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Characterization and therapeutic potential of newly isolated bacteriophages targeting the most common *Salmonella* serovars in Europe

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Despite meticulous monitoring of *Salmonella* spp. throughout the food chain to ensure safer animal food products for consumers, the number of salmonellosis cases in humans continues to rise annually in Europe. Phage therapy emerges as a promising tool for controlling and eradicating *Salmonella* in primary production. This study aimed to fully characterize new phage therapy candidates isolated from animal sources. To achieve this, a phenotypic and genetic characterization of five phage isolates was conducted. The five phages demonstrated physical stability across a wide range of temperatures and pH levels, effectively lysing 12 different *Salmonella* serovars, including the most prevalent ones in the European Union in recent years, as well as multidrug-resistant strains isolated from the field. Additionally, four of the phages exhibited depolymerase production in the host range, with genomic analysis confirming that all five possessed sequences encoding for this activity, suggesting their potential as surface-disinfecting agents. Genetic analysis further revealed that the phages belong to distinct genera: *Felixounavirus*, *Cornellvirus*, *Skatevirus*, *Agtevirus* and *Berlinvirus*. Notably, none of the phages contained harmful sequences that could compromise their future application, such as virulence factors, antibiotic resistance genes or temperate markers. Overall, these five phages show promise as suitable candidates for phage therapy applications or phage-based *Salmonella* eradication strategies, where their integration in the existing biocontrol measures may enhance both food safety and public health.

Keywords *Salmonella*, Bacteriophages, One health, Phage therapy, Genomic analysis, Depolymerase

The World Health Organization (WHO) estimates that around 550 million people contract a gastrointestinal illness related to unsafe food consumption each year, leading to an annual loss of 33 million healthy life years[1–3]. *Salmonella* is a bacterial genus mainly known for causing gastroenteritis in humans and animals and is classified by the WHO as one of the four main causes of diarrhoeal diseases[1–3]. In fact, in 2022, *Salmonella* was the most frequently implicated aetiological agent in foodborne outbreaks (FBO) within the European Union (EU)[4]. Beyond its economic impact on the agricultural sector and public health, *Salmonella* significantly affects human quality of life, with up to 65,208 reported salmonellosis cases, 11,287 hospitalizations and 81 deaths in 2022 in the EU alone[4]. *Salmonella* is most commonly associated with poultry, swine products and their derivatives, though cases linked to other livestock productions are reported annually[4–7].

Among all *S. enterica* serovars detected in the EU, the most frequently implicated in human salmonellosis from 2019 to 2022 were Enteritidis, Typhimurium, monophasic Typhimurium (mST), Infantis, and Derby[4–6,8]. In 2022, *S.* Enteritidis, and *S.* Infantis were more frequently isolated from poultry products, *S.* Typhimurium and

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mST from swine sources and *S.* Derby from bovine matrices[4]. Despite the numerous control measures taken to ensure a safer final product, such as vaccination in the poultry sector against *S.* Enteritidis and *S.* Typhimurium, or the mandatory self-control analysis for farm-level *Salmonella* detection, serovar crossovers between food chain and animal production still occur[9–13]. Thus, from a One Health perspective, no animal production system is entirely free from *Salmonella* contamination, or the antimicrobial resistance (AMR) and virulence factors linked to them, reinforcing the need for innovative control tools to safeguard public health[14–16].

The rise of antimicrobial and multidrug-resistant (MDR) bacteria poses an urgent public health threat[17,18]. The WHO has predicted that by 2050 the main cause of death in humans will come from unsolved infections caused by MDR bacteria[19,20]. This trend is closely linked to the historical overuse and misuse of antibiotics, and exacerbated by horizontal transmission of AMR and virulence factors among bacteria[17,21,22]. *Salmonella* is classified as a high-priority research pathogen by the WHO, due to fluoroquinolone resistance observed in some isolates[23].

In this context, bacteriophages, or phages, are gaining renewed interest as a promising alternative to antibiotics in combating MDR bacteria, including *Salmonella*[24]. Phages are viruses that specifically infect and kill bacteria, depending on them to fulfil their replication cycle[25,26]. These viruses have a unique ability to control bacterial populations in various environments, including animals, surfaces or even the human body[27]. They possess a narrow range of action against different bacterial species, meaning they are highly specific and fatal to their hosts, but have minimal impact on other bacteria in the same environment[28,29]. Furthermore, though such attributes might only seem advantageous, the genetic material they encode can contain information other than that needed for the assembly of new virions[27]. Once phages attach to their host, they introduce their genetic material into the bacteria; when its lifecycle is lytic, such material is read by the host and new phages are released into the environment after the bacteria is lysed[30]. However, this genetic material can potentially integrate into the bacterial genome, leading to lysogeny[31]. Lysogenic phages also end their lifecycle destroying the host when external stress factors trigger phage production within the cell[31]. Nevertheless, while the production of new phages is not triggered, they can transfer additional virulence factors or AMR genes to the bacteria that can enhance the bacteria's pathogenic capacities[31]. Therefore, when a phage is isolated for therapeutic purposes, it is essential to conduct thorough characterization. This ensures that the selected phages follow strict lytic lifecycles and do not carry any AMR genes or other harmful genetic elements, which could be self-defeating for phage therapy[32,33].

The use of phages as prophylactic and therapeutic agents has been described throughout the last century and is known as phage therapy[29,34]. Studies assessing their suitability as prophylactic, disinfecting and therapeutic agents are numerous and widespread in different health fields, including animal production[35–39]. Other studies have also been focused on the limits, or the delayed failure, of phage therapy linked to the host's resistance mechanisms against phages[40,41]. Therefore, due to the changing susceptibility patterns some bacteria can have and the high diversity of *Salmonella*, new phages against different serovars that possess high lytic capacities against the pathogen must be continuously isolated to withhold phages as effective therapeutic agents[26,40–42]. This study aimed to fully characterize newly isolated *Salmonella* phages capable of lysing the most prevalent *Salmonella* serovars in Europe in recent years, and to evaluate them as potential candidates for phage therapy.

Results

Antimicrobial susceptibility test of the challenge strains

Regarding the antibiotic resistance profiles of the challenge strain (CS), 48% (20/42) exhibited resistance to at least one antibiotic, while 17% (7/42) were classified as MDR. Among the MDR strains, *S.* Typhimurium, mST, *S.* Infantis, *S.* Virchow and *S.* Kedougou were the serovars with the highest numbers of resistance, as illustrated in Fig. 1. The antibiotics to which the highest resistances were observed were: tetracyclines (29%), quinolones (26% for ciprofloxacin (CIP) and 26% for nalidixic acid (NAL)), sulphonamides (24%), β-lactams (19% for ampicillin (AMP)), phenicols (12% for chloramphenicol (CHL)) and pyrimidines (7% for trimethoprim (TMP)). Finally, 2% of the strains were resistant to macrolides (azithromycin (AZI)), glycylcycline (tigecycline (TGC)) and aminoglycosides (gentamicin (GEN)). All strains were sensitive to β-lactams, carbapenems (meropenem (MERO)), cephalosporins (ceftazidime (TAZ) and cefotaxime (FOT)), polymyxins (colistin (COL)) and aminoglycosides (amikacin (AMI)).

Phage isolation and propagation

The 120 samples were tested against each of the five propagation strains (PS), performing 600 spot test assays. Phage was detected in 15.83% (29/120) of the samples, where 41.38% (12/29) presented clear morphologies on double layer agar (DLA) plates. Avoiding crossed lytic capacities between phages and PS, 5 phages were selected and further characterized for this study (Table 1). Phages vB_CECAV_048 and vB_CECAV_050 were isolated from the same sample.

Plaque morphology and phage imaging by transmission electron microscopy

Following an overnight incubation, all phages formed distinct plaques on the DLA plates specific to their respective PS (Fig. 2a) (Table 2).

Regarding the micrographs obtained through transmission electron microscopy (TEM), phages vB_CECAV_041 and vB_CECAV_050 were classified as morphologically compatible with Myoviruses; their capsids ranged from 68.6 to 88.4 nm and their tails from 101.6 to 119.6 nm (Fig. 2b). Phages vB_CECAV_044 and vB_CECAV_048 were classified as morphologically compatible with Siphoviruses, with capsid sizes between 53.3 and 84.4 nm and tails from 90.8 to 105.7 nm (Fig. 2b). Phage vB_CECAV_058 was classified as morphologically compatible with a Podovirus and had a capsid size of 78.8 nm (Fig. 2b).

