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Microbial Biofilms in Veterinary Medicine

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ABSTRACT

Microbial biofilms defined as extremely complex ecosystems are considered clinically important for humans. However, the concept and significant roles of microbial biofilms in the progression of disease have seriously lagged in veterinary medicine, when compared with human medicine. Although the importance of biofilms in animal health is just beginning to emerge, limited studies have paid attention that microbial biofilms are clinically important in the field of veterinary medicine, and lead to serious economic losses. In this review, the importance of microbial biofilms causing high economic losses in the livestock industry has been highlighted. Besides, the concept of microbial biofilm, their role in the pathogenesis of the animal diseases, as well as diagnosis approaches and possible therapeutic strategies needed to overcome their detrimental effects in veterinary medicine, have been discussed.

Introduction to microbial biofilms

In the early years of microbiology, microorganisms were mainly characterized as planktonic or freely floating cells. However, scientific observations have dramatically shown that the predominant form of microbial growth is in biofilms that attach to the surface of living and nonliving materials, in an almost irreversible manner (16). Today, biofilm is generally identified as an accumulation of microbial communities which are enclosed in an extracellular matrix (11). According to the recent international consensus statement (23) biofilm is defined as "A structured community of microbes with genetic diversity and variable gene expression (phenotype) that creates behaviors and defenses used to produce unique infections (chronic infection)". Biofilms are characterized by significant tolerance to antibiotics and biocides while remaining protected from host immunity.

Microbial biofilm formation is considered as a complex process with the inclusion of a cascade of molecular, biochemical and physiological events that depend on the type of microorganism, the surface, and environmental factors (1). The multistage development of mature biofilm begins with the primary adhesion between the microorganisms and the abiotic or biotic surfaces. Following the reversible attachment (i), bacteria and biotic surfaces express multiple adhesins factors and receptors for specific adherence (ii). They form aggregates and microcolonies, differentiate by the production of extracellular matrix (iii), and finally, the maturation of biofilms occurs by the attachment of additional microorganisms (iv) (Figure 1). The matrix consists of extracellular polymeric compounds including polysaccharides, proteins, DNA, and lipids, and protects the bacteria from extreme and depleted environments and antimicrobials, and gives mechanical stability (30, 43). Within biofilms, bacterial cells are sheltered against different adverse environmental conditions such as ultraviolet light radiation, osmotic changes, pH variability, dehydration, antimicrobial drugs, disinfectants, and host immune responses (40). Besides, the bacteria in the microenvironment of the biofilm matrix can deploy cell to cell communication by a signal system called quorum sensing (QS) through the production of autoinducers (48). With QS mechanisms, microbial populations in the biofilm matrix coordinate their behaviors and gain advantages compared to planktonic cells (5).

The planktonic microorganisms have precisely been of value in strategies to combat diseases. However, recent studies have shown that microorganisms in biofilms show differences from their planktonic counterparts in terms of behavior, structure, and physiology (43). In addition, biofilms reduced the ability of the antimicrobials to get access to the microorganisms and make them resistant against certain antimicrobials.

The importance of microbial biofilms in animal health

With the increasing role of microbial biofilms in natural environments, it is not surprising that they are responsible for infection in both humans and animals. The Centre for Disease Control and Prevention (CDC) has suggested that 65% of bacterial infections in humans are related with microbial biofilms (2). The National Institutes of Health (NIH) revealed that of among all microbial and chronic infections, 65% and 80% are associated with biofilm formation, respectively (28). Due to the different husbandry and living conditions of animals, the risk of infection as well as biofilm formations presumably much greater in animal species than in humans. Biofilms have been also linked with numerous infectious diseases in animals, including chronic wounds, periodontal diseases, mastitis, and Salmonellosis (Figure 1). In addition to their direct effect, they also have significant indirect effects on the industries (1, 11).

