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RESEARCH REVIEW

A Review of Microbial Biofilms of Produce: Future Challenge to Food Safety

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Abstract Outbreaks of produce-related food-borne pathogens have undergone a sharp increase in last three decades because of high produce consumption. A paradigm of food safety for produce is important due to its susceptibility to microbial attack and biofilms formation. Greater attention should be paid to decontaminating the pathogens in biofilms as they pose a risk to public health. This review will focus on produce-related outbreaks, attachments, quorum sensing, biofilms formation, resistance to sanitizers and disinfectants, and current and emerging control strategies for fresh and minimally processed produce, providing new insight into food safety. The consequences of biofilms formation on produce include the formation of a protective environment that is resistant to cleaning and disinfection. Alternative means of controlling or inhibiting biofilms formation on produce will be explained briefly and we will identify where additional research is needed.

Keywords: biofilm, quorum sensing, disinfection, produce, food-borne pathogenic bacteria

Introduction

The World Health Organization (WHO) cites food safety as one of the top 11 priorities and challenges of this century (1). WHO has undertaken an initiative to know the real global burden of food-borne diseases (2). Currently, food-borne diseases are a primary public health concern in both developing and developed countries. In 2005, WHO (3) reported 1.8 million mortality cases of diarrheal diseases worldwide, while in industrialized countries 30% of people suffered from food-borne diseases. In the USA every year, it has been estimated that 48 million people suffer from food-borne diseases, with 2,612 related deaths (4). Biofilms are involved in over 65% of all microbial diseases according to the US National Health Institute (NIH) and the Centers for Disease Control and Prevention (CDC) (5). It is now well-documented that food-borne pathogens could form biofilms on produce, which are a major cause of outbreaks. Most studies of biofilms formation on produce have revealed that they are resistant to commonly used sanitizers and disinfectants. The objectives of the review are to summarize the efficacy of current disinfectants used to decontaminate microorganisms present in biofilms of produce biofilms and control the produce-related outbreaks by alternative approaches.

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Microbial Ecology of Produce

Generally, the term 'produce' refers to raw and ready-to-eat vegetables, fruits, or goods made from these, which are also called commodities. In general, produce have high contents of carbohydrate and high water activity for the growth of microorganisms. Produce can naturally carry non-pathogenic epiphytic microflora, including bacteria, viruses, fungi, and parasites (6). The surfaces of plants usually have Gram-negative bacteria of Enterobacteriaceae and *Pseudomonas* spp. (7). The presence, growth, and survival of microorganisms on produce depend on nutrients, the characteristics of microflora, and environmental conditions. It is already established that produce may become contaminated with pathogenic microorganisms at different stages: in the fields, during harvesting, transport, processing, distribution, marketing, or even in the home.

Potential pre-harvest sources of contamination include soil, feces (8), water for fungicides and insecticides, dust, insects, contaminated manure (9), irrigation water (10), wild and domestic animals, and human handling (9,11). In a recent review, Jacobsen and Bech (12) demonstrated that manure or irrigation water may contaminate and internalize *Salmonella* spp. in produce.

It is now well-documented that equipment used in harvesting, processing, washing, and transport; human contamination with hands; feces, wild and domestic animals, and water used for washing and cleaning might possibly cause post-harvest contamination of produce (13). Different sources suggest that the most prevalent pathogens on produce are Norovirus, pathogenic *Escherichia coli*, *Salmonella* spp., *Listeria monocytogenes*, *Shigella* spp., *Yersinia enterocolitica*, and *Campylobacter* spp. (14,15). Frequency of diseases, types of pathogens, and produce outbreaks have been revealed in a review by Erickson (16). The frequency and incidence of contamination also depend on extrinsic environmental conditions (17). In Italy, Caponigro *et al*. (18) reported that 27% of samples were found to be *E. coli* positive amongst 1,158 ready-to-eat vegetables, including lettuce, arugula, spinach, and lamb's lettuce. Kim *et al*. (19) isolated *Bacillus cereus*, *Staphylococcus aureus*, *Vibrio parahaemolyticus*, *Clostridium perfringens*, *Yersinia enterocolitica,* and *L. monocytogenes* in Seoul, Korea from various food samples. To understand the ecology of pathogens of produce, Oliveira *et al*. (20) examined *Salmonella* Typhimurium following sequential passage through soils, fresh cut lettuce, and simulated gastrointestinal tracts and observed virulence activity to human tracts.

Outbreaks of Produce-related, Food-borne Illness

The increase in produce consumption has raised the number of food-borne illnesses worldwide (21). Although Americans have a greater awareness of food safety, 218 out of 1,034 food-borne diseases outbreaks were caused by Norovirus, followed by *Salmonella* spp., and shiga toxin producing *E. coli* (STEC) in the USA in 2008 (22). In Europe, 60 (out of 1,408 total food-borne diseases) outbreaks were reportedly due to produce during the period of 1992-1999 (21). Several reports have addressed outbreaks caused by produce (16,21,23,24). The world's biggest produce-related outbreak was caused by *E. coli* O157:H7 and affected 11,000 people in Japan in 1996 (25). The Pan-American Health Organization/World Health Organization (PAHO/WHO) reported 9,180 outbreaks between 1993 and 2010 in 22 countries (26). Gwack *et al*. (27) noted 1,026 outbreaks resulting in 25,310 affected people from 2007 to 2009 in Korea. Most outbreaks with a fresh and minimally processed produce etiology remain unknown as the contamination may originate from different stages of processing. The US Food and Drug Administration (FDA) (14) has proposed 8 principles to minimize the food safety hazards of fresh vegetables that address major potential sources of contamination, such as irrigation water, manure, worker hygiene and sanitation, fields, transport sanitation, and trace back. Between 1996 and 2006, a total of 98, 40, 33, 25, 25, and 24 outbreaks were reported due to fresh vegetables and herbs in the USA, Finland, Poland, Canada, Australia, and Brazil, respectively (24). The US FDA (28,29) reported 82 produce-related outbreaks in the USA from 1996 to 2008, and of these, 28% were from leafy green vegetables. During this period, a total of 949 illnesses and 5 deaths were recorded due to vegetable consumption, and the major pathogens were *E. coli* O157:H7, *Cyclospora,* and *Salmonella* spp. (29,30). Reportedly 13 out of 507 and 14 of 1,927 illnesses were linked to melons and tomatoes, respectively.

