA analysis found that single effects were significant (P < 0.05) apart
311 from pH (Table 3). It is generally accepted that resistance to acidity of *Salmonella* varies
312 between serovarieties and even between strains of the same serovar (Arvizu-Medrano et al.,
313 2005; Berk et al., 2005; Yuk & Schneider, 2006). A limitation of our study relies on the
314 difficulty to quantify the acid sensitivity of each *S. enterica* strain since a cocktail inoculation
315 was performed. Indeed, a combination of environmental factors prior to the storage of
316 strawberries under certain conditions could have influenced the survival of *S. enterica*. In fact,
317 the interaction between mould and pH was significant at 99% level, which explains that the
318 inhibitory effect was attributed to the inoculated mould rather than to the acidic pH of
319 strawberries.

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

327 **4. Conlcusion**

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

342 **Acknowledgements**

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454 **Table 1** – Microbial kinetic parameters (mean ± s.d.) inactivation rate ($k_{max}$, $h^{-1}$) and initial
455 concentration ($log\ N_0$, $log_{10}$ CFU/wound) estimated by the log linear models of *S. enterica* in
456 strawberries inoculated with *B. cinerea* and *R. stolonifer* during storage at 20 and 4ºC at the
457 different studied treatments. CK: control without inoculation of postharvest pathogen, D2, D1
458 and D0: strawberries inoculated with mould suspension 2 days, 1 day before and at the same
459 time as 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 |

460 *Observed values could not be fitted.

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462 **Table 2** – Estimated values and significance level ($P<0.05$) of the fixed effects linear model
463 with interactions for the calculation of the survival of *S. enterica* in strawberries during storage.
464 Treatments D2, D1 and D0: strawberries inoculated with mould suspension 2 days, 1 day before
465 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 |

466 *Significant factors at 95% confidence level
467 **Significant factors at 99% confidence level

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

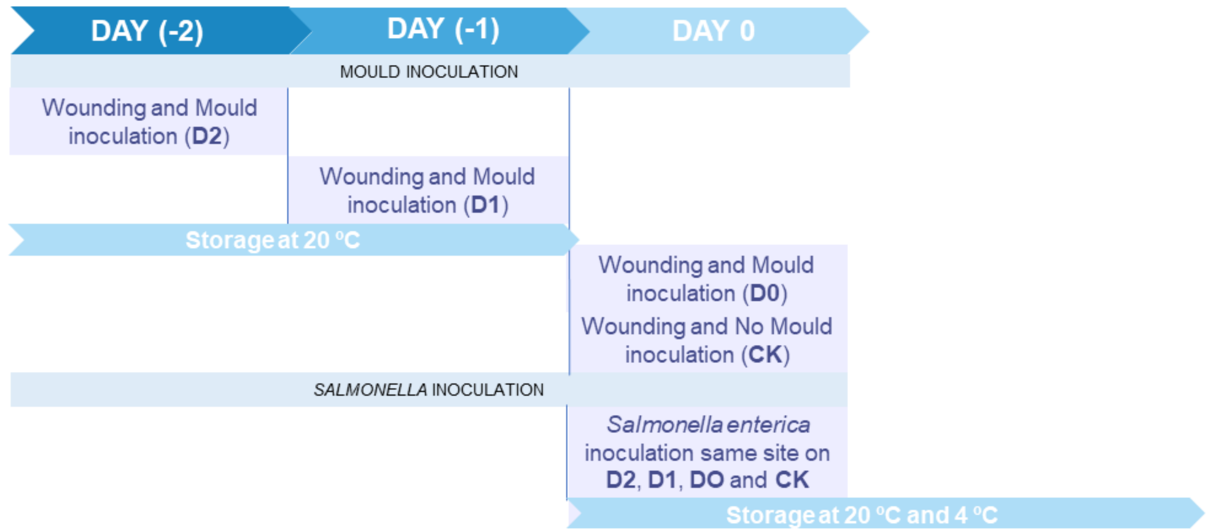
| | **Df** | **Sum. Sq.** | **Mean Sq.** | **F value** | **Pr(>F)** |
|---|---|---|---|---|---|
| **Mould** | 1 | 5.519 | 5.519 | 25.746 | < 0.001[**] |
| **Temperature** | 1 | 29.903 | 29.903 | 139.499 | < 0.001[**] |
| **Treatment** | 3 | 14.445 | 4.815 | 22.4625 | < 0.001[**] |
| **Time** | 1 | 58.392 | 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 | | |

