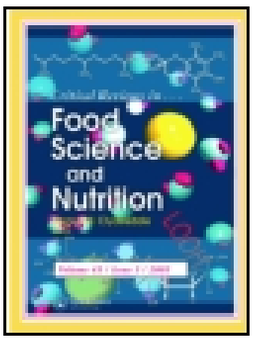

Critical Reviews in Food Science and Nutrition

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/bfsn20

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To cite this article: Unji Kim, Jin-Hee Kim & Se-Wook Oh (2021): Review of multi-species biofilm formation from foodborne pathogens: multi-species biofilms and removal methodology, Critical Reviews in Food Science and Nutrition, DOI: 10.1080/10408398.2021.1892585

To link to this article: https://doi.org/10.1080/10408398.2021.1892585

Published online: 05 Mar 2021.

CRITICAL REVIEWS IN FOOD SCIENCE AND NUTRITION
https://doi.org/10.1080/10408398.2021.1892585

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REVIEW

Review of multi-species biofilm formation from foodborne pathogens: multi-species biofilms and removal methodology

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ABSTRACT

Multi-species biofilms are ubiquitous worldwide and are a concern in the food industry. Multi-species biofilms have a higher resistance to antimicrobial therapies than mono-species biofilms. In addition, multi-species biofilms can cause severe foodborne diseases. To remove multi-species biofilms, controlling the formation process of extracellular polymeric substances (EPS) and quorum sensing (QS) effects is essential. EPS disruption, inhibition of QS, and disinfection have been utilized to remove multi-species biofilms. This review presents information on the formation and novel removal methods for multi-species biofilms.

KEYWORDS

Food microbiology; food safety; extracellular polymeric substances; quorum sensing

Introduction

Biofilms are an aggregation of microorganisms, including bacteria, fungi, viruses, protozoa, and algae (Costerton, Stewart, and Greenberg 1999). In the food industry, biofilms formed by foodborne pathogens are a growing concern. Foodborne pathogens easily form biofilms on the surfaces of fresh cut fruits and vegetables, including cantaloupe melon (Almasoud et al. 2016), lettuce, and cabbage (Zhang et al. 2018). In addition, biofilms are formed on food contact surfaces, increasing the risk of cross-contamination (Lequette et al. 2010). Furthermore, biofilms are more antibiotic-resistant than planktonic cells (Sib et al. 2019) and can lead to serious foodborne diseases (Lee et al. 2014). Therefore, biofilm formation in food-related environments can result in severe public health problems.

Biofilm formation is a primary factor in determining the initiation of bacteria-mediated diseases (Costerton, Geesey, and Cheng 1978). However, biofilms are difficult to eradicate using conventional sanitizers (Pan, Breidt, and Kathariou 2006). In various bacterial species, the production of extracellular polymeric substances (EPS) and quorum sensing (QS) effects (Flemming, Neu, and Wozniak 2007) plays an important role in the biofilm formation process (Parsek and Greengerg 2005). Thus, controlling the formation process of EPS and QS effects is essential in removing biofilms. In the past years, biofilm studies have focused on mono-species biofilms produced by a single pathogen, such as *Escherichia coli* O157:H7 (Niemira and Cooke 2010), *Listeria monocytogenes* (Ölmez and Temur 2010), and *Salmonella* Typhimurium (Wong et al. 2010). However, current studies have focused on dual-species biofilms (Knowles et al. 2005) and multi-species biofilms (Bridier et al. 2015) because of their complexity and ubiquity.

Multi-species biofilms are abundant in the natural environment and food industry (Yuan et al. 2019). The complexity of multi-species biofilms has been linked to their interactions, metabolism, and QS. Furthermore, when pathogens interact and form multi-species biofilms, their resistance to antimicrobial therapies becomes higher compared to the condition of their presence than in mono-species biofilms (Burmølle et al. 2006). Recent studies of multi-species biofilms have been established to elucidate the complexity and develop removal methods. However, the mechanism is not fully understood, and the potential effects have not been stressed in the field of food safety.

This review aims to summarize the current studies of multi-species biofilms formed by various foodborne pathogens and provide the importance of multi-species biofilms in the food industry. This review outlines the formation and removal methodology of mono-species and multi-species biofilms. The removal methods are summarized by controlling EPS and QS effects.

