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Efficacy and safety of phage therapy against *Salmonella enterica* serovars Typhimurium and Enteritidis estimated by using a battery of *in vitro* tests and the *Galleria mellonella* animal model

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ABSTRACT

In light of spreading antibiotic resistance among pathogenic bacteria, the development of novel approaches to combat such microorganisms is crucial. *Salmonella enterica* is pathogenic to humans, however, it can also infect poultry, being a potential foodborne pathogen when poultry-derived food is contaminated by this bacterium. Phage therapy is one of the alternative ways to treat *Salmonella*-infected animals while the establishment of this method and its introduction to a general practice requires detailed studies on safety and efficacy. Here, we present the results of such studies with two previously isolated and characterized bacteriophages, vB_SenM2 and vB_Sen-TO17, and four strains of *S. enterica* belonging to two serovars, Typhimurium and Enteritidis. We demonstrated effective reduction of bacterial cell number and cell culture density when using each phage alone, and in combination (as a cocktail). These phages were also effective in reducing bacterial biofilm. The efficacy of this *in vitro* phage therapy was compared to the action of known antibiotics, as was the efficiency of appearance of bacteria resistant to both these types of antibacterial agents. Safety of the use of bacteriophages was demonstrated using the LAL chromogenic test and the chicken fibroblast viability assay. Finally, the efficacy of phage therapy was assessed with the *in vivo* model of *S. enterica*-infected *Galleria mellonella* larvae, showing a significant improvement in the survival of the animals. In conclusion, we demonstrated high efficacy and acceptable safety profiles of phage therapy against *S. enterica* strains using vB_SenM-2 and vB_Sen-TO17 phages (both alone and in a cocktail). These results open a possibility for a trial with the use of poultry and these phages which might potentially allow to introduce of this method for practical use in poultry farming.

1. Introduction

Consumption of poultry meat is one of the bases of nutrition of the human population, and it is constantly increasing during the last 50 years (Clavijo and Flórez, 2018). The use of growth-promoting antibiotics in aviculture was allowed until recently (for example, see US Food and Drug Administration (FDA) regulations which are available at: https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr= 558.3). Although stabilization of the poultry gut microbiome could be achieved by using subtherapeutic amounts of antibiotics, resulting in higher meat production (Diaz-Sanchez et al., 2015; Dibner and Richards, 2005; Lu et al., 2008) such a practice has been considered one of the major causes of the development of antibiotic resistance by many bacterial pathogens (Diarra et al., 2010; Mehdi et al., 2018; Schwaiger et al., 2012; Singer and Hofacre, 2006; Singh et al., 2010). Such problems have been recognized in European Union (EU) and the USA which either banned the use of antibiotics to promote the growth of animals (EU regulation No 470/2009) or limited such practices (Diaz-Sanchez et al., 2015), respectively. These decisions were due to the development of bacterial drug resistance (Liljebjelke et al., 2017; Mehdi et al., 2018;

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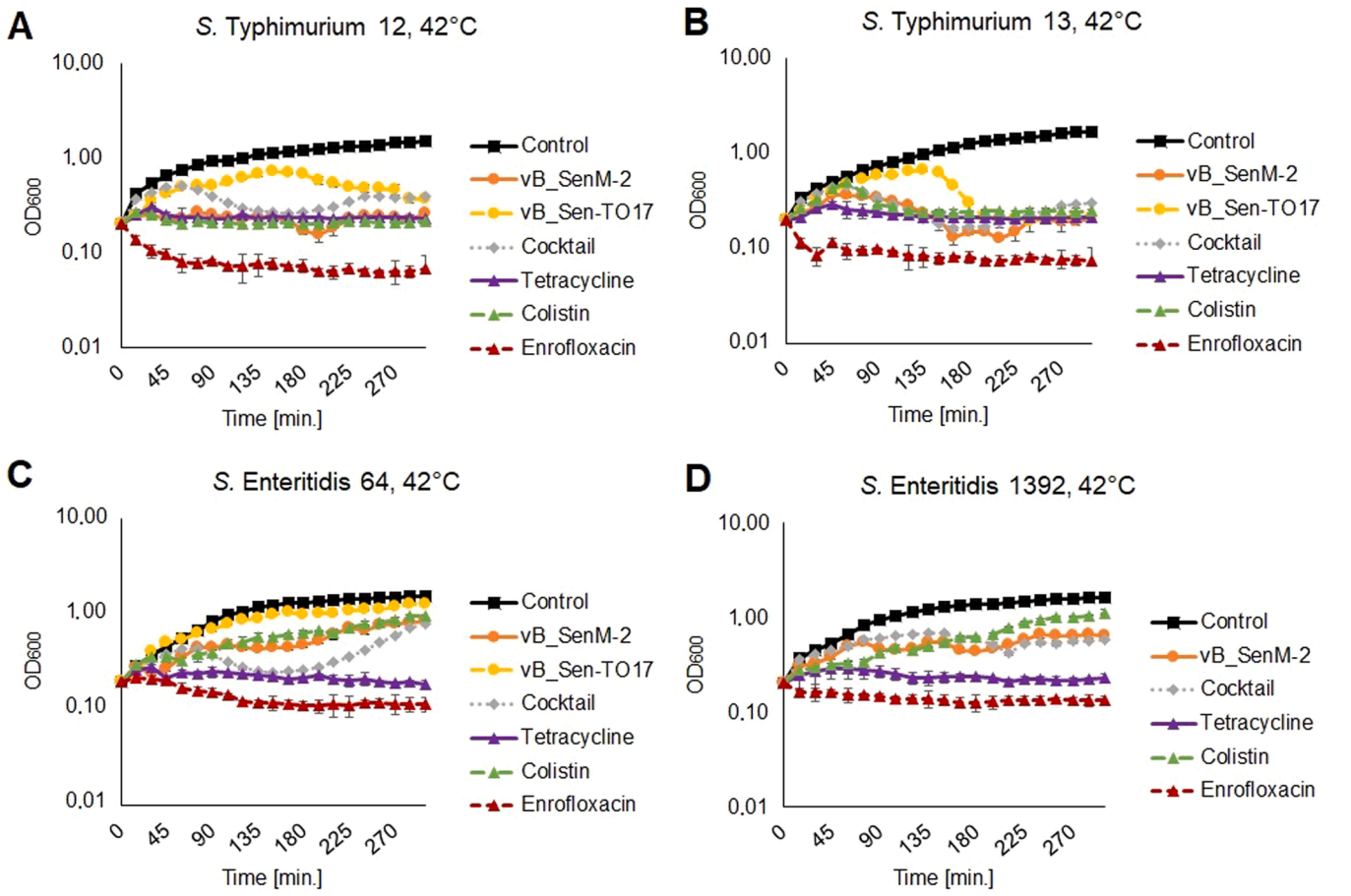

Fig. 1. Changes in optical density of bacterial culture of *S.* Typhimurium 12, (A), *S.* Typhimurium 13 (B), *S.* Enteritidis 64 (C), and *S.* Enteritidis 1392 (D) after infection with phages vB_SenM-2 (orange circles, straight line), vB_Sen-TO17 (yellow circles, dotted line) at 10^9 PFU/ml, with phage cocktail (grey diamonds) at 2×10^9 PFU/ml or after addition of tetracycline (12.5 μg/ml; purple triangles, straight line), colistin (50 μg/ml; green triangles, dotted line) or enrofloxacin (50 μg/ml; red triangles, dotted line) in comparison with control (black squares) at 42 °C. Mean values from three independent experiments are shown, with error bars representing SD. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Nhung et al., 2017), and negative effects of massively used antibiotics on the environment (Gonzalez Ronquillo and Angeles Hernandez, 2017) and human health (Chan, 1999; Kümmerer, 2009; Mehdi et al., 2018).

Among bacteria that can cause foodborne diseases in humans, *Salmonella* and *Campylobacter* are predominant genera, as they can occur in the chicken intestine (Oakley et al., 2014; Atterbury et al., 2007). *Salmonella enterica* is found in the chicken gut rather temporary (Liljebjelke et al., 2017; Oakley et al., 2014), however, human salmonellosis is predominantly caused by the consumption of contaminated chicken meat or eggs (Capparelli et al., 2010; Wegener, 2003). There are over 2500 known serovars of *S. enterica* (Gal-Mor et al., 2014), however, *S.* Typhimurium and *S.* Enteritidis are predominant ones causing disease in humans (Gal-Mor et al., 2014; Feasey et al., 2012; Tindall et al., 2005).

In the light of the problems discussed briefly above, it is necessary to develop alternative methods to prevent infectious diseases in avian farms as well as to protect poultry-derived food against bacterial contamination, where *S. enterica* is definitely one of the most important targets. Therefore, in our previous works, we have isolated and characterized a series of bacteriophages infecting different serovars of *S. enterica*, in order to develop effective and safe phage therapy procedures that might be used in poultry farms (Jurczak-Kurek et al., 2016; Kosznik-Kwaśnicka et al., 2020a, 2020b). Phages vB_SenM-2 and vB_Sen-TO17, infecting *S.* Typhimurium and *S.* Enteritidis, appeared especially effective in preliminary studies (Kosznik-Kwaśnicka et al., 2020a, 2020b). Particularly, these phages were found to be able to infect and lyse host bacteria at 42 °C (equal to normal body temperature of chicken), were stable under different storage conditions, and were shown to tolerate low pH indicating their suitability for oral phage therapy (Jurczak-Kurek et al., 2016; Kosznik-Kwaśnicka et al., 2020a, 2020b). Hence, in this work, we aimed to test their efficacy and safety in both various *in vitro* models and *Galleria mellonella in vivo* animal model.

2. Materials and Methods

2.1. Bacterial strains and culture conditions

S. Typhimurium (strains 12 and 13) and *S.* Enteritidis (strains 64 and 1392) were obtained from The National Salmonella Centre at the Medical University of Gdansk (Gdansk, Poland) and were used in our previous studies (Kosznik-Kwaśnicka et al., 2020a, 2020b). Strains of *Proteus vulgaris*, *Citrobacter freundii*, and *Escherichia coli* came from the Department of Molecular Biology of the University of Gdansk collection of microorganisms (Gdansk, Poland). *Lactobacillus acidophilus* 314™ was purchased from ATCC. Bacteria were cultured at 42 °C or 37 °C. LB-medium (BioShop, Burlington, Ontario, Canada) was used to cultivate Gram-negative bacteria. For *L. acidophilus*, BHI medium (Graso Biotech, Starogard Gdanski, Poland) was used. Bacteriological agar (1%) (BioShop, Burlington, Ontario, Canada) was used in solidified media of LB-agar or BHI-agar. *L. acidophilus* cells were cultivated in microaerophilic conditions using GenBox microaer system (BioMérieux, Marcy l'Etoile, France).

