Comprehensive REVIEWS in Food Science and Food Safety

The Paradox of Mixed-Species Biofilms in the Context of Food Safety

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Abstract: Formation of mixed-species biofilms constitutes a common adaptation of foodborne pathogens and indigenous microbiota for prolonged survival in their food niche. Nevertheless, the potential role of mixed-species biofilms in food safety remains to be elucidated. The formation of mixed-species biofilms on food and food processing surfaces depends on various physical, chemical, and biological processes including species composition, especially of the indigenous microbiota and nutrients, food types, temperature, quorum sensing, extracellular polymeric substance (EPS) production, biofilms maturation, and dispersal steps. Compared to monospecies, mixed-species are highly resistant to antimicrobials, possibly due to higher EPS production, internalization into food, fitness of species, denser and thicker biofilms maturation, and interspecific protection of 1 species by others, although there are much debate among studies. The fitness of mixed-species biofilms populations is suggested to be of a cooperative, competitive, or neutral nature based on the genetic background of the involved species. Currently, various methods using microarray, confocal microscopy, proteomics, and selective media are being explored for the detection of mixed-species biofilms to resolve the conflict issues. Here, we review recent progress in this emerging field in the context of food safety and propose that novel and alternative techniques like antiquorum sensing, antibiofilms, enzymes, hurdle techniques, and bacteriophages will significantly help to control the formation of mixed-species biofilms for enhanced food safety. The next challenge will be to integrate the fitness and resistance patterns of mixed-species biofilms in the laboratory with those of natural settings.

Keywords: antimicrobial resistance, food safety, indigenous microbiota, mixed-species biofilms, public goods, quorum sensing

Introduction

Foodborne diseases are a burden worldwide, yet much remains unknown about them in both industrialized and developing countries (WHO 2012). The Dept. of Food Safety and Zoonoses (FOS) of the World Health Organization (WHO) takes initiatives to measure the global burden of mortality and morbidity caused by foodborne diseases (WHO 2012), while the Global Foodborne Infections Network (GFN) functions to estimate and mitigate the problem of foodborne diseases (WHO 2011). In the U.S.A. alone, 48 million cases of foodborne diseases are reported annually for which 9.8 million are caused by known foodborne pathogens (MMWR 2013). During 1998 to 2008, the Centers for Disease Control and Prevention (CDC) reported 13405 foodborne disease outbreaks, which resulted in 273120 cases of diseases, 9109 hospitalizations, and 200 deaths (MMWR 2013). Almost 45% of the reported cases are caused by bacteria such as *Salmonella* spp., Shiga-producing *Escherichia coli*, *Listeria monocytogenes*, *Vibrio parahaemolyticus*, *Clostridium botulinum*, and *Campylobacter jejuni* (MMWR 2013). A total of 5648 foodborne diseases outbreaks were reported in 69553 cases, 7125 hospitalizations, and 93 deaths in Europe (EFSA 2013), with the most prevalent outbreaks being caused by Shiga toxin-producing/verotoxigenic *E. coli*.). It has been reported that 80% of all microbial diseases, including foodborne illnesses, are caused by microorganisms in biofilms (National Institutes of Health, USA 1997). It has also been estimated that the annual economic burden from biofilm-related infections about 2 decades ago amounted to $6 billion in the U.S.A. (O'Toole 2002).

Biofilms are complex architectural and shelf-organized with altered phenotypic and genotypic functions of different microorganisms, including foodborne pathogens. Biofilms constitute an aggregation of microorganisms in complex 3-dimensional structures that form on surfaces and are surrounded by extracellular polymeric substances (EPSs) (Sutherland 2001). Perhaps, the best definition of biofilm is a microorganisms-derived sessile community of cells that are irreversibly attached to biotic or abiotic surfaces or interfaces or to each other, are embedded in a matrix of EPS and extracellular DNA (eDNA) secreted by the microorganisms, and exhibit an altered phenotype with respect to growth rate and gene transcription (Donlan and Costerton 2002). Foodborne pathogens can form biofilms on food and food contact surfaces and are thus a big concern to food safety (Kumar and Anand 1998;

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doi: 10.1111/1541-4337.12087

Poulsen 1999; Chmielewski and Frank 2003; Brooks and Flint 2008; Shi and Zhu 2009; Simo˜es and others 2010; Jahid and Ha 2012; Srey and others 2013). When biofilms form on food processing surfaces, they exhibit resistance to common disinfectants (Jahid and Ha 2012; Srey and others 2013). Although much research has focused on monospecies and/or pure cultures, in natural environments biofilms consist of multiple bacterial species as well as fungi, algae, and protozoa (Percival and others 2000; Manuzon and Wang 2007; Moons and others 2009). Recently, mixed-species biofilms have gained importance in food microbiology and food safety since they are more resistant to disinfectants and sanitizers compared to monospecies/pure species biofilms. Table 1 presents numerous examples of mixed-species biofilms, their interactions, resistance to antimicrobials, and relevant key findings. This intuitive conflict (Table 1) motivated us to focus on whether research emerging from mixed-species biofilms on foods truly represents the mixed-species biofilms generally, and how it might overcome the problem in the context of food safety. This review highlights the dynamics of mixed-species biofilms, their relationship to indigenous microbiota (IM), the detection of mixed-species biofilms on food and food contact surfaces, their resistance to various disinfectants, jamming of quorum sensing (QS), and comments on prospective future research on mixed-species biofilms for enhanced food safety.