Mono-species biofilms

Mono-species biofilms in the food industry

Biofilms are an accumulation of microorganisms embedded inside EPS (Flemming, Neu, and Wozniak 2007). Foodborne pathogens have the ability to form biofilms on food surfaces and food contact surfaces, thereby posing a threat to the food industry. Biofilms produced by foodborne pathogens affect agricultural products, such as fresh cut fruits and vegetables (Moriarty et al. 2019). *E. coli* O157:H7 is a major foodborne disease-causing pathogen that produces biofilms (Niemira and Cooke 2010) on pores and veins of vegetables (Cui et al. 2020). In Silagyi et al.'s (2009) study, *E. coli*

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O157:H7 form biofilms on poultry, meat, carrot, and ready-to-eat deli. *L. monocytogenes* (Ölmez and Temur 2010) and *Bacillus cereus* (Peng, Tsai, and Chou 2002) are also biofilm-forming foodborne pathogens known to form biofilms on food contact surfaces, such as stainless steel (Borucki et al. 2003). Biofilm formation on food contact environments is closely linked to the cross-contamination of pathogens to the food (Hansen and Vogel 2011). In addition, *S.* Typhimurium form biofilms on the surface of the cantaloupe melon (Fu et al. 2017) and ready-to-eat shrimp (Beshiru, Igbinosa, and Igbinosa 2018). Various kinds of foodborne pathogens, including *Campylobacter* spp. (Li et al. 2017), *Staphylococcus aureus* (Chen et al. 2020), and *Pseudomonas fluorescens* (Wang et al. 2018), can form biofilms in food-related environments. Thus, adequate treatments are required.

Mono-species biofilm formation

Biofilm formation is a multistep process that requires bacterial adhesion, maturation, and dispersion processes. Bacterial adhesion can be divided into primary and secondary stages (Palmer, Flint, and Brooks 2007). In the primary stage, planktonic cells move near the conditioned surface (Silva et al. 2014). In Hori and Matsumoto (2010) and Guttenplan and Kearns (2013) studies, bacterial nanofibers play an important role as adhesin on both biotic and abiotic surfaces. Bacterial nanofibers, such as pili and flagella, regulate the motility of foodborne pathogens, such as *Bacillus*, *Pseudomonas*, *Vibrio*, and *Escherichia*. Polysaccharides, a major component of EPS, also function as a significant mediator of bacterial adhesion. According to Tsuneda et al. (2003) study, as the number of polysaccharides increased, the amount of bacterial adhesion on solid increased as well. Bacterial adhesion during the primary stage is detachable. Therefore, the primary stage is also referred to as a reversible attachment stage.

If the surface conditions are favorable for cells to mature, the primary stage changes into the secondary stage governed by several factors, including SadB (Caiazza and O'Toole 2004), SadC (Merritt et al. 2007), cAMP (Ono et al. 2014), and LapA (Hinsa et al. 2003). In the secondary stage, bacterial adhesion is irreversible (Costerton, Stewart, and Greenberg 1999). After the irreversible attachment stage, bacterial cells on the surface mature and form a microcolony (Prakash, Veeregowda, and Krishnappa 2003).

Bacterial cells undergo QS by communicating using various autoinducers (Hammer and Bassler 2003). QS is a cell-to-cell communication that plays a vital role before and after bacterial adhesion of mono-species and multi-species biofilms (Federle and Bassler 2003). When the level of autoinducers reaches a threshold level, exopolysaccharide production is activated (Tan et al. 2014). Afterward, bacterial species protected by EPS produce polysaccharide enzymes during different growth phases for the EPS dispersal (Prakash, Veeregowda, and Krishnappa 2003). EPS degradation allows bacterial species inside the matrix to either form a new biofilm or flow as a planktonic cell (Wijman et al. 2007). In addition, biofilms can detach via physical mechanisms, such as sloughing (Hunt et al. 2004) and erosion (Telgmann, Horn, and Morgenroth 2004).

Removal methods for mono-species biofilms

Numerous studies of mono-species biofilms have been developed to eradicate biofilms in food-related environments. In this review, the removal methods of biofilms are classified into three categories: control of the EPS formation process, EPS disruption, and inhibition of QS effect.

EPS disruption

EPS is also called the "house of biofilm cells," which inhibits antimicrobial agents from killing microorganisms embedded under EPS (Flemming, Neu, and Wozniak 2007). Thus, controlling the formation and decomposing the structures of EPS have been utilized as anti-biofilm methods. EPS is mainly comprised of polysaccharides, extracellular DNA (eDNA), and proteins (Flemming, Neu, and Wozniak 2007), and each component has distinct functions (Periasamy et al. 2015). In biofilms, EPS is the first layer that must be penetrated by disinfectants for biofilm removal (Rickard et al. 2003).

