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Antimicrobial Usage for the Management of Mastitis in the USA: Impacts on Antimicrobial Resistance and Potential Alternative Approaches

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Abstract

Mastitis is the most frequently diagnosed disease of dairy cattle responsible for the reduction in milk quantity and quality and major economic losses. Dairy farmers use antibiotics for the prevention and treatment of mastitis. Frequent antimicrobial usage (AMU) undeniably increased antimicrobial resistance (AMR) in bacteria from dairy farms. Antimicrobial-resistant bacteria (ARB) from dairy farms can spread to humans directly through contact with carrier animals or indirectly through the consumption of raw milk or undercooked meat from culled dairy cows. Indirect spread from dairy farms to humans can also be through dairy manure fertilized vegetables or run-off waters from dairy farms to the environment. The most frequently used antibiotics in dairy farms are medically important and high-priority classes of antibiotics. As a result, dairy farms are considered one of the potential reservoirs of ARB and antimicrobial resistance genes (ARGs). To mitigate the rise of ARB in dairy farms, reducing AMU by adopting one or more of alternative disease control methods such as good herd health management, selective dry-cow therapy, probiotics, and others is critically important. This chapter is a concise review of the effects of antimicrobials usage to control mastitis in dairy cattle farms and its potential impact on human health.

Keywords: antimicrobial resistance, bovine mastitis, intramammary infection, antimicrobials, mastitis, bovine, dairy cattle

1. Introduction

Since the discovery of antibiotics, microbes have continued to uncover new ways to survive and thrive in the presence of antibiotics [1]. In recent years, the emergence and spread of antimicrobial resistance (AMR) worldwide have increased at an alarming rate [2]. AMR has been detected almost as quickly as newer antibiotics were developed and used [3]. Mastitis, an inflammation of the mammary gland, mainly caused by bacteria, is the most frequent reason for antibiotic use in dairy cattle. Mastitis causes significant economic losses to the dairy industry directly through a reduction in milk yield and quality and indirectly by

increasing the cost of its management [4]. The indirect cost includes heavy use of antibiotics, which contributes to the occurrence of AMR. In addition, some AMR mastitis pathogens can pose public health threats through the consumption of milk and milk products [5].

The rise in AMR occurs mainly due to the imprudent use of antimicrobials which increasingly undermines the sustainable use of antimicrobials. Studies reported that the amount of antimicrobials used (AMU) to treat clinical and subclinical mastitis accounts for nearly twice the quantity of antibiotics used for all other health problems in dairy cows [6, 7]. The United States Department of Agriculture (USDA), National Animal Health Monitoring System (NAHMS) survey of 2013 reported a 24.8% clinical mastitis in all cows involved [8]. The majority (87.3%) of the cows with clinical mastitis were given antibiotic treatment. Nearly three-fourths of the farms (73%) used cephalosporins, 34.4% used first-generation cephalosporins (FGCs), and 38.6% of them used third-generation cephalosporins (TGCs). The NAHMS also reported that out of 21.4% of cows treated for mastitis, the primary treatments given were TGCs (50.7%), lincosamide (24.7%), and FGCs (15.2%). The same report showed that there are seven approved intramammary (IMM) antimicrobial products in the United States but no systemic products for treating clinical mastitis except limited extra-label usage of some products. While one approved IMM antimicrobial product is classified as a lincosamide (pirlimycin) and six IMM antimicrobial products are classified as beta-lactams. The beta-lactams that are used as IMM products include FGCs (cephapirin) and TGCs (ceftiofur), aminopenicillins (amoxicillin and hetacillin), penicillin G, and penicillinase-resistant penicillins (cloxacillin) [9].

Another most common AMU is for dry cow therapy (DCT). Dairy cows are susceptible to intramammary infection (IMI) during the early and late dry period [10–12]. To prevent IMI during the dry period, the National Mastitis Council (NMC) recommends IMM of long-acting IMM antibiotics, also known as dry cow therapy (DCT), as a prophylactic control measure for the management of mastitis. The DCT is routinely used at the end of lactation to cure existing subclinical mastitis so that it will not be carried over to the next lactation and to prevent new infections during the dry period [13]. According to the 2014 NAHMS of dairy herds study, 93% of cows in the U.S. received DCT. Among the operations that used DCT, more than half (58.1%) of them used cephapirin benzathine followed by ceftiofur 27.9%, and procaine penicillin G and dihydrostreptomycin sulfate combination (24.5%). A recent study also reported that beta-lactam antibiotics such as cephapirin, ceftiofur, and penicillin are the top three antibiotics used for DCT on U.S. farms [14].

