Comparison of the effectiveness of antibiotic treatment and phage therapy in eradication of Salmonella enterica responsible for infections of poultry

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Research articles included in this thesis:

Article 1: Kosznik-Kwaśnicka, K.; Topka, G.; Dydecka, A.; Necel, A.; Nejman-Faleńczyk, B.; Bloch, S.; Węgrzyn, G.; Węgrzyn, A. The Use of Bacteriophages in Animal Health and Food Protection. In Phage Therapy: A Practical Approach; Springer: Cham, Switzerland, 2019; pp. 213–256.

Article 2: Kosznik-Kwaśnicka, K, Ciemińska, K, Grabski, M, Grabowski, Ł, Górniak, M, Jurczak-Kurek, A, Węgrzyn, G, and Węgrzyn, A. 2020. Characteristics of a Series of Three Bacteriophages Infecting *Salmonella enterica* Strains, *International Journal of Molecular Sciences* 21, no. 17: 6152. https://doi.org/10.3390/ijms21176152

Article 3: Kosznik-Kwaśnicka, K, Grabowski, Ł, Grabski, M, Kaszubski, M, Górniak, M, Jurczak-Kurek, A, Węgrzyn, G, and Węgrzyn, A. 2020. Bacteriophages vB_Sen-TO17 and vB_Sen-E22, Newly Isolated Viruses from Chicken Feces, Specific for Several *Salmonella enterica* Strains *International Journal of Molecular Sciences* 21, no. 22: 8821. https://doi.org/10.3390/ijms21228821

Article 4: Kosznik-Kwaśnicka K, Stasiłojć M, Grabowski Ł, Zdrojewska K, Węgrzyn G, Węgrzyn A. (2022) 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 *Microbiological Research* Volume 261, 127052, https://doi.org/10.1016/j.micres.2022.127052

Article 5: Kosznik-Kwaśnicka K, Podlacha M, Grabowski Ł, Stasiłojć M, Nowak-Zaleska A, Ciemińska K, Cyske Z, Dydecka A, Gaffke L, Mantej J, Myślińska D, Necel A, Pierzynowska K, Piotrowska E, Radzanowska-Alenowicz E, Rintz E, Sitko K, Topka-Bielecka G, Węgrzyn G and Węgrzyn A (2022) Biological aspects of phage therapy versus antibiotics against *Salmonella enterica* serovar Typhimurium infection of chickens. *Frontiers in Celluar and Infection Microbiolology* 12:941867. doi: 10.3389/fcimb.2022.941867

Poultry is currently the most popular type of meat consumed in the world. In 2020, the average person consumed about 14.9 kg of poultry, in comparison, beef consumption was 6.4 kg/person, and pork 10.7 kg/person (OECD data for 2020, https://data.oecd.org/agroutput/meat-consumption.htm). The poultry meat production currently exceeds 100 million tonnes per annum, and is expected to increase by another 17% by 2030 taking up to 41% of meat products available on the market (OEDC-FAO). To meet the requirements of the growing consumers' needs, breeders focus on increasing the efficiency of production by increasing birds' growth rate and weight gain. For this purpose animal feed is enriched with amino acids, vitamins, enzymes and probiotics, and recently also with plant extracts or essential oils (Zhai et al. 2018; Borda-Molina et al. 2019). Starting from the 50's of the last century, the most popular growth promoters were antibiotics, e.g. tetracycline, bacitracin or penicillin-G (Castanon 2007). Research showed that administration of subtherapeutic doses of antibiotics increased the muscle tissue gain in farming animals by stabilizing the intestinal microbiome, which allowed for better adsorption of nutrients from the feed and thus resulted in an increased weight gain (Chattohyay 2014). However, after years of using antibiotics as feed additive in industrial farming it was reported that they may have a negative influence on the environment and on the human body. Long-term exposure to antibiotics present in meat was shown to result in prolonged activation of human immune system, resulting in oversensitivity and inflammation of the digestive system; moreover it was shown to impact the intestinal microbiome in a negative way (Donoghue 2003; Ramatla et al. 2020; Zhang and in 2021). In addition, it was shown that the usage of antibiotics as feed additives may be one of the reasons for the spread of antibiotic resistance among bacteria and for the spreading of those bacteria in the environment (Castanon 2007; Chattopadhyay 2014). Taking all the above points into consideration, World Health Organisation (WHO) as well as American Food and Drug Agency (FDA) began to push for a radical limitation of antibiotics' use as growth promoters.

