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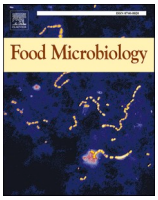

Full-scale industrial phage trial targeting *Salmonella* on pork carcasses

Marta Volpi [a], Michela Gambino [b], Kirsten Kirkeby [c], Anne Elsser-Gravesen [a,*], Lone Brøndsted [b]

[a] ISI Food Protection ApS, Agro Food Park 13, 8200 Aarhus N, Denmark
[b] Department of Veterinary and Animal Sciences, University of Copenhagen, Stigbøjlen 4, 1870 Frederiksberg C, Denmark
[c] Danish Crown, Danish Crown vej 1, 8940 Randers SV, Denmark

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ABSTRACT

Phages have been suggested as promising biocontrol agents in food, but trials demonstrating the efficiency of phage treatment under industrial settings are missing. Here we performed a full-scale industrial trial to evaluate the efficacy of a commercial phage product to reduce the prevalence of naturally occurring *Salmonella* on pork carcasses. A total of 134 carcasses from potentially *Salmonella* positive finisher herds were chosen to be tested at the slaughterhouse based on the level of antibodies in the blood. During five consecutive runs, carcasses were directed into a cabin spraying phages, resulting in a dosage of approximately 2×10^7 phages per cm^2 carcass surface. To evaluate the presence of *Salmonella*, a predefined area of one half of the carcass was swabbed before phage application and the other half 15 min after. A total of 268 samples were analysed by Real-Time PCR. Under these optimized test conditions, 14 carcasses were found positive before phage application, while only 3 carcasses were positive after. This work shows that phage application allows to achieve approximatively 79% reduction of *Salmonella*-positive carcasses and demonstrates that implementation of phage application in industrial settings can be used as an additional strategy to control foodborne pathogens.

1. Introduction

Salmonella is a major cause of foodborne gastroenteritis worldwide, with an estimation of 1.35 million illnesses, 26,500 hospitalizations, and 420 deaths in the United States every year (Centers for Disease Control and Prevention, 2022). In European Union (EU), salmonellosis is the second most reported human zoonoses in 2020 with 52,702 confirmed cases, of which 29.8% are hospitalized and most are caused by foodborne outbreaks (EFSA and ECDC, 2021b). Pork meat and derived products are the second foodborne vehicle of salmonellosis after eggs and derived products (EFSA and ECDC, 2021a), thus highlighting the importance of addressing *Salmonella* contamination in pig farming and pork meat. Derby, Typhimurium monophasic and Typhimurium are the top 3 *Salmonella* serovars involved in human salmonellosis cases in the European Union from pigs (EFSA and ECDC, 2021b).

In pigs, *Salmonella* is primarily located in the gastro-intestinal tract from the oral cavity to the rectum, thus constituting a source of contamination from pig to pig during transport and lairage (Berends et al., 1996; Isaacson et al., 1999). In addition, *Salmonella* from positive pigs may contaminate pork carcasses within the slaughterhouse during the slaughter processes (Hald et al., 2003). Official European surveillance programs show that the proportion of *Salmonella*-positive samples from pork carcasses at slaughterhouses is on average 3.6%, with high variations between countries and abattoirs (EFSA and ECDC, 2021b). According to regulation EU No 2073/2005 on microbiological criteria for foodstuffs, *Salmonella* positive pork carcasses at slaughterhouses are set to max. 6% (European Commission, 2005), but there is so far no agreed strategy in EU on how to achieve it. Furthermore, only few EU member states have implemented monitoring and surveillance programs aiming at reducing *Salmonella* prevalence in pigs and pork meat. Such pre-harvest surveillance programs may aim at *Salmonella* eradication if the prevalence is close to negligible as seen in Sweden, Norway, and Finland (Bonardi et al., 2021). However, for most countries in the EU, with non-negligible *Salmonella* prevalence (including Denmark), it is more realistic to aim for a reduction strategy with focus on ensuring high standards of hygiene at the slaughterhouse (Alban et al., 2012).

According to Regulation (EC) No. 853/2004, use of any substance other than potable water is not permitted for post-harvest reduction of *Salmonella* and other organisms on the surface of pork carcasses in EU (European Commission, 2004). However, Regulation (EC) No. 101/2013 allows the use of lactic acid for reducing microbiological surface contamination on bovine carcasses (European Commission,

* Corresponding author.
E-mail address: aeg@isifoodprotection.com (A. Elsser-Gravesen).

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2013). Thus, there is room for development and application of new decontaminating solutions at pork slaughter, with the notion that such solutions should not replace high standards of hygiene at the slaughterhouse and implementation of fully integrated control programmes throughout the entire food chain (CEP EFSA, 2018). In Danish slaughterhouses, bacterial contamination on pork carcasses is reduced by scraping during dehairing, flaming, polishing, and good hygiene practice in the clean slaughter area. Furthermore, the chilling tunnel and overnight cold storage may reduce *Salmonella* further, as it has been observed for *E. coli*, a marker for bacterial contamination from the digestive tract (Larsen and Christensen, 2010). Still, other solutions contributing to a reduction or even an eradication strategy are not currently available.

In the last decades, Western countries have expressed a growing interest in the use of bacteriophages as an alternative to chemical treatments or antibiotics (Broncano-Lavado et al., 2021; Callaway et al., 2011; Cristobal-Cueto et al., 2021). Bacteriophages (phages) are viruses that only infect bacteria for their propagation, a process leading to death of the infected bacteria. Phages are extremely versatile, they could be used both as food and feed additives, and they can be applied as pre-harvest or post-harvest interventions (Lewis and Hill, 2020). Recent reviews summarize the successful use of phages to control zoonotic pathogens in food-producing animals and food (Gigante and Atterbury, 2019; Lewis and Hill, 2020; Moye et al., 2018; Ushanov et al., 2020, 2020b; Żbikowska et al., 2020). Yet, the use of phages for biocontrol strategies at a commercial scale in the food industry still faces challenges, including lack of knowledge of implementing phage solutions in industrial settings (Gambino and Brøndsted, 2021; García-Anaya et al., 2020).