Although total AMU in the U.S. cattle production, including dairy farming, is lower than that of other food animals such as pigs, most of the antibiotics used are important to treat infections in humans. Of all antibiotics classes approved for use in U.S. dairy cattle, at least eight are medically important (**Table 1**). These antibiotics used in both dairy and human medicine include aminoglycosides, cephalosporins, fluoroquinolones, lincosamides, macrolides, penicillins, sulfonamides, and tetracyclines [19]. These antibiotics are also used to treat other diseases of dairy cattle, such as respiratory and reproductive diseases and foot infections [7]. Some of these antibiotics are categorized by the World Health Organization (WHO) as critically important ones. Quinolones (enrofloxacin and danofloxacin) and extended-spectrum beta-lactams such as third-generation cephalosporins, which are heavily used in U.S. dairy farms for the treatment of mastitis, are considered as “highest priority critically important” classes of antibiotics [19]. The use of these antibiotics in dairy farms can exert selection pressure that may lead

Antimicrobial class	Antimicrobial agent	Indications	Importance for human medicine	References
Cephalosporins	Ceftiofur Cephalothin Cephapirin	DCT, BRD, mastitis, and metritis	Critically important	[8, 15–18]
Fluoroquinolones	Danofloxacin, Enrofloxacin	BRD	Critically important	[16, 17]
Aminoglycosides	Amikacin, Clindamycin Gentamicin, Apramycin Kanamycin, Neomycin	DCT, feet infections	Critically important	[16–18]
Penicillin	Amoxicillin/Clavulanic acid Ampicillin Penicillin Cloxacillin	DCT, mastitis, metritis, and other local infections	Important	[14, 16, 17]
Sulfonamide	Sulfamethoxazole, Sulfadimethoxine, Sulfisoxazole, Trimethoprim/ Sulfamethoxazole, Sulfamethoxazole/ Sulfisoxazole	Calf diarrhea	Highly important	[16–18]
Macrolides	Erythromycin, Tilmicosin Tulathromycin, Tylosin Tulathromycin and Gamithromycin, Tilmicosin,	BRD, foot rot, and metritis	Critically important	[16–18]
Amphenicols	Florfenicol	BRD	Highly important	[16–18]
Tetracyclines	Chlortetracycline Oxytetracycline Tetracyclines	BRD, metritis, bacterial scours, and eye infection	Highly important	[16–18]
Lincosamide	Pirlimycin, Lincomycin	DCT, Mastitis, BRD, and feet infections	Highly important	[16, 17]

BRD: Bovine respiratory disease; and DCT: Dry cow therapy.

Table 1.
Major antimicrobial classes used in the U.S. dairy cattle and their medical importance according to WHO classification.

to the emergence and spread of AMR pathogenic, opportunistic, and commensal bacteria from dairy farms to humans. Transmission may occur through direct contact between cattle and humans or indirectly through the food chain (milk and meat). The horizontal transfer of resistance genes may occur from bacteria of dairy cattle origin to human commensal or pathogenic bacteria in the gut [20]. Thus, the development of AMR that arises from the AMU in dairy farms could seriously impact the management of infectious diseases in the human population using antibiotics [21].

2. Antibiotics use in dairy farms and their implication to human health

There is considerable evidence that supports the view that the development of AMR in food animals such as dairy cattle is linked to the emergence of AMR bacteria that infected humans [22–24]. As one of the major consumers of antibiotics, dairy cattle production farms are likely to contribute to the rise of AMR bacteria in humans. Studies from outside of the U.S. [25–27] showed direct transmission of AMR from dairy cattle to humans through contact on farms or through indirect routes. The most common route of the spread of AMR bacteria and their resistome from dairy cattle farms to humans could be indirect through the food chain. In the U.S., the CC97 methicillin-resistant *S. aureus* (MRSA), the human pandemic clone, which claims the lives of thousands of people every year, was suggested to be originated from the dairy farm [28].

According to the U.S. centers for disease control and prevention (CDC), about 22% of infections (440,000 cases) caused by antibiotic-resistant pathogens in the U.S. are from a food of animal origin, such as milk [29, 30]. Most of these bacteria could be normal microflora that colonizes the gastrointestinal tract of the animal [24], but they could be pathogenic for humans or may also be commensal but may transfer resistance genes to other foodborne pathogens in the human gastrointestinal tract [23]. Additional routes of transmission of AMR bacteria and their resistome to humans is through contaminated dairy farm environments and other wastes entering the environment [31].

