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Isolation and Characterization of a Bacteriophage with Potential for the Control of Multidrug-Resistant *Salmonella* Strains Encoding Virulence Factors Associated with the Promotion of Precancerous Lesions

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Abstract: Background: Antimicrobial-resistant bacteria represent a serious threat to public health. Among these bacteria, *Salmonella* is of high priority because of its morbidity levels and its ability to induce different types of cancer. **Aim:** This study aimed to identify *Salmonella* strains encoding genes linked to the promotion of precancerous lesions and to isolate a bacteriophage to evaluating its preclinical potential against these bacteria. **Methodology:** An epidemiological approach based on wastewater analysis was employed to isolate *Salmonella* strains and detect genes associated with the induction of precancerous lesions. Antimicrobial susceptibility was assessed by disk diffusion method. A bacteriophage was isolated via the double agar technique, and its morphological characteristics, stability, host range, replication dynamics and ability to control *Salmonella* under different conditions were evaluated. The bacteriophage genome was sequenced and analyzed using bioinformatics tools. **Results:** Thirty-seven *Salmonella* strains were isolated, 17 of which contained the five genes associated with precancerous lesions induction. These strains exhibited resistance to multiple antimicrobials, including fluoroquinolones. A bacteriophage from the *Autographiviridae* family with lytic activity against 21 bacterial strains was isolated. This phage exhibited a 20 min replication cycle, releasing 52 ± 3 virions per infected cell. It demonstrated stability and efficacy in reducing *Salmonella* concentration in simulated gastrointestinal conditions, and its genome lacked genes that represent a biosafety risk. **Conclusion:** This bacteriophage shows promising preclinical potential as a biotherapeutic agent against *Salmonella*.

Keywords: Antibiotic resistance; Wastewater epidemiology; Oncogenic *Salmonella*; Lytic phage

1. Introduction

Currently, bacterial infections account for one in every eight deaths worldwide, and this figure continues to rise [1]. Moreover, this problem is aggravated by the emergence of bacteria that are multidrug-resistant to antimicrobials [2]. In the absence of effective preventive measures, projections suggest that this type of bacteria could become the leading cause of death in the coming years [3]. Consequently, experts have emphasized the urgent need for immediate intervention to mitigate this escalating crisis [4,5].

Among the particularly dangerous multidrug-resistant bacteria, considered as priority pathogens by the World Health Organization, *Salmonella* stands out for its high morbidity rate (6). Worldwide, between 200 million to 1 billion infections caused by *Salmonella* are reported annually, leading to approximately 420,000 deaths [7–9].

In addition, growing evidence suggests that *Salmonella* is implicated in the development of different types of cancer in the gastrointestinal tract [10–13]. Although many aspects of *Salmonella*'s in promoting precancerous lesions and tumorogénesis remain unclear, it is known that the oncogenic potential exhibited by certain *Salmonella* strains is directly attributed to the ability to synthesize certain effector proteins, including AvrA, SopB, CdtB, PltA, and PltB [14–17]. These proteins contribute chronic inflammation, DNA damage, and manipulation of host signaling pathways [18,19].

This underscores the pressing need for more effective antimicrobial agents to reinforce public health strategies against these types of bacteria. In this context, there is renewed interest in the use of bacteriophages, also called phages, viruses that infect and lyse bacteria, as therapeutic agents [20–22]. These viruses represent one of the most promising alternatives against resistant bacteria due to their highly specific, which enables the selective targeting and destruction of pathogenic bacteria without harming beneficial ones, a crucial advantage in precision medicine [23]. Furthermore, bacteriophages are generally recognized as safe, as they do not directly affect human or animal health [24]. Their low production costs also make them an attractive option for biopharmaceutical application [25].

However, not all bacteriophages are suitable as therapeutic agents, as some may carry genes encoding pathogenicity factors, which could increase the virulence of the bacteria. Others may contain proteins with allergenic potential, be unstable during storage, or fail to exert their therapeutic effect at the intended site of action [26–28]. Therefore, it is essential to select bacteriophages through comprehensive characterization to assess both their therapeutic potential and biosafety.

Therefore, the main aim of this research is to isolate and characterize a bacteriophage with preclinical potential as an alternative for the treatment for multidrug-resistant *Salmonella* strains, particularly those encoding virulence factors associated with the induction of precancerous lesions.

2. Materials and Methods

2.1. Isolation of Salmonella Strains and Identification of Genes with Oncogenic Potential

Salmonella strains were isolated from 54 urban wastewater samples collected in August-September 2023 from the central zone of the State of Sinaloa, Mexico. Samples were obtained from the sewage system in the municipalities of Culiacan and Navolato. The ultrafiltration method described by Liu et al. (2021) was employed due to its high efficiency to concentrate pathogenic bacteria present in environmental samples, enabling a more effective recovery of bacteria such as *Salmonella* [29]. Subsequently, the bacteria were isolated by inoculating the eluate onto Hektoen enteric agar. The presence of the *inv*A gene was confirmed using polymerase chain reaction (PCR), ensuring accurate identification of each bacterial strain [30]. In addition, PCR analysis also was utilized to detect genes associated with the promotion of precancerous lesions, which are encoded by specific *Salmonella* strains. The genes targeted in this study included *avr*A, *sop*B, *cdt*B, *plt*A, and *plt*B. For this purpose, the oligonucleotides and protocols described by lshaheeb et al., 2023, Hawwas et al., 2022, and Mezal et al., 2014 were used for analysis (Table 1) [31–33].

Table 1. List of oligonucleotide sequences used for the amplification of genes in *Salmonella* strains encoding effector proteins linked to the promotion of precancerous lesions and tumorigenesis.

