



In vitro and in vivo assessment of phage therapy against *Staphylococcus aureus* causing bovine mastitis



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ABSTRACT

Objective: The aim of this study was to assess the efficacy of lytic bacteriophages on *Staphylococcus aureus* causing bovine mastitis, by in vitro and in vivo assays using *Galleria mellonella* and murine mastitis models.

Methods: Between May and December 2016, ten *S. aureus* (five methicillin-resistant and five methicillin-sensitive) isolates were isolated from milk samples of cattle with mastitis in Belgium and Norway. The isolates were assessed in vitro for their susceptibility to four lytic bacteriophages (Romulus, Remus, ISP and DSM105264) and subsequently in vivo in *G. mellonella* larvae and in murine mastitis model.

Results: Romulus, Remus and ISP showed a lytic activity against the *S. aureus* isolates in vitro. A larvae survival rate below 50% was observed at 4 days post-inoculation (DPI) in the groups infected with a methicillin-sensitive *S. aureus* isolate and treated with these three phages in vivo. An incomplete recovery of the mouse mastitis was observed at 48 h post-inoculation (HPI) in the groups infected and treated with the ISP phage in vivo.

Conclusions: The observations are much more pronounced statistically between the infected- phosphate buffered saline (PBS)-treated and infected-phage-treated groups in *G. mellonella* and the murine mastitis model demonstrating an effect of the phages against *S. aureus* associated with bovine mastitis.

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1. Introduction

Staphylococcus aureus is responsible for several infections in humans and animals and represents an important hazard in public health. In humans, it is responsible for a range of illnesses from mild skin disorders to invasive infections and life-threatening in hospital settings [1]. It can also be community-acquired, causing skin and soft tissue infections with moderate to

severe symptoms in healthy and younger people [2]. In animals, *S. aureus* infections impact livestock, companion animals and some wild animals [3,4].

Besides their virulence properties, *S. aureus* can also acquire genes conferring resistance to different antimicrobials. The most widespread and frequent resistant strains are methicillin-resistant *S. aureus* (MRSA) that are resistant to all β -lactams [5]. In the case of bovine species, livestock-associated MRSA causes subclinical and clinical mastitis which can lead to high financial costs [6,7]. To avoid the continuous selection pressure for development and dissemination of antimicrobial resistance by antimicrobial treatment procedures, one strategy could be the use of bacteriophages rather than traditional antimicrobials.

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Table 1
Antibiotic profile of the ten *S. aureus* strains.

ID	Origin	Strain	Cefoxitin	Ampicillin	Amoxicillin + CA	Ciprofloxacin	Clindamycin	Colistin	Erythromycin	Florfenicol	Gentamicin	Lizenoil	Penicillin	Sulfamycin	Sulfa-Thim	Trimethoprim	Tetracycline	Resistance	Multi resistance	
MRSA	Flanders Wallonia	Ani_Gt_111	R	R	S	S	S	S	S	S	R	S	R	S	R	R	R	7	Yes	
		Ani_Lg_017	R	R	S	R	S	S	S	R	S	S	S	R	R	R	R	S	8	Yes
		Ani_Lg_020	R	S	S	R	S	S	R	S	S	S	S	R	R	R	R	S	7	Yes
		Ani_Lg_027	R	R	S	R	S	S	R	S	S	S	S	R	R	R	R	S	8	Yes
MSSA	Flanders Wallonia Norway	Ani_Lg_010	R	R	S	R	S	R	S	S	S	S	R	S	R	R	S	8	Yes	
		Ani_Gt_110	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	0	No	
		Ani_Gt_117	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	0	No	
		Ani_Lg_028	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	0	No	
		Ani_Os_001	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	0	No	
		Ani_Os_002	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	0	No	

CA, clavulanic acid; ID, identification; MRSA, methicillin resistant *S. aureus*; MSSA, methicillin sensitive *S. aureus*; R, resistant; S, susceptible.

Bacteriophages are viruses able to infect and replicate within bacteria after injection of their genetic material [8]. They are functionally classified into strictly lytic and temperate phages. These strictly lytic phages are of interest for therapy [9]. To assess the potential of phage therapy, studies generally rely on in vitro or in vivo approaches using mammalian models. The lack of realistic conditions of in vitro assay, the expensive costs and the ethical requirements for in vivo models have led to the use of alternative models such as *G. mellonella* larvae [10]. In this study, the efficacy of lytic bacteriophages on *S. aureus* isolated from cows with mastitis in Belgium and Norway was assessed in vitro and in vivo with *G. mellonella* and murine mastitis models.

2. Methods

2.1. Bacterial and bacteriophage selection

Ten *S. aureus* isolates including five MRSA and five methicillin sensitives (MSSA) were isolated in Belgium and Norway between May and December 2016 from quarter milk samples of cows with clinical or subclinical mastitis and stored at -80°C with 50% (v/v) glycerol until further use (Table 1). Four well-defined lytic bacteriophages were used: Romulus, Remus, ISP [11,12] and DSM105264 (phage K) [13].