Mixed-Species Biofilms and the Food Safety Paradox

Although microbiological research began with studies of pure cultures of nonaggregated (planktonic) cells by Robert Koch in 1876, it is now well accepted that the biofilms life cycle is part of a dominant survival strategy in natural niches (Costerton and others 1978). After the discovery of biofilms, monospecies-based research was primarily conducted; however, such monocultural biofilms are rarely observed in foods and environmental and industrial settings where different microorganisms and surfaces exist in close proximity (Percival and others 2000; Sutherland 2001). The biofilms theory and its importance were proposed in 1978 (Costerton and others 1978). To our knowledge, the significance of biofilms in food safety was first reviewed in 1994 (Zottola and Sasahara 1994). Since then, almost 3 decades is going to pass which multiple studies have been carried out on biofilms, their molecular structure, and disinfectant resistance, as well as novel and alternative techniques to combat their growth on food and food contact surfaces. According to a Pubmed search, approximately 23300 articles with the keyword "biofilm" and approximately 3300 articles on "resistance to biofilms" have been published (as of: 2014.02.11), along with several reviews that address the importance of biofilms on foods and food contact surfaces (Kumar and Anand 1998; Poulsen 1999; Brooks and Flint 2008; Shi and Zhu 2009; Simo˜es and others 2010; Jahid and Ha 2012; Srey and others 2013). Since most foodborne pathogens form biofilms in food and are resistant to disinfectants and sanitizers, optimization of novel and alternative techniques is necessary to control the biofilms mode of growth (Simo˜es and others 2010; Jahid and Ha 2012; Srey and others 2013). These techniques include biofilms disruption, hurdle technology, phage-based technology, electrolyzed water, essential oils, cold oxygen plasma, molecular brush, surface modifications, photosensitizing agents, quorum quenching (QQ), and antibiofilm compounds (Brooks and Flint 2008; Simo˜es and others 2010; Jahid and Ha 2012; Olaimat and Holley 2012; Srey and others 2013). The significance of biofilms in the food industry has been reviewed in terms of general foods (Hood and Zottola 1995; Kumar and Anand 1998; Brooks and Flint 2008; Shi and Zhu 2009; Srey and others 2013), specific foods such as meat (Sofos and Geornaras 2010; Giaouris and others 2013a), fresh produce (Jahid and Ha 2012), and dairy manufacturing (Marchand and others 2012; Anand and others 2014). Most of these reviews and studies have explored the molecular and physiological interactions of monospecies biofilms. However, the interactions of multiple microorganisms in biofilms on food and food contact surfaces in different niches, and their contribution to the spread of foodborne pathogens, have remained elusive for a long time. Although various studies have highlighted the importance of mixed-species biofilms and interspecies interactions in foods (Table 1) (reviewed in Manuzon and Wang 2007; Moons and others 2009), mixed-species biofilms research is still in its infancy. Table 1 highlights the paradox: mixed-species biofilms are more resistant than monospecies biofilms for similar microbiota and food conditions. Given the diversity of biofilms in food niches, it is likely that mixed-species biofilms play a vital role in food safety and resistance to disinfectants and sanitizers. Mixed-species biofilms have been reported to inhibit pathogens (Guillier and others 2008), enhance the survival of pathogens through culturable (Ica and others 2012) and commensal interactions (Cowan and others 2000), provide higher resistance to disinfectants compared to monospecies (Lee and others 2013; Jahid and others 2014b), provide equal resistance to pathogens (Lebert and others 2007; Chorianopoulos and others 2008; Kay and others 2011; Kostaki and others 2012), internalization of pathogens (Jahid and others 2014b), or inhibit the internalization of pathogens (Figure 2). The obtained conflicting results can be attributed to different laboratory conditions, organisms studied, food and food contact surfaces, niches, types of disinfectants, and experimental methods used. Thus, significant research is necessary to address questions such as: what is the reality in nature and what actions should be taken for mixed-species biofilms in foods and the food industry? How could we control resilience in the mixed-species biofilms and its link to foodborne outbreaks?

Mixed-Species Biofilms and the IM in Food

It is well documented that every food and food contact surface comprises a specific niche and contains diverse microorganisms. IM biofilms have also been studied and are now well accepted (Figure 1; Morris and others 1997, 1998; Fett 2000; Rayner and others 2004; Manuzon and Wang 2007; Moons and others 2009; Cleto and others 2012; Jahid and others 2014b). Laboratory-based experiments with IM isolated from natural settings have shown that mixed-species biofilms can be formed by enteric pathogens such as *Salmonella* spp. (Jahid and others 2014b). In addition to the artificial laboratory methods, mixed-species biofilms in natural conditions exhibiting fungal hyphae and excessive EPS in tomatoes, carrots, mushrooms, cutting boards, and kitchen sponges have been observed by cryoscanning electron microscopy (Rayner and others 2004). Liu and others (2013) have also demonstrated that fresh produce contains approximately 23 different genera including soil bacteria, plant-related bacteria, coliforms, and opportunistic plant- or human-pathogenic bacteria, with *Pseudomonas fluorescens*, *Rahnella aquatilis*, and *Ralstonia insidiosa* being the most prevalent species. It has been noted that dual-species biofilms composed of resident microbiota from fresh-cut produce processing plants and *E. coli* enhanced the dual-species biofilms (Liu and others 2014). Most of the isolates were shown to be positive for biofilms formation, and 30% of these were moderate biofilms producers. Natural plant or soil microbiota were found to exert inhibitory or stimulatory effects on colonization or biofilms formation by

Table 1–Summary of the research conducted on mixed culture biofilms and the effectiveness of disinfectants

