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Bacteriophages' action against mastitis-causing bactéria

Atuação de bacteriófagos em bactérias causadoras de mastite

Rendimiento de bacteriófagos en bacterias causantes de mastitis

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Abstract

Mastitis is a breast tissue disease with a high incidence in dairy cows and implications ranging from the health of the animals to the economy of the sector. Although antibiotic therapy is widely used, the search for new perspectives in the management and treatment of this disease is necessary. Although phage research preceded the discovery of antibiotics, with the appearance of antibiotics and their efficiency in treating infections, phage therapy fell into disuse. However, phage therapy has now re-emerged as an alternative for combating multidrug-resistant bacteria. The relationship between phages, bacteria, and the immune system is unique, generating a wide range of opportunities, some of which have yet to be studied. Thus, the objective of this review was to analyze the use of bacteriophages in the control of bovine mastitis and its association with other natural products today. Phages have been shown to exert effective antibacterial and anti-biofilm activity, and their interactions with other substances of natural origin could be a viable path for treating disease. Despite being little explored, phages are already being considered as an alternative for treatment against the main bacterial agents of mastitis. In this review, the safety and future pathways of phage therapy are addressed in order to indicate points where research still needs to progress and the main advantages and difficulties in this area.

Keywords: Antibacterial action; Anti-biofilm action; Natural products; Phage; Endolysin.

Resumo

A mastite é uma afecção do tecido mamário que tem alta incidência no rebanho leiteiro com implicações que vão desde a saúde até a economia do setor. O uso de antibioticoterapia é amplamente aplicado, no entanto, a busca por novas perspectivas no manejo e tratamento desta doença é necessária. As pesquisas com fagos são anteriores a descoberta dos antibióticos, mas com o aparecimento dessas substâncias e a sua eficiência no tratamento das infecções, essa terapia ficou por um tempo esquecida vindo à tona novamente como alternativa no combate as bactérias multirresistentes. A relação entre fagos, bactérias e sistema imune é única, gerando um amplo range de oportunidades, algumas delas ainda pouco estudadas. Dessa forma, o objetivo desta revisão foi analisar o uso de bacteriófagos no controle da mastite bovina e sua associação com outros produtos naturais na atualidade. Pesquisas tem demonstrado efetiva atividade antibacteriana e anti-biofilme e, interações de fagos com outras substâncias de origem natural aparecem como um caminho viável ainda pouco explorado para a doença, mas já vislumbrado frente aos principais agentes causadores da mastite. Questões sobre a segurança e caminhos para o futuro da terapia fágica também são abordados com intuito de indicar pontos onde a pesquisa ainda deve avançar e as principais vantagens e dificuldades nesta área.

Palavras-chave: Ação antibacteriana; Ação anti-biofilme; Produtos naturais; Fago; Endolisina.

Resumen

La mastitis es una enfermedad del tejido mamario que tiene una alta incidencia en el hato lechero con implicaciones que van desde la salud hasta la economía del sector. El uso de la terapia con antibióticos es de amplia aplicación, sin embargo, es necesaria la búsqueda de nuevas perspectivas en el manejo y tratamiento de esta enfermedad. La investigación en fagos es anterior al descubrimiento de los antibióticos, pero con la aparición de estas sustancias y su eficacia en el tratamiento de infecciones, esta terapia quedó en el olvido por un tiempo y resurgió como una alternativa para combatir las bacterias multirresistentes. La relación entre los fagos, las bacterias y el sistema inmunológico es única y genera una amplia gama de oportunidades, algunas de las cuales aún están poco estudiadas. Así, el objetivo de esta revisión fue analizar el uso de bacteriófagos en el control de la mastitis bovina y su asociación con otros productos naturales en la actualidad. Las investigaciones han mostrado una

actividad antibacteriana y antibiopelícula efectiva, y las interacciones de los fagos con otras sustancias de origen natural aparecen como una vía viable aún poco explorada para la enfermedad, pero que ya se vislumbra en relación a los principales agentes causantes de mastitis. También se abordan cuestiones sobre la seguridad y los caminos para el futuro de la terapia con fagos con el fin de señalar los puntos en los que la investigación aún debe avanzar y las principales ventajas y dificultades en esta área.