Biofilms

What are biofilms? It is difficult to describe biofilms briefly, as they have many forms and mechanisms, ecologies, physiological and genetical heterogeneities, and resistance to sanitizers, disinfectants, and antimicrobials. Biofilms are architecturally complex assemblies of microorganisms on or in biotic or abiotic surfaces and interfaces, characterized by interactions between populations. They have exopolymeric substances (EPS) and survive as self-organized, 3-dimensional structures that exhibit altered phenotypic and genotypic characters.

Mechanisms of biofilms formation of produce Bacterial colonization of produce is a stepwise, dynamic process and different physical, chemical, genetic, and biological processes are involved in the final maturation of biofilms. Though it is not clearly defined, more or less 5 steps are involved in biofilms formation. The steps are: (i) reversible attachment to a produce surface, (ii) irreversible attachment through producing quorum sensing and EPS, (iii) microcolony formation, (iv) colonization or maturation steps, and (v) dispersal.

***Attachment*:** Few genetical mechanisms have known about the process of attachment steps of produce biofilms while much research has done on biofilms of different sectors. Several studies have suggested that a microorganism's cellular surface charge, Van der Waals forces, surface hydrophobicity, produce hydrophobicity, and electrostatic forces, simultaneously interact and adhere to the surface (31,32). It might be speculated that under distinct

environmental conditions, bacteria exploit diverse pathways for biofilms formation on produce (33). In general, force-generating movements might be required to form attachments to produce (33).

The attachments depend on pathogenic and nutritional conditions of the plants, hydrophobicity, cellular surface charge, a plant is injured or intact, waxy materials on the produce, electron donation and acceptance with bacteria, and even bacteria to bacteria interactions (34). Flagella, fimbriae, and pili might play a major role in the attachment to produce (35). For example, *agfA*, curli subunits, *agfB* genes, bacterial cellulose synthesis (*bcsA*), and O-antigen capsule assembly and translocation (*yihO*) are involved in the attachment of *Salmonella enterica* serovar Enteritidis to alfalfa sprouts (36,37). It has also been reported that wild type *E. coli* (curli aggregative fimbriae positive) attach in greater numbers than mutant strains on cabbage and iceberg lettuce (38), though other researchers have disagreed on the necessity of curli to attach with stainless steel (39).

The leaves of most plants contain cutin, suberin, and waxes, which make accessible the surface hydrophobicity and increase attachment. Han *et al*. (40) noted that waxy materials on an uncut surface repressed attachments, wherein the hydrophilic, injured surface promotes the adherence of *E. coli* O157:H7. In another study, Reina *et al*. (41) suggested that *S.* Typhymurium was more adhesive to cucumbers than *L. monocytogenes*. The authors noted that bacterial adherence depended on inoculum size, bacterial species, concentration, and time of contact. In contrast, through examining 24 *Listeria* spp. with cut and intact cabbage, other researchers showed that pathogens attach more quickly to an injured site than an intact surface, regardless of the strain used (42).

In another study, a strong correlation was found between surface charges and the hydrophobicity of *E. coli* (both O157:H7 and non-O157:H7), *Salmonella* spp., and *L. monocytogenes* with cantaloupe melon (34). Hassan and Frank (43) argued that only capsule production is significantly associated with attachments of *E. coli* O157:H7 to apples and lettuce leaves. More recently, Patel and Sharma (44) suggested that extrinsic environmental cues are linked to the produce attachment strength of *S. enterica*. In light of all these researches, we might suggest that attachments depend on bacterial genetic interactions, hydrophobicity, and produce surface properties.

***Quorum-sensing (QS) signals*:** When bacteria aggregate and attach, they later express some particular kinds of molecules to communicate or coordinate with one another (called quorum-sensing) for gene regulation (45). Microbial communication-related research is called sociomicrobiology (46). It appears that bacteria attach to biotic or abiotic surfaces and express their QS molecules. Thus far, 4 kinds of QS systems that control biofilms formation have been identified. Of these, Gram-negative bacteria have autoinducer-1 (AI-1) that secretes *N*-acylhomoserine lactones (AHLs) (47) and autoinducer-3 (AI-3) (45) while Gram-positive bacteria have auto-inducing peptide (AIP) types of signaling pathways for intra-species communication (47). Both types of microorganisms express autoinducer-2 (AI-2) QS molecules furanosyl-borate-diester to communicate with inter-species populations (47).

It has been stated that certain extrinsic environmental cues and intrinsic food factors might induce to express QS molecules of many food-borne pathogens, spoilages, and nonpathogens in plant's roots microorganisms (48,49). In general, when a certain level of auto-inducer molecules is reached in the environment of a food, QS molecules might modulate the targeting gene or genes for growth and survival for stresses, food spoilage, virulence genes expression, nodulation, biofilms formation, antibiotics production, bacteriocins secretion, motility, toxins control, sporulations, and antimicrobial and disinfectant resistance (49). AHL (3-oxo-C6-HSL) controls protease and pectinase production, as well as the siderophore production, of Enterobacteriaceae and *Pseudomonas* spp. on beans sprouts (50).

However, QS molecules has been detected from bacterial supernatants of food ingredients using suitable reporter such as *V. harveyi* BB170 (51) or *Serratia plymuthica* RVH1 (52). Recent sophisticated methods such as HPLC, GC-MS, and NMR spectroscopy, can be used in quantification and structure determination of QS molecules (53,54). Lu *et al*. (55) noticed the modulation of AL-2 molecules on produce under refrigerated conditions on tomatoes. In other studies, AHL molecules were detected in contaminated, cold smoked salmon (56) and raw vegetables with *S. plymuthica* (57).