Multiple studies have linked the outbreak of foodborne AMR pathogens to animal and their products, including milk [25–27, 32]. Despite these reports, it should be noted that direct proof for AMR transmission through foods of animal origin or directly through contact is limited, especially from dairy cattle [33]. In the U.S., strong evidence for transmission of AMR isolates between dairy cattle and humans is not yet proven. Previous reviews that attempted to discern any linkage between AMU in dairy cows and AMR development in veterinary and human pathogens showed the absence of scientific proof to support this assumption [34]. However, there is ample evidence that the use of antibiotics in food-producing animals contributes to increased AMR [35]. Published literature showed that the risk of getting an infection from AMR zoonotic dairy pathogens seems less likely [36].

However, the absence of direct evidence of AMR bacteria or resistant determinant transmission does not mean there is no transmission between dairy cattle and humans. For instance, the current and future risk of acquiring AMR bacteria from milk is an important human health concern as the consumption of raw milk is increasing in some states in the U.S. [32]. Due to the presence of antibiotic-resistant foodborne or zoonotic bacteria in raw milk [29, 30], *an increasing trend in the consumption of raw milk* in the U.S. and other countries indicates public health risk [37]. Similarly, AMR bacteria present on meat from culled dairy cows should also be seen as an important human health risk since it can cause life-threatening infection if undercooked meat is consumed [34]. It is also unknown if pasteurization of milk or proper cooking of meat will prevent the AMR gene transfer especially in the gastrointestinal tract where horizontal gene transfer may occur.

2.1 Antimicrobial resistance in mastitis pathogens

Antibiotics are regularly used for the prevention and treatment of mastitis in dairy cows. Some review articles showed such uses had not been associated with a high risk of developing resistance in mastitis-causing pathogenic bacteria [7, 34]. The previous review on the impact of antibiotic use in adult dairy cows on antimicrobial resistance of veterinary and human pathogens concluded that common

AMU in dairy farms did not lead to the widespread occurrence of resistance among mastitis pathogens against antibiotics frequently used in dairy production [34]. Nevertheless, there is no doubt that AMU in food-producing animals such as dairy cows contributes to the rise in AMR [7]. Recently Abdi et al. [38] reported a high prevalence (34.3%) of resistant *S. aureus* isolates from different dairy farms in Tennessee, U.S. suggesting a potential increasing trend of antimicrobial resistance in *S. aureus* isolates against some antibiotics.

Only a handful of studies investigated the impact of treatment of clinical or subclinical mastitis on AMR development. A controlled study by Levy et al. [39] measured AMR changes after antimicrobials were administered to a host; however, this study lacks mastitis treatment procedures [7]. However, some studies showed that AMU for mastitis treatment is linked to AMR development and changes in the diversity of mastitis pathogens [40, 41]. Pol and Ruegg [4] found a positive relationship between AMU such as pirlimycin, ampicillin, erythromycin, and tetracycline and increased resistance among gram-positive mastitis pathogens. Another U.S. study also reported a higher proportion of resistant mastitis pathogens recovered from conventional dairy farms than organic dairy farms [42], suggesting the effect of AMU.

3. Alternative approaches for the management of mastitis

There were no specific AMU data collected from U.S. dairy farms. Thus, it is not possible to know the doses of each antibiotic given to dairy cattle, the length of the treatment, and the diseases for which antibiotics were prescribed. However; there is no doubt that antibiotics have been administered for a considerable proportion of dairy cattle's lifetime in a farm, and dairy farm consumes a huge quantity of antibiotics, especially those of the medically important ones. The United States Food and Drug Administration (FDA) report showed more than 16,155 kg of medically important antimicrobials intended for IMM therapy were sold in 2019 [19].

The major concern is the use of critically important antibiotics for human medicine in dairy farms such as third-generation cephalosporins and fluoroquinolones. Both qualitative and quantitative studies that analyzed the risk of AMR in food animals such as dairy farms indicated that the continued use of these antimicrobials would increase the number and types of AMR bacteria and worsen the public health and animal health issues in the U.S. and beyond [43]. It is no longer deemed appropriate that antibiotics should be the only remedy to prevent disease, especially when other alternative disease control measures exist. Thus, it is important to look for potential alternative strategies that help to reduce AMU and prevent disease without heavily relying on antibiotics [7]. Some of the alternative approaches that can be explored to mitigate the rise of AMR bacteria include but are not limited to selective dry-cow therapy (SDCT) [44], good herd health management [45], vaccination [46], phage therapy [47], probiotics [48] antibacterial peptides [49], and nucleic acid-based antibacterial treatments such as CRISPR-Cas system [50].