Palabras clave: Acción antibacteriana; Acción anti-biofilm; Productos naturales; Fagos; Endolisina.

1. Introduction

Over the years, the effects of mastitis in dairy cows have had a negative impact on the agricultural economy and animal welfare. The epidemiology of mastitis has changed, changing as a result its management and treatment (Ruegg & Petersson, 2018). If previously strategies mainly focused on the control of *Streptococcus agalactiae* and *Staphylococcus aureus*, today the research is more focused on opportunistic pathogens from environmental sources and the rational use of antimicrobials (Ruegg, 2017).

Currently, antibiotic administration is the most common method for treating bovine mastitis. However, this type of strategy has several disadvantages, including a low cure rate, increased occurrence of resistance, and presence of residues in the milk and meat of treated cows. In most cases, the selection of multidrug-resistant strains is due to the indiscriminate use of antimicrobials, underdosing, or the interruption of prescribed treatments, making the search for effective alternatives to combat infections important (Silva et al., 2013).

Several alternatives to antibiotics have been investigated by various groups of researchers in order to find an efficient approach for the management of mastitis. Vaccines, nanoparticles, cytokines, bacteriophages, isolated compounds of plants, animals, and bacteria are some examples of valid substitutes for antibiotics. Although bacteriophages are a promising alternative both to mastitis-causing bacterial strains and to biofilm formation, further research needs to be conducted in order to harness their full potential in the control and treatment of disease (Gomes & Henriques, 2016).

Bacteriophage therapy has been used for a long time, with bacteriophages being considered potential therapeutic agents since 1931, when its lytic activity in specific bacteria was discovered (D´Herelle, 1931). Prior to the discovery and widespread use of antibiotics, bacterial infections were prevented and/or treated by the administration of bacteriophages.

However, over time, bacteriophage therapy became a secondary issue, used only for research purposes. Now, with the emergence of multidrug-resistant strains in recent years, interest in the use of bacteriophage therapy as a method to combat various pathogens has re-emerged (Patel et al., 2015).

Bacteriophage therapy is performed through the targeted application of bacteriophages (or phages). When the phages encounter specific pathogenic bacteria, they can infect and destroy them (Burrowes et al., 2011). In this context, there are two forms of bacteriophage replication: the lysogenic cycle, where the phages integrate their DNA with the genetic material of the host, and the lytic cycle, where the host is destroyed immediately after virion replication (Drulis-Kawa et al., 2012).

Furthermore, phage components, the so-called lysins, are known to exert bactericidal activity in the treatment of infections caused by bacteria. Lysins are phage enzymes that can digest the walls of bacterial cells and can have lysozyme activity (muramidase), with endopeptidase or glucosaminidase, in this case, having specific action against Gram-positive bacteria (Waldor et al., 2005; Fischetti, 2016).

The aim of this review was to address the use of bacteriophage therapy in the current scenario, with an emphasis on bacteria that cause bovine mastitis, the antibacterial activity of the association of phages and/or their products with natural antimicrobials, as well as the future prospects for this area in the field.

2. Methodology

A qualitative literature review was conducted (Pereira et al., 2018), where the search was based on articles available on the Google Scholar online platforms and on the PubMed database, between December 2017 and March 2019.

For this purpose, the key words were used: "phage", "mastitis", "antimicrobial", "bacteria", "bacteriophages" and "endolysin" in both Portuguese and English.

As an inclusion criterion, articles related to bacteriophages used in combating mastitis were selected studies conducted *in vitro* and *in vivo*. Articles that were not available in full were excluded.

3. Development

3.1 Mastitis

Mastitis is an inflammatory response resulting from a breast tissue disease that can occur in different animal species. It is the most frequent pathology of dairy cattle, with important economic impacts associated with the production of milk, such as reduced production, changes in composition and quality, and the potential fatality of milk-producing animals. Control measures and the development of effective therapeutic alternatives for mastitis are a continuous and urgent field of research (Gomes & Henriques, 2016).