Types of microorganisms or cell extracts	Biofilm formation on surface of foods	Interaction	Mixed-species differentiation methods	Types of antimicrobial	Key findings	References
Salmonella typhimurium and cultivable natural microflora from lettuce	Stainless steel (SS) and lettuce	Competitive	Brilliant green agar (BGA) with nalidixic acid and novobiocin for *S. typhimurium* selection	UV-C irradiation	Resistance to UV-C by mixed-culture biofilms compared to mono-cultures on lettuce but not on SS	Jahid and others (2014)
Escherichia coli and Gram-negative species isolated from fresh produce-processing facilities	Microtiter plate and glass surface	Both neutral and stimulatory	Sorbitol MacConkey agar (SMAC) *for E. coli selection*, GFP with fluorescence microscopy	ND*	Environmental isolates enhance or remain neutral to biofilms of pathogenic bacteria	Liu and others (2014)
Acylated homoserine lactones-containing chicken breast muscle broth from *Pseudomonas aeruginosa*	Microtiter plate with chicken broth	Competitive	ND	ND	Inhibition of *P. aeruginosa* biofilms by quorum sensing compound- containing broth	Zhang and others (2014)
Listeria monocytogenes, *Pseudomonas fluorescens*, *Serratia proteamaculans,* or *Shewanella Baltica*	SS surface	Inhibitory or stimulatory	*Listeria* selective agar base for *Listeria, Pseudomonas* agar base for *Pseudomonas*, Pseudomonas agar base, *S. proteamaculans* was enumerated on violet red bile glucose (VRBG) agar, using scanning electron microscopy (SEM)	ND	More EPS, enhanced survival against desiccation and distinct biofilm structures	Alavi and Hansen (2013)
P. aeruginosa and *E. coli*	Silicon coupons	Inhibitory	MacConkey agar for *E. coli* and Pseudomonas isolation agar for *P. aeruginosa* and peptide nucleic acid-fluorescence *in situ* Hybridization combined with confocal laser scanning microscopy (PNA-FISH)	ND	*P. aeruginosa* outnumbered *E. coli* after 48 h	Cerqueira and others (2013)
Commensal *E. coli* with pathogenic *E. coli,* and *Klebsiella pneumoniae*	Microferm-entor glass slide and mouse model intestinal colonization	Inhibitory	LB with selective antibiotic	ND	Mixed-species biofilms express different genes than single species, and commensal species reduce pathogens biofilms	Da Re and others (2013)
L. monocytogenes and *Pseudomonas putida*	SS surface	Competitive, 90% of population was *P. putida*	Selective media	Benzalkonium chloride	Resistance to benzalkonium chloride	Giaouris and others (2013b)
E. coli and *P. aeruginosa*		Competitive		ND	Mixed-species biofilms have greater mass than monoculture biofilms	Kuznetsova and others (2013)
P. aeruginosa, Pseudomonas protegens, and *K. pneumonia*	Three-channel flow cells	Mixed biofilm formation delayed than single biofilm	Fluorescent-tagged bacteria and confocal laser scanning microscopy (CLSM), flow cells, 16S RNA	Tobramycin	Mixed-species biofilm compact structure and resistance to antibiotic	Lee and others (2013)
Stenotrophomonas rhizophila, Xanthomonas retroflexus, Microbacterium oxydans, and *Paenibacillus amylolyticus*	Microtiter plate with peg	Synergistic	Quantitative PCR based on SYBR Green I fluorescence with specific primers	ND	Strong synergistic interactions in a 4-species biofilm model, more than 3-fold increase in biofilm formation and strong dominance of 2 strains, *Xanthomonas retroflexus* and *Paenibacillus amylolyticus*	Ren and others (2013)

(*Continued*)

Table 1–Continued.

Types of microorganisms or cell extracts	Biofilm formation on surface of foods	Interaction	Mixed-species differentiation methods	Types of antimicrobial	Key findings	References
E. coli, Enterobacteriaceae cloacae, P. aeruginosa, Stenotrophomonas maltophilia Variovorax sp., Cupriavidus respiraculi Bradyrhizobium sp., Sediminibacterium sp., Novosphingobium subterraneum Sphingomonas sp., Blastomonas natatoria, and Methylobacterium isbiliense Mycobacterium sp.	Calgary biofilm device (CBD)	ND	DC agar *for E. coli and E. cloacae*; OFL for *P. aeruginosa* and *S. maltophilia* and CLSM	Chlorine	Mixed species are 50- to 300-fold more resistant compared to single-species biofilms, and distinct 3-D structures are observed for multispecies biofilms	Schwering and others (2013)
Shiga toxin-producing *E. coli* and *Salmonella enterica* serovar Typhimurium		Competition	Selective media	Common disinfectants	Mixed-species form more EPS, and are more resistant to sanitizers	Wang and others (2013b)
P. aeruginosa and *S. enterica* from meat processing plants	Polystyrene microplate	Competitive	ND	ND	Cell-free supernatant inhibits biofilms of *S. enterica* at exponential phase but not at stationary phase	Wang and others (2013a)
Burkholderia cepacia and *P. aeruginosa*	Silicone tubing	Competitive	Colony differentiation	Chlorine dioxide	Mixed biofilms are more resistant compared to monocultures	Behnke and Camper (2012)
Bacillus subtilis and *Staphylococcus aureus*	Polystyrene 96-well microtiter plates	Cooperative	GFP-tagged confocal electron microscopy and selective media	Peracetic acid	Mixed biofilms are more resistant because *B. subtilis* protects *S. aureus* isolates in mixed biofilms with higher EPS production	Bridier and others (2012)
L. monocytogenes and *P. putida*	Stainless steel and polypropylene sheets	Competitive	*P. putida* in VRBG agar with glucose; *L. monocytogenes* was determined by plating in PALCAM agar and SEM	benzalkonium chloride (BAC)	Mixed biofilms form more complex and dense structures,causing resistance to BAC	Ibusquiza and others (2012)
Campylobacter jejuni and *P. aeruginosa*	Flat plate flow reactor	Positive	Modified charcoal cefoperazone deoxycholate agar with an antibiotic supplement that contains cefoperazone and amphotericin B for *C. jejuni*; CLSM with live and dead kit	ND	*C. jejuni* survive better compared to mono-culture due to oxygen consumption by *P. aeruginosa*	Ica and others (2012)
S. aureus, L. monocytogenes and *Salmonella Enteritidis*	Polystyrene microplate	ND	ND	Surfactin from *B. subtilis* and rhamnolipids from *P. aeruginosa*	Mixed culture is more resistant compared to monocultures	Valle Gomes and Nitschke (2012)
Aeromonas hydrophila and *Flavobacterium* sp.	Chitin surface	Competitive	Colony differentiation (*A. hydrophila* formed smooth whitish colonies while *Flavobacterium* sp. formed structured orange colonies)	ND	*Flavobacterium* sp. outcompetes *A. hydrophila* in chitin biofilm	Jagmann and others (2012)
L. monocytogenes and *S. enterica*	Stainless steel	None	Xylose lysine deoxycholate agar for *S. enterica* and PALCAM Listeria selective agar for *L. monocytogenes*, pulsed-field gel electrophoresis (PFGE)	Benzalkonium chloride, sodium hypochlorite, peracetic acid, and a mixture of hydrogen peroxide and peracetic acid	Different strains show different ability to form biofilms in mixed culture and monocultures, mixed cultures are equally sensitive	Kostaki and others (2012)