The use of phages for reducing *Salmonella* in pork meat production represents a sustainable alternative to antibiotics or decontaminating agents, as they are specific and only kill the target bacteria (Ojha and Kostrzynska, 2007). Pre-harvest addition of dried phages to feed has been shown to be an effective measure to reduce *Salmonella* shedding in pig faeces (Seo et al., 2018) and colonization of the pig gut (Thanki et al., 2022b). In addition, being natural, nontoxic, and relatively inexpensive, phages have the potential to be used in the clean zone at pig slaughterhouses. According to literature, a *Salmonella* reduction up to and above 2 log/cm^2 can be achieved following application of bacteriophage cocktails on pork skin models in laboratory settings (Hong et al., 2016; Hooton et al., 2011; Spricigo et al., 2013; Wang et al., 2017). Yet, phages targeting *Salmonella* have not been tested on pork carcasses in a slaughterhouse.

Here we aimed to evaluate the efficacy of a commercial phage product in reducing the prevalence of naturally occurring *Salmonella* on pork carcasses in a full-scale industrial trial, providing the first documentation of implementation of phage application at a slaughterhouse. We report the process for selecting the phage product for the industrial trial, the validation procedure performed to detect *Salmonella* from swabs of phage-treated carcasses, the pre-test to optimize phage application by spraying with a spray cabin at the production line, and the results of the actual industrial trial. Finally, we discuss our findings and detailed considerations relevant for industrial implementation of phage applications.

2. Materials and methods

2.1. Phage products

Three commercial phage products were used in this study: product A (6 phages), product B (2 phages), and product C (2 phages). For the industrial trial, product B (total concentration of 2×10^{11} plaque forming units (pfu)/ml) was used in a 2% solution in drinking water by adding 2 L of phage product to a tank containing 100 L in total. Based on pre-tests, it was estimated that 5 μl phage solution was sprayed per cm^2 surface of the carcass, corresponding to a dosage of 2×10^7 pfu per cm^2.

2.2. Host range analysis by plaque assay

Wild type strains of the prevalent *Salmonella* serotypes isolated from pork meat between 2011 and 2015 have been used for host range analysis (Table 1). Ten-fold serial dilutions of the cocktails in SM buffer (0.1 M NaCl, 8 mM $MgSO_4$*$7H_2O$, 50 mM Tris-HCl, pH 7.5) were prepared and three drops of 10 μl each were spotted on bacterial lawns as in (Gencay et al., 2019). Briefly, 100 μl of overnight cultures of each strain grown in LB (Lysogeny Broth, Merck) at 37 °C were mixed with 3 ml of molten overlay agar (LB with 0.6% agar bacteriological no.1, Oxoid), spread on a LA (LB with 1.2% agar) plate, dried and spotted. Following incubation, plaques were counted and plaque forming units per ml (pfu/ml) were calculated for each strain. Plaque assays were performed in at least two independent replicates.

2.3. Warm pork skin model for testing phage products against Salmonella

A warm pork skin model was designed and used for identifying the phage product with the best killing efficacy and determine if the killing efficacy was dose dependent. The phage products were tested at two different concentrations (recommended by the suppliers), against multiple *Salmonella* strains (10 strains: EGS1, EGS90, EGS284, EGS28, EGS38, EGS24, EGS48, EGS15, EGS20 and EGS165), grown overnight in LB (Lysogeny Broth, Merck) at 37 °C. Pork skin squares of 3 × 3 cm^2 were first incubated at 37 °C to reach a target temperature of 30 °C on the skin surface, then inoculated with 9 μl of the strain to reach a concentration of 10^4 cfu/cm^2 on the pork skin, and spread evenly. The inoculated skin squares were incubated for 15 min at 30 °C to allow bacterial attachment. Skin squares were treated with 90 μl of phage product, diluted to reach a target concentration of 1×10^7 pfu/cm^2 (multiplicity of infection, MOI, 1000) or 5×10^6 pfu/cm^2 (MOI 100) on the pork skin (each phage product and each dilution was tested in triplicate) and distributed evenly with a Drigalski spatula. Additional skin squares were treated with SM buffer and used as untreated controls. Treated and untreated skin squares were incubated at 30 °C for 5 min to simulate application of phages on warm carcasses, then packed tightly each into one stomacher pouch, protected from drying out, and retrieved after 24 h at 5 °C for enumeration of *Salmonella* using SCA (*Salmonella* Chromogenic agar, Oxoid CM1092, with *Salmonella* selective supplement, Oxoid SR0194), after dilution in 100 ml SPO (8.5 g/l of sodium chloride, 1.0 g/l of peptone, 1 L of demineralized water). Results from triplicates were averaged and log reduction was calculated as the log difference from the untreated control. The same model was tested on additional *Salmonella* strains. A total of three sensitive strains (EGS84, EGS90 and EGS307) were selected to test that 1 log reduction per phage product and phage dilution is achievable on warm pork skin contaminated by a pool of different *Salmonella* strains.

2.4. Industrial trial design

The overall prevalence of *Salmonella* in pork carcasses from Danish slaughterhouses is low, maximum 1%. Therefore, in order to enhance the probability of recovery of *Salmonella*, three main precautions were followed: 1) selection of flocks based on the finisher herd index, which is determined from *Salmonella* antibodies in meat juice samples as part of the Danish surveillance for *Salmonella* in pig production (Danish Agriculture and Food Council, 2021), 2) a large swabbing area on the carcass (as described below), and 3) swabbing before the completion of the slaughter process, while standard surveillance is done on the final product on the day after slaughter and overnight cold storage (Fig. 1).