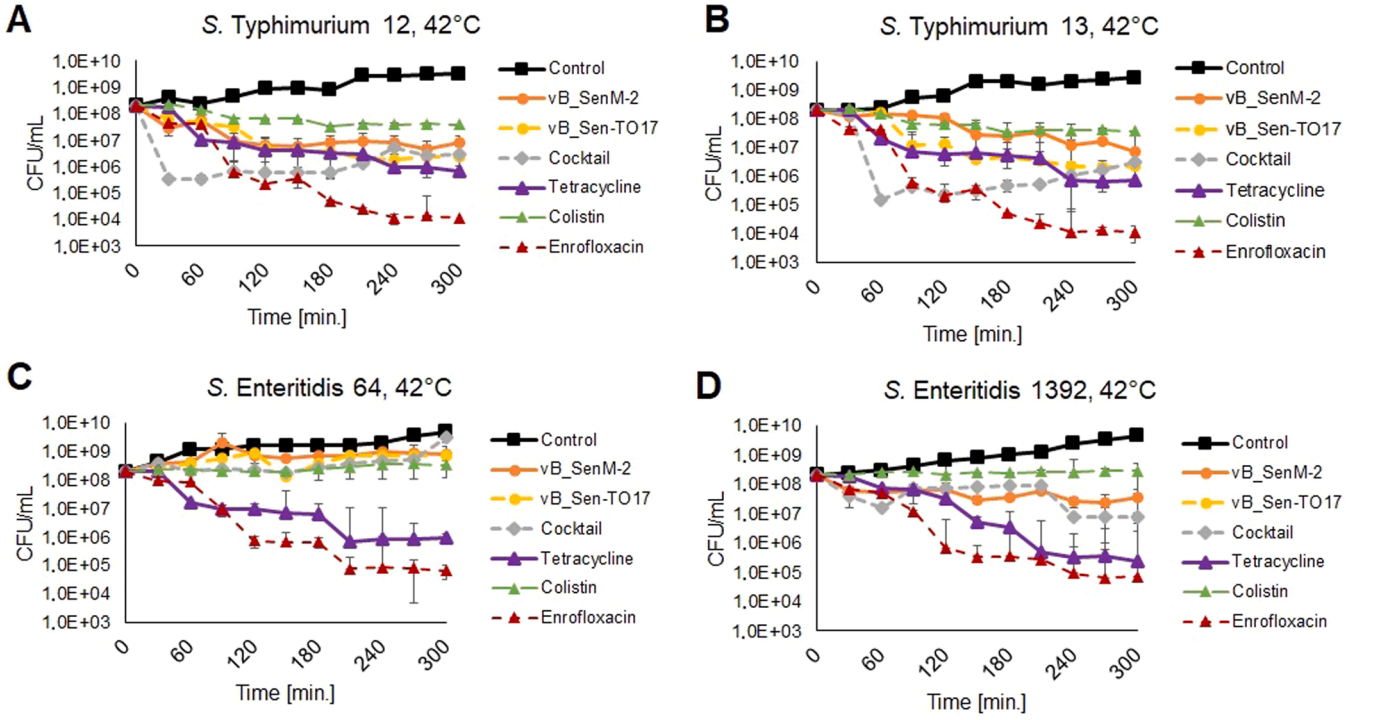

Fig. 2. Changes in CFU/ml (CFU/ml) of bacterial culture of *S*. Typhimurium 12, (A), *S*. Typhimurium 13 (B), *S*. Enteritidis 64 (C), and *S*. Enteritidis 1392 (D) after infection with phages vB_SenM-2 (orange circles, straight line), vB_Sen-TO17 (yellow circles, dotted line) at 10^9 PFU/ml, with phage cocktail (grey diamonds) at 2×10^9 PFU/ml or after addition of tetracycline (12.5 μg/ml; purple triangles, straight line), colistin (50 μg/ml; green triangles, dotted line) or enrofloxacin (50 μg/ml; red triangles, dotted line) in comparison with control (black squares) at 42 °C. Mean values from three independent experiments are shown, with error bars representing SD. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.2. Bacteriophages, phage cocktail and antibiotic solutions

Bacteriophages vB_Sen-TO17 and vB_SenM-2 were characterized previously by Kosznik-Kwaśnicka et al. (2020a), (2020b). The phage cocktail used in this work was a mixture of both phages in equal ratio and the final concentration used was 2×10^9 PFU/ml (1×10^9 PFU/ml of each phage). Phage lysates were stored at 4 °C (revealing the highest stability in the long-term storage, though a relative high stability of both phages was demonstrated previously during a short-term storage in the range of temperatures between −80 and 60 °C; see Jurczak-Kurek et al., 2016; Kosznik-Kwaśnicka et al., 2020a, 2020b). Following antibiotics were used at indicated concentrations: tetracycline, colistin, enrofloxacin.

2.3. Selective media used for multispecies biofilm experiments

In order to differentiate each of the bacterial species used in multispecies biofilm (*E. coli*, *P. vulgaris*, *C. freundii*, *L. acidophilus*, and *S. enterica*) experiments and to calculate changes in the colony-forming units (CFU/ml), two selective media were used: CHROMagar SALP (Graso Biotech, Starogard Gdanski, Poland) and MRS (Graso Biotech, Starogard Gdanski, Poland). The morphology of bacterial colonies was interpreted according to the manufacturer's instruction.

2.4. Phage propagation

Ten ml of bacterial host strain, grown overnight in LB medium, were added to 1 l of LB medium and incubated at 37 °C with agitation at 150 rpm. At an optical density of $OD_{600} = 0.15$ (SmartSpec PLUS, BIO-RAD, California, USA), the bacteria were infected with phages at a multiplicity of infection (m.o.i.) of 0.5 and incubated at 37 °C until lysis occurred. For phage purification, polyethylene glycol (PEG) 8000 (BioShop, Burlington, Ontario, Canada) was added to a final concentration of 10% and stirred (Carl Roth, Karlsruhe, Germany) overnight at 4 °C. The precipitate was collected by centrifugation at 10,000 × *g* for 30 min at 4 °C (Avanti JXN-26, rotor JLA-8000, Beckman Coulter, Indianapolis, USA) and suspended in 0.89% NaCl (Alchem, Torun, Poland). PEG8000 was removed by adding 2 ml of chloroform (Alchem, Torun, Poland) and centrifugation at 4000 × *g* for 15 min, 4 °C (Avanti JXN-26, rotor JS-13.1, Beckman Coulter, Indianapolis, USA). The procedure was repeated until no PEG8000 precipitate could be observed. Obtained lysates were then purified by centrifugation in CsCl (Sigma Aldrich, Saint Louis, Missouri, USA) gradients at 95,000 × *g* (Optima XPN-100, rotor SW32.1 Ti, Beckman Coulter, Indianapolis, USA) for 2.5 h (Green et al., 2012).

2.5. Effects of phage, phage cocktail or antibiotic treatment on bacterial liquid culture

The experiments were performed in accordance with previously described protocols (Abedon, 1992; Łubowska et al., 2019). Bacteria were cultivated to $OD_{600} = 0.2$ at 42 °C (to resemble conditions occurring in the chicken gut). Then, phage stock solution or phage cocktail was added to the bacterial culture to a final concentration of 10^9 PFU/ml (in the case of phage cocktail, each phage was added to 1×10^9 PFU/ml, giving total concentration of 2×10^9 PFU/ml), and antibiotics were added to following concentrations (chosen on the basis of the therapeutic dose sgiven to poultry during antibiotic therapy; these doses did not affect stability of phage lysates): tetracycline, 12.5 μg/ml; colistin, 50 μg/ml; enrofloxacin, 50 μg/ml. Bacterial growth was monitored by measuring the OD_{600} in 15 min intervals for 300 min. The number of bacterial cells per ml (CFU/ml) was determined by titration. One hundred μl of bacterial culture were collected every 30 min, serial dilutions were prepared in 0.89% NaCl and overlaid onto LB agar plates in order to assess the number of cells surviving the infection. The CFU/ml value was calculated based on the counted colonies after overnight incubation

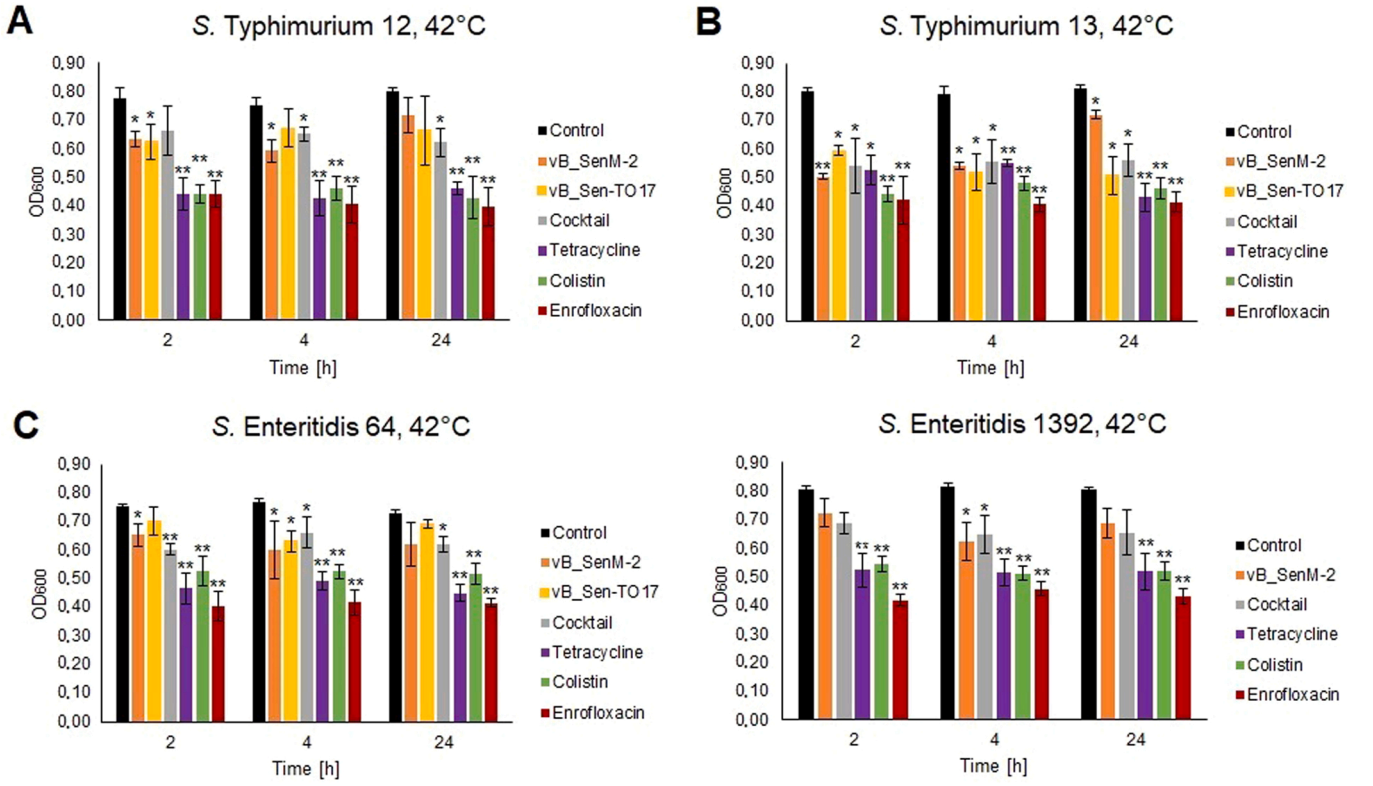

Fig. 3. Optical density of biofilm formed by *S.* Typhimurium 12, (A), *S.* Typhimurium 13 (B), *S.* Enteritidis 64 (C) and *S.* Enteritidis 1392 (D) after infection with phages vB_SenM-2 (orange bars), vB_Sen-TO17 (yellow bars), with phage cocktail (grey bars) (2×10^9 PFU/ml) or after addition of tetracycline (12.5 μg/ml; purple bars), colistin (50 μg/ml; green bars) or enrofloxacin (50 μg/ml; red bars) in comparison with control (black bars). Mean values from three independent experiments are shown, with error bars representing SD. Statistical analysis was performed using t-test, where $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

at 42 °C. The samples were overlaid onto a double-layer agar plate. The phage titer was determined by counting single plaques. The experiments were repeated at least 3 times.