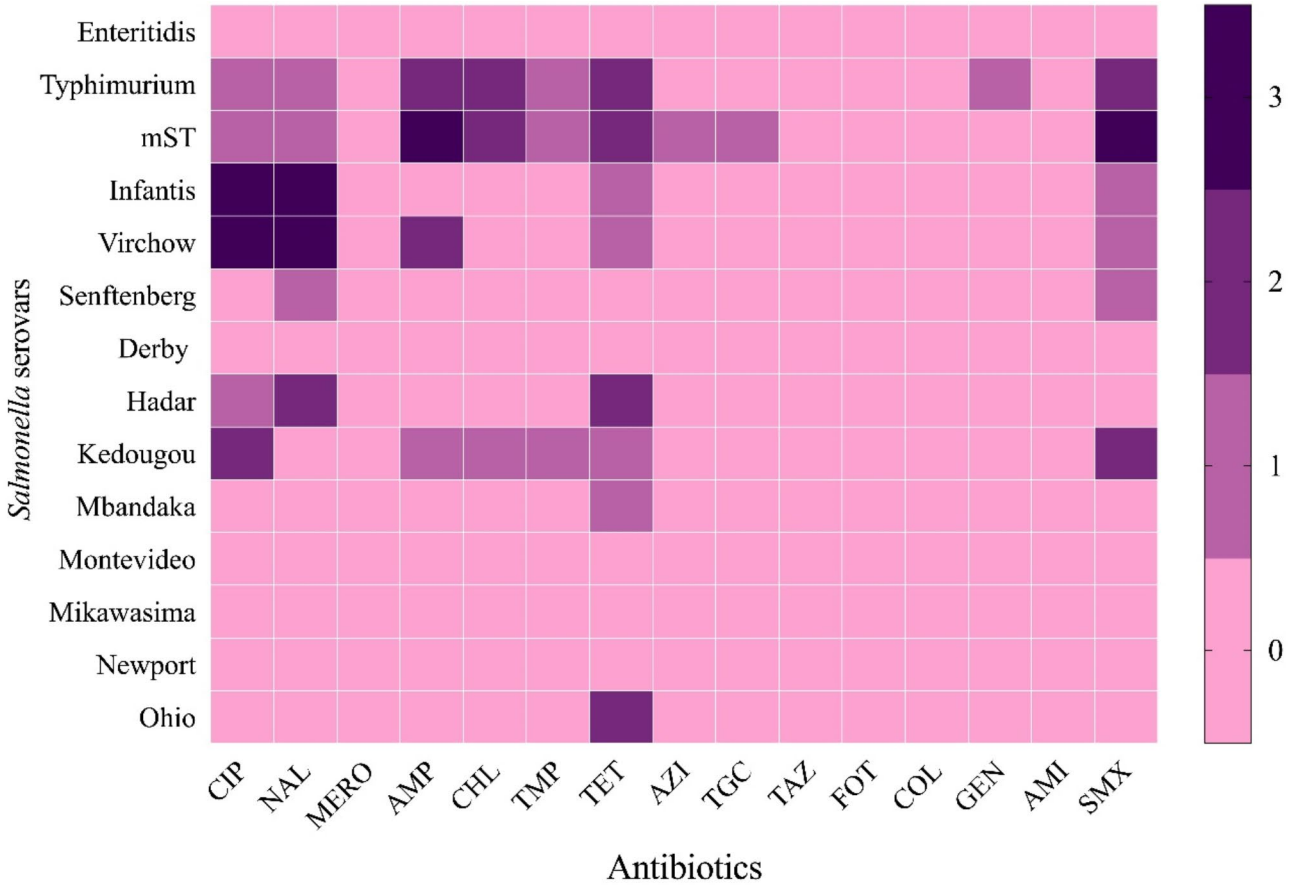

Fig. 1. Antimicrobial resistance patterns in the CS. Heat map illustrating the number of AMR strains detected per antibiotic in each serovar from the CS. Each serovar was compounded of three challenge strains ($n = 3$). CIP: ciprofloxacin. NAL: nalidixic acid. MERO: meropenem. AMP: ampicillin CHL: chloramphenicol. TMP: trimethoprim. TET: tetracycline. AZI: azithromycin. TGC: tigecycline. TAZ: ceftazidime. FOT: cefotaxime. COL: colistin. GEN: gentamicin. AMI: amikacin. SMX: sulfamethoxazole.

Phage ID	Propagation strain (PS)	Source sample
vB_CECAV_041	*S.* Kedougou	Broiler faeces
vB_CECAV_044	*S.* Infantis	Broiler faeces
vB_CECAV_048	mST var. Copenhagen	Waste water from a pig slaughterhouse
vB_CECAV_050	*S.* Typhimurium var. Copenhagen	Waste water from a pig slaughterhouse
vB_CECAV_058	*S.* Mikawasima	Layer faeces

Table 1. Phages selected per PS and their source.

Host range and efficiency of planting

To evaluate the host range and efficiency of planting (EOP) of the candidates, a total of 210 assays were carried out to challenge the 42 CS against the 5 phages. Although only 59 assays tested positive for bacterial lysis, out of the 42 *Salmonella* CS, 80.95% (34/42) were lysed by at least one of the phages (Fig. 3). Phage vB_CECAV_041 had lytic capacities against 10/14 serovars of the CS, vB_CECAV_058 against 6/14 serovars, vB_CECAV_048 and vB_CECAV_050 against the same 4/14 serovars and vB_CECAV_044 against 2/14 serovars. Only two groups of serovars, Hadar and Senftenberg, were not lysed by the candidates.

Thermal and pH stability

No significant differences in phage stability across the tested temperatures ($p\text{-}value > 0.05$) were observed. All phages remained stable between −80°C and 60°C (Fig. 4). For all five phages, a marked decrease in titres occurred only at temperatures of 70°C and above.

Regarding pH stability assays (Fig. 4b), vB_CECAV_041 proved to be stable from pH 4 to pH 11, where titres slightly decreased at pH 10 to 11. Phage vB_CECAV_044 was stable from pH 3 to pH 12, also with slight drop in