[**]Significant factors at 99% confidence level

479 **Figure 1** - Chronological representation of experimental design. D2: mould was inoculated two

480 days before *Salmonella*; D1: mould was inoculated one day before *Salmonella*; D0: mould was

481 inoculated at the same day as *Salmonella*.

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

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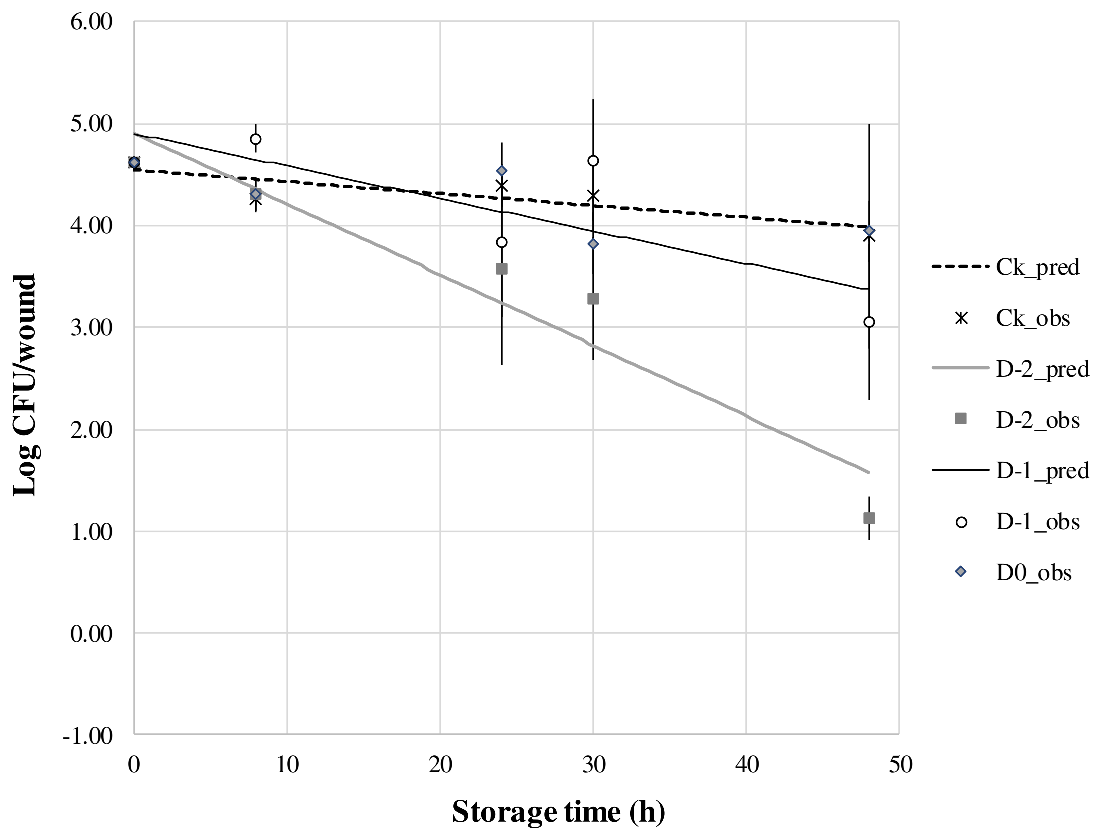