2.6. Estimation of effects of phage, phage cocktail or antibiotic treatment on bacterial biofilm using CFU/ml count

Biofilm cell biomass after phage infection was determined according to the protocols described by Ramachandran et al. (2016) with some modifications. For biofilm cell culture, *S. enterica* was grown at 42 °C in a 24-well polystyrene plate (Nest Scientific Biotechnology, Wuxi, China) with 0.5 × LB medium for 24 h. After the incubation period, the liquid medium containing planktonic cells was removed and the biofilm was washed with 1 ml of distilled H_2O. In the next step, phage lysate or phage cocktail was added to each well, except for controls, to the final titer of 10^9 PFU/well. Antibiotics were added to following concentrations: tetracycline, 12.5 μg/ml; colistin, 50 μg/ml; enrofloxacin, 50 μg/ml. The plates were then incubated at 42 °C for 2 h, 4 h and 24 h. In the case of control wells, LB medium was added instead of phage lysate. Following the incubation, phage lysate was removed, biofilms were washed twice with 1 ml of distilled H_2O and then resuspended in 1 ml of distilled H_2O. Ten-fold dilutions were prepared and overlaid on the top of the LB-agar medium and left to dry down. The plates were incubated at 42 °C overnight and then checked for bacterial colonies. The experiment was done in triplicates.

2.7. Assessment of biofilm biomass by crystal violet staining after treatment with phage lysate, phage cocktail or antibiotics

Biofilms were prepared according to the procedure described in the preceding subsection. Phage lysates or phage cocktail were then added to formed biofilm and incubated for 4 h at 42 °C, the phages were then removed, biofilms were gently washed with 1 ml of distilled H_2O and antibiotics were added to biofilm; the biofilms were then incubated for additional 4 h at 42 °C. Alternatively, antibiotics were added to formed biofilm and incubated for 4 h at 42 °C, these compounds were then removed, biofilms were washed with 1 ml of distilled H_2O and phage lysate or phage cocktail was added; the biofilms were then incubated for additional 4 h at 42 °C. After incubation with phage lysate, phage cocktail or antibiotic solution, the liquid medium was removed, and surface-attached cells were treated with 1 ml of 0.1% crystal violet solution (Sigma-Aldrich, Missouri, USA). Plates were incubated in the dark for 20 min at 42 °C. Crystal violet was then carefully removed and biofilms were washed 2 times with 1 ml of distilled H_2O. Then, biofilms were fixed by additional incubation at 42 °C for 20 min. Afterwards, crystal violet was dissolved by the addition of 0.5 ml of 96% ethanol (Alchem, Torun, Poland). The absorbance was measured in a plate reader at 600 nm (VICTOR, PerkinElmer, Waltham, MA, USA) in order to assess biofilm biomass.

2.8. Analysis of phage and antibiotic activities on multispecies bacterial biofilm

Biofilms were prepared according to the procedure described above. Phage lysate or phage cocktail was added to the formed biofilm and incubated for 4 h at 42 °C. The phages were then removed, biofilms were gently washed with 1 ml of distilled H_2O and antibiotics were added to the biofilm. Alternatively, treatment with antibiotic preceded that with phages. In studies on multicultural biofilms, overnight cultures of *E. coli* MG1655, *P. vulgaris*, *C. freundii*, *L. acidophilus* 314™ and *S.* Typhimurium or *S.* Enteritidis were diluted in fresh BHI medium at a ratio of 1:200, and 2 ml of mixed bacterial culture was placed in a well of 24-

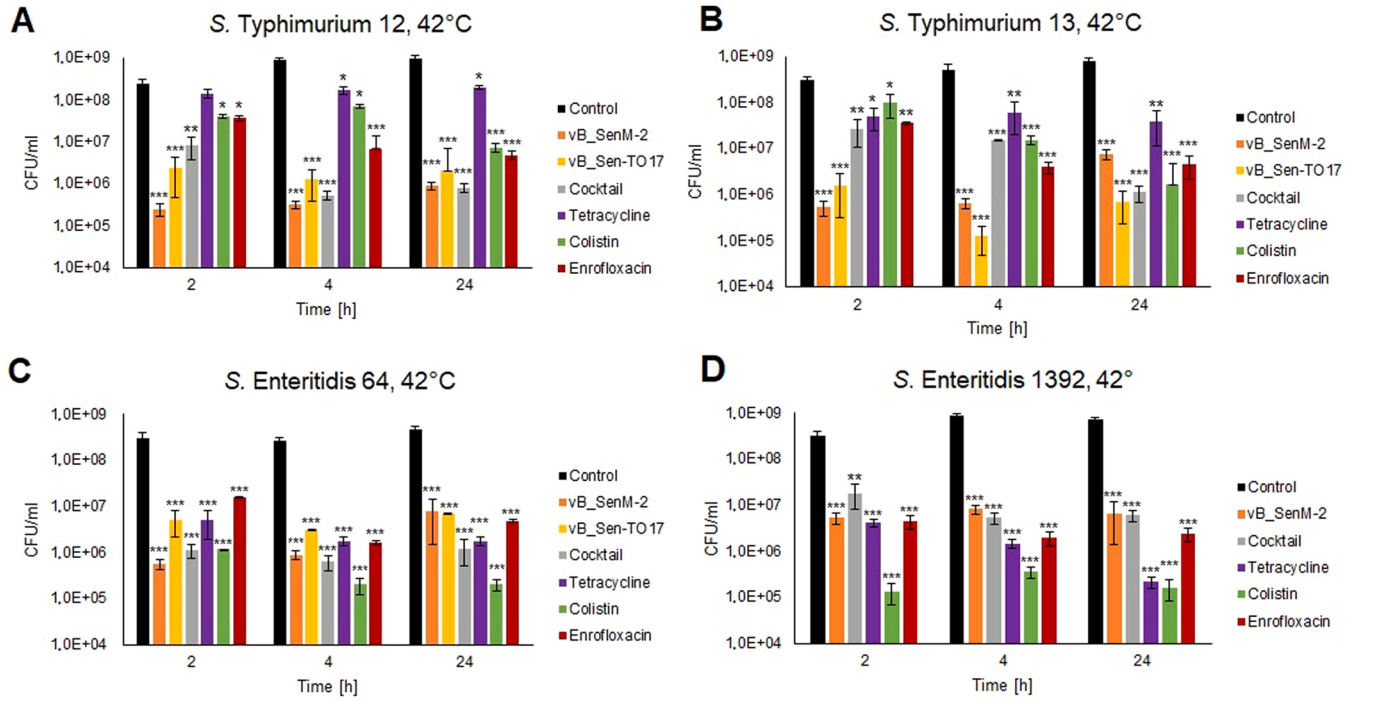

Fig. 4. Reduction in number of bacteria in biofilm formed by *S.* Typhimurium 12, (A), *S.* Typhimurium 13 (B), *S.* Enteritidis 64 (C), and *S.* Enteritidis 1392 (D) after infection with phages vB_SenM-2 (orange bars), vB_Sen-TO17 (yellow bars), with phage cocktail (grey bars) (2 ×109 PFU/ml) or after addition of tetracycline (12.5 μg/ml; purple bars), colistin (50 μg/ml; green bars) or enrofloxacin (50 μg/ml; red bars) in comparison with control (black bars) at 42 °C. Mean values from three independent experiments are shown, with error bars representing SD. Statistical analysis was performed using t-test, where $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

well polystyrene plate (Nest Scientific Biotechnology, Wuxi, China). The biofilms were then incubated for an additional 4 h at 42 °C. In the case of control wells, LB or BHI medium was added. Following the incubation, the antibiotic was removed, biofilms were washed twice with 1 ml of distilled H_2O and then resuspended in 1 ml of distilled H_2O. Ten-fold dilutions were prepared and overlaid on the top of the LB-agar medium and left to dry down. The plates were incubated at 42 °C overnight and then scanned for bacterial colonies. For alternative assessment of the biofilm, surface-attached cells were treated with 1 ml of 0.1% crystal violet solution. Plates were incubated in the dark for 20 min, at 42 °C. Crystal violet was then carefully removed, and biofilms were washed 2 times with 1 ml of distilled H_2O. Then, biofilms were fixed by additional incubation at 42 °C for 20 min. Afterwards, crystal violet was dissolved by the addition of 0.5 ml of 96% ethanol. The absorbanc was measured in a plate reader at 600 nm in order to assess biofilm biomass. Each experiment was repeated 3 times.

2.9. Determination of rate of development of bacterial resistance to phages and antibiotics

In order to determine the average number of bacteria resistant to the antibiotic in bacterial population, cells derived from 10 ml, 100 ml and 1000 ml of liquid culture at $OD_{600} = 0.1$, obtained after centrifugation and suspension in a small volume of LB, were spread on LB agar containing antibiotics at indicated concentrations. For bacteriophages, bacteria were mixed with 10^9 PFU/ml of phage lysate and 4 ml of top agar and poured onto LB-agar plates. Samples were then incubated overnight at 42 °C. After incubation, the number of bacterial cells resistant to antibiotics and phages was determined.

For the rate of resistance development, bacteria were cultivated to $OD_{600} = 0.2$ at 42 °C. Then, phage stock solution or phage cocktail was added to the bacterial culture at a total concentration of 10^9 PFU/ml and antibiotics were added to indicated concentrations. Bacterial growth was monitored by measuring OD_{600} every 30 min, with intervals throughout 13 h.