3.1 Selective dry cow therapy (SDCT)

The number one reason for AMU in the U.S. dairy industry is to control mastitis. Studies showed that almost all U.S. dairy farms treat all cows in the farm (blanket dry cow therapy-(BDCT) with long-acting antibiotics at drying off to prevent mastitis during the dry period. The ideal dry period, the period between the end of the current lactation and the beginning of the next, for a profitable dairy producer is usually 60 days or 8 weeks [51]. A USDA survey of dairy farms reported that 85%

of conventional dairy farms used BDCT [15]. A study suggests that BDCT accounts for approximately one-third of the total AMU on conventional dairy farms in the U.S. [52].

Selective dry cow therapy (SDCT), unlike BDCT, uses a specific strategy to avoid treating every cow with antibiotics at dry off. In SDCT, only animals with IMI or high somatic cell count or cows with a health record showing a high probability of developing mastitis receive antibiotics. A teat sealant is applied to all cows at drying off. Using an internal teat sealant prevents entry of mastitis pathogens and decreases the prevalence of clinical mastitis, reducing the need for treatments for clinical cases [44]. To determine cows that require SDCT, bacterial culture, or somatic cell count (SCC) data of individual animals are required. A cow with a composite milk high SCC of $\geq 200,000$ cells/mL of milk indicated the presence of subclinical mastitis and is eligible for IMM antibiotic infusion [52]. Studies [44, 53] showed that internal teat sealants, alone or when used with antibiotics can decrease the risk of acquiring new IMI after calving by as much as 25%. Internal teat sealants lowered the risk of IMI by 73% compared with cows that do not have teat sealants suggesting its potential use for managing mastitis [44].

3.2 Evidence-based treatment of mastitis

Before administering antibiotics, it is crucially important to isolate and identify mastitis-causing agents from infected udder quarters. Bacterial isolation and identification should be attempted at least in large dairy operations to make an evidence-based decision on whether to use antibiotics. Some investigations have confirmed that on-farm bacterial identification can decrease AMU by as much as 50% [40] since the use of antibiotics is not justified in some infections caused by gram-negative bacteria such as *E. coli* with high “spontaneous self-cure” [54, 55]. Another study also showed that the majority of (as high as 57%) milk samples collected from quarters of cows with negative culture results did not have bacterial DNA [56] suggesting that environmental factors such as trauma or viral infection may trigger an inflammatory response or infected animal was already fully recovered during sample collection. Failure to detect bacterial DNA could be due to bacteria elimination from the udder quarters by the host immunity [7]. In general, the possibility of a natural cure without the use of antibiotics against some bacterial pathogens is well documented in dairy cattle [57–61], and it is an important alternative to consider before deciding on antibiotic use.

3.3 Good dairy herd health management

Dairy herd health management is an essential component in the fight against AMR. The objectives of herd health management are to prevent and control mastitis and other diseases using appropriate hygienic and management practices [62]. AMU can be reduced by improving hygiene, frequent physical examination of animals, regular herd testing for common diseases, and quarantining all-new replacement animals before mixing with the herd [45]. In addition, dairy cattle should be managed to reduce stress and promote their welfare and immunity by providing suitable housing (good ventilation, appropriate humidity, low stocking densities, and good hygienic practices). Studies showed that hard flooring, poor bedding, and overcrowded conditions increase the chance of cows developing mastitis, lameness, and respiratory diseases [63, 64]. All efforts made to maximize herd health and welfare will enhance the host immune function and considerably reduce mastitis and other common dairy cattle diseases, reducing the need for antibiotics [65].

3.4 Vaccination

Vaccination against mastitis pathogens is recommended as one of the most important strategies to prevent new infections, which in turn reduce AMU in dairy farms [46]. Vaccination against mastitis-causing bacteria induces the cow's immune response that fights against subsequent infection and disease. Effective vaccine enhances adaptive humoral (antibody-mediated Th2 immunity) and cellular (cell-mediated- Th1 and Th17 immunity) immunity against mastitis pathogen that inhibits or restricts bacterial growth or kills bacteria upon its invasion of a mammary gland. The enhanced immunity cures the infection or reduces the number of invading bacteria, which reduces pathogen damage to milk-producing tissues and lessens the clinical severity of disease and production losses [66].