***EPS formation*:** The EPS of biofilms consist of polysaccharides, proteins, S-layer glycoproteins, and glycolipids, as well as extra-cellular DNA, metal ions, divalent cations, and other surface-active components (58). When microorganisms secrete a critical concentration of auto-inducers molecules, they form EPS at the surface of bacterial aggregates, thereby sheltering cells as well as binding the cells of the plant's surfaces with epiphytic bacteria (59). Unlike with abiotic surfaces, epiphytic bacteria make bio-surfactants and syringomycin, indole-3-acetic acid, auxin, and cytokinin to first promote bacteria to colonize on phyllosphere. The polysaccharide, flagella, pili, or fimbriae of bacteria anchor to the produce surface. The bacterial aggregates form an EPS, thus sheltering the microorganisms inside and protecting them from stresses such as antibiotics, disinfectants, and irradiation under natural conditions (60).

Although the role of EPS for biofilms has controversy and species specific, the authors reported that EPS

facilitated adherence to cells of biotic or abiotic surfaces, microcolony formation, and the 3-dimensional structure of the maturation steps of biofilms (61,62). The review (62) also demonstrated that colanic acid, *N*-acetyl-D-glucosamine and cellulose production is related to biofilms production of *E. coli* O157:H7 to alfalfa sprouts but not the adhesion of *Staphylococci* spp. Rayner *et al*. (63) detected large amounts of EPS on fresh produce and household surfaces.

***Microcolony formation*:** When bacteria attach on produce, they begin to communicate with one another, forming QS molecules and multiplying. At a certain level of QS molecules, environmental cues stimulate the formation of EPS and multiply inside the EPS as well as form the microcolony formation. Analyses of biofilms have revealed that formation of EPS on produce, most of the bacteria slow down to produce of flagella, pili, and fimbriae, and focus on the maturation of biofilms. The microcolony consists of multi-species and nutrient cycling may occur in biofilms (64).

***Colonization or maturation steps*:** If the conditions are favorable, the final stage of biofilms formation is maturation, wherein the biofilms develops a self-organized structure in a microenvironment. The final arrangement may be in monolayers, a 3-dimensional structure, or mushroom- or tulip-like shapes. The final biofilms consist of non-motile bacteria surrounded by EPS, which have channels for nutrients and water flow (65). *Pseudomonas* spp. express alginate to protect themselves from desiccation and help to develop the biofilms' architecture (66).

***Dispersal*:** Biofilms forming microorganisms may detach due to various reasons, such as presence of QS molecules, nutrient accessibility, surface character changes, as well as physical forces from the produce (67). Some plant surfaces have hydrophobic cuticle layers, which play an important role in attachment, EPS formation, and biofilms formation, as well as detachment from the surfaces of plant leaves. Bacterial growth, different enzymes produced by bacteria, external environmental influences, and human interactions might be reasons for the dispersal of cells from biofilms. Nutrient deprivation and bacterial autocidal activity might be attributed to the dispersal of biofilms from produce (68,69).

Biofilms formation on produce Biofilms on produce might be different from biofilms on food contact surfaces and dental biofilms, as there are numerous interactions present between bacteria and a plant's phyllosphere. In natural environments such as plants leaves, biofilms may represent 80% of total microbial populations (70). In one study, natural biofilms on endives and parsley made up about 10-40% of the total population (71). Morris *et al*. (59) explained that multi-species biofilms of Gram-positive and Gram-negative bacteria with prominently filamentous fungi (*Penicillium* spp. and *Cladosporium* spp.) of 20 μm depths and 1 mm lengths were found on the leaves of different fresh vegetables (spinach, lettuce, Chinese cabbage, celery, leeks, and parsley). Fett (72) reported naturally-occurring biofilms on alfalfa, broccoli, clover, and sunflower. Rudi *et al*. (73) used culture-independent methods of 16S ribosomal DNA array and identified the population dynamics of *Enterobacteriaceae*, *Pseudomonas* spp., *Oxalobacter* spp., and lactic acid bacteria on ready-to-eat vegetable salads.

In contrast to natural biofilms, laboratory-based biofilms have been reported in many studies on lettuce (74,75), apples (76), spinach (77), and cabbage (42,44). The stomata, trichomes, veins, and cell wall junctions are the main locations on leafy produce for microbial biofilms formation (77,78) Warner *et al*. (79) observed *S*. Thompson on spinach leaves and greater numbers were in the stomata and veins. Produce often have punctures, cuts, splits, and cracks due to injury during pre-harvesting and post-harvesting handling, and bacteria can attach and assemble at the injury sites to form biofilms (76). The pathogens can also enter tomatoes (55), apples (76), and oranges (80). Fransisca *et al*. (81) noticed the presence of *E. coli* biofilms on the surfaces of alfalfa sprout roots, cotyledon, and hypocotyls. Liao and Cooke (82) showed that attachment of *Salmonella* spp. on pepper disks generally occurred on the surfaces of injured tissue, but rarely on unbroken skin. Khalil and Frank (83) suggested that the damaged parts of leafy green vegetables supported the growth of pathogens and found a distinct growth niche that elicits different microbial responses in various types of leafy greens. However, another study (84) tested aggregates of *E. coli* O157:H7 on lettuce leaf surfaces using a confocal laser electron microscope (CLSM) and found entrapped *E. coli* O157:H7 located 20 to 100 μm below the surface, and that it preferred cut edges over stomata. It is noteworthy that in nature, most pathogens do not form biofilms, as they are fastidious (85). Produce, however, might be contaminated with pathogens at some point between farm and fork, and growth would be enhanced due to the presence of pre-existing biofilms of epiphyte microorganisms on mainly damaged or injured parts, and may survive longer periods on the surface or injured parts of the produce.

Methods of biofilms studies on produce To better understand produce biofilms *in situ*, previous studies have used direct epifluorescence microscopy (59,86), atomic force microscopy (87), scanning electron microscopes (59,77,87), episcopic differential interference contrast microscopy (79), confocal laser electron microscope (40,88), and cryostage scanning electron microscopy (63). New electron microscopic techniques such as laser scanning microscopy, magnetic resonance imaging, and

scanning transmission X-ray microscopy, could also be employed to visualize produce biofilms *in situ* (89).

