Potential therapeutic application of bacteriophages and phage-derived endolysins as alternative treatment of bovine mastitis

Potentiële therapeutische toepassing van bacteriofagen en faag-afgeleide endolysinen als alternatieve behandeling van boviene mastitis

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ABSTRACT

The increase in bacterial drug resistance causes major difficulties in the clinical treatment of a growing number of bacterial infections worldwide. Consequently, there is an urgent need to develop novel anti-bacterial agents to control these resistant pathogens and to complement the currently used antibiotics. Mastitis is the most prevalent disease impacting dairy cattle, and therefore one of the costliest diseases in the global dairy industry. The excessive use of curative as well as preventive antibiotics in this sector entails a real risk for the emergence of antimicrobial resistance. Moreover, these traditional antimicrobial agents are often ineffective and lead to residues in the milk, which can affect dairy product consumers. As an alternative therapeutic approach, bacteriophages and phage-encoded endolysins have been proposed and are currently (re)investigated as potential antibacterial agents against mastitis.

SAMENVATTING

De toename van antimicrobiële resistentie veroorzaakt wereldwijd grote problemen bij de klinische behandeling van een groeiend aantal bacteriële infecties. Daardoor is er een dringende behoefte aan nieuwe antibacteriële middelen als aanvulling op de huidige antibiotica om deze resistente pathogenen onder controle te houden. Mastitis is de meest voorkomende ziekte bij melkvee en veroorzaakt de grootste economische verliezen in de mondiale zuivelindustrie. Het overmatig gebruik van curatieve en preventieve antibiotica in deze sector brengt een reëel risico met zich mee voor het ontstaan van antimicrobiële resistentie. Bovendien zijn deze traditionele antimicrobiële middelen vaak ineffectief en leiden ze tot residuen in de melk die negatieve gevolgen hebben voor de consument van zuivelproducten. Als alternatieve therapeutische benadering worden momenteel bacteriofagen en faag-gecodeerde endolysinen (her)onderzocht als potentiële antibacteriële middelen.

INTRODUCTION

Mastitis

Mastitis is an inflammatory process of the udder in response to an intramammary infection, which can have either a clinical or subclinical outcome. In clinical mastitis, the cow is generally ill (e.g. fever, depression) and the affected udder quarter shows redness and swelling. Milk derived from the inflamed quarter often looks abnormal. In contrast, no visual abnormalities are present in the milk derived from an udder quarter with subclinical mastitis. Therefore, subclinical mastitis can easily be diagnosed through measuring an increase of inflammatory cells (somatic cell count, SCC) in the milk (Sadek et al., 2017; Pyörälä, 2003). Other diagnostic methods are the California mastitis test (CMT) and in-line electrical conductivity tests (IECT) (Ruegg, 2002). The CMT involves a colorimetric reaction with the DNA of the inflammatory cells in milk, whereas the IECT measures the electric resistance of milk.

The main cause of mastitis is the penetration of bacteria, yeasts and/or fungi through the teat orifice.

Today, more than hundred organisms are known to cause bovine mastitis (De Vliegher, 2017). Two main approaches are used to stratify these pathogens. A first classification is cow-adapted versus environmental pathogens. Cow-adapted germs survive and propagate mainly on the animal and are transmitted through direct or indirect contact. The most important cow-related bacteria are: non-aureus *Staphylococci spp.*, *Staphylococcus aureus* (*S. aureus*) and esculin-positive cocci. Their prevalence in Flanders is shown in Table 1 (Piepers et al., 2007). Other important cow-adapted bacteria are *Streptococcus dysgalactiae* (*S. dysgalactiae*), *S. agalactiae* and *Trueperella pyogene*s, although their prevalence is significantly lower (Gill et al., 2006; Piepers et al., 2007) (Table 1). In contrast, environmental germs survive mainly in the stable. These pathogens are harder to eradicate and are therefore considered more important than the cow-adapted germs. *Escherichia coli* (*E. coli*), *Klebsiella* and *S. uberis* infections are most common and arise from manure, wood shavings and straw, respectively (De Vliegher, 2017; Gonggrijp et al., 2017). A second classification differentiates 'major' from 'minor' pathogens. Major pathogens cause a high increase in milk SCC and severe clinical mastitis, whereas these characteristics are rather mild in so-called minor pathogens. In contrast to the first classification, this approach does not include the spread of the different pathogens on a dairy farm. Although the latter aspect is important in breaking the transmission cycle, the first approach is internationally more preferred (De Vliegher, 2017).