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Table 1–Continued.

Types of microorganisms or cell extracts	Biofilm formation on surface of foods	Interaction	Mixed-species differentiation methods	Types of antimicrobial	Key findings	References
S. aureus and *E. coli*	Polypropyl-ene coupons	Competitive	Eosin methylene blue agar to count *E. coli* and Baird Parker agar for *S. aureus*	Essential oils (EOs) from citronella and lemon	Dual species are more resistant than single-species biofilms	Millezi and others (2012)
S. enterica serovar Thompson or Newport or with *P. fluorescens*	SS surface	Competitive	Live/Dead BacLight Bacterial Viability Kit with CLSM, XLT-4 agar (*Salmonella*) or Pseudomonas F agar (*P. fluorescens*)	T-128 (phosphoric acid and propylene glycol) and chlorine solution	Increase in resistance of *Salmonella* sp. when it forms mixed-species biofilms with *P. fluorescens*	Shen and others (2012)
S. enterica, *E. coli*, and *L. monocytogenes*	Glass, polypropylene, polyethylene, polyvinyl chloride, copper, silicone rubber, and stainless steel)	Competitive	MacConkey agar, that discriminates between *S. enterica* and *E. coli* based on each species' ability to consume lactose, and the Oxford agar for *L. monocytogenes* counts using PNA FISH with CLSM	ND	Top layers by *E. coli* and bottom layers by *S. enterica* and *L. monocytogenes*	Almeida and others (2011)
S. typhimurium and *Aspergillus niger*	Fungal hypae act as biotic surfaces	*S. typhimurium* forms biofilm on hyphae of *A. niger*	LB broth amended as appropriate with streptomycin, kanamycin, gentamycin, or ampicillin for bacteria; potato dextrose agar with tetracycline and cefoperazone for fungi; and Epifluorescence microscopy	ND	Bacteria form biofilms on hyphae using cellulose and chitin interaction	Brandl and others (2011)
E. coli and *P. aeruginosa*	Silicone rubber	Neutral	Selection by resistance to different antibiotics	Bacteriophage	Phage can penetrate inside both monoculture and mixed-culture biofilms, which act as reservoir for phage	Kay and others (2011)
L. monocytogenes and *P. aeruginosa*	CBD	Neutral	Selection of *L. monocytogenes* by PALCAM agar	Alkyl amine acetate, liquid alka-line/chlorine, powder alka-line/chlorine, phosphoric acid	Formation of mixed-culture biofilms shows lowered susceptibility at lower temperature	Lourenço and others (2011)
L. monocytogenes and *Lactobacillus plantarum*	Polystyrene microtiter plates	Almost neutral, a slightly lower count in mixed culture for *L. monocytogenes*	BHI agar containingerythromycin for *L. monocytogenes* and MRS agar containing kanamycin for *L. plantarum*. For microscopy differentiation; fluorescence protein DsRed was used for *L. monocytogenes* and GFP was used for *L. plantarum*.	Benzalkonium chloride and peracetic acid	Mixed-species biofilms are more resistant compared to monospecies biofilms	van der Veen and Abee (2011)
P. aeruginosa and *Candida comprising Candida albicans, C. glabrata, C. krusei, C. tropicalis, C. parapsilosis,* and *C. dubliniensis*	Polystyrene surface	Inhibitory	Sabouraud dextrose agar for *Candida* spp. and MacConkey for *P. aeruginosa* selection, CLSM, SEM	ND	Mixed-species form scanty biofilms while single-species form dense biofilms	Bandara and others (2010)
Cell-free supernatant of *Hafnia alvei* and *Salmonella enterica* serovar Enteritidis	SS coupons	Inhibitory	ND	ND	Cell-free supernatant reduces the biofilm formation of *Salmonella enterica* serovar Enteritidis but not artificial quorum sensing compounds	Chorianopoulos and others (2010)
Resident microflora from feed industry and *Salmonella enterica*	SS coupons	Synergistic and neutral	Laser-scanning confocal microscopy (LSCM) with GFP tagging	ND	Synergistic effect found for *Pseudomonas* and *Staphylococcus* spp.	Habimana and others (2010a)

(*Continued*)

Table 1–Continued.