The first regulation to delegalize the use of antibiotics in animal feed and as water additive was the European Union directive no. 1831/2003 that went into effect on 1st of January 2006 (Bedford 2000; Castanon 2007). Further regulation have later been introduced as the European Parliament and Council regulation no. 470/2009 regarding the limits of active compounds of pharmaceuticals in animal-based food products and EU regulation no. 2019/6 that banned the use of most antibiotics from use in veterinary medicine, including their use for therapeutic purposes.

Introduction of the regulations mentioned above regarding the use of antibiotics in industrial farming was meant to slow down the spread of antibiotic resistance among pathogenic bacteria and to improve overall quality of the obtained meat (Attia i in. 2011; Chattopadhyay 2014). However, introduction of this strict regulations also meant that farmers were left without any means of prevention against colonisation of their animals by pathogenic bacteria (Bedford 2000). Although, the enforcement of aforementioned restrictions resulted in drastic decrease in the use of antibiotics in animal farming among the European Union member countries, it has also brought up the need to search for ecological means of eradication of pathogenic bacteria from farming animals.

Salmonellosis is the disease that is most commonly associated with poultry meat and other produce of avian origin i.e. eggs. Salmonellosis is a disease caused by bacteria belonging to one of more than 2,500 serovars of the *Salmonella enterica* species, that differ in the level of pathogenicity. Serovars most commonly responsible for salmonellosis are *S.* Typhimurium i *S.* Enteridіdis (Gal-Mor i in. 2014) [**article 1**]. The infections with bacteria belonging to *Salmonella* genus differ in clinical symptoms and their severity. Among most frequent, there are stomach aches, diarrhoea (sometimes with blood in stool), fever and sometimes vomiting. In severe cases, the intestinal barrier can get compromised and the bacteria may enter the bloodstream resulting in severe complications, like sepsis or meningitis (Cianflone 2008; Sánchez-Vargas i in. 2011). It is estimated that each year around 100 million *S. enterica* infections occur with 150,000 deaths. In Europe, the number of cases is estimated as 690 per 100,000 yearly (Sánchez-Vargas i in. 2011; Gal-Mor i in. 2014). For most *S. enterica* serovars, birds act as carriers, without showing any signs of infections. Under favourable conditions, *S. enterica* colonizes chicken's gastrointestinal tract and later on, if the intestine get compromised during meat processing, the content may spill contaminating the meat (Fries 2002; Adeyanju i Ishola 2014).

Due to the fact that salmonellosis is one of the leading foodborne diseases many countries, including Poland, they implement the so called "zero tolerance" policy. It consists of, among other things, bird termination if during the veterinary control *S. enterica* is detected in the flock. The birds are not subjected to treatment due to aforementioned law restrictions, but also due to the fact that broad-spectrum antibiotics used in veterinary medicine have been proven to be highly toxic, with severe adverse effects. They also have long grace period after the use due to potential toxic effects on human meat consumers (Grabowski i in. 2022).

Since poultry farming and meat production industries are fast developing branches of world's food industry, and Poland is one of the leading countries in European Union in poultry meat production and export, there is a need to formulate alternative methods of prevention against *S. enterica* colonisation of poultry gastrointestinal tract (Pawłowska i Sosnówka-Czajka 2019). One of the proposed alternatives is the use of phage therapy, i.e. the use of preparations containing bacteriophages.

