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Bacteriophages | Full-Length Text

Functional and practical insights into three lactococcal antiphage systems

Andriana Grafakou,[1] Cas Mosterd,[1] Paul P. de Waal,[2] Irma M. H. van Rijswijck,[2] Noël N. M. E. van Peij,[2] Jennifer Mahony,[1] Douwe van Sinderen[1]

AUTHOR AFFILIATIONS See affiliation list on p. 13.

ABSTRACT The persistent challenge of phages in dairy fermentations requires the development of starter cultures with enhanced phage resistance. Recently, three plasmid-encoded lactococcal antiphage systems, named Rhea, Aristaios, and Kamadhenu, were discovered. These systems were found to confer high levels of resistance against various *Skunavirus* members. In the present study, their effectiveness against phage infection was confirmed in milk-based medium, thus validating their potential to ensure reliable dairy fermentations. We furthermore demonstrated that Rhea and Kamadhenu do not directly hinder phage genome replication, transcription, or associated translation. Conversely, Aristaios was found to interfere with phage transcription. Two of the antiphage systems are encoded on pMRC01-like conjugative plasmids, and the Kamadhenu-encoding plasmid was successfully transferred by conjugation to three lactococcal strains, each of which acquired substantially enhanced phage resistance against *Skunavirus* members. Such advances in our knowledge of the lactococcal phage resistome and the possibility of mobilizing these protective functions to bolster phage protection in sensitive strains provide practical solutions to the ongoing phage problem in industrial food fermentations.

IMPORTANCE In the current study, we characterized and evaluated the mechanistic diversity of three recently described, plasmid-encoded lactococcal antiphage systems. These systems were found to confer high resistance against many members of the most prevalent and problematic lactococcal phage genus, rendering them of particular interest to the dairy industry, where persistent phage challenge requires the development of starter cultures with enhanced phage resistance characteristics. Our acquired knowledge highlights that enhanced understanding of lactococcal phage resistance systems and their encoding plasmids can provide rational and effective solutions to the enduring issue of phage infections in dairy fermentation facilities.

KEYWORDS *Lactococcus*, lactic acid bacteria, bacterial immunity, phage defense, Abi

The production of a plethora of fermented dairy foods, such as cheese, buttermilk, and sour cream, has long depended on the employment of *Lactococcus lactis* and *Lactococcus cremoris* (1, 2). These economically and industrially important starter bacteria are members of the lactic acid bacteria (LAB), which is a group of Gram-positive, non-spore-forming, micro-aerophilic bacteria (3). Many LAB, including *L. lactis* and *L. cremoris*, enjoy a generally recognized as safe (GRAS) status according to the Food and Drug Administration due to their historic safe application in food products. As dominant components of many dairy starter cultures, their primary functions in milk fermentations are to decrease milk pH by producing lactic acid, metabolize milk proteins, and, in certain cases, produce antimicrobial substances such as bacteriocins, thus contributing to the organoleptic properties and safety of the final product (1, 4).

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Address correspondence to Douwe van Sinderen, d.vansinderen@ucc.ie.

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However, the intensive application of strains of these two lactococcal species is accompanied by the persistent challenge of infecting (bacterio)phages (5). A phage-infected starter culture may partially or completely lyse, leading to delayed or failed fermentations with major economic and environmental consequences (6). Among the described lactococcal phages (7, 8), members of the *Skunavirus* genus are the most frequently encountered and problematic for the dairy industry (9). The continuous threat posed by phages in the dairy fermentation environment has inspired major research efforts to discover and implement lactococcal antiphage mechanisms. Until recently, described antiphage systems of *Lactococcus* spp. encompassed various Type I, II and III Restriction-Modification (RM) systems (10–12), a Type III-A Clustered Regularly Interspaced Short Palindromic Repeats and CRISPR-associated protein (CRISPR-Cas) system (13), five Superinfection exclusion (Sie) proteins [Sie_{2009}, Sie_{IL409} or Sie_{309}, $Sie_{F7/2AB}$ or Sie_{285}, $Sie_{mg2/312}$, and Sie_{T712} (14, 15)], and 23 abortive infection (Abi) systems (4).

Abi systems are among the most prevalent lactococcal antiphage strategies. Upon activation, the infected cell prevents a crucial step in the phage propagation process which typically affects its own viability yet preventing completion of the phage life cycle and consequently averting infection of neighboring cells (16, 17). Most of the currently known lactococcal Abi systems are encoded by one or two plasmid-borne genes and have been shown to affect different points of the phage reproduction cycle, including DNA replication (AbiA, AbiF, AbiK, AbiP, AbiR, and AbiT), transcription (AbiB, AbiG, AbiU, and AbiV), and translation (AbiC) (4, 16). Recently, seven additional lactococcal antiphage systems have been described, of which five behave as Abi systems (Rhea, Aristaios, Kamadhenu, Rugutis, and Hesat), while the classification/mode of action for the remaining two (Fliodhais, Audmula) is not yet known. Furthermore, the antiphage activity of five additional systems (PARIS, type II and II CBASS, Lamassu, and Septu), whose activity had not been previously established in lactococci, was recently reported (18).

There is a technological need for phage-robust starter cultures which simultaneously satisfy consumer demands for products with specific organoleptic features (1, 19). Although genetically modified organisms (GMOs) may address such requirements (20), their use by the food industry faces considerable consumer opposition and is subject to strict and varying regulatory conditions in various jurisdictions, such as the European Union regulatory framework for deliberate release of GMOs (21). Nonetheless, many desired traits are encoded by conjugative or mobilizable plasmids that by natural processes can be transferred or co-transferred between strains (22). Conjugation is a natural process that involves transfer of genetic material from a donor to a recipient cell via a conjugative apparatus through direct cell-to-cell contact (23). Since conjugation is not classified as genetic modification under Directive 2001/18/EC and is exempt from labeling requirements in the United States, it can be employed to generate phage robust dairy starter cultures which are not considered GMOs (24).

The aim of the current study was to characterize and evaluate mechanistic details of the recently described plasmid-encoded Rhea, Aristaios, and Kamadhenu lactococcal antiphage systems which in particular confer high resistance against many members of the most prevalent lactococcal phage genus *Skunavirus* (18), rendering these systems of particular interest to the dairy industry. In the current study, we also apply this resource of transferable phage resistance for the development of phage robust strains.