Vaccines can be classified into inactivated/killed, live/attenuated, chimeric live attenuated, subunit, and nucleic acid-based (DNA or mRNA) vaccines, each with advantages and disadvantages [66]. Live vaccines contain attenuated disease-causing agents capable of replicating within the host but do not cause disease because of attenuated pathogenicity. Modified live vaccines (MLV) are usually developed from the naturally occurring pathogen by (1) attenuation in cell culture, (2) use of variants from other species, and (3) development of temperature-sensitive mutants. Recombinant live attenuated vaccines include: (1) live attenuated vectored vaccines—pathogen's antigenic parts incorporated into a harmless carrier virus or bacteria, (2) chimeric live attenuated vaccines—genes from the target pathogen substituted for similar genes in a safe, but closely related organism, and (3) nucleic acid (DNA or mRNA) vaccines—a DNA vaccine is an immunogenic product encoding gene (DNA) cloned into a plasmid that can be injected into the host, where it will be transcribed and translated into an immunogenic product. The mRNA vaccine contains a messenger RNA (mRNA) molecule that encodes antigen that induces an immune response [67].

Inactivated/killed pathogen vaccines contain whole pathogens that have been inactivated with agents, such as phenol (bacteria) and formalin or beta-propiolactone (viruses). Inactivated/killed vaccines lack pathogenicity and can neither replicate nor spread between hosts and require multiple doses and regular boosters. The efficacy of inactivated/killed vaccines depends on the use of potent adjuvants. Bacterin is one of the killed/inactivated vaccines in which a suspension of killed whole bacterial cultures is used as a vaccine. Protein vaccines—include naturally produced proteins of pathogens and induce less injection site reactions than products containing the entire pathogen. Recombinant subunit vaccines—contain synthetically produced antigens that induce immunity to a specific pathogen. Adjuvants are one of the components of killed/inactivated vaccines that function to modulate and amplify the host immune response to the accompanying antigen and are critical to the success of inactivated vaccines.

Live-attenuated bacteria can multiply in the host, expressing a complete range of antigens [68]. However, the most important shortcomings of the live vaccine are their persistence in the animal body for an extended time, limited shelf life, potential for contamination, may cause abortion in pregnant animals, and safety concerns as the attenuated organism may revert to full virulence [69]. On the other hand, killed vaccines are safe, induce good colostral (lactogenic) immunity, have longer shelf lives but may interfere with passive immunity and are less immunogenic, and need adjuvants to enhance immune responses [70].

There is no effective vaccine against mastitis pathogens, and results of vaccine efficacy studies showed limited efficacy against mastitis-causing bacterial pathogens [66]. The most targeted udder pathogens for vaccine development include *S. aureus* [71–81], *Streptococcus uberis* [82, 83], *Streptococcus agalactiae* [66], and

E. coli [84–86]. Among mastitis pathogens, most vaccine trials were conducted against *S. aureus*, a major mastitis pathogen with a low cure rate by antibiotics, and remain undetected in the subclinical form in dairy cows [47, 87, 88]. Currently, there are two commercially available bacterin vaccines against *S. aureus* mastitis. These are Lysigen® (Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO) in the United States and Startvac® (Hipra S.A, Girona, Spain) in Europe and some other countries. Several staphylococcal vaccine efficacy trials showed that vaccination with bacterin vaccines induced increased antibody titers associated with partial protection in the blood and milk in some studies [71, 74, 78, 81] or no protection at all in some other studies [72, 79, 80]. Neither of the two commercial vaccines against *Staphylococcus aureus* mastitis on the market, Lysigen®, and Startvac® [79] confers protection under field trials and controlled experimental studies [71–74]. Some studies reported that Lysigen® reduced somatic cell count (SCC), clinical mastitis, and chronic intramammary infection (IMI) [89–91], whereas other field-based studies concluded no such effect [72, 73, 75–77]. Similarly, some studies reported vaccination with Startvac®, reduced incidence, severity, and mastitis duration in vaccinated cows compared to non-vaccinated control cows [71, 74, 78]. Contrary to these observations, other studies failed to find an effect on improving udder health or showed no difference between vaccinated and non-vaccinated control cows [79, 80]. Overall, effective intramammary immune mechanisms against staphylococcal mastitis are still poorly understood.