To assess the viable microorganisms present before and after exposure to disinfectants, flourescein isothiocyanate (FITC) conjugated antibody, carboxyfluorescein diacetate (CFDA) (90), resazurin (91), mutant-expressing green fluorescent protein (GFP) (92,93), live and dead viability staining (40,86), 5-cyano-2,3-ditolyl tetrazolium chloride with 4'-6 diamino-2 phenylindole (CTC-DAPI) staining (94), and flow cytometry (95) can be applied to produce biofilms. To study the efficacy of disinfectant biofilms strains, many researchers have used the Calgary Biofilms Device, MBEC^{TM} assay, as it can support reproducible biofilms for liquid media (96). Most researchers also evaluate biofilms formation by standard plate count methods. Although, the methods used are gold standard methods to identify the microorganisms, in recent times denaturing gradient gel electrophoresis (DGGE) and 16rRNA genes PCR are promising alternatives for biofilms studies as culture-independent methods (73). Very recently, Almeida *et al*. (97) examined mixed culture biofilms of *E. coli*, *L. monocytogenes*, and *S. enterica* using peptide nucleic acid fluorescence *in situ* hybridization methods. They noticed that mixed culture biofilms were thicker because of high EPS production, and survived extended periods as compared to single species biofilms. To know the biofilms community, both culture-dependent and -independent procedures should be employed to identify other bacterial genera (98).

Paradigm of Produce Biofilms Disinfection Resistance

It is the highest priority for food-processing industries to apply efficient sanitation programs to lessen the risk of biofilms contamination (99). At present, the evaluation of sanitizers used on produce do not cover microorganisms present in biofilms, as the cells are 100-1,000 times more resistant than their planktonic siblings (100,101). The present standard methods are based on the planktonic state of microorganisms (102,103), though one standard method exists for testing disinfectant efficiency of biofilms (104).

Although chlorine is the most widely used disinfectant for produce, it might possible that chlorine could make carcinogenic compounds while used to decontaminate produce. Chlorine at the desired concentrations can reduce the bacterial load from produce biofilms to less than 5.0 log (Table 1). Niemira and Cooke (77) reported on the inefficacy of biofilms formation by *E. coli* on spinach and lettuce through chlorine washing (300 and 600 ppm). It was also demonstrated that washing with 1,000 ppm chlorine or 5% hydrogen peroxide to decontaminate *Salmonella* Stanley from melons and inoculating for 72 h reduced contamination by less than 1 log unit, and pathogens were detected on the produce after treatment (114).

Liao and Cooke (115) examined the efficacy of trisodium phosphate on green peppers and noticed that bacterial populations on the produce were reduced by 10 to 100-fold. This study showed that the surfaces of injured fruit tissue are the principal sites for attachment, and that bacteria attached to the tissue are resistant to sanitizing treatment.

Lapidot *et al*. (93) studied biofilms formation and the survival of *S.* Typhimurium on parsley and evaluated chlorinated water containing sodium hypochlorite at concentrations of 100, 200, 800, and 1,600 mg/L, and evaluated the resistance to disinfectants after maturation of biofilms incubated at 4 and 25ºC for 7 days. Different commercial acid-based cleaners were used for cleaning biofilms from apple surfaces, which reduced microbe populations by 2.86, 3.11, 2.48, and 0.73 log CFU/apple for *E. coli* O157:H7, *S. muenchen*, aerobic mesophiles, yeasts, and molds, respectively (116).

Other research has shown the ineffectiveness of produce biofilms on lettuce leaves, alfalfa, radish, tomatoes, cantaloupes, and honey melons (Table 1). There are many others studies related to produce disinfection; however, but we are summarizing papers that are directly related to produce biofilms and disinfectants.

Factors Affecting the Efficacy to Disinfect Microorganisms in Produce Biofilms

Disinfectants that we are referring to consist of physical and chemical agents that can kill microbial pathogens, but might not kill the spores (117). Standard disinfectants, according to guidelines, should reduce microorganisms by 5 log in the presence of an organic load (102,103). Standards sanitizers should kill 5 log of bacteria within 30 s (117). The effectiveness or efficacy of sanitizers and disinfectants depends on the interval between contamination and sanitizer application, contact time, attachment site, internalization of bacteria, temperature, pH, bacterial load, and age of the produce biofilms.

Inoculum size, microorganisms, and organic loads Most produce contains large amounts of carbohydrate and if the concentrations are high, disinfectants cannot work effectively. Efficacy also depends on the type of microorganisms present and its load on and in the produce. To reduce food-borne illness from sprouts during pre-harvesting and post-harvesting, the US FDA recommends sanitizing seeds with 20,000 ppm of free chlorine from $Ca(OCl)_2$, applied for 15 min, to reduce *E. coli* O157:H7 and *Salmonella* spp. (118).

Table 1. A summary of research on the efficacy of disinfectants for inactivation of microorganisms in produce biofilms