MATERIALS AND METHODS

Strains, bacteriophages, cultivation media, and growth conditions

Bacterial strains, relevant plasmids, and bacteriophages used in this study are listed in Table 1. Lactococcal strains were grown at 30°C in M17 broth (Oxoid, United Kingdom) supplemented with 0.5% (wt/vol) glucose (GM17) for 16–20 hours. GM17 was supplemented with either chloramphenicol (5 µg/mL, to select for strains carrying pNZ44 or pJP005 and their derivatives, Sigma-Aldrich, Ireland) or streptomycin (500 µg/mL, to

TABLE 1 Bacterial strains and bacteriophages used in this study

Bacterial strains and bacteriophages	Characteristics[a]	Source or reference
L. cremoris NZ9000::pNZ44	Laboratory strain, host of the antiphage systems and for phage sk1	(25, 26)
L. cremoris 3107::pNZ44	Dairy starter strain, host of the antiphage systems and for phage 62601	(26, 27)
L. cremoris NZ9000/3107::pNZ44 + Rhea	Antiphage system constitutively expressed in *L. cremoris* NZ9000/3107::pNZ44	(18)
L. cremoris NZ9000/3107::pNZ44 + Aristaios	Antiphage system constitutively expressed in *L. cremoris* NZ9000/3107::pNZ44	(18)
L. cremoris NZ9000/3107::pNZ44 + Kamadhenu	Antiphage system constitutively expressed in *L. cremoris* NZ9000/3107::pNZ44	(18)
L. cremoris NZ9000::pNZ44 + AbiP	Antiphage system constitutively expressed in *L. cremoris* NZ9000/3107::pNZ44	(18, 28)
L. cremoris UCCL624	Donor for conjugation experiments carrying among other plasmids pUCCL624B (antiphage systems-encoding conjugative plasmid)	NCBI accession numbers SAMN40630563 and PP556517
L. cremoris NZ9000::pJP005	Laboratory strain, recipient for conjugation experiments, Cm^r	(25, 29)
L. cremoris MG1614	Plasmid-free laboratory strain, used as recipient for conjugation experiments, Str^r	(30)
L. lactis UCCL643	Industrial strain, recipient for conjugation experiments	Strain previously isolated from the dairy environment
L. cremoris NZ9000::pJP005::pUCCL624B	Transconjugant	This study
L. cremoris MG1614::pUCCL624B	Transconjugant	This study
L. lactis UCCL643::pUCCL624B	Transconjugant	This study
sk1	*Skunavirus* propagated on *L. cremoris* NZ9000::pNZ44	(31)
62601	*Skunavirus* propagated on *L. cremoris* 3107::pNZ44	(32)
p2	*Skunavirus* propagated on *L. cremoris* NZ9000	(33)
712	*Skunavirus* propagated on *L. cremoris* NZ9000::pNZ44	(34)
jj50	*Skunavirus* propagated on *L. cremoris* NZ9000	(35)
phUCCL643.2	*Skunavirus* propagated on *L. lactis* UCCL643	Phage previously isolated from the dairy environment
phUCCL643.3	*Skunavirus* propagated on *L. lactis* UCCL643	Phage previously isolated from the dairy environment
phUCCL643.4	*Skunavirus* propagated on *L. lactis* UCCL643	Phage previously isolated from the dairy environment
phUCCL643.5	*Skunavirus* propagated on *L. lactis* UCCL643	Phage previously isolated from the dairy environment

[a]Cm^r, chloramphenicol resistant; Str^r, streptomycin resistant.

select for *L. cremoris* MG1614, Sigma Aldrich). Propagation of bacteriophages, spot and plaque assays, and phage titration were performed as previously described by Grafakou et al. (18). Efficiency of plaquing (EOP) was estimated from three biological replicates by spotting appropriate dilutions of the phage lysate on a relevant sensitive host.

One-step phage growth curves for ECOI and burst size determination

One-step phage growth curves were conducted as described by Moineau et al. (36) with some modifications. Two milliliters of bacterial culture [optical density at 600 nm (OD_{600nm}) of ~0.8] was harvested by centrifugation (7,000 × *g* for 2 minutes) and resuspended in 900 µL of GM17 supplemented with 10 mM $CaCl_2$ (GM17 + $CaCl_2$). Phage sk1 lysate was added at a multiplicity of infection (MOI) of 0.1. After 5 minutes of phage adsorption at 30°C, bacteria were harvested as previously described and washed twice in 1 mL of GM17 + $CaCl_2$ to remove free phages. The suspension was serially diluted 10,000-fold in GM17 + $CaCl_2$ to reduce the number of bacteria available for residual free

phage adsorption and incubated at 30°C. Samples were withdrawn immediately (time zero, t_0) and every 15 minutes for 75 minutes and plated on the phage-sensitive host (*L. cremoris* NZ9000::pNZ44) after appropriate dilution. The initial titer (t_0) defined the number of centers of infection (COI) formed. The efficiency of COI formation (ECOI) for each sample was calculated as the COI from the test strain divided by the COI obtained from the reference strain *L. cremoris* NZ9000::pNZ44. The burst size of sk1 on a test strain was determined as [phage titer at the end of the single step growth curve (60 minutes) – initial titer (15 minutes)]/initial titer (15 minutes). Data are presented as mean ± standard deviation (SD) from three biological replicates.

DNA replication assay

Quantification of intracellular sk1 DNA in phage-infected cells of *L. cremoris* NZ9000::pNZ44 and its phage-resistant derivatives expressing the three antiphage systems (Table 1) was evaluated using quantitative PCR (qPCR) as previously described with some modifications (37). Ten milliliters -of GM17 broth inoculated with 2% of fresh overnight culture of relevant strains was incubated at 30°C until an OD_{600nm} of 0.2 was reached. The culture was subsequently supplemented with a final concentration of 10 mM $CaCl_2$. Phage lysate was added at an MOI of 0.03. The infected cultures were incubated at 30°C, and 1-mL samples of the culture were taken after 5, 15, and 25 minutes and centrifuged in pre-chilled tubes at 14,000 × *g* for 2 minutes at 4°C. The supernatant was discarded to avoid quantifying residual phages. The pellet was resuspended in 1 mL of ice-cold 1/4 strength Ringer's solution (Merck, Ireland), flash frozen in ethanol (70%, −80°C), and stored at −80°C prior to DNA extraction. Subsequently, the frozen samples were thawed on ice and the pellets were washed once in 1/4 strength Ringer's solution. Total DNA was extracted using the Purelink Genomic DNA extraction kit (Invitrogen) according to the manufacturer's instructions. Three biological replicates were performed for each strain.

LightCycler 480 SYBR Green I Master (Roche, Ireland) and a LightCycler 480 Instrument (Roche) were used to perform the qPCR assays. Previously optimized qPCR primers targeting the sk1 small terminase-encoding gene (ORF1, NCBI Taxonomy ID 2905675) were used (38). Three technical replicates (15 µL reaction mixture) containing 5 µL of the sample or sk1 DNA (for the construction of the standard curve, see below) and 500 nM of each primer were prepared for each sample. The initial denaturation step was performed at 95°C for 5 minutes, followed by 40 cycles of (i) denaturation at 95°C for 15 s, (ii) annealing at 55°C for 30 s, and (iii) extension at 72°C for 30 s. Negative controls without template DNA were included in the analysis.

