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Isolation, Characterization, and Application of Bacteriophages against *Salmonella enterica.*

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Graphical Abstract

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Abstract.

Salmonella infection is an important food borne consumer health concern that can be mitigated during food processing. Bacteriophage therapy imparts many advantages over conventional chemical preservatives including pathogen specificity, natural derivation, potency, and providing a high degree of safety. The objective of this study aimed to isolate and characterize phages that effectively control Salmonella food contamination. A total of 36 bacteriophages infecting *Salmonella enterica* were isolated, tested at different pH ranging from 3 to 11, and high temperatures from 37 ° C to 70 ° C, then tested against 11 strains in order to define their host range. The kinetics of phages have been studied in order to understand their lysis process. The genomic restriction profile of the isolated phages was interpreted following the action of 5 restriction enzymes (BamHI, EcoRI, HindIII, and EcoRV and NdeI). Tests of the application

	of these phages were carried out on a food matrix in order to evaluate their ability to fight against *Salmonella enterica*. The results obtained are very encouraging, showing the possibility of using the bacteriophages isolated against *Salmonella enterica*, which can have a significant socio-economic impact.

Introduction *(optional)*

The Gram-negative bacterial genus *Salmonella* belongs to the family Enterobacteriaceae, order Enterobacteriales, class Gammaproteobacteria and phylum Proteobacteria. *Salmonella* is the most common cause of the acquired bacterial foodborne illness named as Salmonellosis. Almost all strains of Salmonella are pathogenic and are predominately harbored in eggs, meats, animal products such as milk, or contaminated vegetables causing disease in human beings consuming the contaminated food. Foodborne illness due to *Salmonella*-contaminated pork products is an important public health problem, causing economic losses, because the presence of this pathogen can limit meat exports from pork-producing countries (Rostagno and Callaway, 2012).

Bacteriophages (phages) are natural predators of bacteria and are ubiquitous in the environment (Rohwer and Edwards, 2002). The use of bacteriophages is an alternative to antibiotics that has been increasingly used on animal production experiments, and it was suggested for prophylactic control and reduction of pathogens (Mahony et al., 2011). The extension of phage biocontrol to food applications has been investigated for a long time until now (Greer, 2005; Kazi M and Annapure US, 2016).

Keeping in view the great efficacy of bacteriophages in controlling pathogens, the present study aims to isolate and characterize bacteriophages which effectively target human pathogenic Salmonella. Moreover, we seek to establish the potential of candidate phages to control Salmonella contamination in ready to eat foods including milk.

Materials and Methods *(optional)*

Sample collection: Samples were taken in the city of Sfax-Tunisia, wastewater collection point / station, Route Matar Ceinture Bourguiba, wastewater Industrial Zone Sidi Salem, Route Gabes and chicken excrement. The samples were taken at the beginning of June 2020. Samples were taken from untreated wastewater 15 - 20 cm deep. The samples were distributed into sterile transparent glass vials, leaving an air volume of approximately 1/10 of the vial volume and transported as quickly as possible to the laboratory under isothermal conditions of 4 ° C and manipulated the same day of collection.

Bacteriophage isolation, purification, and concentration: Samples were centrifuged at 10,000 g for 10 min to remove solid particles and bacteria were excluded using a 0.22 mm sterile filter. As for chicken excrement, samples were dissolved in 10 mL/g of PBS before centrifugation.

For enrichment, *Salmonella enteric* strain were used as the host strain was grown 8–10 h at 37°C in tryptic soy broth (TSB) to obtain pure bacterial cultures. Two hundred microliter overnight cultures were inoculated into 10 mL TSB and incubated at 37°C shaker at the speed of 160 rpm for 6–8 h to reach the exponential growth phase. 10 mL Salmonella cultures were mixed with 40 mL TSB media and 10 mL filtered sample to amplify the collected phages. Amplified phages were isolated by centrifugation at 8000 g for 15 min and filtration using 0.22 mm sterile filter. Both large and small phage plaques were picked. To do so, dilution series of isolated phage samples were assessed on plates covered in a lawn of target bacteria. Individual plaques were picked and re-purified for three consecutive passages.