Industrial testing with the phage product B was performed over a period of two months (October–December 2018), during which five test rounds were conducted. The number of carcasses per test round were 29, 32, 24, 16 and 33, respectively, giving a total of 134 carcasses. Carcasses were taken out from the flow after the weighing and before the chilling tunnel (Fig. 1), marked individually with a number striped to the foreleg

Table 1
Host range analysis of the three commercial phage products A, B and C. *Salmonella* strains are grouped according to their serotypes and infectivity is expressed as plaque forming units per ml (pfu/ml). Dark green: Titres in the range of 10^8-10^{11} pfu/ml; light green: titres in the range of 10^5-10^8 pfu/ml.

Serotype	Lab ID	Product A [pfu/ml]	Product B [pfu/ml]	Product C [pfu/ml]
Derby	EGS1	1.0E+08	5.0E+10	0.0E+00
	EGS33	1.1E+07	1.1E+10	0.0E+00
	EGS84	2.2E+08	2.3E+10	1.7E+08
	EGS90	6.0E+07	4.0E+10	0.0E+00
	EGS141	2.1E+07	4.8E+10	2.0E+08
	EGS142	1.5E+06	1.9E+10	0.0E+00
	EGS146	4.7E+06	2.3E+10	3.3E+08
	EGS173	2.2E+07	2.2E+10	0.0E+00
	EGS200	3.8E+07	5.6E+10	5.0E+07
	EGS225	3.0E+07	0.0E+00	0.0E+00
	EGS252	4.2E+06	4.5E+10	3.3E+09
	EGS256	3.0E+07	2.7E+10	0.0E+00
	EGS284	1.2E+08	2.1E+08	0.0E+00
	EGS310	3.2E+07	3.1E+10	0.0E+00
Typhimurium	EGS28	2.0E+08	2.3E+10	4.2E+09
	EGS38	8.0E+07	3.5E+10	0.0E+00
	EGS42	2.3E+08	5.6E+10	0.0E+00
	EGS43	1.0E+07	2.1E+10	0.0E+00
	EGS121	1.0E+08	2.2E+10	0.0E+00
	EGS136	4.0E+07	5.8E+10	0.0E+00
	EGS191	1.1E+08	3.4E+10	0.0E+00
	EGS304	4.0E+06	2.7E+09	5.0E+09
Dublin	EGS24	5.6E+06	4.6E+10	0.0E+00
	EGS40	2.1E+07	4.0E+10	0.0E+00
	EGS244	1.8E+07	4.2E+10	0.0E+00
	EGS306	1.9E+07	7.5E+10	0.0E+00
Enteritidis	EGS45	1.1E+05	0.0E+00	0.0E+00
	EGS47	1.4E+09	7.0E+09	2.0E+08
	EGS48	2.2E+05	2.2E+08	5.0E+07
Infantis	EGS15	4.0E+07	7.5E+10	0.0E+00
	EGS160	2.1E+07	8.2E+10	0.0E+00
	EGS208	4.9E+07	7.4E+10	5.0E+07
4.12:i:-	EGS20	1.0E+08	2.3E+09	2.0E+08
	EGS135	3.7E+07	5.4E+10	0.0E+00
	EGS162	3.0E+07	4.0E+09	2.8E+09
	EGS206	2.8E+07	2.7E+10	0.0E+00
	EGS235	1.0E+06	2.0E+08	1.0E+08
4.5.12:i:-	EGS165	2.3E+06	1.0E+10	0.0E+00
	EGS156	1.0E+07	1.0E+09	0.0E+00
	EGS167	1.2E+07	2.0E+10	0.0E+00
	EGS169	3.0E+07	2.8E+10	0.0E+00
	EGS193	2.0E+07	4.1E+10	0.0E+00
	EGS287	1.1E+08	5.0E+10	0.0E+00
	EGS307	4.8E+07	2.7E+10	0.0E+00
4.[5].12:i:-	EGS248	1.1E+08	2.9E+10	0.0E+00
Goettingen	EGS30	3.0E+07	0.0E+00	0.0E+00
Livingstone	EGS172	4.0E+07	5.7E+10	0.0E+00
London	EGS183	4.0E+06	0.0E+00	6.0E+07
Rissen	EGS203	2.0E+05	0.0E+00	1.0E+07
Brandenburg	EGS300	2.0E+05	3.2E+10	0.0E+00
Bradford	EGS319	9.0E+07	2.0E+11	0.0E+00

before entering a specific test loop including the spray cabin. The test was performed as described below, and subsequently the carcasses were reintroduced into the normal production line entering the chilling tunnel. Next day, carcasses were washed off with drinking water, according to the agreements with the Danish Authority (as described under the paragraph "Approval" below).

2.5. Spray equipment

Spray equipment HCSS-1000 ACID SPRAY CABINET, FIELD WELDING AND ASSY (CHAD EQUIPMENT LLC) was set up off-line, allowing one carcass to be sprayed at a time using 2.5 bar for approximately 20 s.

2.6. Sampling during the industrial trial

Samples were collected from one half of each carcass before directing it into the spray cabin (untreated control sample) and from the other half 15 min after phage treatment, alternating between left and right on consecutive carcasses. The samples were taken by swab with a gauze cloth moistened in BPW (Buffered Peptone Water, Oxoid CM0509). The swab procedure was performed covering the inside and the perimeter of the pelvis of about 5 cm on the rind side, the sternum incl. 5 cm of the rind, the inside of the jaw, the rind side of the jaw, and the outside of the foreleg. The swabbed area is shown in Fig. 2; the total area was approx. 2800 cm^2. For comparison, the area swabbed as part of the normal surveillance program (Fig. 1) amounts to 400 cm^2. Analyses of the samples taken for the full-scale industrial trial for presence or absence of *Salmonella* were performed by the Danish Crown lab in Horsens (Denmark). Swabs collected during the day were packed inside plastic pouches and kept at a temperature lower than 6 °C until the next day, when the enrichment was performed.