Types of microorganisms or cell extracts	Biofilm formation on surface of foods	Interaction	Mixed-species differentiation methods	Types of antimicrobial	Key findings	References
E. coli O157:H7 and *Acinetobacter calcoaceticus* from meat industry	Sealed glass coverslip	Synergistic	GFP-tagged by *E. coli* O157:H7	ND	*E. coli* cells embedded and covered by *A. calcoaceticus* cells in mixed-species biofilms	Habimana and others (2010b)
E. coli O-:H4, *E. coli* O157:H7, *Salmonella* spp., *P. aeruginosa*, *Citrobacter* spp., *Serratia liquefaciens*, and *B. subtilis*	Glass microscope slides	Positive	Brain heart infusion with streptomycin antibiotic to differentiate *E. coli* O157:H7	Hydrogen peroxide	*E. coli* O157:H7 enhances the biofilm formation with dual-species biofilms as well as resistance	Uhlich and others (2010)
Dekkera bruxellensis, Saccharomyces cerevisiae, Saccharomycodes ludwigii, Schizosaccharomyces pombe, Acetobacter aceti, and *Lactobacillus hilgardii*	CBD	Both neutral and stimulatory	Selective medium with antibiotics	ND	*D. bruxellensis* is neutral with *A. aceti* while *A. aceti* forms 3-fold higher biofilms with *L. hilgardii*	Tristezza and others (2010)
Enterococcus faecalis, E. coli, P. aeruginosa, Salmonella agona, Staphylococcus simulans, and *C. jejuni*	Polystyrene microtiter plate	Both neutral and stimulatory	Modified cefoperazone charcoal deoxylate agar as selective medium for *C. jejuni*	ND	*E. faecalis and S. simulans* enhance while *P. aeruginosa and S. agona* are neutral to formation of mixed-species biofilms	Teh and others (2010)
L. monocytogenes and Staphylococcus epidermis	Polystyrene microtiter plate	Synergistic	SEM and PALCAM for *Listeria* selection	ND	Higher and stronger biofilms formed for mixed culture	Zameer and others (2010)
E. coli O157:H7 and resident flora from meat processing plants	Polyurethane conveyor belt	Synergistic or neutral	To differentiate from Gram-positive, *E. coli* O157: H7 CFUs were counted on TSA plates supplemented with novobiocin or acriflavin. To differentiate from Gram-negative, TSA with anaerobic system has been applied	ND	Resident microflora show favorable effect on biofilm formation of *E. coli* O157:H7	Marouani-Gadri and others (2009)
Serratia plymuthica and *E. coli*	Microscope glass coverslip	Competitive	LB with selective antibiotic for each strain and fluorescent protein with CLSM	ND	Wild-type shows more competitive interaction than deficient mutant of *Serratia plymuthica* with *E. coli*	Moon and others (2006)
L. monocytogenes serotypes 2a and 4b	SS coupons	Synergistic	Serotype-specific quantitative PCR and propidium monoazide treatment	ND	Mixed-culture forms more biofilms than single-culture	Pan and others (2009)
S. simulans and Lactobacillus fermentum, P. putida, Salmonella enterica, and *L. monocytogenes*	SS coupons	Competitive	Selective media	Essential oil of *Satureja thymbra* (1%), as well as its hydrosol fraction (100%)	Both monoculture and mixed-cultures show same effectiveness	Chorianopoulos and others (2008)
L. monocytogenes and biofilms natural microflora on wooden shelves used in the ripening of a soft and smear cheese	Glass fiber filters (GFF) deposited on sterile smear cheese	Competitive, natural microflora inhibit *L. monocytogenes* growth and biofilm formation	PALCAM agar for *Listeria* differentiation and SEM	ND	Natural microflora inhibit the biofilm formation of *L. monocytogenes*	Guillier and others (2008)
L. monocytogenes and *S. aureus* from dairy industry	SS coupons	Stimulatory, inhibitory, and neutral	Acriflavine Ceftazidime Agar supplemented with acriflavine; ceftazidime *for L. monocytogenes* counts and mannitol salt agar for *S. aureus* counts and SEM	ND	Effect is strain-dependent and supernatant also shows the effect	Rieu and others (2008)

(*Continued*)

Table 1–Continued.