Enzymes can be used for the inhibition of EPS production and penetration of EPS polymers. eDNA, one of the components of EPS, functions as a factor of initial attachment and aggregation of planktonic cells. DNase I is an enzyme that degrades eDNA. Das et al. (2010) demonstrated that the presence of DNase I reduced bacterial adhesion on surfaces, thereby inhibiting biofilm formation. In addition, DNase I inhibited the initial formation of EPS of *Pseudomonas aeruginosa* biofilms (Whitchurch et al. 2002). Protease, which promotes proteolysis, can be used for the breakdown of EPS. Molobela, Cloete, and Beukes (2010) found out that protease enzymes showed high degrading effects of *P. fluorescens* biofilms due to the high amount of protein in EPS produced by *P. fluorescens*. In addition, enzyme treatments showed a higher removal effect of biofilms on food contact surfaces than clean-in-place treatment and reduced water loss, energy loss, and use of chemical compounds (Lequette et al. 2010).

Essential oils (EOs), which have long been used as antimicrobial agents in the food industry (Gutierrez, Barry-Ryan, and Bourke 2008), showed effects on biofilm eradication. Oregano EOs eliminated *S. aureus* biofilms formed on stainless steel slides with not even destroying the surface of the food contact environment (dos Santos Rodrigues et al. 2018). EOs of *Cymbopogon citratus* (de Oliveira et al. 2010), limonene, and linalool inhibited biofilm formation (Valeriano et al. 2012). In recent years, EO encapsulation has been investigated to protect antimicrobial agents (Girardi et al. 2017). Cui et al. (2020) encapsulated clove EOs [clave oil solid liposomes (SLPs)] for the eradication of biofilms formed on cucumber skin and lettuce. As a result, SLPs reduced the number of bacteria from 6.377 to 2.431 Log CFU/cm^2 and decreased the concentration of protein

Table 1. Methods of mono-species biofilms removal by targeting EPS disruption.

Foodborne pathogens	Biofilm forming surfaces	Anti-biofilm methods	Reference
P. fluorescens	Glass wools and microtiter plates	Savinase, everlase, amyloglucosidase, polarzyme, protease, and amylase	Molobela, Cloete, and Beukes (2010)
B. cereus and 24 bacterial isolates from food environments or processed milk	Microtiter plates and stainless steel slides	Serine protease P2 and P4, papain P1 and P3, α -amylase S1, polysaccharidase mix A (cellulase and hemicellulase), and polysaccharidase mix B (cellulase/ hemicellulase, α-amylase S1, and β-glucanase)	Lequette et al. (2010)
Staphylococcus epidermidis	Glass and dimethyldichlorosilance	DNase I	Das et al. (2010)
S. aureus	Microtiter plates, stainless steel slides	Oregano EO and carvacrol	dos Santos Rodrigues et al. (2018)
E. coli O157:H7	Cucumber skin, lettuce	Clove oil SLPs	Cui et al. (2020)
B. cereus	50 mL conical tubes with stainless steel, glass, polyethylene, and polypropylene or wood	Chlorine, chlorine dioxide, alcohol, and quaternary ammonium compound	Kim et al. (2019)
E. coli O157:H7 and *S.* Typhimurium	Fresh spinach and cantaloupe melon	Lactic acid, malic acid, and a combination of lactic and malic acids	Almasoud et al. (2015)
E. coli O157:H7, *L. monocytogenes* EGD-e, and *S. aureus* SC-01	Microtiter plates	Carvacrol	Espina et al. (2017)
S. epidermidis	CDC biofilm reactor	Pac3 and Vmh2	Artini et al. (2017)
E. coli and *S.* Typhimurium	Stainless steel slides and microtiter plates	*L. brevies* DF01 bacteriocin isolated from kimchi	Kim et al. (2019)

and polysaccharides, thereby inhibiting the secretion of extracellular substances. In addition, SLPs extended the storage period.

Bacteriophages, lyase viruses for a specific bacterium, have been utilized as a removal factor of biofilms (Soni and Nannapaneni 2010). In Kim, Kim, et al. (2019) study, *Lactobacillus brevis* DF01 bacteriocin isolated from kimchi efficiently inhibited mono-species biofilms of *S.* Typhimurium and *E. coli*. The inhibition mechanism was affected by cell wall synthesis, thereby inhibiting EPS production.

The control of food contact surfaces by hydrophobin coatings with Pac3 and Vmh2 (Artini et al. 2017) and organic acid treatments, such as malic and lactic acids (Almasoud et al. 2015), was evaluated. Studies on the removal of mono-species biofilms by targeting EPS disruption are listed in Table 1.