Bacteriophages are viruses that are able to infect and multiply inside bacterial cells. This feature of phage biology is the basis for their use in treatment of bacterial infections. Phage therapy in human have been successfully used for over 100 years in Georgia, where phage-based preparations are available as Off-The-Counter (OTC) medicines (Parfitt 2005). In countries belonging to European Union, including Poland, phage therapy is available as an experimental therapy. However, some countries have decided to formulate regulations enabling it's use as one of the standard method of treatment of bacterial infections (Pirnay i in. 2018). Bacteriophage properties have also been appreciated by different branches of the industry, including food industry. Year 2006 was the crucial year in the history of the use of bacteriophages in prevention of bacterial diseases. The US Food and

Drug Administration and US Department of Agriculture have approved several bacteriophage products to be used for food protection against *Listeria monocytogenes*: ListShield™ (Intralytix Inc.) and LISTEX (Micreos) [**article 1**]. Successes of those two preparations allowed for other phage-based products to be introduced to the market, including preparations against *S. enterica*. Currently, there are three phage-based products available on the market that are used for food protection against *S. enterica* contamination: SalmoFresh™ and SalmoLyse®, manufactured by Intralytix Inc., and Salmonellex™, manufactured by Micreos [**article 1**]. Additionally, many experiments have been conducted in order to evaluate the efficacy of bacteriophage-based preparations used in poultry farming in order to reduce the risk of the birds being colonized by *S. enterica*. Ahmadi et al. (2016) reported that phage administration for 3 days removed the biofilms formed by *S.* Enteritidis strains from the tonsils of quails. The use of the mixture of phage and probiotic by Torro et al. (2005) and Borie et al. (2009) resulted in statistically significant reduction in the number of *S.* Typhimurium and *S.* Enteritidis in chicken intestine, especially if the treatment was applied shortly after hatching [**article 1**]. Proteon Pharmaceuticals, a Polish company, have developed a phage cocktail that can be used as feed additive and is available on Russian, American and Ukrainian markets. The product, BAFASAL®, is a cocktail consisting of phages effectively infecting *S. enterica* serovars Enteritidis, Typhimurium, Typhi, and Paratyphi. During product testing *in vivo* in a group consisting of 220 broiler chickens, it was proven that the cocktail significantly reduced the number of *S.* Enteritidis in gastrointestinal tract of the birds in comparison with untreated control (Wójcik i in. 2020) [**article 1**].

The data presented by multiple research groups from all over the world indicated that the use of phage-based preparations might be a potential alternative to antibiotics in preventing the spread of infections. However, limitations of this form of treatment have also been observed. We are currently aware that the effectiveness of phage therapy is influenced by number of factors such as the serovar responsible for the infection, the type of phage used, different adaptation mechanisms developed by bacteria as well as the treatment scheme: the number of doses and cocktail formulation and administration. Furthermore, the efficacy of phage preparations is limited by the host range of bacteriophages in the cocktail, as phages can have a narrow host range. Some experiments have also been reported showing that the therapeutic effect lasted for a short period of time, as the emergence of phage-resistant bacteria was observed (Wernicki et al. 2017). There is also a limited number of research comparing the efficacy of phage therapy with other methods (e.g. antibiotic therapy). Furthermore, currently, we observe an increase in number of reports of phages interacting with eukaryotic cells. Phages are able to enter the bloodstream and penetrate to other internal organs (Podlacha et al. 2021). Therefore, the question is whether phage therapy is safe for potential consumers. Can bacteriophages impact human health and wellbeing in a negative way? Furthermore, it is unknown how long bacteriophages can prevail inside the organism receiving phage therapy. Such knowledge could help with determining the grace period, after which the meat would be considered safe to eat. All these reports clearly indicated that it is necessary to conduct comprehensive analysis of the effectiveness and safety of phage formulations preventing the colonization of the digestive system by *S. enterica* in poultry.

The aim of my dissertation was to create an experimental phage cocktail effective against the most common *S. enterica* serotypes: Typhimurium and Enteritidis, and to compare its effectiveness with antibiotics employed in veterinary medicine, by using various research models. Additionally, I have checked how long do bacteriophages prevail inside the animal organism after the end of the treatment, and whether they penetrate inside various internal organs. I have also wanted to determine whether phage-resistant strains of *Salmonella* can appear in response to phage treatment after finalizing the procedure.

