Food safety concerns of biofilm-forming zoonotic bacterial pathogens

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Abstract

Transmission of zoonotic pathogens from livestock to humans through contaminated meat, dairy, and egg products presents significant food safety and public health concerns. Bacterial pathogens form biofilms as an integral part of their life cycle. Biofilms also aid in microbial persistence and disease progression in livestock and become a major source for zoonotic infection. Zoonotic pathogens of food safety concern include but are not limited to *Listeria monocytogenes*, *Staphylococcus aureus*, *Clostridium perfringens*, *Yersinia enterocolitica*, *Salmonella enterica*, *Campylobacter* spp., and *Escherichia coli*. Several bacterial virulence factors are recognized to have dual functions in biofilm formation and pathogenicity. Implementing strategies to prevent and control biofilms through inactivation of signaling molecules, cyclic-di-GMP (guanosine monophosphate) and quorum sensing would be an effective robust approach for reducing zoonotic diseases, food safety and public health concerns.

Key words: Biofilm, Food safety, Livestock, Quorum sensing, Zoonotic pathogens

Highlights

- Biofilm helps in pathogen colonization, persistence and disease progression.
- Biofilms protect pathogens from antibiotics and immune cells.
- Several bacterial virulence factors have dual functions in biofilm formation and pathogenicity.
- Prevention and control of biofilms are effective approaches for reducing zoonotic infection and foodborne diseases.

Introduction

Bacterial biofilms, zoonotic pathogens and food safety: Zoonotic pathogens not only affect the health of livestock, but also humans due to their transmission via meat, dairy products, eggs, other animal by-products, and indirectly through fruits and vegetables due to environmental contamination. Zoonotic diseases also affect food security, economic loss, and farmers' livelihood (Gebreyes *et al.*, 2020). Zoonotic pathogens of food safety importance include *Listeria monocytogenes*, *Staphylococcus aureus*, *Clostridium perfringens*, *C. difficile*, *Mycobacterium paratuberculosis*, *Bacillus anthracis*, *Brucella abortus*, *Salmonella enterica*, *Escherichia coli*, *Yersinia enterocolitica*, and *Campylobacter*

spp. (Bhunia, 2018; Abebe *et al.*, 2020; Gebreyes *et al.*, 2020). These pathogens, in part, are responsible for approximately 2 billion illnesses and over one million deaths globally (Kirk *et al.*, 2015) and about 48 million illnesses, and 3000 deaths in the United States (Scallan *et al.*, 2011) annually. Therefore, understanding the pathophysiology of zoonotic diseases by biofilm-forming pathogens in livestock and their dissemination to food provides an opportunity to improve food safety and reduce human foodborne illnesses.

Bacterial biofilms are a well-structured protective microbial community that allows the microbial cell to resist any unfavorable threats, such as the immune system, antibiotics and

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antimicrobials in a host or food processing environments, such as sanitizers, desiccation, ultraviolet radiation, and metallic cations (Flemming and Wingender, 2010; Ren *et al.*, 2015; Bai *et al.*, 2021b). Steps involved in biofilm formation include bacterial attachment, microcolony formation, maturation and detachment or dispersion. Microorganisms express fimbriae, curli, flagella, adhesion proteins, and capsules to firmly attach to a surface to initiate and form biofilms (Flemming and Wingender, 2010; Nadell *et al.*, 2016). In biofilms, bacteria release an extracellular polymeric substance (EPS) consisting of polysaccharides, proteins, and extracellular DNA (e-DNA) forming a three-dimensional biofilm scaffold (Nadell *et al.*, 2016). Bacteria also secrete signaling molecules such as 3',5' cyclic di-guanosine monophosphate (cyclic-di-GMP), and quorum sensing (QS) molecules, which regulate gene/protein expression necessary for biofilm formation and virulence (Cotter and Stibitz, 2007; Landini *et al.*, 2010; Römling *et al.*, 2013). QS molecules help bacterial communication with other cells (cell-to-cell communication) when they grow in close proximity. The QS molecules include N-acyl homoserine lactone (AHL), autoinducer-2 (AI-2), AI-3, autoinducing peptides (AIPs), indole, pyrones, quinolones and several other small molecules. The QS molecules help biofilm formation and also regulate gene expression for bacterial survival, growth, cell density, motility, resistance to antimicrobials, tolerance to desiccation, and pathogenesis (Riedel *et al.*, 2009; Landini *et al.*, 2010).

Regulating biofilm formation by manipulating cyclic-di-GMP and QS production is an intriguing approach in controlling infection and pathogenesis (Caly *et al.*, 2015; Kim *et al.*, 2017; Almeida *et al.*, 2018; Aswathanarayan and Vittal, 2018; Defoirdt, 2018). In addition, natural antimicrobials, microorganisms, or nanotechnology approaches could be employed to inactivate microbes in biofilms to prevent pathogen transmission (Singh *et al.*, 2018; Bhunia, 2019; Liu *et al.*, 2021; Rao *et al.*, 2021).