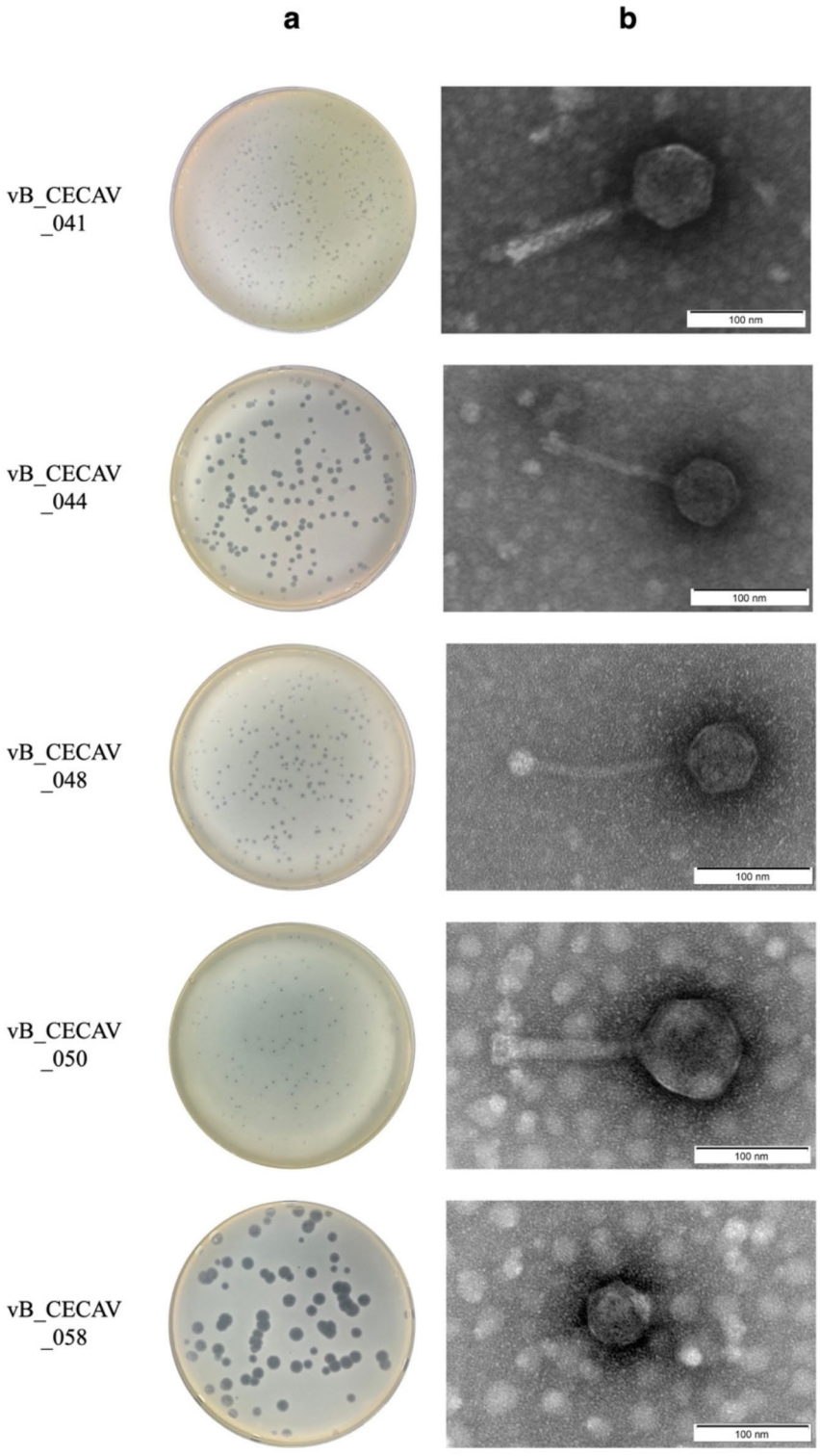

Fig. 2. Macro and micro morphology of the phages. (**a**): plaque morphology on DLA of their PS. (**b**): TEM imaging of the bacteriophages.

Phage ID	Plaque diameter (mm)	Depolymerase
vB_CECAV_041	0.81 ± 0.18	–
vB_CECAV_044	2.24 ± 0.15	+
vB_CECAV_048	1.24 ± 0.17	+
vB_CECAV_050	0.82 ± 0.12	+
vB_CECAV_058	3.61 ± 0.49	+

Table 2. Plaque morphology, metrics and depolymerase expression on their PS.

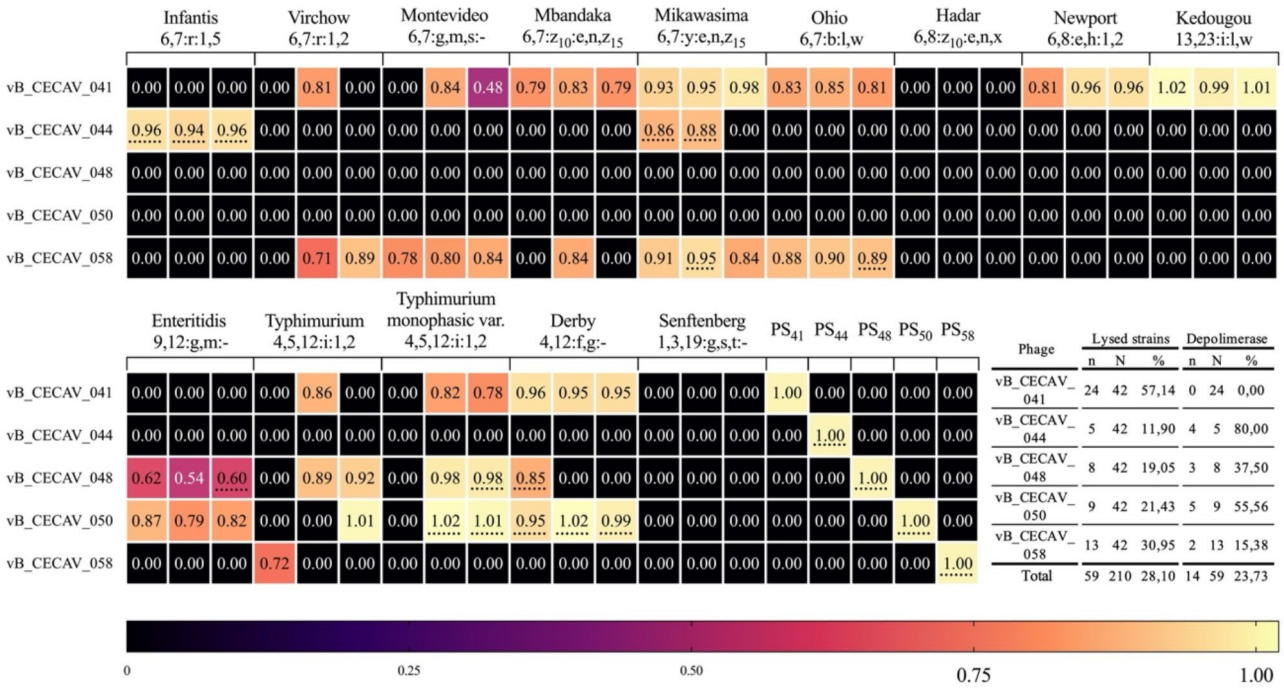

Fig. 3. Host range and EOP of the phages against the 42 *Salmonella* field strains and their own PS. EOP was established relative to the phage titres with their PS. The PS of the phages belonged to serovars Kedougou (PS_{41} for vB_CECAV_041), Infantis (PS_{44} for vB_CECAV_044), mST var. Copenhagen (PS_{48} for vB_CECAV_048), Typhimurium var. Copenhagen (PS_{50} for vB_CECAV_050) and Mikawasima (PS_{58} for vB_CECAV_058). Dotted underlining (…) indicates the expression of depolymerase halos around the lytic plaques. N: whole group number. n: number of positive outcomes.

titres at pH 3, 11 and 12. Phage vB_CECAV_048 could withstand a pH of 5 to a pH of 11 and vB_CECAV_050 endured pHs from 4 to 10. As for vB_CECAV_058, it was stable at pH levels 5 to 10.

Infection kinetics

The kinetics of lytic development for each phage are shown in Fig. 5a. All phages were able to delay the exponential growth phase of the PS from 2 to 6 h. No differences were observed in the timing of the exponential growth phase of the PS based on the multiplicity of infection (MOI) used.

One-step growth

One-step growth assays revealed variations in burst size and lysis time among the phages (Fig. 5b). On one hand, the burst size of each phage was established through the formula previously described, with phage vB_CECAV_058 being the one with the biggest burst size, ~1,525 PFU/cell, followed by vB_CECAV_050 with ~1,385 PFU/cell, vB_CECAV_048 with ~314 PFU/cell, vB_CECAV_041 with 75 PFU/cell and vB_CECAV_044 with 33 PFU/cell. On the other hand, phages vB_CECAV_041, vB_CECAV_044 and vB_CECAV_048 were able to complete a lifecycle within 30 min after the inoculation, while phages vB_CECAV_050 and vB_CECAV_058 took 50 and 70 min, respectively. The latency period for all phages was approximately 20 min, accounting for the 10 min required to enter the exponential growth phase and an additional 10 min allotted to ensure proper attachment.

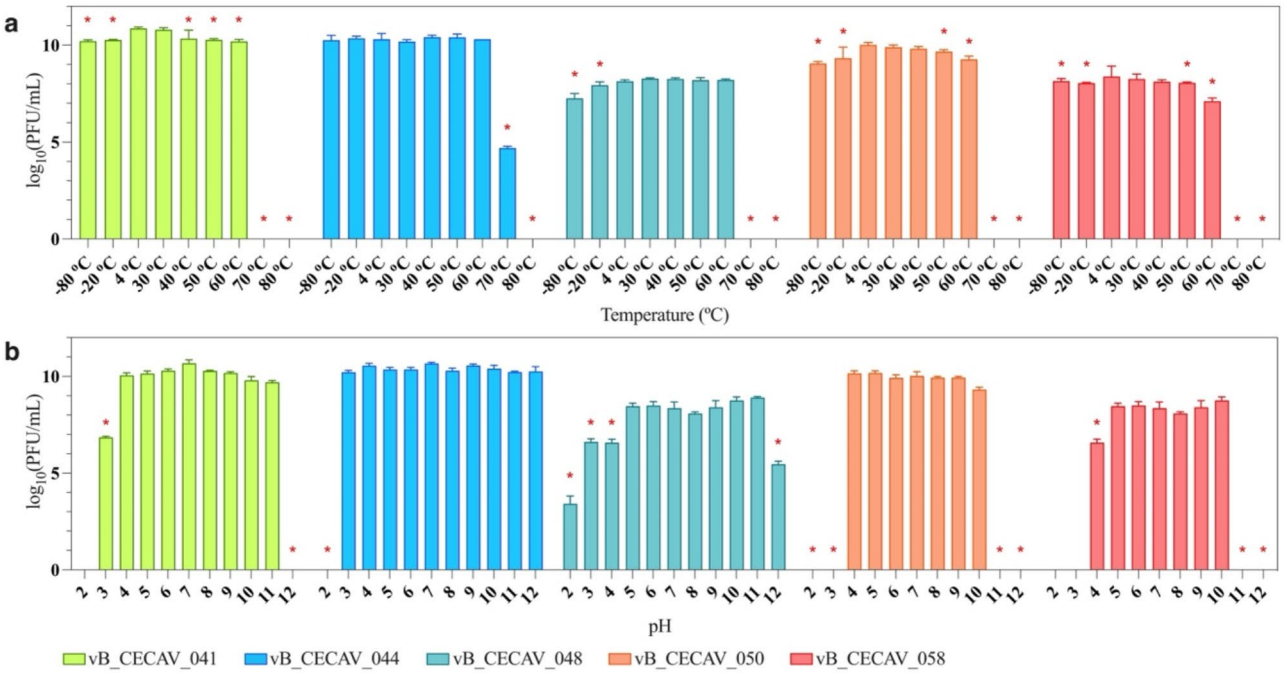

Fig. 4. Stability assays of five phages. (**a**): thermal stability tests. (**b**): pH stability tests. *: titres statistically different from the baseline titre (*p-value* < 0.05), pH 7 and 4°C respectively.