This disease may have an infectious or non-infectious etiology. Infectious pathogenesis is the most prevalent etiology due to the presence of bacteria, viruses, mycoplasma, yeasts, and algae (Janosi et al., 2001; Spanamberg et al., 2009; Cheng et al., 2010; Nicholas et al., 2016; Moreira et al., 2019).

Even though it is a multifactorial disease, bacteria are the main cause of mastitis. Among the numerous bacteria causing mastitis are staphylococci, streptococci, and enterobacteria. Infections can be clinical, when physiological symptoms and changes occurs in the milk and breasts, and subclinical, when there are no clinical signs. Depending on the primary reservoir and mode of transmission of this pathology, the pathology can be contagious or environmental. In the case of a contagious pathology, where the main source is the mammary glands of the infected cows, *Staphylococcus aureus* and *Streptococcus agalactiae* are the most prevalent bacteria. The main source of environmental pathogens related to the cow's habitat are *Streptococcus uberis*, *Escherichia coli*, and *Klebsiella* spp. (Bogni et al., 2017; Ashraf & Imran, 2018).

The diagnosis can be divided into two stages. The disease is detected in the first stage. For this purpose, somatic cell counts (SCC), California Mastitis Test (CMT), and Surf field mastitis tests are commonly used. In the second stage, the causative agent is identified using microbial culture, polymerase chain reaction (PCR), and tests with proteins or protein-antibody association (Rossi et al., 2018).

As the most prevalent etiological agents, treatment with antimicrobials has been the most widely used alternative and the main reason for the use of antibiotics in adult cows. However, it is necessary to evaluate the need for and benefits of this practice in mild cases, using specific drugs for the causative pathogens and assessing the host immune system response (Ruegg, 2018). In this context, in addition to bacteria, the formation of bacterial

biofilm as a virulence factor with a direct impact on breast health stands out. Biofilms favor the permanence of bacteria in the intramammary environment, hindering antimicrobial access and decreasing the susceptibility of bacteria to treatment (Tremblay et al., 2014; Srednik et al., 2017).

Due to the importance of mastitis in milk production, the search for new treatments for its control is essential since the continuous use of antibiotics has led to the appearance of resistant microorganisms and has a negative impact on the health of animals.

3.2 Bacteriophages

Bacteriophages, or viruses that infect bacteria, were discovered around 1920. Despite their therapeutic potential, they have long been neglected by science. Recently, phages have aroused the interest of public health as an interesting option against the emergence of bacteria resistant to multiple antimicrobial agents (Chan et al., 2013).

Bacteriophage therapy involves the use of phages or their products as bioagents for the treatment or prophylaxis of bacterial infectious diseases. This type of therapy has several advantages compared to antibiotic therapy, including: efficacy against multi-resistant pathogenic bacteria, due to its different mode of action than antibiotics; high specificity by target bacteria; quick response to the appearance of resistant mutants, since phages can mutate; lower cost to develop a new drug; and, finally, their lack of adverse effects, whereby phages and their products do not affect eukaryotic cells (Matsuzaki et al., 2005).

Bacteriophages are classified in 13 families according to their morphology, type of nucleic acid, and presence or absence of an envelope or lipid. About 96% of the reported phages are “tailed phages” composed of an icosahedral head and tail, and all have double-stranded DNA as their genome. Phages can also be classified into two groups according to their life cycle: lytic phages, when their proliferation leads to the destruction of bacteria (lytic cycle) and lysogenic phages. In the lysogenic cycle, the genetic material of the phage integrates with that of bacteria and multiplies cooperatively without destroying it, i.e., these bacteria are resistant to infection by phages. For this reason, lytic phages are more appropriate than lysogenic phages for use in therapy (Yamaguchi et al., 2000; Matsuzaki et al., 2005).

For the lysis of bacteria, phages use a mechanism that begins with bacterial infection, usually starting in a host cell protein or surface sugar. After the entry of the phage DNA into the bacterial cytoplasm, it is replicated and synthesized, and the several copies of DNA are then collected in the capsule, which is rebuilt during the late stage of the phage infection. he

downstream particles are completed by the coupling of a tail filled with DNA. Finally, the progeny phages are released by the coordinated action of two proteins, holin and endolysin, which create pores in the membrane, allowing the release of the new phages to the neighboring bacterial cells, culminating in the lysis of the bacterial population. Fortunately, most phages are only able to adsorb specific strains, and target bacteria without affecting the normal microbiota (Young, 1992; Wang et al., 2000).