The data summarized in Table 1 (Piepers et al., 2007) probably have become outdated, as the percentage of cow-adapted infections can be expected to have decreased during the past decade due to more effective control campaigns in Flanders. Indeed, compared to the distribution of the main udder pathogens isolated from clinical milk samples in 2014 (data from the Milk Control Centre of Flanders (MCC Vlaanderen)), the following two observations are important: 1. a decrease in the cow-adapted non-aureus *Staphylococci spp.* and *S. aureus* from 19.1% to 10.6% and 56.6% to 7.8%, respectively; 2. an increase to 18.1% and 18.7% for *S. uberis* and *E. coli*, respectively. These results indicate that in Flanders, environmental-bound pathogens have become more important than cow-adapted pathogens.

Table 1. Prevalence of mastitis and the isolated bacteria in Flemish dairy farms from 2000 till 2002 (Piepers et al., 2007).

	2000	2001	2002	Overall
Number of cows sampled	16,432	16,270	11,965	44,667
% culture positive cows	39.0	41.7	43.0	41.1
Number of quarters sampled	65,728	65,080	47,860	178,668
No. Of culture-positive samples	10,602	11,236	8,637	30,475
% quarters culture-positive for:				
Staphylococcus aureus	19.1	19.1	16.6	18.4
Esculine-positieve cocci	15.5	14.8	18.0	16.0
Streptococcus dysgalactiae	2.8	2.4	1.8	2.4
Streptococcus agalactiae	0.3	0.3	0.3	0.3
non-aureus staphylococci	56.6	57.5	57.4	57.2
Corynebacterium bovis	0.9	1.0	0.6	0.8
Coliforms	1.0	0.6	0.9	0.8
Contaminated samples	2.9	3.4	4.0	3.4
Other	1.0	1.0	0.3	0.8

Bovine mastitis is typically treated with antibiotic preparations, which are administered intramammarily. A division between preparations for dry and lactating cows is consistent in the currently used drugs. In this article however, the possible application of phages and phage-derived endolysins in the future treatment of bovine mastitis is focussed. Current treatments have been described by Royster E. and Wagner S. (2015).

Bacteriophages and phage-derived endolysins

Bacteriophages, or shortly phages, are viruses that infect bacteria. Each phage can infect only one bacterial species (or even strain) because it recognizes an adhesion molecule on the cell wall of the bacterium. When the phage is attached, it infects its host by injecting its genetic material through the production of endolysins. These endolysins are enzymes, which are capable of digesting the bacterial cell wall. Subsequently, there are two possibilities: 1. the phage genome can pass a lysogenic cycle, in which the genome is incorporated into the bacterial DNA (prophage) and will replicate together with the bacterium, 2. the phage genome can start a lytic cycle, in which it uses the bacterial replication mechanisms to multiply its DNA (Figure 1). The genome then assembles with the viral proteins to create a new virion. Due to the massive production of virus particles, the bacterium eventually undergoes lysis. It is this feature which gives phages their ability to kill bacteria and therefore, they are proposed as an alternative to antibiotics. Phage-derived endolysins have also been suggested as a novel antimicrobial agent, because of their ability to lyse bacterial cell walls (Weber-Dąbrowska, 2016).

The One Health Initiative

The One Health Initiative is a movement to forge co-equal, all-inclusive partnerships between physicians, veterinarians, and other scientific-health related experts, recognizing that the health of people is connected to the health of animals and the environment (http://www.onehealthinitiative.com/). The overuse of antibiotics in dairy farming leads to resistant bacteria and antimicrobial residues in milk (Oliver et al.,