2.10. Assessment of endotoxin level in phage lysates using LAL chromogenic test

In order to assess the purity of phage lysates, levels of endotoxins were measured. Purified Thermo Scientific™ Pierce™ LAL Chromogenic Endotoxin Quantitation Kit (no. 12117850, Thermo Fisher Scientific Inc., Paisley, UK) was used in accordance with the manufacturer's protocol. Endotoxin Standard Solutions were prepared in accordance with the manufacturer's guidelines and the standard curve was formulated. Fifty μl of purified phage lysate were then dispensed in a microplate well, with the microplate maintained at 37 °C. At time 0, 50 μl of LAL reagent were added to each well. The plate was then briefly removed from the heating block, tapped several times to facilitate mixing, and returned to the heating block to incubate at 37 °C for 10 min. After incubation, 100 μl of prewarmed Chromogenic Substrate solution was added to each well, followed by incubation at 37 °C for 6 min. At time 6 min, 100 μl of Stop Reagent was added. The plate was removed from the heating block and the absorbance was measured at 410 nm at EnSpire Multimode Plate Reader (PerkinElmer, Inc. Waltham, Massachusetts, U.S). The level of endotoxin was determined using a formulated standard curve. The experiment was performed three times.

2.11. Chicken cell cultures

Immortalized spontaneously transformed chicken fibroblast (UMN-SAH/DF-1) were obtained from ATCC, Manassas, Virginia, USA (CRL-12203TM). Fibroblasts were cultured on 15-cm^2 plates (Corning Inc., New York, USA) in DMEM medium (Thermo Fisher Scientific Inc., Paisley, UK) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific Inc., Paisley, UK) and 1% antibiotic/antimycotic solution (Sigma Aldrich Co. LLC., St. Louis, USA) at 37 °C in a humidified atmosphere of 95% air, 5% CO_2, in the Galaxy 170 S incubator (New

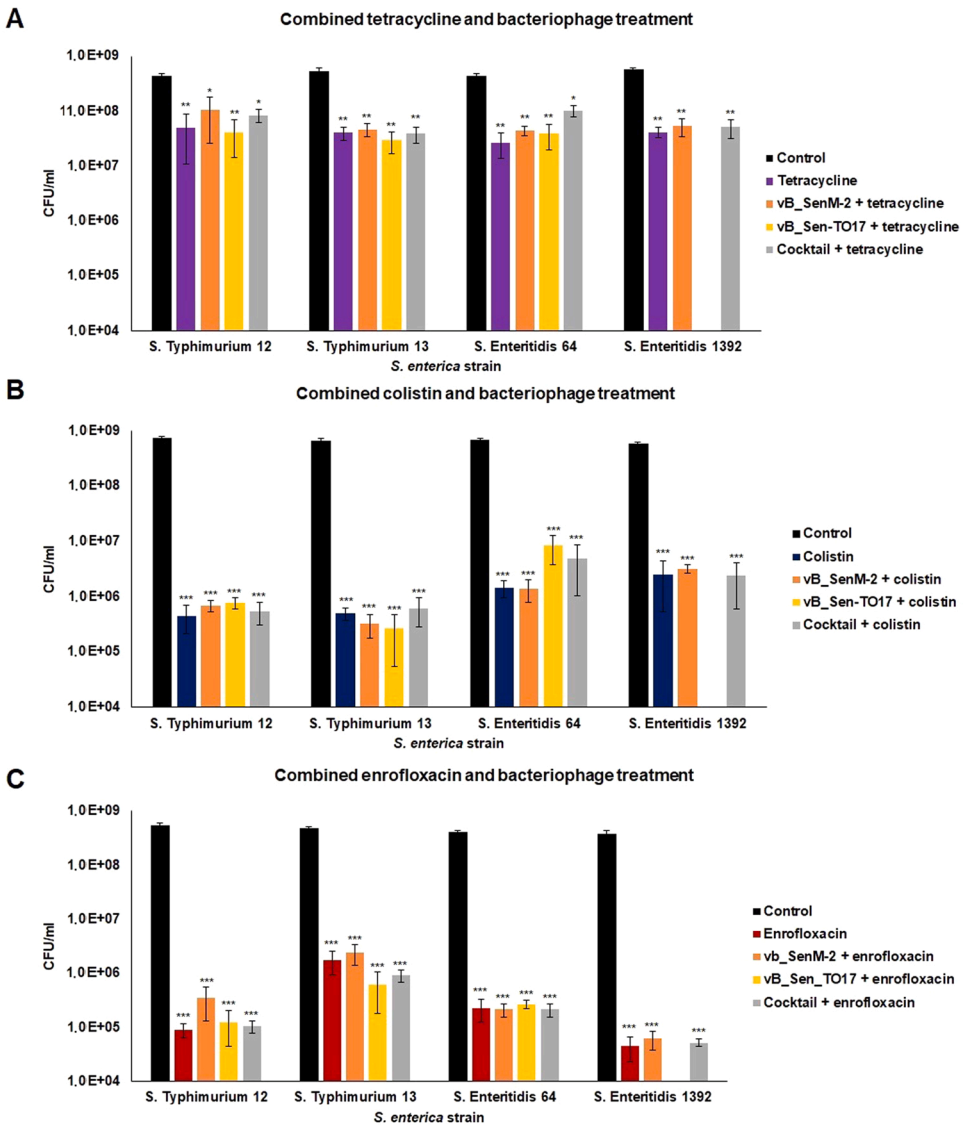

Fig. 5. The effect of combined treatment with antibiotics (tetracycline (12.5 μg/ml) (A), colistin (50 μg/ml) (B), and enrofloxacin (50 μg/ml) (C)) added first, and bacteriophages (vB_SenM-2 (orange bars), vB_Sen-TO17 (yellow bars), and phage cocktail (grey bars)) supplemented 24 h later (2×10^9 PFU/ml), on number of bacteria in biofilm. The results are compared with the control (black bars) and single antibiotic treatment: tetracycline (purple bars), colistin (green bars) or enrofloxacin (red bars). Mean values from three independent experiments are shown, with error bars representing SD. Statistical analysis was performed using t-test, where $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Brunswick Scientific, Edison, New Jersey, USA).

2.12. Neutral red cytotoxicity assay

UMNSAH/DF-1 cells were plated into 96-well tissue culture plates (Nest Scientific Biotechnology, Wuxi, China) at a density of 2×10^4 per well and allowed to attach for 24 h in DMEM supplemented with 10% FBS and 1% antibiotic/antimycotic solution. After 24 h, the medium was removed, and 10% DMSO (Sigma-Aldrich Co. LLC., St. Louis, USA) treated cells were used as a positive control, while non-treated cells were considered a negative control. Cells were treated with phage cocktail (2×10^5, 2×10^6, 2×10^7, 2×10^8 and 2×10^9 PFU/ml), enrofloxacin or colistin suspended in the DMEM medium. Antibiotics were added to indicated concentrations. Cells were incubated for 24, 48, 72, 96 or 120 h at 37 °C in a humidified atmosphere of 95% air/5% CO_2. In experiments with cells treated for 96 h and 120 h, the medium was changed after 72 h of treatment. Following incubation, the supernatants were discarded and replaced with 100 μl of culture medium containing 0.33% neutral red (Sigma-Aldrich Co. LLC., St.Louis, USA) diluted 1:40. After 45 min incubation at 37 °C, the neutral red medium was removed, and cells were washed twice with 100 μl of PBS buffer per well. Cells were then treated with 100 μl of a solution containing 50% methanol (Alchem, Torun, Poland), 20 mM HCl (Alchem, Torun, Poland) and 30 mM sodium citrate (Alchem, Torun, Poland), to extract the dye into solution. Absorbance was measured at 490 nm (VICTOR, PerkinElmer, Waltham, MA, USA).

2.13. Effectiveness of phage therapy on the Galleria mellonella infection model

For the *Galleria mellonella* infection experiments, an overnight culture of *S.* Typhimurium 13 strain was diluted 1:100 in LB and grown to $OD_{600} = 0.1$ (approximately 1×10^8 CFU/ml, assessed by serial dilutions and plating). Bacteria were harvested by centrifugation, washed twice with PBS buffer and diluted to a final concentration of 5×10^5 CFU/ml. Bacteriophages were diluted in TM buffer to a final concentration of 5×10^5, 5×10^6 and 5×10^7 PFU/ml (corresponding to an m.o.i. of 1, 10 and 100, respectively). *G. mellonella* larvae were obtained from Biosystems Technology (Crediton, UK) or Egzotic Room (Plewiska, Poland). Only larvae with no signs of pigmentation, weighing between 300 and 400 mg, were selected for the experiment. The larvae were stored at 17 °C and used within one week. Each *G. mellonella* larva was surface-sterilized with a 70% ethanol rinsed cotton swab. The larvae were inoculated using a 10 μl Hamilton syringe with a blunt tip needle (Merck, Germany). 10 μl of the bacterial suspension containing 5×10^3 bacterial cells were inoculated via the last left proleg. One hour after the