Genomic analysis and taxonomy of phages

BLAST comparison of the putative phage genomes against the nucleotide database revealed that vB_CECAV_041 was closely related to phages in the *Felixounavirus* genus (undefined family), while vB_CECAV_044 showed strong similarity to *Cornellvirus* (undefined family). Likewise, vB_CECAV_048 exhibited high similarity to *Skatevirus* (undefined family), vB_CECAV_050 aligned most closely with *Agtrevirus* (*Ackermannviridae* family) and vB_CECAV_058 was found to be highly similar to *Berlinvirus* (*Autographiviridae* family) (Table 3).

Genome reordering resulted in final genome sizes ranging from 38.9 kb to 152.5 kb, with GC content varying between 39.0% and 50.8%. Functional and structural annotations identified between 47 and 194 coding sequences (CDS). Furthermore, tRNA genes and pseudogenes were identified in vB_CECAV_041, vB_CECAV_048, and vB_CECAV_050 within the host, while vB_CECAV_044 and vB_CECAV_058 lacked these elements (Fig. 6) (Table 3).

In terms of antibiofilm properties, Pharokka annotation identified several tail-related proteins across the genomes of the five phages (Table 4). Further analysis with DePP indicated that some of these proteins had a 90% probability of being depolymerases. Specifically, vB_CECAV_041 encoded a tail fibre protein (ORF 74) on the positive strand. Phage vB_CECAV_044 contained two tail-related proteins: a tail protein (ORF 65) and a tail spike protein (ORF 66), both on the positive strand. In vB_CECAV_048, a tail protein (ORF 67) was identified. Phage vB_CECAV_050 encoded a hypothetical protein of unknown function (ORF 84) on the negative strand, while vB_CECAV_058 encoded a tail protein (ORF 37) on the positive strand.

PhageLeads and PhaTYP analyses predicted that all five phages undergo a strictly lytic infection cycle, with no evidence of temperate markers, virulence factors or AMR genes. These findings were corroborated by ABRICATE results, which confirmed the absence of AMR genes in all five phages.

The combined results from whole-genome and single-gene phylogenetic analysis based on the *terL* gene confirmed the previous taxonomic classification (Figs. 7 and 8). Thus, vB_CECAV_041 was confidently classified as part of *Felixounavirus*, with high intergenomic similarity to phage FelixO1 (86.9%), and its placement within this genus was strongly supported by the phylogenetic analysis. Similarly, vB_CECAV_044 was confirmed to belong to the *Cornellvirus* genus, showing 77.0% similarity to phage FSL SP-031, with the phylogenetic tree reinforcing this grouping. Phage vB_CECAV_048 was placed in the *Skatevirus* genus, with 70.7% similarity to phage_Skate, also consistent between both analyses. In turn, vB_CECAV_050 was similarly aligned with the *Agtrevirus* genus, showing 77.8% similarity to phage_Ag3, and the phylogenetic tree corroborated this placement. Finally, vB_CECAV_058 was confirmed as part of *Berlinvirus*, with 85.7% similarity to phage Berlin, as supported by both analysis methods. VipTree analysis further validated these classifications, with all five phages clustering as expected within their respective genera, based on whole-genome comparisons (see Suppl. Figure S1).

Discussion

Despite rigorous cleaning and disinfection protocols, biosecurity-compliant workflows and self-sampling controls on farms and end products[43–45], *Salmonella* remains one of the leading causes of FBO in Europe[4].

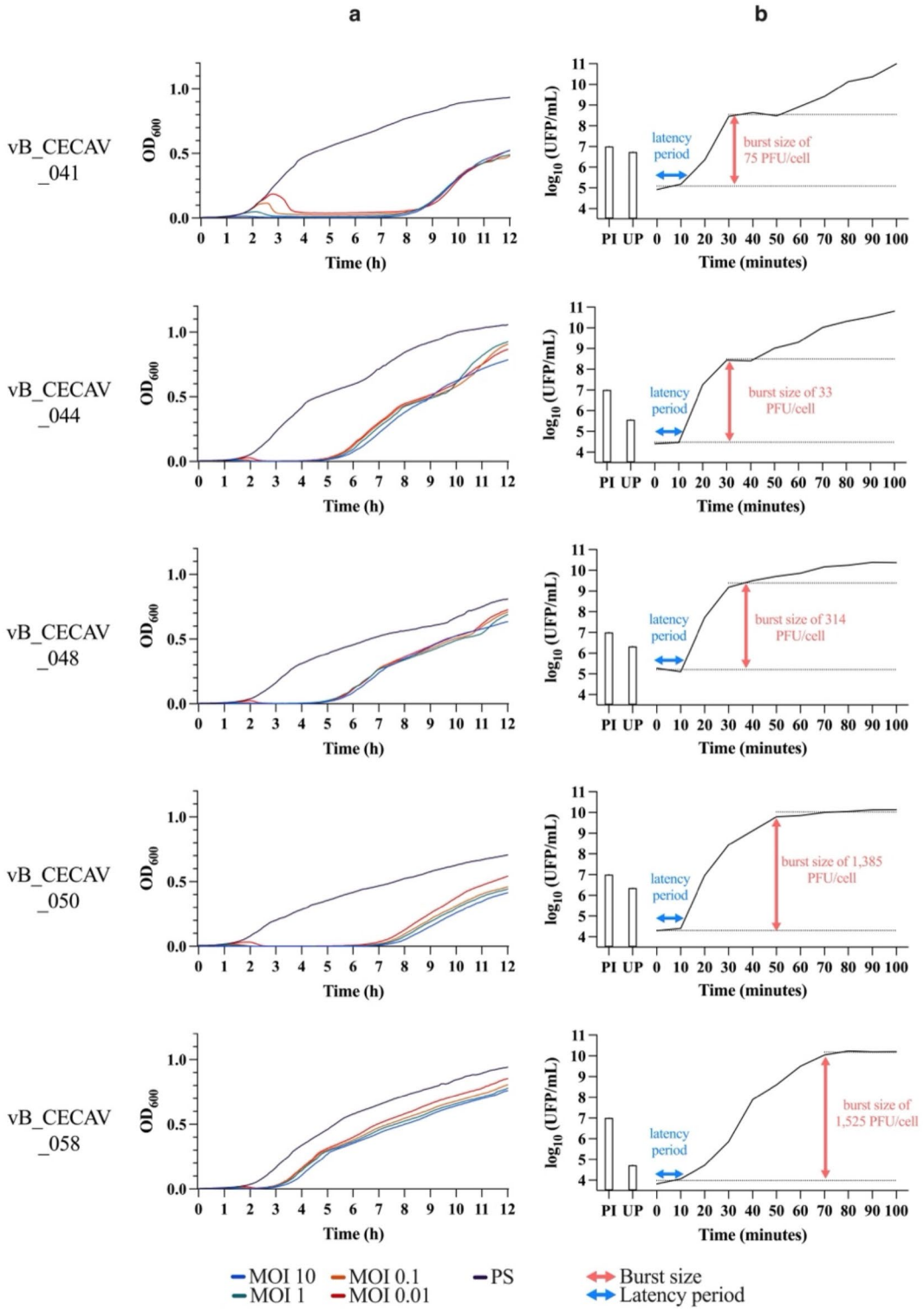

Fig. 5. Multiplicity dynamics of the phages. (**a**): infection kinetics. (**b**): one-step growth curves. PI: phage inoculated, UP: unattached phages.