QS inhibition

QS is a vital step in biofilm formation. Therefore, inhibition of QS effects can regulate biofilm formation (Hentzer et al. 2002), and there are several pathways for the inhibition of QS effects. Removing autoinducers using antibodies and enzymes, inhibiting the synthesis of autoinducers, and blocking autoinducer receptors (Jang et al. 2013) have been used for QS inhibition.

QS interrupting molecules, also called quorum quenching (QQ) molecules, have been investigated to control biofilms. QQ enzymes penetrate and destroy autoinducers, which are known as important molecules in communication. Acylated homoserine lactone (AHL-)-lactonase, AHL-acylase, and oxidoreductase are known as major QQ enzymes (Fetzner 2015).

Bacteriocin, a peptide produced in the bacterial ribosome, interrupted cell-to-cell communication during biofilm formation. Melian et al. (2019) investigated the effect of lactocin AL 705 on the inhibition of QS effects using *Vibrio harveyi* BB170 as a biosensor. Accordingly, bacteriocin represented useful methods as food processing environments.

EOs, which have been used for EPS disruption, can also be used as a QS inhibitor (Zhang et al. 2019). Amrutham, Sundar, and Shetty (2017b) isolated *E. coli* and *S.* Typhimurium from fresh fruits and vegetables and evaluated the anti-QS effect of spice oil nanoemulsions using violacein. The reduction of violacein production from *Chromobacterium violaceum* indicates the inhibition of QS activity. As a result, EO of cumin showed a high anti-QS effect by inhibiting 42.2% of violacein production. Additionally, Algburi et al. (2020) demonstrated that EO of black cardamom inhibited the QS of *E. coli* O157:H7 and *S.* Typhimurium with the capacity of being an antioxidant.

Besides, organic acids including lactic acids, malic acids (Almasoud et al. 2016), acetic acids (Amrutham, Sundar, and Shetty 2017a), cinnamic acids (Zhang et al. 2019), and edible coatings of oregano EOs (Alvarez et al. 2014) on various products and environments inhibited QS effect, thereby inhibited the formation of biofilms on food surfaces and food contact surfaces. Studies associated with the removal of mono-species biofilms by targeting QS effect disruption are listed in Table 2.

Multi-species biofilms

Multi-species biofilms in the food industry

Multi-species biofilms are produced by more than two different kinds of microorganisms. In the food industry, multi-species biofilms formed by different kinds of foodborne pathogens cause problems in various aspects and are found everywhere (Yuan et al. 2019). The production of multi-species biofilms reduces the efficacy of antimicrobial agents (Pang, Yang, and Yuk 2017) and promotes the cross-contamination of foodborne pathogens from food contact surfaces to food surfaces (Liu et al. 2014). In Alonso, Harada, and Kabuki (2020) study, *L. monocytogenes* isolates from

Table 2. Methods of mono-species biofilms removal by targeting QS effect inhibition.

Foodborne pathogens	Biofilm forming surfaces	Anti-biofilm methods	Reference
L. monocytogenes	Stainless-steel slides	Bacteriophage P100	Soni and Nannapaneni (2010)
L. monocytogenes isolates from foods	Microtiter plates	Lactocin AL 705	Melian et al. (2019)
E. coli and *S.* Typhimurium isolated from fresh fruits and vegetables	Microtiter plates	EO nanoemulsions of cumin and pepper	Amrutha, Sundar, and Shetty (2017b)
E. coli O157:H7 and *S.* Typhimurium	Microtiter plates	Black cardamom EO	Algburi et al. (2020)
E. carotovora and *P. fluorescens*	Chinese cabbage petiole, lettuce leaf	Hexanal	Zhang et al. (2018)
E. coli O157:H7 and *S.* Typhimurium	Homogenates of spinach and cantaloupe melon	Lactic acid and malic organic acids	Almasoud et al. (2016)
E. coli and *Salmonella* spp	Cucumber	Lactic and acetic acids	Amrutha, Sundar and Shetty (2017a)
E. carotovora and *P. fluorescens*	96 well microtiter plate	Carvone, hexanal, carvacrol, citral, geraniol, salicylic acid, cinnamic acid, thymol, eugenol, and cinnamaldehyde	Zhang et al. (2019)
E. coli O157:H7, *S.* Choleraesuis, *S. aureus,* and *L. monocytogenes*	Fresh shrimp and cucumbers	Edible coatings of oregano EO and pectin-oregano EO	Alvarez et al. (2014)

dairy products formed multi-species biofilm with *B. cereus*, demonstrating that adequate treatments for eradicating multi-species biofilms for the dairy industry are required. In addition, foodborne pathogens isolated from fish (Wang et al. 2020), meat, and seafood (Puga et al. 2018) could all form multi-species biofilms.