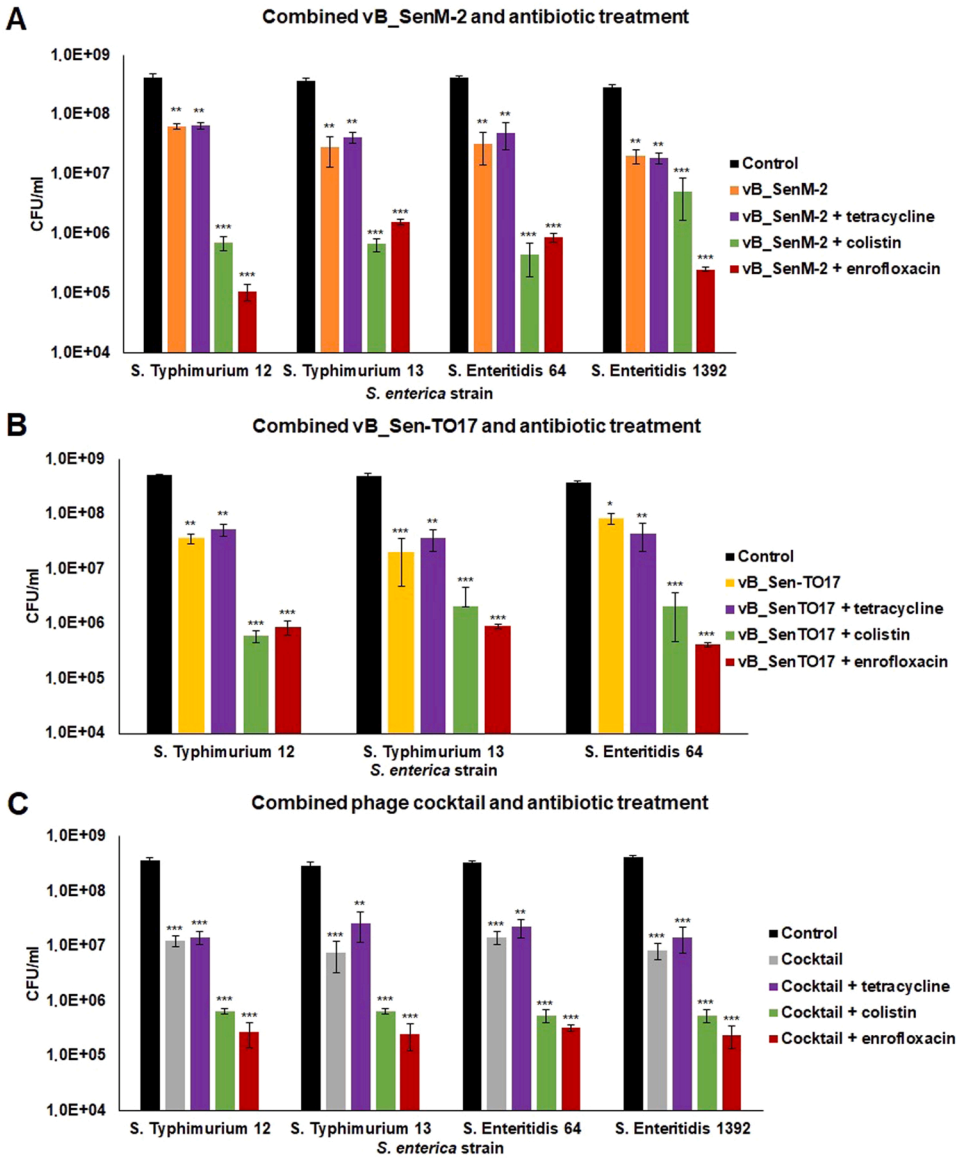

Fig. 6. The effect of combined treatment with bacteriophages (vB_SenM-2 (A), vB_Sen-TO17 (B), phage cocktail (C)) added first (2×10^9 PFU/ml), and antibiotics (tetracycline (12.5 μg/ml; purple bars), colistin (50 μg/ml; gray bars) or enrofloxacin (50 μg/ml; red bars)) supplemented 24 h later on number of bacteria in biofilm. The results are compared with the control (black bars) and phage treatment with vB_SenM-2 (orange bars) vB_Sen-TO17 (yellow bars) or phage cocktail (grey bars). Mean values from three independent experiments are shown, with error bars representing SD. Statistical analysis was performed using t-test, $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

injection of the bacteria, 10 μl of a phage suspension at a concentration of 5×10^5, 5×10^6 or 5×10^7 PFU/ml (m.o.i. of 1, 10 or 100, respectively) were inoculated via the last right proleg. Control larvae were inoculated with 10 μl of PBS buffer followed by 10 μl of TM buffer (injection control) or with 10 μl of PBS followed by 10 μl of phage suspension at the concentration of 5×10^7 PFU/ml (phage control). Following injections, larvae were incubated at 37 °C in the dark for five days, and their survival was monitored at 12-h intervals. The larvae were considered dead when they blackened and showed no movement in response to touch. Each group consisted of 30 larvae.Kaplan-Meier survival curves and Log-rank (Mantel-Cox) statistical test were performed using GraphPad Prism (GraphPad Software, San Diego, CA, USA).

2.14. Statistical analysis

In the figures, data are presented as mean values ± SD. For the statistical analysis of the results, SPSS21.0 (SPSS Inc., Armonk, USA) software was used. The normality of the distribution of variables was checked by the Kolmogorov-Smirnov test. The homogeneity of the variances was analyzed using the Levene test. Comparisons between two groups were performed by the Student's *t*-test. Comparisons between several groups were analyzed by the Kruskal-Wallis test followed by Dunn's multiple comparison test for values with the nonparametric distribution. In all tests, the values of $p<0.05$ were considered statistically significant.

3. Results

3.1. Efficacy of phages in inhibiting the growth of S. enterica cultures

To test the effects of investigated phages on *S. enterica* culture growth, we have used strains *S.* Typhimurium 12 and 13, and *S.* Enteriditis 64 and 1392, demonstrated previously to be sensitive to one or both these viruses (Kosznik-Kwaśnicka et al., 2020a, 2020b). Bacteria were grown in LB medium at 42 °C (to mimic temperature conditions occurring in the chicken gut), and phage vB_SenM-2or vB_Sen-TO17 or both (a mixture called cocktail) were added to m.o.i. = 5. Bacterial culture growth was monitored by measurement of OD_{600}. Bacteria treated with a buffer were used as a negative control, and antibiotics (tetracycline at 12.5 μg/ml, colistin at 50 μg/ml and enrofloxacin at 50 μg/ml) were used as positive controls. As expected, treatment with

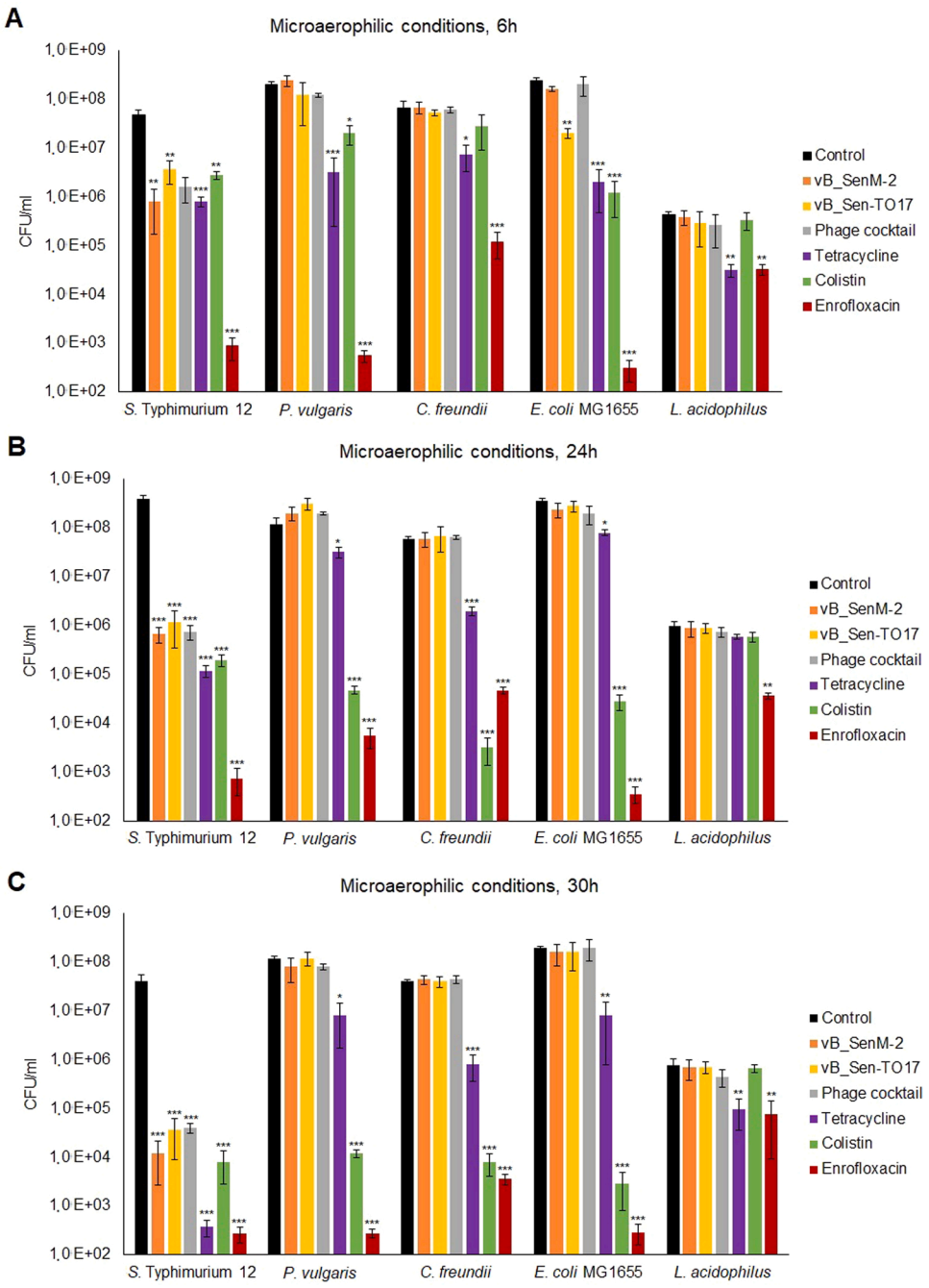

Fig. 7. Reduction in the number of bacteria in multispecies biofilm formed with *S.* Typhimurium 12 after 6 h (A), 24 h (B) and 30 h (C) of incubation with phages vB_SenM-2 (orange bars), vB_Sen-TO17 (yellow bars) and phage cocktail (grey bars) (2×10^9 PFU/ml) or after addition of tetracycline (12.5 μg/ml; purple bars), colistin (50 μg/ml; green bars) or enrofloxacin (50 μg/ml; red bars) in comparison with control (black bars) at 42 °C. Mean values from three independent experiments are shown, with error bars representing SD. Statistical analysis was performed using t-test, where $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

antibiotics strongly inhibited the growth of all tested *S. enterica* strains (Fig. 1). Both tested phages also caused significant slowing down of the bacterial growth, though the effects were dependent on the specificity of the phage-bacteria interactions. Nevertheless, a phage cocktail was always more efficient than any single phage used alone (Fig. 1).

In the same kind of experiments, we have monitored the number of living cells. Both antibiotics and phages were effective in killing bacteria, while the efficacy differed depending on *S. enterica* strains, and antibiotics caused generally more pronounced effects (Fig. 2). In conclusion, we found that both tested phages and their mixture revealed acceptable efficacy in combating *S. enterica* strains, though phage-strain specificity must be considered, and the phage cocktail was more efficient than single phages. Although antibiotics were evidently more effective in reducing the number of living bacterial cells, the phages were still sufficiently effective in this parameter to inhibit the growth of *S. enterica* cultures.

3.2. Effects of phages on single- and multi-species biofilms

To test the effects of phages on bacterial biofilms, these structures were allowed to form first in wells of polystyrene plates, then, phages, antibiotics or control buffer were added, and the incubation was prolonged up to 24 h. We observed a significant reduction of the bacterial biomass (assessed by measurement of OD_{600}) after treatment with antibiotics (Fig. 3). Effects of phages were less pronounced, though still significant in some combinations of phages and hosts (Fig. 3). On the other hand, bacteriophages were either as efficient as antibiotics in reducing the number of living *S. enterica* cells in the biofilm or even more efficient in the case of some strains (Fig. 4).