Characterization of selected phages: pH, thermal stability and morphology of phages were tested using the method of Huang et al., (2018) to determine the stability of phages.

DNA analysis of phages: The nucleic acid of the phage was extracted according to a previously described method (Sambrook and Russel, 2001) using 10% SDS and proteinase K (10 mg/mL). BamHI, EcoRI, EcoRV, HindIII and NdeI were chosen to use as the enzyme for restriction enzyme digestion.

Application and assays in Milk: Fresh skim milk was prepared using the skim milk powder from BD-Difco Company, United States and was sterilized according to manufacturer's instructions. 100 mL phage lysates (10^6 CFU/mL) were added to milk inoculated with 10 mL *Salmonella enterica* an MOI of 1 (10^7 CFU/mL) or MOI of 100 (10^5 CFU/mL). Equal volume of SM buffer was added to the milk in the control group. Samples were incubated at 4 or 28°C. After 0, 1, 2, 4, and 6 h of incubation, aliquots were drawn to determine viable bacterial counts (CFU/mL) and phage concentrations (PFU/mL). Recoverable bacteria were enumerated by serial plating. Phage concentration was assessed by centrifuging the aliquot at 11,000 g for 10 min and determining phage present in the supernatant.

Results and Discussion *(optional)*

Isolation and evaluation of host range of phages: a total of 36 phages against *Salmonella enterica* were isolated from various environments in Sfax (wastewater fromRoute Matar Belt Bourguiba, chicken excrement, and wastewater from Industrial Zone Sidi Salem, Route Gabes) and exhibited a variety of bacteriophage morphologies. Firstly, the three collections of bacteriophages are tested for their ability to infect a broad spectrum of hosts. The spot test method, adapted from the double layer method (Adams 1959), was retained for this first part of the screening. It allows on the one hand identifying which strains is sensitive for each phage and on the other hand to estimate their effectiveness against these strains. Only 28 phages were included in the host range analysis because the other 8 phages did

not reach a titer of 10^9 PFU / ml required for host range analysis (Fong et al. 2017; 2019), suggesting a non-Salmonella host or a Salmonella host other than *S. enterica*.

The host range of a phage is affected by a number of factors. The phage must first and foremost be able to adsorb to the cell surface to be able to initiate infection and the absence or masking of a compatible receptor will prevent this initial interaction (Drulis-Kawa, Majkowska-Skrobek, and Maciejewska 2015). If surface adsorption is successful, entry of phage DNA into the bacterial cell can then be blocked by exclusion systems (Lu and Henning 1994). The modification / restriction systems (Tock and Dryden 2005) and CRISPR block infection by degrading phage DNA shortly after entering the cell cytoplasm (Fineran and Charpentier 2012; Shabbir et al., 2016). All these factors naturally govern and limit the number of phage hosts.

In our case, using phages at high titer (10^9 PFU / ml), we screened these phage isolates against two Salmonella serotypes to determine their host range, such as lysis of a wide range of Salmonella strains is essential for biological control applications (Goodridge et al. 2018). The two strains representing the Typhimurium serovar were lysed by all phages, which is important given the worldwide association of this serotype with salmonellosis (Andino and Hanning 2015; Tarabees et al. 2017). Regarding the Abony serovar, which was isolated in 2016 from alfalfa sprouts "Alfalfa sprouts" (Oh and Park 2017), all phages show lytic activity against this serovar. Of all the phages tested, no phage crossed the genus border like *Escherichia coli*, *Listeria monocytogenes* and *Erwinia amylovora*, showing that these had the potential to be used for effective control of minimally disturbing *Salmonella* other microflora present in food. The strict host range is in line with previous phages isolated by different researchers, proving that phages are safe and well-targeted candidates for application in different foods (Huang et al. 2018). The similarity between host ranges indicates that these 28 phages can recognize similar hosts (Kalatzis et al. 2016).