The five newly isolated phages in this study were capable of lysing 12 different *Salmonella* serovars, including Enteritidis, Typhimurium, monophasic Typhimurium, Infantis and Derby, which were the five most prevalent serovars in the EU in 2019, 2020, 2021, and 2022 and which currently lack biocontrol tools. These phages provide five new, natural-source tools to control a wide range of *Salmonella* serovars across the poultry, swine and bovine food chains, aligning with the "Farm to Fork" strategy[4–6,8].

ColorInfoStart FFFFFFCharacterizationColorInfoEnd FFFFFF		vB_CECAV_041	vB_CECAV_044	vB_CECAV_048	vB_CECAV_050	vB_CECAV_058
Size		86,103	41,614	45,195	152,465	38,913
GC content (%)		39.0	50.8	46.6	49.0	48.8
Coding sequences		131	67	89	194	47
tRNA and pseudogenes		20	0	1	5	0
Hypothetical protein (%)		62	36	69	48	17
Accession number (Genbank)		PQ064465.1	PQ186186.1	PQ220373.1	PQ306799.1	PQ356389.1
Most similar by Blast	Name	OQ845959.1 *Salmonella* phage NJ12	PP664540.1 *Salmonella* phage pJS4	OP745620.1 *Salmonella* phage INT55	OQ973471.1 *Escherichia* phage AV101	OP185508.1 *Salmonella* phage JSS1,
	Length (bp)	87,366	41,754	44,945	156,759	40,070
	Coverage (%)	92.0	98.0	90.0	91.0	96.0
	E-value	0.0	0.0	0.0	0.0	0.0
	Identity (%)	95.43	99.90	99.27	97.59	96.84
Taxonomy	Class	*Caudoviricetes*	*Caudoviricetes*	*Caudoviricetes*	*Caudoviricetes*	*Caudoviricetes*
	Family	–	–	–	*Ackermannviridae*	*Autographiviridae*
	Genus	*Felixounavirus*	*Cornellvirus*	*Skatevirus*	*Agtrevirus*	*Berlinvirus*

Table 3. Genomic and taxonomic characteristics of isolated phage genomes.

Although phages are highly specific by nature[35], overlap in host range allowed the five new phages to effectively lyse 80.95% (34/42) of the CS. Among the stains with MDR patterns, 71.43% (5/7) were lysed by the candidate phages, including Typhimurium, Infantis and Kedougou serovars. This suggests that these phages could play a dual role: not only effectively reducing zoonotic *Salmonella* strains, thus lowering FBO risk, but also limiting the transmission of AMR *Salmonella* to humans[35]. Regarding the fluoroquinolone-resistant CS, the candidates were capable of lysing 72.73% (8/11) of the strains resistant to CIP. As CIP is one of the main antibiotics recommended for treating *Salmonella* infections in humans, and fluoroquinolone-resistant strains have been isolated more frequently in public health in recent years, the phage candidates could also help alleviate the pressing need for alternatives to these antimicrobial drugs[23,46]. Furthermore, each phage exhibited a well-defined range of action, bound to the antigenic profiles from the PS, a pattern consistent with previous studies that associated the range of action of phages with the antigenic formula of the hosts[47,48]. Notably, phage vB_CECAV_041, belonging to the *Felixounavirus* family, demonstrated the broadest host range, infecting 10 out of 14 *Salmonella* serovars, regardless of the antigenic formula. This agrees with previous research identifying *Felixounavirus* as a broad-host-range genus capable of infecting multiple serovars[49,50]. The use of reference strains from publicly accessible repositories to characterize phages for future phage therapy uses is well described in the literature[51,52]. However, this study characterized phages using field-isolated clinical strains from the most relevant serovars in the EU in recent years. This approach offers a more accurate assessment of their potential against the current strains present in animal production.

While some phages might exhibit strong lytic capacities, their success in phage therapy could be hindered by a lack of physical stability, as they may only survive within narrow ranges of environmental conditions[53]. Nevertheless, the phenotypical characterization of the phages showed that all phages tolerated temperatures ranging from −80 °C to 50 °C, confirming their potential viability as stable disinfecting agents on surfaces in various environments, as well as their adaptability to different thermal conditions, including those similar to animal body temperatures. Their physical stability supports their potential use in reinforcing cleaning and disinfection practices on poultry farms to eliminate *S.* Infantis, as described by Sevilla-Navarro[36], as well as their applicability in phage therapy as water or feed additives to control *S.* Typhimurium and *S.* Infantis[54,55]. Furthermore, their stability under different pH conditions and their capacity to endure temperatures below 0ºC also ensures they could undergo lyophilization or encapsulation processes, different storage and administration formulas used for phage therapy[56,57]. Moreover, the detailed study of their lytic patterns, through the kinetics of infection and one-step growth assays, provides sturdier grounds for any future application of the candidates[58]. Although in-vitro and in-vivo results may vary, assessment of the exponential growth phase delays in the PS and the latency period and burst size of the phages is essential to forecast and understand future in-vivo results[58,59].

As for phages vB_CECAV_048 and vB_CECAV_050, their isolation from the same wastewater sample and their highly similar host range could have led to mislabelling the isolates as the same phage in the early stages of the phenotypical characterization. Only after TEM imaging and genome sequencing could they be properly identified as different phages, enhancing the importance of an in-depth and full characterization of any isolated phages. Although they were completely different phages, where vB_CECAV_048 was a *Skatevirus* and vB_CECAV_050 was an *Agtevirus*, their similarity in host range could have been driven by their original source. The wastewater deposit from the pig slaughterhouse was an enclosed environment where the two phages could have had to compete for suitable hosts, entering into a co-evolutionary competitive dynamic between them. Brockhurst et al. (2006) described in their study that the population of two different *Pseudomonas* phages cultured with a mutual host were self-regulated by the adaptative changes of the bacteria towards one of them[60]. Such adaptive changes between phage and host are also known resistance mechanisms, which lead to co-