Dental biofilms in oral health: Dental/oral biofilms are one of the most studied microbial biofilms in humans which is responsible for dental caries and periodontal diseases. As in humans, the oral microbiota of animals is structured in a variety of aerobic, facultative, or strictly anaerobic bacteria (61). Dysbiosis of oral microbiota leads to oral infections caused by dental plaques/biofilm bacteria which are highly prevalent in periodontal disease, soft tissue infections, and dental caries (59). Among oral infections, dental caries is a rare occurrence in pet animals (61). However, periodontal diseases as a chronic bacterial infection caused by microbial biofilms of mixed-species are one of the most common diseases of adult dogs and cats, and effect up to 80% of animals (31, 61). According to the American Veterinary Dental College, it is estimated that the majority of pets show symptoms of dental or periodontal diseases starting with dental biofilms, by three years of age. The formation of dental biofilm in the oral cavity of animals is a multi-stage process and mostly related to microorganisms in the oral cavity. *Porphyromonas cangingivalis* and oral protozoa such as *Entamoeba gingivalis* and *Trichomonas tenax* are the most common cariogenic microorganisms in canine dental biofilms which play an important role in canine periodontal disease (31). Borsanelli et al. (8) indicated that the significant antagonistic interactions between the *Petrimonas* spp., *Porphyromonas* spp., *Prevotella*spp., and *Fusobacterium* spp. species in the oral microbiota of shep are the key factors for dental biofilm associated with ovine periodontitis. An interesting data obtained by Perez- Serrano et al. (44) showed that dental biofilm can be considered as a source and reservoir of antibiotic resistance genes (ARG) and can be shared between humans and pets living in a household. Their results showed that dogs seem to play an important role in the transference of ARG, and the children appear to be the most affected.

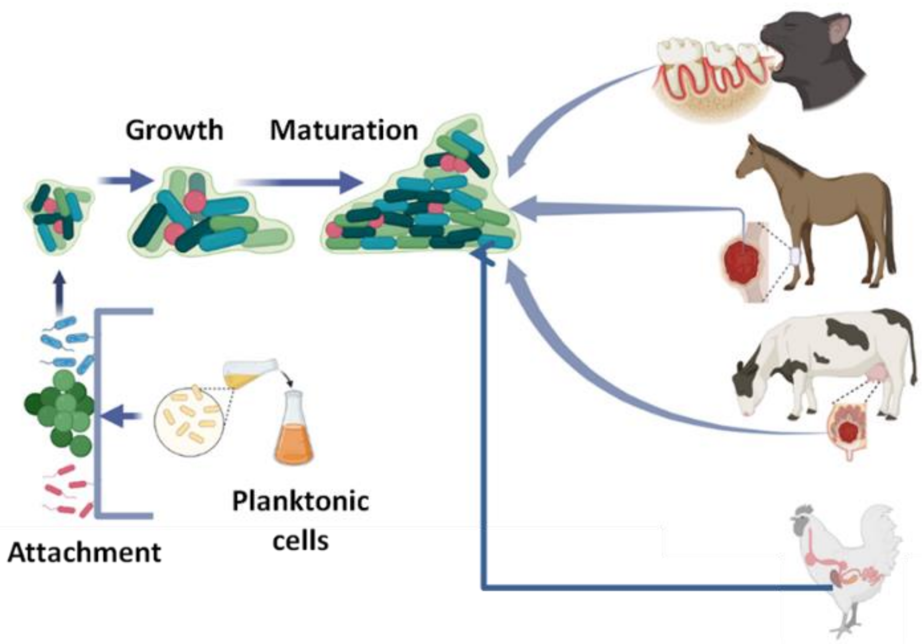

Figure 1. Schematic representation of the stages of microbial biofilm formation and its implications in the veterinary medicine. Figure created with BioRender.com (accessed on 26 March 2022).

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As with humans, dental biofilms in animals are highly important not only for oral health but also for their overall health. In addition to tooth loss, biofilm linked infections in the oral cavity may relate to various local consequences such as oral-nasal fistula, perioendo abscess, pathologic fracture because of chronic periodontal loss which weakens the bone in affected areas, inflammation close to the orbit which potentially leads to blindness, oral cancer and chronic osteomyelitis (61). The implications of "broken mouth" periodontitis especially affect the sheep grazed on rough pasture, and involves periodontal infection of the incisor teeth. In addition to being a painful condition, it reduces the efficiency of grazing of sheep and consequently leads to economic problems for sheep farmers (59).