The study of the effect of different incubation temperatures / different pH on the stability of phages: Some bacteriophages can be resistant to physical and chemical factors, such as low and high temperatures, pH, salinity and ions (Jończyk et al. 2011). Thus, they can settle and persist in extreme environments (Luhtanen et al. 2014). However, depending on the phage used, unsuitable conditions can inactivate the virus by damaging its structural (head, tail, and envelope) and genetic (DNA / RNA) elements. The variation of a factor can by itself modify the sensitivity of phages to other factors (Müller et al. 2011). Maintaining their activity is however important to ensure the stability of phage preparations (Fister et al. 2016).

The thermal and pH stability of the phages was determined based on the phage titers under various conditions. In this study, we have chosen to determine the work on phages showing a phage titer greater than or equal to 1011. That is to say to eliminate around 10 phages (5 phages from the 1st collection: M3, M9, M12, M15 and M16 and also 5 phages from the 2nd collection: P1, P5, P10, P11, P12).

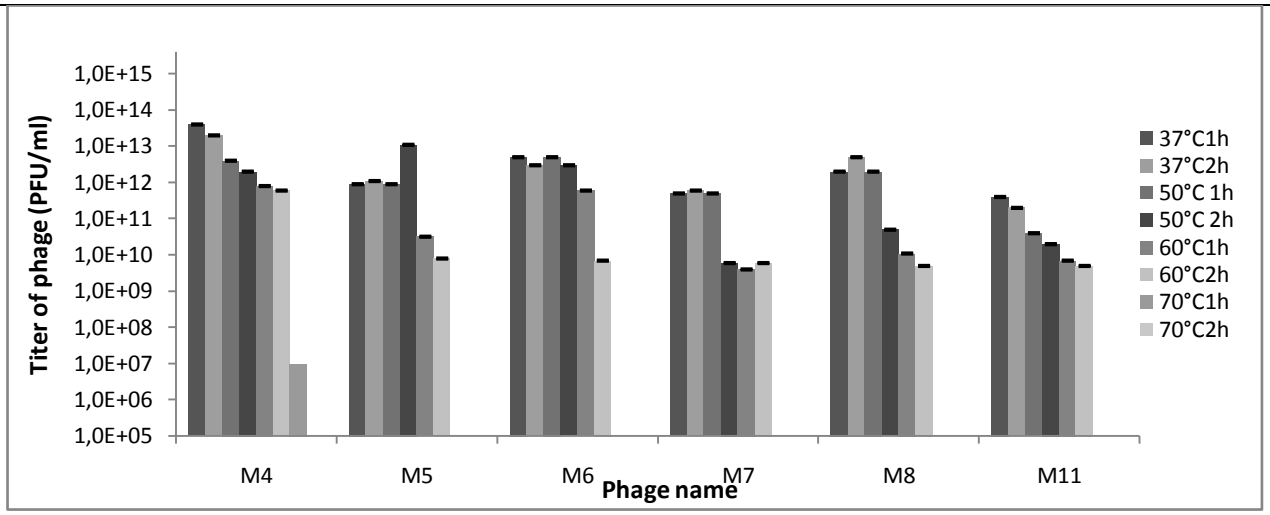

Figure 1: Stability of the phages of the first collection at different temperatures

Beginning with the first collection (wastewater from Route Matar Belt Bourguiba), the stability of phages at pH was also achieved at 37 ° C for 2 h and showed the highest stability at pH 9 (Fig 2). The phages showed stability at pH 5, 7.9 and 11 while at pH 3 no formation of lysis plaque was observed.

The stability of the phages at temperature is tested, for 2 h, from 50 °C to 70 °C with a control at 37 °C. The phages showed good stability after 1 hour incubation at 50 °C and 60 °C. Except at 70 °C, only phage M4 is present with a phage titer equal to 10^7 PFU / ml. After 2 hours of incubation, a slight decrease in the titer of the phages compared to the control (less than 10^2 PFU / ml) is marked at 50 °C and 60 °C. The non-resistance of phage M4 during the second hour of incubation at 70 °C (Figure 1).