Biofilms can be comprised of single-species or mixed-bacterial species. Often, a strong biofilm-forming strain can provide shelter to other pathogens including the weak biofilm former within the biofilm scaffold to promote pathogen survival and persistence. The mixed culture biofilm can also facilitate genetic exchange between bacteria which may often perpetuate the spread of antibiotic resistance (Madsen *et al.*, 2012; Stalder and Top, 2016).

During infection, pathogens also form biofilms to aid in adhesion for colonization on biotic surfaces including feathers, skin, oral cavity, respiratory tract, intestine, and urogenital tract. Pathogens also form biofilms on abiotic surfaces such as floors, walls, slaughterhouse or dairy processing equipment, food-contacting surfaces and drains. This implies that pathogens must express molecules that can promote biofilm formation and persistence in the environment and host. Virulence factors that are involved in both biofilm formation and pathogenesis are discussed below for major zoonotic bacterial pathogens.

Virulence factors involved in host cell interaction and biofilm formation by zoonotic bacterial pathogens

***Listeria monocytogenes*:** *Listeria monocytogenes* is a rod-shaped, Gram-positive, facultative intracellular pathogen and is ubiquitously distributed in nature. Improperly produced silage is a major source of *L. monocytogenes* in ruminants, while ready-to-eat meat, dairy products, fruits and vegetables are the major source for humans. *L. monocytogenes* being an etiologic agent for mastitis in cows, milk is one of the major sources (Winter *et al.*, 2004). The major route of pathogen transmission to humans and animals is oral. Therefore, the gastrointestinal phase of infection is central to its systemic dissemination (Drolia and Bhunia, 2019; Lopes-Luz *et al.*, 2021). As a foodborne pathogen, crossing the intestinal epithelial barrier is a critical step for disease progression.

L. monocytogenes uses three major invasive pathways: (a) *Listeria* adhesion protein (LAP), (b) Internalin A (InlA), and (c) M-cell-mediated translocation to cross the intestinal barrier (Drolić and Bhunia, 2019). It then systemically disseminates to the spleen, liver, mesenteric lymph node, gall bladder, brain, and placenta in pregnant women. Epithelial cell invasion and cell-to-cell movement allow bacteria to maintain an intracellular lifestyle aided by numerous virulence factors (Lopes-Luz *et al.*, 2021). *L. monocytogenes* invades the central nervous system to cause meningitis and encephalitis as manifested by circling disease (due to the loss of balance and gait), common in ruminants. The bacterium also causes abortion, premature birth, and stillbirth in the ovine and humans. Three major virulence factors including actin polymerization protein (ActA), *Listeria* adhesion protein (LAP), and Teichoic acid are shown to play a central role in biofilm formation. ActA, regulated by PrfA facilitates bacterial cell-to-cell movement by rearranging host cell cytoskeletal proteins. It also promotes bacterial aggregation and biofilm formation on intestinal epithelial cells and aids in disease progression (Travier *et al.*, 2013).

Listeria teichoic acid is a structural component of the cell wall (peptidoglycan) consisting of alternating phosphate and ribitol or glycerol. Teichoic acid helps bacterial attachment to the host cells and also contributes to biofilm formation in *L. monocytogenes* (Zhu *et al.*, 2018). Tunicamycin (antibiotic) inhibits teichoic acid biosynthesis, thus interferes with *L. monocytogenes* adhesion and invasion to intestinal epithelial cells and biofilm formation (Zhu *et al.*, 2018).

The involvement of *Listeria* adhesion protein (LAP) (Drolić *et al.*, 2018) in biofilm formation has been demonstrated in recombinant *Lactobacillus casei* expressing LAP from *L. monocytogenes* in a cultured cell model and mouse intestinal villi (Drolić *et al.*, 2020). Though biofilm-forming *L. monocytogenes* has attenuated infectivity in a cell culture model, it is highly infective in a mouse

model after oral dosing (Bai *et al.*, 2021a) suggesting management of biofilm can potentially reduce the zoonotic spread of the disease.

***Staphylococcus aureus*:** *Staphylococcus aureus* is a Gram-positive opportunistic pathogen and is commonly found in water, soil, animal mucus membrane, udder, anterior nares and on skin (Balasubramanian *et al.*, 2017; Miao *et al.*, 2017). It is also salt tolerant and as a commensal skin bacterium, often becomes a source of the contaminant in foods that are heavily handled. It also causes mastitis; hence milk and dairy products serve as a vehicle. Livestock-associated methicillin-resistant *S. aureus* (LA-MRSA) presents the major challenge due to its resistance to antibiotics. Antibiotic treatment for skin infection is under heightened scrutiny because of biofilm formation and widespread antibiotic resistance.