To quantify sk1 DNA, a standard curve was constructed by extracting DNA from 1 mL pure phage lysate (Norgen Biotek, Canada) according to the manufacturer's instructions with slight modifications. Prior to DNA extraction, a phage lysate was treated with 2 µL of DNAse I (Roche) for 30 minutes followed by ethylenediaminetetraacetic acid (EDTA; 5 mM) inactivation for 20 minutes at room temperature. The DNA was quantified using a Qubit 1× dsDNA High Sensitivity (quantitation range 0.1 to 120 ng) (Thermo Fischer Scientific, United Kingdom), then serially diluted, and analyzed by qPCR as described above. To generate the best-fit line, the obtained cycle threshold (C_T) values were plotted against the range of known quantities of DNA that were converted to DNA copies (per mL of standard sample) considering the molecular weight of phage sk1 as 17,654.67 kDa (39). Data are presented as mean ± SD from three biological replicates, and an unpaired *t*-test was performed to determine statistically significant differences (P value < 0.01).

Transcription assay

Quantification of intracellular sk1 RNA was evaluated in sk1-infected cells of *L. cremoris* NZ9000::pNZ44 and its phage-resistant derivatives expressing the three antiphage systems (Table 1) by RT-qPCR. Bacterial strains were cultivated and infected as described above for the replication assays apart from adding sk1 phage lysate at MOI of 0.1. Infected cultures were initially incubated at 30°C, and after 10 and 25 minutes, 1-mL

samples were centrifuged in pre-chilled tubes at 14,000 × *g* for 2 minutes at 4°C as an initial screening. To obtain a more detailed profile of *L. cremoris* NZ9000::pNZ44 carrying the Aristaios antiphage system, another round of infection was performed along with the reference strain and samples were collected at 5-, 10-, 15-, 25-, 35-, and 60-minute time points. Pellets were resuspended and stored in 500 µL of RNA*later* (Invitrogen). Prior to RNA extraction, 500 µL of 1/4 strength Ringer's solution was added to the samples, which were then centrifuged at 10,000 × *g* for 5 minutes to remove RNAlater. Total RNA was extracted using the High Pure RNA Isolation Kit (Roche) according to the manufacturer's instructions with the modification of adding 12 µL of DNAse mix (DNAse and incubation buffer included in the kit) to each sample and incubating at 30°C. Three biological replicates were performed for each strain. cDNA was synthesized using SuperScript III reverse transcriptase (RT; Invitrogen) according to the manufacturer's instructions. For each 20-µL reaction, 250 ng of random oligomeric primers (Invitrogen) and 5 µL of total RNA were used. Reactions containing the same mix but replacing the RT enzyme with water were included as controls to verify appropriate DNA digestion.

LightCycler 480 SYBR Green I Master (Roche) and a LightCycler 480 Instrument (Roche) were also employed for the RT-qPCR experiments. According to the time of their appearance, phage transcripts have been divided into three categories, corresponding to early-, middle-, or late-expressed regions (31). Primers targeting the middle and late transcripts were used in this study. Primers annealing to the predicted Holliday junction endonuclease-encoding gene of sk1 (NCBI Taxonomy ID 2905675; open reading frame 53 [ORF53]; sk1_hol_F ACGTTCAAGTCTAAGGGAAAC and sk1_hol_R TGGTACATCAACAT AGCCAATG) were designed to quantify the middle transcripts. Primers were validated and optimized by determining the primer efficiency (89.8%) and the R^2 (0.99) during a standard curve construction as described in the previous paragraph. Optimized qPCR primers targeting the sk1 small terminase-encoding gene were applied (38) to quantify the late transcripts. Three technical replicates were prepared and analyzed as described for the qPCR experiments with slight modifications as follows: 5 µL of cDNA added for each reaction, and the extension step was performed at 72°C for 20 s. Phage sk1 cDNA in infected lactococcal cells was quantified and interpreted as described for the replication assay.

Protein overexpression and antibody generation

Phage sk1 capsid-, tail-, and receptor binding protein (RBP)-encoding genes (ORF6, ORF11, and ORF18, respectively; NCBI Taxonomy ID 2905675) were amplified from a fresh lysate using Phusion Green High-Fidelity DNA Polymerase (Thermo Fisher Scientific) according to the manufacturer's instructions. The NZYEasy Cloning & Expression Kit I (NZYTech, Portugal) and *Escherichia coli* vector pHTP1 were selected, since it contains two poly-histidine (6xHis) sequences allowing subsequent recombinant protein purification by affinity chromatography. Therefore, primers (Table S1) were designed by omitting the start codon for the forward primer and including an in-frame stop codon to the reverse primer to exclude the C-terminal His-tag according to the manufacturer's instructions. The purified PCR-generated fragments were cloned into the linearized pHTP1 *E. coli* vector. Ligation mixtures were introduced into competent *E. coli* BL21 (DE3) (40) according to the manufacturer's instructions. The integrity of recombinant plasmid sequences was verified by Sanger sequencing (Eurofins Genomics, Germany) using vector-specific primers (Table S1). *E. coli* BL21 (DE3) harboring a plasmid of interest was inoculated (1%) to 100 mL of LB media (Thermo Fisher Scientific) supplemented with 50 µg/mL kanamycin (Sigma), and cultures were incubated with agitation (200 rpm) at 37°C until an OD_{600nm} of 0.4–0.5 was reached. Induction with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; PanReac, United States) followed, and cultures were incubated at 30°C for 4–6 hours. Cells were harvested at 4,600 × *g* for 30 minutes, and the pellet was resuspended in 10 mL lysis buffer (10 mM Tris-HCl pH 7.5, 300 mM NaCl, 50 mM $CaCl_2$, 10 mM imidazole, and 25 mg/mL lysozyme). The cell suspensions were lysed by sonication, and recombinant proteins were purified as previously described

by Lavelle et al. (41). Antibodies targeting the purified and dialyzed phage sk1 capsid, tail, and RBP were generated with a standard rabbit immunization protocol (63 days) executed by Davids Biotechnologie GmbH (Germany, https://www.davids-bio.com/pages/polyclonal-rabbit-antisera.html).