Evidence is emerging that biofilm-related periodontal infections are also associated with serious systemic diseases, in animals. Therefore, the microbial and matrix composition of dental biofilms and their mechanisms in animals is now considered important to understand their role in animal diseases. However, comparable studies of dental biofilms in animals are relatively limited, despite the fact that similar infections also occur in the case of humans.

Biofilms in chronic wounds: In humans, microbial biofilms related to wound infections lead to chronic inflammation and delayed management (30). Although there are many biofilm-related researches conducted in animal models including rodents, rabbits, pigs, dogs, horses, etc., limited studies have been directly carried out for the determination of the clinical importance of microbial biofilms in the field of veterinary medicine. However, similar to humans, animals suffer from chronic wounds which are the common sites for biofilm formation in veterinary clinics (30). Recent limited studies have been identified the prevalence of microbial biofilms in wounds of dogs, cats, and horses. Nevertheless, their significance and the factors that modulate and stimulate their formation are still unknown (33).

In veterinary medicine, horses are particularly at risk from chronic non-healing wounds of the lower limb similar to venous leg ulcers seen in humans (55). The first study which identified the microbial biofilms in the chronic wounds of horses was carried out by Cochrane et al. (12). In a recent experimental wound model of equine with bacterial inoculation, Jorgensen et al. (29) showed that microbial biofilms have a negative impact on wound healing of distal limb wounds but not another part of the body wounds. Although there are no investigations focused on the effect of microbial biofilms for wound healing of horses in the literature (30), the presence of biofilms in equine wounds partly explains the reluctance of many lower limb wounds to heal. The prevalence of biofilm in traumatic wounds of horses makes them important due to the non-healing limb wounds leading to well-documented welfare and economic concerns (58). In addition to horses, the first report of microbial biofilms in dog chronic wounds has been reported by Swanson et al. (54). A 4-year-old spayed female Mastiff was evaluated for the treatment of chronic non-healing pressure wounds, and biofilm reformation was prevented by treatment with antimicrobials. Although the microbial composition of biofilms in chronic wound infections of animals is still unclear, the majority of the microorganisms in chronic wounds of dogs were found belonging to the *Propionibacteriaceae*, *Porphyromonadaceae*, *Deinococcaceae*, *Nocardiaceae*, *Methylococcaceae*, and *Alteromonadaceae* which may not be cultured by conventional microbiological methods under laboratory conditions (33).

In addition to the direct effect of biofilms on wound infections, the formation of biofilms on surgical implants has also a major role in chronic wound infection, in veterinary medicine (34). Based on the initial researches of microbial biofilms in wounds or on surgical implants, the role of microbial biofilm in wound management is dependent on various factors, mainly the wound bed and its microenvironment. However, additional knowledge is still needed to investigate the prevalence and etiology of biofilms in animal chronic wounds.

Mastitis and microbial biofilms: Mastitis is one of the most important and multi-factorial disease affecting many species of animals including sheep, pigs, dogs, cats, goats, and horses. Bovine mastitis defined as an inflammation of the udder generally caused by microbial biofilm-related infection has a high incidence, worldwide (21). Besides, it is responsible for major economic losses on dairy farms with decreasing in milk production, increasing in health care costs, and leading to serious public health considerations (6, 21).