My work begun with the characterization of three bacteriophages: vB_SenM-1, vB_SenM-2, and vB_SenS-3 infecting various *S. enterica* serotypes, that were part of the collection of the Department of Molecular Biology of the University of Gdańsk (Jurczak-Kurek et al. 2016). I have observed that these bacteriophages have a broad host spectrum and are stable at a variety of temperatures, including 42°C (bird body temperature) [**article 2**]. In order to evaluate their potential

use in the therapy, I have performed a detailed characterization of these bacteriophages, including adsorption rate, the time of a single life cycle, and the phage burst size at three different temperatures: 25°C, 37°C and 42°C, using four *S. enterica* strains from the collection of the National Salmonella Center (NSC) in Gdańsk: *S.* Typhimurium 12, *S.* Typhimurium 13, *S.* Enteritidis 64, and *S.* Enteritidis 1392. I have observed that all three bacteriophages effectively adsorbed on all tested strains and at all tested temperatures within 15 minutes. The most efficient adsorption process was detected for the vB_SenM-1 phage on *S.* Typhimurium 13 at 25°C and *S.* Enteritidis 1392 at 42°C, for the vB_SenM-2 phage on *S.* Typhimurium 12 at 25°C and *S.* Enteritidis 1392 at 25°C and 42°C, and vB_SenS-3 on *S.* Typhimurium 12 at 42°C and on *S.* Enteritidis 64 at 37°C [**article 2**]. During the "one-step growth" experiments, I have determined the kinetics of a single life cycle of a bacteriophage and the burst size. I have observed that the vB_SenS-3 phage developed poorly at 25 and 42°C when the host was *S.* Typhimurium 13 and *S.* Enteritidis 64, and vB_SenM-2 and vB_SenS-3 phage development was less efficient in *S.* Enteritidis 1392 regardless of temperature [**article 2**]. All three bacteriophages produced the largest burst size, ranging from about 100 to even 500 progeny virions, at 37°C, regardless of the host in which they have multiplied. Moreover, the phage yield between 100 and 400 progeny virions was observed for vB_SenM-1 on *S.* Typhimurium 12 and 13 at 25°C and *S.* Enteritidis 64 at 42°C, and for vB_SenM-2 on *S.* Typhimurium 12 at 25°C and *S.* Enteritidis 64 at 42°C [**article 2**]. The analysis of the lysis profile of the infected bacterial culture and the analysis of the biomass reduction and bacterial titer in the bacterial biofilm confirmed that these bacteriophages are able to reduce the titer of *S. enterica* under laboratory conditions. I have observed that all used m.o.i. values (0.1; 1.0; and 10) caused a significant decrease in optical density (OD_{600}) of the culture as well as bacterial titre (CFU / ml), but the most effective were m.o.i = 1.0 and 10, between which there was no statistically significant differences. In studies on bacterial biofilms, I have observed that each tested phage was able to reduce the bacterial titer by 47% -99% CFU/mL (depending on the host and incubation temperature) [**article 2**]. Analysis of vB_SenM-1, vB_SenM-2, and vB_SenS-3 genomes did not show the presence of toxin genes or integrase genes, which could suggest that these bacteriophages were temperate phages, a feature that would exclude their use in therapy. The lytic nature of these bacteriophages was further confirmed by experiments in which no formation of lysogenic bacteria was observed [**article 2**].

In addition to the characterisation of bacteriophages already being a part of collection of the Department of Molecular Biology of the University of Gdańsk, I have isolated new bacteriophages from chicken faeces and wastewater samples. I have isolated about twenty bacteriophages, but only two showed the greatest therapeutic potential and were subjected to detailed molecular characterization. The characterization of vB_Sen-TO17 and vB_Sen-E22 bacteriophages was performed at 42°C to assess their performance in the gastrointestinal tract [**article 3**]. The vB_Sen-TO17 bacteriophage was characterized as a narrow host range, and a detailed analysis of the adsorption kinetics and its life cycle showed that it multiplies effectively on *S.* Typhimurium 12 and 13 strains. The adsorption time to these strains was approx. 8 min, and the phage burst size ranged from 50 to 80 progeny virions per cell, while for *S.* Enteritidis 64 it was only about 10 virions per cell. The vB_Sen-E22 phage had a broader host spectrum than the vB_Sen-TO17 bacteriophage, but it was more sensitive to environmental factors, such as pH. For example, the vB_Sen-E22 bacteriophage was inactivated at pH = 2.2, while the vB_Sen-TO17 phage was inactivated at pH = 2.0. The adsorption and life cycle analysis showed that the adsorption time, life cycle length and phage burst size were similar to those of phage vB_Sen-TO17. Analysis of the vB_Sen-E22 phage genome showed that this bacteriophage does not carry genes encoding toxins or integrases in its genetic material, and its life cycle was classified as lytic [**article 3**].