We asked if the combined action of antibiotics and phages might be

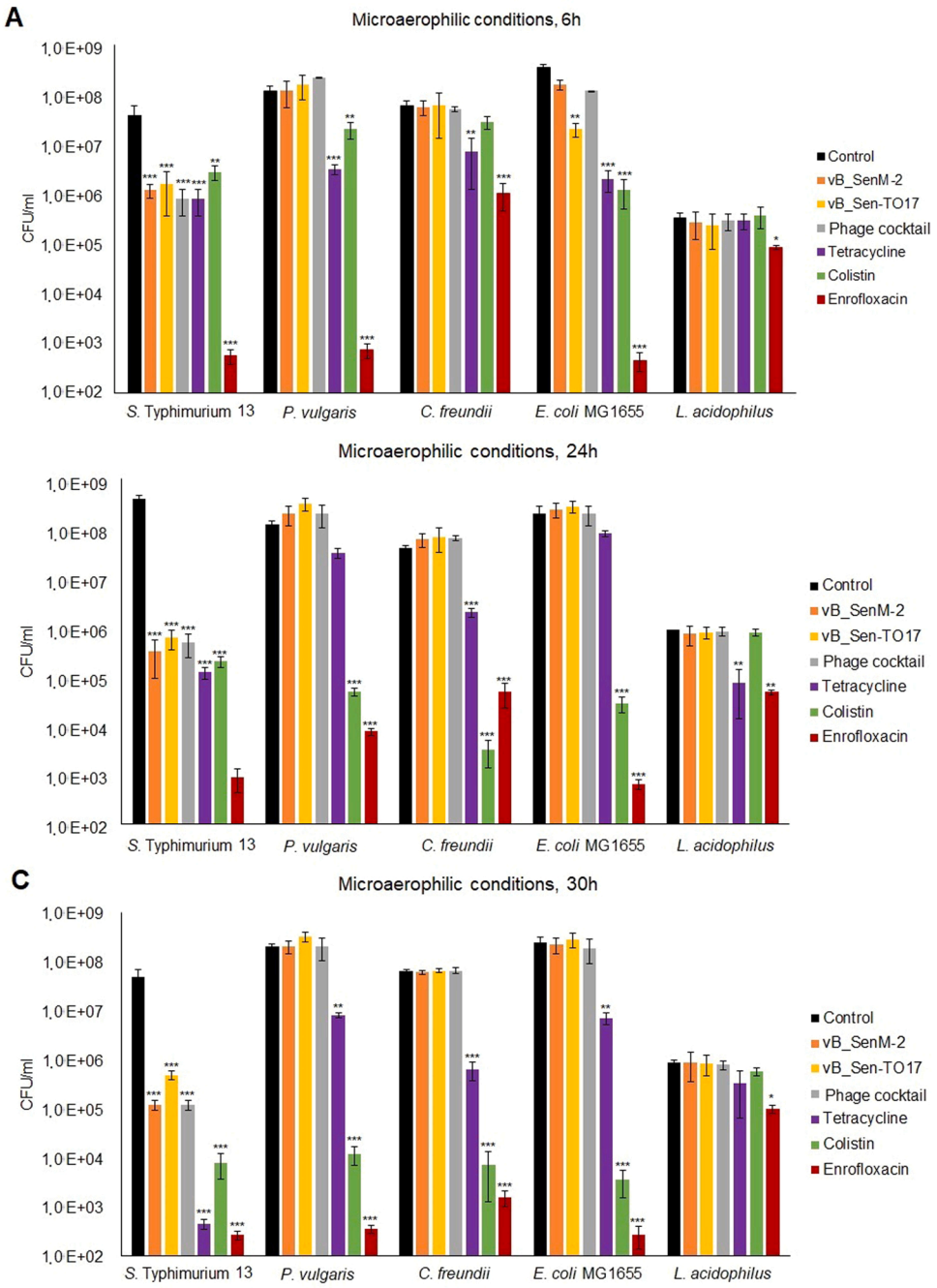

Fig. 8. Reduction in number of bacteria in multispecies biofilm formed with *S.* Typhimurium 13 after 6 h (A), 24 h (B) and 30 h (C) of incubation with phages vB_SenM-2 (orange bars), vB_Sen-TO17 (yellow bars) and phage cocktail (grey bars) (2×10^9 PFU/ml) or after addition of tetracycline (12.5 μg/ml; purple bars), colistin (50 μg/ml; green bars) or enrofloxacin (50 μg/ml; red bars) in comparison with control (black bars) at 42 °C. Mean values from three independent experiments are shown, with error bars representing SD. Statistical analysis was performed using t-test, where $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

more efficient in the treatment of biofilms relative to the effects of these agents used alone. Thus, *S. enterica* biofilms were treated first with antibiotics and then phages were added. However, no improvement in the efficacy of antibiotics was observed if these compounds were supplemented with phages (Fig. 5). Similarly, when the order of addition of antibacterial agents was reversed (i.e. phages added first, and then supplemented with antibiotics) the effects on biofilms were not enhanced relative to those of phages or antibiotics used alone (Fig. 6).

Although biofilms can be formed by one bacterial species, in nature, multi-species biofilms are predominant. Therefore, we have tested the effects of phages and antibiotics on biofilms built by *S. enterica*, *P. vulgaris*, *C. freundii*, *E. coli* and *L. acidophilus*. Microaerophilic conditions were used to form such biofilms and to better resemble conditions found in the chicken gut. Bacteriophages vB_SenM-2 and vB_Sen-TO17 were effective in reducing the number of living *S. enterica* cells (with efficacy dependent on the host strain, Figs. 7–10) while, as expected, they could not reduce the numbers of viable cells of other bacteria (Figs. 7–10). On the other hand, various antibiotics revealed different efficiency in killing *S. enterica*, *P. vulgaris*, *C. freundii*, *E. coli* and *L. acidophilus* present in biofilms (Figs. 7–10).

3.3. The emergence of phage- and antibiotic-resistant bacteria

We have tested the kinetics of the appearance of *S. enterica* cells resistant to tested phages and an antibiotic (colistin). In these experiments, bacterial cultures were either infected with phages or treated with the antibiotic as described in the first subsection of this chapter, however, the cultures were grown as long as 780 min. By monitoring OD_{600} of the cultures, we found that cultures that initially lost their density due to actions of phages or the antibiotic, started to re-grow

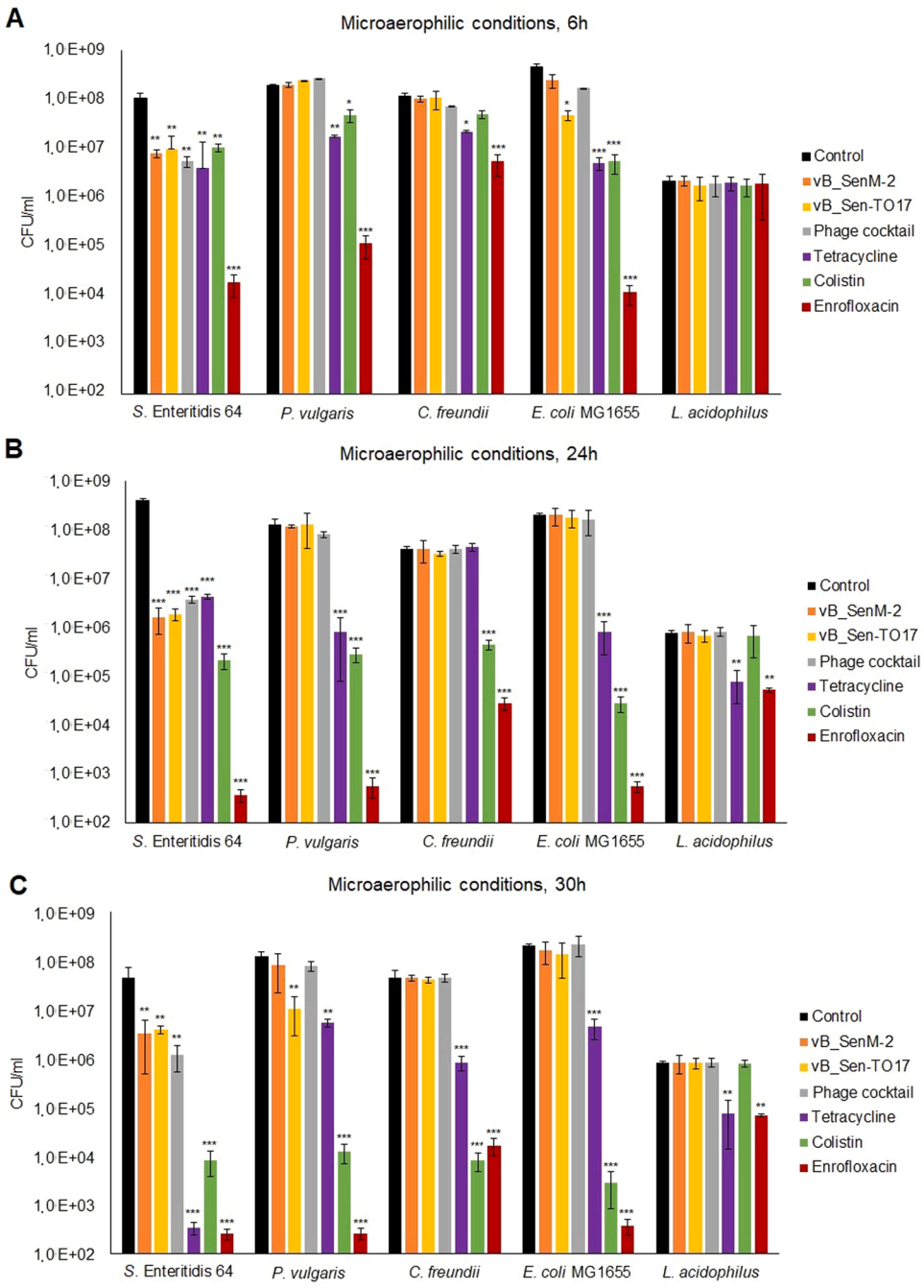

Fig. 9. Reduction in number of bacteria in multispecies biofilm formed with *S.* Enteritidis 64 after 6 h (A), 24 h (B) and 30 h (C) of incubation with phages vB_SenM-2 (orange bars), vB_Sen-TO17 (yellow bars) and phage cocktail (grey bars) (2×10^9 PFU/ml) or after addition of tetracycline (12.5 μg/ml; purple bars), colistin (50 μg/ml; green bars) or enrofloxacin (50 μg/ml; red bars) in comparison with control (black bars) at 42 °C. Mean values from three independent experiments are shown, with error bars representing SD. Statistical analysis was performed using t-test, where $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

several hours later (Fig. 11). This indicated that efficiencies of the emergence of bacteria resistant to vB_SenM-2 and vB_Sen-TO17 phages and to colistin are comparable.

3.4. Safety of the use of bacteriophages vB_SenM-2 and vB_Sen-TO17

The safety of phage lysates used in this study was assessed first with the LAL chromogenic test which allows to estimate the level of endotoxins. We have determined that lysates of both vB_SenM-2 and vB_Sen-TO17, prepared as described in Materials and Methods, revealed low levels of endotoxins, 0.156 ± 0.062 and 0.085 ± 0.043 EU/kg/h, respectively, which were within norm values (negative control had <0.05 EU/kg/h, while the upper acceptable limit of the enterotoxin concentration is 5.0 EU/kg/h).

Effects of phages and antibiotics on chicken cells were tested using the line of UMNSAH/DF-1 fibroblasts and the neutral red cytotoxicity assay. We found that vB_SenM-2 and vB_Sen-TO17 had no significant effects on the viability of chicken fibroblasts at all tested amounts (up to 2×10^9 PFU/ml) (Fig. 12). Similarly, colistin also did not affect the viability of these cells at concentrations up to 160 μg/ml. However, strong cytotoxic effects of enrofloxacin, which acted in a dose-response manner, were determined even at the dose as low as 5 μg/ml after 120 h incubation while the severe decrease in viability of cells was observed at 20 or 40 μg/ml even after 24 h (Fig. 12).