Bovine mastitis is characterized by the infection of the mammary gland epithelium. In the pathogenesis of mastitis, biofilm formation is also considered another important virulent factor and also a selective advantage for mastitis-causing pathogens such as *Staphylococcus aureus*, *S. epidermidis*, *S. uberis*, and *S. dysgalactiae*. In addition to staphylococci, coliforms, enterococci, and streptococci are also the common isolated genus from cows which suffer from mastitis (53). During the last decade, over 200 studies have been published focused on the *in vitro* biofilm forming potential of bovine mastitis pathogens, especially to *S. aureus*, on their molecular mechanism to form a biofilm, and their potential treatments (41). However, the main role of microbial biofilms in the pathogenesis of bovine mastitis is still unclear. To investigate their actual role in bovine mastitis,

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in vivo studies of biofilms in infected udders have to be carried out. Overall, it is considered very important to understand the role of biofilm in bovine mastitis to apply the best control strategies in veterinary medicine. It has to be also the main necessity in order to reduce economic problems in the dairy global market, and to ensure milk safety and quality, as well as animal welfare.

Salmonella biofilms in poultry: Salmonellosis is an infection caused by different serotypes of *Salmonella* spp. live in the intestinal tracts of domestic animals, and cause ranging in the severity of symptoms such as diarrhea and enteritis to systemic syndrome (42). As an emerging zoonotic bacterial threat in the poultry industry, the infection of *Salmonella* spp. leads to important global public health problems (26). *Salmonella*-contaminated animal-derived food products resulted in 3% of the bacterial food-borne disease in all around the world, with approximately 80 million infections and 155.000 deaths, per year (3, 18, 35). According to the Interagency Food Safety Analytics Collaboration (27), 14% of outbreaks of Salmonellosis are estimated to be related with chicken meat and egg, which are contaminated by chicken intestinal contents (9, 45). In addition to public health concerns, Salmonellosis affects meat and egg production and results in a huge economic loss in the poultry industry (39).

Although it consists of more than 2500 serologically different variants or serotypes, *Salmonella enteritidis* and *Salmonella typhimurium* are the most common serotypes isolated from Salmonellosis outbreaks and infected poultry products (7, 20). *Salmonella* infection is transmitted horizontally and vertically in poultry, with high incidence in one-day-old chicks (51), and the contamination with these bacteria in poultry-derived meat/eggs products can occur at multiple stages along the food chain (17, 39). *Salmonella* strains often exist not only as planktonic cells but also in biofilms formed on various surfaces (50). *Salmonella* spp. are able to adhere to abiotic and biotic surfaces and form biofilms. The formation of biofilm may lead to a direct interaction between the contaminated of food products in food processing environments (39). Approximately, 50% of the *Salmonella* strains isolated on poultry farms were able to produce biofilms (37). The biofilms formed by *Salmonella* strains provide them more resistance to antimicrobials, chemical, physical and mechanical stresses, and host immune systems (42), thus playing an important role in the survival of planktonic cells under unsuitable conditions, such as poultry farms and slaughterhouses (56). Therefore, special attention must be paid to the prevention and management of Salmonellosis (39). Since biofilms protect the bacteria from antimicrobial agents, sanitizers, as well as other environmental factors (4), *Salmonella* biofilms, represent a major problem, especially in the feed and food industry (dairy, fish, and meat industry) (50). As a consequence of the importance of biofilm-forming *Salmonella* spp., combating *Salmonella* infections gains importance not only for the public health but also for the poultry industry. Therefore, the multi-factorial and complex phenomenon of the biofilm formation has to be identified under laboratory and, *in vivo* conditions as well as in farm environments (42). Moreover, current approaches are still necessary to develop a control strategy to eradicate the biofilm formed by *Salmonella* spp.

***Medical device-associated biofilms*:** Medical devices can be suitable abiotic surfaces for biofilm formation of various microbial species (15). However, there are relatively few studies on biofilm infections from medical devices in the field of veterinary medicine. According to a study, catheterized dogs developed urinary tract infections more frequently than non-catheterized dogs and *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas*, *Enterococcus*, and *Klebsiella* species were commonly described in catheter-related infections in domestic cats and dogs (10).