2.7. Detection of Salmonella from swabs

Detection of *Salmonella* was performed using Real-Time PCR (Bio-Rad, iQ Check® *Salmonella* II), as this method was accredited by ISO 17025 at the participating laboratories. Briefly, DNA is extracted after enrichment in BPW, and a Real-Time PCR is performed for detection of *Salmonella* spp. specific genes. Swabs from untreated reference samples are enriched in a total volume of 100 ml BPW. Swabs from phage treated samples were enriched in a total volume of 2000 ml BPW, to dilute phages below the level where they may prevent recovery of sensitive *Salmonella* cells. To compensate for the increased volume, incubation time for enrichment for treated samples was extended by 2 h. The duration of the extension was estimated using the software CombBase (2012) for predictive modelling, with a reduction by factor 20 in the cell count, and with temperature, pH and a_w chosen to reflect the incubation conditions in BPW buffer (37 °C, 7 and 0.993 respectively); the prediction showed a 2 h delay in the exponential growth phase. Analyses for validation of the analytical procedure was performed by ISI Food Protection as presented in the Results section. Following detection, isolation of *Salmonella* strains from positive samples and strain sensitivity testing was performed by ISI Food Protection (Denmark).

2.8. Approval

The Danish Veterinary and Food Administration approved the execution of the trial on 06.02.2018, journal number 2017-28-39-00548. The conditions for approval were as follows: 1) no more than 1000 carcasses would be included in the study; 2) the slaughterhouse's own-check must ensure that meat from the treated carcasses is marketed on the Danish market; 3) all processed carcasses are subsequently checked for fresh meat characteristics; 4) discoloured areas are cut off and discarded; 5) the carcasses involved in the experiment have been approved for human consumption by the local meat inspection unit, that means that the treatment only occurs after the carcasses have been

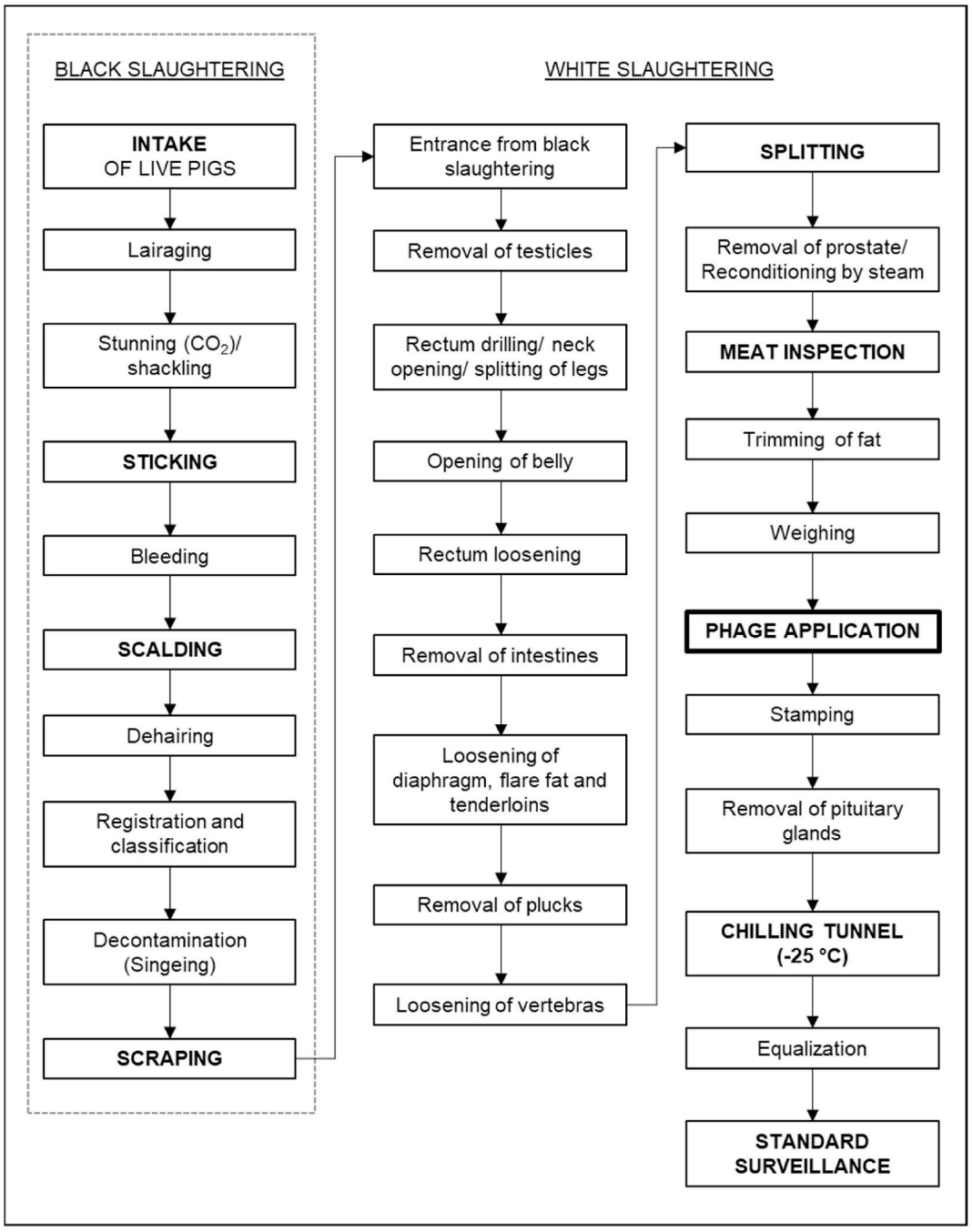

Fig. 1. Schematic flow diagram of the different stages of habitual processing lines in the slaughterhouse.

stamped with health marks; 6) the treated carcasses are washed off with drinking water, after cooling. Furthermore, the slaughterhouse chose to redirect all the carcasses used in the test to production of heat-treated products.