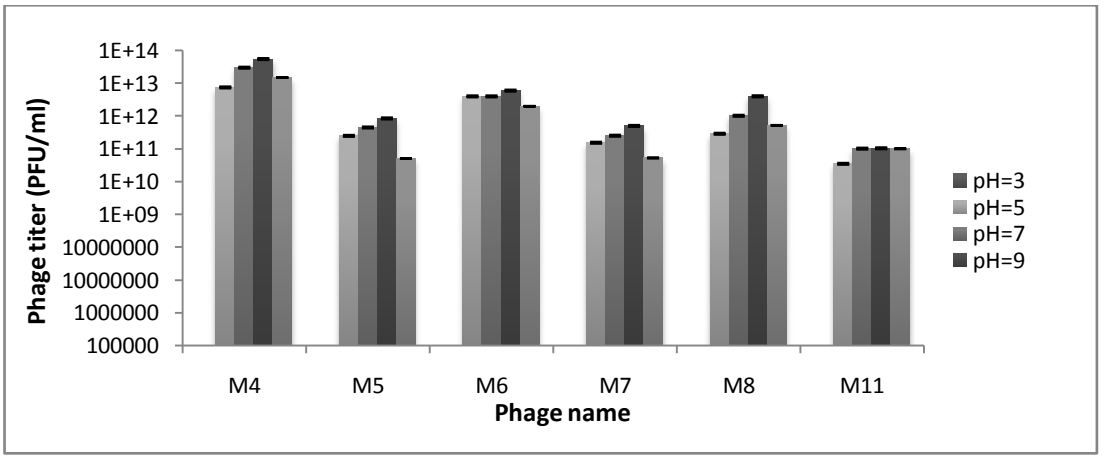

Figure 2: Stability of phages from the first collection at different pH

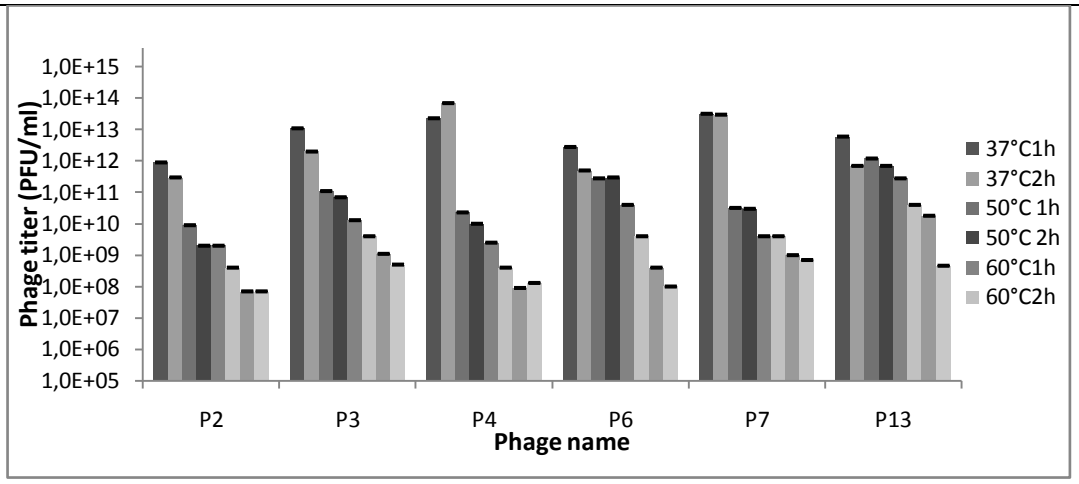

Figure 3: Stability of phages from the second collection at different temperatures

For the collection of phages isolated from chicken excrement, the bacteriophages tested are very stable showing a resistance to pH ranging from 5 to 11 after 2 h. The recoverable phage titers remained active throughout pH 5–11 (Figure 4). The titer of the phages decreased at pH3 (the titer recorded between 10^8 PFU / ml and 10^9 PFU / ml). And as regards the temperatures, at 50 °C and 60 °C, the phages of this collection are stable over time (after 1 and 2 h) and showing a slight difference compared to the control. At 70 °C, the phages are stable over time with the phage titer around 10^8 PFU / ml (Fig 3).