Detection of intracellular phage proteins during infection

Fresh overnight cultures of *L. cremoris* NZ9000::pNZ44 and its derivatives (Table 1) were added (1%) to 100 mL of GM17 and incubated at 30°C until an OD_{600nm} of ~0.2 was reached. Cells were harvested by centrifugation (at 2,600 × *g* for 5 minutes), and the obtained cell pellet was resuspended in 10 mL of GM17 supplemented with 10 mM $CaCl_2$ and subsequently infected with phage sk1 at an MOI of 1. Two-milliliter samples were taken at 0, 15, 25, 30, and 60 minutes post infection and centrifuged at 14,000 × *g* for 2 minutes at 4°C. The obtained cell pellets were flash-frozen (−80°C) and analyzed for intracellular phage protein production by western blotting. Cell pellets were resuspended in 80 µL of sodium dodecyl sulfate loading buffer (10% glycerol, 3% SDS, 0.0625 M Tris pH 6.8, and 0.01% bromophenol blue) and were incubated at 100°C for 5 minutes prior to SDS-PAGE gel loading. The samples were centrifuged (at 14,000 × *g* for 1 minute), and 20 µL protein solution was separated at 160 V for 90 minutes on a 12% SDS-PAGE gel. The proteins were electroblotted (100 V for 30 minutes) onto a nitrocellulose membrane (0.45 µm, Thermo Fisher Scientific) using a 1× CAPS solution (pH 10.5; Sigma) as transfer buffer in a Mini-PROTEAN 3 Cell apparatus (Bio-Rad, Ireland). The membranes were subsequently soaked in PBS (Sigma) for several minutes and then treated with Intercept (PBS) blocking buffer (LI-COR, United Kingdom) for 1 hour on a platform shaker STR6 (Stuart Scientific). Membranes were then incubated (1 hour, gentle shaking) with primary antibody, having been diluted 1:5,000 (in the case of antibodies directed against the major capsid or tail protein) or 1:2,500 (for antibodies against the RBP) in blocking buffer supplemented with 0.25% Tween-20 (Sigma). After four washes (5 minutes each with gentle shaking) with PBS supplemented with 0.1% Tween-20, the membrane was incubated (1 hour, shaking) with secondary antibody (IRDye 680RD Goat anti-Rabbit IgG; LI-COR) diluted 1:7,500 in blocking buffer. This was followed by four washes with PBS supplemented with 0.1% Tween-20 (5 minutes each with gentle shaking) and a final rinse in PBS to remove residual Tween-20. The protein bands were visualized with IRDye 680RD application and an optimal auto-exposure time using ChemiDoc MP imager (Bio-Rad).

Milk acidification assay

L. cremoris 3107::pNZ44 and derivative strains expressing either of the three antiphage systems (Table 1) were grown overnight at 30°C in M17 supplemented with 0.5% lactose (LM17). Fresh overnight cultures (2%) were added directly to 10% wt/vol Reconstituted Skimmed Milk (RSM, Carbery Milk Products, Ireland) supplemented with 5 µg/mL chloramphenicol, and the cultures were grown overnight at 30°C. Subsequently, a 2% inoculum was added to 10 mL of fresh 10% RSM and cultures were then incubated at 30°C for 3 hours at which point they were infected with 100 µL of *Skunavirus* 62601 ($1*10^8$ PFU/mL). Uninfected cultures were included as controls and were supplemented with 100 µL of sterile-filtered overnight culture (*L. cremoris* 3107::pNZ44, LM17) instead of phage lysate. The pH was recorded every 3 hours for a total of 9 hours, with a final reading after 24 hours. Data are presented as mean ± SD from three biological replicates.

Conjugation experiments

Conjugation was performed using the spread solid mating approach as described by Ortiz Charneco et al. (42). Agar plates were supplemented with streptomycin, chloramphenicol, or nisin to select for recipient cells, i.e., *L. cremoris* MG1614, *L. cremoris* NZ9000::pJP005, and *L. lactis* UCCL643, respectively.

The presence of pUCCL624B in *L. cremoris* MG1614, *L. cremoris* NZ9000::pJP005, and *L. lactis* UCCL643 was verified by colony PCR using Phusion Green High-Fidelity

DNA Polymerase (Thermo Fisher Scientific) according to the manufacturer's instructions and pUCCL624B- and NZ9000/MG1614/UCCL643-specific primers (Table S2), confirming conjugative transfer of pUCCL624B to the recipient strain.

RESULTS

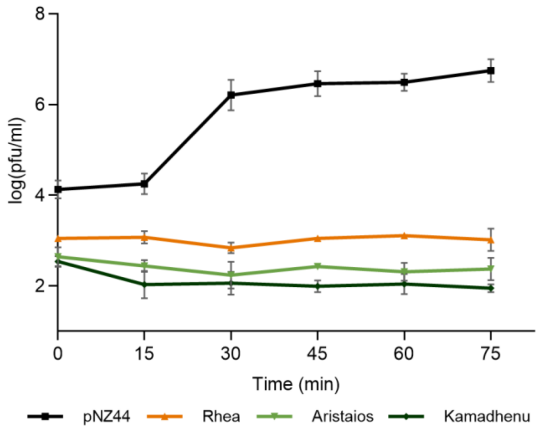
Rhea, Aristaios, and Kamadhenu antiphage systems dramatically reduce the burst size and ECOI of phage sk1

To date, in addition to the previously established 23 lactococcal abortive infection systems [AbiA to AbiZ (4)], 10 additional antiphage systems that behave as Abi systems (Rhea, Aristaios, Kamadhenu, Hesat, Rugutis, PARIS, type I and II CBASS, Lamassu, and Septu) have been described to be present in representatives of this genus (18). The objective of the current study was to further characterize the plasmid-encoded Rhea, Aristaios, and Kamadhenu lactococcal antiphage systems. The effectiveness of Rhea, Aristaios, and Kamadhenu antiphage systems against many members of the most prevalent lactococcal phage genus *Skunavirus* and specifically against *Skunavirus* sk1 in solid and liquid growth medium had previously been demonstrated (18). To further evaluate the impact of these systems on phage development, the burst size and the ECOI of phage sk1 on *L. cremoris* NZ9000::pNZ44 and its derivatives expressing each of the three antiphage mechanisms (Table 1) were determined using one-step phage growth curves (Fig. 1A). Infected cells of *L. cremoris* NZ9000::pNZ44 release approximately 180 progeny sk1 virions per infected cell (Fig. 1B). However, the strains containing the three antiphage systems produce no detectable phage progeny (burst size close to zero) (Fig. 1B). Similarly, an 11-fold (for Rhea) and approximately 30-fold (for Aristaios and Kamadhenu) ECOI reduction was observed in the presence of these antiphage systems relative to the control (Fig. 1B).

