Salmonella Infantis and *Salmonella* Enteritidis specific bacteriophages isolated form poultry faeces as a complementary tool for cleaning and disinfection against *Salmonella*

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ABSTRACT

Salmonellosis represents an important public health concern. Several authors point out the inefficiency of the cleaning and disinfection protocols to remove the bacteria from the field. For this reason, innovative techniques, as bacteriophages, could be implemented to control the bacteria. The main objectives of this study were to assess the effect of bacteriophages against *Salmonella* Infantis and *Salmonella* Enteritidis on farm surfaces, and to evaluate bacteriophage procedure application as sanitiser against *Salmonella* in field conditions. Thus, most prevalent serovars in poultry production were selected (*Salmonella* Infantis and *Salmonella* Enteritidis) to contaminate farm facilities. Then, two specific bacteriophages isolated from poultry faeces were applied against them. Results showed *Salmonella* Infantis and *Salmonella* Enteritidis decreased of 4.55 log_{10}CFU/mL and 3.85 log_{10}CFU/mL, respectively; the maximum reduction in *Salmonella* was the 5th day, after 10^8 PFU/mL and 10^3 PFU/mL bacteriophage application. These results highlight bacteriophages as a promising tool together with cleaning and disinfection.

Keywords: *Salmonella,* poultry, bacteriophages, cleaning and disinfection, sanitiser

1. INTRODUCTION

Salmonella is widely recognised as one of the most important zoonotic pathogens with economic impact in animal and humans. There are roughly 550 million gastrointestinal cases worldwide, and *Salmonella* is one of the main pathogens in these disease outbreaks [1]. In the United States, this is a significant public health concern, and *Salmonella* causes around 1.2 million cases and 450 deaths every year [2]. In Europe, salmonellosis was responsible for 91,662 cases in humans, of which 9,426 were reported in Spain the same year [3]. The main sources of infection are poultry products, particularly meat and eggs [3]. Main serovars involved in these food outbreaks are *Salmonella* Enteritidis (*S*. Enteritidis), *Salmonella* Typhimurium and monophasic *Salmonella* Typhimurium. However, last year *Salmonella* Infantis (*S*. Infantis) prevalence increased considerably, being the most prevalent serovar in broilers [3].

These zoonotic bacteria represent an important public health concern and controlling the disease has become a vital challenge in most countries [3,4]. Thus, *Salmonella* NCP (National Control Programmes), in accordance with Regulation (EC) Nº 2160/2003 [5], together with biosecurity measures, cleaning and disinfection protocols and prophylactic measures, have resulted in a decreased prevalence at field level [6]. However, despite all these measures, new cases of salmonellosis emerge every year and survival of the bacteria is still being demonstrated in some poultry farms [7]. Several authors have pointed out the inefficiency of the hygiene programmes and cleaning and disinfection protocols [8] not only because of incorrect practice, but also due to the bacteria's resistance to disinfectants [9]. For this reason, innovative techniques applied at farm level, such as the use of bacteriophages or phages, must be implemented to complement the cleaning and disinfection protocols [10,11].

Bacteriophage are ubiquitous agents that infect and replicate in the prokaryotic cells [12-14]. These viruses only attack bacteria, altering them until they are destroyed. Their success lies in their high specificity against the target bacterium, their self-amplification and auto-limiting nature and their evolving capacity against antimicrobial resistant bacteria [10]. The effectiveness of bacteriophage therapy depends on the individual bacteria, on the given

bacteriophage concentration, the adaptive mechanism of the bacteria and time of applications [11]. These characteristics make bacteriophages a very promising tool for the elimination of *Salmonella* in those farms where disinfectants fail to eradicate it [11,15]. Although some bacteriophage products are already being commercialized, no precise official guidelines of bacteriophage production have been made, for this reason bacteriophage production is still in development and there are some challenges to overcome [16,17]. Cocktails of bacteriophages, are the most often used against the bacteria, however resistance against the diana bacteria can occur and there is the possibility to produce auto-bacteriophages, when the cocktails are not active against the field isoltes [11,16,18].

Few articles describe the use of bacteriophages as a sanitiser at field level in poultry farms, although it appears as an emergent measure in the food industry, where it is applied as sanitiser against biofilm bacteria [19,20,21].

Therefore, the main objectives of this study were to assess the effect of bacteriophages against *S.* Infantis and *S*. Enteritidis on farm surfaces, and to evaluate bacteriophage-procedure application as sanitiser against *Salmonella* in field conditions.