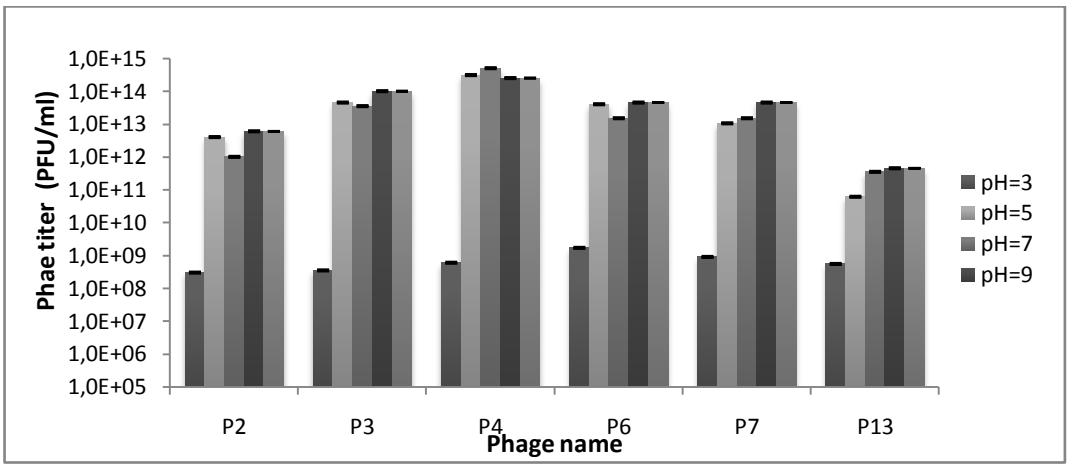

Figure 4: Stability of phages from the second collection at different pH

Finally, the third collection of phages from wastewater from the industrial area of Sidi Salem, after incubation at pH ranging from 3 to 11, all phages showing strong stability even at extreme pH (acidic: pH3; basic: pH11) (figure 6).

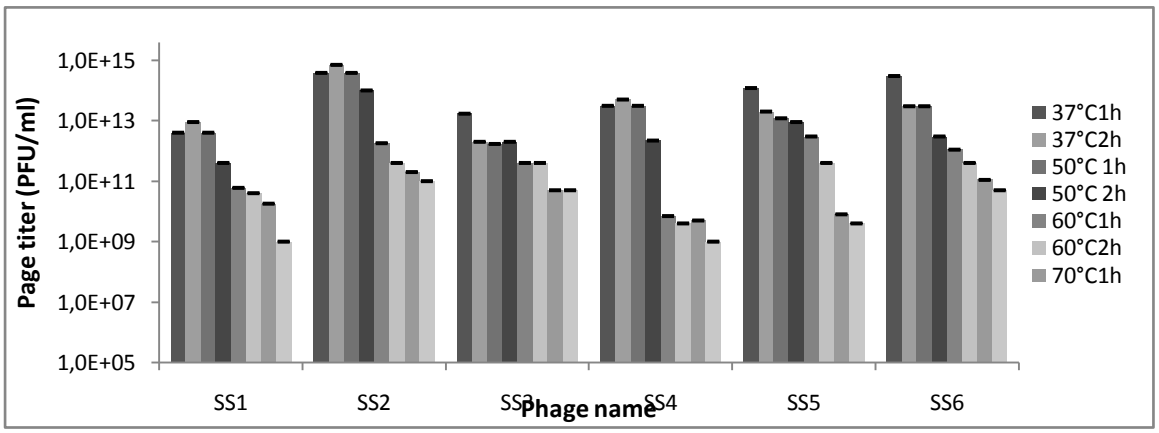

Figure 5: Stability of phages from the third collection at different temperatures

Likewise for the different temperature degrees tested, all phages are stable during the two hours of incubation. The titer of the phages ranging from 10^9 PFU / ml to 10^{11} PFU / ml at 70 °C after 2 hours (fig 5).