Type (concentration)	Contact time	Pathogens applied	Plants or parts	Procedures used	Efficiency	References
Chlorine (100 and 200 ppm) or short chain fatty acid formulation (0.4%)	222°C for 2 min on an orbital shaker	*E. coli* O157:H7	Romaine lettuce	Incubation at 4°C for 18 to 24 h	<2.0 log CFU/g	Keskinen and Annous (105)
Calcinated calcium (0.04 and 0.4%) with 20,000 ppm NaOCl	20 min room temperature	*E. coli* 157:H7	Radish sprouts	Germination for 72 h in the dark at room temperature	1.65 log CFU/mL reduction	Fransisca *et al.* (81)
Slightly acidic electrolyzed water (5.8±0.5) and NaOCl (107±3.7 mg/L)	5 min	*E. coli* or *Salmonella* spp.	Chinese celery, lettuce, daikon sprouts	Inoculated with pathogens for 15 min and then dry 1 h	<3.0 log CFU/g	Issa-Zacharia *et al.* (106)
Ozone (2 mg/L), chlorine (100 mg/L), organic acid (0.25 g/100 g citric acid plus 0.50 g/ 100 g ascorbic acid)	10°C for 2 min	*E. coli* and *L. monocytogenes*	Green lettuce leaves	Inoculated with pathogens 60 min at room temperature and incubation 6, 24, and 48 h	ca. 4.0 log CFU/g after 6 h incubation, about 3.0 log CFU/g, after 24 h, <2.0 log CFU/g after 48 h	O¨lmez and Temur (74)
Sodium hypochlorite solution (300, or 600 ppm), irradiation (0, 0.25, 0.5, 0.75, or 1 kGy)	3 min	*E. coli* O157:H7	Romaine lettuces, spinach leaves	Pieces were submerged for 2 min and incubate 4°C 24, 48, or 72 h	1.3 log CFU/g for baby spinach, 1.8 log CFU/g for chlorine, <3.0 log CFU/g for 1 kGy after 72 h	Niemira and Cooke (77)
Caprylic acid and monocaprylin (25, 50, and 75 mM)	10 to 90 min	Cocktails of *E. coli* O157:H7 and *Salmonella* spp.	Alfalfa seeds	Bacteria with seeds incubated for 24 h under different conditions	<2 log CFU/mL for biofilms, >7.0 log CFU/mL planktonic cells	Chang *et al.* (107)
Chlorine dioxide (3 mg/L), sodium hypochlorite (100 mg/L)	1 min	*E. coli*	Lettuces	Stored for 3 days at 4°C plus 4 days at 7°C	1.3 to 0.3l log CFU/g for hypochlorite and 0.8 to 0.01 log CFU/g for chlorine dioxide	López-Gálvez *et al.* (108)
Chlorine (20-200 ppm), aqueous chlorine dioxide (20-200 ppm chlorite ion concentration, pH 8.0, TriNova), acidic electrolyzed water (50 ppm chlorine, pH 2.6)	2 min	*E. coli* O157:H7	Romaine or iceberg lettuce leaves	Fresh cut lettuce leaves were inoculated with pathogens for 5 min, dried 2 h and then 24 h at 4°C	ca. 1.0 log CFU/g reduction	Keskinen *et al.* (75)
Chlorine (200 ppm), irradiation (0.75 kGy)	10 min	*E. coli* O157:H7	Lettuce plants	Inoculated lettuce seedlings were planted in contaminated Hoagland's nutrient solution and incubated 23°C and harvested after 14 days	<5 log CFU/mL reduction for chlorine, 7 log CFU/mL reduction for irradiation	Nthenge *et al.* (109)

Table 1. Continued

Type (concentration)	Contact time	Pathogens applied	Plants or parts	Procedures used	Efficiency	References
Sodium hypochlorite sanitizing solution (300 or 600 ppm) or ionizing radiation (0.25 to 1.5 kGy)	3 min for chemical sanitizer	*E. coli* O157:H7	Romaine lettuce, baby spinach		<1.0 log for chemicals, 3-4 log for ionization	Niemira (110)
Irradiation (0.75 and 0.95 kGy)		*Salmonella* spp.	Tomatoes	Incubate 15 days at 4°C	1.8 log CFU/g for low dose, 2.2 log CFU/g high dose	Schmidt *et al.* (111)
Hydrogen peroxide (2.5%) alone or hydrogen peroxide (1%) in combination with nisin (25 mg/mL), sodium lactate (1%), and citric acid (0.5%)	5 min	*E. coli* O157:H7 or *L. monocytogenes*	Cantaloupes, honeydew melons	Incubate 5°C for 7 days	<3.0 log CFU/cm^2 for hydrogen peroxide, 3-4 log CFU/cm^2 for mixed disinfectants	Ukuku *et al.* (112)
Nisin (50 μg/mL), EDTA (0.02M), sodium lactate (2%), potassium sorbate (0.02%), individually or in various combinations	5 min for intact and 1 min for cut pieces	*Salmonella* spp.	Cantaloupe	Incubate 5°C for 7 days	ca. 2.0 log CFU/cm^2	Ukuku and Fett (113)
ClO2 (0.62 or 1.24 mg/L)	30 min at 22°C and 90-95% relative humidity	*E. coli* O157: H7	Green peppers	Inoculated and dried 2 h, added disinfectants, and again incubated 12 to 24 h	<3.1 for low dose, >6.0 log for high dose	Han *et al.* (40)

Concentration of disinfectants and exposure time A sufficient concentration of cleaning agent must be applied to achieve pathogen-free produce, although the scenarios are different for sessile cells that are on and within plants. It is well known that higher concentrations of sanitizers and disinfectants reduce the microorganisms of planktonic cells more efficiently than lower doses. Increasing concentrations or exposure times do not lead to greater reductions in pathogens from produce. Han *et al*. (40) demonstrated that 0.60 and 1.2 mg/L ClO_2 decreased the *E. coli* populations of intact and injured green peppers by 7.27 and 6.5 log, respectively. Most studies report an inefficacy of disinfectants on produce biofilms at higher concentrations.

Internalization of microorganisms to the plant tissues As we mentioned earlier, bacterial cells found in stomata, trichomes, calyx, stems, and damaged and cut edges of produce might be inaccessible to disinfectants (74,77,81, 84,108,119). Bacteria hold shelter inside the stomata and from there, defend from disinfectants (108). In these cases, the standard cleaning and sanitation procedures used on fresh produce are not fully efficient in eliminating biofilms (74,81). The waxy cuticles, internal leaf tissue, and other polysaccharides also protect pathogenic bacteria by keeping disinfectants away from them. Brandl and Mandrell (119) also suggested that *Salmonella* Thompson was present at high densities within some lesions on cilantro plants and opposed disinfectants after gaining access through the disrupted cuticle of the damaged regions. However, other researchers have demonstrated that hydrophobic surfactants are unable to remove pathogens from the cutting sites on lettuce (43).

High temperatures and excess relative humidity during the sprouting of produce create incredibly vulnerable conditions for food-borne pathogens to internalize, as well as forming biofilms and protect from disinfectants (120,121).

Interval between contamination and washing Generally, the earlier the events of contamination occur, the more difficult it is to disinfect the produce. There is a consequence of increasing probability that, given more time, the contaminating bacteria may tightly attach to inaccessible locations, be incorporated into biofilms, or even internalize within the fruit or vegetable interior.

