USE OF BACTERIOPHAGES TO CONTROL Salmonella Enteritidis IN FECAL FERMENTATION

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Abstract

Salmonella Enteritidis (SE) is one of the main serovar of Salmonella enterica involved in foodborne infections. When intestinal infection by SE requires treatments using antimicrobial, cases can be aggravated if the cause bacteria are resistant to the drugs used. A possible alternative to control these antimicrobial resistant bacteria is phagotherapy, which is characterized by the use of bacteriophages that will lead to the lysis of the target bacteria. The objective was to evaluate the in vitro efficacy of a bacteriophage cocktail for the inactivation of SE in fecal fermentation. Fecal samples were collected from healthy donors and the fecal fermentation preparation was carried out. For the tests, a pool with three different SE isolates was used, and the bacteriophage cocktail was elaborated with the phages UPF_BP1, UPF_BP2 and UPF_BP3. Four different treatments were evaluated: the group called ASF, where the action of the bacteriophage cocktail against a pool of SE in fecal fermentation was tested; the CP1 group (positive control 1), in which only SE was inoculated in fecal fermentation; the CP2 group (positive control 2), in which the bacteriophage cocktail was inoculated in the fecal fermentation; and the CN group (negative control), containing only fecal fermentation. In all treatments the quantification of SE was performed, and in the ASF group, the quantification of phages was also performed. In the CP1 group there was a growth of 3,76 log_{10} UFC/mL, while in the ASF group there was a decrease in Salmonella showing 0,77 log_{10} UFC/mL. The results showed that the use of the bacteriophage cocktail against SE in fecal fermentation was able to reduce the amount of Salmonella in the sample. Despite not showing a

significant difference (p=0.059), this in vitro sudy demonstrates the ability of phages to act against Salmonella in fecal fermentation, bringing positive evidence that corroborates the continuity of the study for future uses of phage therapy.

Keywords: Bacteriphage cocktail. *Salmonella*. Feces. Fecal fermentation.

Introduction

Salmonella spp. is a facultative anaerobic Gram-negative bacterium belonging to the family Enterobacteriaceae. The genus Salmonella has two species, S. enterica and S. bongori. *Salmonella enterica* has about 2,659 serovars (Eng et al 2015), six subspecies, and can be divided into two different categories: typhoid species (typhoid fever) and nontyphoid species (diarrhea-enterocolitis) (Nair et al 2018, Pradhan et al 2019).

Salmonella enterica infections in humans are transmitted through food ingestion, and popularly known as salmonellosis. The disease is characterized by gastroenteritis, with fever, abdominal pain, vomiting, and diarrhea, affecting 98.3 million people a year, resulting in 155,000 deaths annually. *Salmonella* enterica subspecies enterica serovar Enteritidis, popularly known as *Salmonella* Enteritidis (SE), is one of the main serovars related to foodborne diseases (Reem et al 2021, Morel et al 2020).

The most indicated treatment for this disease is the use of antimicrobials such as ampicillin, ceftriaxone, and azithromycin. The indiscriminate use of antimicrobials for prophylaxis and therapy is a public health problem in Brazil (Zenghai et al 2019), because it leads to the development of pathogen resistance through the transfer of plasmid genes among bacteria, and places Salmonella in the group of multiresistant drugs (Hsu et al., 2019).

The control of antimicrobial resistant bacteria can be influenced by phage therapy, by using bacteriophages, viruses that infect bacteria and lyses the host bacteria, which can control or prevent an infection (Moelling et al 2018, Febvre et al 2019, Wang et al 2019).