Oligonucleotide	Sequence	Gene	Fragment size (bp)
AvrA-F	CCTGTATTGTTGAGCGTCTGG	*avr*A	422
AvrA-R	AGAAGAGCTTCGTTGAATGTCC		
SopB-F	TCAGAAGRCGTCTAACCACTC	*sop*B	517
SopB-R	TACCGTCCTCATGCACACTC		
CdtB-F	GAAACAAGTCAGGCATTGCC	*cdt*B	819
CdtB-R	GAATGGCTCATAAACACGCC		
PltA-F	GTGGGACTATCATCGTGCAG	*plt*A	729
PltA-R	AGGGTGATCAACGTAACCAC		

PltB-F	GCCGGAAGTACCTGTGTTAT	*plt*B	414
PltB-R	AGTAGTGAAAACCCATCGCG		

2.2. Antibiotic Susceptibility Tests

The disk diffusion method was used to evaluate the *in vitro* susceptibility of the isolated bacterial strains to 12 antibiotics, representing six distinct families and five mechanisms of action. The antibiotics included beta-lactams (ampicillin, amoxicillin/clavulanic acid, cephalothin, imipenem), which inhibit cell wall synthesis; quinolones (ciprofloxacin, nalidixic acid), targeting DNA synthesis; aminoglycosides (gentamicin, amikacin), phenicols (chloramphenicol), and tetracyclines (tetracycline), which inhibit protein synthesis; polymyxins (colistin), which disrupt the cell membrane; and sulfonamides (sulfamethoxazole/trimethoprim), inhibiting folic acid synthesis. This selection covers key biochemical pathways to provide a comprehensive profile of antimicrobial resistance. Antibiotics were chosen based on their clinical relevance in treating *Salmonella* infections and their usage in the local agricultural sector, the predominant activity in the sampling area. For this purpose, 6 mm discs impregnated with standard antibiotic concentrations, as recommended by the International Committee for Laboratory Standards (CLSI), were used. The bacterial inoculums were adjusted to a turbidity equivalent to 0.5 on the McFarland scale, and the plates were incubated at 37°C for 24 h. After incubation, the diameter of the inhibition zones was measured with a calibrated Bernier [34]. *Salmonella* ATCC 14028 was used as the reference strain.

2.3. Bacteriophage Isolation and Purification

For bacteriophage isolation, urban wastewater samples were filtered through aluminum oxide nanofibers (NanoCeram-Argonide Corp), due to their high efficiency in retaining viral particles, using a MasterFlex peristaltic pump (Cole-Palmer, USA) at a constant flow rate of 500 ml/min [35]. The eluate was recovered, and the double agar layer technique was used to determine the presence of bacteriophages with lytic activity on *Salmonella*. Briefly 1 ml of the overnight bacterial culture grown in trypticasein soy broth (TSB) was mixed with 200 μl of the eluate and 3 ml of 0.4% TSB-agarose, and the mixture was poured onto Petri dishes containing trypticasein soy agar (TSA). The plates were incubated at 37 °C for 24 h, after which lysis plaques were identified, excised with a Pasteur pipette, and transferred to microcentrifuge tubes containing sterile distilled water. An aliquot of 100 μl was taken, and the double agar layer technique was repeated. Plates were again incubated for 24 h at 37 °C [36]. This process was repeated five times to ensure the recovery of a single phage type. The bacteriophage was propagated following the protocol described by [37]. Purification of the bacteriophage suspension was achieved via dialysis using the 20,000 MWCO Slide-A-Lyzer system (Thermo Fisher, USA), followed by endotoxin removal with the EndoTrap HD system (Lionex). Subsequently, the suspension was filtered through a polyvinylidene difluoride membrane with 0.22 μm pores.

2.4. Transmission Electron Microscopy

For electron microscopy, 30 μl of the purified bacteriophage suspension were placed on a Formvar-coated copper grid with 400-mesh carbon baking and allowed to absorb for 10 min [38]. Subsequently, the grid was placed in a vacuum evaporator (JEE400, JEOL Ltd. Tokyo, Japan), stained with 2% (w/v) phosphotungstic acid (pH 7.2) for 1 min, and air-dried in a dust-free environment. The samples were examined using a transmission electron microscope (JEM-1011, JEOL Ltd. Tokyo, Japan) operating with an accelerating voltage of 80 kV.

2.5. One-Step Replication Curve

To evaluate the replication kinetics of bacteriophage, a colony of the host strain was resuspended in 50 ml of TSB medium and incubated at 37 °C with shaking at 100 rpm. When the optical density at 600 nm reached 0.1, the bacteriophage was added at a multiplicity of infection (MOI) of 0.1. Every five minutes, two aliquots were collected from de culture, one treated with chloroform and the other

untreated. The aliquots were centrifuged at 10,000 × *g* for 1 min, and the supernatant was used to determine the bacteriophage concentration through serial decimal dilutions and the double agar layer method by [37]. This procedure was performed in triplicate for each time point to ensure reproducibility and accuracy of the results.

2.6. Host Range

The host range of the bacteriophage was evaluated by the agar double-layer method using a purified suspension of the phage at a concentration of 1×10^3 PFU/ml. The assay included 37 *Salmonella* strains, 8 *Escherichia coli* strains, and 5 probiotic bacterial strains known to be part of human gut microbiota and considered beneficial, including *Bacillus clausii*, *Bacillus coagulans*, *Lactobacillus paracasei*, *Lactobacillus rhamnosus* and *Limosilactobacillus reuteri*. Double-layer agar plates were prepared for each strains according to the method described in section 2.3. The plates were inspected for the formation of lysis plaques on the agar, which indicated the lytic activity of the bacteriophage against the tested strains.