Based on the research presented above, from the bacteriophages isolated by me and those available in the collection of the Department of Molecular Biology, I have chosen two of which I have formed an experimental phage cocktail: vB_Sen-TO17 and vB_SenM-2. In the next stage of my work, I have focused on comparing the effectiveness of a phage cocktail with the effectiveness of a single phage and antibiotics used in veterinary medicine: tetracycline, colistin and enrofloxacin. I have

conducted my research with the use of various research models, both *in vitro* and *in vivo* [**articles 4 and 5**]. At the first stage of this part of my work, I have analysed the lysis profiles of bacterial cultures infected with bacteriophages (10^9 PFU/ml, phage cocktail (2×10^9 PFU/ml) and with the addition of antibiotics: tetracycline (12.5 μg/ml), colistin (50 μg/ml) or enrofloxacin (50 μg/ml). I observed that the decrease of the optical density of the culture (OD_{600}) for *S*. Typhimurium 12 and 13 infected with phage cocktail was larger than those for the *S.* Enteritidis 64 and 1392 strains. The single phage, phage cocktail, and antibiotics colistin and tetracycline inhibited bacterial growth at similar rate. However, bacterial titer analysis showed that for *S.* Typhimurium 12 and 13, the phage cocktail reduced bacterial titer to a greater extent than a single phage did. The phage cocktail had effectiveness comparable to the treatment with tetracycline, and greater than colistin, but it was less effective than enrofloxacin. In the case of serotype *S.* Enteritidis, strains 64 and 1392, the cocktail, single phage, and colistin treatment had shown no significant differences. The antibiotics tetracycline and enrofloxacin were more effective in this experiment [**article 4**]. At the next stage of my work, I have analysed the influence of the aforementioned factors on the reduction of bacterial biomass and the bacterial titre in the biofilm. I have used this research model due to the fact that in the natural environment, including the intestines, bacteria rarely exist in a planktonic form. Signirficantly more often, they form complex structures consisting of bacterial cells and a mixture of exopolysaccharides, lipids and DNA, called a biofilm (Muhammad et al. 2020). During my experiments, I have observed that, as it was the case with a liquid culture, the phage cocktail was more effective in reducing the biofilm formed by *S*. Typhimurium strains 12 and 13 than in the case of *S*. Enteritidis strains 64 and 1392, and it was most effective after 24 h of incubation [**article 4**].

To mimic the growth conditions of the chicken intestine even more accurately, I have created a model of a multicultural biofilm, grown in microaerophilic conditions and containing other non-pathogenic bacteria from the Enterobacteriaceae family that may be present in birds' digestive system: *Escherichia coli*, *Citrobacter freundii*, and *Proteus mirabilis*, and a representative of lactic acid producing bacteria: *Lactobacillus acidophilus*. I have observed that bacteriophages and the phage cocktail showed similar effectiveness in reducing the titer of *S. enterica*, especially in the case of *S.* Typhimurium strains 12 and 13. However, they had no effect on the titer of other Enterobacteriaceae or on the titer of *L. acidophilus* [**article 4**]. The antibiotics tested, apart from a significant reduction in the titer of *S. enterica*, also significantly reduced the number of *E. coli*, *C. freundii* and *P. mirabilis*. Additionally, enrofloxacin showed a bacteriostatic effect against *L. acidophilus* [**article 4**].

Based on the conducted research, I have concluded that the phage cocktail was effective in reducing the bacterial titer on various models of bacterial culture. In some cases, its effectiveness was comparable to the antibiotics tetracycline and colistin. However, unlike antibiotics, phages and the cocktail did not affect other species of bacteria, which may suggest that they will be safer to use and will not have a negative effect on the intestinal microbiome of chickens.