Bacterial infections cause de-biosis in the gut microbiome, causing a homeostatic imbalance, and the use of antimicrobials contributes to a decrease in the beneficial microbiota, facilitating the development of diseases more easily. To reorganize the intestinal microbiota and increase the diversity of beneficial bacteria, the use of bacteriophages can be chosen, which act on specific bacteria, leading to the establishment of the microbiome, and can become a favorable alternative, such as the use of probiotic bacteria (d'Humières C et al 2019). The human intestine is composed of a vast community of commensal bacteria, mostly from the phylum Firmicutes and Bacteroidetes (Hsu et al 2019). It is estimated that there are around 1,000 to 7,000 species of beneficial bacteria and that 109-1012 viruses can be found per gram of feces, which makes the contribution of bacteriophages to the balance of the intestinal microbiota and its effects on the human host important (d'Humières C et al 2019), since the use of antimicrobials alters this beneficial microbiota.

In this context, this study aimed to investigate the "in vitro" efficacy of using a bacteriophage cocktail against SE in fecal fermentation samples.

Method

Research Ethics

This research was approved by the Research Ethics Committee of the University of Passo Fundo under number 4.415.862.

Salmonella Enteritidis

In this study, three different SE samples were used. The ATCC 13076 strain of *Salmonella* enterica subspecies enterica serovar Enteritidis, and two different isolates of SE, named Salmonella Enteritidis 24 (SE24), from coproculture of patients involved in outbreaks with food of poultry origin, and Salmonella Enteritidis 69 (SE69), isolated from mayonnaise salad with potatoes, both kindly provided by Prof. Dr. Eduardo César Tondo from UFRGS (Pottker et al 2020, Sillankorva 2018), are from the bacterioteca of the Laboratory of Bacteriology and Mycology of the Veterinary Hospital of FAMV/UPF.

SE24 and SE69 were selected because they had been tested in previous experiments performed by the research group (Oliveira et al 2019, Pottker et al 2020, Sillankorva 2018, Silva 2014), and SE ATCC 13076 because it was a reference strain. They were stored frozen at -20±1ºC in Brain-Heart Infusion Broth (BHI, HiMedia®) with 20% glycerol, were reactivated in selective media, and purity was verified by colony pattern, biochemical and serological confirmation.

For the experiment the three isolates of SE were used in a pool, homogenized in the same proportion, after cultivation in BHI broth at 37±1ºC for 18h to 24h.

Bacteriophage Coktail

To prepare the cocktail, three bacteriophages were used in the study, named UPF_BP1, UPF_BP2 and UPF_BP3, previously isolated and characterized by our research group (Pottker et al 2020). The plaque assay method was used for the recovery of the phages, which were preserved in an ultrafreezer, based on the semi-solid medium overlay technique according to Sillankorva et al. (2008), with modifications.

For recovery, the bacteriophages UPF_BP1, UPF_BP2 and UPF_BP3 were amplified using the host bacteria S. Brandenburg, S. Bredeney and S. Enteritidis, respectively, the same used in the isolation, which were stored frozen at -20±1ºC in Brain-Heart Infusion Broth (BHI, HiMedia®) with 20% glycerol. They were reactivated in selective media, and purity was verified by colony pattern, biochemical and serological confirmation. After reactivation, a characteristic colony was selected to be inoculated again in BHI broth and incubated at 37±1ºC for 18h.

Bacteriophage amplification, to increase the number of phages to be worked on, was performed from a 125 mL solution of Soy Tryptone Broth (TSB) with double concentration, in which 50 mL of the respective host bacterium was inoculated individually at a concentration of 107 CFU/mL, and 25 mL of the stock phage was added, then incubated for 18 hours at 37±1ºC. After the incubation period, 20 mL of chloroform P.A. solution was added to the tube and centrifuged under refrigeration at 4°C at 8000 rpm for 5 to 10 minutes (min). Then, the supernatant was filtered in a syringe with 0.22 µm filters and transferred to sterile vials, from which 20 µL was withdrawn for inoculation in 5 mL of BHI broth,

incubated for 18 to 24 hours at 37±1°C to confirm the absence of bacterial multiplication. Afterwards, the stock solution of purified phages was stored at 4°C (Sillankorva et al 2008).