2.7. Stability of the Bacteriophage

Accelerated stability test. The accelerated stability test of the phage was conducted according to the protocol described by Xu et al. (2023), with modifications outline below [39]. A purified suspension of bacteriophage was incubated at 60, 65, and 70 °C, its concentration was continuously monitored at 1 or 2 h intervals. The data on viral concentration were analyzed using three reaction kinetic models: zero-order ($kt = M_0 - M$), first-order ($kt = \ln(M_0 / M)$), and second-order ($kt = 1 / M - M_0$), respectively. The kinetic model that exhibited the lowest coefficient of determination (r^2) was selected for subsequent calculations, indicating the best fit to the experimental data. The thermodynamic temperature (T) and rate constant (k) obtained from the selected kinetic model were applied to the Arrhenius equation ($-\ln(k) = [Ea/RT] - \ln[A]$), where *Ea* is the activation energy and R is the gas constant. The values of Ea and A were determined experimentally by a nonlinear fit of the viral concentration data. By combining the Arrhenius equation with the selected reaction kinetics model, was used to calculate the relationship between time (t) and the actual phage concentration (M), which was the model for predicting phage lifetime. To validate the model′s applicability under realistic conditions, it was tested at 28 °C, with bacteriophage concentration measured monthly during six months.

Stability in simulated gastrointestinal environment. The simulated gastrointestinal system was composed of three phases: oral phase, gastric phase and intestinal phase. The composition of each phase is detailed in Table 2. Electrolyte solutions were sterilized at 121 °C for 15 min at 1 atm pressure. After sterilization, the corresponding enzymes and bile solution, previously filtered with 0.45 μm filters, were added. The bacteriophage was then inoculated into the oral phase at a concentration of approximately 1×10^6 CFU/ml, with final pH adjusted to 7. The mixture was incubated at 37 °C with agitation at 100 rpm for 2 min. Subsequently, the contents of the oral phase were mixed in a 1:1 ratio with the gastric phase, adjusting the final pH of 3, and incubated under the same conditions for 2 h. Finally, the contents of the gastric phase and the intestinal phase were mixed in a 1:1 ratio, adjusting the final pH to 7, and the solution was incubated for 2 h at the described conditions [40]. At each phase of the experiment, the bacteriophage concentration was determined through serial decimal dilutions and the agar double-layer method. Results were expressed as means ± standard deviation of the bacteriophage concentration, and values of $p < 0.01$ were considered significant.

Table 2. Composition of fluids in each phase of the simulated gastrointestinal tract.

Reagent	Oral phase (mmol/l)	Gastric phase (mmol/l)	Intestinal phase (mmol/l)
KCl	15.1	6.9	6.8
KH_2PO_4	3.7	0.9	0.8
$NaHCO_3$	13.6	25	85
NaCl	-	47.2	38.4

MgCl$_2$(H$_2$O)$_6$	0.15	0.12	0.33
(NH$_4$)$_2$CO$_3$	0.06	0.5	-
CaCl$_2$(H$_2$O)$_2$	1.5	0.15	0.6
Enzymes			
α-amylase	150 U/ml	-	-
Pepsin	-	4,000 U/ml	-
Lipase	-	120 U/ml	-
Pancreatin	-	-	200 U/ml (based on trypsin activity)
Bile salts	-	-	10 mM

2.8. Bacteriolytic Activity of the Bacteriophage

In culture medium. The *Salmonella* strain exhibiting the highest antibiotic resistance and coding for all five evaluated virulence genes was cultured in TSB medium at 37°C for 24 hours. Afterward, 1 ml of this culture was added to four separate flasks, each containing 200 ml of TSB, and incubated at 37°C in a shaker at 100 rpm. Bacterial growth was monitored by measuring the optical density at 600 nm (OD$_{600}$). When the OD$_{600}$ reached 0.5 (~2 × 10^8 CFU/ml), varying concentrations of purified bacteriophage suspension were added: 100 μl to the first flask to final concentration of 1 × 10^7 PFU/ml, 1 × 10^6 PFU/ml to the second, and 1 × 10^5 PFU/ml to the third. The fourth flask served as a control without bacteriophage. All cultures were incubated under the same conditions, and optical density measurements were taken hourly [41].

In simulated gastrointestinal system. The bacteriolytic capacity of the bacteriophage was evaluated using the *in vitro* system described in section 2.7, with modifications to incorporate a food model similar to that proposed by Akritidou et al. (2023), designed to promote bacterial survival, was provided [42] . To simulate a microbiome, the five bacterial species described in section 2.6 were used. All strains were mixed in an equal proportion and used immediately [43]. In the simulated intestinal fluid, each of the following treatments were inoculated: (1) beneficial bacteria consortium (1×10^6 CFU/ml) + 50 μl of sterile water, (2) *Salmonella* (1×10^6 CFU/ml) + 50 μl of sterile water, (3) beneficial bacteria consortium (1×10^6 CFU/ml) + 50 μl of purified bacteriophage suspension (1×10^4 PFU/ml), (4) *Salmonella* (1×10^6 CFU/ml) + 50 μl of the purified bacteriophage suspension (1×10^4 CFU/ml), (5) *Salmonella* (1×10^6 CFU/ml) + ciprofloxacin 0. 5 mg/ml and (6) beneficial bacteria consortium (1×10^6 CFU/ml) + 0.5 mg/ml. The bacterial concentration of each treatment was quantified by plaque extension. Results were presented as the mean concentration ± standard deviation, with p-values < 0.01 considered statistically significant.