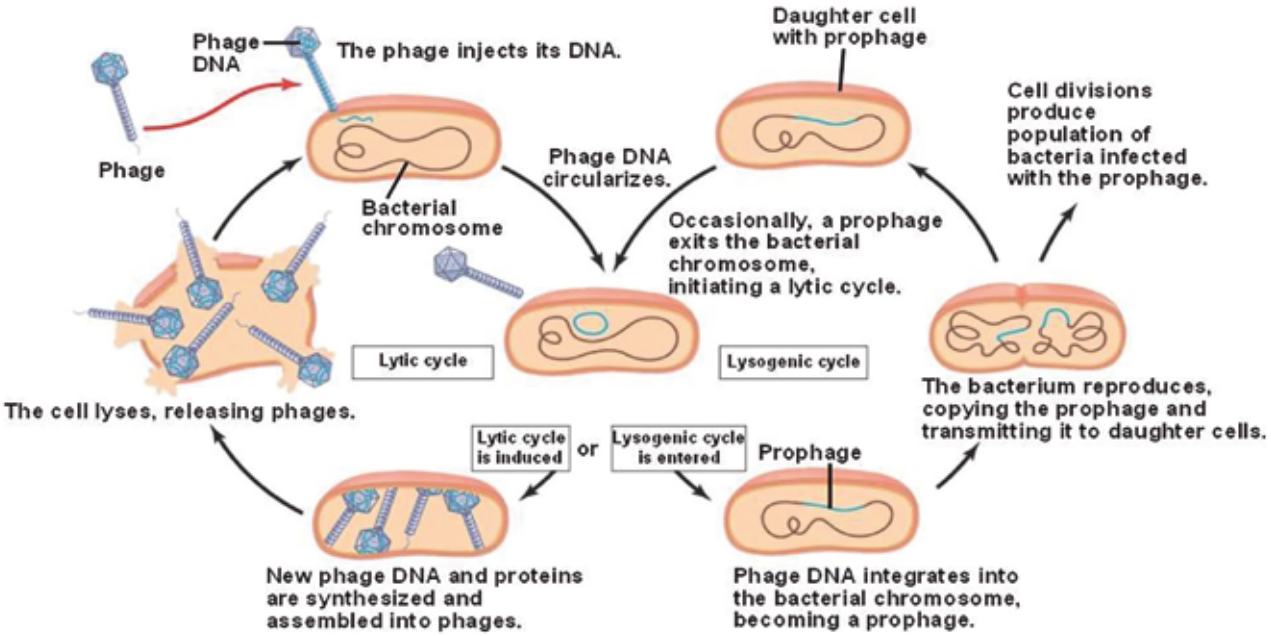

Figure 1. The lysogenic and lytic cycle of bacteriophages. The lysogenic and lytic cycle can pass into one another (https://kullabs.com/classes/subjects/units/lessons/notes/note-detail/8287).

2012). If zoonotic pathogens become resistant, they can no longer be treated with conventional antibiotics when causing disease in humans. Residues of antimicrobial agents in milk and/or meat cause an increase of resistance genes in the commensal intestinal flora. If this resistance is plasmid-mediated, the intestinal flora is considered a source of resistance to possible pathogens (Carattoli et al., 2013).

Methicillin resistant *S. aureus* (MRSA) is known to cause mastitis, but also skin, soft tissue, bone, joint and implant infections, pneumonia and septicemia in both humans and animals. Approximately 20-30% of the human population carries MRSA asymptomatically, resulting from nosocomial or community-acquired transmissions. An important transmission route of community-acquired MRSA is through contact with intensively antibiotic-treated livestock. Close contact with veal calves and pigs is a major risk factor for the transmission of livestock-associated MRSA to humans, because its prevalence is highest in these production animal sectors. In veterinary medicine, MRSA causes its most significant economic losses in the context of bovine mastitis (Holmes et al., 2011; Bosch et al., 2013).

A MINI-REVIEW OF THE BACTERIOPHAGE AND BOVINE MASTITIS LITERATURE

Since bacteriophages and phage-derived endolysins were first proposed as a new potential antimicrobial drug in the treatment of bovine mastitis, promising literature has been published. For this mini-review, the results from approximately ten international peer-reviewed papers are included. Although in vitro and in vivo tests with bacteriophages or phage-derived endolysins have only seldom been reported to treat bovine mastitis, this strategy has been intensively discussed over the past five years, which is indicative of the current and growing interest in bacteriophages and endolysins as alternatives for the traditional antimicrobial agents.