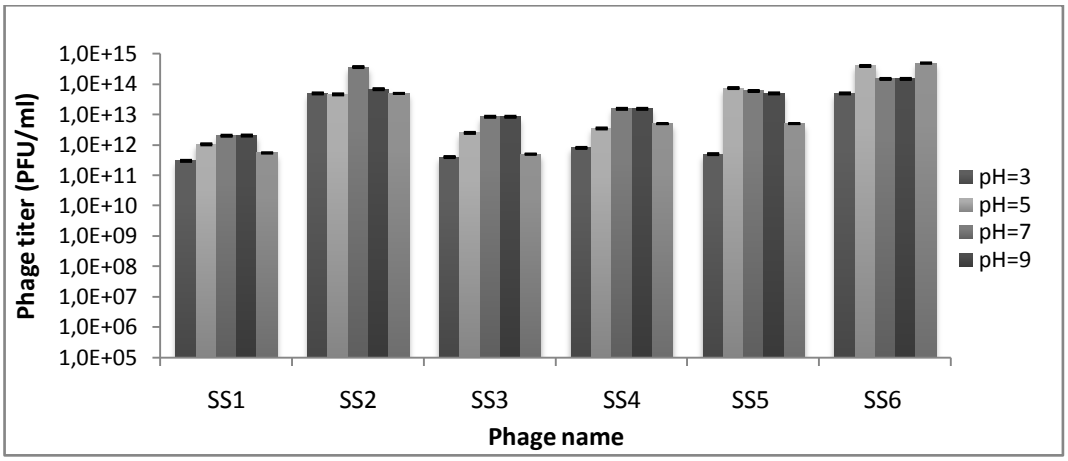

Figure 6: Stability of phages from the third collection at different pH

All phages are active and stable at pH 3 - 5 - 7 - 9 and 11 and also at elevated temperatures 37 °C, 50 °C, 60 °C and 70 °C, with the exception of phages M4, M5, M6, M7, M8 and M11 are not active at pH3 and temperature 70 °C (only phage M4 resists 70 °C for 1 hour of incubation with a phage titer equal to 10^7 PFU / ml). The phages of the third collection are found to be the most stable at extreme pH conditions and high temperatures. As a result, phages can be applied in a wide range of foods due to their high pH stability (pH 3–11) and thermal stability (37 °C - 70 °C).

The tolerance of bacteriophages to pH and temperature were similar or even better than previously reported phages having a pH of 4 to 10 and 60 °C, pH of 4 to 11 and 70 °C (Bao et al. 2015) and pH of 4 at 11 and 60 °C reported by (Kim et al. 2019) for phages applied against *S.* Enteritidis. Applying phages with a better range can give a wider window of application in food products with lower or higher pH and with heat treatment. Indeed, phages which resist high temperature have considerable potential to control or eliminate *Salmonella* spp. during pasteurization, thus allowing more efficient control of bacterial contamination (Kim et al. 2019) as the application of heat and pH resistant phages adds an advantage to the treatment against pathogens, as only heat or pH cannot completely kill pathogens. This argument is supported by the data provided (Lambertz et al. 2012) showing the existence of pathogens even after heat treatment of meat products. Some studies show a prevalence of pathogens below 100 CFU/g (Modzelewska-Kapituła and Maj-Sobotka 2014) but some contradict this and even show more than 100 CFU/g (Garrido, García-Jalón, and Vitas 2010). In addition, the phages of the first collection were found to be sensitive to acidic conditions (pH3) and high temperatures (70 °C), and therefore, its application can be controlled. Phages can be completely inactivated if necessary by adjusting pH and temperature (Huang et al. 2018).

Restriction profile of phage DNA; Each more efficient phage in its collection: M4 of the first collection, P3 of the second collection, and SS2 of the third are chosen for the digestion of the DNA of isolated bacteriophages, the action of 5 restriction enzymes was tested (BamHI, EcoRI, HindIII, EcoRV and NdeI). Digestion of genomic DNA from phages P3, M4 and SS2 by HindIII shows restriction profiles that are not clearly visible and are difficult to interpret. However, those obtained by the use of BamHI, EcoRI, EcoRV and NdeI do not show bands.

Application of a phage cocktail on milk: To simulate food processing conditions before packaging and storage, temperatures 28 °C and 4 °C were chosen in the present study to represent ambient temperature and storage temperature, respectively (Huang et al. 2018). In addition, the experiment was designed to replicate the interaction between bacteria and bacteriophages in food within 6 hours, due to the limited time for processing phages in food processing (Parveen et al. 2017).