3. Results

3.1. Coverage of commercial phage products targeting Salmonella in vitro

Three commercial phage products designated A, B and C were kindly donated by phage companies. To determine the ability of the phage products to kill diverse *Salmonella*, we determined plaque formation against 51 different *Salmonella* strains isolated from Danish pigs and pork meat, representing the serotype diversity associated with pigs and pork meat in Denmark. While product C could infect only 15 strains (29%), product A infected 51 strains (100%) and product B 46 strains (90%). Despite the slightly broader host range of product A, product B infected the strains with a high titer, ranging from 2×10^8 to 2×10^{11} pfu/ml, compared to the product A (from 1.1×10^5 to 2×10^8 pfu/ml) (Table 1). This demonstrates that product A and B kill a broad range of *Salmonella* strains likely present on pork carcasses.

3.2. Killing efficacy of commercial phage products determined in the warm pork skin model

To identify the killing efficiency and dose response among the products showing the broadest coverage (A and B), we used a warm pork skin model. The two products were used to determine killing efficiency

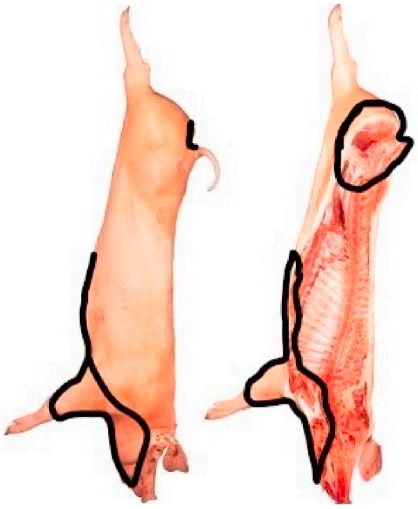

Fig. 2. Depiction of the area sampled by swabbing.

of 10 *Salmonella* strains identified as sensitive to the phage products. We demonstrated that both products reduce *Salmonella* on pork skin, and that killing is dose dependent (Fig. 3). On average, regardless the dose, product A showed higher efficacy in reducing *Salmonella* on pork skin (1.0 log reduction) than product B (0.6 log reduction) (Supplementary Table S1). However, strains that are less sensitive (returning less than 1 log reduction, strains EGS24, EGS28, EGS20, EGS15) showed a less pronounced difference in killing efficacy of the two products (on average, 0.55 log reduction for product A and 0.51 log reduction for product B), indicating a more comparable ability to reduce *Salmonella*. Moreover, the efficacy of product A and B against a pool of 3 strains on pork skin showed comparable log reductions among test replicates. A reduction of 1 log was achieved for both phage products with the higher dosage (1×10^7 pfu/cm^2), and a reduction of 0.6 log was achieved for both phage products with the application at a lower dosage (5×10^6 pfu/cm^2) (data not shown). We concluded that a reduction of 1 log on the pork skin is achievable, and that the efficacy of the two products is overall comparable, and selected product B for a full-scale industrial trial.

3.3. Validation of procedure for detection of surviving Salmonella on phage-sprayed carcasses

Detection of *Salmonella* in samples containing phages is a challenge, as phages may kill *Salmonella* during the enrichment needed for detection by Real-Time PCR. We initially attempted to remove phages by sterile filtration and subsequently detect *Salmonella* following enrichment of the filter in BPW for 21 h. Laboratory testing using a swab containing 2×10^9 pfu of phages and spiked with 1000 cfu of *Salmonella* Typhmurium LT2 confirmed that *Salmonella* could indeed be detected following filtration, but not in the unfiltered samples (data not shown). However, subsequent test of swabs of pork carcasses showed that the high content of blood, fat and other tissue made filtration impossible. Therefore, an alternative strategy was used, increasing the volume of the enrichment step which decreases the chances of the phages to collide with and thus infect and kill *Salmonella*. To validate this strategy, swabs of untreated carcasses (phages were added directly to the swab) were spiked with intermediate levels of *Salmonella* (800 cfu pr. swab) and enriched in 1000 or 2000 ml BPW instead of the 100 ml BPW in the original procedure, in triplicates. As control, enrichment in 100 ml BPW was performed. In this setup, *Salmonella* could be detected from all the three replicates enriched in 2000 ml, and from 2 out of 3 replicates enriched in 1000 ml (Table 2a). *Salmonella* was also detected in the 100 ml enrichment, however with a high cycle threshold (ct = 45, positive ct values are usually ct $\leq$ 40, as described under the validation procedure in the iQ-Check *Salmonella* spp. II kit User Guide) (Table 2a). Consequently, recovery of *Salmonella* may be challenging in samples with high phage titres using 100 ml enrichments. Therefore, we repeated the analysis using 14 swabs from carcasses sprayed with phages in the factory and spiked with three concentrations of *Salmonella* (high, medium, or low) and enriched in 2000 ml BPW. As control, enrichment in 100 ml BPW was performed on two swabs spiked respectively with the highest and the lowest concentrations of *Salmonella*. The results show that *Salmonella* can be detected in most of the swabs spiked with high and medium level of *Salmonella* and enriched in 2000 ml, but recovery of the bacteria becomes challenging when the level of *Salmonella* is low. Enrichment in 100 ml did not allowed detection of *Salmonella* in any of the two swabs (Table 2b). Based on these results, enrichment in 2000 ml was chosen as procedure for the industrial trial.