After 24 h, *E. coli*-inoculated apples had essentially all bacteria firmly attached and did not eliminate more than 3 log by washing with 1% hydrogen peroxide; treatment time and temperature had little or no effect (122). Nonetheless, it might be complicated to trace back produce biofilms maturity or attachment age because it had a chance to contaminate from the seedling to consumption stages.

Formation and maturity of biofilms As we mentioned earlier; once attached, bacteria become incorporated into biofilms, an extracellular polysaccharide matrix that holds the cells together and attaches to the surface of produce.

The ecology and physiology of planktonic cells and biofilms are different. Biofilms-forming cells are typically more resistant to antimicrobial agents, sanitizers, cleaning agents, biocides (100,101,123,124), and heat (125) than planktonic cells. A chlorine treatment regime (10 ppm, 10 min) is sufficient to kill 6 log of *Salmonella* spp. planktonic cells, but a less than 1 log decline in biofilms-associated cells (123). To kill the biofilms-associated *S. aureus*, the required concentrations of benzalkonium chloride (BAC) and sodium hypochlorite were 50-600 times that needed for planktonic cells (126). Robbins *et al*. (127) demonstrated that 0.25 ppm ozone decreased the number of *L. monocytogenes* planktonic cells by 8.29 log CFU/mL, while biofilms cells only dropped by 1.48 log CFU/chip.

When biofilms mature, nutrient, and gaseous gradients from the outside to inside change gradually, and growth rate as well as disinfectant susceptibility might change (33). Several authors have correlated susceptibility to disinfectants and age of biofilms (128,129). Mangalappalli-Illathu *et al*. (130) demonstrated that the protein and fatty acids expression of planktonic and biofilms states of microorganisms exposed to benzalkonium chloride (BC) are dissimilar due to the biofilms cells' increased survival under stress conditions.

Multi-species biofilms and subsequent interactions with produce Most laboratory studies of produce biofilms and the efficacy of disinfectants are based on monocultures; such types of biofilms are rarely present in nature (72, 131,132). As we know, biofilms in nature consist of multiple species, including pathogens, as produce are more susceptible to contamination from different sources. Biofilms of multi-species have shown less susceptibility to sanitizers and disinfectants due to their complex architectures and inter-species and intra-species interactions (133-137). A mixed culture of *Burkholderia cepacia* and *P. aeruginosa* inactivates with 80 ppm chlorine, while only 30 ppm is sufficient to kill a single species (124).

In general, multi-species biofilms have been found to have individual capabilities and synergistic inter-species interactions, physiological heterogeneity as well as antimicrobial resistance (136,138,139). Chorianopoulos *et al*. (140) tested mixed culture populations with chemical and bio-surfactants and reported the greater susceptibility of mono-species over mixed-species biofilms. However, the presences of naturally occurring biofilms on produce enhance the ability of pathogens to survive, as well as resist disinfectant effects (72). To better understand mixed culture behavior on dental plaque, Shu *et al*. (141)

employed 10 oral species of wild and mutant strains (urease negative) in a constant-depth film fermenter with urea and monitored the microbial community for 14 days. They reported the variations in the microbial community in biofilms and the results support biofilms physiology of multi-species interactions within microenvironments. As stated in the experiments of Shu *et al*. (141), if acid-tolerant or antimicrobial-resistant microorganisms form biofilms initially under stress conditions, could favor further growth of other organism to favorable microenvironments created by initial population. In mono-species and mixed populations, bacteria stay in close contact with one another, communicate by producing QS of inter-species or intra-species, resist gene transfer within species, and thus combat sanitizers as a whole (46,133).

Resistant Mechanisms of Microorganisms in Produce Biofilms

Microorganisms in produce biofilms are notoriously tolerant of various conventional physical and chemical disinfectants (60,100). Several authors have described the activity, action, and resistance mechanisms to antiseptics and disinfectants of planktonic cells (142,143). The high tolerance and resistance of bacteria that form biofilms in produce are due to: (a) limited diffusion of disinfectants and sanitizers through the biofilms' extracellular carbohydrate complexes, (b) physiological heterogeneity of the populations within biofilms, (c) development of resistance phenotypes by resistant gene transfer, (d) cross resistance of antimicrobials, disinfectants, and sanitizers, and (e) persister cells of biofilms.

Limited diffusion by biofilm matrix To inactivate microorganisms, bactericides must achieve a satisfactory concentration at the target site to perform their antimicrobial actions. Slime layers, capsules, and the EPS of biofilms might create hurdles layers to penetrate sanitizer, biocides, and disinfectants in contact with sessile microorganisms (144,145). At the final maturation stage, the EPS forms a relatively thick wall to provide a barrier for other cell components such as nucleic acids, proteins of cell membranes, and lipids (146). Therefore, disinfectants with a single target have to face many targets in the EPS of produce biofilms. EPS also shows charge properties by its components and acts as ion exchanging resins and keep away the disinfectants from microorganisms (147). Biofilms matrix are also known to protect bacterial cells from chemical sanitizers (101,145).

Enterobacteriaceae and Gram-positive *Streptomyces* have been shown to form amyloids, which are important components of the extracellular matrix of most biofilms, and might be more resistant to sanitizers (148). Ryu and Beuchat (39) noticed that curli and EPS contributed to a barrier for chlorine resistance. Resistance to biofilms is also enhanced by carrying the degrading enzymes of disinfectants present in EPS (60). Lapidot *et al*. (93) reported that the EPS of *S*. Typhimurium was irrelevant in chlorine resistance on parsley. In conclusion, EPS provides shelter for the microorganisms by creating physical, chemical, and biological barriers to disinfectants.

Physiological heterogeneity within biofilms It has already been reported that sessile cells need diverse genotypes and phenotypes that express distinct metabolic pathways for stress survival in biofilms. Cells within biofilms in multiple cell layers of microenvironments have a distinct environmental heterogeneity of nutrients, oxygen, growth factors, chemicals, and toxins (149,150). Bacteria express genetic heterogeneity such as microscale chemical gradients, adaptation to local environmental conditions, stochastic gene expression and mutations, gene transfer, and other genetic variations (151). The underlying cells of biofilms microenvironments are generally slower growing and more cohesive than the outer cells (149,151). The heterogeneity of cells in biofilms are optimized to withstand treatment with disinfectants as most of these agents function at the growing stages of the cells (152-154).