Phages can be found in almost all environments, such as soil, water, the ocean bed, sewers, and the gastrointestinal tract of animals, with a high specificity for their hosts (Gorski et al., 2003; Othman et al., 2008). Thus, several bacteriophages are reported to inhibit the pathogens that cause mastitis.

Bai et al. (2016) isolated phages of *Streptococcus agalactiae* from milk and environmental samples and from the induction of lysogens with mitomycin C. The four phages obtained were found to promote the lysis of 12 to 42 isolates of bovine *S. agalactiae*. In another study with phages isolated from the milk of cows with mastitis, phages also showed specific activity against bovine *S. agalactiae* and were characterized as belonging to the Siphoviridae family; genome analysis revealed a high similarity with *S. pyogenes* indicating lateral gene transfer between these phages (Bai et al., 2013).

Kwiatek et al. (2012) isolated milk bacteriophages from a cow with mastitis for one year. During this period, they found about 25 lytic phages with specific activity against strains of *Staphylococcus aureus.* Moreover, they found that one of the phages, with a more detailed description, was effective against staphylococcal pathogens, associated with bovine mastitis, and antibiotic-resistant *S. aureus*, associated with human infections. Subsequently, other studies also investigated the efficiency of phages against *S. aureus*, demonstrating their potential in the treatment and prophylaxis of mastitis associated with staphylococci, including those resistant to methicillin (Li & Zhang, 2014; Aldoori et al., 2015; Saglam et al., 2017).

Amiri-Fahliyani et al. (2018) isolated and identified lytic bacteriophages from public effluents and observed a 97% reduction in the growth of *Klebsiella oxytoca* isolated from milk samples after 12 hours of incubation, demonstrating the potential of these phages in the biocontrol of bovine mastitis. Ribeiro et al. (2018), who also investigated enterobacteria, observed that the phage EcoM017, isolated from sewage system samples, reduced bacterial growth and biofilm formation by *Escherichia coli* in 90.0% and 87.5% polystyrene plates, respectively. Bacteriophage therapy combining several phages (cocktail) also proved to be a potential alternative in the prevention of *E. coli* mastitis by reducing their adhesion and intracellular survival in breast tissue. In the strains where there was total inhibition by phage

cocktail, there was no statistically significant difference compared to 10 µg/mL of ceftiofur over a period of 12 hours (Porter et al., 2016).

Despite the lytic efficiency of bacteriophages, host bacteria have several mechanisms of antiviral resistance. However, this selective pressure leads phages to develop multiple tactics to avoid, bypass, or subvert these mechanisms in order to establish themselves in most environments (Klaenhammer & Fitzgerald, 1994). Conversely, some studies report that bacteriophages may add virulence factors to their host and increase bacterial virulence or/and exert selective pressure, allowing for the proliferation of strains with reduced virulence due to the presence of receptors on the surface of the bacterial cell (León & Bastías, 2015).

Phage therapy requires further study to determine how bacteria acquire resistance. Mutations in bacterial membrane proteins, chromosomal mutations, antiviral mechanisms, reduction in the phage adsorption system, and gene regulation of the restriction/modification system provide some indications of how this resistance occurs (Duerkop et al., 2016; Zago et al., 2017).

3.3 Bacteriophages associated with natural antibacterial substances

Table 1 provides a list of studies that have tested the association between natural substances and bacteriophages. Among the substances presented are essential oils, essential oil components, nisin, and honey.

Table 1 - Survey of studies that addressed the association between bacteriophages or phage products and natural antimicrobials.