3.5. Efficacy of phage therapy in the treatment of S. enterica-infected Galleria mellonella larvae

The efficacy of phage therapy with vB_Sen-TO17 and vB_SenM-2 phages to treat *S. enterica* infection was assessed *in vivo* using the

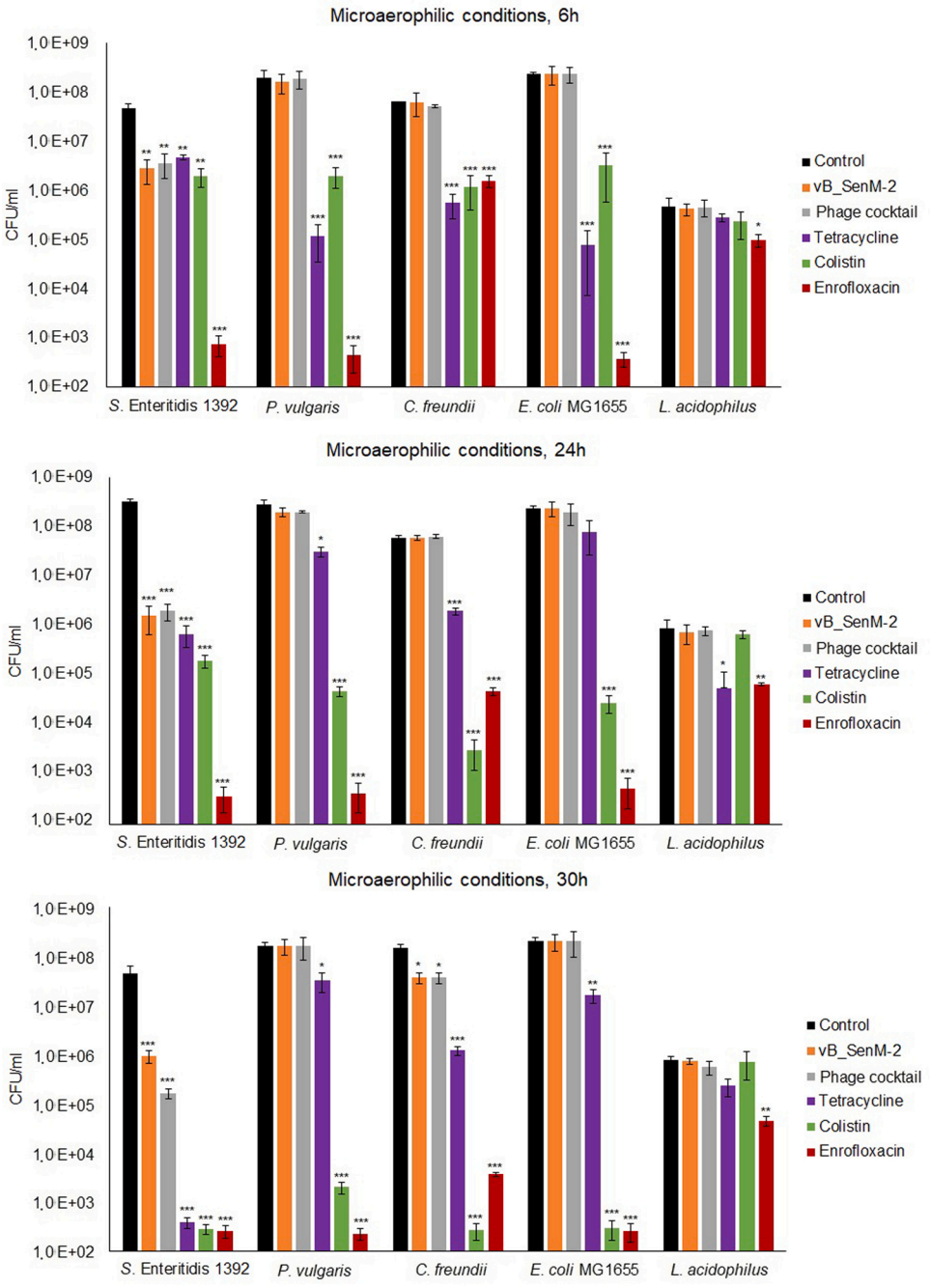

Fig. 10. Reduction in number of bacteria in multispecies biofilm formed with *S.* Enteritidis 1392 after 6 h (A), 24 h (B) and 30 h (C) of incubation with phages vB_SenM-2 (orange bars), vB_Sen-TO17 (yellow bars) and phage cocktail (grey bars) (2×10^9 PFU/ml) or after addition of tetracycline (12.5 μg/ml; purple bars), colistin (50 μg/ml; green bars) or enrofloxacin (50 μg/ml; red bars) in comparison with control (black bars) at 42 °C. Mean values from three independent experiments are shown, with error bars representing SD. Statistical analysis was performed using t-test, where $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Galleria mellonella infection model. This model was successfully used in previously described studies on efficacy of both phage therapy and various antimicrobial molecules at the pre-clinical stages of *in vivo* testing of different therapeutical options, including but not limited to investigations of potential drugs for chickens, as it was found that experiments on such a simple animal can give reliable results in a relatively short time while allowing to reduce the use of vertebrates in invasive experiments (Lacharme-Lora et al., 2019; Mohsin et al., 2019; Nale et al., 2021). The larvae were infected with *S.* Typhimurium 13 strain at the dose of 5×10^3 CFU per animal. One hour after bacterial infection, larvae were treated with a suspension of bacteriophage vB_Sen-TO17 or vB_SenM-2, or a cocktail of both phages. Bacteriophages were tested at m.o.i. values 1, 10, and 100. The results are presented in Fig. 13.

In the infection control group (injection with *S.* Typhimurium 13 followed by injection of the buffer), the survival rate was 40% and 10% at 24 and 48 h post-infection, respectively, while all infected animals died after 108 h. Treatment with vB_Sen-TO17 led to a significant increase in the survival rate at all tested m.o.i. values. In contrast, vB_SenM-2 treatment was less effective and resulted in an evidently increased survival rate only when phage was used at m.o.i. of 100. The highest increase in the survival rate of larvae was observed in the group

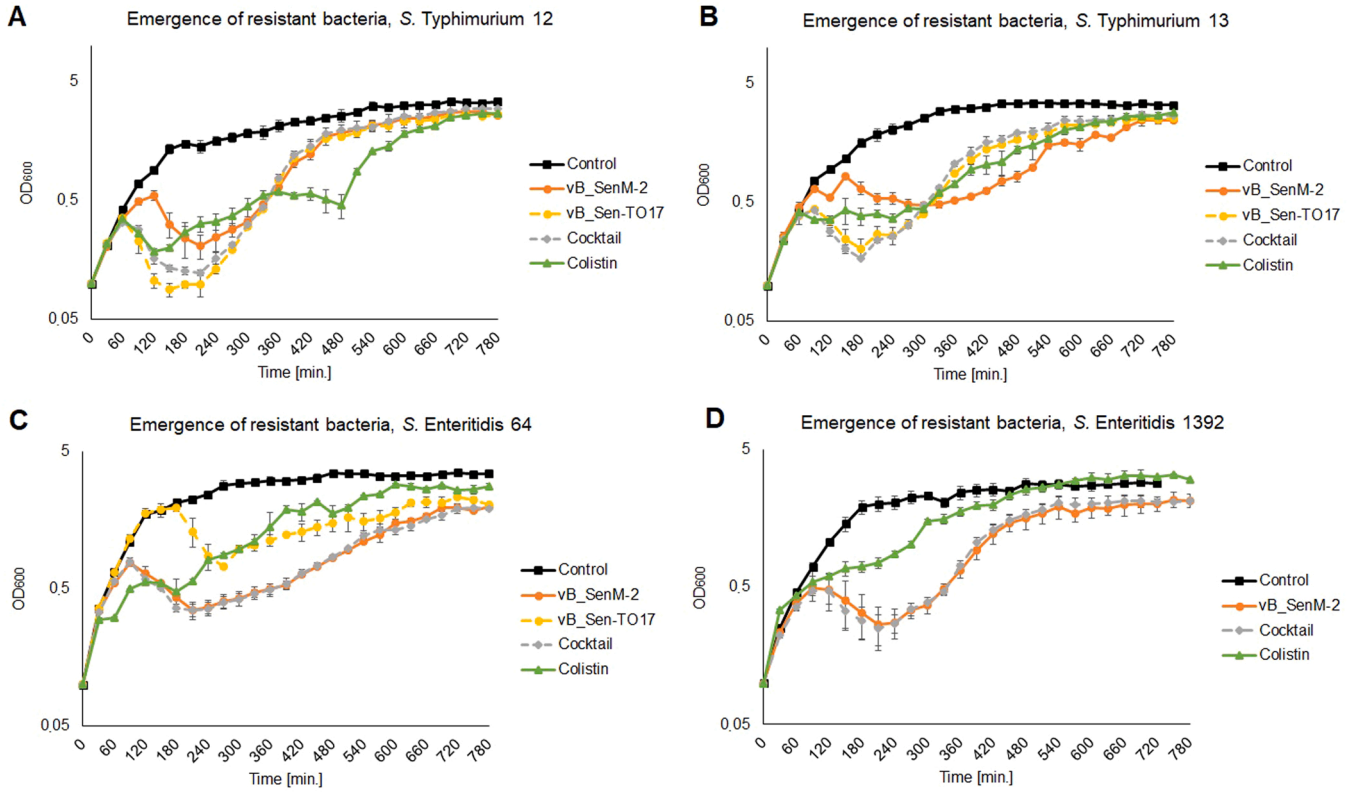

Fig. 11. Development of resistance to phages vB_SenM-2 (orange circles, straight line), vB_Sen-TO17 (yellow circles, dotted line), phage cocktail (grey diamonds) (2×10^9 PFU/ml) and colistin (50 μg/ml; green triangles) by *S.* Typhimurium 12 (A), *S.* Typhimurium 13 (B), *S.* Enteritidis 64 (C), and *S.* Enteritidis 1392 (D). Mean values from three independent experiments are shown, with error bars representing SD. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of animals treated with the cocktail of vB_Sen-TO17 and vB_SenM-2 at all tested m.o.i. In all negative control groups (larvae treated with either the buffer or all combinations of phages (vB_Sen-TO17 or vB_SenM-2 or the cocktail), survival was maintained at 100% throughout the whole experiment.

These results indicated both the safety of tested phages to *Galleria mellonella* larvae and the efficacy of treatment of *S. enterica*-mediated infection in the animal model. Therefore, further studies on the use of phage therapy with vB_Sen-TO17 and vB_SenM-2 in aviculture are substantiated.