The probability of physiological heterogeneity might be more attributable to the formation of biofilms at produce injury sites, as these sites have many layers and nutritional inconsistencies. On the produce surface, biofilms cells exhibit nutrient limitations, and the formation of toxic byproducts favor the expression of the stress-induced genes of alternative sigma factor (sigB*)*. SigB shows resistance to benzalkonium chloride and peracetic acid for *L. monocytogenes* (155) although there is debate as to the necessity of *sig*B for biofilms formation (156). *Rpo*S is expressed higher on the outer surface and less in the inners layers of biofilms, and mediates the disinfectant resistance of produce biofilms (134,157).

Horizontal gene transfers Horizontal gene transfer in natural environments occurs by plasmids, transposons, bacteriophages, and integrons to adapt to stress conditions. Gene transfer rates are more common in biofilms cells as they are attached strongly with each other and constantly adapt to stress conditions. Horizontal gene transfer has been shown to occur by conjugation or non-conjugation in biofilms on produce (133,158-160). Ando *et al*. (158) examined plasmid transfer directly to foods (meat, fresh vegetables) and found lateral gene transfer even within a day. Reisner *et al*. (159) demonstrated the transfer of conjugative plasmids to mixed biofilms populations of *E. coli*. The transfer of conjugative plasmid from *Pseudomonas*

putida to residence microflora has also been observed in alfalfa sprouts (160). Horizontal transfers of IncP-1 plasmid from Gram-negative and Gram-positive bacteria were detected in barley rhizospheres (161). If naturally present biofilms have the resistance gene to disinfectants, it would transfer to pathogens of produce as well. The pathogens then might be resistant to the disinfectants used for decontamination.

Cross resistance to sanitizers for biofilm-forming populations Many antimicrobials, sanitizers, and disinfectants have a common mode of action. In general, they bind to specific target sites of microorganisms such as cell walls, plasma membranes, ribosomes, nucleic acids, viral envelopes, and spores cortexes etc. Cross-resistance is a common phenomenon as many sanitizers and antibiotics share the same target sites (135,162,163). Clearly, some researchers have identified the quaternary ammonium compounds and β-lactum antibiotics resistant shared same genes of qacA/B genes of *Staphylococcus* spp. (164).

The frequent use of antimicrobial agents in humans and animals develops increased cross resistance to disinfectants and sanitizers, which is a challenge to food safety. The reason for resistance to biofilms might also be the presence of disinfectant-degrading enzymes in one species of microorganisms that degrade the disinfectants and support the growth and survival of other species within the biofilms. As multi-species biofilms are more common in produce and produce are more vulnerable to contamination by various organisms, cross-resistance between disinfectants and antibiotics on produce might be a serious issue for food safety.

Resistance due to persister cells Persister cells comprise a small fraction of the population of planktonic or sessile cells that might be defective in programmed cell death (136,165), express toxin-antitoxin systems and other genes (166), and might be modulated by a stochastic process (167). Significant phenotypic changes in biofilms communities and their resistance to disinfectants may be explained by persister cells (168,169). Phenotypic changes can block the target sites of disinfectants, represent resistance to disinfectants, and again show their susceptibility after re-growth and exposure to disinfectants. Keren *et al*. (167) suggested that persister cells are not resistant cells of antimicrobial agents, injured or dead. They are unusually slow growers and present in very small numbers with other microbial populations. EPS also protects the persister cells in biofilms and enhances survival with sanitizers and disinfectants (168).

Biofilms Control on Produce: An Alternative Novel Approach

Phages-based control Bacteriophages are omnipresent in the environment, and many phages are lytic, as well as organism-specific and nontoxic to humans. Bacteriophages would be one alternative means to control biofilms, as they have to penetrate the EPS of host cells to replicate (170). Several successful studies of bacteriophages and biofilms control on produce are already published (171,172). In a recent report, Jassim *et al*. (173) observed a >3 log reduction in *E. coli* biofilms populations in lettuce, cabbage, meat, and egg using phages. Commercial-based phages for control of *L. monocytogenes* in meat and cheese food applications are already in progress in the Netherlands (www.ebifoodsafety.com) (174). A study by Hanlon *et al*. (175) revealed that bacteriophages were shown to penetrate alginate EPS of *P. aeruginosa* biofilms, producing enzymes and reducing by 2 log CFU/mL the biofilms population. *In vitro* tests of *L. monocytogenes*-specific phages controlled the pathogens on melons, but not on apples (176).

Removal of single species biofilms of *Enterobacter cloae* was unsuccessful using 3-phage cocktails, although complete inhibition of the biofilms was demonstrated when both phages and disinfectants were applied (177). Kocharunchitt *et al*. (178) questioned the efficacy of *Salmonella* spp. bacteriophages on sprouts. A significant decrease of biofilms populations by phages is dependent on environmental conditions, populations of the biofilms (host and non-host), phage titer, temperature, pH, and nutrient concentrations of the produce. Further research is needed to successfully use phages as biocontrol agents for biofilms on produce.

QS inhibitors Many bacteria, plants, and fungi can secrete anti-quorum sensing molecules that can inhibit the production or degrade the QS of food-borne pathogens. AHLs degradation enzymes (*N* acyl-homoserine lactonases) have been found in many symbiotic bacteria and fungi (179-181). A significant number of α, β, γ proteobacteria and Gram-positive bacteria, particularly *Bacillus* spp., can secrete enzymes (AHL lactonase and AHL acylase) called quorum-quenching enzymes, to degrade AHL molecules (182). Genes can be cloned to transgenic plants, and pathogens might not be able to cause spoilage and biofilms formation (183,184).

Ponnusamy *et al*. (185) noticed vanillin inhibited approximately 60% production of AHLs from *Aeromoas hydrophila* biofilms. Furanones isolated from marine algae,

Dalisea pulchra, exude halogenated furanones to inhibit QS (186). A microarray-based study also confirms that bromofuranones modulate the metabolism, motility, drug resistance, and stress response of *S.* Typhimurium (187).