2.2. Phage preparation

The four phages were amplified using the *S. aureus* PS47 (NCTC 8325) strain for Romulus, Remus and ISP phages, and the *S. aureus* DSM105272 (DSMZ) strain for the DSM105264 phage, as propagation strains. Phage lysates (100 μL) were added to 5 mL of the propagation strain culture [optical density (OD_{600}) = 0.2–0.3] in Lennox broth (LB; Alfa Aesar, UK) [complemented with 1 mM of calcium chloride (CaCl_2) and magnesium sulfate (MgSO_4)] and incubated at 37°C , with shaking at 100 revolutions/min (rpm) until the solution became transparent. Then, this solution was added again to 500 mL of bacterial culture (with OD_{600} = 0.2–0.3) and incubated overnight at 37°C with shaking at 100 rpm. After addition of 1/20 of chloroform and incubation at 37°C for 10 min with shaking at 100 rpm, the solutions were placed at 4°C until the chloroform fraction separated. The solutions were then centrifuged at 4°C and 4600 rpm for 30 min and the supernatants were filtered through a superposed filter of 0.45 μm and 0.22 μm . Phage titration was performed in triplicate on a bacterial overlay of the propagation strain (OD_{600} 0.2–0.3) spread on an LB agar plate (Applichem, Germany). To obtain high concentrated phage solutions, a polyethylene glycol (PEG) precipitation was performed [14]. After centrifugation (4600 rpm for 40 min at 4°C), the pellets resulting from 50 mL of phage lysate were re-suspended in 1 mL of phage buffer [10 mM Tris-hydrochloride (HCl), 10 mM MgSO_4 , 150 mM NaCl, pH7.5] and stored at 4°C until further use.

2.3. In vitro assays

2.3.1. Host spectrum and efficiency of plating

Ten mastitis-causing *S. aureus* isolates were screened for their susceptibility to the four phages (Romulus, Remus, ISP and DSM105264) on LB agar by the ‘spot-on method’ after serial dilutions [15]. Lysis were recorded as no lysis (–), semi-confluent lysis (SCL) or confluent lysis (CL). The efficiency of plating (EOP) was then calculated (Table 2) [16].

2.3.2. Phage activity in LB

A total of 100 μL of the bacterial suspension at 10^6 colony-forming units (CFUs)/mL and 100 μL of the phages (Romulus, Remus, ISP, mix of the three latter phages or DSM105264) at 10^9

Table 2
Host spectrum test and EOP.

ID	Origin	Strain	Romulus		Remus		ISP		DSM_105264	
			Lysis	EOP	Lysis	EOP	Lysis	EOP	Lysis	EOP
MRSA	Flanders	Ani_GT_111	–	/	–	/	–	/	–	/
		Wallonia	Ani_LG_017	–	/	–	/	CL	6.80E-01	–
	Ani_LG_020		–	/	–	/	CL	9.10E-01	–	/
		Ani_LG_027	–	/	–	/	CL	1.10E+00	–	/
		Ani_LG_010	–	/	–	/	CL	6.80E-01	–	/
MSSA	Flanders	Ani_Gt_110	–	/	SCL	5.30E-01	CL	1.30E+00	–	/
		Ani_Gt_117	–	/	SCL	1.10E-01	CL	3.30E-01	–	/
	Wallonia	Ani_Lg_028	–	/	SCL	7.40E-01	CL	4.50E-01	–	/
		Norway	Ani_Os_001	CL	3.57E+00	CL	1.90E-01	CL	1.30E+00	–
			Ani_OS_002	–	/	–	/	CL	9.10E-01	–

CL, confluent lysis; EOP, efficiency of plating; ID, identification; MRSA, methicillin resistant *S. aureus*; MSSA, methicillin sensitive *S. aureus*; SCL, semi-confluent lysis.

plaque-forming units (PFUs)/mL were added to 4 mL of LB broth (complemented with 1 mM of CaCl₂ and MgSO₄) in triplicates. A positive control containing 4 mL of LB broth with 100 µL of bacteria and negative controls with 4 mL of LB broth only or LB broth + 100 µL of phage were also tested. The broth was incubated for 24 h at 37 °C with shaking at 100 rpm and lysis was followed by measuring the OD₆₀₀ of the solutions every 3 h.

2.4. In vivo assays

2.4.1. *G. mellonella* larva model

Larvae of *G. mellonella* were obtained from 'La tourterelle des bois' (Comines-Warneton, Belgium) and stored at 4 °C for a maximum of 1 week. The larvae were inoculated into the haemolymph behind the last right and/or left proleg by using a 30-gauge (G) insulin syringe (U-100) from BD Micro-Fine™ (Franklin Lakes, NJ, USA).

2.4.1.1. Preliminary experiments. Based on the in vitro assay results, one mastitis-causing *S. aureus* isolate (Ani_OS001) was chosen for a first preliminary experiment to assess infectivity. An aliquot of 10 µL of the bacterium was inoculated in three groups (A, B, C) of ten larvae, each at different bacterial titres (A = 10⁶ CFUs/10 µL, B = 10⁴ CFUs/10 µL, C = 10² CFUs/10 µL). Another group (D) of ten larvae was inoculated with 10 µL of PBS and served as a negative control. The mortality rate of the larvae was followed until 4 days post-inoculation (DPI) to choose the optimal titre of isolate Ani_OS001 that would kill at least 75% of the larvae at DPI 4. During a second preliminary experiment, four other groups (E, F, G, H) of ten larvae each were inoculated with isolate Ani_OS001 at the optimal titre and with different concentrations of gentamicin (Sigma-Aldrich, Germany) (E = 2.5 µg/10 µL, F = 5 µg/10 µL, G = 7.5 µg/10 µL, H = 10 µg/10 µL) to choose the concentration that would give 100% of larva survival at DPI 4.

2.4.1.2. Phage efficacy assessment. The first principal experiment consisted of the inoculation of isolate Ani_OS001 at the optimal

titre (10⁴ CFUs/10 µL) with and without phages (Romulus, Remus and/or ISP) at the multiplicity of infection (MOI) 1000, performed in triplicate. The gentamicin (7.5 µg/10 µL) and the PBS were also inoculated as positive and negative controls, respectively (Table 3). Bacteria and treatments were inoculated 1 h apart. Mortality rate was followed for 4 DPI and surviving larvae were sacrificed at DPI 4.