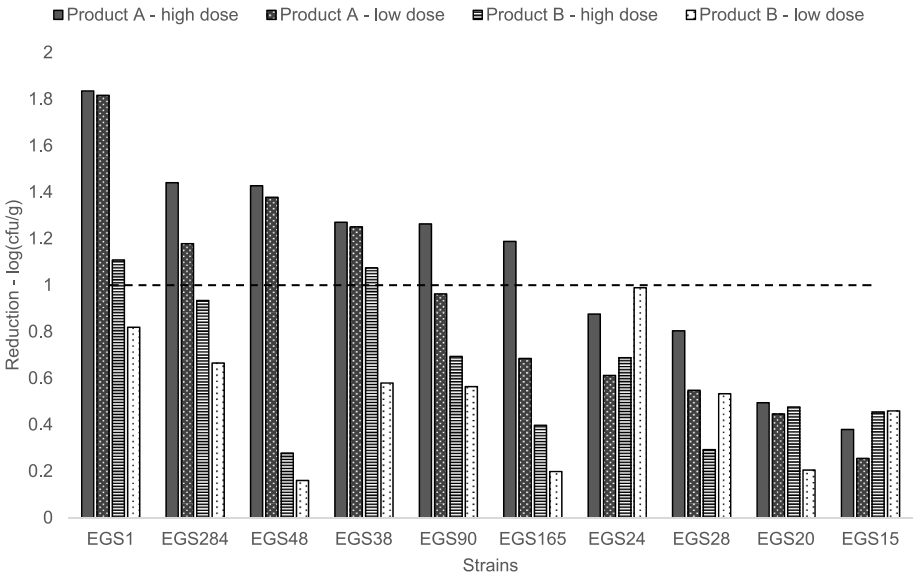

Fig. 3. Dose response of 10 *Salmonella* strains to commercial phage products A and B on a pork skin model. The dashed line indicates the 1 log reduction target. High dose corresponds to 1×10^7 pfu/ml, low dose to 5×10^6 pfu/ml. Log reductions are calculated from the untreated controls (Supplementary Material, Table S1).

Table 2
Validation of procedure for detection of *Salmonella* on carcasses. A) Detection of *Salmonella* on spiked swabs following artificial addition of phages on the swabs (2×10^7 pfu/swab); *, positive qPCR results but high ct, ct = 45. B) Detection of *Salmonella* on spiked swabs from carcasses sprayed with phages. No. of pos/tot, number of positive on total samples.

a)

Swab from test round no.	***Salmonella* inoculation level (cfu/swab)**	**Enrichment volume (ml)**	**Theoretical phages concentration in the enrichment (pfu/ml)**	**No. of pos/tot**
2	800	2000	1.0 E+04	3/3
2	800	1000	2.0 E+04	2/3
2	800	100	2.0 E+05	1/1*

b)

Swab from test round no.	***Salmonella* inoculation level (cfu/swab)**	**Enrichment volume (ml)**	**Phages concentration in the enrichment based on titration (pfu/ml)**	**No. of pos/tot**
4	1200	2000	1.5 E+05	2/3
5	335	2000	1.4 E+06	2/3
4	120	2000	1.5 E+05	1/3
4	1200	100	1.0 E+07	0/1
4	120	100	1.0 E+07	0/1

3.4. Pre-test of phage application by spraying

Before the full-scale industrial trial, we evaluated the distribution of the phage product on the carcass as well as the stability of the phages during spraying at pressure settings of 2.0, 2.5 and 3.0 bar. To determine the stability of the phages, liquid samples taken from the spray tank and from the nozzles at the top and bottom of the spray cabin were analysed for the content of phages using a plaque assay (Table 3). The results show that all samples have comparable level of phages, thus demonstrating that the phages were not inactivated by the pressure and shear in the spray system. To determine the distribution of phages on the carcass at each pressure setting, we collected duplicate samples from 6 representative positions on the carcass by swabbing an area of 10×10 cm^2. The number of recovered phages per swab was enumerated by titration and the results showed an acceptable distribution of the phages on the carcass (Fig. 4). A visual assessment revealed that the carcasses exposed to spraying at 2 bars remained somewhat dry, while spraying at 3 bars resulted in a considerable run-off of excess liquid. The setting of 2.5 bar was therefore selected for the full-scale industrial trial. After cooling through the chilling tunnel, the treated carcasses were checked for fresh meat characteristics and discolouration; no visual effects were observed on any of the carcasses from pre-test or from the five industrial test rounds.

3.4.1. Industrial trial

For the full-scale industrial trial, 134 carcasses were selected according to the level of *Salmonella* antibodies in the blood as determined during the ongoing surveillance program. In five consecutive runs, the carcasses were directed into a cabin spraying phage product B for 20 s. Untreated control samples were collected from the carcass before the spray cabin. Results are summarized in Table 4. Despite the low prevalence of *Salmonella* in pork carcasses from Danish slaughterhouses (<1%), about 10.5% of the carcasses selected for the trial were *Salmonella*-positive, thus highlighting that the precautions utilized to enhance *Salmonella* recovery were successful. The number of positive samples for *Salmonella* was reduced from 14 before phage application to three after phage application, corresponding to a 78.6% reduction in prevalence. *Salmonella* was detected in all 14 untreated samples, whereas *Salmonella* could only be recovered from one out of the three phage treated *Salmonella*-positive samples, namely from the positive treated sample in test round 3.

Table 3
Stability of phages during spraying.