EPS destruction Different bacteria, fungi, and plants secrete anti-adhesion polysaccharides or proteins that could reduce biofilms on produce. Although bio-surfactants are widely used for oil recovery, emulsifying cosmetics, and demulsifying waste, very few applications exist for controlling biofilms on produce (188). Detachment-promoting agents (DPAs) of polysaccharide depolymerases, esterases, and dispersin B, might rupture the EPS, and disinfectants may then affect the biofilms cells (189). Lu and Collins (190) constructed engineered bacteriophages that were able to penetrate the biofilms by expressing enzymes against EPS, and successfully reduced the populations. Investigations should continue into the nature of anti-adhesive molecules that may be alternatives to control biofilms (191).

Use of biological compounds in anti-biofilms activity Many plants and microorganisms, particularly marine bacteria, secrete anti-biofilms compounds that might be applied after harvesting to decrease the contaminations of food-borne pathogens. Yanti *et al*. (192) reported that *Curcuma xanthorrhiza* has anti-biofilms activity against methicillin-resistant *Streptococcus mutants, Streptococcus sanguis*, and *Actinomyces viscosus* oral biofilms. Furukawa *et al*. (193) noticed the sugar fatty acids reduce the biofilms of *S. aureus* and *E. coli*. Another food additive, chitosan, also appears to reduce biofilms formation *in vitro* (194). Another article showed the anti-biofilms activity of selected culinary herbs and medicinal plants against *L. monocytogenes* (195). In a recent study, Jiang *et al*. (196) observed the anti-biofilm activity of marine *Vibrio* spp. to *P. aeruginosa*, but not to *S. aureus*. Sayem *et al*. (197) isolated EPS from *Bacillus licheniformis* and found anti-biofilms activity to *E. coli* and *Pseudomonas fluorescens*. Khan *et al*. (198) isolated a naphthalene derivative, which showed an anti-biofilm role with *Streptococcus mutants*. Dheilly *et al*. (199) reported that a culture supernatant of *Pseudoalteromonas* spp. had anti-biofilm activity to *S. enterica*, *E. coli*, and *P. aeruginosa*. Novak and Fratamico (200) reported the inhibition of Al-2 activity and growth, and sporulation of *Clostridium perfriengens* with ascorbic acids. Recently, Lee *et al*. (201) stated that flavonoid phloretin inhibits the biofilms formation of *E. coli* O157:H7.

Control by photosensitizing agents Photosensitization is a non-thermal treatment involving photosensitizers, light, and oxygen application to produce after harvesting and during storage. Porphyrins, phthalocyanines, porphycenes, chlorins, and pheophorbides could be used as photosensitizers (202,203). Luksiene *et al*. (204) used photosensitization to kill the spores or biofilms of food-borne pathogens. In another study, Luksiene and Paskeviciute (205) reported a 1.8 log CFU/mL reduction of *L. monocytogenes* from strawberries using Na-chlorophyllin (1 nmol/L) as a photosensitizer.

Control using synergism of different disinfectants and bio-sanitizers The synergistic effect of 2 or more disinfectants are hurdles for produce biofilms as the technology provides more stresses, acts at multiple target sites, and changes the homeostasis of pathogens (206). Hurdle technologies, such as disinfectants and ionizing and nonionizing radiation, could reduce food-borne pathogens better than single treatments (207). Although radiation-based foods are questionable to consumers, the US FDA has approved low dose irradiation (1.0 kGy) for disinfection of produce (208). Irradiation of seed sprouts also could minimize food-borne diseases (209). Nonionizing UV light might be effective in reducing populations from the surface of leaves (210,211). The synergistic effect of UV-C radiation and modified atmospheric packaging seems to be useful in control psychrophilic bacteria, coliforms, and yeast microflora (210). Mattson *et al*. (212) noticed that carvacrol (CAR), *trans*-cinnamaldehyde (TC), eugenol (EUG), and β-resorcylic acid (BR) decreased *Salmonella* spp. counts on tomatoes by about 6.0 log CFU/mL. Although there is much controversy over using phages, Viazis *et al*. (213) reported the efficacy of a combination of bacteriophages and *trans*-cinnamaldehyde for the reduction of *E. coli* O157:H7 on lettuce and spinach leaves. Kim *et al*. (214) examined the effect of oscillation on food-borne pathogens on lettuce and spinach, and found a >3.0 log reduction. The combination of oscillation and other disinfectants might be an excellent way to decontaminate pathogens from produce. Gopal *et al*. (215) demonstrated that silver (5 ppm) and hydrogen peroxide (0.4 ppm) reduced pathogens on iceberg lettuce. The synergistic effects of ultrasound (216) and irradiation with disinfection might be another alternative strategy to control produce biofilms. The combined efficacy of nisin or periodecin with sodium lactate, potassium sorbate, phytic acid, and citric acids, also demonstrated the high reduction ability of *L. monocytogenes* on beans, cabbage, and broccoli (217). Two or 3 physical or chemical methods can be applied to biofilms for produce decontamination (218-220). There might be many alternatives to study that effectively decrease produce biofilms populations.

Miscellaneous alternative techniques One study has described the naturally occurring bacteria and yeast that cause antagonistic activity against food-borne pathogens *L.*

monocytogenes and *S. enterica* serovar Poona on apples (221). Simões *et al*. (222) stated that ortho-phthalaldehyde (OPA) killed the persister cells of *P. fluorescence* biofilms. Other techniques that we have not addressed, as they are not directly related to produce biofilms, include vacuum infiltration, vapor-phase treatments, surface pasteurization, intense light pulses, super-atmospheric oxygen with modified atmospheric packaging, and innovative gas treatments (223-226).

Conclusions

The higher consumption rates of produce are raising the outbreaks worldwide. Produce are susceptible to microbial attack from different sources and biofilms might be one of the great concerns in the current outbreaks in produce. It is now well-established that current sanitizers and disinfectants do not show efficacy to the biofilms of pathogens. The risk of pathogenic biofilms development depends on produce injury, cracks, or damage, and the presence of natural biofilms. Greater attention should be paid to these issues to lessen produce outbreaks. Research into novel, alternative, and sustainable applications would also lessen the current emerging problems with produce-related outbreaks.

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