In vitro and in vivo evaluation of bacteriophages in the context of bovine mastitis

Since 2010, several lytic phages derived from mastitis-affected cows have been proposed as potential therapeutic drugs (Han et al., 2013; Kwiatek et al., 2012). The phages are able to effectively infect and kill bovine *S. aureus* including methicillin-resistant strains in vitro. Both bacteriophages are morphologically classified as Myoviridae and exhibit 1. rapid adsorption, i.e. the time needed for free phages to attach to the bacterium, 2. a short latent period, i.e. the time needed for lytic infection, and 3. a relatively small burst size, i.e. the average number of phages needed to cause bacterial lysis. Due to these three characteristics, the bacteriophages have been found eligible for therapeutic use. Another *S. aureus* bacteriophage (*SPW*-phage), also belonging to the Myoviridae family, has recently been isolated from lactating dairy cattle (Li et al., 2014). The three previously mentioned characteristics were likewise evaluated, whereby this *SPW*-phage may indeed have a potential use in future *S. aureus* mastitis therapy.

Recently, a cocktail consisting of four different bacteriophages has been proposed against *E. coli* through several in vitro tests (Porter et al., 2016). A 3.3 to 5.6 log reduction of growth in raw milk was observed when *E. coli* was co-incubated with this phage cocktail for twelve hours. Moreover, bacterial growth decreased with 1.6×10^3 CFU/mL (colony forming

units/mL) when tested against a mastitis-derived *E. coli* strain.

A phage K solution was administered intramammarily (1.25×10^{11} PFU/mL (plaque forming units/mL)) to 24 lactating Holstein cows with a persistent *S. aureus* infection (Gill et al., 2006). While none of the negative control saline-treated quarters were cured, *S. aureus* could not be isolated in only three of the eighteen phage-treated quarters samples, which were consecutively collected at 2 to 7 days, 9 to 14 days, 16 to 21 days, and 23 to 28 days after the end of local phage treatment. The success rate of this phage K therapy is therefore regarded as limited.

A selected phage cocktail for the treatment of *S. aureus*-associated (Newbould 305) bovine mastitis has recently been tested in vitro and in a mouse model (Breyne et al., 2017). First, different cultures were verified in the presence or absence of IgG and the phage cocktail. *Staphylococcus aureus* could not be isolated from any of these cultures to which the phage cocktail was added, whether or not in the presence of IgG. These promising in vitro results were partly confirmed in an in vivo pilot study using a mouse model for bovine mastitis. Mammary glands of lactating mice were inoculated with the same bovine mastitis isolate (N305) of *S. aureus*. Subsequently, a first group of mice was inoculated intramammarily with cefalonium, a first-generation cephalosporin (used as positive control). A second group of mice received no treatment, but only saline (negative control). A third group was injected with the phage cocktail. Breyne et al. (2017) reported that *S. aureus* could not be isolated from the mice which were injected with cefalonium. In contrast, *S. aureus* was still present in the phage-treated group although the number of colony forming units (CFU) was significantly lower when compared to the negative control group. In addition, a clinical score was given to the different mammary glands after infection. Both the cefalonium and phage-treated groups scored significantly better than the negative control group.

In vitro and in vivo evaluation of phage-derived endolysins in the context of bovine mastitis

In a study by Zhou et al. (2017), a recombinant, lytic enzyme (LysKΔamidase) was constructed out of a staphylococcal phage lysin, in which a broad lytic activity of LysKΔamidase was observed against 137 methicillin-resistant and methicillin-susceptible staphylococcal strains isolated from human hospital patients and cows with bovine mastitis. In addition, this lytic enzyme was also found to disrupt the normal structure of biofilms, which are protective structures produced by bacteria (i.e. *S. aureus*) that consist of DNA, proteins and carbohydrates. The in vitro potential of endolysins was demonstrated to combat MRSA and other antimicrobial-resistant, biofilm-forming staphylococcal strains associated with bovine mastitis.

In a study by Donovan et al. (2006), an endolysin derived from a *S. aureus* bacteriophage phi11 was purified and its effectiveness was demonstrated against *S. aureus* and non-aureus staphylococci. Its lytic activity was maintained at the pH (6.7) and Ca^{2+} concentration (3 mM) of milk, making phi11 endolysin a potential candidate as antimicrobial protein.

In a study by Fan et al. (2016), another recombinant endolysin (Trx-SA1) from a *S. aureus* bacteriophage was derived by cloning it into the pET-32a bacterial expression vector. Subsequently, an efficacy trial of its effectiveness against bovine mastitis was conducted. When Trx-SA1 was added to the host bacteria in early growth stages, a complete bacterial lysis was observed after nine hours. Preliminary results of a proof-of-concept therapeutic trial in cow udders showed that Trx-SA1 could effectively control mild clinical mastitis caused by *S. aureus*.