2.9. Genomic Sequencing and Bioinformatic Analysis

Bacteriophage genetic material was extracted according to the proteinase K/SDS protocol [44]. Genomic libraries were generated using the MGIEasy DNA library Prep Universal System, and nucleotide sequencing was conducted on the MGISEQ-2000 platform utilizing the DNBSEQTM (nanoballs DNA) system. Assembly and bioinformatics analysis was performed according to the guidelines proposed by Philipson et al. (2018) and Turner et al. (2021) [45,46]. Random sampling of reads (50,000 to 100,000) was performed using the Seqtk Toolkit tool until a coverage depth of ~100× was achieved. After analyzing the quality of reads with FastQC, low quality adapters and sequences (Phred index < 30) were removed using Trimmomatic. *De novo* assembly of reads was performed with SPAdes, employing k-mer of 21, 33 and 55. Open reading frames (ORFs) were identified using Glimmer, Genemark, Genemark.hmm, Genemark S, Prodigal, RAST and MetaGene. Promoters were identified with PhagePromoter and PHIRE, while FindTerm and RNAold were used to identify Rho-independent terminators. The tRNAs were determined by ARAGORN and tRNAscan-SE. The ability of the bacteriophage to establish lysogenic cycles was evaluated using PhageAI and PHACTS. The presence of genes associated with antibiotic resistance was analyzed in the CARD platform (https://card.mcmaster.ca) and AMRFinderPlus, while virulence factors were identified through

VFDB   (www.mgc.ac.cn/VFs/search_VFs.htm)   and   VirulentPred
(http://203.92.44.117/virulent/submit.html).

2.10. Statistical Analysis

All statistical analyses were conducted using Minitab 19. Normality assumptions were verified with the Shapiro-Wilk test, and homogeneity of variance was assessed with the Levene's test. Differences in bacteriophage stability under simulated gastrointestinal conditions and bacteriolytic activity were analyzed by repeated measures ANOVA and two-factor ANOVA, respectively, both followed by Tukey's *post hoc* test. Significance was set at $p \leq 0.01$. All assays were performed in triplicate.

3. Results

3.1. Isolation of Salmonella and Identification of Genes Associated to Cancer Induction

A total of 37 *Salmonella* strains were isolated from the wastewater samples, with an average isolation rate of 43%. This high prevalence of this bacterium suggests a significant occurrence of *Salmonella* in urban wastewater, underscoring the importance of monitoring its presence due to its potential impact on public health risks. These findings are congruent with previous studies that have documented the presence of *Salmonella* in urban wastewater systems as an indicator of fecal contamination and insufficient water treatment efforts [47,48].

Notably, 17 of the 37 isolates harbored five virulence genes associated with potential cancer induction, specifically *avr*A, *sop*B, *cdt*B, *plt*A, and *plt*B (Table 3). According to numerous studies, *Salmonella* strains that encode these genes produce effector proteins implicated in chronic inflammation and the induction of precancerous lesions. Although several aspects remain unclear, it is known that the AvrA protein modulates the β-catenin signaling pathway, potentially influencing the regulation, differentiation, and proliferation of intestinal epithelial cells [49]. In contrast, SopB promotes intracellular invasion of *Salmoenlla* and inhibits apoptosis [50,51]. Additionally, the cytolethal distending toxin, composed of CdtB, PltA, and PltB proteins, induces DNA damage, which results in genomic instability and a proinflammatory environment associated with precancerous lesions [52]. Consequently, future research will aim to evaluate the pathogenic potential of these strains in cancer promotion.

Table 3. Antimicrobial resistance profiles and virulence factors of *Salmonella* strains isolated from urban wastewater.

Bacterial strain	Antimicrobials and average inhibition diameter (mm)												Virulence genes associated with the potential to induce precancerous lesions				
	IMP	SXT	GEN	AMP	CIP	NAL	CHL	TET	COL	AMC	CFP	AMK	*avrA*	*sopB*	*cdtB*	*pltA*	*pltB*
Salmonella **sal01**	26	21	16	15	16	14	24	19	8	21	24	17	-	+	+	-	+
Salmonella **sal02**	26	24	18	19	25	21	25	22	9	10	24	18	+	+	+	+	+
Salmonella **sal03**	22	18	19	16	22	21	17	19	8	17	22	19	+	-	+	+	+
Salmonella **sal04**	25	24	18	14	22	17	22	18	7	18	21	18	+	-	+	-	+
Salmonella **sal05**	29	26	21	22	31	21	23	20	8	22	24	19	+	+	+	+	+
Salmonella **sal06**	26	22	18	21	30	21	23	20	9	22	17	17	+	-	-	-	+
Salmonella **sal07**	27	25	18	21	31	21	31	19	20	17	8	16	+	+	+	+	+
Salmonella **sal08**	15	18	17	15	14	12	21	27	8	22	19	19	+	-	+	+	+
Salmonella **sal09**	29	25	23	31	22	24	29	9	24	19	25	19	+	+	+	+	+
Salmonella **sal10**	26	24	18	23	30	22	26	22	9	22	24	19	+	+	+	-	-
Salmonella **sal11**	26	25	16	21	29	20	24	19	9	22	26	19	+	+	+	+	+
Salmonella **sal12**	26	24	18	21	25	21	25	22	9	23	24	18	+	+	+	+	+
Salmonella **sal13**	22	22	19	21	30	21	24	19	9	21	22	19	+	-	+	-	+
Salmonella **sal14**	25	23	18	22	32	22	22	18	9	22	28	18	+	-	-	+	+
Salmonella **sal15**	29	20	21	22	31	21	23	20	8	22	24	19	+	+	+	+	+
Salmonella **sal16**	26	24	18	21	30	21	23	20	9	22	23	17	+	+	+	+	-
Salmonella **sal17**	21	19	12	21	22	21	31	21	25	18	8	22	+	-	+	+	+
Salmonella **sal19**	25	26	17	22	32	20	21	27	8	22	23	19	+	+	+	+	+
Salmonella **sal20**	22	18	16	21	19	20	18	19	10	18	26	20	+	+	+	+	+
Salmonella **sal21**	26	24	18	21	25	21	24	22	9	23	24	18	+	+	+	+	+
Salmonella **sal21**	22	24	19	21	30	21	22	19	9	21	22	19	+	-	+	+	-
Salmonella **sal23**	25	24	18	22	32	22	22	18	9	22	28	18	+	+	+	+	+
Salmonella **sal24**	29	26	21	22	31	21	23	20	8	22	24	19	+	-	+	+	+
Salmonella **sal25**	26	24	18	21	30	21	23	20	9	22	23	17	+	+	+	+	+
Salmonella **sal26**	21	19	18	21	15	21	31	21	15	18	8	21	+	-	+	+	+
Salmonella **sal27**	22	26	17	18	17	12	21	27	8	22	23	19	+	-	+	+	+
Salmonella **sal28**	12	14	13	14	9	12	20	9	8	10	14	13	+	+	+	+	+
Salmonella **sal29**	26	24	18	23	30	22	26	22	9	22	24	19	+	-	+	-	+