S. aureus is a strong biofilm former and helps bacterium to persist and cause chronic infection (Moormeier and Bayles, 2017; Di Domenico *et al.*, 2019). A large number of surface molecules, collectively referred to as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) help bacterial adhesion and biofilm formation (Corrigan *et al.*, 2007; Moormeier and Bayles, 2017). The major biomolecules that are specifically involved in both biofilm formation and host cell adhesion have redundant functions. Those include biofilm-associated protein (Bap), fibronectin-binding proteins (FnBP), *S. aureus* surface protein G (SasG), *S. aureus* protein A (SpA), cell wall-anchored clumping factor (ClfA), polysaccharide intercellular adhesin (PIA), and teichoic acids (TA) (Zapotoczna *et al.*, 2016; Bai *et al.*, 2021b).

***Clostridium perfringens*:** *Clostridium perfringens* is a Gram-positive, rod-shaped, spore-forming, obligate anaerobe (aerotolerant) and inhabits human and animal intestines. This organism produces multiple toxins and is responsible for gas gangrenes, enteritis

necroticans (pigbel), dysentery, enteritis, and enterotoxaemia in livestock. In humans, as a foodborne pathogen, it causes food poisoning, diarrhea, and antibiotic-associated diarrhea similar to *Clostridium difficile*. Biofilm formation and resistance to multiple antibiotics are important attributes of persistence and pathogenesis in livestock (Hu *et al.*, 2018). *C. perfringens* Agr-like (CpAL) molecule is one of the major quorum-sensing molecules and regulates biofilm formation and toxin secretion (Vidal *et al.*, 2015). Biofilm formation is aided by Type IV pili and CcpA protein. Furthermore, biofilm also protects cells from antibiotic and oxygen-induced damage (Varga *et al.*, 2008).

***Escherichia coli*:** *E. coli* is a Gram-negative, facultatively anaerobic commensal bacterium of the intestinal tract of animals and humans. Flagella, fimbriae or pili, and curli and lipopolysaccharides are important accessory molecules that help bacterial motility and colonization in the intestine. Only a few strains of *E. coli* strains are pathogenic, which are classified as enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC), enteroinvasive *E. coli* (EIEC), adherent invasive *E. coli* (AIEC), and Shiga toxin-producing *E. coli* (STEC) that include enterohemorrhagic *E. coli* (EHEC) (Croxen *et al.*, 2013). Toxin production and epithelial adhesion, colonization are essential traits of pathogens. ETEC is responsible for piglet and calf diarrhea. Animals suffer from dehydration and electrolyte loss, which may be fatal. Survivors may experience poor growth. In humans, ETEC causes traveler's diarrhea, which may be fatal in severe cases. STEC strains, especially the EHEC strains can cause lethal foodborne diseases leading to hemorrhagic colitis and hemolytic uremic syndrome, often associated with kidney failure and death (Newell and La Ragione, 2018). Raw meat or meat products (Currie *et al.*, 2019) and fresh produce (Yaron and Romling, 2014; de Oliveira Elias *et al.*, 2019) are major sources of STEC.

E. coli form biofilms in the gastrointestinal tract employing adhesion molecules, and extracellular matrix including curli and cellulose (Rossi *et al.*, 2018). Curli is consisted of amyloid protein nanofibers and is responsible for cell-to-cell and cell-to-surface attachment (Patel *et al.*, 2011; Nguyen *et al.*, 2014). Cellulose is the major exopolysaccharide consisting of (1–4)- β -linked linear glucose chain. Cellulose production is dependent on the activation of bacterial cellulose synthesis (BcsA) by cyclic-di-GMP (Römling *et al.*, 2013). Some *E. coli* strains (EAEC) can form a thick aggregating biofilm (stacked-brick) using aggregative adherence fimbriae (AAF) on the intestinal mucosal surface and cause diarrhea (Boll *et al.*, 2017).