To confirm the titration of the bacteriophages to be used, individually for each of the three, serial decimal dilution and an inoculation of 100 µL of the phage stock solution into microtubules with 900 µL of saline solution (0.85%) was used, up to dilution 10-10. After dilution, the microtubules were left immobile for a period of 15 min to 30 min for pre-adsorption of the phages (Sillankorva et al 2008). For quantification, Petri dishes were prepared with a thin layer of Soy Tryptone Agar (TSA), and on top of the layer, added 100 µL of the host bacterium respectively used for phage isolation, which had been grown overnight, and 100 µL of the phage dilution. Immediately after, 5 mL of semi-solid agar was poured, and movements were performed for homogenization. After drying the agar, the plates were incubated at 36 ± 1 ºC for 24 h. Halos or phage plaques were counted and titration was determined in Plate Forming Units per milliliter (PFU/mL), according to the equation below (Sillankorva et al 2008):

Bacteriophage titer (PFU/mL) = Number of lysis plates x dilution factor/Volume of bacteriophage sample (mL).

To prepare the cocktail, at the time of the experiment, the different phage stock solutions were homogenized in equal parts in a 1:1:1 ratio to form the cocktail, which was used for inoculation into the fecal fermentation.

Preparation of the fecal fermentation

Four donors of fecal samples were used, apparently healthy, with a mean age of 34 years, normal Body Mass Index (BMI) (18.5 kg/m2 < BMI < 23.9 kg/m2), without any known gastrointestinal pathology, or treatment with antimicrobials, during the last three months prior to collection. The informed consent form (ICF) for the research was read and signed by the donors.

After the feces were collected they were immediately placed inside an anaerobic chamber, and all procedures were performed within 4 hours of collection.

For the fecal fermentation preparation, 25g of fresh homogenized fecal samples and carbonate-phosphate buffer, sterilized in an autoclave at 121°C for 20 min, were used and placed in an anaerobic chamber overnight before use (Gaspar et al 2015, Gaspar et al 2013).

To prepare the fecal paste, the four fecal samples were homogenized with carbonate-phosphate buffer in a 1:3 (w/v) ratio, and 4 mL of carbonate-phosphate buffer was added to the anaerobic tubes.

Treatments used

Four different treatments were used, named ASF, CP1, CP2 and CN. The ASF treatment received the inoculum from the SE pool and the bacteriophage cocktail; the CP1 treatment (positive control 1) received only the SE pool; the CP2 treatment (positive control 2) received only the bacteriophage cocktail; and the CN treatment was the negative control, which received neither the SE pool nor the bacteriophage cocktail. Three tubes with fecal fermentation were used for each of the treatments, characterizing three repetitions in each one.

After inoculation, all replicates of each treatment in fecal fermentation were incubated at 37°C for 24 h (Gaspar et al 2013). S. Enteritidis was quantified in all treatments, and phage quantification was also performed in the ASF group.

Methodology for quantification of Salmonella Enteritidis in fecal fermentation

After 24 hours of incubation, from each tube of each treatment, an aliquot of the fecal fermentation solution was taken for SE quantification. Serial decimal dilutions of each tube were performed, starting with a 100 µL aliquot of each treatment individually, up to 10-7 dilution. For quantification, 100 µL of each dilution, individually, was inoculated on the surface in deoxycholate-lysine-xylose (XLD) agar and incubated at 37º C for 24 h to obtain the number of colony forming units (CFU) per milliliter of fecal fermentation.

Methodology for quantification of bacteriophages in fecal fermentation

For the quantification of bacteriophages per mL of fecal fermentation, in the ASF treatment, a similar methodology to that of titration was used. Serial decimal dilution was performed from a 100 µL aliquot of each treatment, in microtubes with 900 µL of saline solution (0.85%), up to 10-7 dilution. Then, in Petri dishes containing thin layer of TSA agar, 100 µL of each dilution and 100 µL of the SE pool used in the experiment was added. Then, 5 mL of semisolid agar was poured over the inocula, and homogenized. After drying the agar, the plates were incubated at 36 ºC ± 1 ºC and the halos (phage plates) were quantified after 24 hours of incubation (Sillankorva et al 2008).