Formation of multi-species biofilms

Bacterial adhesion

Like the mono-species biofilms, the formation of multi-species biofilms initiates with a reversible attachment stage of bacterial adhesion, followed by irreversible attachment, maturation, and dispersion (Jahid and Ha 2014). The basic procedures of multi-species biofilm formation are same as those of mono-species biofilm formation. However, multi-species biofilm formation requires more complex mechanisms, such as bacterial types and interactions, compared to mono-species biofilm mechanisms (Røder, Sørensen, and Burmølle 2016; Liu et al. 2016).

The attachment stage of biofilm formation requires physical and biological properties (Van Houdt and Michiels 2010). Physical properties include electrostatic and van der Waals forces (Van Houdt and Michiels 2010). Biological properties responsible for the interaction between the bacteria and surface include flagella (Lemon, Higgins, and Kolter 2007), fimbriae (Schroll et al. 2010), curli (Boyer et al. 2007), pili (Klausen et al. 2003), and surface polysaccharides (Wang, Preston, and Romeo 2004). In Carter et al. (2016) study, multi-species biofilms formed by spinach-leaf isolated bacteria and *E. coli* O157:H7 were mediated by fimbriae. Additionally, curli increased the amount of attachment of multi-species biofilms to the spinach-leaves.

The major difference between mono-species biofilms and multi-species biofilms is that interspecies interactions occur during multi-species biofilm formation. Interspecies interaction is an important factor in forming multi-species biofilms, and it can be synergistic (Habimana et al. 2010), antagonistic (Simões et al. 2008), or neutral (Kay et al. 2011). Synergism occurs when the species inside the EPS matrix cooperate (Christensen et al. 2002) or coaggregates (Rickard et al. 2003). In Liu et al. (2014) study, *E. coli* O157:H7 synergistically formed a dual-species biofilm with *Ralstonia insidiosa*, an environmentally isolated bacterium. Owing to the synergistic effect, the thickness of *R. insidiosa* increased from 10 to $>$ 20 µm. Burmølle et al. (2006) reported that when a synergistic interaction between pathogens occurs in multi-species biofilms, antimicrobial resistance increases more than individually formed biofilms. Antagonism occurs when bacterial interspecies compete or inhibit growth because of the metabolic and environmental effects of an antibacterial agent (Liao 2007; Winkelströter, Tulini, and De Martinis 2015). Tait and Sutherland (2002) researched the antagonistic effect of bacteriocin-producing bacteria in multi-species biofilms. They found that bacteriocin worked as an antagonistic compound and formed a competitive interaction. In *P. aeruginosa*, an AHL-containing culture worked as an antagonistic compound (Zhang, Ye, et al. 2014).

Maturation

After irreversible attachment, the maturation of multi-species biofilms begins (Jahid and Ha 2014). The maturation of multi-species biofilms is controlled by specific bacterial species (Almeida et al. 2011). In Ibusquiza et al.'s (2012) study, multi-species biofilms formed by *L. monocytogenes* and *Pseudomonas putida* were dependent on the bacterial species involved. Kostaki et al. (2012) found that the interaction between *L. monocytogenes* and *Salmonella* enterica in multi-species biofilms resulted in the alteration of the population and resistance. During maturation, EPS secretes, biofilms accumulates, and the complexity increases (Ramasamy and Zhang 2005).

QS also plays an important role in the maturation of biofilms. QS requires a chemical signal called an autoinducer and is dependent on the bacterial species and adhering positions (Waters and Bassler 2005). When the bacterial population increases to a quorum level, an autoinducer adheres to the transcription regulator and activates or represses the target gene (Smith, Fratamico, and Novak 2004).

The mechanism of QS between Gram-positive and Gram-negative bacteria is different. For Gram-positive bacteria, QS is regulated by specific autoinducer peptides (Kleerebezem et al. 1997). However, for Gram-negative bacteria, QS is regulated by LuxI-LuxR regulation homologs (Miller and Bassler 2001). In recent studies of QS, autoinducer-2 (AI −

Table 3. Methods of multi-species biofilm removal by targeting EPS disruption.