2. MATERIAL AND METHODS

2.1 *S.* Infantis and *S*. Enteritidis bacteria

The two *Salmonella* isolates (*S.* Infantis and *S*. Enteritidis) employed in this study were isolated in the Poultry Quality and Animal Nutrition Centre of the Valencia Region (CECAV) from the *Salmonella* NCP [5]. The isolates were isolated following the ISO 6579:2017 [22] and serotyped according to the Kauffman-White-Le Minor technique [23]. They have been stored at -80 ºC for further studies.

2.2 Bacteriophage Isolation

2.2.1 *S.* Infantis Bacteriophage Isolation

Bacteriophage against *S.* Infantis was isolated from faeces collected from different farms. Briefly, 25 g of faeces were homogenised and diluted 1:10 in LB (Luria-Bertani, VWR

Chemicals, Barcelona, Spain). Samples were centrifuged at 16,000 x g for 5 min. The supernatant was then filtered through a 0.22 µm membrane. Bacteriophage detection was performed by spotting samples on *S.* Infantis lawns as described by Kropinski et al. [24] These plates were incubated overnight at 37ºC. After incubation, a clear zone in the plate resulting from the lysis of host bacteria cells indicates the presence of a specific bacteriophage [21]. A single lysis plaque from each positive sample was purified by serial dilutions and plated to LB agar supplemented with $MgSO_4$ and $CaCl_2$ (Luria-Bertani, VWR Chemicals, Barcelona, Spain). To do so, 200 mL from the host culture and 100 mL of bacteriophage containing sample were mixed with 5 mL of 0.6% LB agar and overlaid onto 1.5% LB agar plates, then the mix was incubated overnight at 37ºC. Lysates of single plaques from a single bacteriophage were mixed in PBS (Phosphate Buffered Saline, VWR Chemicals, Barcelona, Spain) and centrifuged at 5,000 x g for 5 min. Bacteriophage suspensions were recovered and filtered using membranes with a pore size of 0.45 µm and 0.22 µm. Bacteriophages were stored at 4ºC. Bacteriophage titre was analysed by successive dilutions of the bacteriophage suspension performed in PBS. One hundred µL of each dilution together with 100 µL of the respective bacterial host suspension were mixed with 5 mL of LB 0.6% top agar layer and placed over a 1.5% LB agar bottom layer. Plates were incubated overnight at 37ºC. Bacteriophage titration was performed three times [25].

2.2.2 *S.* Enteritidis Bacteriophage Isolation

Regarding the *S.* Enteritidis bacteriophages used in this study, it was previously obtained and characterised by Sevilla-Navarro et al. [11].

2.3 Bacteriophage Purification

Finally, the bacteriophage was multiplied until a concentration of 10^{12} PFU/mL, 10^{8} PFU/mL and 10^{3} PFU/mL were reached and stored at 4ºC until use [26]. For this purpose, 400 mL of host culture (SI and SE, respectively) was grown to Optical Density $(OD)_{600}$ = 0.2 at 37ºC. Bacteriophage lysate was added to a Multiplicity of Infection (MOI) = 0.1. The sample

(bacterium and phage) was incubated under agitation (180 rpm) and every hour up to 8 h, 10 mL of the sample was taken, and several dilutions were prepared into LB. After, 10 µL of each fold dilution were spotted onto double agar layer and incubated overnight at 37ºC. The bacteriophage titre was calculated on the basis of counted plaques. Bacteriophage titres of 10^{12} PFU/mL, 10^{8} PFU/mL and 10^{3} PFU/mL were selected for the *in situ* trial.

2.4 Bacteriophage Phenotyping

Morphologic plate characteristics were performed to characterise phenotypically whether the bacteriophages were lytic or lysogenic according to Jurczak-Kurek et al. [27].

Likewise, the bacteriophages were studied in terms of size and morphology by transmission electron microscope as described in previous studies [11]. To this end, 10 µL from the bacteriophage with a concentration of 10^{8} PFU/mL was fixed in an aqueous solution of paraformaldehyde (2%). A 7.2 V glow was discharged on samples placed on the MESH Cooper grid and incubated in the grids for 15 min. Then, samples were washed in phosphate buffered 0.1 M for 2 min and fixed with glutaraldehyde (1%). Samples were negatively stained with uracil acetate and incubated with methyl cellulose (1%) for 30 sec. Samples were dried until use.