Statistical Analysis

The results obtained were tabulated in an Excel spread sheet, and the CFU/mL Salmonella variable was transformed using the log10 function (CFU/mL Salmonella + 1). For the comparison of the groups, it was decided to develop a resampling with repetition (n=1000), employing, from this, an independent t test, for non-homogeneous variances, being considered significant the value of $p \leq 0.05$.

Results

Bacteriophage sterility test and titration

In BHI broth, it can be seen that there was no bacterial growth in the positive sterility test for phage stocks UPF_BP1, UPF_BP2 and UPF_BP3.

Table 1 shows the bacteriophage titers against the different serovars studied, as well as their corresponding serovar, which confirms their effectiveness and confirms their viability for use in phage pools, as they are more efficient.

Table 1

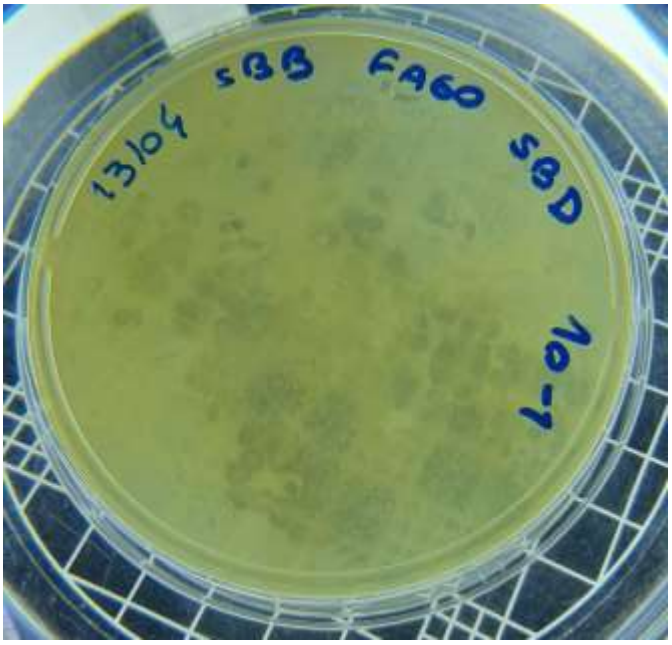
Bacteriophage titration (UFP/mL).

Bacteriophage titration (UFP/mL)			
	Sorovars		
Phages	*S.* Brandenburg	*S.* Bredeney	*S.* Enteritidis
UPF_BP1	5,6 x 10^{10}	-	-
UPF_BP2	2,6 x 10^{10}	4,0 x 10^{10}	-
UPF_BP3	-	-	1,8 x 10^{10}

The formation of phage halos can be observed on the quantification plates when different strains of *Salmonella* are used, as shown in figure 1, where lytic action of the phage UPF_BP2 against S. Brandenburg was identified.

Figure 1

Titration test.



Quantification of Salmonella Enteritidis in fecal fermentation

In treatments CP2, in which only the bacteriophage cocktail was inoculated, and CN, only the fecal fermentation, no SE was recovered.

In treatment CP1, where the SE pool was inoculated, it was possible to recover, after 24 hours of incubation in fecal fermentation, an average of 5,900 CFU of SE per mL of fecal fermentation, corresponding to 3,76 log10 CFU/mL.

In the ASF treatment, in which the SE pool and the bacteriophage cocktail were inoculated, it was possible to recover, after 24 hours of incubation in fecal fermentation, an average of 67 CFU of Salmonella Enteritidis per mL of fecal fermentation, corresponding to 0.77 log10 CFU/mL, lower than in CP1.