Phage DNA replication is not impaired in the presence of Rhea, Aristaios, and Kamadhenu

Previously, it was shown that the three antiphage systems do not interfere with phage adsorption or DNA injection and exhibit an Abi-like phenotype (18). To further assess which step of the phage life cycle is targeted by the Rhea, Aristaios, and Kamadhenu antiphage systems, we investigated the replication, transcription, and translation process of phage sk1 in sensitive host cells (*L. cremoris* NZ9000::pNZ44) and cells harboring the antiphage systems (*L. cremoris* NZ9000::pNZ44 expressing Rhea, Aristaios, Kamadhenu, or AbiP antiphage systems; Table 1). AbiP was previously described to interfere with phage DNA replication and was therefore used as a positive control (28). DNA replication

A



B

Strain	ECOI	Burst size
NZ9000::pNZ44	1	180 ± 65
NZ9000::pNZ44+Rhea	0.088 ± 0.035	0.16 ± 0.40
NZ9000::pNZ44+Aristaios	0.032 ± 0.002	-0.22 ± 0.29
NZ9000::pNZ44+Kamadhenu	0.029 ± 0.018	0.16 ± 0.57

FIG 1 (A) One-step phage growth curve of phage sk1 and (B) ECOI and burst size of phage sk1 on *L. cremoris* NZ9000::pNZ44 and its phage-resistant derivatives harboring Rhea, Aristaios, and Kamadhenu antiphage systems. Experiments were performed in biological triplicate, and data are presented as means ± SD.

of phage sk1 was studied by quantifying the sk1 *terS* (small terminase-encoding) gene using qPCR 5, 15, and 25 minutes post phage infection. Phage sk1 DNA was already detected in all samples 5 minutes post phage infection (Fig. 2). After 15 minutes, a more than a 10-fold increase of *terS* was observed for all samples except for the culture expressing AbiP (positive control). After 25 minutes, similar levels of DNA copies/mL were recorded for all samples except for the culture expressing AbiP. Additionally, AbiP was shown to prevent *terS* increase after 25 minutes compared with the 15-minute time point. Therefore, in contrast to AbiP, lactococcal cultures expressing Rhea, Aristaios, and Kamadhenu antiphage systems do not interfere with sk1 phage replication and allow DNA multiplication at a level similar to the reference strain.

Aristaios interferes with phage transcription

Transcription of phage sk1 in sensitive cells (*L. cremoris* NZ9000::pNZ44) and cells harboring phage resistance systems (*L. cremoris* NZ9000::pNZ44 carrying Rhea, Aristaios, and Kamadhenu antiphage systems; Table 1) was examined by quantifying the sk1 *hol* (encoding a predicted Holliday junction endonuclease; part of the middle transcript) and *terS* (encoding the presumed small terminase; part of the late transcript) cDNA with RT-qPCR 10 and 25 minutes post phage infection (Fig. 3A). Phage sk1 middle and late transcript cDNA was detected for all assessed samples 10 minutes post phage infection followed by an increase at 25 minutes in strains harboring Rhea and Kamadhenu, but not for the Aristaios antiphage system. A detailed profile for the Aristaios antiphage system (Fig. 3B) confirmed that at 25 minutes post infection, there were approximately 10 times fewer copies of cDNA (for both middle and late transcripts) when this system was expressed compared with the reference strain. Interestingly, the additional time point at 60 minutes showed an increase in the cDNA copies for the reference strain, indicating a second round of infections, compared with the strain carrying Aristaios where at 60 minutes, a decrease is observed that could be explained by the activation of the antiphage system and cell lysis without release of phage progeny, confirming

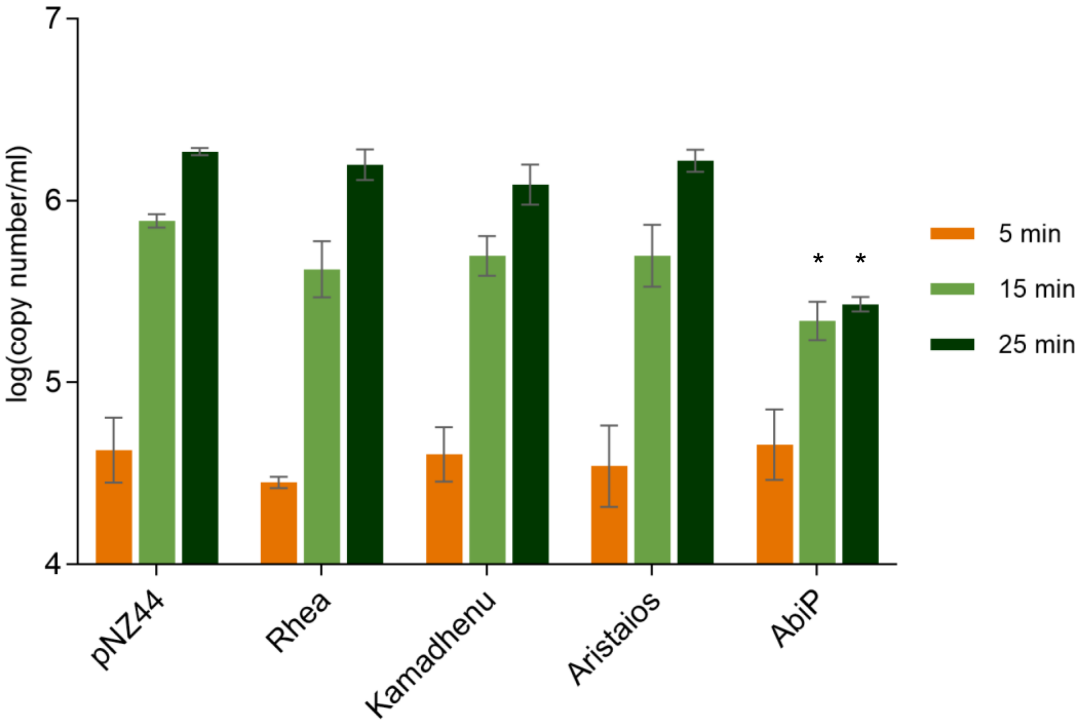


FIG 2 Quantification of phage sk1 DNA on different time intervals by qPCR in *L. cremoris* NZ9000::pNZ44 and its phage-resistant derivatives harboring Rhea, Aristaios, Kamadhenu, and AbiP antiphage systems. Experiments were performed in biological triplicate, and data are presented as means ± SD. Asterisks mark statistically significant differences between the phage-resistant derivatives and the reference strain at respective time points (unpaired *t*-test, *P* value < 0.01).