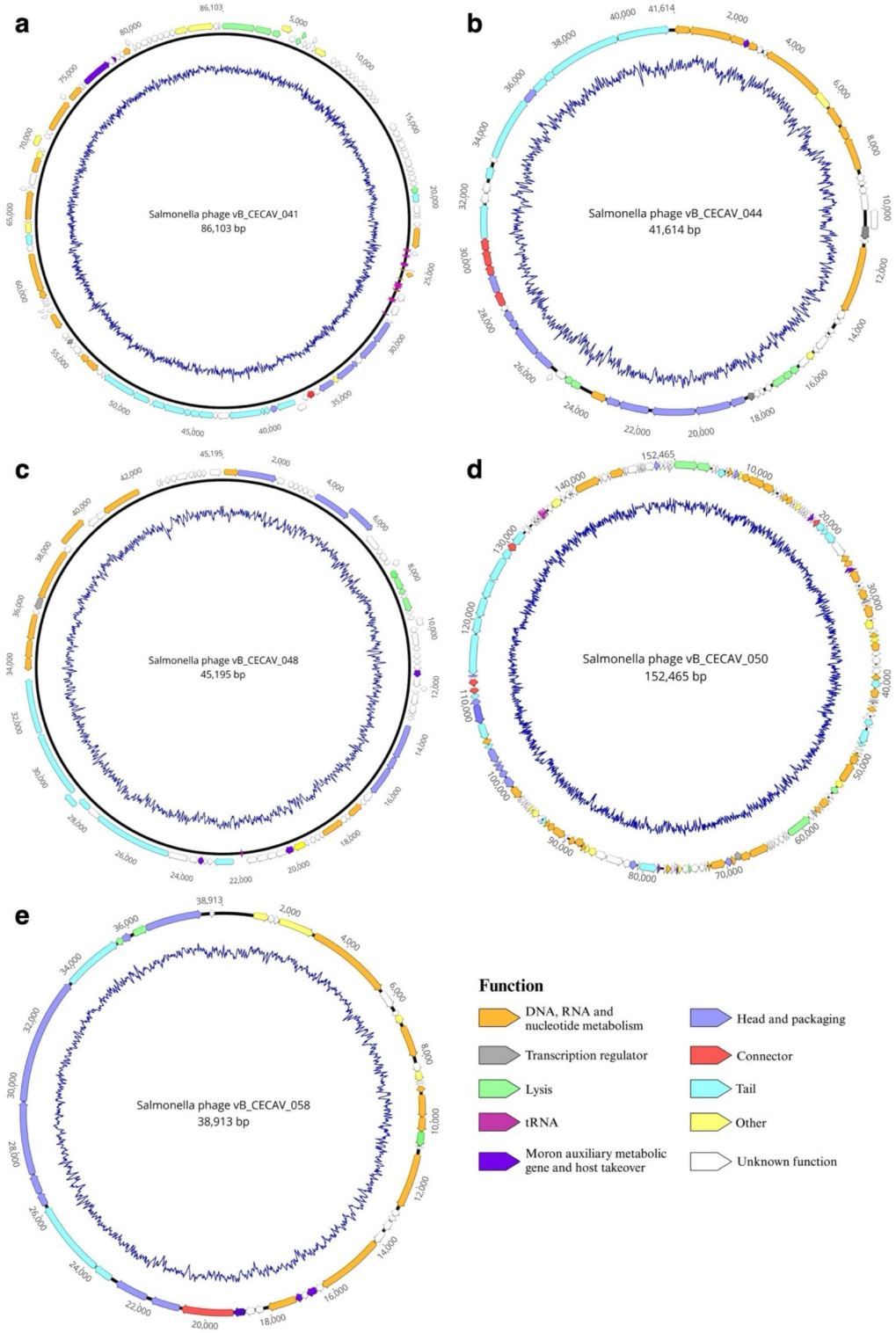

Fig. 6. Circular genome maps of phages. (**a**) vB_CECAV_041, (**b**) vB_CECAV_044, (**c**) vB_CECAV_048, (**d**) vB_CECAV_050 and (**e**) vB_CECAV_058. Generated using Geneious Prime 2024.0.7. The inside rings represent GC percentage content and predicted coding sequences, identified by *Pharokka*, are represented as coloured arrows based on functional groups as defined by Phrog.

Phage	ORF	Locus	Start	End	Strand	Function	Product
vB_CECAV_041	74	cecavS41_074	49,628	51,970	+	Tail	Tail fibre protein
vB_CECAV_044	65	cecavS44_065	37,074	39,620	+	Tail	Tail protein
	66	cecavS44_066	39,632	41,431	+	Tail	Tail spike protein
vB_CECAV_048	67	cecavS48_067	28,897	31,386	+	Tail	Tail protein
vB_CECAV_050	84	cecavS50_084	61,586	58,512	−	Unknown function	Hypothetical protein
vB_CECAV_058	37	cecavS58_037	23,762	26,143	+	Tail	Tail protein

Table 4. Predicted depolymerases in the phage genomes by DePP.

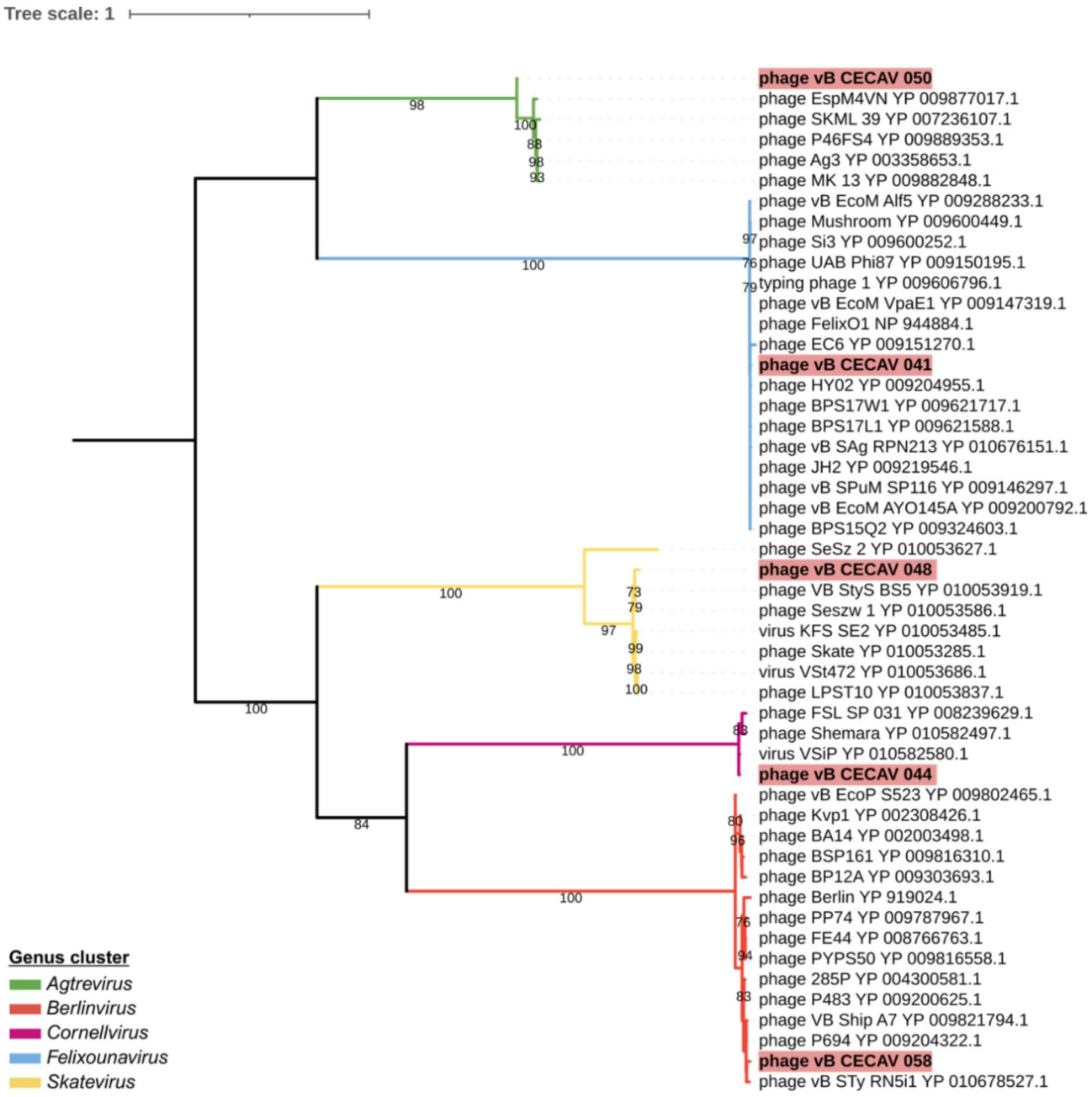

Fig. 7. Taxonomic classification of isolated phages based on terL gene comparison. Maximum likelihood phylogenetic analysis of the five isolated phages and closely related reference phages. Figure generated with iTOL v.6.