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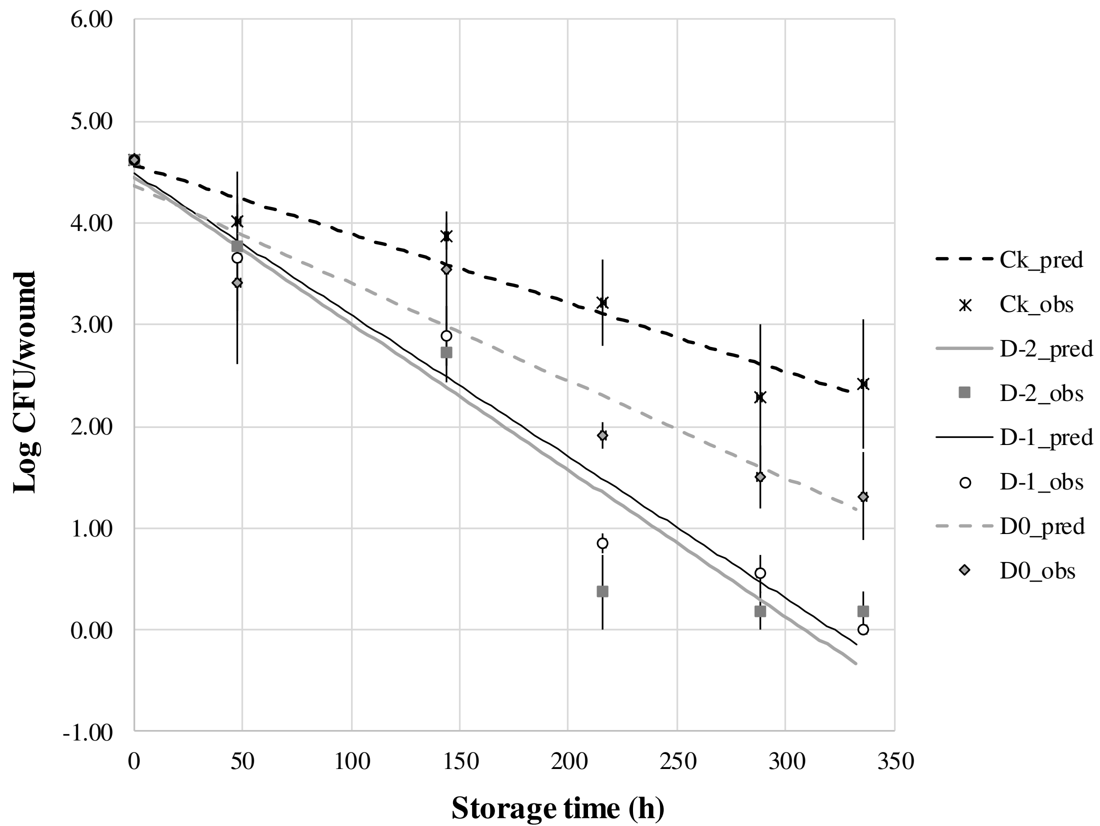
496 **Figure 3.** Observed values and estimations provided by the $\log_{10}$ linear models for the survival
497 of *S. enterica* in strawberries inoculated with *B. cinerea* at 4 ºC. CK: control without inoculation
498 of postharvest pathogen, D2, D1 and D0: strawberries inoculated with mould's suspension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