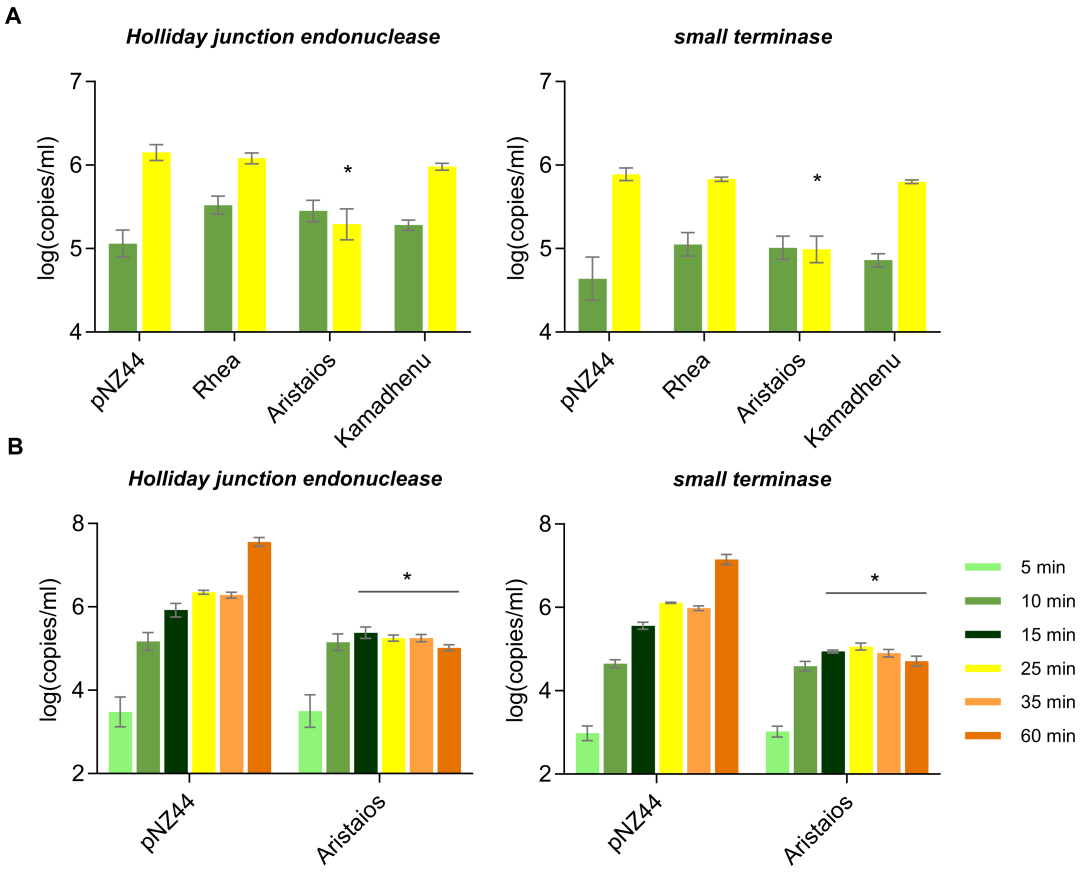


FIG 3 Quantification of phage sk1 cDNA on different time intervals by qPCR in *L. cremoris* NZ9000::pNZ44 and its phage-resistant derivatives harboring Rhea, Aristaios, and Kamadhenu antiphage systems. Experiments were performed in biological triplicate, and data are presented as means ± SD. Asterisks mark statistically significant differences between the phage-resistant derivatives and the reference strain at respective time points (unpaired *t*-test, *P* value < 0.01).

the previous one-step phage growth curve result. Therefore, the Aristaios antiphage system appears to interfere with sk1 transcription, in contrast to Rhea and Kamadhenu antiphage systems for which no impact was observed.

Aristaios downstream impact on phage translation

The effect of Rhea, Aristaios, and Kamadhenu antiphage systems (Table 1) on the production of phage structural proteins was investigated at different time intervals by using polyclonal antibodies specific to sk1 capsid, tail, and RBP proteins (Fig. 4). In cell extracts of the reference strain (*L. cremoris* NZ9000::pNZ44), sk1 phage proteins were detected 15 (capsid and tail) or 30 minutes (RBP) post phage infection, followed by an increased signal at the next time point (Fig. 4). A similar profile was recorded for the cell extracts of Rhea- and Kamadhenu-expressing strains. Consistent with the finding that Aristaios interferes with phage sk1 transcription, sk1 capsid, tail, and RBP proteins were not detected at any time point (capsid; Fig. 4A) or there was a substantially reduced signal detection (tail and RBP; Fig. 4B and C) in the presence of Aristaios. These results indicate that Rhea and Kamadhenu do not hinder the production of sk1 capsid, RBP, and tail protein and likely do not interfere with phage mRNA translation. In contrast, Aristaios seems to directly or indirectly interfere with transcription of *Skunavirus* sk1 with a consequent impact on the produced protein levels.

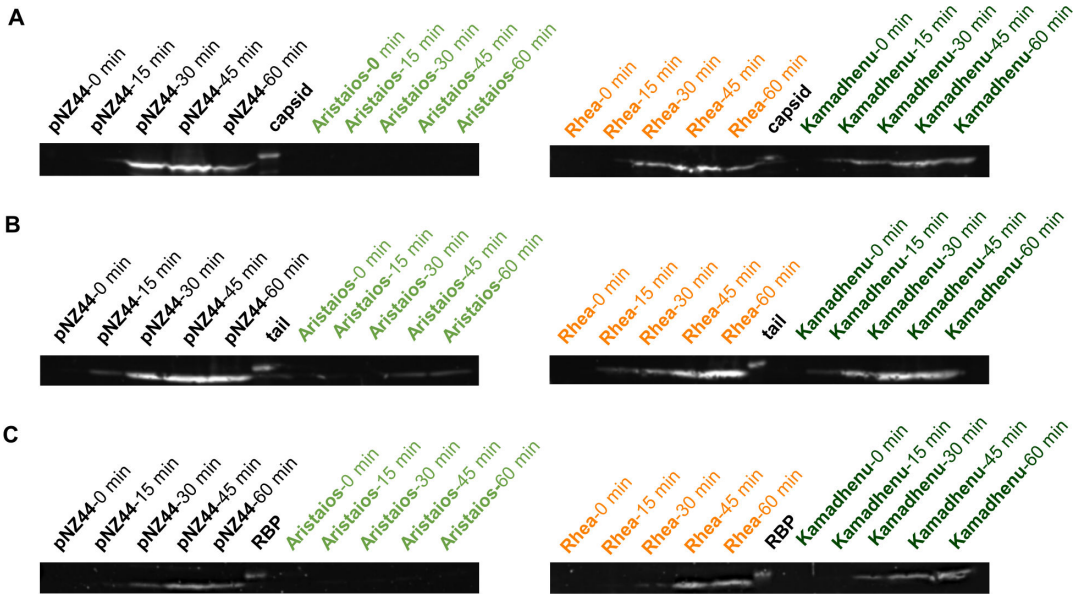


FIG 4 Detection of sk1 phage capsid (A), tail (B), and RBP (C) in *L. cremoris* NZ9000::pNZ44 and its phage-resistant derivatives harboring Rhea, Aristaios, and Kamadhenu antiphage systems with western blot in different time points.