In parallel, independent groups of ten larvae (inoculated as described) were blended at DPI 2 and DPI 4, respectively, to assess the evolution of the bacterial and phage titres over time. A 10× phosphate-buffered saline (PBS) dilution of the blended suspension of each larvae group was spotted (10 µL) in triplicate on Chapman agar (selective for staphylococci; VWR, Belgium) to assess the bacterial titres. The same dilutions were spotted on LB agar to estimate the phage titre after addition of 100 µL of lysostaphin (to lyse the staphylococci; 0.1 mg/mL; Sigma-Aldrich, Belgium), incubation at 37 °C for 1 h, centrifugation at 46 000 rpm for 45 min and filtration at 0.2 µm.

2.4.2. Murine mastitis model

The experimental protocol was approved by the Ethical Committee of the University of Liège (approval number 14-1719) and the experiments were conducted in a biosafety level 3 laboratory at the Faculty of Veterinary Medicine of the University of Liège. Female BALB/cJrJ specific-pathogen-free (SPF) mice (Janvier, Saint Berthevin Cedex, France) of approximately 30 g were used at 13 days of lactation. The pups were removed and euthanised just before inoculation of the mammary glands. The fourth abdominal mammary glands pair (left and right) were inoculated using a 30-G insulin syringe (U-100 from BD Micro-Fine™, Franklin Lakes, NJ, USA).

2.4.2.1. Preliminary experiment. The Ani_OS001 strain and the ISP phage (chosen based on its in vitro lytic activity) were assessed, respectively, for their infectivity and for their safety. Three groups (A', B', C') of four mice each were inoculated (50 µL) with 10⁵ CFUs of Ani_OS001 (inoculum titre based on previous results, data not shown), 10⁸ PFU of ISP phage and PBS (negative control),

Table 3
Experimental design of the phage efficacy assessment in *G. mellonella* larvae performed in triplicate.

Groups ^a	S	S_ROM	S_REM	S_ISP	S_MIX	S_Gent	ROM	REM	ISP	Gent	PBS
Bacteria (10 ⁴ CFUs/10 µL)	×	×	×	×	×	×					
Phage (10 ⁷ PFUs/10 µL)		×	×	×	×		×	×	×		
Gentamicin (7.5 µg/10 µL)						×				×	
PBS	×						×	×	×	×	2×

^a S, bacteria and PBS; S_ROM, bacteria and Romulus; S_REM, bacteria and Remus; S_ISP, bacteria and ISP; S_MIX, bacteria and the mix of the phages Romulus, Remus and ISP; S_Gent, bacteria and Gentamycin; ROM, Romulus and PBS; REM, Remus and PBS; ISP, ISP and PBS; Gent, Gentamycin and PBS; PBS, PBS 2 times; ×, 10 µL of inoculum.

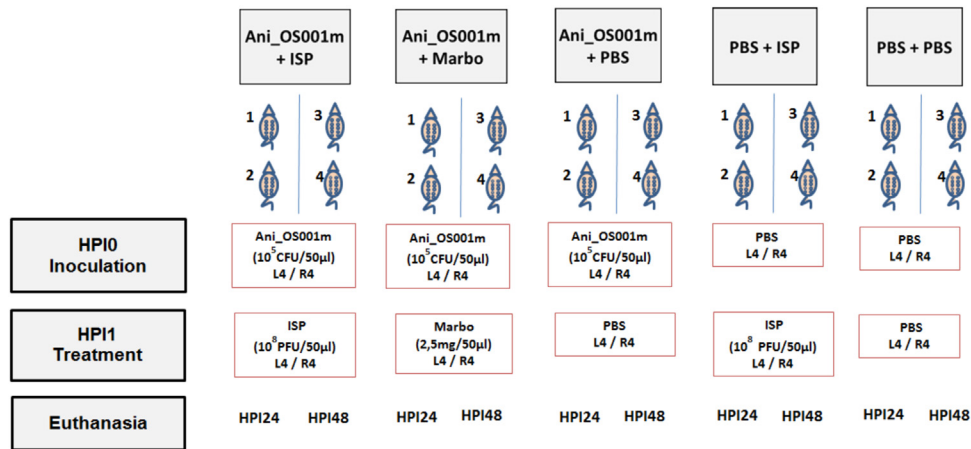


Fig. 1. Experimental design of the phage efficacy assessment in a murine mastitis model. HPI, hour post-inoculation; L4/R4, left/right fourth abdominal mammary gland pair; Marbo, marbofloxacin.

respectively, in one experiment. One hour post-inoculation (HPI 1), all the mice were further inoculated with 50 μL of PBS. Two mice were euthanised in each group after 24 h (HPI 24) and 48 h (HPI 48) post-inoculation, respectively, their blood was collected and their mammary glands were dissected for histopathological analysis then blended with a Tissue lyser II (Qiagen, Hilden, Germany) for bacterial counts. The *S. aureus* strain (Ani_OS001m) resulting from the preliminary experiment was purified and used as an inoculum for the phage efficacy assessment. The mouse presenting an infection with the highest bacterial titre was selected to isolate the strain (Ani_OS001m) to be used for the main experiment in order to maximise the infection success.

2.4.2.2. Phage efficacy assessment. The strain Ani_OS001m was used in one experiment after being checked phenotypically (antimicrobial profile) and genetically (whole genome sequencing) (Fig. 1). Five groups of four mice were used and 1 h was left between two inoculations in the same mammary gland. Marbofloxacin (antibiotic) and the PBS were used, respectively, as positive and negative treatment controls. The mammary glands were dissected and blended for bacterial and phage counting at HPI 24 and HPI 48 after dilutions in the PBS as described. The blood was also collected for bacterial and phage titrations.

2.4.2.3. Mammary gland gross pathology and histopathology. The mammary glands were observed for gross pathology using a score from I (no inflammation) to V (severe inflammation) [17]. Then, small samples were fixed in 4% buffered paraformaldehyde, dehydrated with ethanol, cleared with xylene and embedded in paraffin for histopathological studies. Tissue sections of 4-μm thickness were stained with haematoxylin and eosin (H&E), and examined by light microscopy at different magnifications. Each sample was evaluated for the presence of necrosis, lymphocytic inflammation, inflammatory cells (polymorphonuclear neutrophils, granulocytes) and bacteria with a score from 0 (absent) to 4 (severe) [17].