Mastitis vaccine research has been conducted over the past several years, but to date, developing an effective vaccine has been a challenge due to the nature of the disease and the pathogens involved [92, 93]. For instance, an increased immune response may not always be beneficial in bovine mastitis unless increased immunity is followed by a decreased number of infecting pathogens, as the presence of a large number of bacteria in the presence of fighting immune cells is considered as an indication of mastitis which decreases milk quality [93]. Successful vaccination is challenging because the volume of milk present in the gland dilutes the number of immune effector cells available to fight off infection [92, 93]. In addition, fat and casein in the milk reduce the bactericidal abilities of the immune cells [93].

The development of an effective vaccine against mastitis pathogens is one of the sustainable alternatives to antibiotics. However, it may not be practically possible to develop an effective vaccine against all bacteria that cause mastitis [68]. Thus, combining effective vaccines with other infection control measures may considerably reduce the incidence of IMI and thereby reduce the need to use antibiotics [66].

3.5 Immunostimulants

Immunostimulants are compounds that activate any components of the host's innate immune system and help to enhance disease resistance. Immunostimulants directly stimulate innate immune responses by activating immune cells (phagocytes), complement system, and increased lysozyme activity [94, 95]. Currently, immunostimulants are increasingly used as an alternative to antibiotics [96]. Immunostimulants are broad ranges of substances including minerals (selenium and zinc); amino acids (leucine, arginine, and ubenimex); vitamins (A, E, C); plants and plant polysaccharides, bacterial components (β -glucan, peptidoglycan, lipopolysaccharide); hormones and hormone-like substances; nucleic acid preparations; chemical synthetics (imiquimod, cimetidine, levamisole, polyinosinic acid, pidotimod, and others); and biological cytokines (transfer factor, interferon, immune globulin, and interleukin) [68, 95, 97].

Bricknell and Dalmo [98] reported that the addition of immunostimulants in animal feed could enhance their innate defense and prevent infection during a period of high stress. Another group of researchers, Gertsch et al. [99], stated that applying plant-derived immunostimulants in animal feed boosts the immune system though they did not specify the mechanism. Similarly, Li et al. [100] administered polysaccharide chitosan to cattle and noted improved immune response and antioxidant activity. In 2010, Thacker [101] reported cytosine-phosphate-guanine (CpG), an oligo deoxynucleotides immune-stimulant, stimulating B-cell proliferation, cytokine production, and enhanced cytokines production and NK cell cytotoxic activity.

3.6 Cytokines

Cytokines are crucial for normal tissue functions, but their over- or under-expression is linked with pathological conditions [102]. They play a significant role in initiating, sustaining, and controlling the innate immune response and suggesting that they may have an excellent therapeutic effect for infectious disease treatment [103]. Toll-like receptors (TLRs), surface receptors that identify the structure of pathogens, also indirectly contribute to the secretion of cytokines by inducing a signaling cascade that leads to the secretion of cytokines controlling the adaptive immune response [104].

Cytokines, such as IL-6, TNF- α , and INF- γ , have also been proposed to treat bovine mastitis and endometritis. Hossain et al. [105] reported that cytokines alone or in combination with antibiotics significantly improve the rate of cure of bovine mastitis. Daley et al. [106] infused the mammary gland with recombinant bovine cytokines ((IL-1 and IL-2) and observed a rise in the proliferation of polymorphonuclear cells, with increased formation of oxygen radicals in the milk. The investigators also observed that the induced host natural defense system could prevent *S. aureus* infection in cattle. This suggests recombinant bovine cytokines are a promising candidate. Thus, further investigation is needed to identify the therapeutic potential of the cytokines for mastitis treatment and their possible use as an alternative to antibiotics.

3.7 Phage therapy

Phage therapy, which treats bacterial infections with bacteriophages, has been considered one strategy to manage mastitis [47]. Results of several studies showed that bacteriophages had antibacterial activity against a range of antibiotic-resistant bacteria with a considerable degree of specificity and potency [107]. Thus, the use of bacteriophages and their derivatives such as endolysins signifies a possible alternative for treating mastitis [108].