The phage products of Salmonella have been approved for application to meat and poultry products as well as to fresh and processed fruits and vegetables (Sharma 2013). In addition, previous studies have demonstrated the effectiveness of bacteriophages as biological control agents in fresh produce, such as lettuce (Spricigo et al. 2013), milk (Guenther et al. 2012), (Huang et al., 2018) and meat (Hungaro et al. 2013).

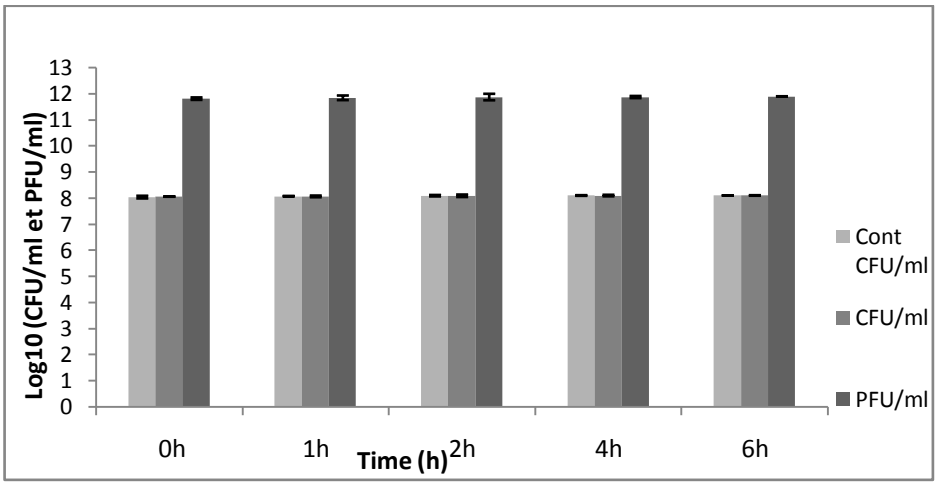

Figure 7: The application of phage cocktail (M4, P3 and SS2) at 4 °C with MOI = 1

When applied to a food matrix (milk) at an MOI of 1 at 4 °C, the cocktail did not confer an appreciable change in the viable statistics of *Salmonella* CFU or of PFU phage replication. The low temperature is one of the reasons for the low virulence of the phage because the low temperatures hamper the growth of microbes and the phages depend on the multiplication of its host (Figure 7).

However, when applied at an MOI of 1 at 28 °C, the titer of the phage cocktail increased during 6 h of incubation at 13.8 log10 PFU/mL (Figure 8). The reduction in the number of bacteria was observed consistently following phage infection, it was evident at 28 °C and an MOI of 1. Administration of the cocktail reduced recoverable Salmonella by 2.42 log10 CFU / mL compared to the excluded phage control (efficiency reached 25.4% (control = 9.5 log10 CFU / mL; test = 7.08 log10 CFU / mL)). This may be due to the exposure of phages to a high density of bacterial cells, resulting in an increase in

phage titer during replication. The phage cocktail largely suppressed the proliferation of *Salmonella* resulting in a decrease of 0.72 log10 CFU/mL. In the absence of phage treatment, *Salmonella* was multiplied by 1.51 log10 CFU/mL. The reason for the increase in the number of *Salmonella* during incubation in milk at 28 ° C can be attributed to favorable replication conditions. This argument is consistent with a related study in which the incubation of chocolate milk was artificially spiked with 103 CFU/mL of *Salmonella* Typhimurium. No detectable bacterial multiplication was observed for up to 6 days after incubation at 8 ° C. However, when incubated at 15 °C, *Salmonella* Typhimurium multiplied rapidly after 48 h after inoculation (Guenther et al. 2012). On the other hand, the storage temperature can also influence the stability of the phage or the kinetics of replication. As detailed in another study, several distinct phages were applied in milk against *Salmonella* at 4 and 25 °C, resulting in varying trends (Bao et al. 2015).