2.5. Statistical analysis

Statistical analyses were performed on in vivo data with GraphPad Prism8, (GraphPad Software, Inc., San Diego, CA, USA). Kaplan–Meier survival curves and log-rank (Mantel–Cox) tests were performed to analyse the *G. mellonella* larva survival curves. Bonferroni's multiple comparisons test was used to compare the variations of bacterial and phage titres inside *G. mellonella* larvae and murine mammary glands.

2.6. Whole genome sequencing comparison of Ani_OS001 and Ani_OS001m

Staphylococcus aureus DNA was extracted from one colony of Ani_OS001 and one colony of Ani_OS001m (recovered from the mouse model preliminary experiment) using a protocol based on lysostaphin followed by chloroform/isoamyl alcohol extraction. Genomic libraries were prepared according to the manufacturer's instructions using the Nextera XT DNA library preparation kit and sequenced by the NovaSeq 6000 Sequencing System (Illumina, San Diego, CA, USA). The raw reads sequences were assembled into contigs with the pipeline shovill 1.0.4 including trimmomatic 0.38 for the cleaning and annotated using Prokka 1.13.3 [18]. The comparison was performed by alignment of Ani_OS001m reads on Ani_OS001 shovill assembly using bwa and VarScan variant calling (-varFreq option set to 0.6 to ensure majority variants) [19].

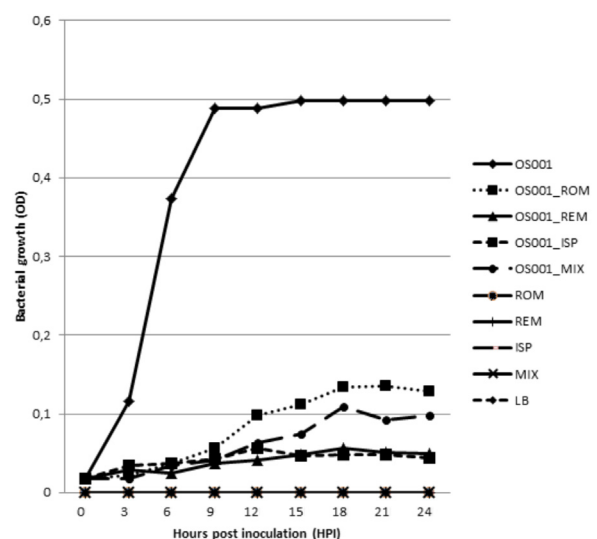


Fig. 2. Activity of the phages on Ani_OS001 in LB broth. OD₆₀₀, optical density at 600 nm; OS001, Ani_OS001 and LB broth; OS001_ROM, Ani_OS001, Romulus and LB broth; OS001_REM, Ani_OS001, Remus and LB broth; OS001_ISP, Ani_OS001, ISP and LB broth; OS001_MIX, Ani_OS001, the mix of the phages (Romulus, Remus and ISP) and LB broth; ROM, Romulus and LB broth; REM, Remus and LB broth; ISP, ISP and LB broth; MIX, mix of phages and LB broth; LB, LB broth only.

3. Results

3.1. In vitro assay

CL and SCL were observed on LB agar for four MRSA and five MSSAs with the phages Romulus, Remus and/or ISP (Table 2). Conversely, no lysis was observed with the phage DSM105264. High EOP (>1) were observed for two MSSAs (Ani_OS001 and Ani_GT110) and one MRSA (Ani_LG027). The Ani_OS001 isolate that was lysed by the three phages was therefore chosen for the *G. mellonella* experiments.

The same three phages, alone or together, had efficient lytic activities on Ani_OS001 isolate in LB broth compared with positive and negative controls (Fig. 2).

3.2. In vivo experiments

3.2.1. G. mellonella model

After inoculation of the larvae with the Ani_OS001 isolate, 100% mortality was observed in group A, 80% in group B and 40% in group C at DPI 4 during the first preliminary experiment (determination of the infectious dose). In the second preliminary experiment (determination of the therapeutic dose of gentamicin) 30% of survival was observed in group E, 70% in group F and 100% in groups G and H at DPI 4. Based on these results, 10⁴ CFUs/10 μL (group B) and 7.5 μg/10 μL (group G) were chosen as the optimal bacterial infectious dose and optimal gentamicin therapeutic dose, respectively, for subsequent experiments.

During the principal experiment, a 10% survival rate was observed in the larvae inoculated with Ani_OS001 isolate and PBS, whereas 90% of the larvae inoculated with Ani_OS001 isolate and gentamicin survived at DPI 4. Survival rates at DPI 4 ranged from 20% to 35% in the groups inoculated with Ani_OS001 isolate and the phages (Romulus, Remus, ISP or the mix of the three phages) (Fig. 3).

High *S. aureus* titres (5 × 10¹¹ CFUs/mg and 2 × 10¹² CFUs/mg) were obtained at DPI 2 and DPI 4, respectively, from the blended larvae inoculated with Ani_OS001 isolate and PBS. These titres were lower than the larvae inoculated with Ani_OS001 and phage (s) (Fig. 4A). The phage titres increased between DPI 2 and DPI 4 in the larvae inoculated with Ani_OS001 isolate and stayed very stable in the groups inoculated only with the phage(s) (Fig. 4B).