Multis-species biofilm producing pathogens	Biofilm forming surface	Anti-biofilm method	Reference
L. monocytogenes and *P. fluorescens* *L. monocytogenes* and *Pseudomonas* spp. *L. monocytogenes* and *Staphylococcus saprophyticus* *L. monocytogenes* and *Carnobacterium* spp. *L. monocytogenes* and *E. coli*	24 well microtiter plate	Pronase, cellulase, pectinase, DNase I, lysozyme, phospholipase, peroxidase, β-glucanase, and chitinase	Puga et al. (2018)
Comamonas, Enterobacteriaceae, *Pseudomonas, Stenotrophomonas, Nakamurella, Clostridium, Azospira, Sphingomonas*, and *Ferribacterium*	6 well polystyrene plates, glass slides, and polymethyl methacrylate	Vanillin with proteinase K or DNase I	Si and Quan (2017)
L. monocytogenes and *E. coli* *L. monocytogenes* and *P. fluorescens*	24 well microtiter plate, stainless steel slides	Pronase, cellulase, or DNase I alone or combine with benzalkonium chlorine	Rodriguez-Lopez et al. (2017)
P. fluorescens and *Staphylococcus lentus* (both strains were isolated from a dairy plant)	Stainless steel slides and microtiter plates	*S. lentus* phage φIBB-SL58B and *P. fluorescens* phage φIBB-PF7A	Sillankorva, Neubauer, and Azeredo (2010)
E. coli O157:H7 and *S.* Typhimurium	96 well polystyrene plates	Vanquish and chlorine solution	Wang et al. (2013)
E. coli and *S. aureus*	96 well microtiter plate	Rutin	Al-Shabib et al. (2017)
E. coli O157:H7 and *Salmonella* strain 457-88	96 well polystyrene plates	Levulinic acid with sodium dodecyl sulfate	Chen, Zhao, and Doyle (2015)
Burkholderia cepacian and *P. aeruginosa*	Biofilm tubing reactor	Chlorine	Behnke et al. (2011)
P. aeruginosa and *Listeria* innocua FI2 *S. aureus* FI2 and *L.* innocua FI2	24 well polystyrene plates and polycarbonate coupons	Quaternary ammonium compound-based disinfectants, tertiary alkyl amine-based disinfectants, and chlorine-based disinfectants	Kocot and Olszewska (2020)
L. monocytogenes, *E. faecium*, and *E. faecalis*	Stainless steel coupon	Anionic tensioactive cleaning, acid-anionic tensioactive cleaning with disinfection, anionic tensioactive cleaning with disinfection, acid-anionic tensioactive cleaning with disinfection, and chlorinated alkaline cleaning	da Silva Fernandes, Kabuki, and Kuaye (2015)
E. faecium, E. faecalis, and *B. cereus*	Stainless steel slides	Anionic tensioactive cleaning, acid-anionic tensioactive cleaning, disinfection, anionic tensioactive cleaning + disinfection, acid-anionic tensioactive cleaning + disinfection, and chlorinated alkaline cleaning	da Silva Fernandes et al. (2017)
L. monocytogenes and *S.* Typhimurium	Small polystyrene Petri dish	CAP	Govaert et al. (2019)

2; H. Zhang, Ye, et al. 2014), autoinducer-3 (AI-3; Walters, Sircili, and Sperandio 2006), and AHL (Lee et al. 2013) have been used as QS molecules. In multi-species biofilms formed from *Streptococcus mitis* and *P. aeruginosa* PAO1, the AI-2 system from *S. mitis* played an important role by promoting the biofilm formation of PAO1 (Wang et al. 2016). AI-2 in multi-species biofilms formed from *E. coli* and *Enterococcus faecalis* increased the stress resistance and growth rates of *E. coli* microcolonies (Laganenka and Sourjik 2017).

Dispersion

When detachment occurs, bacterial species inside EPS release and either form a new colony or flow as a planktonic cell (Jahid and Ha 2014). The dispersion of the attached cells is caused by various intrinsic and extrinsic factors (McDougald et al. 2012), including shear effects (Rochex et al. 2008) and fluid dynamics (Brugnoni, Lozano, and Cubitto 2007). For dispersion, bacterial species inside the matrix make polysaccharide enzymes (Flemming and Wingender 2010). For example, *P. fluorescens* makes lyase as a polysaccharide enzyme (Allison et al. 1998). *P. aeruginosa* produces alginate lyase for the degradation of alginate, a secreted polysaccharide (Franklin et al. 2011). As described above, the dispersion of a biofilm is divided into erosion (Telgmann, Horn, and Morgenroth 2004) and sloughing (Hunt et al. 2004).

Removal methods for multi-species biofilms

Recent studies have focused primarily on the removal method of mono-species biofilms (Shi and Zhu 2009). However, multi-species biofilms are ubiquitous in natural environments, and multi-species biofilms are more resistant to anti-biofilm agents (Rickard et al. 2003). Thus, studies focused on the removal of multi-species biofilms need to be evaluated. In this review, the removal methods of multi-species biofilms are classified into two categories: EPS disruption and QS inhibition.

Table 4. Methods of multi-species biofilm removal by targeting QS effect disruption.