Rhea, Aristaios, and Kamadhenu antiphage systems are active in milk-based growth medium

Previously, it was shown that the three antiphage systems are effective against multiple *Skunavirus* members in three different lactococcal strains (*L. cremoris* NZ9000, *L cremoris* 3107, and *L. lactis* IL1403) on solid semisynthetic medium (18). To assess the effectiveness of these systems in a milk-based medium, which is more relevant for dairy applications, *L. cremoris* 3107::pNZ44 and its phage-resistant derivatives expressing Rhea, Aristaios, and Kamadhenu (Table 1) were grown and infected with *Skunavirus* 62601 in reconstituted skimmed milk (Fig. 5). *L. cremoris* 3107 is a dairy starter strain with plasmids encoding the ability to utilize lactose (27), while *Skunavirus* 62601 can infect *L. cremoris* 3107, unlike *Skunavirus* sk1. Therefore, it allows growth in milk-based medium in contrast to the laboratory strain *L. cremoris* NZ9000 that was used for the remainder of this study. The pH of the milk was measured at different time points starting at the point of inoculation. After 6 hours, all infected cultures exhibited similar acidification profiles, except for the infected reference strain, where acidification was notably affected. Phage resistance of strains expressing either of the three antiphage systems was maintained overnight, as shown by a further pH decrease. Therefore, under the tested conditions, the Rhea, Aristaios, and Kamadhenu antiphage systems were effective against this member of the *Skunavirus* genus in a milk-based medium at a level that was equivalent to that observed in synthetic liquid laboratory medium (18).

Phage-resistant strains generated by natural transfer of Kamadhenu antiphage system encoding plasmid

Previously, it was reported that Aristaios and Kamadhenu antiphage systems are encoded on pMRC01-like conjugative plasmids (18), among other antiphage systems, based on BLASTP alignment (43) against the two prevalent lactococcal conjugation systems on pNP40 and pMRC01 (42). Therefore, these antiphage systems could potentially be transferred to starter strains of interest by conjugation, to generate natural derivatives with enhanced antiphage characteristics that could be used in the food industry.

To evaluate this possibility, plasmid pUCCL624B (Kamadhenu-encoding plasmid; Table 1) was selected as a representative for conjugation experiments, as it encodes

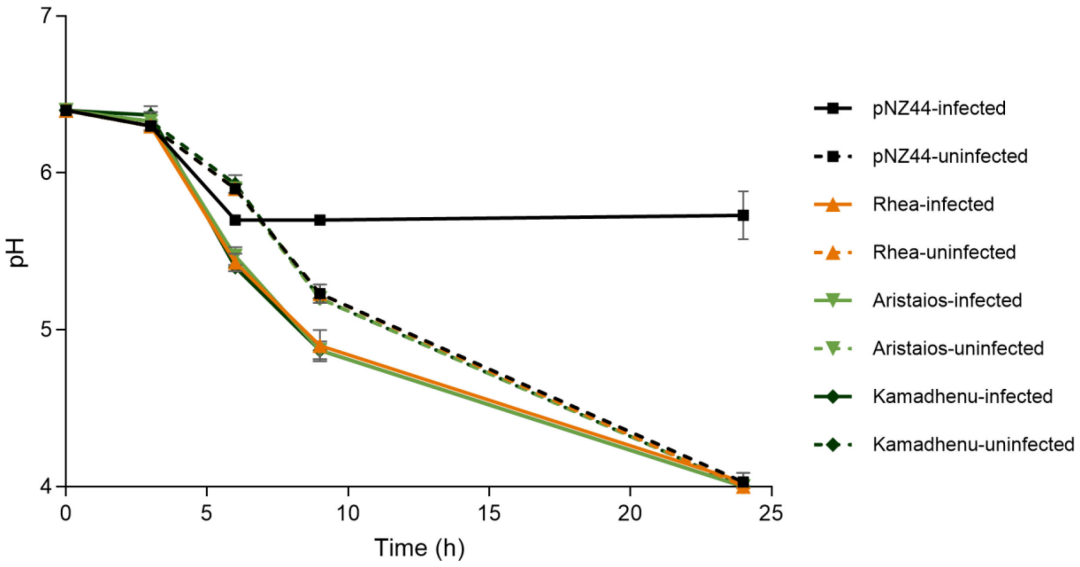


FIG 5 Milk acidification curves of *L. cremoris* NZ9000::pNZ44 and its phage-resistant derivatives harboring Rhea, Aristaios, and Kamadhenu antiphage systems. Experiments were performed in biological triplicate, and data are presented as means ± SD.

several additional (predicted) antiphage systems including AbiA, AbiZ, and Kamadhenu, as well as an RM system (Fig. 6). Indeed, pUCCL624B was successfully transferred by conjugation to three strains (*L. cremoris* NZ9000::pJP005, *L. cremoris* MG1614, and *L. lactis* UCCL643; Table 1). The presence of pUCCL624B in *L. cremoris* MG1614, *L. cremoris* NZ9000::pJP005, and *L. lactis* UCCL643 was further verified by PCR using pUCCL624B/NZ9000/MG1614/UCCL643-specific primers (Table S1).

The three transconjugants (Table 1) were subsequently challenged against skunaviruses that infect the recipient strain (i.e., sk1, p2,712, and jj50 for *L. cremoris* NZ9000::pJP005 and MG1614 and phages phUCCL643.2–5 for *L. lactis* UCCL643). Indeed, the transconjugants provided either over five log EOP reduction (*L. cremoris* NZ9000::pJP005::p60975B and *L. cremoris* MG1614::pUCCL624B) or three to five log EOP reduction (UCCL643::pUCCL624B) against the above-mentioned phages of the genus *Skunavirus* that infect the respective strains (Table S2). Therefore, it was confirmed that pUCCL624B can be transferred by conjugation delivering associated antiphage traits.

DISCUSSION

Three recently discovered lactococcal antiphage systems, namely, Rhea, Aristaios, and Kamadhenu, were observed to behave as Abi systems and to be highly effective against all tested phages of the genus *Skunavirus* (18). Members of the *Skunavirus* genus are the most problematic lactococcal phages (9), rendering the Rhea, Aristaios, and Kamadhenu antiphage systems particularly interesting to the dairy fermentation industry.

In the current study, the burst size and the ECOI of phage sk1 was observed to drop dramatically in the presence of these systems, resembling similar observations for AbiG, AbiQ, AbiU, and AbiT (44–47). Severe ECOI and burst size reductions are typical of Abi systems, confirming the Abi phenotype for the Rhea, Aristaios, and Kamadhenu antiphage systems. Furthermore, it was previously shown that the three systems allow sk1 phage adsorption/transduction and are therefore active post phage DNA injection (18). Here, phage DNA replication was observed to proceed unimpaired in the presence of the described antiphage systems. In contrast, AbiP was observed to affect DNA replication, consistent with previous literature reports (28).