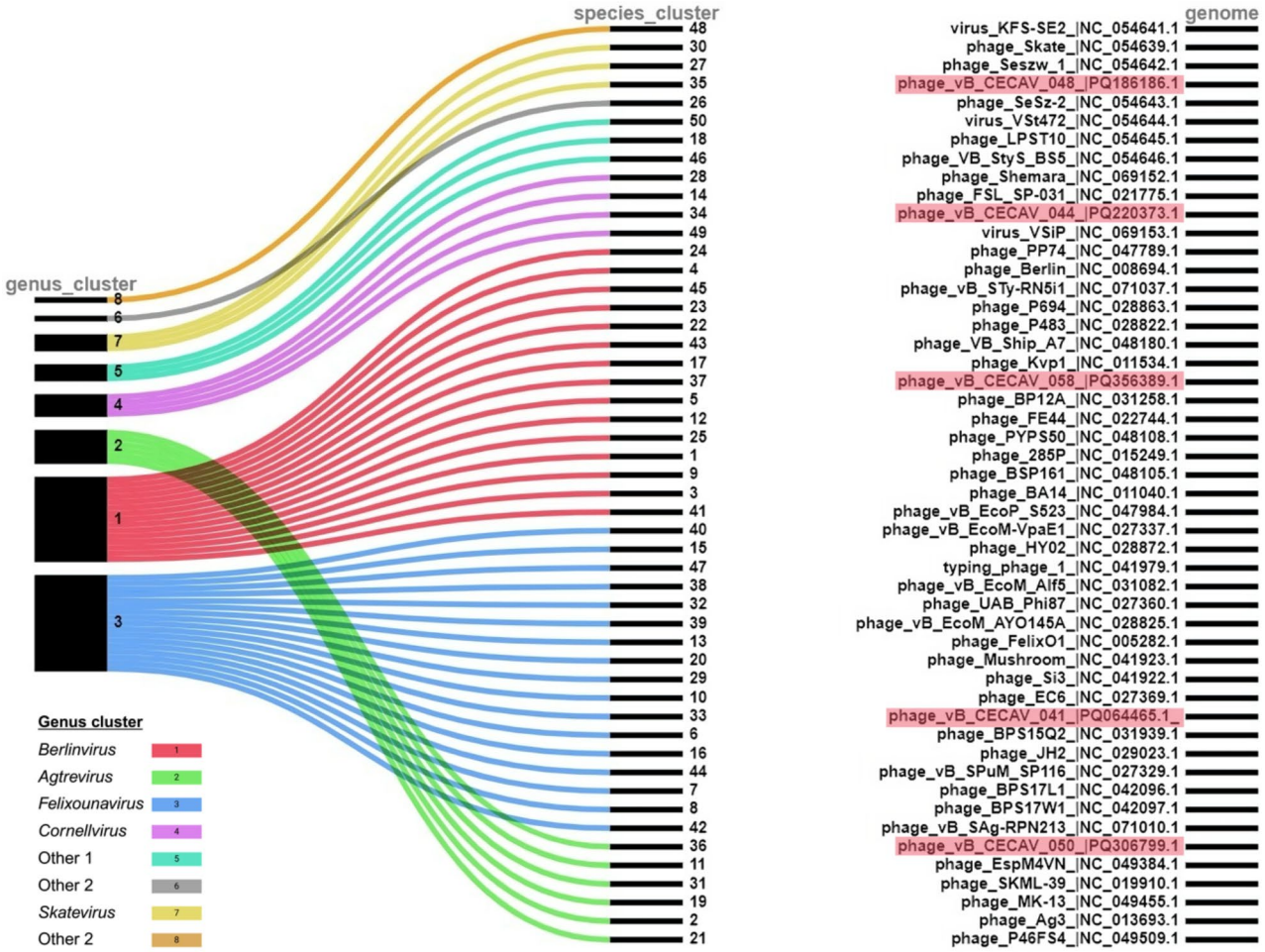

Fig. 8. Alluvial diagram of the intergenomic similarity of the five new phages. The image was generated with RawGraphs.

evolution[61,62]. Together, prolonged times in the same enclosure and the co-evolution dynamics could have led to the convergence in host range of two different phages, though further in-vitro assays should be carried out to confirm this hypothesis.

Genomic analysis of the five phages revealed key features that underline their potential as controlling tools against *Salmonella*. All phages exhibit strictly lytic behaviour and lack temperate genes, AMR determinants or virulence factors, making them safe and suitable for both biocontrol and therapeutic strategies. This could ensure more predictable results driven by the utilization of agents that do not enhance AMR or bacterial pathogenicity, and would not compromise the expected outcome of any future application[63,64]. Moreover, though only four of the phages showed depolymerase activities when cultured with the CS, the genetic analysis of the candidates suggests the presence of these enzymes in all of them. Biofilms, notoriously resistant to traditional disinfectants, can be effectively targeted by phages with depolymerase activity, facilitating the eradication of bacteria from multiple surfaces prone to contamination[36,65]. As depolymerases can degrade polysaccharides in bacterial capsules and biofilms, the capability to produce such enzymes also features the five phages as advantaged agents against biofilm-producing bacteria. From a taxonomic standpoint, genomic and phylogenetic analyses confirmed that the phages vB_CECAV_041, vB_CECAV_044, vB_CECAV_048, vB_CECAV_050, and vB_CECAV_058 belong to the genera *Felixounavirus*, *Cornellvirus*, *Skatevirus*, *Agtrevirus* and *Berlinvirus*, respectively. Additionally, given their intergenomic similarities below 95%, we propose these phages as new species within these genera. However, intergenomic similarities below 70% with some reference genomes suggest the need for taxonomic revision within the *Skatevirus* genus[49,51,66–68].

This study presents a comprehensive genotypic and phenotypic characterization of five new bacteriophages targeting *Salmonella*, including those resistant to antibiotics and relevant to public health. Our findings emphasise the need for detailed characterization, as seemingly similar phages can differ significantly in their infection kinetics and host range. Notably, the identified phages were able to lyse approximately 81% of *Salmonella* strains isolated from diverse sources. Therefore, establishing a thoroughly characterized collection of bacteriophages, taking into account genomic data as well as environmental stability under different temperature and pH conditions, is necessary to evaluate their potential as therapeutic agents in animal production to effectively control *Salmonella*. Moreover, while vaccination against serovars like Enteritidis or Typhimurium is already

practiced as a control measure, the lytic capacities of the phages proved their potential for integration into control strategies targeting other relevant serovars in animal production, such as Infantis, Virchow or Derby, which currently lack complementary biocontrol tools[4–6,8–10].

The five phages demonstrated the ability to lyse a wide range of *Salmonella* serovars while proving to be physically stable and genetically safe. This positions them as promising candidates for phage-based control of *Salmonella* in animal production, contributing to innovative biocontrol strategies. Their application could address the growing challenge of AMR, and their integration into existing biocontrol protocols may significantly reduce *Salmonella* transmission, contributing to safer animal food products and, consequently, reinforcing human health from a One Health perspective.

Materials and methods

Bacterial strains and culture conditions

In total, 47 *Salmonella* field strains isolated from different poultry productions in Spain were recovered from CECAV's repository for the study. The serovars selected were the most prevalent ones in the EU in 2020, 2021 and 2022[4–6]. Five of the *Salmonella* isolates, from serovars mST Copenhagen variant, Typhimurium Copenhagen variant, Infantis, Kedougou and Mikawasima, were used as PS while the other 42 strains were used as CS (Suppl. Table S1). The PS were selected and assigned after phage isolation, aiming to avoid cross effectivity of the chosen phage isolates among PS. As for CS, all of them were selected from different locations from Spain's geography, different years and different animal productions, to avoid working with clonal strains within serovars.

Isolation of all strains was carried out using the gold standard ISO 6579-1:2017[69] and serotyped following the White-Kauffmann-Le Minor scheme[70]. All strains were preserved in 20% glycerol at -80ºC and revived in Luria Bertani (acc. Miller) (LB) agar medium at 37ºC overnight.

Antimicrobial susceptibility test of the challenge strains

Salmonella CS were characterized according to the ISO 20,776–1:2006[71], by commercially available microtitre plates, Sensititre™ EUVSEC3 (Thermo Scientific, East Grinstead, United Kingdom). To this end, *Salmonella* Sensititre Plates (Gram Negative MIC Plate) were used to assess antimicrobial susceptibility of the CS. The selected antibiotics were those from the antimicrobial panel determined by the Commission Implementing Decision of November 2020 on the monitoring and reporting of AMR in zoonotic and commensal bacteria[72]. This panel included CIP (0.015–8 µg/mL) and NAL (4–64 µg/mL) from the quinolone family; two β-lactams: MERO (0.03–16 µg/mL), a carbapenem, and AMP (1–32 µg/mL), a penicillin; one phenicol: CHL (8–64 µg/mL); one pyrimidine: TMP (0.25–16 µg/mL); one tetracycline: tetracycline (TET, 2–32 µg/mL); one macrolide: AZI (2–64 µg/mL); one glycylcycline: TGC (0.25–8 µg/mL); two cephalosporins: TAZ (0.25–8 µg/mL) and FOT (0.25–4 µg/mL); one polymyxin: COL (1–16 µg/mL); one potentiated sulphonamide: sulfamethoxazole (SMX, 8–512 µg/mL); and two aminoglycosides: GEN (0.5–16 µg/mL) and AMI (2–64 µg/mL).

Epidemiological cutoff values were taken to determine resistance against the antibiotics analysed. The values taken were those set by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and recommended by EU Commission Decision 179/2020[73]. The values not covered by this legislation (AZI and SMX) were assessed following National Committee for clinical Laboratory Standards (CLSI) criteria[74]. MDR was defined as acquired resistance to at least one agent in 3 or more antimicrobial families[75].

Phage isolation and purification

A total of 120 samples, faeces from poultry production farms ($n = 110$) and wastewater from a pig slaughterhouse ($n = 10$), were used for isolation of the phages. The faecal samples were collected from 26 different poultry farms, including rearing facilities for broilers, layers, turkeys and quail. Only the phages that presented clear morphologies on DLA were considered for selection; from these, one phage was selected per PS, avoiding phages that presented cross activity between propagation hosts.

Phages were isolated and purified as previously described by Sevilla-Navarro, with slight modifications[36]. Samples were diluted 1:10 with LB broth, w/v for faeces and v/v for water samples. Then the diluted samples were inoculated with the five PS and incubated overnight at 37ºC. After incubation, individual aliquots were recovered, centrifuged at 8,000 x g for 5 min and the supernatant was filtered through 0.22 µm membranes. Bacterial lawns of each of the five PS were prepared with the DLA method and tested for phage presence through spot test assays. Briefly, 5 mL of LB soft agar (0.6% agar) tempered at 50ºC were mixed with 200 µL of individual *Salmonella* inoculum at an optical density at 600 nm (OD) of 0.2 (~10^8 CFU/mL) and poured onto a basal layer of LB agar (1.5% agar). Then, 10 µL of each sample were laid on the bacterial lawns and after an overnight incubation at 37°C, phage presence was evaluated.