Salmonella **sal30**	26	25	16	21	29	20	24	19	9	22	26	18	+	+	+	+	+
Salmonella **sal31**	19	20	18	17	14	19	17	22	9	19	16	18	+	+	+	+	+
Salmonella **sal32**	18	16	17	18	18	16	20	19	9	19	22	19	+	-	+	+	+
Salmonella **sal33**	24	24	18	22	32	22	22	18	9	22	28	18	+	-	+	+	+
Salmonella **sal34**	26	22	21	22	31	21	23	20	8	22	24	19	+	+	+	+	+
Salmonella **sal35**	26	24	18	21	30	21	23	20	9	22	23	17	+	+	+	-	+
Salmonella **sal36**	21	25	18	21	31	21	31	21	25	18	8	22	+	+	+	+	+
Salmonella **sal37**	25	18	17	22	32	14	18	16	8	22	23	12	+	+	+	-	+

The detection of *Salmonella* strains harboring genes associated with cancer induction in wastewater highlights the urgent need for stricter water treatment policies to mitigate the spread of these pathogens and their long-term public health implications

3.2. Antibiotic Sensitivity Tests

The antimicrobial sensitivity of the 37 *Salmonella* strains was evaluated against 12 antibiotics commonly used for treating bacterial infections. The results revealed that four (sal01, sal08, sal27, and sal28) of the strains exhibited resistance or intermediate sensitivity to at least three different classes of antibiotics, categorizing them as multidrug-resistant (Table 3). A significantly higher incidence of resistance was observed for colistin (11 strains), followed by nalidixic acid (9 strains), tetracycline (7 strains), and ampicillin (6 strains). The resistance to ciprofloxacin and colistin, two critical antibiotics used the treating severe *Salmonella* infections, and other bacterial infections, is particularly alarming. These findings are consistent with recent reports from other Latin America regions, which also indicate a rising prevalence of antibiotic-resistant bacterial strains [53,54]. The identification of multidrug-resistant *Salmonella* strains highlights the need for new strategies to combat antimicrobial resistant. In this context, research into therapeutic alternatives, such as the use of bacteriophages, may offer a promising approach for addressing this growing public health challenge [22].

3.3. Isolation of the Bacteriophage

A bacteriophage, designated Phylax-28 (from the Greek "guard" or "protector), was successfully isolated, producing clear and well-defined lysis plaques, with a diameter of approximately 1.2 cm (Figure 1A). The plaque size generated by Phylax-28 is notably larger than typical *Salmonella* phages, which are generally reported to form plaques ranging between 0.5- 3.5 mm in diameter [55–58].

In addition, Phylax-28 induces the formation of an inhibition halo, a semitransparent zone surrounding the lysis plaques. This phenomenon is consistent with findings by Jurczak-Kurek and coworkers, who noted that phages that produce clear plaques are often associated with a strong lytic activity against bacteria. The appearance of the halo is attributed to the activity of some enzymes, encoded by certain bacteriophage, that degrade the cell wall, thereby enhancing the phage's potential for bacterial elimination [59].

3.4. Bacteriophage Morphology

Analysis of transmission electron micrographs of 20 virions of the Phylax-28 revealed that it has an icosahedral-isometric capsid 28 ± 0.2 nm in diameter, along with a thin, short, non-contractile and rigid tail 8.2 ± 0.1 nm in length (Figure 1B). Based on these morphological features and the criteria established by the International Committee on Viral Taxonomy, the bacteriophage Phylax-28 is classified as a new member of the family *Autographiviridae*.