Types of microorganisms or cell extracts	Biofilm formation on surface of foods	Interaction	Mixed-species differentiation methods	Types of antimicrobial	Key findings	References
C. jejuni and bacteria collected from a saline rinse of poultry processed broiler chicken carcasses	SS	Neutral effect	Campy-Cefex agar for Campylobacter differentiation and GFO tagged with Campylobacter	ND	*C. jejuni* can form biofilms on preexisting biofilms on SS	Sanders and others (2008)
Lactobacillus casei and *S. cerevisiae*	Both glass slide and microtiter plate	Synergistic	Morphology differentiation by light microscopy and SEM	ND	Enhanced biofilm formation with yeast cells with distinct morphology for mixed biofilms	Kawarai and others (2007)
Staphylococcus equorum, Staphylococcus succinus and Lactobacillus sakei as monoculture and *S. aureus* with *L. monocytogenes and Pseudomonas fragi* with *E. coli* as mixed cultures	Glass fibre filter	Inhibitory	*P. fragi* on cetrimide–fucidin–cephaloridine agar; *E. coli* on BHIA with ampicillin; *S. aureus* on Baird-Parker agar base supplemented with rabbit plasma and bovine fibrinogen; *L. monocytogenes* on *Listeria* agar	Brillo, PE 270% to 30 0.5%, *S. thymbra* essential oil	Antimicrobials have similar effects on both mono and mixed cultures	Lebert and others (2007)
L. monocytogenes, Yersinia enterocolitica, Salmonella enterica, E. coli O157:H7, *Pseudomonas marginalis*, and native microflora recovered from fresh peeled baby carrots	Baby carrot	Inhibitory	PALCAM agar for *L. monocytogenes*, tryptic soy agar supplemented with nalidixic acid for *Y. enterocolitica* or *E. coli* O157:H7, xylose-lysine-Tregitol 4 agar for *S. enterica*, and Difco Pseudomonas agar F supplemented with rifampicin for *P. marginalis*.	ND	Inhibitory effect caused due to antagonistic effect of associated microflora and pathogens	Liao (2007)
Microbacterium phyllosphaerae, Shewanella japonica, Dokdonia donghaensis, and *Acinetobacter lwoffii*	Polystyrene	Synergistic	16S rRNA PCR	Hydrogen peroxide and tetracycline	Multispecies form more biomass than single-species biofilms, also show resistance to antimicrobials	Burmølle and others (2006)
E. coli, *P. putida*, and *Staphylococcus epidermidis*	Glass tube	Synergistic and neutral	Quantitative PCR and FISH analysis	ND	Coadhesion mechanisms stimulate biofilms of *E. coli*	Castonguay and others (2006)
S. typhimurium and *E. coli*	Epithelial cells	Inhibitory	GFP and RFP with CLSM	ND	*S. typhimurium* displaces *E. coli* biofilms	Esteves and others (2005)
L. monocytogenes and resident microflora from food industry	SS coupons	Stimulatory, inhibitory, and neutral, depending on strains	PALCAM agar for *Listeria* enumeration	ND	Bacterial strains or supernatant have the positive effect on biofilms of *L. monocytogenes*	Carpentier and Chassaing (2004)
C. albicans and *S. epidermidis*	Catheter disks	Synergistic	Morphology by SEM	Fluconazole and vancomycin	Mixed-species, that is, fungi and bacteria protect each other from antibiotics	Adam and others (2002)
Shewanella putrefaciens and *P. fluorescens*	SS coupons	Inhibitory	rRNA oligonucleotide with fluorescent probe	ND	Both species form biofilms and inhibit the growth of *S. putrefaciens*	Bagge and others (2001)
Pseudomonas sp., *P. putida*, and *E. coli*	Glass surface	Commensal	CLSM	ND	Tower-like biofilm structure and mixed-culture forms higher EPS	Cowan and others (2000)
P. putida Acinetobacter sp., and 5 other Gram-positive bacteria	Four-channel flow cells	Synergistic	GFP expression with *in situ* hybridization with fluorescence-labeled 16S rRNA targeting probes	ND	Thick biofilms form and degrade toluene, rather than monocultures	Møller and others (1998)

*ND—not done.

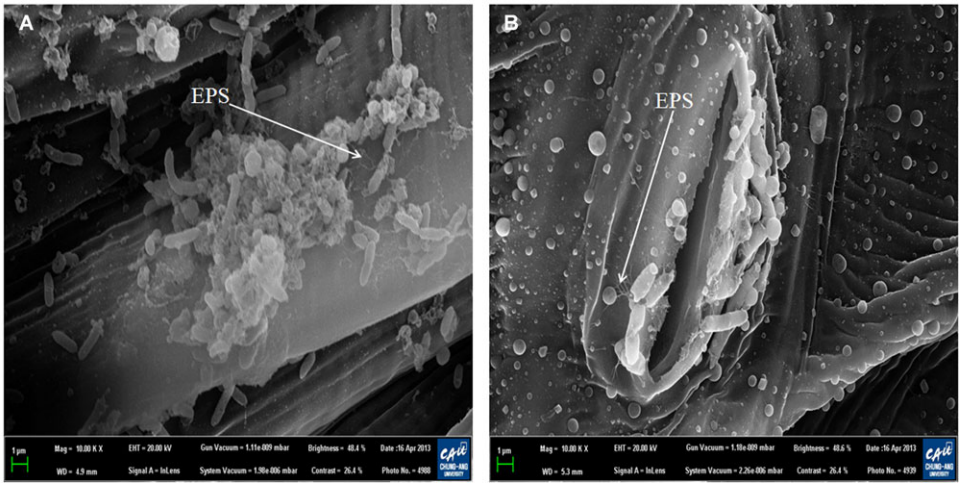

Figure 1–Field emission scanning electron microscopy images of extracellular polymeric substance (EPS) in lettuce leaves colonized by cultivable indigenous microbiota (CIM) and *Salmonella typhimurium*. The white arrows indicate EPS production. (A) EPS by CIM and (B) EPS by *S. typhimurium.*