The selected phages were recovered from the DLA and resuspended in 50 µL of phosphate buffered saline; 1:10 serial dilutions were performed and 10 µL of each dilution were plated using the DLA method. To ensure phage isolation, the 10 µL of the phage aliquot were added to 200 µL of *Salmonella* inoculum at an OD of 0.2, incubated at room temperature for 15 min, mixed with 5 mL of LB soft agar (0.6% agar) and then poured onto a basal layer of LB agar (1.5% agar). The procedure was repeated three times to guarantee proper phage isolation and purification. Only the phages that showed clear lytic plaques on DLA plates after the third purification were further propagated and stored for the study.

Plaque morphology and phage imaging by transmission electron microscopy

Following isolation, plaque morphology of each phage on its PS was registered. Plaque measurements were taken ($n = 10$) and the presence of depolymerases was evaluated.

Phages were visualized by TEM (FEI Tecnai G2 Spirit Biotwin, 120 KW) in the Centro de Investigación Principe Felipe (CIPF, Valencia, Spain). To this end, 10 µL of the phages at a 10^8-10^{10} PFU/mL concentration

were fixed in an aqueous solution of paraformaldehyde (2%). A 7.2 kV glow was discharged onto the samples placed on a MESH copper grid and incubated for 15 min. The samples were then washed in phosphate saline 0.1 M for 2 min and fixed with glutaraldehyde (1%). Samples were negatively stained with uracil acetate (1%) and incubated with methyl cellulose (1%) for 30 s. Samples were dried and stored until use[36,76]. Regarding the micrographs obtained, phages were classified following International Committee on Taxonomy of Viruses (ICTV) guidelines[77].

Host range and efficiency of planting

The host range and EOP of the phages were simultaneously assessed against their own PS and the 42 CS[76]. Serial 1:10 dilutions of the phages were carried out, and each dilution was tested by DLA method. After an overnight incubation at 37ºC, both lytic and titre capabilities were determined. EOP towards the CS was calculated relative to titres the phage reached with its own PS, only when clear and individual plaques were observed.

Thermal and pH stability

To assess the survival of the phages under different field conditions, their stability was tested under different temperatures and pH conditions. For the thermal stability assay, 100 µL of the original stocks of each phage were added to 900 µL of sterile LB broth and heated to 30 ºC, 40 ºC, 50 ºC, 60 ºC, 70 ºC and 80 °C for 60 min[59]. Stability was also evaluated at -20 ºC and – 80 ºC[78]. The titre of the stocks at 4 ºC was used as the baseline value to evaluate the stability of the phages at different temperatures. Phage titres were established by the DLA method. The assays were performed in triplicate.

Regarding pH stability tests, 100 µL of the original stocks of each phage were also taken and diluted into 900 µL SM buffer ($MgSO_4 \cdot 7H_2O$ 2 g, NaCl 5.8 g, and 50 mL l mol/L Tris–HCI (pH 7.5) in 1 L) (1:10 dilution) adjusted with NaOH and HCl to different pH values ranging from 2, 3, 4, 5, 6, 8, 9, 10, 11 to 12[59]. After 2 h of incubation at 37ºC, the aliquots' titres were assessed by DLA method. The values obtained for pH 7 were used as baselines to evaluate the stability of the phages at different pH levels. All assays were performed in triplicate.

Infection kinetics

Kinetics of lytic development were assessed to reach MOI levels of 10, 1, 0.1 and 0.01 for each phage, as previously described by Khan (2024)[58]. To do so, 96 microtitres well plates were loaded with: (i) a blank, with 200 µL of sterile LB broth; (ii) positive control, with 180 µL of LB broth and 20 µL of an inoculum at an OD of 0.2 of the PS; and (iii) treatment groups, with 180 µL of LB, 20 µL of PS inoculum at OD of 0.2 and different phage aliquots. OD measures were taken every 5 min up to 12 h at 37ºC with continuous shaking using a microplate spectrophotometer (Multiskan SkyHigh, ThermofisherThermo Fisher Scientific™). All the values obtained were later corrected with blank values.

One-step growth

The methodology followed for the one-step growth assays was as described by Sattar (2022), with slight modifications[53]. In brief, 500 µL of an overnight culture of its PS was mixed with 50 mL of sterile LB broth and incubated at 37ºC for 60 min. Once the culture reached an OD of 0.2 (~ 10^8 CFU/mL), the phage was added to reach a MOI of 0.1, incubated at 37ºC for 10 min and afterwards centrifuged at 12,000 x g for 10 min. Once centrifuged, the supernatant was removed and the pellet was resuspended with 50 mL of sterile LB broth. The supernatant was filtered through 0.2 µm filters and PFU/mL quantified by DLA method to establish the unattached phages after the 10 minutes' incubation. The pellet was resuspended into 50 mL of sterile LB broth, homogenized and incubated at 37ºC for 100 min. Every 10 min, aliquots from the resuspended pellet were taken, filtered through 0.2 µm filters and phage concentration established by DLA method. The burst size was established following the formula: *burst size = (average of free phage after burst – average of free phage before burst) / (phage administered– unattached phages).*

Genomic analysis of the phages

DNA extraction of the phages was done with 200 µL of high titre phage aliquots and the QIAamp® cador® Pathogen Mini Kit (Quiagen™) (Werfen, Barcelona, Spain), for isolation of viral RNA and DNA and bacterial DNA from animal samples. The library was prepared with the Nextera XT Library prep kit and used for sequencing using Illumina MiSeq technology (250 bp paired-end reads).

Raw sequencing data was processed using FastQC v0.11.9 and fastp v0.20.1 for quality control and adaptor removal[79–81]. The filtered reads were then assembled with SPAdes v.3.15.5 (only-assemble mode) using the following k-mers list: 33, 55, 77, 99 and 127[82,83]. The assembled contigs were analysed using Geneious Prime 2024.0.7, and for each phage, the contig with the highest coverage and length was selected as the putative genome. BLAST was performed against the nucleotide collection database to find the closely matching phages and assign a preliminary taxonomic classification[36]. The reference sequences (RefSeq) of type-phages from each genus were used to manually rearrange the genomes using Progressive Mauve[36,84,85].

Read mapping was performed using BBMap.sh v38.84 to evaluate coverage of the trimmed reads against the assembled contigs[86]. The assembly was further refined using Pilon v1.20[36,87]. For structural and functional annotation, Pharokka v1.4.1 was used with default parameters and each sequenced coding was associated with a Phrog functional group[36,88]. Protein sequences were manually cross-checked with BLASTp searches[89,90].

Pharokka and PhageLeads were used to assist in the prediction of therapeutic suitability while Abricate was used to identify AMR and virulence genes[91,92]. Phage lifestyle prediction was performed using PhaTYP[93], and the presence of depolymerase enzymes was predicted using the DePolymerase Predictor (DePP, web version 1.0.0) machine learning tool, considering proteins with a probability greater than 90% as potential depolymerases[94].

Taxonomic classification of the phages was validated using three complementary approaches[36,89,95]. First, a phylogenetic tree was constructed based on the large terminase subunit (terL) sequences from RefSeq phages, with sequence alignment performed in Clustal Omega v1.2.2[96], and phylogeny inferred using IQ-TREE v1.6.12 under the maximum likelihood method with 1000 bootstraps[97,98]. The tree was later visualized in iTOL v.6. Second, intergenomic similarity was calculated using VIRIDIC v1.1, applying a 95% similarity threshold for species classification and 70% for genus classification[99]. Genus clustering was represented using an alluvial diagram created with RawGraphs v2.0[100]. Finally, a phylogenetic analysis was conducted by comparing the five phages against the ViPTree database, integrating both proteomic profiles and intergenomic similarity to confirm their taxonomic relationships[101].

Statistical analysis

Phage concentration results obtained in the assays were treated and expressed as normalized values (log_{10}(PFU/ml)). All data are expressed as the mean ± standard deviation. Phage stability data were analysed using two-way ANOVA, Dunnett's test, to check for statistical differences with the baseline titres. A significance level of *p-value* < 0.05 was adopted to denote statistical significance in all analyses. All statistical analyses were performed using GraphPad Prism 10.

Data availability

Data is provided within the manuscript and supplementary information files.

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Declarations

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The authors declare no competing interests.

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