Usually, bacteriophages with short tails or absent tails, such as Phylax-28, are typically associated with increased resistance to harsh environmental conditions [60,61]. These morphological traits suggest that Phylax-28 may maintain stability under aggressive bio-physicochemical conditions, such as those prevailing in the gastrointestinal system. However, further experimental data are required to confirm this hypothesis. Subsequent sections will detail the experimental results of phage stability under these conditions.
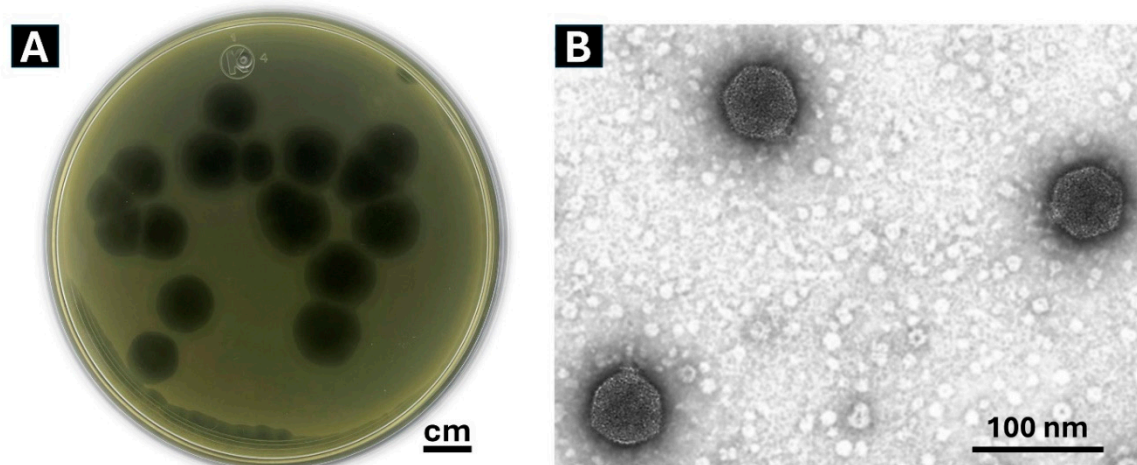
Figure 1. (A) Morphology of lysis plaques and (B) structural features of Phylax-28 bacteriophage virions. The clarity and size of the plaques reflect the lytic activity and efficacy of the bacteriophage against *Salmonella*. The virions exhibit an icosahedral structure, characterized by a capsid composed of 20 equidistant triangular faces.

3.5. Bacteriophage Replication Curve

Within 5 minutes of exposure, approximately 80% of Phylax-28 virions were adsorbed to the bacterial surface (Figure 2). Phylax-28 presents a latency period of 15 min, with bacterial lysis occurring 20 min post-infection, releasing 52 ± 3 virions per bacterial cell. This burst size observed in Phylax-28 is relatively large compared to other *Salmonella*- phages, which typically produce between 34 and 37 virions per cell [62,63]. Some *Salmonella*-infecting phages, however, have been reported to produce larger burst size but with longer latency periods [64,65]. The short latency period of Phylax-28 suggest a competitive advantage over other phages, as they can produce enough virions to lyse the bacterium in a short period of time, making it a promising candidate for *Salmonella* control.
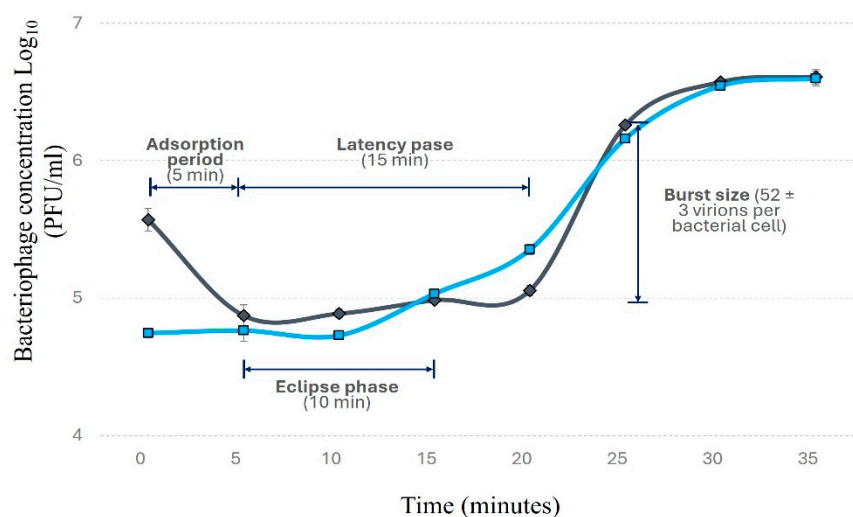
Figure 2. Replication curve of bacteriophage Phylax-28 using *Salmonella* as the host bacterium. The replication dynamics are depicted under two conditions: with chloroform (blue line), which indicates the formation of intracellular virions, and without chloroform (black line), which shows the release of virions into the extracellular medium.

3.6. Host Range

Phylax-28 showed the ability to lyse 21 of the 37 *Salmonella* strains and 3 strains of *E. coli*. Bacteriophages capable of lysing two or more bacterial species or diverse strains within a bacterial species, like Phylax-28, are considered to have a broad host range [66]. These bacteriophages are more likely to be enhances as therapeutic agents [67]. However, the therapeutic efficacy of a phage also depends on its specificity. It is essential that phages selectively target pathogenic bacteria without harming bacteria that perform beneficial functions [68]. Notably, Phylax-28 did not exhibit lytic activity against probiotic strains such as *Bacillus clausii, Bacillus coagulans, Lactobacillus paracasei, Lactobacillus rhamnosus* and *Limosilactobacillus reuteri*, an attribute that could prove advantageous foro control the population of pathogenic bacteria during infection without affecting beneficial bacteria.

3.7. Stability of the Bacteriophage

3.7.1. Storage stability. An essential criterion for selecting bacteriophages as therapeutic is their stability during storage and under the biophysicochemical conditions prevailing at the site of action [39]. For this reason, the storage stability of the Phylax-28 phage was assessed through an accelerated stability assay and validated by monitoring the decrease in phage concentration at 28 °C over a six-months.

The experimental data from the accelerated stability revealed that the reduction in Phylax-28 concentration follows first-order kinetics, with a correlation coefficient (r) of 0.991 at 70 °C (Figure 3). The coefficient of determination (r^2) for the first-order kinetics was 2.80, higher than the values obtained for the zero-order (2.40) and second-order (2.63) reactions, confirming that the first-order model best describe the changes in Phylax-28 concentration. The degradation constant (k), calculated from the experimental data obtained for the stability of Phylax-28 at a temperature of 28 °C over six-months, was estimated to be 0.045 days^{-1}, while the activation energy (Ea) was 158.6 kJ/mol.