The last stage of my research using *in vitro* models was the comparison of resistance development by *S. enterica* bacteria to bacteriophages and to colistin and enrofloxacin. I have observed that during the 24-hour incubation of bacteria with enrofloxacin, no resistant bacteria have emerged. In the case of the vB_Sen-TO17 and vB_SenM-2, phage cocktail and colistin, the rate of development of resistance by bacteria was comparable [**article 4**].

Due to the observed difference in effectiveness of the phage cocktail against different serotypes of *S. enterica*, I have decided to conduct further *in vivo* tests using only the Typhimurium 13 serotype. In the future, an analysis of the effectiveness of the cocktail should be carried out with the remaining strains of bacteria of the genus *Salmonella*, but it requires a large financial outlay and additional approvals of the ethics committee.

The first *in vivo* experiment I have carried out was the assessment of phage cocktail efficacy using the *Galleria mellonella* model. This model has already been successfully used to assess the effectiveness of phage therapy and other bactericidal substances, as it has been observed that experiments implementing this simple research model can produce reliable results in a short period of time, limiting the use of vertebrates at the same time (Grygorcewicz et al. 2020; Kaźmierczak et al.

2022). The larvae were infected with *S.* Typhimurium 13 strain at a dose of 5×10^3 CFU/animal. One hour after infection, the larvae were administered with vB_Sen-TO17 or vB_SenM-2 bacteriophage lysate or phage cocktail at m.o.i = 1, 10 or 100. In the control group, the survival was 40% and 10%, respectively, after 24 and 48 hours of infection, and after 108 hours, all animals were reported dead. The administration of vB_Sen-TO17 led to a significant increase in the survival rate of the larvae in comparison to the control group, regardless of the dose used. In the case of the vB_SenM-2 phage, the use of m.o.i = 1 showed no therapeutic effect, and a statistically significant difference in survival was observed when this phage was administered at m.o.i = 100. The highest percentage of survival (between 65% and 75%) was observed when using the phage experimental cocktail. I did not find statistically significant differences depending on the used m.o.i. These results confirmed that the use of the cocktail gives better therapeutic effects than the use of single phages, even if the effect was not so clearly visible during *in vitro* studies [**article 4**].

The last stage of my work was to experimentally check the efficacy of experimental phage cocktail in the elimination of *S.* Typhimurium 13 using a domestic chicken, *Gallus gallus*, as a model. The research was carried out in the Pavilion of Experimental Bird Infections at the Department of Bird Diseases, Faculty of Veterinary Medicine at the University of Warmia and Mazury in Olsztyn. Chickens at the 7^{th} day of life were divided into 8 groups consisting of 25 individuals each. On the first day of the experiment, birds from groups 3-8 were infected with 1 ml of *S.* Typhimurium suspension with a titer of 10^6 CFU/ml in 0.89% NaCl. Twenty four hours post infection, group 6 has been treated with a phage cocktail at the titer of 2×10^9 PFU/ml (1×10^9 PFU/ml of each phage) in 20 mM $CaCO_3$ for 14 days. Groups 4 and 5 received an antibiotic for 5 days: enrofloxacin at a dose of 10 mg/kg (group 4) and colistin; 120,000 IU/kg (group 5). Group 1 was the negative control which received 0.89% NaCl for 14 days. Group 2 received a phage cocktail for 14 days, while group 3, infected with *S.* Typhimurium, was a positive control and, similarly to the first group, received 0.89% NaCl. Group 7 received the first dose of phage cocktail 2 days after detection of *S.* Typhimurium in the faeces and Group 8 - 4 days after detection of *S.* Typhimurium. In both cases, the full cycle of phage therapy lasted 14 days. In order to determine the titer of *S.* Typhimurium and bacteriophages, samples of chicken faeces were collected daily. Additionally, each day cloaca swabs were collected from five randomly selected chickens in each group. On the 7^{th} day of the experiment, after the end of antibiotic administration to the chickens, 5 animals from each group were terminated and their organs were examined for the presence of phages and bacteria. On day 21, after completion of the phage therapy cycle, another 5 animals from each group were anesthetized. Two further terminations were made on days 28 and 35 of the experiment. The entire experiment lasted 35 days and corresponded to a full breeding cycle of a broiler [**article 5**].