2.5 *In situ* Assay

The floor contamination procedure was performed inside an experimental poultry house at the Animal Research Centre (CITA, Segorbe, Spain) to mimic the real conditions of poultry production. To assess *Salmonella* status of the experimental house before the trial, surface samples were taken in accordance with ISO 6579-1:2017 [22]. The material of the bacteriophages application were tested on the cement floor of the house, as house floors have shown a high tendency to resist *Salmonella* disinfection [8,28].

Two experiments were performed. Bacteriophage concentration and times of application were assessed in the first one, and *Salmonella* reduction counts throughout the week after bacteriophage application were evaluated in the second.

2.5.1 Experiment 1 - Definition of Bacteriophage Concentration and Time of Aplication

Each bacteriophage (against *S*. Infantis and *S*. Enteritidis) was tested at different concentrations (10^{12}, 10^{8}, 10^{3} PFU/mL) and number of applications (1, 2 or 3) against *S.* Infantis and *S.* Enteritidis, respectively. Each treatment (bacteriophage x concentration x application) was evaluated twice. A negative control (only bacteria) was included in the study per concentration, application and session.

For experimental contamination of the house, 80 cm^2 squares were marked on the cement floor. Each square was an experimental unit. First, *Salmonella* (1 mL) was inoculated in each area (SI or SE) at a concentration of 10^{8} CFU/mL and spread with a sterile swab. All test areas were allowed to dry under environmental conditions for 3 days [8].

Before bacteriophages were applied, the negative control was swabbed to establish initial *Salmonella* growth according to Commission Regulation (EU) No 2160/2003 [5]. Then, each bacteriophage was applied on each area at different concentrations and number of applications. To avoid cross contamination between bacteriophages and different concentrations, squares were covered with a plastic cover. Finally, each area was swabbed 24h after bacteriophages application and *Salmonella* counts were determined according to ISO 6579-2:2017 [29].

2.5.2 Experiment 2 - *Salmonella* count monitoring

As cleaning and disinfection procedures are applied with a maximum duration of one week during the downtime in Spanish broiler production [30], the experiment was performed over one week. In accordance with results obtained in experiment 1, the optimum combination of concentration of each bacteriophage and number of bacteriophages applications were selected for on-farm application. *Salmonella* contamination of the house and the bacteriophage application were performed as reported above (Experiment 1). A total of 14 samples per bacteriophage were taken and *Salmonella* counts were determined (1 sample x 7 days x 2 sessions) (ISO 6579-2:2012) [29]. Moreover, each negative control per experimental unit was assessed as reported above (Experiment 1).

2.6 Statistical Analysis of the *Salmonella* counts

A General Linear Model was used to compare the effect of bacteriophage application on *Salmonella* counts, including as fixed effect the number of applications (1, 2 or 3), concentration (10^{12}, 10^{8} and 10^{3} PFU/mL) and number of sessions (n=2). Sessions were not significant and were excluded from the final model (p=0.127). The optimal result obtained in *Salmonella* reduction (concentration x application) was used to assess the evolution of *Salmonella* decreasing during the week. A *P value* less than 0.05 was considered to indicate a statistically significant difference. All statistical analyses were carried out using SPSS 16.0 software.

3. RESULTS

3.1 Bacteriophage Phenotyping

Phenotypic characterisation showed a *S.* Infantis-phage and a *S.* Enteritidis-phage with a size of 200 nm and an isometric head, which could correspond to the *Myoviridae* family (Figure 1). Moreover, the presence of lytic plaques suggested that both were lytic bacteriophages [27].

3.2 Experiment 1 – Definition of Bacteriophage concentration and time of application

Regardless of the bacteriophage assessed and the concentration applied, statistical differences were found between the number of bacteriophage applications and *Salmonella* reduction (p<0.05). However, no statistical significant differences were shown between concentration of bacteriophages and *Salmonella* reduction.

According to the results obtained after *S.* Infantis-phage application (Table 1), the highest *Salmonella* reduction was obtained after two applications of the bacteriophage at a concentration of 10^{8} PFU/mL (p<0.05). In addition, no statistical differences were observed after the third application. For 10^{12} PFU/mL, the highest *Salmonella* counts reduction was also observed after the second application of the bacteriophage, showing no differences after the third application. Finally, at a concentration of 10^{3} PFU/mL, no significant differences were found in *Salmonella* reduction despite the number of applications (p<0.05).