Table 2 shows the results of the statistical analysis performed with the averages of the results obtained in log10 CFU/mL. The groups were compared after resampling with repetition (n=1000). From the resampling, the independent t test for non-homogeneous variances was performed. When evaluating the difference in means, the results were considered significantly different (p=0.059), not significant for our study.

Table 2

Statistical analysis between CP1 and ASF treatments.

Treatments	Average difference	Lean[a]	Standard Error	Value of *p*[b]
CP1 vs. ASF	-3,00	0,042	0,62	0,059

Legend: [a] Based on 963 samples, [b] Independent t-test for in homogeneous variances, t = -3,90.

Quantification of bacteriophages in fecal fermentation

In figure 2 we have the demonstration of phage plaque formation, observed in the lysis area, the clearest halos, which demonstrate the recovery of viable phages after incubation of the fecal fermentation of the ASF treatment. Table 3 presents the results obtained.

Figure 2

Presence of halos, demonstrating areas of phage lysis.

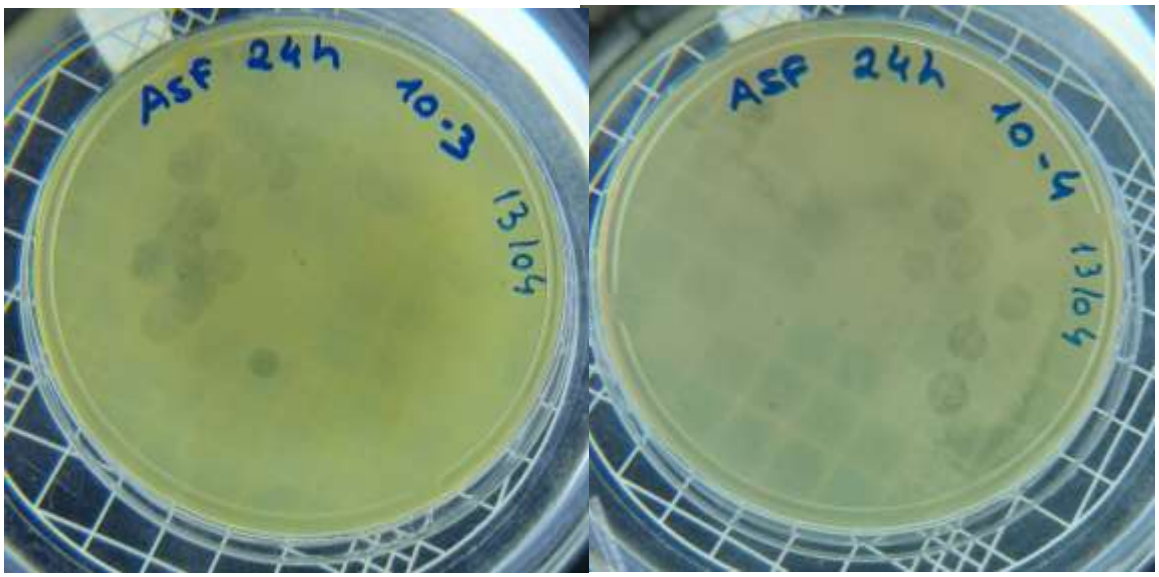


Table 3

Quantification of bacteriophages in the ASF treatment.

Treatment	Bacteriophage Quantification	
	Average UFP/mL	Mean log10 UFP/mL
ASF	**586**	**2,768**

Discussion

The use of this pool of phages in the study was determined because they have presented efficacy in other works carried out by the research group, with lytic action in different serovars. The use of a single bacteriophage has a lower lytic effect on the host bacterium, as demonstrated in previous studies by our group (Pottker et al 2020).

The sterility test and the titration prove that no bacterial growth occurred, which confirms that the use of the phages tested is effective for carrying out the tests, as shown in figures 1 and 2, and may be a viable option in combating other bacterial strains.