***Salmonella enterica*:** *Salmonella enterica* is a Gram-negative rod-shaped bacterium and has a broad host range infecting animals, poultry, and humans. There are over 2500 serovars of *Salmonella enterica* and some are host-specific. Among the *Salmonella enterica* serovars, Enteritidis, Typhimurium, Newport, Heidelberg, Javiana, Dublin, and Choleraesuis are representative of commonly occurring zoonotic serovars (Boore *et al.*, 2015; Snyder *et al.*, 2019). These serovars cause severe foul-smelling diarrhea and septicemia in animals. Besides meat, milk, poultry, and eggs, *Salmonellae* also are transmitted via fresh produce, nuts, spices, and flours. Salmonellae infection in the ovary of laying hen is responsible for trans-ovarian transmission and contamination in eggs. Non-typhoidal *Salmonella* (NTS) also known as gastroenteritis-causing salmonellae are responsible for 93.8 million illnesses and 155,000 deaths in humans worldwide (Majowicz *et al.*, 2010; Kirk *et al.*, 2015). Salmonellae also form biofilms on human and animal intestines (Steenackers *et al.*, 2012) and biofilm formation is facilitated by curli, flagella, Bap, cellulose, e-DNA, c-di-GMP, and quorum sensing (Almeida *et al.*, 2017; Harrell *et al.*, 2020; Lories *et al.*, 2020; Rana *et al.*, 2021). c-di-GMP signaling network also supports the transition between virulence

(invasion) and biofilm formation in *Salmonella* (Ahmad *et al.*, 2011). The QS inhibitors have been considered potential therapeutics for *Salmonella* infection (Almeida *et al.*, 2018; Aswathanarayan and Vittal, 2018)

***Campylobacter* spp.:** *Campylobacter* sp. is a Gram-negative, curved or spiral (helical) rod. Its cork-screw motility is facilitated by a single polar flagellum. There are four major species; *C. jejuni*, *C. coli*, *C. fetus*, and *C. lari*. *Campylobacter* causes abortion in animals and as a zoonotic organism; it can be transmitted from animals to humans via food or contact. Poultry, eggs, raw milk are common sources of this organism. After ingestion, this organism invades and causes cell damage in the lower small intestine. It produces a cytolethal distending toxin (CDT) and the infection results in severe pain mimicking acute appendicitis. It also causes fever, diarrhea, meningitis, endocarditis, pancreatitis, and reactive arthritis. Sequelae of this disease are Guillain Barre's Syndrome, manifested by a paralytic neurological disorder. *Campylobacter* produces firm biofilms. Flagella, capsular polysaccharides, lipo-oligosaccharide, e-DNA, cell-binding proteins, Peb1A (CBF1), and Peb4 (CBF2) which assist in tissue adhesion and biofilm formation (Kim *et al.*, 2015; Tram *et al.*, 2020). Quorum sensing molecules including AI-2 and LuxS play important role in biofilm formation (Plummer *et al.*, 2011).

***Yersinia* spp.:** The genus *Yersinia* is a Gram-negative coccoid rod and is considered a major zoonotic pathogen. Three major species including *Y. enterocolitica*, *Y. pseudotuberculosis*, and *Y. pestis* are important and share common O antigens. In addition, all three species also share *Yersinia* outer membrane proteins (YOPs), V (immunogenic protein), and W (nonprotective lipoprotein) antigens. *Y. enterocolitica* and *Y. pseudotuberculosis* are enteropathogenic and responsible for gastroenteritis and *Y. pestis* is responsible for the plague. Pig serves as the primary reservoir

for *Y. enterocolitica* and *Y. pseudotuberculosis* while rodents and flea are for *Y. pestis*.

All three pathogens carry chromosomal and plasmid (pVY)-encoded virulence factors, which are required for adhesion, invasion, and colonization of intestinal epithelial cells and lymph nodes, growth inside macrophages, macrophage apoptosis, and serum resistance. The pVY plasmid-encoded virulence factors are also involved in biofilm formation (Wang *et al.*, 2019). Flagella and exopolysaccharides are important factors regulated by RpoS in biofilm formation (Guan *et al.*, 2015).

Conclusions

Zoonotic bacterial pathogens express several molecules that are essential for biofilm formation, quorum sensing, and adhesion to host cells and tissues for colonization. Many of these molecules have redundant functions. Therefore, in the absence of one or more molecules, bacteria still are capable of forming biofilm for colonization, persistence, and infection. Quorum sensing and c-di-GMP network are critical for biofilm formation. Hence, strategies to inhibit QS and c-di-GMP provide an opportunity for pathogen control in livestock and humans as an alternative to antibiotics.

Conflict of interest: Authors have no conflict of interest in this study.

ACKNOWLEDGMENTS

The research in the authors' laboratory is supported in part by a grant from the National Academy of Science (NAS) and USAID (AID-263-A-15-00002), Korean Food Research Institute (KFRI grant # E0142102-03), the U.S. Department of Agriculture, Agricultural Research Service, under Agreement No. 59-8072-6-001, and the USDA National Institute of Food and Agriculture (Hatch accession no. 1016249). Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the view of the USDA, USAID, or NAS.

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Received – 30.09.2021, Accepted – 10.11.2021, Published – 01.12.2021

Section Editors: Prof. S. Biswas and Dr. A. K. Das, Members, Editorial Board