The predictive model suggests that the bacteriophage suspension will retain its stable concentration for approximately 92 days at 28 °C before undergoing a 1-logarithm reduction. This prediction is based on the application of the first-order kinetic model together with the Arrhenius equation.

Notably, experimental results support the accuracy of this model: after three months of storage at 28°C, only a 1 logarithm reduction in phage concentration was observed, consistent with model predictions at 94% accuracy. According to Xu et al. (2023), this level of accuracy is considered highly. However, the full validation of the model remains ongoing.

Figure 3. Kinetics of Phylax-28 bacteriophage concentration reduction at various temperatures. (A) No significant reduction was observed at 60 °C (black line) and 65 °C (blue line). However, at 70 °C (green line), the reduction follows first-order kinetics with an r^2 value of 0.991. (B) Experimental results on the stability of Phylax-28 at 28 °C, measured monthly over six months, were compared with the model's predicted stability. The model's accuracy was expressed as a percentage.

3.7.2. Stability in simulated gastrointestinal system. Phage Phylax-28 exhibits a remarkable stability under simulated gastrointestinal conditions. In the oral phase, no significant reduction in viral concentration was observed, and at the gastric level, the decrease was limited to 1.1 logarithms. Furthermore, the concentration remained stable at the intestinal level in relation to the gastric phase (Figure 4). These finding stand in contrast to those report for coliphage Ace, which experienced a 4 logarithms reduction compared to the initial dose under similar conditions [69]. Additionally, when five *Salmonella* phages, Dlamini et al. (2023) documented reductions ranging from 4.86 to 5.55 logarithms, concluding that these phages would require encapsulation in CaCO3 for therapeutic viability [70]. Similarly, bacteriophage ZCEC5 showed comparable reductions, with authors recommending microencapsulation in chitosan-alginate for stability [71]. In comparison, Phylax-28 demonstrates superior stability under gastrointestinal conditions, suggesting its potential as a more robust therapeutic agent.
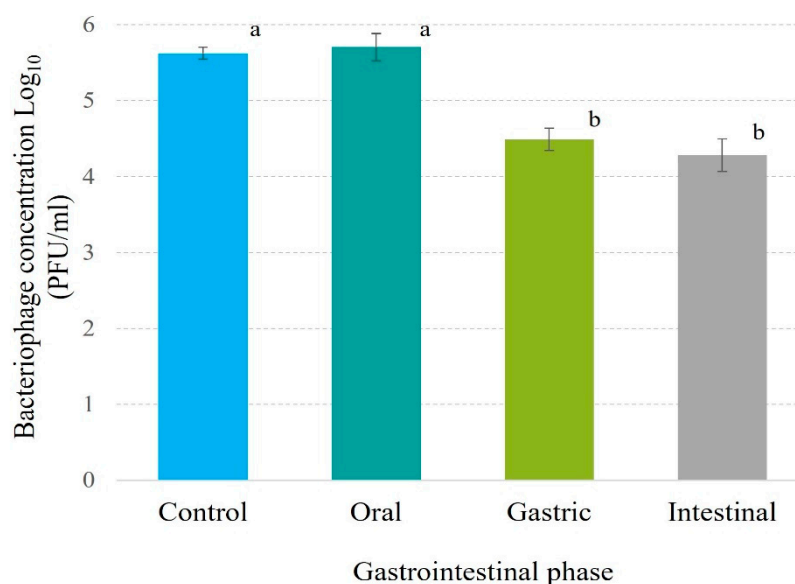
Figure 4. Stability of Phylax-28 bacteriophage under simulated gastrointestinal conditions. In the oral cavity, no significant reduction in phage concentration was observed, indicating high stability in this phase. A slight loss of viability was noted in the gastric environment, but the phage retained functionality. In the intestinal phase, the phage concentration remained stable with no significant changes. Error bars represent the standard deviation of the measurements, reflecting variability across replicate experiments.

3.8. Capacity of the Bacteriophage to Control Salmonella

3.8.1. In culture medium. Under these conditions, Phylax-28 exhibits bacteriolytic activity against *Salmonella*, achieving a significant reduction in bacterial concentration within 60 minutes of inoculation compared to normal bacterial growth ($p < 0.01$). This effect was observed even at low multiplicities of infection (MOI) of 0.01 and 0.001 (Figure 5). These findings are noteworthy, as many phages demonstrate rapid bacteriolytic activity only at higher MOIs. For instance, [72] reported a significant reduction in bacterial load only when using MOIs of 10 and 100. Phylax-28's bacteriolytic activity at low MOIs presents a significant advantage for therapeutic applications, as it suggests that lower phage doses may effectively control *Salmonella* populations. This reduces the potential risk of side effects commonly associated with higher phage doses, offering a safer and more efficient approach to phage therapy.