While monitoring the titer of *S.* Typhimurium in the faeces and cloaca swabs, I did not observe the presence of *S.* Typhimurium in any of these materials in the case of the groups that received the antibiotic and the phage cocktail 24 hours after the infection. In the remaining groups, *S.* Typhimurium have appeared in the samples between 4 and 6 days after infection, and its mean titer was approx. 10^5 CFU/ml in faecal samples and approx. 5.5×10^4 CFU/ml in the cloaca swabs. For the groups that received phage therapy after developing *S.* Typhimurium infection, I have stopped detecting bacteria in the faeces between days 10 and 13 of the experiment and after 10 days in the cloaca swabs. In the case of the control group No. 3, the titer of *S.* Typhimurium fell below the detection level around the 13^{th} day of the experiment. In this case, the infection probably continued, but I was not able to detect it in biological materials, as the biochemical parameters of the chicken blood tested by other team members indicated inflammation and ongoing bacterial infection (Grabowski et al.. Analysing the bacteriophage titer, I have observed that in the groups that received the cocktail (Groups 2, 6, 7, 8) both bacteriophages were present in both tested biological materials (stool samples and cloaca swabs) for up to 25 days, if they were in contact with the host (14 days of therapy included). Bacteriophages remained in the faeces (Groups 6, 7, 8) for about 1.5-2 weeks after the end of therapy. In the case of group 2, which was not infected with bacteria, I have failed detecting bacteriophages in biological materials about 7 days after finalizing the therapy.

The next stage of my work was to analyse bacteriophage penetration into the internal organs of chickens. I have noticed that bacteriophages were present only in the organs of birds which were terminated shortly after the end of phage administration or during the administration of a treatment. I have found that bacteriophages were mainly present in the stomach and intestines of chickens. Moreover, the presence of phages was most often observed in organs such as liver, spleen and kidneys, which is consistent with the literature data (Dabrowska et al. 2005; Podlacha et al. 2021). I have also observed that phage penetration into internal organs depended on the kind of bacteriophage (vB_Sen-TO17 phage penetrated into organs to a greater extent than vB_SenM-2 phage) and individual characteristics of each individual chicken [**article 5**].

In my work, I have also checked whether the bacteriophage therapy had any effect on the composition of the intestinal microbiome of chickens in comparison with the untreated control groups and groups that have received antibiotic therapy. For this purpose, genetic material was isolated from the content of chicken intestines collected during dissection, and after preparation in accordance with proper standards for sequencing, the V3-V4 regions of the gene encoding the 16S rRNA were amplified and sequenced. Analysing the obtained data, I have noticed that in the case of chickens from the control group, after the microbiome have stabilised, bacteria from the Lactobacillaceae and Lachnospiraceae families were the most abundant, with a significant share of Rumincoccaceae. *S.* Typhimurium infection resulted in a significant increase in the number of Enterobacteriaceae, but additionally, the percentage of Enterococcaceae in the intestinal microbiome also significantly increased. Treatment with antibiotics, enrofloxacin or colistin, resulted in a significant increase in the percentage of Enterococcaceae and Ruminococcaceae, and in subsequent terminations, I have also observed a significant dominance of Lactobacillaceae over other bacterial families. The use of bacteriophages initially increased the percentage of other bacterial phyla, including Lactobacillaceae in comparison with the control, but the microbiome stabilized very quickly and in the later stages of the experiment, I have not observed statistically significant differences between the groups receiving phage therapy and the control group. In the case of the groups receiving antibiotic therapy, the microbiome showed statistically significant differences until the very end of the experiment, if compared with the control group, with the dominant phyla being Enterococcaceae, Lactobacteriaceae and Enterobacteriaceae [**article 5**].