Peritoneal dialysis is a procedure that has been used for many years in dogs with acute and chronic renal failure. Not surprisingly, *S. aureus* and *S. epidermidis*, which are commonly isolated in catheter-related biofilm infections, are also present in peritoneal catheters used in the treatment of animals. This is because staphylococci that migrate into the catheter from the skin microbiota, and can form biofilms on the catheter surface (38, 46). It is highly possible to encounter *Pseudomonas*, *S. aureus*, coagulase-negative staphylococci, and meticillin-resistant Gram-positive bacteria, which are also common in humans with catheter-associated bloodstream infections as well as in animals (25). In addition to these medical devices associated with biofilm, polyurethane or silicone surfaces of gastronomy tubes that are essential for animals if they can no longer feed themselves, are suitable environments for the bacterial adherence. Various Gram-positive and Gram-negative bacteria can form biofilms in the lumen of these tubes (60).

Diagnosis of microbial biofilms

Diagnosis of biofilms in animals is very complicated due to the complex lifestyle of planktonic cells, the lack of evident clinical signs, and the requirements for advanced methods (30). Numerous approaches based on different methods have been developed for the phenotypic, biochemical and genotypic analysis of biofilm formation. These techniques aim to determine the viability of microorganisms (quantification of viable cells) and to analyze the components of biofilm matrix and biomass

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(39). For the phenotypic identification of biofilm-producing strains, the most common methods are based on microtitre-plate analysis (13). Macroscopic and quantitative estimation of bacterial biofilm on different surfaces can be determined by crystal violet staining assay, and the Congo red agar test (19) which lead to direct analysis of the colonies and the detection of slime-forming strains and non-slime-forming strains (39).

It is also challenging to achieve an accurate diagnosis of the heterogenetic distribution of bacteria in biofilms through the conventional culture and isolation methods. Standard microbiological culture techniques can allow to detect only the culturable microorganisms but not the unculturable ones. To detect the microorganism composition of the biofilms, molecular techniques including RT-PCR (Real-Time Polymerase Chain Reaction), 16S ribosomal ribonucleic acid (rRNA) gene sequencing, next gene sequencing (NGS) give deeper information. However, they are not considered as the gold standards from the point of biofilm detection. When bacterial infection progresses to biofilm, there is an urgency to develop more accurate diagnostic tools for analyzing the biofilm biomass (14). Considering this, scanning electron microscopy (Figure 2), confocal laser microscopy, and its combination with fluorescent in situ hybridization using different probes can be successfully used in the detection of microbial biofilms (47, 49). In addition to optical imaging techniques, nuclear and ultrasonic imaging techniques and their combination with other methods have been explored in order to detect and quantify early and mature microbial biofilms (14). However, microbial biofilm detection still possesses a challenge for the scientific community. Potential diagnostic markers which able to utilize the difference between planktonic and biofilm cells, are still needed.