194 According to the results obtained after *S.* Enteritidis-phage application (Table 2), the optimum
195 reduction in *Salmonella* was obtained after 2 consecutive applications of the bacteeriophage
196 at a concentration of 10^3 PFU/mL. In the same line, for 10^{12} PFU/mL and 10^8 PFU/mL *S.*
197 Enteritidis-phage, 2 applications were necessary to reach the maximum reduction and no
198 statistical differences were found after the 3rd application.
199
200 **3.3 Experiment 2 - *Salmonella* count monitoring**
201 In accordance with the results obtained in Experiment 1, *Salmonella* counts during a week
202 were analysed after 2 consecutive applications of bacteriophage at different concentrations
203 (10^8 PFU/mL and 10^3 PFU/mL, for *S.* Infantis-phage and *S.* Enteritidis-phage, respectively).
204 After bacteriophage application, the highest reduction for both serovars (*S*. Infantis and *S*.
205 Enteritidis) was observed after 5th day of application (7 $\log_{10}$ CFU/mL and 4.1 $\log_{10}$ CFU/mL)
206 (p<0.05). However, for S. Infantis, no statistical signigicant differences were shown between
207 the bacteria decrease on days 3th, and 5th, rising again days 4th, 6th an 7th. Similar to this
208 happens with *S.* Enteritidis, were no statistical significant differences were shown between the
209 bacteria reduction in days 5th and 7th, showing a rise back up at day 6th. Results obtained were
210 summarised in Figure 2.
211
212 **4. DISCUSSION**
213 To the best of our knowledge, this is the first study to assess the efficacy of bacteriophage as
214 sanitiser against *Salmonella* in poultry farm facilities.
215 Nowadays, *S.* Infantis and *S.* Enteritidis are the main significant serovars in meat and egg
216 production, respectively [3]. Due to the impact of these serovars, over the past few years the
217 poultry sector has focused its effort on controlling *Salmonella* in farms. However, the measures
218 are not effective enough, and the bacteria remain in some facilities [15]. In this context,
219 effective and cost-effective solutions for cleaning and disinfection protocols are seen as a
220 necessary measure for the elimination of *Salmonella* from poultry farms [31]. For this reason,
221 bacteriophages have garnered high interest as a potential measure to reduce *Salmonella*

222 contaminations in commercial poultry farms. Bacteriophages are useful in a wide range of
223 applications, from health facilities to agriculture and foodstuff industries, to combat bacterial
224 infections [21, 32].
225 The results of our study showed *S.* Infantis and *S.* Enteritidis decrease of 4.55 log_{10} CFU/mL
226 and 3.85 log_{10} CFU/mL, respectively, from the surfaces of farm facilities after consecutive
227 bacteriophage application. Similar results were reported by Woolston et al. [33], showing
228 reductions of 4.3 log CFU/surface and 3.0 log CFU/surface after the application of a specific
229 *Salmonella*-phage cocktail.
230 Moreover, after 2 consecutive bacteriophage applications, the optimal reduction of *S.* Infantis
231 and *S.* Enteritidis (4.55 log_{10} CFU/mL and 3.85 log_{10} CFU/mL, respectively) was reached for
232 10^8 PFU/ mL and 10^3 PFU/mL, respectively. By comparison, these results are consistent with
233 results obtained by Sevilla-Navarro et al. [11], where the highest *S.* Enteritidis reduction was
234 reached after 2 consecutive bacteriophage applications. Furthermore, some authors applied
235 a single bacteriophage dose in their studies; however, after the trial they hypothesised that a
236 second application could produce better results [34]. In contrast, Fiorentin et al. [35] had
237 significant reductions with the use of a single dose of bacteriophage in animals than with
238 repeated bacteriophage administration, arguing that continuous administration of
239 bacteriophage may lead to resistant *Salmonella.*
240 No statistically significant differences were found between bacteriophage concentration used
241 and *Salmonella* reduction in our study. Different hypothesis could explain this results. Wernicki
242 et al. [10] explained bacteriophages could reach a maxium antimicrobial activity and Carvalho
243 et al. [36] showed that increasing the titer of the bacteriophage used could increase the
244 bacteria resistance. Conversely, the bacterium can adopt resistances after bacteriophage
245 treatment over time [37,38]. However it is not cernaily know the time or the bacteriophage
246 dose that could induce this change [39,40].
247 For this reason, strategies to address the problem of resistance, could be the use of cocktails
248 of bacteriopahges, changes in the bacteriophage composition and, therefore, personalizing
249 the phage therapy. The different bacteriophages present in the cocktail would target different