Bacteriophage/ Origin	**Target**	**Interaction**	**Reference**
Phage PAØ (Wastewater)	*Pseudomonas aeruginosa* – biofilm	*Thymus vulgaris* essential oil	Said et al. (2017)
Phage K (ATCC 19685-B1)	*Staphylococcus aureus*	Essential oil compounds (alpha-pinene, 3-carene, (+)-limonene, (1S)-(-)-β-pinene)	Ghosh et al. (2016)
Bacteriophage cocktail, BEC8 (previous studies)	Enterohemorrhagic *Escherichia coli* (EHEC) O157: H7	Trans-cinnamaldehyde	Viazis et al. (2011)

Phage SA97/Endolysin LysSA97 (Skin)	*S. aureus*	Carvacrol	Chang et al. (2017)
Phage LH7 (Human sewage effluent)	*Listeria monocytogenes*	Nisin	Dykes & Moorhead (2002)
Phage mixtures LM-103 and LMP-102 (Commercially obtained)	*L. monocytogenes*	Nisin	Leverentz et al. (2003)
Phage phi-SauS-IPLA88/endolysin LysH5 (Dairy products)	*S. aureus*	Nisin	García et al. (2010)
Phage Fmb-p1 (Sewer)	*Salmonella* Typhimurium	Nisin and potassium sorbate	Wang et al. (2017)
Phage P100 (Obtained commercially)	*L. monocytogenes*	Nisin and sodium lactate	Figueiredo & Almeida (2017)
Phage P100/Endolysin PlyP100 (Not informed)	*L. monocytogenes*	Nisin	Ibarra-Sánchez et al. (2018)
PS1 phage (Sewer)	*E. coli, S. aureus, MRSA* and *MDR P. aeruginosa*	Honey, polyvinyl alcohol, chitosan nanofibers, bee poison, propolis	Sarhan & Azzazy (2017)
EC3a (Sewer)	*E. coli* – biofilm	Honey	Oliveira et al. (2018)
PAO1-D (Obtained commercially)	*P. aeruginosa*	Honey	Oliveira et al. (2018)
EC3a phage (Sewer)	*E. coli* – biofilm	Honey	Oliveira et al. (2017)

Source: The authors.

The analysis of these studies showed that the interaction of bacteriophages with products of natural origin is focused on the area of food and that factors such as temperature, acidity, lipid content, pH and ions affect antibacterial activity. It has already been reported

that the presence of fat, carbohydrates, proteins and salt, as well as the pH, may influence the antibacterial efficacy of essential oils (Holley & Patel, 2005).

Essential oils are secondary metabolism products of plants that play an important role as antiviral, antifungal, insecticidal, and antibacterial agents (Bakkali et al., 2008). While analyzing the potential of thyme essential oil in wastewater treatment, Said et al. (2017) found that lithic phages reduced bacterial density and, consequently, limited intracellular communication (*sensing quorum*), inhibiting biofilm formation and the expression of other virulence factors. Thyme essential oil showed anti-biofilm activity by inhibiting the mobility of planktonic cells and fixation on an inert surface, resulting in the deterioration of the biofilm structure (Said et al., 2017).

The effect of alpha-pinene, 3-carene, limonene, and β-pinene on the control of *S. aureus* was tested. Alpha-pinene exhibited the best activity in the diffusion disc test, however, when the compounds were tested for growth inhibition, all showed activity. The combination of phages and natural compounds had an inhibitory activity against *S. aureus*, especially with the combination between alpha-pinene and phage K. However, phages only showed antimicrobial activity at 37°C (Ghosh et al., 2016).

Bacteriophages and their endolysins used in natural or recombinant form show efficacy in the control of several forms of infections. Endolysin LysSA97 has lytic activity against several strains of *Staphylococcus* and has exhibited significant synergistic effects when combined with carvacrol. While LysSA97 (376 nM) and carvacrol (3.33 mM) showed a reduction of 0.8 ± 0.2 and 1.0 ± 0.0 log CFU / mL in *Staphylococcus aureus* cells, respectively, the cocktail containing both at the same concentrations applied alone in a bacterial culture showed a bacterial decrease of 4.5 ± 0.2 log CFU/mL. When LysSA97 and carvacrol were used in combination in food, synergistic activity was influenced by total lipid content, and skim milk bacteria were inactivated more dramatically than whole milk bacteria. The synergistic activity between the endolysins and essential oil-derived compounds, which act at distinct target sites, i.e., the bacterial peptidoglycan layer and the cytoplasmic membrane, should be explored in further studies with other Gram-positive bacteria (Chang et al., 2017).