Bar	Tank (pfu/ml)	Bottom of spray cabin (pfu/ml)	Top of spray cabin (pfu/ml)
2	1.5 E+08	1.3 E+07	5.5 E+07
2.5	1.6 E+08	1.5 E+08	1.3 E+08
3	1.5 E+08	1.2 E+08	7.0 E+07

To understand if *Salmonella* present on the carcasses were sensitive to the phage product, we performed titration of the phage product B on the isolated strains (Table 4). The phage product led to formation of single plaques on strains isolated from test round 1, 2, and 5, whereas no single plaques could be detected on *Salmonella* isolates retrieved from test round 3. These data confirm the efficacy of the used phage product, and highlighting a difference in phage sensitivity of the strains naturally present on the carcasses (Table 4).

4. Discussion

In this study, we describe the procedure for advancing phage application targeting *Salmonella* on pork carcasses from the laboratory through simplified model systems to testing in full scale at a slaughterhouse. Testing application of phages under industrial settings is key for understanding the challenges in the development of sustainable alternatives for food safety and security. Moreover, by documenting the results of this case study, we gather evidence for the authorities on the efficacy and safety of phage-based approaches. In a scenario of low *Salmonella* prevalence, resulting from the high focus Denmark put into ensuring hygiene at the slaughterhouse, we were able to detect 14 positive carcasses out of 134 selected carcasses and observed 3 positive carcasses following phage application. Despite data uncertainty, we demonstrated a reduction in the range of 79% of *Salmonella* prevalence following phage application on pork carcasses with a low prevalence of *Salmonella*. In the following, we share our learnings and technical challenges, and highlight what industrial producers should consider for utilizing phages along the production line.

Currently, the number of phage products available on the market is quickly expanding. With more than 2600 serotypes, *Salmonella* is a highly diverse pathogen (Gal-Mor et al., 2014) and serotype prevalence differs between foods. For example, in EU, *S.* Enteritidis is primarily associated with broiler, layers and eggs, while the monophasic *S.* Typhimurium (1,4, [5],12:i:) is related both to pig and to broiler sources (EFSA and ECDC, 2021b). A good understanding of the bacterial strains to target is particularly relevant when applying phages, as serotype diversity reflects the surface diversity of the bacteria (Liu et al., 2014) and phages must recognize and bind receptors on the bacterial surface to start their infection cycle. This and the further steps of the phage infection cycle make phages very specific for their bacterial host (Ross et al., 2016). Previous studies reported both about *Salmonella* phages able to infect across serotypes, and others that are instead extremely specific for one serotype (for example, Gencay et al., 2019; Moreno Switt et al., 2013; Thanki et al., 2022). To choose the phage product to be used for our industrial trial, we selected three commercially available *Salmonella* targeting phage products, and tested their ability to kill diverse *Salmonella* strains reflecting the diversity of pork-associated *Salmonella* serotypes in Denmark. We observed that one product out of three had a narrower host range among the *Salmonella* strains used, while two other products performed similarly, with only a little gap between them in terms of efficiency. In addition, the efficacy of the chosen phage product was confirmed after the trial by the observed sensitivity of the *Salmonella* isolates from phage-treated and un-treated positive carcasses. Our results confirmed that the phage product was effective on most naturally occurring *Salmonella* strains, and underlines differences in phage sensitivity among naturally occurring *Salmonella* strains.

When applying phages in food, the product matrix may affect the efficacy of the phages (Lewis and Hill, 2020). Here we used a pork-skin

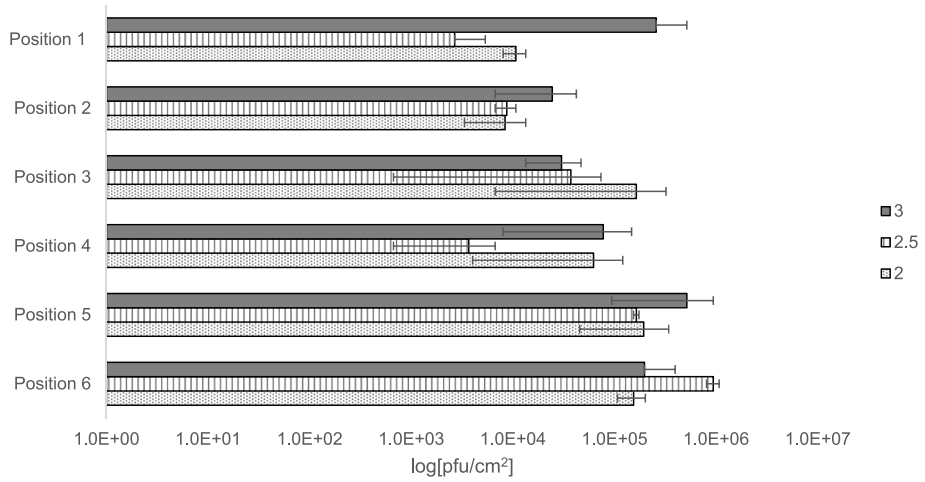

Fig. 4. Distribution of phages after spraying on carcasses, according to spraying pressure (2, 2.5 or 3). Position 1 to 6 indicates the swabbed areas, from the upper part to the lower part of the hanging carcass (Position 1: side of tail, top part carcass; Position 2: side of the tail; Position 3: middle body, upper part carcass; Position 4: middle body, lower part carcass; Position 5: upper front leg, lower part carcass; Position 6: shoulder and neck).

Table 4
Industrial trial of a commercial phage product B against *Salmonella* on pork carcasses.