The bacteriophage works by inserting its genome into the bacterial cytoplasm, thereby the phage genome will incorporate itself into the host genome and reproduce along with the bacteria and produce endolysin, which break-down the bacterial cell wall and induce a cascade of bacterial lysis [108, 109]. Phages and endolysins are also known to destroy biofilms produced by major gram-positive and gram-negative mastitis pathogens, including *Staphylococcus* species, *E. coli*, *Klebsiella pneumonia*, and others [110].

Currently, interest in bacteriophages for the treatment of mastitis is rapidly growing [80]. Results from several *in vitro* experiments indicated that this method of treating mastitis is a viable option as phage therapy shows promising effectiveness against some mastitis pathogens, such as *S. aureus* [107, 108, 110–114]. However, a handful of clinical studies to evaluate the efficacy of bacteriophage for the treatment and prevention of mastitis showed limited efficacy of this approach, suggesting the

need for further study to improve its effectiveness [47, 112, 115]. Moreover, the practical use and broad application of phage therapy are limited by several factors. These include high specificity of phages, low effectiveness in eliminating the population of pathogenic bacteria, the need for a high dose of phage for effective therapy and its degradability in milk, and the emergence of phage resistance bacterial strains [108, 116]. Further clinical studies are needed to address these limitations and exploit the full potential of phage to prevent and treat mastitis.

3.8 Use of probiotics for the treatment of mastitis

The rise of AMR against antibiotics used in dairy farming demands the search for other alternative disease control measures. In this regard, probiotics have lately been considered a potential alternative for treating mastitis [49]. Probiotics are living microorganisms that give a health benefit to the recipient when given in sufficient amounts. This less precise definition includes several different well-identified microorganisms, safe for intended use, have proven health benefits when used in appropriate amounts and through the correct routes [117, 118].

Two mechanisms of action were suggested for mammary gland probiotics. The first mode of action is through the interactions between probiotics and the local microbiota (indirect mode) [48]. This model assumes that cows develop mastitis due to a lack of balance between the normal mammary gland microbiota and pathogenic bacteria causing mastitis. Therefore, modification of this imbalance with probiotics is suggested as an option to AMU [119]. The second proposed mode of action is a direct one, where probiotics interact directly with mastitis pathogen. Probiotic bacteria generate a range of antimicrobial substances such as short-chain fatty acids, lactic acid, nitric oxide, hydrogen peroxide, and bacteriocins, all of which may inhibit the growth and multiplication of mastitis-causing bacteria [120]. Rainard and Gilles [48] reviewed the use, mechanism of action, and *in vitro* and *in vivo* efficacy studies on probiotics used in mastitis treatment.

The selection and prophylactic or therapeutic use of mammary gland probiotic strains depend on the production of substances affecting the growth or survival of mastitis pathogens, the absence of known virulence factors, the absence of antibiotic resistance, and the ability to colonize mammary gland epithelium cells. The bacteria that meet these conditions are deemed promising for use as mammary probiotics [121]. Most studies investigated lactic acid bacteria as a potential probiotic for mastitis treatment and prevention. Few of these studies reported that probiotics are as effective as antibiotics for treating clinical mastitis [122]. In contrast, most other studies reported that the probiotics elicit a strong inflammatory response in the mammary gland or are neither effective nor safe [123, 124]. The current reports on the safety and efficacy of intramammary probiotics are generally conflicting, necessitating the need for further research to develop a conclusive recommendation on the use of probiotics for the management of mastitis.

3.9 Antimicrobial peptides

Antimicrobial peptides (AMPs), also known as cationic host defense peptides, are potent naturally occurring antibacterial agents with a broad spectrum of activities against both gram-negative and gram-positive bacteria. AMPs are found in all forms of life, from prokaryotes to eukaryotic cells. In contrast to most conventional antibiotics, AMPs often work in direct and indirect ways. They may directly kill the bacteria by disrupting cell membranes, thereby creating trans-membrane channels. They indirectly may also enhance host immunity as immunomodulators so that the host can clear the pathogen [49].

In vertebrates AMPs promote natural immunity and are a component of the first line of defense against pathogenic microorganisms. The crucial role of AMPs as innate immune modulators was shown in an experimental study in which the *cnlp* gene (encoding CRAMP) knockout mutant mice, a gene coding mouse analog of human LL-37 (encoded by *camp*) antimicrobial peptide, were very susceptible to infection [125]. In prokaryotes such as bacteria, the production and release of AMPs give a competitive advantage in a given environment by AMPs-mediated killing of other bacteria [126].