3.2.2. Murine mastitis model

3.2.2.1. Preliminary experiment. Mammary gland inflammations were observed in group A' with a bacterial titre up to 4.2 × 10⁴ CFUs/mg at HPI 48, whereas neither inflammation nor infection

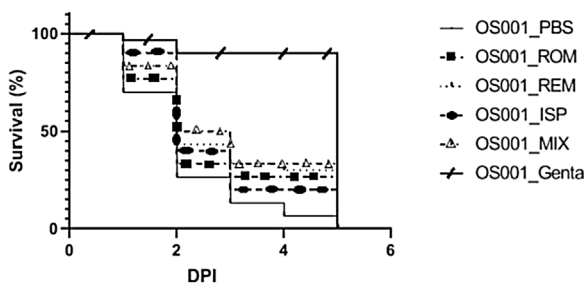


Fig. 3. Kaplan–Meier survival curves of *G. mellonella* larvae. OS001_PBS, Ani_OS001 and PBS; OS001_ROM, Ani_OS001 and Romulus; OS001_REM, Ani_OS001 and Remus; OS001_ISP, Ani_OS001 and ISP; OS001_MIX, Ani_OS001 and the mix of Romulus, Remus and ISP; OS001_Genta, Ani_OS001 and gentamicin; DPI, days post-inoculation.

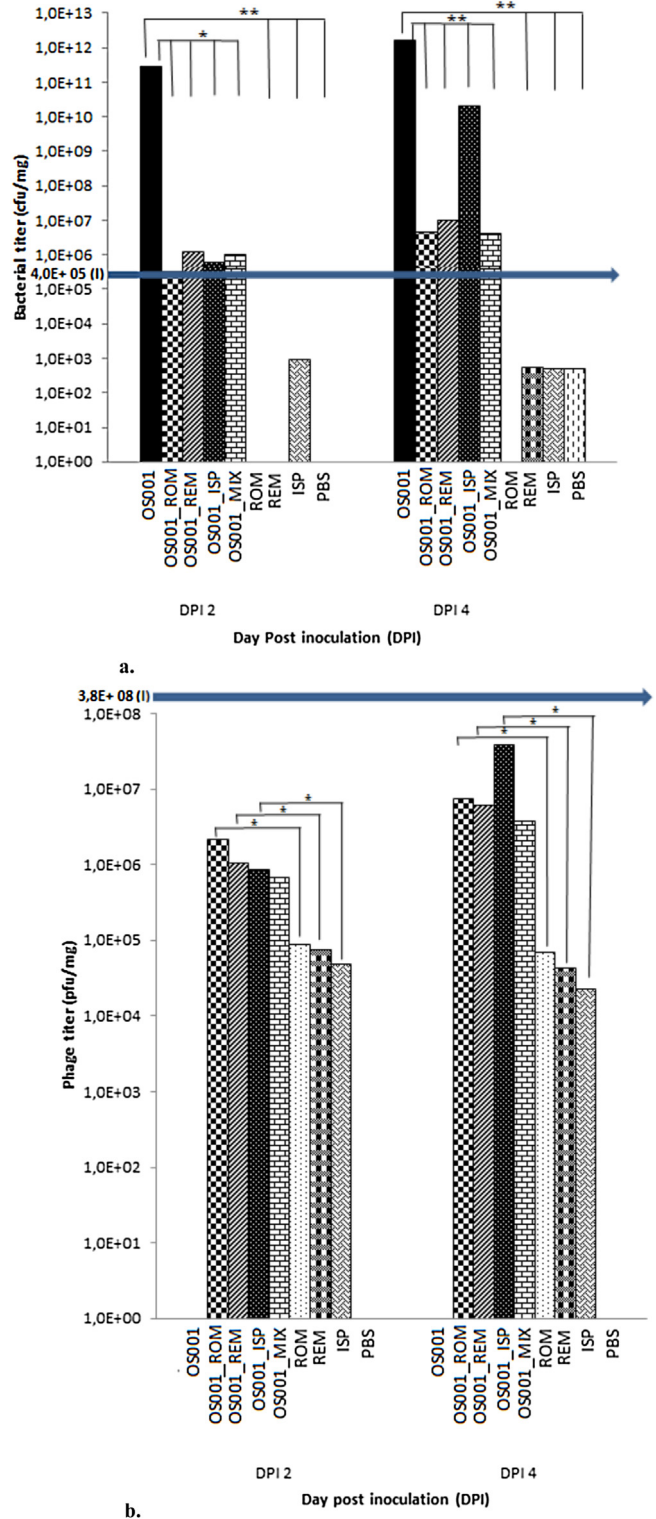


Fig. 4. (A) Bacterial titres in blended *G. mellonella* larvae.^a (B) Phage titres in blended *G. mellonella* larvae.^a OS001_PBS, Ani_OS001 and PBS; OS001_ROM, Ani_OS001 and Romulus; OS001_REM, Ani_OS001 and Remus; OS001_ISP, Ani_OS001 and ISP; OS001_MIX, Ani_OS001 and the mix of the phages Romulus, Remus and ISP; ROM, Romulus and PBS; REM, Remus and PBS; ISP, ISP and PBS; PBS, PBS 2 times; DPI, days post-inoculation; I, inoculum titre; statistically significant difference between values (**P* < 0.05; ***P* < 0.01 (determined by Bonferroni multiple comparison test)).

Table 4
Results of the preliminary experiment (mice model).^a

Groups	Mean bacterial titres of the blended mammary glands (CFUs/mg)		
	A(Ani_OS001 +PBS)	B(ISP +PBS)	C(PBS +PBS)
HPI24	2.80E+04	0.00E+00	0.00E+00
HPI48	4.20E+04	0.00E+00	0.00E+00

^a HPI, hour post-inoculation.

was present in the groups B' and C' (Table 4). No bacterial infection was detected in the mouse blood.

3.2.2.2. Main experiment. At necropsy, gross pathology scores were established for the 40 mammary glands of the 20 mice (Fig. 5). Score values of III and V with redness and slimy exudate of the gland were observed in the mammary glands inoculated with Ani_OS001m isolate and PBS, whereas the mammary glands inoculated with Ani_OS001m isolate and the ISP phage had score values of II and III with slightly red glands. The other three groups of mice had score values between I and II with very little redness of the glands.