Infection of the small intestine by *Salmonella* spp, is influenced by the production of short chain fatty acids. It is identified in some works that feces are mostly composed of fatty acids, which facilitates their utilization and provides a favorable enrichment medium for proliferation, as proposed in our work (Huang et al 2008, Ser et al 2021, Xin et al 2021).

In our study it was observed that when SE was added to fecal fermentation samples, without the use of bacteriophages (treatment CP1), it was able to multiply, in interaction with the other bacteria in the feces, obtaining an average of 5,900 CFU/mL of SE (3,76 log10 CFU/mL), corroborating the data presented by Gaspar (2013), assuming that bacterial growth may have been influenced by the "in vitro" environment performed in the study.

In the ASF treatment, where the bacteriophage cocktail was inoculated, in addition to SE, there was a control of bacterial growth, obtaining an average of 67 CFU/mL of SE (0,7 log10 CFU/mL), that is, less SE than in CP1, demonstrating the possibility that the bacteriophage cocktail used is beneficial in the control of gastroenteritis caused by SE.

Feces have a favorable pH and environment for the development of bacteria, present a large amount of the commensal intestinal microbiota of the colon, and influence the infectivity capacity of different pathogenic serovars, which makes the use of biological control a viable option (Karin 2018).

The commensal microbiota of feces is composed of numerous bacterial species, including E. coli. It is known that the presence of commensal bacteria in the in vitro sample may have influenced the SE count through competition between different species, as observed in the effect of the presence of E.coli by Gaspar (2015), explained by being the result of a specific inhibition mechanism mediated by toxin production, which may have caused the growth of the strains studied.

Phages are used as therapy options in the treatment of many pathologies, as demonstrated by several studies (Marzanna et al 2017, Kristin et al 2015). Phages are seen to be used in biological control of foods (Endersen et al 2020), as well as in prevention and restoration of the gut microbiota (Guitiérreze et al 2020, Sutton et al 2019).

The presence of the phage halos in the ASF group samples in our study demonstrates that the SE pool was susceptible to the bacteriophage cocktail, and that these phages remained viable for the incubation period in the fecal fermentation.

Dallal et al. (2019) analyzed 24 mice, of which were infected with 1,5x107 CFU/mL of SE via oral administration, in group A and B, subsequently 1,63x108 CFU/mL was found in the stool samples and 2,1x107 CFU/mL in the liver sample of group B, in which all died because they presented clinical findings in the spleen and liver. In group A, the phage was administered 2x108 CFU/mL, concluding that there was no change in the liver and spleen samples, so the oral administration of phages for Salmonella was effective, which corroborates our study.

In another study, chicken carcass was contaminated with SE at a concentration of 1x105 CFU/cm². These samples were treated with a cocktail of five phages and, as a result, a decrease of 1 log CFU/m² was observed. The use of bacteriophages in the control of poultry carcass contamination is a good option to be used for the control of Salmonella in food (Hungaro et al 2013), corroborating the effectiveness of phages in various treatments against Salmonella.

In recent years, the use of antibiotics in Brazil has increased due to the resistance of pathogens in infections, which makes the use of phage therapy a viable and beneficial option to reduce the indiscriminate use and adverse effects caused by antimicrobials (Brazilian 2017). In our "in vitro" study, we evidenced that phages reduced the population of SE, considered resistant pathogens.

Thus, it is observed that, although there is a reduction in the number of SE in the treatment with bacteriophages, further studies are needed, with a greater number of repetitions, and different serovars, as well as "in vivo" studies to evaluate whether the phages, in addition to reducing Salmonella, do not alter the commensal microbiota.

Conclusion

We can conclude that the bacteriophage cocktail used reduced the SE population in a fecal fermentation environment. However, further studies are needed on the use of these bacteriophages as phage therapy, for possible treatment and/or prevention of intestinal infections by *Salmonella*.

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