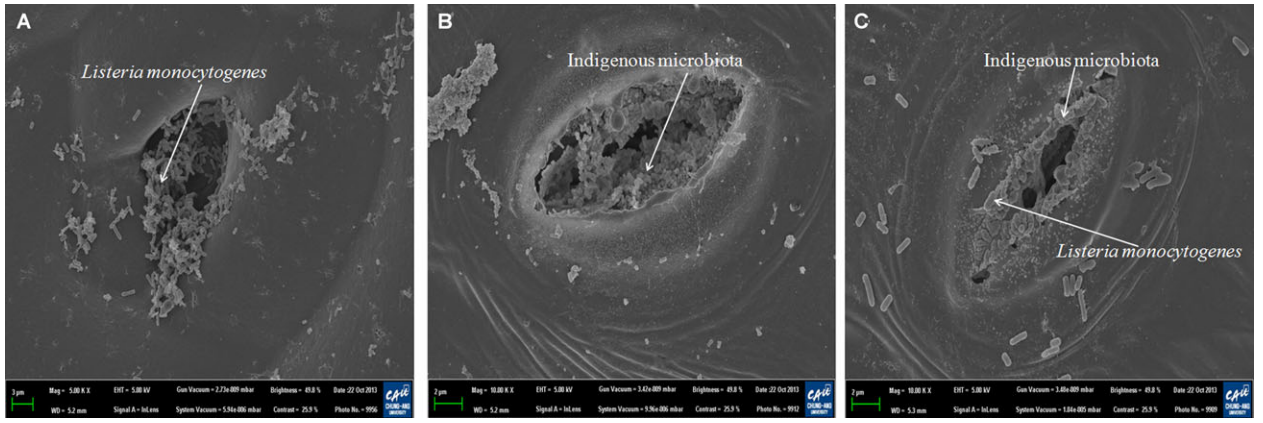

Figure 2–Field emission scanning electron microscopy images of stomatal colonization of lettuce leaves by *L. monocytogenes* and indigenous microbiota (IM) monocultures and mixed cultures. The white arrows indicate biofilm formation. (A) *L. monocytogenes* monospecies biofilms, (B) IM biofilms in lettuce, and (C) *L. monocytogenes* and IM mixed colonies in lettuce stomata.

enteric pathogens such as *E. coli* O157:H7 and *Salmonella* spp. in the rhizosphere and phyllosphere of fresh produce by flagella and fimbriae (Critzer and Doyle 2010). It has been observed by scanning electron microscopy (SEM) that alfalfa and other types of sprouts are abundant with rod-shaped and cocci-shaped bacterial mixed-species biofilms (Fett 2000). Another SEM study revealed that mixed-species of bacteria and yeast biofilms are abundant, while filamentous fungi are absent in mung bean sprouts (Fett and Cooke 2003). Culture-independent methods using pyrosequencing have demonstrated that fresh produce harbors diverse bacteria of the phyla Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria; the families are identified as Enterobacteriaceae, Leuconostocaceae, Moraxellaceae, Rhodobacteraceae, Bacillaceae, Micrococcaceae, Rhizobiaceae, and others (Leff and Fierer, 2013), and the dominant bacterial species are *Pantoea*, *Klebsiella/Raoultella*, *Pectobacterium*, *Janthinobacterium*, *Pseudomonas*, *Bacillus*, and *Acinetobacter* (Leff and Fierer 2013). In another study, *Bacillus* spp., *Pseudomonas* spp., *Erwinia* spp., and *Pantoea* spp. were shown to form the common IM in lettuce (Hou and others 2013). Morris and others (1998) demonstrated that 10% to 40% of the total population of leaves of endives and parsley are biofilms producers. Morris and others (1997) observed multispecies biofilms of Gram-positive and Gram-negative bacteria with prominent filamentous fungi on the leaves of various fresh vegetables (spinach, lettuce, Chinese cabbage, celery, leeks, and parsley). Rudi and others (2002) also reported mixed-species biofilms on lettuce where the dominant IMs were *Pseudomonas* spp., Enterobacteriaceae, and lactic acid bacteria (LAB), yeasts, and molds. Mathematical modeling studies, using pathogens at low densities, have predicted that IM in fresh produce enhances biofilms of *L. monocytogenes* and inhibits those of *Salmonella typhimurium* (Manios and others 2013). In contrast, research from our laboratory showed that IM inhibits the stomatal colonization of *L. monocytogenes* if the IM forms biofilms earlier at the stomata (Figure 2) (unpublished data). As shown in Figure 2(A), *L. monocytogenes* forms monospecies biofilms when IM is absent, whereas the IM forms biofilms inside the stomata when *L. monocytogenes* is absent. When these populations are mixed (Figure 2C), the IM forms biofilms inside the stomata and *L. monocytogenes* attaches to the edge of the stomata.

Multiple studies have demonstrated that milk processing plants have IM that enhances the biofilms formation by pathogens (Rieu and others 2008; Cleto and others 2012), the predominant species being *Pseudomonas* spp., *Serratia* spp., *Staphylococcus sciuri*, and *Stenotrophomonas maltophilia* (Cleto and others 2012). Many of these isolates are strong biofilms and siderophore producers. *Pseudomonas* spp. isolates produce either proteases or lecithinases at high levels, while *Serratia* spp. and *Pseudomonas* spp. are also positive for antimicrobial production (Cleto and others 2012).