Histopathology was performed on the 40 mammary glands and an average score was established for each group (Fig. 6). The group

inoculated with Ani_OS001m isolate and PBS had severe tissue necrosis and high numbers of polymorphonuclear neutrophils (PMN), lymphocytes and bacteria. In the group inoculated with Ani_OS001m isolate and ISP phage, the lesions were moderate and in the three others groups, they were very weak with no bacteria and no tissue necrosis. Moderate amounts of macrophages were also present in all mice.

A high bacterial titre was observed in the blended mammary glands of the group inoculated with Ani_OS001m isolate and PBS at HPI 24 and HPI 48, whereas a progressive decrease of the bacterial titres was observed between HPI 24 and HPI 48 in the group inoculated with Ani_OS001m isolate and ISP phage. A drastic decrease was observed in the group inoculated with Ani_OS001m isolate and treated with marbofloxacin at HPI₂₄ leading to the absence of bacterial detection at HPI 48. No bacterial growth was observed in the other two groups (Fig. 7A, Table 5). No bacteria or phages were detected in the mouse blood.

A progressive decrease of the phage titres was observed between HPI 24 and HPI 48 in the two groups inoculated with phage. The decrease of the phage titre was more rapid and drastic in the group inoculated with Ani_OS001m isolate and ISP phage leading to the absence of phage detection at HPI₄₈ than in the group inoculated with ISP phage and PBS (Fig. 7B, Table 5). No phage was detected in the other three groups.

Group	Number of mammary glands										
	OS001_PBS		OS001_ISP		OS001_Marbo		ISP_PBS		PBS_PBS		
	24	48	24	48	24	48	24	48	24	48	
HPI											
I					3	4			4	4	
II			2	1	1		4	4			
III	2		2	3							
IV	2	1									
V		3									
Total	4	4	4	4	4	4	4	4	4	4	4

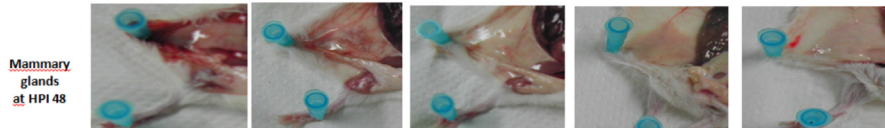


Fig. 5. Gross pathology of the mammary glands. OS001_ISP, Ani_OS001m and the ISP phage; OS001_Marbo, Ani_OS001m and marbofloxacin; OS001_PBS, Ani_OS001m and PBS; ISP_PBS, ISP phage and PBS; PBS_PBS, PBS twice, HPI, hour post-inoculation. Clinical score: I, no inflammation; II, mild inflammation; III, moderate inflammation; IV, highly moderate inflammation; V, severe inflammation.

Group	OS001_PBS				OS001_ISP				OS001_Marbo				ISP_PBS				PBS_PBS			
	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
Observation																				
0										x			x	x	x		x	x		x
1											x									x
Score			x		x		x	x				x				x				
3																				
4	x	x		x																

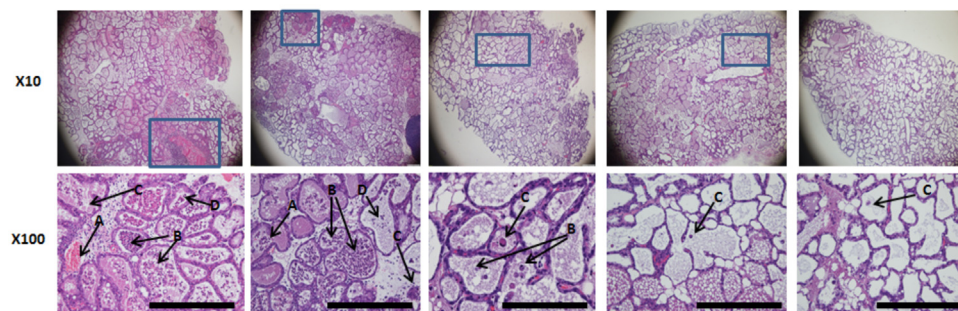


Fig. 6. Mammary gland histopathology. OS001_ISP, Ani_OS001m and ISP phage; OS001_Marbo, Ani_OS001m and marbofloxacin; OS001_PBS, Ani_OS001m and PBS; ISP_PBS, ISP phage and PBS; PBS_PBS, PBS twice, A, tissue necrosis (degeneration); B, polymorphonuclear neutrophils (PMNs) and lymphocytes; C, macrophages; D, bacteria; Score: 0, absent, 1, minimal, 2, mild, 3, moderate, 4, severe. 10×, ten-fold magnification; 100×, 100-fold magnification.