The individual and combined effect of a bacteriophage cocktail (BEC8) and trans-cinnamaldehyde was assessed on strains of enterohaemorrhagic *Escherichia coli* (EHEC). The treatment was applied to vegetables and assessed at different temperatures. Isolated bacterial inhibition occurred after 24 hours at 23 and 37°C. However, when the two treatments were combined, growth was not detected at all temperatures after 10 minutes,

which indicates that the combination was highly effective in inactivating EHEC in both green leaves. This combination could be used as an antimicrobial to inactivate EHEC (Viazis et al., 2011).

Nisin is an antibacterial peptide produced by the bacteria *Lactococcus lactis* and is widely used in food. The effect of nisin and phage LH7 was assessed, alone and in combination, on the growth and survival of *Listeria monocytogenes*. Nisin alone reduced or inhibited the growth of bacteria. Conversely, the listeria phage LH7 alone had no effect on the broth under the conditions studied. Remarkably, the mixture of nisin and listeria phage substantially reduced the number of cells, showing an increased effect (Dykes & Moorhead, 2002).

Treatments with phages combined with nisin were assessed by Leverentz et al. (2003). When combined with nisin, phages reduced *L. monocytogenes* populations by up to 5.7 log units in melon slices and up to 2.3 log units in apple slices compared to the control. Phage titer was stable in melon slices but decreased rapidly in apple slices, indicating that, in fruits with lower pH, the addition of nisin complements phage activity in controlling *L. monocytogenes* (Leverentz et al., 2003). Subsequently, the efficacy of bacteriophage P100, nisin, and sodium lactate in inhibiting *L. monocytogenes* was evaluated. Phage P100, when used as an antimicrobial treatment, proved to be very effective in inactivating and inhibiting the growth of *L. monocytogenes* in sliced pork ham. Under refrigerated conditions, the combination of P100 bacteriophage and nisin bacteriocin was more effective as a bio-conserver against *L. monocytogenes* than nisin alone (Figueiredo & Almeida, 2017). More recently, the synergistic potential between endolysin, PlyP100, and nisin has also been assessed for anti-Listeria activity. The combination resulted in the bacteria in fresh cheese being undetectable after 4 weeks under refrigerated storage, with a high stability and dose-dependent antilisterial activity (Ibarra-Sánchez et al., 2018).

The ionic requirements for optimal lytic activity of LysH5, the endolysin coded by staphylococcal bacteriophage phi-SauS-IPLA88, were assessed. LysH5 activity was inhibited by Mn^{2+} and Zn^{2+} and increased by Ca^{2+}, Mg^{2+}, and NaCl. The combination of LysH5 and nisin generated a strong synergistic effect, with the minimum inhibitory concentrations of nisin and LysH5 being reduced 64- and 16-fold, respectively. In challenge assays in pasteurized milk contaminated with *S. aureus*, the complete elimination of the pathogen was achieved only by the combined activity of both antimicrobials after 6 h of incubation (García et al., 2010).

The effect of the combinations of bacteriophage, nisin, and potassium sorbate on *Salmonella* was assessed. The combined treatment significantly reduced the total viable counts of refrigerated pork during the storage period, extending the shelf life of the refrigerated food up to 14 days. However, no significant synergistic activity in reducing the *Salmonella* count was observed among the three antimicrobials (Wang et al., 2017).

Sarhan & Azzazy (2017) have proposed the development of natural wounds dressing using antibacterial substances such as honey, polyvinyl alcohol, chitosan nanofibers loaded with bee poison, propolis, and/or bacteriophages against Gram-positive and Gram-negative bacteria. Among the different formulations of nanofibers, honey, and polyvinyl alcohol, chitosan-bee poison/bacteriophage exhibited the most potent antibacterial activity against all the bacterial strains tested. *In vivo* tests showed improved wound healing results and cytotoxicity tests showed improved biocompatibility.