3.8.2. In simulated gastrointestinal system. *Salmonella* tends to colonize and invade the human intestine [73], making it crucial to evaluate the bacteriophage's ability to control this pathogen under such conditions. In this regard, the results indicate that Phylax-28 significantly reduces *Salmonella* concentration in simulated intestinal environment ($p \leq 0.01$). Moreover, no significant changes were observed in the population of beneficial bacteria due to phage activity (Figure 6). In contrast, in the treatment with ciprofloxacin did not reduce *Salmonella* concentration compared to the control, and there was a marked reduction in the population of beneficial bacteria caused by the antibiotic. While in the beneficial bacteria there is a marked reduction in the concentration due to the action of the antibiotic. These findings are highly relevant, as they suggest that Phylax-28 could potentially control *Salmonella* without adversely affecting the microbiome, positioning it as a promising tool in precision medicine. However, further studies are necessary to validate these results.
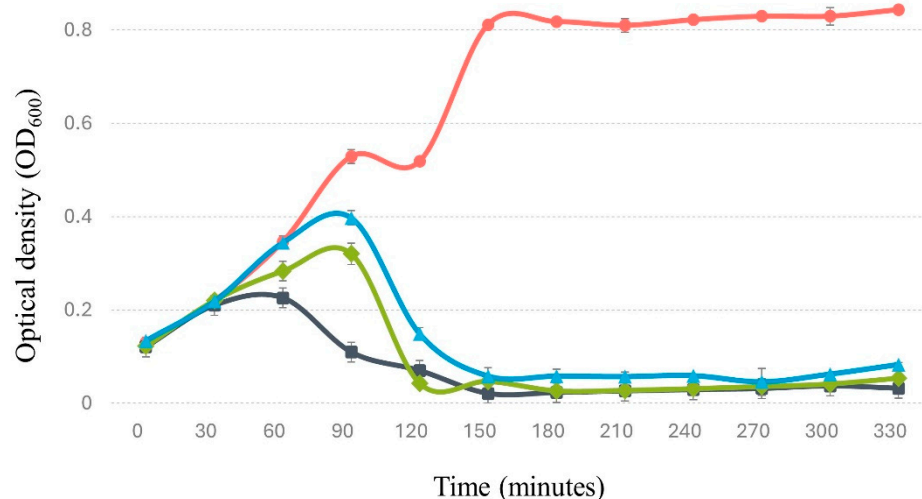
Figure 5. The figure demonstrates the lytic activity of bacteriophage Phylax-28 against Salmonella in trypticase soy broth (TSB). The red line indicates the normal growth of Salmonella without bacteriophage treatment, serving as the control. The black, green, and blue lines represent treatments with Phylax-28 at multiplicities of infection (MOI) of 0.1, 0.01, and 0.001, respectively. A significant reduction in bacterial growth is observed across all MOI levels, with Phylax-28 showing pronounced efficacy in reducing Salmonella concentrations, even at the lowest MOI.
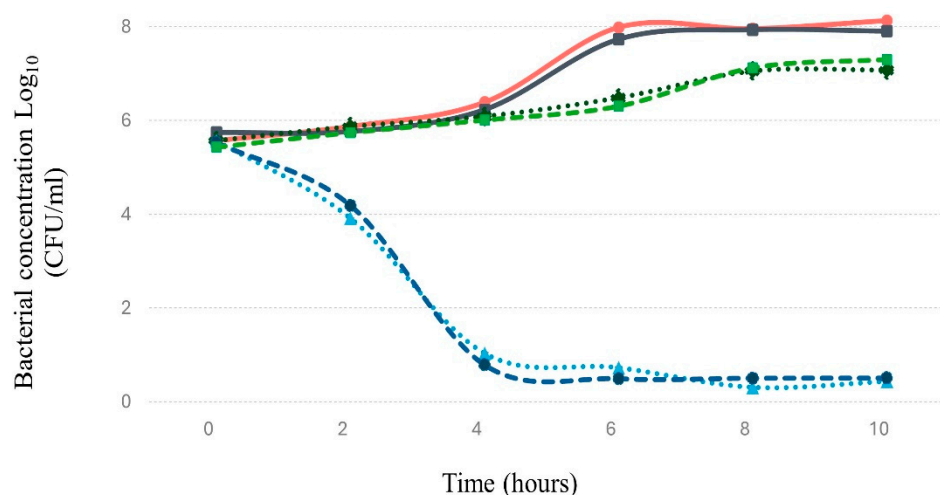
Figure 6. Effect of bacteriophage and ciprofloxacin on the concentration of *Salmonella* and probiotic bacteria in a simulated intestinal environment. The graph displays *Salmonella* growth (red line) in the absence of antibiotic, *Salmonella* in the presence of antibiotic (black line), probiotic bacteria without antibiotic (dashed green line), probiotic bacteria with antibiotic (dashed dark blue line), *Salmonella* in the presence of bacteriophage (dotted light blue line), and probiotic bacteria with bacteriophage (dotted dark green line). The magnitude of error bars, represented as standard deviation, is not discernible in the graph.

3.9. Bacteriophage Genomic Analysis

The genome of the bacteriophage Phylax-28 consists of linear double-stranded DNA, with a molecular size of 40,989 bp and GC content of 57.81%. It encodes 50 genes, none of which are associated with tRNA. Gene functions were annotated using the NCBI BLAST database. Gene functions were identified with an e-value of $<10^{-5}$, sequence coverage exceeding 50%, and identity greater than 85%. The genome is organized into distinct functional modules: a morphogenesis module, containing ten genes responsible for the synthesis of virion structural proteins; a packaging

module, comprising four genes involved in DNA encapsulation within the capsid; a DNA processing module with 13 genes regulating the replication and transcription of genetic material; and a lysis module, consisting of three genes responsible for cell wall degradation and bacterial lysis; the remaining genes encode hypothetical proteins. Critically, for biosafety considerations, no genes related to virulence factors, antimicrobial resistance, or allergenic responses were identified. Moreover, Phylax-28 was classified as strictly lytic, a desirable trait for bacteriophages intended for therapeutic applications [74]. The complete genome sequence of phage Phylax-28 was deposited in GenBank/EMBL/DDBJ under the accession number PQ306468.

5. Conclusions

Phylax-28 exhibits promising potential as a therapeutic agent against multidrug-resistant *Salmonella* strains that encode virulence factors associated with the development of precancerous lesions. Although further research is necessary to confirm its safety and efficacy in clinical settings, preliminary data suggest that this bacteriophage could become a viable biopharmaceutical option in the near future.

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