Multi-species biofilm producing pathogens	Biofilm forming surface	Anti-biofilm method	Reference
B. cereus and *P. fluorescens* (both isolated from sanitization solution)	96 well polystyrene tissue culture microtiter plates	Ferulic acid and salicylic acid alone or in combination	Lemos et al. (2014)
P. fluorescens and *S. aureus* (isolated from raw yellow croaker)	Stainless steel slides	Carvacrol	Wang et al. (2020)
Serratia sp. JSB1 and *Rhodococcus* sp.BH4 *Bacillus* sp. JSB2 and *Rhodococcus* sp.BH4 *Aeromonas* sp. JSB4 and *Rhodococcus* sp.BH4 Str.JSB11 and *Rhodococcus* sp.BH4 *Bacillus* sp. JSB13 and *Rhodococcus* sp.BH4 *Bacillus* sp. JSB22 and *Rhodococcus* sp.BH4	96 well polystyrene plates	*Rhodococcus* sp.BH4	Maddela and Meng (2020)
Comamonas, Enterobacteriaceae, Pseudomonas, Stenotrophomonas, Nakamurella, Clostridium, Azospira, Sphingomonas and *Ferribacterium*	6 well polystyrene plates, glass slides, and polymethyl methacrylate	Vanillin, proteinase K, and DNase I	Si and Quan (2017)
P. aeruginosa PAO1, *E. coli* TG1 and *E. coli* BL21	96 well polyvinyl chloride microtiter plates	Engineered T7 bacteriophage	Pei and Lamas-Samanamud (2014)

EPS disruption

Enzymes have been investigated as a removal factor for both mono-species and multi-species biofilms (Puga et al. 2018). In addition, the enzymes that could be used for removing EPS include pronase, cellulase, chitinase, phospholipase, pectinase, peroxidase, lysozyme, and β-glucanase. However, as the antimicrobial resistance of multi-species biofilms increases because of the production of complexed EPS, combination methods have been studied as novel methods. Si and Quan (2017) investigated the effect of proteinase K or DNase I combined with vanillin. The combination of enzyme treatments inhibited the multi-species biofilm formation by decreasing the number of exopolysaccharides and exoprotein productions. The use of pronase, cellulase, or DNase I combined with benzalkonium chloride has also been evaluated. The combination of an enzyme and benzalkonium chloride efficiently destroyed multi-species biofilms formed by *L. monocytogenes* and *E. coli* (isolated from fish processing plants) and *P. fluorescens* (Rodriguez-Lopez et al. 2017). In the combination methods of enzymes and benzalkonium chloride, enzymes penetrated the matrix of EPS, and benzalkonium chloride treatment performed bactericidal activity.

Bacteriophage treatments have been studied to remove mono-species biofilms, and bacteriophages have been used as an anti-biofilm agent of multi-species biofilms. Bacteriophages are viruses that lyse specifically targeted pathogens (Sulakvelidze, Alavidze, and Morris 2001). In Sillankorva, Neubauer, and Azeredo (2010) study, both single and combined phage treatments to a dual-species biofilm, formed by two bacterial strains isolated from dairy plants, were examined. Both single and dual treatments decreased the number of bacterial cells, and the combined phage treatment even reduced the number of planktonic cells released from biofilms.

Ready-to-eat foods are easily contaminated by food environments. In Wang et al. (2013) study, a phytocompound called rutin was investigated as a removal factor of multi-species biofilms formed of *E. coli* and *S. aureus*. As a result, rutin inhibited EPS production in individual bacteria and impaired multi-species biofilms. By analyzing the structure of biofilms using scanning electron microscopy images, a significant disturbance of EPS in multi-species biofilms was observed.

Combinations of levulinic acid and dodecyl sulfate have been evaluated to eradicate multi-species biofilms of *E. coli* O157:H7 and *Salmonella* strain 457-88 (Chen, Zhao, and Doyle 2015). Enumerating and visualizing multi-species biofilms by confocal laser scanning microscopy demonstrated that the combination was efficacious for removing multi-species biofilms (Chen, Zhao, and Doyle 2015).

Combined sanitation methods have been studied to eradicate multi-species biofilm of *Enterococcus faecium*, *E. faecalis*, and *B. cereus* (da Silva Fernandes et al. 2017). Sanitation procedures, including anionic tensioactive cleaning, acid-anionic tensioactive cleaning, disinfection, anionic tensioactive cleaning combined with disinfection, acid-anionic tensioactive cleaning combined with disinfection, and chlorinated alkaline cleaning, were performed. In da Silva Fernandes et al. (2017) study, disinfection procedures included sodium hypochlorite, peracetic acid, quaternary ammonium, biguanide, and chlorinated alkaline. Among the sanitation agents, peracetic acid was the most effective and sodium hypochlorite was the least effective in eradicating multi-species biofilm formation, like the findings in a previous study analyzing that analyzed effects on biofilms formed by *E. faecium*, *E. faecalis*, and *B. cereus* (da Silva Fernandes et al. 2017).