4. Discussion

The appearance of antibiotic-resistant bacteria led to the so-called "antibiotic crisis" (Larsson and Flach, 2021). The problem is vital not only in the treatment of infected humans, but also in veterinary medicine, both in combating infectious diseases and the protection of farm animals. Phage therapy appears to be one of the promising alternatives to the use of antibiotics (Górski et al., 2020). Nevertheless, introducing bacteriophages as therapeutic or protective agents requires establishing a large collection of these viruses, due to their specificity to bacterial species, and often also to serovars or strains. Such phages must be characterized in details as they should not bear genes coding toxins, should not lysogenize bacterial cells, and should effectively and rapidly kill host cells during lytic development (Górski et al., 2022).

Since *S. enterica* is one of the major pathogenic bacterium causing foodborne diseases in humans, and poultry-derived products contaminated with this bacterium are the major sources of infections that might be potentially combated with phages (Capparelli et al., 2010), we aim to develop effective phage therapy for chickens infected with *Salmonella*. We focus on serovars which are predominant in causing foodborne infections in humans, *S.* Typhimurium and *S.* Enteritidis (Gal-Mor et al., 2014). In the literature, such attempts have already been described, to list only the most recent (Gomez-Garcia et al., 2021; Huang et al., 2022; Lamy-Besnier et al., 2021; Lorenzo-Rebenaque et al., 2022; Pelyuntha et al., 2021; Sabzali and Bouzari, 2021; Shakeri et al., 2021; Sui et al., 2021; Tao et al., 2021; Thanki et al., 2022; Wottlin et al., 2021). However, since the potential success of phage therapy depends on possessing large collections of phages, or so-called phage biobanks, characterization of newly isolated phages is still desirable (Lin et al., 2021). Moreover, even if a phage is well characterized genetically, it is still necessary to assess its efficacy and safety in phage therapy. Therefore, in this work, we aimed to test these features of two previously isolated phages, vB_Sen-TO17 and vB_SenM-2, infecting *S.* Typhimurium and *S.* Enteritidis.

Our results indicated that tested phages were effective in inhibiting the growth of bacterial cultures and reducing the number of viable cells. Importantly, they could act effectively on bacterial biofilms, both uni- and multi-species, though affecting only *S. enterica* cells, in contrast to antibiotics that killed bacteria belonging to various species. An important finding was that tested phages were safe to chicken cells and *Galleria mellonella* larvae. Moreover, the lysates used in this work revealed a favorable safety profile in the LAL chromogenic test, indicating that they were not contaminated by endotoxins. Interestingly,

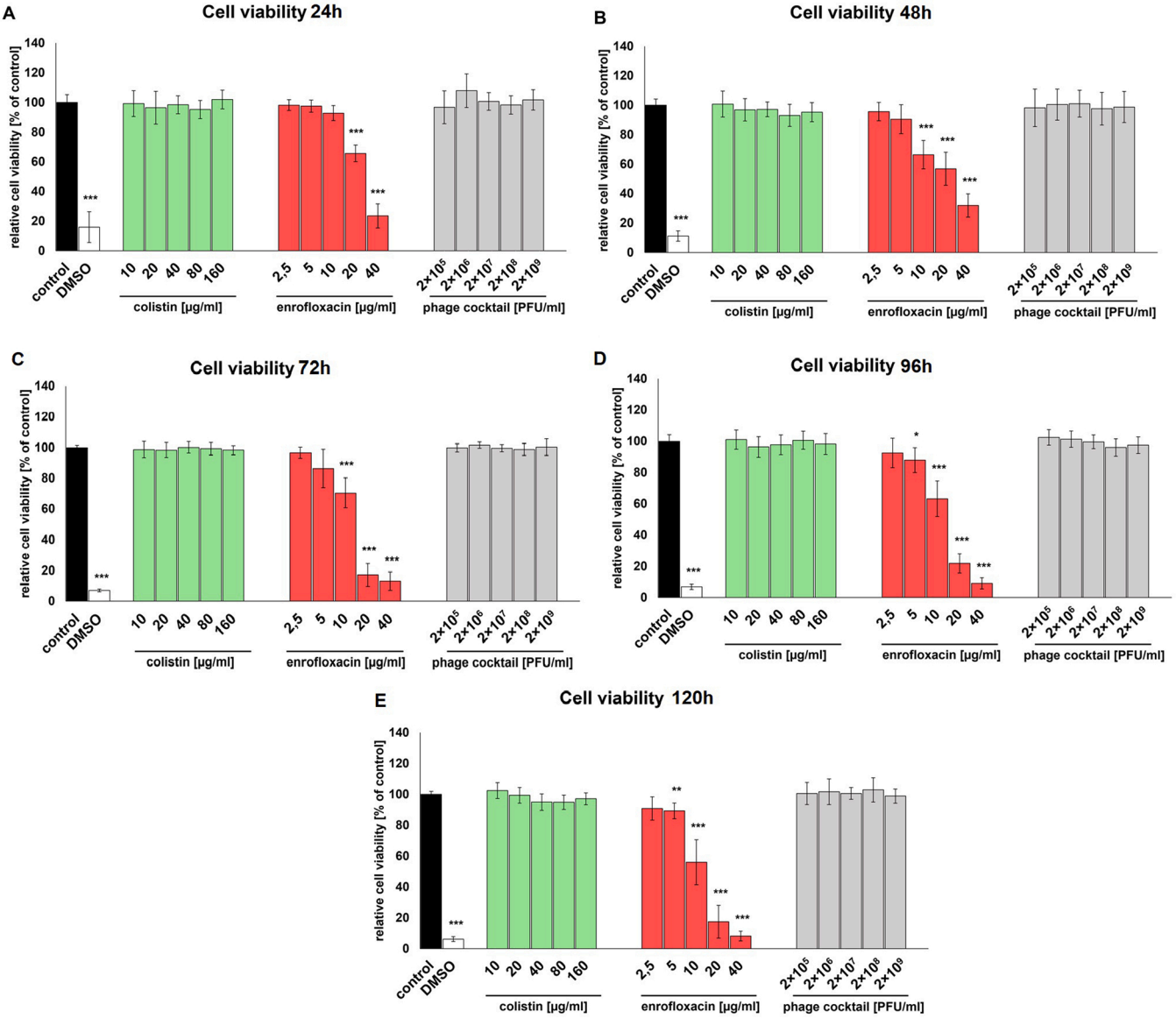

Fig. 12. Viability of UMNSAH/DF-1 cells after 24 h (A), 48 h (B), 72 h (C). 96 h (D) and 120 h (E) in comparison to untreated and 10% DMSO-treated cells. Mean values from three independent experiments are shown, with error bars representing SD. Statistical analysis was performed using the Kruskal-Wallis test followed by Dunn's multiple comparison test for values with nonparametric distribution, with $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***).

when comparing the safety of phages to that of different antibiotics, colistin revealed no cytotoxic effects on chicken fibroblasts while enrofloxacin caused dramatic loss of viability of these cells even at relatively low concentrations. The high toxicity of the latter antibiotic is especially important in light of its common use on a large scale in avian farms (Shang et al., 2018). Therefore, the safety profile of bacteriophages is evidently higher than that of enrofloxacin, pointing to the desired replacement of the treatment of animals with this antibiotic with phage therapy in the future. Finally, the efficacy of the phage therapy using vB_Sen-TO17 or vB_SenM-2 or a cocktail of these two phages in the *Galleria mellonella* animal model clearly showed that these phages have a therapeutic potential which should be next verified in studies with poultry.

In conclusion, the results presented in this study demonstrated high efficacy and favorable safety profile of tested bacteriophages in a battery of *in vitro* tests and in *in vivo* studies with an animal model. Thus, further large-scale studies with these phages as potential therapeutics in combating infections of poultry with *S. enterica* are substantiated.

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CRediT authorship contribution statement

Katarzyna Kosznik-Kwaśnick: Conceptualization, Investigation, Methodology, Visualization, Writing – review & editing. **Małgorzata Stasiłojć:** Investigation, Writing – review & editing. **Łukasz Grabowski:** Investigation, Writing – review & editing. Karolina Zdrojewska:

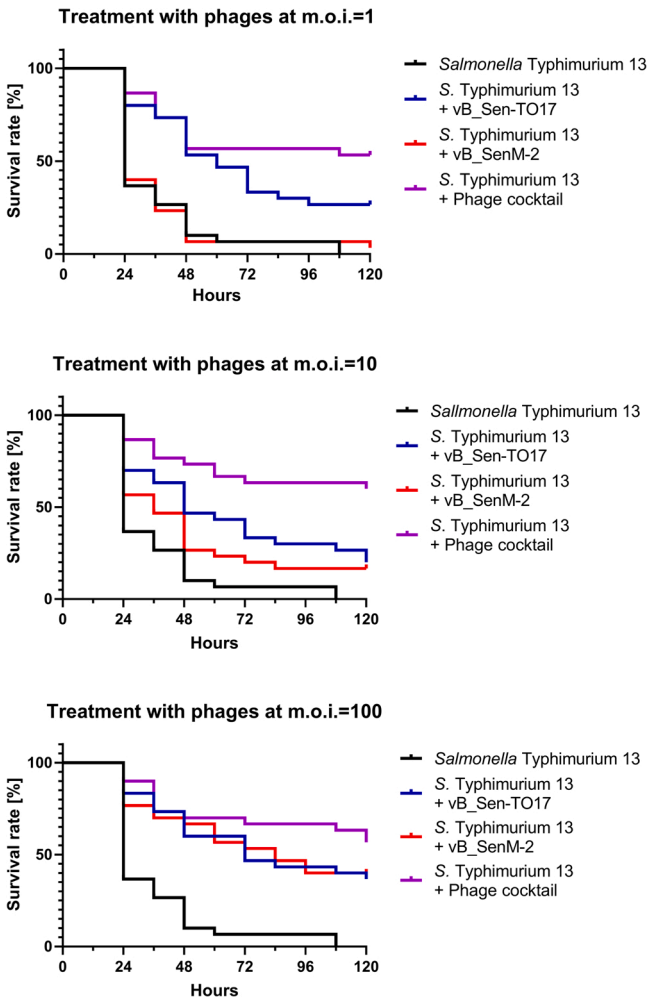

Fig. 13. Survival of *Galleria mellonella* larvae during *S. enterica* infection and phage therapy. Treatment of *S.* Typhimurium 13-infected larvae (10^3 PFU/animal) with phages vB_SenM-2 (red line), vB_Sen-TO17 (blue line) and phage cocktail (purple line) at m.o.i.= 1 (A), m.o.i.= 10 (B), and m.o.i.= 100 (C), in comparison to phage-untreated, *S.* Typhimurium 13-infected larvae (black line). Survival of larvae treated with a buffer or phage alone was 100% (these lines were skipped for clarity). Kaplan-Meier graph is shown, with values determined based on analysis of 30 animals in each group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Investigation, Writing – review & editing. **Grzegorz Węgrzyn:** Supervision, Writing – original draft, Writing – review & editing. **Alicja Węgrzyn:** Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing.

Declaration of interest

The authors declare no conflict of interest.

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