Rhea and Kamadhenu do not evidently block any of the investigated steps of the phage lytic cycle (adsorption, DNA injection, DNA replication, transcription, and translation). However, these systems could interfere with other steps, such as DNA

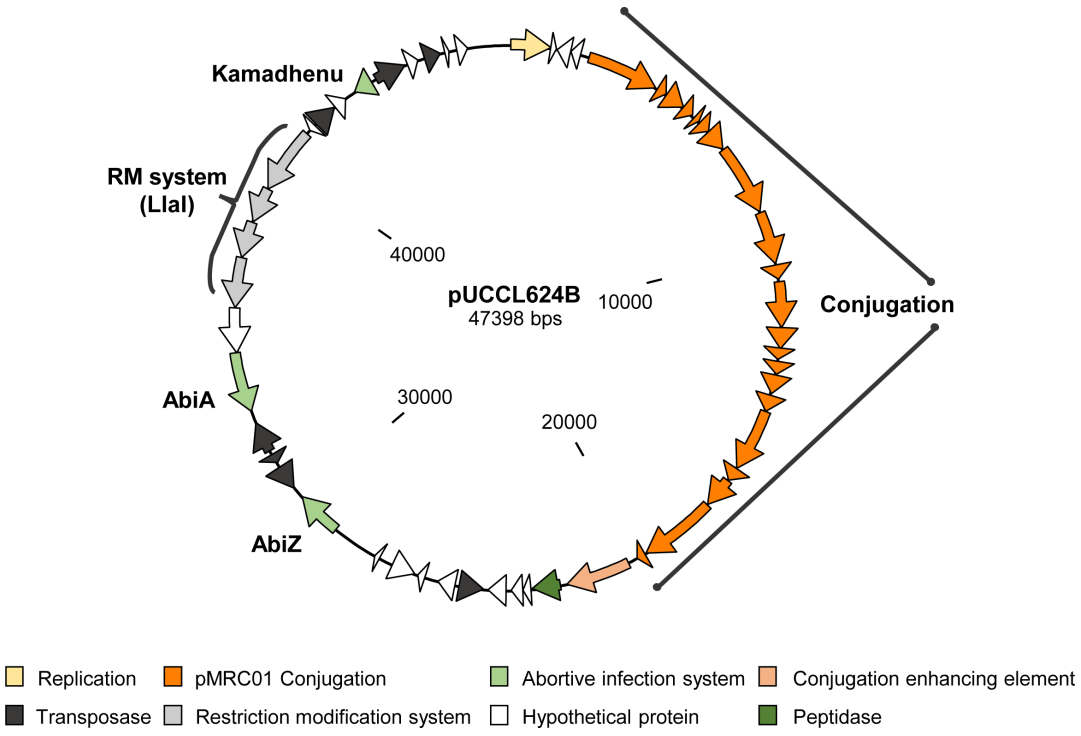


FIG 6 Plasmid map of the pMRC01-like conjugative plasmid pUCCL624B carrying Kamadhenu antiphage system among other antiphage systems.

packaging, virion assembly, or host lysis, which were not investigated in the current study. Aristaios was found to (directly or indirectly) inhibit sk1 transcription with a consequent impact on phage protein production. This finding supports the previously proposed hypothesis that the Aristaios protein hinders an essential host or phage function as it resembles an ADP-ribosyltransferase (18), a domain also found in bacterial toxins (48) including a toxin-antitoxin antiphage system (49). Therefore, these results confirm that these antiphage systems are active post phage DNA injection. Notably, several (i.e., six) characterized lactococcal Abi systems have been demonstrated to interfere with phage DNA replication, while four and one of them have been shown to interfere with transcription and translation, respectively (4).

Furthermore, it is known that phages may bypass antiphage systems through phage-specific mutations. Future isolation of phage mutants that escape these antiphage systems may provide further mechanistic details (i.e., activation) as demonstrated for antiphage systems in other bacterial species (50). Therefore, thoroughly characterized antiphage systems with diverse mechanisms could be combined to develop starter cultures with stronger phage resistance that are harder to be bypassed by phages through single point mutations. Interestingly, the synergistic protection of combined systems, such as Zorya II with Druantia III or ietAS and tmn with Gabija, Septu I, or PrrC, has recently been demonstrated in *E. coli* (51).

Additionally, along with the high effectiveness of the three antiphage systems against members of the genus *Skunavirus* in synthetic medium, the three antiphage systems were found to retain their protective action against a member of the *Skunavirus* genus in a milk-based medium, highlighting their industrial significance. This knowledge can be applied to improve dairy fermentations by either employing strains carrying (any of the) antiphage systems as starters or transferring the plasmids encoding these mechanisms to strains of interest. Interestingly, two of the three systems of this study are encoded on a pMRC01-like plasmid. Plasmid pMRC01 has previously been successfully transferred to over 30 different lactococcal strains, including commercial starter strains (52). Here, the Kamadhenu-encoding plasmid (which also encodes AbiA, AbiZ, and an RM

system) was successfully transferred to three lactococcal stains, including laboratory and industrial strains, offering protection against all tested *Skunavirus* members. This finding demonstrates that genetically transferable antiphage activities can be harnessed for the development of phage robust strains.

Although more than 30 genetically distinct lactococcal Abi systems have been described, their mechanisms of action appear to be diverse, affecting different critical stages of the phage multiplication cycle, such as DNA replication, transcription, and translation. However, antiphage systems can be overcome through phage-specific mutations, resulting in delayed fermentations and downstream economic sequences. This highlights the necessity of expanding the arsenal of antiphage systems by discovering previously unknown antiphage systems and understanding their diverse mechanisms that may be combined for the generation of more robust starter cultures.

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A.G., J.M., and D.V.S. led the study. A.G. performed the experiments and the analyses. C.M. performed and analyzed the milk acidification assay. P.P.D.W., I.M.H.V.R., and N.N.M.E.V.P. contributed with meaningful discussion of the results. J.M. and D.V.S. supervised and examined the results of the experiments. The initial concept manuscript was written by A.G. All authors contributed to editing the manuscript.

AUTHOR AFFILIATIONS

[1]School of Microbiology & APC Microbiome Ireland, University College Cork, Cork, Ireland
[2]dsm-firmenich, Taste, Texture & Health, Center for Food Innovation, Delft, the Netherlands

AUTHOR ORCIDs

Andriana Grafakou http://orcid.org/0000-0003-3803-4479
Cas Mosterd http://orcid.org/0000-0002-5369-8938
Douwe van Sinderen http://orcid.org/0000-0003-1823-7957

DATA AVAILABILITY

This study did not generate new unique reagents or sequencing data. Any additional information required to reanalyze the data reported in this paper is available from the corresponding author upon request.

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Supplemental tables (AEM01120-24-s0001.docx). Tables S1 and S2.

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