Phage-derived λSA2 and B30 endolysins were tested in vitro and in a mouse mastitis model against bovine streptococci (Schmelcher et al., 2015). Lytic activities were observed to be optimal at ionic strengths, pH, and Ca^{2+} concentration consistent with those in cow milk. Moreover, λSA2-endolysins were demonstrated to reduce in vitro the amount of *S. agalactiae*, *S. dysgalactiae* and *S. uberis* in cow milk by a log 2, 3.5, and 4 CFU/mL, respectively. Interestingly, although the B30 endolysin alone turned out to be less effective, a strong synergy appeared with the λSA2-endolysin. When further tested in a mouse model for bovine mastitis, a significant decrease in CFU was observed after intramammary inoculation of these endolysins in vivo.

CRITICAL COMMENTS AND FUTURE PROSPECTS

With only about ten promising in vitro and few in vivo studies reported till date, more research should be performed, especially in vivo, on the clinical applicability of either bacteriophages or their endolysins for the curative treatment of udder infections. Nevertheless, it may be summarized that most of these current experiments show the effectiveness -at least in vitro- of phage therapy against *S. aureus*. There is an urgent demand for alternative therapies against this Gram-positive mastitis pathogen, because staphylococcal intramammary infections are typically difficult to combat with classic antibiotics (Holmes et al., 2011). Persistence of this germ in the mammary gland results in chronic, subclinical mastitis. In addition, resistance against *S. aureus* is quickly established and persistent (Kadlec et al., 2012).

Recently, several researchers have claimed that promising candidate bacteriophages and endolysins should now be tested in vivo to evaluate the effects of this novel treatment strategy in mammary gland of rodents, but preferably in the target species i.e. the cow (Schmelcher et al., 2015; Porter et al., 2016). How-

ever, even if the envisaged phage therapy confirms to be promising in these follow-up studies, several practical hurdles will raise during its development. It is known that raw milk inhibits staphylococcal phage K proliferation due to the formation of bacterial clusters associated with fat globules and/or the presence of IgG (O'Flaherty et al., 2005; Tanji et al., 2015). In addition, Phage K has been reported to cause an increase in the SCC of healthy quarters (Gill et al., 2006). Most strains belonging to the group of Gram-positive mastitis pathogens are also known to cause biofilms, a property associated with their difficult eradication by traditional antimicrobial drugs. It should be remarked that biofilm-formation also occurs in some Gram-negative mastitis-causing bacteria such as *Klebsiella*. As mentioned above, phages and endolysins have been described to have the unique characteristic to digest these protective structures and are expected to be able to infect and lyse these problematic biofilm-forming, mastitis-causing bacteria (Latka et al., 2017; Zhou et al. 2017; Gerstmans et al. 2016; Gutiérrez et al., 2014).

Although some bacteria can be naturally resistant to bacteriophages due to the lack of required adhesion molecules on their bacterial cell wall, induced resistance has not yet been described. If resistance would nevertheless occur, supplementing the phage therapy with endolysins could be a useful tactic. Due to the broader spectrum of action, resistance selection among the pathogens against the used phage type may be expected to be counteracted. Still, it has been stated that phage therapy could give rise to antibiotic resistance as bacteriophage therapy is not capable of breaking down plasmids (Colavecchio et al., 2017). After lysis, these plasmids could easily be taken up by other bacteria. If they contain resistance genes, then this induced resistance may spread between the surviving bacteria (De Vliegher, 2017).

In addition, in the in vivo and some in vitro studies mentioned above, only a reduction of the bacterial count was observed, not a complete killing. This incomplete lysis of bacteria confirms the suboptimal effectiveness of current bacteriophages as well as phage-derived endolysins, even after enhancement of the latter by genetic engineering. Moreover, it should be noted that the delay time between experimental infection and administration of either bacteriophages or phage-derived endolysins in the used mouse mastitis model is very short, i.e. typically thirty minutes. Consequently, this set-up probably does not allow the mastitis pathogens to enter intracellularly nor to form biofilms. These experiments should be adapted to provide more relevant conditions for bovine mastitis as occurring on dairy farms.