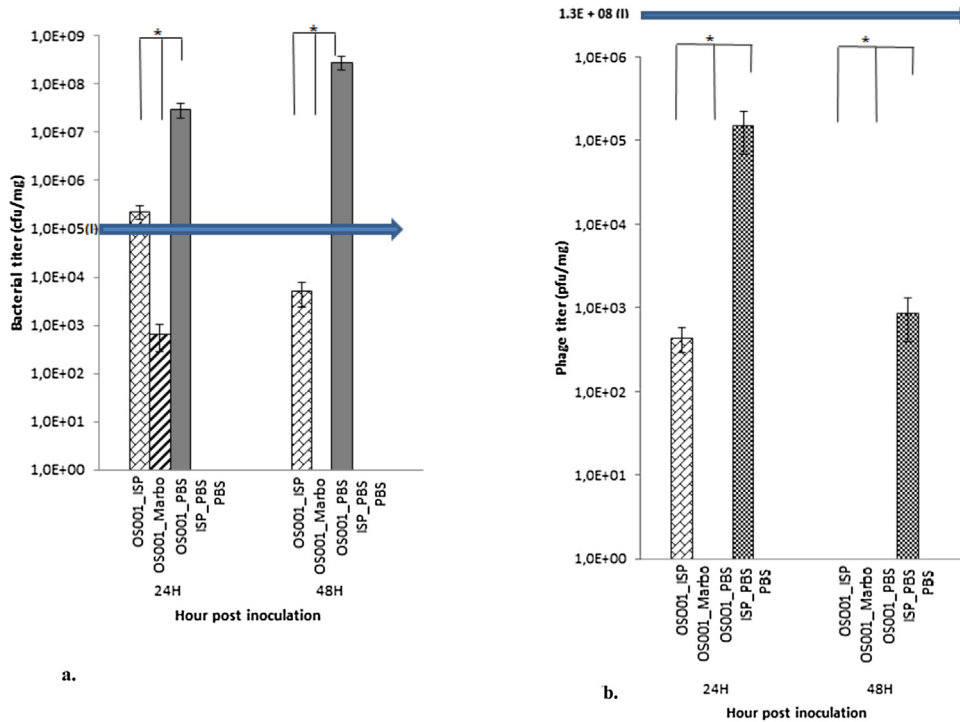


Fig. 7. (A) Mean of the bacterial titres in the mammary gland.^a (B) Mean of the phage titres in the mammary gland.^a. ^aOS001_ISP, Ani_OS001m and the ISP phage; OS001_Marbo, Ani_OS001m and marbofloxacin; OS001_PBS, Ani_OS001m and PBS; ISP_PBS, ISP phage and PBS; PBS, PBS twice. Each bar on the graphs represents the mean of the bacterial or phage titres in four mammary glands (two mice per group). I, Inoculum; statistically significant difference between values [^{*}*P* < 0.05 (determined by Bonferroni multiple comparison test)]. Error bars represent standard error.

Table 5
Results of the phage efficacy assessment in murine mastitis model.^a

Groups	OS001_ISP				OS001_Marbo				OS001_PBS				ISP_PBS				PBS_PBS			
	HPI 24		HPI 48		HPI 24		HPI 48		HPI 24		HPI 48		HPI 24		HPI 48		HPI 24		HPI 48	
Mice	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13	M14	M15	M16	M17	M18	M19	M20
Bacterial titres (CFU/mg)	L4	2.10E+03	1.43E+03	-	-	2.63E+03	-	-	4.30E+06	6.25E+06	6.89E+08	3.71E+07	-	-	-	-	-	-	-	-
	R4	5.66E+05	3.28E+05	-	2.03E+04	-	-	-	2.42E+07	8.35E+07	3.24E+08	7.09E+07	-	-	-	-	-	-	-	-
Phage titres (PFU/mg)	L4	3.39E+02	1.17E+03	-	-	-	-	-	-	-	-	-	5.83E+05	1.31E+02	1.39E+02	-	-	-	-	-
	R4	-	2.31E+02	-	-	-	-	-	-	-	-	-	-	3.39E+03	1.93E+02	-	-	-	-	-

^a OS001_ISP, Ani_OS001m and the ISP phage; OS001_Marbo, Ani_OS001m and marbofloxacin; OS001_PBS, Ani_OS001m and PBS; ISP_PBS, ISP phage and PBS; PBS_PBS, PBS twice; M, mouse; L4/R4=Left and right fourth abdominal mammary gland pair; -, no bacterial or phage titre; HPI, hour post-inoculation.

3.2.3. Statistical analysis

A significant difference was observed between the survival curves of the infected-gentamicin-treated, on the one hand, and the infected-phage-treated and infected-PBS-treated *G. mellonella* larvae groups, on the other hand, as determined by log-rank (Mantel Cox) test (*P* < 0.0001). A significant difference was also detected between the bacterial titres of the infected-PBS-treated and the infected-phage-treated larva groups as determined by the Bonferroni multiple comparison test (*P* < 0.05). This test also showed a higher significant difference between the bacterial titres of the infected-PBS-treated and the non-infected larva groups (*P* < 0.01). In the murine mastitis model, the Bonferroni multiple comparison test showed a significant difference between the bacterial titres of the infected-PBS-treated and the infected-phage-treated mammary glands (*P* < 0.05). In both *G. mellonella* and mouse models, a

significant difference (*P* < 0.05) was observed between the phage titres of the infected-phage-treated and the ‘non-infected’-phage-treated groups.

3.2.4. Genome comparison of Ani_OS001 and Ani_OS001m

The draft genome of Ani_OS001 and Ani_OS001m are available from GenBank, accession numbers VVKU01000000 and VVKV01000000, respectively. The VarScan analysis revealed only two single polymorphism nucleotide (SNPs) between the two strains. One is associated with a region without any annotation according to Prokka (position 25 of contig OS001-00027 C → T). The second SNP (G → T) occurred in position 60 157 of contig OS001-00001. The Prokka annotation revealed *SdrE*, a gene coding for a member of the microbial surface components recognizing adhesive matrix family molecule (MSCRAMM). This mutation resulted in a G899V mutation at the protein level.

4. Discussion and conclusions

This study highlights the therapeutic potential of bacteriophages against *S. aureus* associated with bovine mastitis by the in vitro assessment of the phage activities and the in vivo assessment of the phage efficacy in *G. mellonella* and murine mastitis models.

Different lytic activities were observed on ten *S. aureus* isolates: the phage Romulus was active against only one isolate, the phage Remus against four isolates and the phage ISP against nine isolates. In previous studies, the Romulus and Remus phages were active against more isolates tested (70%) [11], whereas the lytic activity of the ISP phage was observed against a similar percentage (87%) of the tested *S. aureus* isolates, including MRSA [12,20]. Conversely, none of the ten isolates tested was susceptible to the DSM105264 phage, although it was demonstrated to be active against ca. half of the previously tested isolates (human strains) [13]. The difference of susceptibility observed could be explained by the difference of the *S. aureus* origins (bovine vs. human).