In the food industry, cold atmospheric plasma (CAP) is used as a sanitizing agent for abiotic surfaces. CAP methods do not require additional disinfection materials. In addition, CAP methods can damage the biofilm structure. In Govaert et al. (2019) study, CAT treatment was performed to eradicate multi-species biofilms formed by *L. monocytogenes* and *S.* Typhimurium. The age of multi-species biofilms increased the treatment time of CAP, although it was still effective in

the eradication. Studies on the removal of multi-species biofilms by targeting EPS disruption are listed in Table 3.

QS inhibition

AI-2 is a QS signaling molecule (Galloway et al. 2011). In multi-species biofilm formation, AI-2 collects and aggregates bacterial species together (Laganenka and Sourjik 2017). Therefore, AI-2 interruption can inhibit multi-species biofilm formation (Laganenka and Sourjik 2017).

Chemical compounds have been developed as anti-QS effects. In Jang et al. (2013) study, D-ribose bonded to the AI-2 receptor and inhibited the QS effects (Wang et al. 2016). Lemos et al. (2014) evaluated the QS inhibitory effect of phenolic acids, including ferulic acid and salicylic acid, by evaluating bacterial motilities. In Lemos et al. (2014) study, salicylic acid inhibited QS by showing a significant reduction of swimming motility compared to the control, and salicylic acid also demonstrated antimicrobial activity against multi-species biofilms formed of *B. cereus* and *P. aeruginosa*. Carvacrol was also efficient in inhibiting AI-2. Wang et al. (2020) investigated the formation and removal of multi-species biofilms cultivated in fish juice by detecting AI-2 and AHLs. As a result, polysaccharide production increased as two different pathogens interacted, but carvacrol was still efficient in removing multi-species biofilms. Studies on the removal of multi-species biofilms by targeting QS effect disruption are listed in Table 4.

Discussion

In this review, the formation and removal methods of mono-species biofilm and multi-species biofilm were discussed. This review expanded on previous reviews and added additional removal methods, such as EPS disruptors and QS effect inhibitors. Overall, enzymes, bacteriophage, EOs, and chemical compounds were used to control multi-species biofilm formation in the food industry. In this review paper, anti-biofilm mechanisms of the treatments were mainly separated as two target points: EPS disruption and QS effect inhibition. The treatments used for the removal of multi-species biofilms were all efficient. However, some points need to be improved.

First, growth media are known to play an important role in biofilm formation. However, interactions of growth conditions and the ability of multi-species bacteria to form biofilms are still lacking. To promote the multi-species biofilm formation, numerous factors, including growth medium, temperature, and pH are essential and these factors have been studied in the field of food safety. However, the effects of different growth media and various nutrient components in growth media have not been studied well.

Second, the number of foodborne pathogens present in foods is regulated by law. As for biofilms, there is no regulation for the number of biofilms present in the food products. Both mono-species and multi-species biofilms can cause serious foodborne diseases by cross-contaminating to food products. However, the relation between the number of biofilms and risk of developing foodborne diseases is still veiled.

Finally, the ability of bacterial species inside EPS to cause a threat to the food products is not evaluated. During the final stage of multi-species biofilm formation, dispersion of the matrix occurs, and bacterial species inside EPS either attach to another surface or flow as planktonic cells. Thus, there is a possibility of planktonic pathogens inside EPS to cause foodborne illnesses.

Studies have found that biofilms are present in fresh cut fruits and vegetables, meat, shrimp, fish processing plants, and dairy plants. Biofilms formed on both food surfaces and food contact surfaces can induce cross-contamination, thereby causing threats in the food industry. The main problem of biofilms is that biofilms are resistant to both physical and chemical sanitization methods. Moreover, the resistance of multi-species biofilms, which are ubiquitous in the food industry than mono-species biofilms, to antimicrobial agents is higher compared to the resistance of mono-species biofilms to antimicrobial agents. Recent studies of multi-species biofilms have demonstrated the danger of multi-species biofilms to the food industry. However, specific risk factors have not been elucidated. Therefore, research needs to be performed on multi-species biofilm formation mechanisms, focusing on bacterial adhesion, interspecies interaction, EPS biosynthesis, and QS pathways.

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