Chronic wounds are a hostile environment in which damaged tissues are susceptible to bacterial proliferation, resulting in the colonization of the wound by bacteria, making it a fertile environment for the formation of biofilms. The control of *E. coli* biofilm with phages and honey alone and in combination was assessed by Oliveira et al. (2017). Two Portuguese types of honey with different floral origins and a specific *E. coli* phage (EC3a) were tested against 24 h and 48 h biofilms. Honey activity at 25% (w/v) was increased by the addition of phages and the resulting antimicrobial effect was similar to the 50% (w/v) of honey applied to biofilms. Honey resulted in small changes in the membrane and the subsequent discharge of cytoplasmic content, while the phage destroyed all the cells, leaving only structures similar to vesicles and debris. The microbial resistance to honey has never been reported and its antiviral effect is advantageous compared to phage therapy alone, reducing the emergence of phage insensitive mutants (Oliveira et al., 2017).

The effects of honey on wounds and biofilm formation were also assessed in combination with *E. coli* and *P. aeruginosa* phages. The combined formulation of honey and phages resulted in an increased efficacy, possibly due to the honey's ability to damage the bacterial cell membrane and penetrate the biofilm matrix, promoting and improving subsequent phage infection (Oliveira et al., 2018).

Up to the time of this review, no studies showing these associations in the treatment or prophylaxis of mastitis were found. As such, this represents an interesting line of research for future studies to explore, since the results are often advantageous compared to their isolated use. Moreover, food is a more complex medium than the culture medium used in other

studies, which reinforces the antibacterial and anti-biofilm capacity of the combined use of phages and natural products.

3.4 Bacteriophages today: possibilities and limitations

The advantages of phage therapy include specificity, unlimited supply, evolutionary capacity, and safety, particularly when compared to conventional therapy with antimicrobial agents. Other advantages include the possibility of combining phages with antibacterial compounds to increase their efficacy or combat drug-resistant bacteria (Torres-Barceló, 2018).

While information regarding the adverse effects of antibiotic therapy are available, information about the safety of phages is scarce and recent. The oral administration of phage therapy, for example, could include risks in the translocation of phages to the intestinal epithelium, and there is still controversy about a possible adverse reaction due to the development of a potential immune response to phages. Since results in animal models are still limited, it is likely that phages in different species elicit different responses (Lin et al., 2017). It has been shown that both innate and adaptive immunity are involved in the elimination of phages from the body. Immune reactions are related to administration method and may vary depending on the type of bacterial virus. For this reason, it is fundamental for the immune response of each phage to be tested (Cisek et al., 2017). Another important safety concern is the horizontal transmission of genes among phage-mediated bacteria. Bacteria are known to acquire antibiotic resistance due to new mutations that may occur spontaneously via horizontal transfer between cells of the one or several species. Phages are one of the vehicles of this genetic exchange. Mobilization of bacterial resistance genes (chromosomes or plasmids) with the incorporation of host genes may occur by mistake, a mechanism called transduction. However, this phenomenon occurs due to a series of errors and at extremely low rates (Torres-Barceló, 2018).

Phages are naturally present in the human body. Despite their close co-existence, there is currently no evidence that phages infect host cells and are therefore considered biologically safe for use in mammalian organisms. Much has been learned about the genetic diversity of phages since their discovery, and this has recently led to their re-emergence as novel therapeutic strategies for the prevention, diagnosis, and treatment of diseases (Domingo-Calap et al., 2016).

The use of phages as biological products in the control pathogens, among others, requires adjustments to their structure to confirm their *in vitro* activity and allow for their use in clinical medicine. Moreover, techniques that facilitate the use of these agents in infection, such as microencapsulation in liposomes and nanoencapsulation, may optimize their antibacterial activity and prolong their viability (Cinquerrui et al., 2018; El Haddad et al., 2018).

There is no doubt that phage therapy is an attractive solution to combat the increasing rates of resistance to antibiotics. However, there is still a lack of scientific evidence to prove its safety and make its therapeutic use feasible.

4. Conclusions

The advantages and possibilities of the use of phages in different infections are undeniable, as well as the capacity of these agents to combat bacterial resistance, which is currently on the rise. Significant progress has been made since the discovery of phages, however, due to their great genetic diversity, both positive and negative generalizations will require further research in order to improve our understanding of their activity.

New research should focus on assessing the body's response to phage therapy based on studies with animal and genetic models. In this way, data on the safety of bacteriophages can be generated, enabling the safe use of bacteriophages.

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