250 receptors on the bacterial surface, resulting in a lower statistical chance of bacterial co-
251 resistance [18].
252 With respect to *S.* Enteritidis and *S.* Infantis decrease throughout the week, our results were
253 consistent with those published by Shao and Wang [41], which reported significant differences
254 in the decrease in *Salmonella* spp. as the week progressed, the 5th day showing the highest
255 reduction in *Salmonella* counts.
256 Due to antimicrobial and disinfectant resistance, *Salmonella* spp. have become a worldwide
257 concern [31]. Some authors have described the use of additional tools to improve the cleaning
258 and disinfection results and reduce the persistence of pathogens on farm facilities [42]. There
259 are some products for the application of bacteriophages as disinfectants in food industry
260 facilities, however, non literature describe the use of bacteriophages at field level. For this
261 reason, further studies are needed to study the effect of bacteriophages on diverse floor
262 surfaces.
263 The development of bacteriophage therapy as non-toxic to humans, environmentally friendly
264 and cost-effective, holds good prospects for the future as a useful measure of cleaning and
265 disinfection in livestock facilities [20].
266 Nevertheless, due to bacteriophage therapy specificity to the host bacteria, bacteriophage
267 strategies should not be used alone, but in combination with cleaning and disinfection [33].
268 This way, it could be possible to reduce the infective pressure (3 and 4 logarithms after 2
269 bacteriophage applications) before applying the detergents and disinfectants, achieving an
270 optimal result of the cleaning and disinfection process. Nevertheless, it is important to highlight
271 that if the bacteria remains in the environment or enters again with a new flock into the farm,
272 it will be necessary to apply th bacteriophages in combination with an accurate cleaning and
273 disinfection. In this sense, we recommend to start the procedure of cleaning and disinfection
274 with the removal of any remain of dust and faeces through a dry cleaning followed by a wet
275 cleaning with detergent. Subsequently, on dried facilities, a two bacteriophages applications
276 will be performed in 24h intervals. Finally, a double disinfection will be applied, firstly by contact
277 and then by nebulization.

These promising results showed a new, safe and effective measure to minimise the persistence of pathogens in farm facilities; however, further studies are needed to prove the efficacy of bacteriophage in combination with commercial cleaning and disinfection protocols at field level.

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Table 1. SI log $_{10}$ CFU/mL reduction according to BP concentrations and time of application.

	BP Concentrations (PFU/mL)								
	10^{12}			10^{8}			10^{3}		
	Log_{10} CFU/mL counts	Log_{10} CFU/mL R	SE	Log_{10} CFU/mL counts	Log_{10} CFU/mL R	SE	Log_{10} CFU/mL counts	Log_{10} CFU/mL R	SE
Applications									
C	8.00	0.00[a]	0.11	8.00	0.00[a]	0.16	8.00	0.00[a]	0.21
1	5.02	2.98[b]	0.18	5.10	2.90[b]	0.26	4.93	3.07[b]	0.35
2	4.12	3.88[c]	0.20	3.45	4.55[c]	0.28	4.31	3.69[b]	0.37
3	3.93	4.07[c]	0.22	3.24	4.76[c]	0.31	4.03	3.97[b]	0.41

C: Control group (concentration control group remained constant along the study); [a, b, c] Means with different superscripts in a column are statistically different (p<0.05); SE: Standard error; R: reduction.

Table 2. SE log $_{10}$ CFU/mL reduction according to BP concentrations and time of application.

	BP Concentrations (PFU/mL)								
	10^{12}			10^{8}			10^{3}		
	Log_{10} CFU/mL counts	Log_{10} CFU/mL R	SE	Log_{10} CFU/mL counts	Log_{10} CFU/mL R	SE	Log_{10} CFU/mL counts	Log_{10} CFU/mL R	SE
Applications									
C	8.00	0.00^{a}	0.12	8.00	0.00^{a}	0.15	8.00	0.00^{a}	0.14
1	5.64	2.36^{b}	0.20	5.52	2.48^{b}	0.24	5.50	2.50^{b}	0.23
2	4.63	3.37^{c}	0.22	4.83	3.17^{bc}	0.26	4.15	3.85^{c}	0.25
3	4.53	3.47^{c}	0.24	4.61	3.39^{c}	0.29	4.91	3.09^{b}	0.27

C: Control group (concentration control group remained constant along the study); $^{a, b, c}$ Means with different superscripts in a column are statistically different (P<0.05); R: reduction; SE: standard error.