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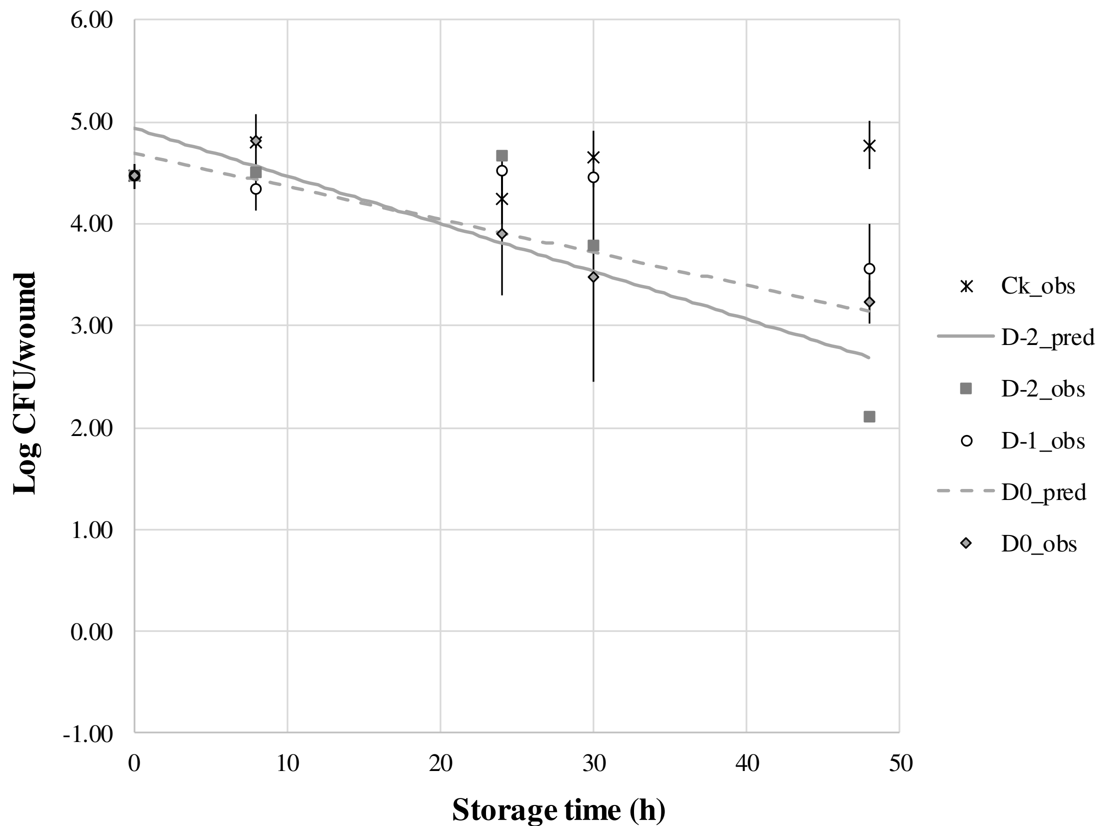
508 **Figure 4.** Observed values and estimations provided by the $log_{10}$ linear models for the survival
509 of *S. enterica* in strawberries inoculated with *R. stolonifer* at 20 ℃. CK: control without
510 inoculation of postharvest pathogen, D2, D1 and D0: strawberries inoculated with mould's
511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

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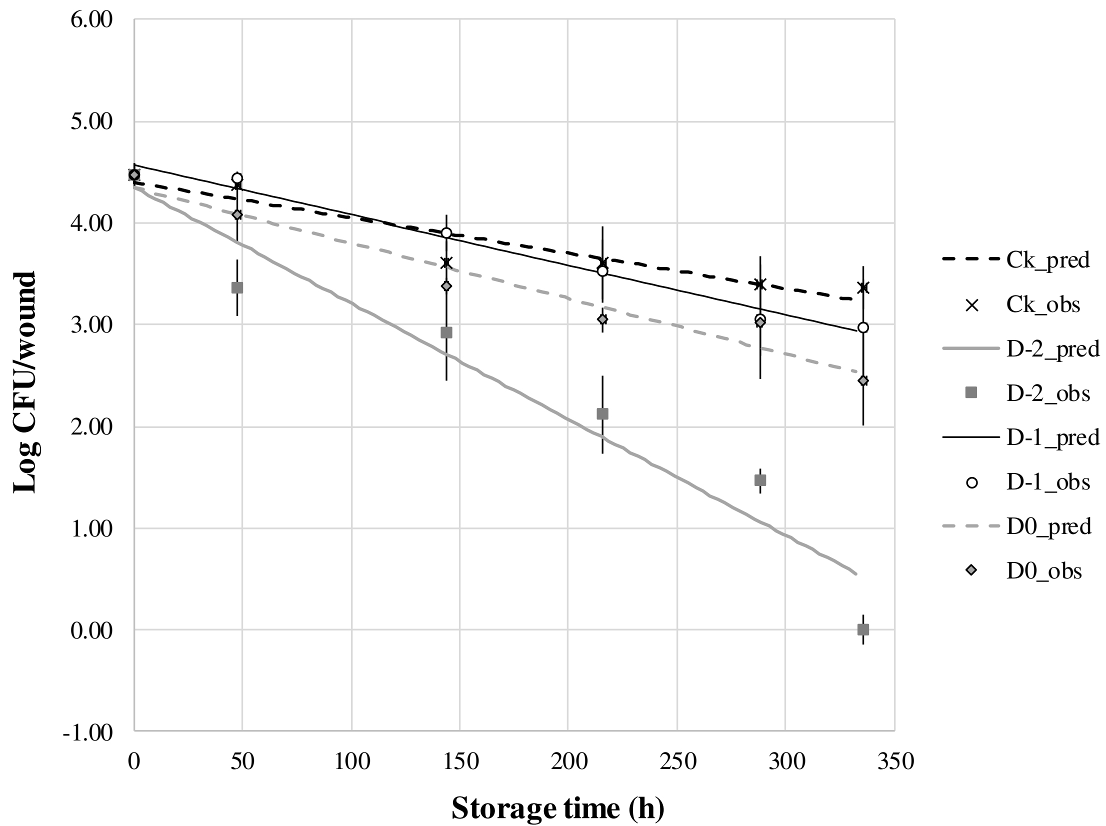
523 **Figure 5.** Observed values and estimations provided by the $log_{10}$ linear models for the survival
524 of *S. enterica* in strawberries inoculated with *R. stolonifer* at 4 ºC. CK: control without
525 inoculation of postharvest pathogen, D2, D1 and D0: strawberries inoculated with mould's
526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