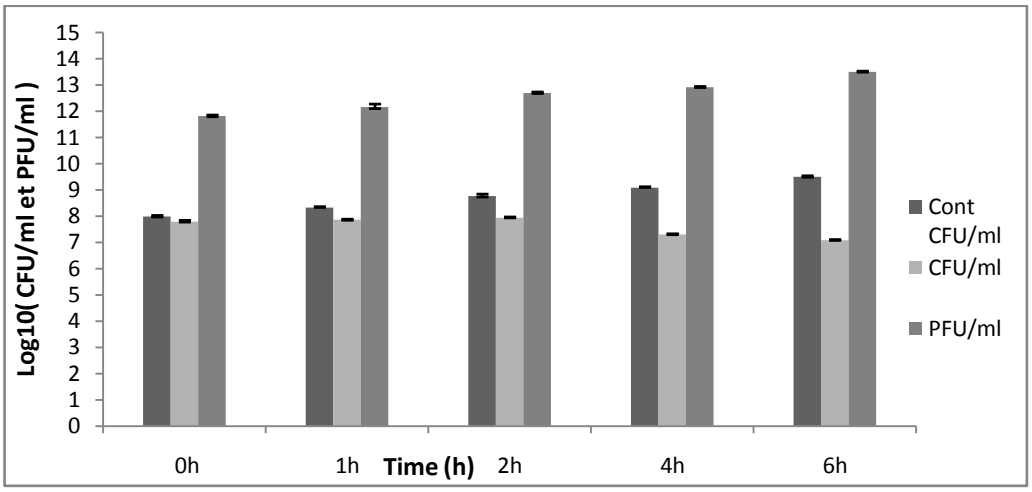

Figure 8: The application of phage cocktail (M4, P3 and SS2) at 28 °C with MOI = 1

The effectiveness of the phage cocktail was found to be relatively lower at 4 °C, compared to that at 28 °C. A similar result was also reported in a previous study on the efficacy of phage LPSTA1 applied to milk, lettuce and sausage at 4 ° C and 28 ° C (Huang et al. 2018), and phage P7 in beef at 5 °C and 24°C (Bigwood et al. 2008), indicating a smaller reduction in the number of bacteria at 4-5 °C compared to that at 28-24 °C. Nevertheless, it was revealed that storage at low temperature (5 °C) could prevent the re-growth of bacteria after phage treatment (Guenther et al. 2012), and that the reduction in the number of bacteria persisted during storage even after 10 days (Soni, Nannapaneni, and Hagens 2010).

Conclusions *(optional)*

Salmonellosis is a foodborne infection of global importance. It is a zoonotic bacterial disease of national and international health and economic significance.

The application of phages as a means of bio-control is recognized to be one of the safest approaches today. This study was focused on the biological control of *Salmonella* in the food industry. The overall objective of this topic was to isolate and characterize phages infecting salmonella and to evaluate their efficacy as a means of controlling *S. enterica*. For the isolation, 3 environmental samples were used to

isolate a total of 36 bacteriophages. Only phages with a high titer reaching 10^9 PFU/ml (Fong et al., 2017; Karen Fong, 2019), were subsequently tested by the spot method (Adams, 1959) against 11 strains in order to define his host specter. A prerequisite for the industrial use of the phages selected in this work is the characterization of their genomes and their biological properties. For this, 18 phages (with a titer reached 10^{11} PFU/ml) were tested at different pHs ranging from 3 to 11, and high temperatures of 37 ° C to 70 ° C, marking encouraging results showing their stability under extreme conditions. The genomes of the three isolated phages show restriction profiles are not clearly visible and are difficult to interpret due to the action of 5 restriction enzymes (BamHI, EcoRI, HindIII, and EcoRV and NdeI).

Finally and in order to assess the ability of bacteriophages M4, P3 and SS2 to fight against *S. enterica*, an application on a food matrix (semi-skimmed milk of delight) was made at two different temperatures: 4°C to simulate the storage temperature and 28°C to simulate the ambient temperature. The efficiency of the phage cocktail was found to be relatively lower at 4°C, compared to that at 28°C which showed a removal efficiency of *S. enterica* reaching 25.4%.

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