In summary:

Due to the emergence of drug-resistant *S. enterica* strains, the prevalence of salmonellosis and restrictions on the use of antibiotics in poultry farming, it becomes necessary to search for alternative methods to combat infections caused by these bacteria. Bacteriophages are one of the proposed alternatives, and although there are many reports on the effectiveness of phage therapy in chickens (Wernicki et al. 2017) [**article 1**], there are no studies comparing the effectiveness of this method of treatment with the antibiotic therapy that was available so far. Therefore, the aim of my work was to create an experimental phage cocktail and performance of a comprehensive analysis of its effectiveness implementing various research models. I have also aimed to compare phage cocktail effectiveness with antibiotics used in veterinary medicine. In my work, I have characterized a number of bacteriophages infecting various *S. enterica* serotypes in order to determine their therapeutic potential [**articles 2 and 3**]. From the bacteriophages I have characterized, I chose two, vB_SenM-2 and vB_Sen-TO17, and I have created an experimental phage cocktail consisting of those phages. Then, I have compared the activity of this cocktail and antibiotics: tetracycline, colistin and enrofloxacin, using two serotypes of *S. enterica*: *S.* Enteritidis and *S.* Typhimurium, with employing different laboratory models [**article 4**]. During *in vitro* experiments, I have noticed that the phage cocktail showed a similar antibacterial activity to the antibiotics: tetracycline and colistin. Using a multispecies biofilm model, I have observed that bacteriophages were more selective than antibiotics and did not reduce the titer of other members of Enterobacteriaceae [**article 4].** On the basis of *in vitro* experiments, I have concluded that the cocktail I have created has a therapeutic potential, and I have decided to conduct further research using two *in vivo* animal models: *Galleria mellonella* and *Gallus*

gallus. Using the *Galleria mellonella* model, I have observed that the phage cocktail significantly increased the survival rate of the larvae, regardless of the applied dose. When used alone, bacteriophages were not as effective, and their therapeutic effect depended on the m.o.i. used [**article 4**]. During the experiments with a chicken model (*Gallus gallus*), I have observed that administration of the phage cocktail shortly after infection prevented the development of infection. Thus, the phage cocktail was as effective as the antibiotics used in veterinary medicine. Bacteriophages administered after the appearance of *S.* Typhimurium in the faeces were able to control the infection, showing that bacteriophages could be potentially used to treat infections in poultry in the future. However, the current legal regulations do not allow the treatment of chickens infected with *Salmonella* strains, hence the preparation can be successfully used in the prevention of infections and as a disinfectant (after the amendment of EU law on the use of preparations based on phages). In addition, I have observed that bacteriophages remained in the chicken feces for about 10-14 days after the end of the treatment with formulation containing bacteriophages. I have isolated phages from internal organs only if chickens were treated during termination or if termination took place several days after the treatment have ended. Additionally, I did not isolate the phage-resistant strains of bacteria of the genus *Salmonella* after the end of the therapy. The study of the intestinal microbiome of chickens confirmed my observations made during *in vitro* tests on a multispecies model of bacterial biofilm. During the therapy, there were changes in the percentage of individual bacterial families compared to the control group (probably due to the lysis of *S. enterica* bacteria an ecological niche was free to inhabit by other bacteria), but after the end of the treatment, the percentage of individual families in the intestinal microbiome stabilized and did not differ from the control group in a significant way [**article 5**]. The research I have carried out suggested that phage therapy may be an effective method of preventing *S. enterica* infections in poultry, if it is administered from the first days of life, as a preventive measure. As bacteriophages are removed from the organism of chickens within a few to several days after the end of therapy, they should not cause concern to consumers.

From the safety point of view, it is also important to analyse the appearance of mutations in the phage genome and the theoretical possibility of transferring bacterial or eukaryotic DNA elements. Therefore, it is important that the genetic material isolated by me from bacteriophages after the end of therapy should be subjected to a detailed analysis in search for foreign genetic material and genome rearrangements.

Bacteriophages are one of the most promising tools to combat bacterial infections in animals, but before their introduction to widespread use, it is necessary to collect as much data as possible on the effectiveness and safety of this therapy, and to create a treatment schedule, including the dosage and duration of therapy and the possibility of production of antibodies against bacteriophages, which could significantly reduce the effectiveness of the therapy and should be taken into account as well.

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