Test round[a]	Sample[b]	Total sample no.	No. *Salmonella* positive by RT-PCR	No. *Salmonella* strains retrieved by culturing	Strain ID	Strain sensitivity (pfu/ml)[c]
1	untreated control	29	4	4	3426	2.1 E+10
					3427	1.0 E+10
					3428	6.5 E+10
					3429	5.1 E+10
	treated samples	29	0	n.d.	n.d.	n.d.
2	untreated control	32	1	1	3430	3.8 E+10
	treated samples	32	1	0	n.d.	n.d.
3	untreated control	24	4	4	3431	Lysis zones
					3432	Lysis zones
					3433	Lysis zones
					3434	Lysis zones
	treated samples	24	1	1	3435	Lysis zones
4	untreated control	16	0	n.d.	n.d.	n.d.
	treated samples	16	0	n.d.	n.d.	n.d.
5	untreated control	33	5	5	3439	2.3 E+09
					3440	8.7 E+07
					3441	8.7 E+09
					3442	3.5 E+08
					3443	4.2 E+08
	treated samples	33	1	0	n.d.	n.d.
TOTAL	untreated control	**134**	**14**	**14**		
	treated samples	**134**	**3**	**1**		

[a] Test round dates: 1 (30-10-2018), 2 (21-11-2018), 3 (28-11-2018), 4 (5-12-2018), 5 (11-12-2018).
[b] The samples were taken from one half carcass before the spray cabin (untreated control sample). The carcasses were subsequently run through the spray cabin, and the other half carcass of each pig was sampled 15 min after the treatment (treated sample).
[c] The *Salmonella* strains isolated from pork carcasses before and after phage treatment were tested for resistance to the phage product. N.d = not determined, because *Salmonella* strains were not detected or not retrieved. Lysis zones: Only found in high concentration of phages, but no single plaques were observed at any concentration of phages.

model to simulate industrial settings in terms of food matrix, temperature, and application time, and demonstrated that 1 log reduction of *Salmonella* on the pork skin matrix is achievable. In comparison, larger reductions were seen in laboratory media (data not shown). Similar approaches have been used to test for example the efficacy of phages targeting *Salmonella* (Aguilera et al., 2022) or *Campylobacter* on chicken skin (Zampara et al., 2017) and *Listeria monocytogenes* on ready-to-eat foods (Perera et al., 2015) and sliced pork ham (Figueiredo and Almeida, 2017). Thus, the efficacy of phage products should be demonstrated in appropriate models for implementing phage applications in industrial settings.

For industrial applications, the phage concentration is important for a successful outcome. In general, the higher the concentration of phages, the more significant reduction of the targeted bacteria (Lewis and Hill, 2020). In addition, phage concentration must be at a level that ensures economic viability for the food industries (Hagens and Loessner, 2010). In this industrial trial, a concentration of 4×10^9 plaque forming units (pfu/ml) was reached by adding 2 L of phage product to a tank containing 100 L in total. A 2% application appears to be economically sustainable for industrial treatment of carcasses via spray application, but further investigations are required to determine the viability on a daily base.

In addition to phage concentration, phage killing efficacy also depends on the latent period, namely the time necessary for effectively reducing viable cells, from phage binding to cell lysis (Wang and Dykhuizen, 1996). The duration of phage application should be planned considering the target organism, the daily routine at the industrial plant and a feasible integration of the phage application in the production chain. In this trial, recovery of *Salmonella* after phage application via spray equipment was designed to take place 15 min following phage treatment, and before the carcass entered the chilling tunnel. However, leaving the phages on the carcasses during their stay in the chilling tunnel have the potential to further reduce the level of *Salmonella* contamination on the carcasses (Supplementary Material). Further optimization may consider testing phage application at different locations in the production line which would ensure longest possible latent times.

A positive outcome of phage application in the food industry may also depend on the method employed for phage application (Lewis and Hill, 2020). A technical challenge for industrial application of bacteriophages via spray equipment concerns its optimization. The United States Food and Drug Administration (US FDA, Washington, DC, USA) has approved phage use in food processing environments, applied via spray or dip. Yet, information on which spray parameters should be used for successful implementation as well as studies that provide guidance on parameters for a suitable industrial spray application system are lacking. Recently, Wessels et al. investigated the combined effect of pressure, strainer, and nozzle diameter on phage survival of a commercial cocktail of phages, showing that dilution of the phage product in unchlorinated water, as well as a pressure of 3 Bar, resulted in the highest phage concentration through the nozzle and strainer tested (Wessels et al., 2022). Here we focused on determining the distribution of phages on the carcass according to the spray pressure, using the inbuild nozzle and strainer of the spray cabinet. We observed that application of phages using pressure at 3 bar resulted in a run-off of excess liquid, therefore choose the pressure of 2.5 bar for the trial. However, further optimization may allow reduction of water and energy used, minimise phage product loss and thereby limit the cost and environmental impact of industrial running to meet industrial cost-benefit targets. Moreover, spraying of an increased numbers of carcasses will require to consider changes in the production line to suit swift and sustainable production.

In summary, we tested a commercial *Salmonella* phage cocktail with broad killing spectrum in a slaughterhouse and observed that phage application reduced *Salmonella*-positive carcasses by approximatively 79%. We conclude that phages can contribute to control foodborne pathogens in industrial settings.

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Credit author contribution

Conceptualization: LB; AEG; Methodology: LB; AEG; MG; KK; MV; Investigation; MV; MG; KK; Formal analysis: LB; AEG; MG; MV; Writing – original draft: LB; AEG; MG; MV; Funding acquisition: AEG; LB; Resources: AEG; KK; LB; Supervision: AEG; LB.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. The co-author Kirsten Kirkeby was employed at Danish Crown at the time of the industrial trial. Danish Crown had no influence on data analysis and interpretation of data. The companies providing the phage products were not involved in the design nor in the execution of the laboratory work or the industrial test.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2023.104240.

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