The lytic activities of the three different phages against the Ani_OS001 *S. aureus* strain in broth medium confirm the results observed on agar medium. However, a higher efficiency has been observed for the phages ISP and REM compared with those of the phage mix, itself more efficient than the phage ROM alone. As showed in the literature, the mix of phages usually leads to synergistic effect in vitro in terms of a broad host range [21,22]. This study showed a probable lower effect of the phages mix compared with those of individual phages when using them against the same bacteria.

In the *G. mellonella* model, using only the Ani_OS001 strain, no significant difference was observed between the survival rate of the infected-phage-treated and infected-PBS-treated larvae group, whereas this difference was significant in comparison to the infected-gentamicin-treated group. The higher bacterial titre observed at DPI 4 in the blended larvae (group OS001_ISP) with the ISP phage compared with those observed with Romulus and Remus, although its higher lytic activity in vitro can be explained by its initial adsorption and biofilm-degradation that are slower than those of Romulus and Remus phages [11]. Another explanation could be the occurrence of a mutation in the bacterial genome during the phage treatment making a part of the Ani_OS001 isolate population resistant to the ISP phage [23,24]. No SPF larvae exist, and natural carriage of *S. aureus* could affect the results of the blended larvae bacterial titres. The very weak impact of natural carriage on our results has been confirmed by the Bonferroni multiple comparison test showing a higher significant difference ($P < 0.01$) between the bacterial titres of larva groups with natural carriage (REM, ISP, PBS) compared with those of the infected-PBS-treated larva groups. Moreover, a decrease in phage titre was observed for the blended larvae at DPI 2 and DPI 4. This could be explained by the single dose of phages inoculated and their possible inactivation as demonstrated in recent studies where bacteriophages have been shown to be efficient in *G. mellonella* larvae after multiple inoculations in the presence of the bacteria and depending on the MOI [25,26]. The decrease of the phage titre observed in this study is slower in the infected-phage-treated groups compared with the 'non-infected'-phage-treated groups meaning that a simultaneous replication of the phage and bacteria occurred inside the larvae. This study reports for the first time results on lytic bacteriophages assessment on *S. aureus* in the *G. mellonella* model, whereas many other studies were already performed, but on other pathogen bacteria [10,25,27]. However, the results observed in this model need to be confirmed in other infectious models in mammals, such as in the murine mastitis model.

An increase of the virulence gene expression after a previous in vivo passage is often observed as reviewed by Angelichio and

Camilli [28]. In the murine mastitis model, Ani_OS001m showed a more efficient infection than Ani_OS001. The WGS of both strains revealed a mutation in the *SdrE* gene of Ani_OS001m. *SdrE* is presented as a key factor for *S. aureus* being invasive and has already been described in an *S. aureus* strain showing higher virulence in mastitis cases [29].

A reduction of the inflammation in mice mammary glands was obtained in the infected-phage-treated group compared with those observed in the infected-PBS-treated group with the strain Ani_OS001m. These observations have also been described in recent studies on the therapeutic assessment of phage against *S. aureus* in a murine mastitis model [17,30]. An increase of the bacterial titre (from 7×10^4 to 2×10^8 CFUs/mg) observed in the infected-PBS-treated group and a decrease of the bacterial titre (from 7×10^4 to 5×10^3 CFUs/mg) observed in the infected-phage-treated group highlight the efficacy of the phage against the *S. aureus* strain in the mice mammary glands, as already shown with other phages in previous studies [30,31]. Nevertheless, this phage efficacy was less important than the one obtained with antimicrobials in the murine model [17]. Moreover, a decrease of the phage titre was observed in the 'non-infected'-phage-treated group (from 1.3×10^8 to 9×10^2 PFUs/mg) and more drastically in the infected-phage-treated group (from 1.3×10^8 to 0 PFUs/mg), suggesting an elimination or a degradation of the phage in the mouse mammary gland. Studies show that the stimulation of the immune response (macrophages, serum complement system) is more important when bacteria are inoculated with phages in the mice mammary glands, which can explain the drastic decrease of the phage titre in the infected-phage-treated groups [17,23,32]. The intra-mammary route can be favourable to the phage inactivation by some proteins and lipids presents in the milk leading to a decrease of the phage titre in the mammary gland [23,33]. Conversely, no decrease of the phage titre was observed in the mice mammary glands showing moderate inflammation as described by Breyne et al. [17], who assessed a phage cocktail effect on *S. aureus* at MOI 10^5 . This contradiction with our study could be explained by the use of a single phage inoculation at a lower MOI (10^3), which would have been quickly eliminated.

This study highlights the lytic activity of three bacteriophages (Romulus, Remus and ISP) against *S. aureus* associated with bovine mastitis in vitro and their efficacy in vivo materialised by a larvae survival rate below 50% at DPI 4 in the groups infected and treated (with the three phages) and an incomplete recovery of the mouse mastitis at HPI 48 in the groups infected and treated (with the ISP phage). Moreover, significant differences were observed between infected-PBS-treated and infected-phage-treated groups in *G. mellonella* and murine mastitis model demonstrating an effect of the three phages against *S. aureus* associated with bovine mastitis. The weak efficacy of phage observed in vivo (low larvae survival rate, incomplete mammary gland recovery) could be related to the unique bacteriophage dose used, the ratio phage/bacteria (MOI), the role of the immune system and the route of inoculation as described in previous studies [32,34]. Further studies are required to assess these parameters in order to confirm the phage therapy efficacy against *S. aureus* causing bovine mastitis.

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Competing interests

None.

Ethical approval

Ethical Committee of the University of Liège (approval number 14-1719).

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