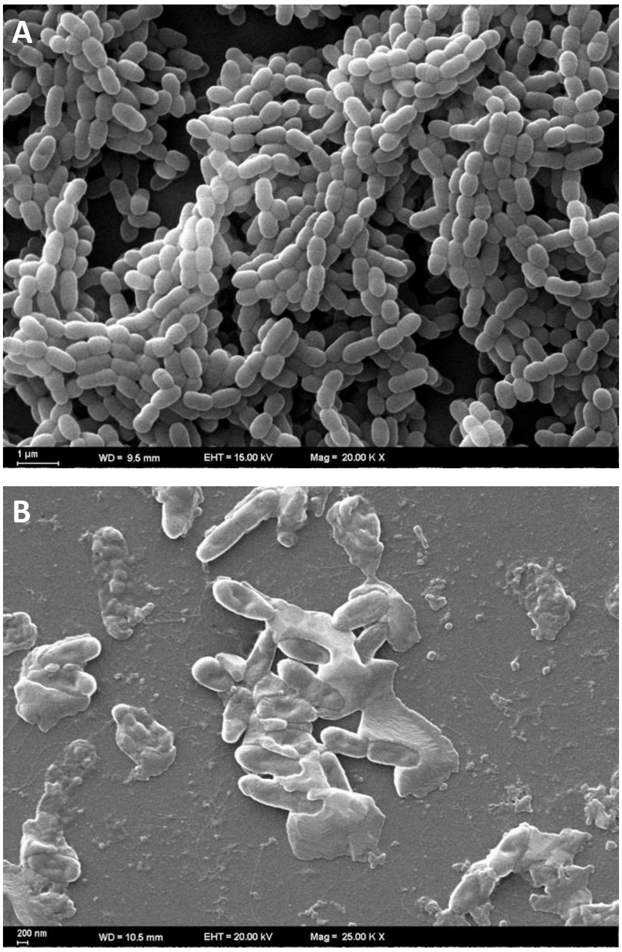

Figure 2. Scanning electron microscopy micrographs of biofilm formation by *Streptococcus mutans* (a, cariogenic pathogen related with dental biofilm progress), and *Pseudomonas aeuroginosa* (b, pathogen related with wound infections).

Novel strategies to control microbial biofilms

Microbial biofilms are highly resistant to antimicrobial drugs (antibiotics, disinfectants, or antifungals) as well as the host immune response when compared to free-flowing planktonic bacteria. Although antimicrobial drugs often eliminate the planktonic cells that are released from the matrix, they have minimal effects on eradicating microbial biofilm that are formed (52). This is because that antimicrobial agent cannot gain access to the pathogens due to the impermeable nature of the biofilm extracellular matrix. This situation makes their treatment increasingly problematic (1, 14). Therefore, an effective treatment has to aim for the complete eradication of microbial biofilm of the pathogenic bacteria, not only to their planktonic form. Thus, alternative and effective strategies play a pivotal role to reach better animal health and bio-safety in veterinary medicine.

Natural or synthetic substances such as chlorhexidine, polyethylemine, silver, nitric oxide, honey, plant extracts, probiotics, matrix-degrading enzymes, are in use as potential antibiofilm treatments (14, 24, 30, 49). Since the QS is a key regulatory system in the pathogenesis of various bacterial infections, applications of these compounds targeted the blocking of QS mechanisms may also provide novel strategies to combat with microbial biofilms (48). Novel therapeutic applications include the combination of conventional antimicrobial agents with ultrasound devices, electric current, phage therapy, or drug delivery systems (1). Ultrasounds enhance the bactericidal activity of the antimicrobials through their passage of non-invasive acoustic energy waves from the skin to the site of biofilm. The electromagnetic impulse increases the antimicrobial activity of cationic agents against bacterial biofilms (32). Although it is not commonly applied in veterinary biofilm therapeutics, phage therapy has the potential for the hydrolyzation and degradation of the extracellular matrix of biofilms (57). With the drug delivery system,

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antimicrobials are incorporated into nano-carriers such as phosphatidylcholine or polyamidoamine, and display their mechanisms by prolonging the effect of the active molecules which is delivered to the appropriate action site (32). Another approach for microbial biofilm treatment especially in chronic wounds is using debridements combined with other antibiofilm strategies, although there are no specific guidelines exist for animals (36).

Despite intense researches in animal models, the optimal antibiofilm treatment in veterinary science has not yet been identified. The use of new technologies as a treatment strategy can provide useful tools in veterinary medicine for the control of biofilm infections, in the future.

Conclusions

Considering the prevalence of microbial biofilms formed by microorganisms in different part of ecosystems in all over the world, it is not surprising that they are one of the main contributors causing serious medical complications in humans and animals. In the light of scientific knowledge that microbial biofilm formation is responsible for many infectious diseases affecting humans, the effect of microbial biofilms in veterinary medicine should not be ignored. Most of the biofilm-based infections are related with animal injuries, oral health, and mastitis cases which are similar to that of humans. It is also known that more than 50% of human biofilm infections are zoonotic origin. Despite the limited number of the studies focused on the importance of biofilm in veterinary medicine, their results highlight the need to develop an eradication treatment and preventive plan to combat the biofilm development in animals. Without effective diagnostic and treatment protocols for veterinary biofilms, their impact will remain a significant challenge. Therefore, additional researches are still needed to unravel the mystery of microbial biofilms in veterinary medicine.

Conflicts of interest

The authors declared that there is no conflict of interest.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

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