A treatment consisting of only one bacteriophage can never provide a broad-spectrum effect due to the species specificity inherent to phages (Nilsson, 2014). In contrast, for endolysins, the spectrum can be enhanced through genetic engineering. Indeed, endolysins derived from bacteriophages that target Gram-positive bacteria feature a modular design, consisting of enzymatically active domains and cell wall binding domains. This modular architecture enables the creation of chimeric fusion proteins with novel enzymatic and antimicrobial properties. In two parallel recent studies by Becker et al. (2016) and Rodríguez-Rubio et al., (2016), an engineered staphylolytic and streptolytic fusion protein have been reported, respectively, with improved activities. Furthermore, the additional fusion to positively charged peptides significantly enhanced both the ability to kill intracellular mastitis pathogens and biofilm eradication, and reduced the incidence of resistant strain development against these engineered endolysins. It is therefore important to pursue research for more potent, novel bacteriophages and to genetically improve their derived endolysins, to obtain an effective and fast-acting engineered fusion endolysins with broad spectrum effect and a minimal induction of resistance.

If phage therapy consists of applying only one phage type, the treatment is targeted against one specific type of bacterium. For the application in veterinary practice however, it is more interesting to obtain a broader spectrum of activity. Bacteriological culture of milk samples from dairy cows is not done routinely and includes an additional cost for the dairy farmer. It may therefore be useful to develop cocktails consisting of different bacteriophages or to supplement the phage therapy with endolysins. The expansion of the working spectrum has already been tested and confirmed by two recent studies (Porter et al., 2016; Breyne et al., 2017). The main disadvantage of the classical endolysins remains their Gram-positive spectrum. Despite the lack of potential to kill Gram-negative germs, it should be emphasized that most of the problematic (intracellular and biofilm-forming) mastitis pathogens are indeed Gram-positive bacteria (Piepers et al., 2007). Moreover, as already demonstrated in human medicine, artificial modification of existing endolysins, so-called Artilysins® (Lysando AG, Germany), may provide a broader spectrum of activity. These Artilysins® specifically attack Gram-negative bacteria (Gerstmans et al., 2017). In addition, it has been demonstrated that artilysation of currently known endolysins also improves the lytic activity against Gram-positive germs (Rodríguez-Rubio et al., 2016).

From the point of view of the dairy practitioner, a broad spectrum, long-acting, intramammary preparation is needed if phage and/or endolysin therapy is envisaged to be used in the local treatment of bovine mastitis. The principal advantage gained from a novel phage/endolysin cocktail would be the reduced use of antibiotics, more specifically, those that are critical for human health. Residues in milk may then be avoided, implying that the dairy farmer does not have to discard milk derived from phage-treated animals as is now obligatory for antibiotic-treated animals. It can also be noted that the in vivo therapeutic concentration and the treatment interval are unknown fac-

tors that depend on the type of bacteriophages and/or endolysins used. A pharmacokinetic study was conducted, in which bacteriophage therapy was tested in subclinical *S. aureus* mastitis in lactating dairy cattle (Gill et al., 2006). Phage K persisted 36 hours within an infused quarter, but the effective concentration was significantly lower than predicted by simple dilution in produced milk. This implicates that more pharmacokinetic studies are also mandatory in the development of phage and/or endolysin-based mastitis drugs.

Finally, in contrast to human medicine, phage therapy has not yet been incorporated into the European legislation for veterinary medicinal products. As registered veterinary medication based either on bacteriophages or on endolysins does not yet exist, their current therapeutic use is considered as a magistral preparation. This implicates that their use is justified for the individual treatment of an animal to avoid unnecessary suffering, after prescription by a veterinarian and preparation by a pharmacist. From the point of view of veterinary practice, this is not desirable because the dairy farmer cannot start therapy immediately when detecting mastitis in one of his animals. Moreover, it does not make sense to apply individual mastitis therapy using a magistral preparation.

CONCLUSION

In conclusion, currently reported data clearly indicate that bacteriophages and phage-derived endolysins constitute a potential therapeutic alternative in the treatment of bovine mastitis. Nonetheless, these studies were predominantly carried out in a preclinical context. Further research should now evaluate whether this promising therapy is also active in the complex bovine mammary gland. If these results are confirmed, bacteriophages and phage-derived endolysins could indeed fulfil their promise by reducing 1. the excessive use of antibiotics in the dairy industry and 2. the common antimicrobial resistance in mastitis-causing pathogens. Furthermore, this alternative treatment would also become an important strategy to counteract the antimicrobial resistance in human pathogens as viewed from the 'One Health' perspective.

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