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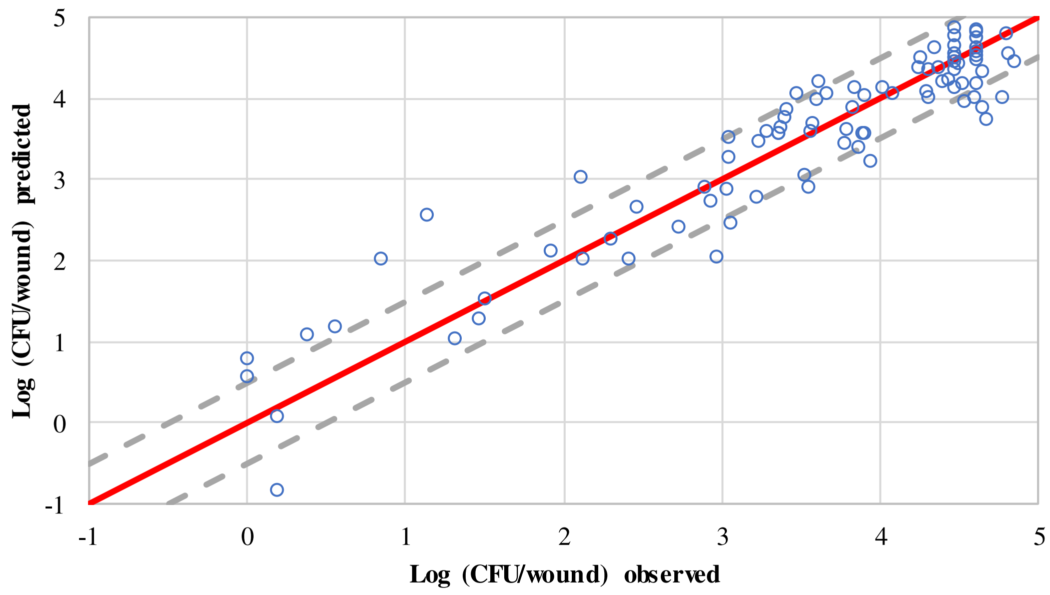
537 **Figure 6.** Graphical representation of the $\log_{10}$ counts predicted vs observed provided by the
538 fixed effect linear model. The dashed lines define the Acceptable Simulation Zone (ASZ) of ±
539 0.5 $\log_{10}$ CFU/wound.

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