The mode of action of AMPs is recently reviewed [127] and seems different and related to the target bacterial pathogen. The positively charged AMPs interact with the negatively charged membranes of bacteria (lipopolysaccharides in gram-negative bacteria) and teichoic acids (in gram-positive bacteria). This strong electrostatic interaction between opposing charges (between AMPs and bacterial surface membranes) is the basis of the specificity of the action of AMPs on bacteria over other higher organisms. The “amphipathic” characteristics of AMPs help them to bind and penetrate the bacterial inner membrane causing leakage of bacterial cell contents and leading to cell death [128].

Currently, AMPs are considered as one of the promising classes of therapeutic agents as an alternative to conventional antibiotics. Several AMPs have been used as therapeutic agents for intravenous administration and topical application in human medicine owing to their short half-lives [129]. A recent study investigating the efficacy of specific AMPs against the AMR *S. aureus* in the mammary epithelial cells reported a very promising result. The study examined the intracellular activities of H₂ in the bovine mammary epithelial and mouse mammary glands infected with methicillin-resistant *S. aureus* (MRSA) and multidrug-resistant *S. aureus*. Results showed a 99% intracellular inhibition rate of the resistant *S. aureus* strains after treatment with the AMPs. The study finally concluded that H₂, the AMPs used in the study, “can be used as a safe and effective candidate for treating *S. aureus*-induced mastitis” [130]. This is an indication that AMPs-based treatment approaches may be used as one of the tools that may help in the fight against AMR pathogens. However, more studies are needed to generate information on the development of resistance to AMPs, challenges to their widespread use in dairy cattle.

3.10 Use of CRISPR-Cas system

The CRISPR-Cas system is a bacterial immune system that gives resistance to foreign genetic elements such as those that exist within plasmids and bacteriophages and provides a form of adaptive immunity [131]. In recent years, the use of the CRISPR-Cas system to treat AMR bacteria has received a considerable level of interest as the approach that can readily kill AMR bacteria in the same way as an antibiotic-sensitive bacterium [132]. Additionally, this system can be designed specifically so that it can only target pathogenic bacteria without disturbing commensal bacteria in the microbiota [50]. This bacterial immune system is commonly used for “genome editing” as it can selectively eliminate virulence and antimicrobial resistance genes from bacterial populations. The system uses small RNAs (sRNA) to detect and destroy specific sequences of DNA, including phages, transposons, and plasmids [133].

Nucleic acid-based antibacterial treatments can be used to control infections caused by resistant bacteria [134], including mastitis-causing pathogens. However, although *in vitro* studies on some resistant pathogens showed successful and promising results, *in vivo* study to treat mastitis pathogen has not yet been carried out [135]. Besides, despite its current potential, the sustainable application of CRISPR-Cas technology is complex. It needs an efficient delivery vector, developing an appropriate wide host

range vector, and using a multiplex method that includes CRISPR-Cas targeting different sequences to reduce the occurrence of resistance possibilities [136].

4. Conclusion

Mastitis is the most prevalent and economically important disease of dairy cattle responsible for the largest antibiotics used in the dairy industry. Most dairy farms in the United States use similar antibiotics used to treat various diseases in humans. Several studies have linked AMR to antibiotic use. Thus, the use of these classes of antibiotics in dairy cattle may speed up the development of AMR, which can also affect the successful treatment of infection in humans. Every effort must be made to avoid unnecessary use or reduce the use of antibiotics to prevent mastitis. Dairy farmers need to be educated on the importance of improving herd and udder health so that the incidence of clinical and subclinical mastitis will decrease, reducing the need to use antibiotics. The use of vaccines, probiotics, antimicrobial peptides, phage therapy, and CRISPR-Cas system are among the promising alternative options for mastitis management. To maintain dairy cattle health and productivity and preserve the effectiveness of antibiotics, these alternative approaches to antibiotic use must be thoroughly investigated and implemented for sustainable management of mastitis. *In vitro* studies showed promising results on the potential use of these approaches, but further *in vivo* studies are needed to make specific recommendations on their use. Research should focus on identifying good alternatives to antibiotics with important characteristics including but not limited to effectiveness against the target pathogens, safety toward the host, ease of elimination from the body, less harmful to normal flora, degradability in the environment, and cost. Thus, it is strongly recommended that researchers and funding organizations invest their resources and focus their effort on developing innovative and sustainable control tools that are easily adoptable by producers such as effective vaccines, probiotics, and others coupled with good herd health management practices.

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