Cleaning and sanitization procedures constitute common practices in the food industry to ensure hygienic conditions of food and food contact surfaces. Inappropriate sanitation can potentially form mixed-species biofilms by the resident microbiota in the food, particularly in the meat industry. Formation of meat biofilms by foodborne pathogens and their roles in the meat-processing environment have been reviewed (Giaouris and others 2013a). It can be speculated that biofilms of resident microbiota may enhance the cross-contamination of pathogenic bacteria by protecting mixed-species biofilms, thus posing a threat to food safety (Pérez-Rodríguez and others 2008). Mixed-species biofilms in the meat industry and on meat contact surfaces have been documented by several authors (Marouani-Gadri and others 2009; Habimana and others 2010a,b). As noted previously, resident microorganisms from the feed industry enhanced the formation of biofilms of *Salmonella* spp. (Habimana and others 2010a), while *Acinetobacter calcoaceticus*, an isolate from meat-processing environments, enhanced the biofilms formation of *E. coli* O157:H7 (Habimana and others 2010b). The representative IM in meat processing plants was composed of *Bacillus*, *Staphylococcus*, *Corynebacterium*, *Staphylococcus*, *Aeromonas*, *Brevibacterium*, *Micrococcus*, and *Pseudomonas* species, among others. Similarly, *Pseudomonas* spp. were noted as the dominant biofilms population in ground beef samples (Jay and others 2003). The IM was found to enhance the biofilms formation by *E. coli* O157:H7 from 0.37 to 1.11 log colony-forming units (CFU)/cm^2 for the EDL 933 strain and from 0.19 to 1.38 log (CFU/cm^2) for the Sakaï strain (Marouani-Gadri and others 2009). In contrast, IM as well as oxygen availability could modulate the *Listeria innocua* biofilms formation in minced chicken breasts (Noriega and others 2010). The above findings suggest that IM may enhance or reduce the biofilms formations of pathogens on meat and meat contact surfaces, thereby contributing to cross-contamination during meat processing. On the contrary, the IM could also act as a microenvironment for the residing pathogens and increase their resistance to disinfectants and sanitizers (Figure 3). The SEM images shown in Figure 3(A) are monospecies biofilms produced by *S. typhimurium* on chicken skin, and Figure 3(B) shows the biofilms produced by IM, whereas Figure 3(C) clearly demonstrates the mixed-species biofilms by both *S. typhimurium* and IM. The inhibitory or stimulatory effects of the planktonic state of natural flora on pathogens have been described by Al-Zeyara and others (2011). IM isolated from packaged mixed-leaf fresh salad, French Brie cheese, Camembert cheese, goat cheese, Italian salami, pasteurized chicken pâté, fresh minced beef, and packaged smoked salmon had been reported to inhibit the growth of *L. monocytogenes* in broth (Al-Zeyara and others 2011). Although it is important to consider the fitness of the planktonic state of bacteria, that discussion is beyond the scope of this review.

Collectively, the diverse microorganisms present in specific food niches and the natural mixed-species biofilms in food have an impact on the cross-contamination of pathogens or other microorganisms. Rendueles and Ghigo (2012) suggest that in multispecies biofilms, 1 species may act as unfriendly and competitive neighbor, whereas conflicting reports indicate that different species may cooperate (Elias and Banin 2012). Three types of interactions, neutral, positive, and inhibitory, have been reported between *L. monocytogenes* and the IM in the catering, meat, milk, and cheese industries (Carpentier and Chassaing 2004). Thus, it is essential to identify the IM that are "unfriendly" or "friendly" neighbors to pathogens in specific food industries as well as their resistance to antimicrobials such asdisinfectants, sanitizers, and antibiotics.

Mixed-Species Biofilms and Food Fermentation

Another important food industry where mixed-species are a primary concern is the fermentation process since diverse microorganisms are required to ferment and produce appropriate tastes and flavors by changing pH and food ingredients (reviewed in Smid and Lacroix 2013). Although this review focused on the planktonic state, it also highlighted aspects of QS, mixed-species metabolism, and fitness with diversified microbes. Diverse natural populations, such as the LAB, have been identified using the culture-independent polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis (DGGE) method during the fermentation process of naturally fermented Aloreña green table olives (Abriouel and others 2011). The identified microorganisms were *Gordonia* sp./*Pseudomonas* sp. and *Sphingomonas* sp./*Sphingobium* sp./*Sphingopyxis* sp., *Thalassomonas agarivorans*, halophilic archaea (mainly haloarchaeon/*Halosarcina pallida*, and uncultured archaeon/uncultured haloarchaeon/*Halorubrum orientalis*), and yeasts (*Saccharomyces cerevisiae* and *Candida* cf. *apicola Pichia* sp., and *Pichia manshurica*/*Pichia galeiformis*), and some LAB including *Lactobacillus pentosus*/*Lactobacillus plantarum*, and *Lactobacillus vaccinostercus*/*Lactobacillus suebicus*, *Lactobacillus paracollinoides*, and *Pediococcus* sp. Although these results were based on the planktonic state of microorganisms, it is possible that these microbes form biofilms in fermentation tanks and thus control the quality of fermented foods.

The fermentation and production of bacteriocin are largely dependent on LAB. The growth and biofilms formation of LAB have been evaluated using QS methods (Maldonado and others 2009) and through cocultivation with other microorganisms (Ruiz-Barba and others 2010). Ruiz-Barba and others (2010) noted that *Enterococcus faecium* and *Pediococcus pentosaceus* induced the growth and survival of *L. plantarum*. Natural biofilms formed by different strains could protect biofilms formation by pathogens such as *L. monocytogenes* (Guillier and others 2008). Therefore, it is possible that formation of mixed-species biofilms in the fermentation industry could protect foods from sources of pathogens and cross-contamination of bacterial pathogens by inhibiting the growth and biofilms formation by pathogens. Goria and others (2011) found both AI-2 positive and negative species in smears of surface-ripened cheeses and identified the bacterial strains as *Arthrobacter nicotianae*, *Corynebacterium ammoniagenes*, *Corynebacterium casei*, *Microbacterium barkeri*, *Microbacterium gubbeenense*, *Staphylococcus equorum* subsp. *linens*, *Brevibacterium casei*, and *Brevibacterium linens*. Mixed-species biofilms of *L. pentosus* and yeast with EPS were observed using SEM during Spanish-style green table olive fermentation (Domínguez-Manzano and others 2012); a change in yeast populations with no effect on *L. pentosus* at the final stage of fermentation was observed.

Thus, we hypothesize that the success of fermentation and the quality of fermented products depends on mixed-species biofilms populations and their interactions.

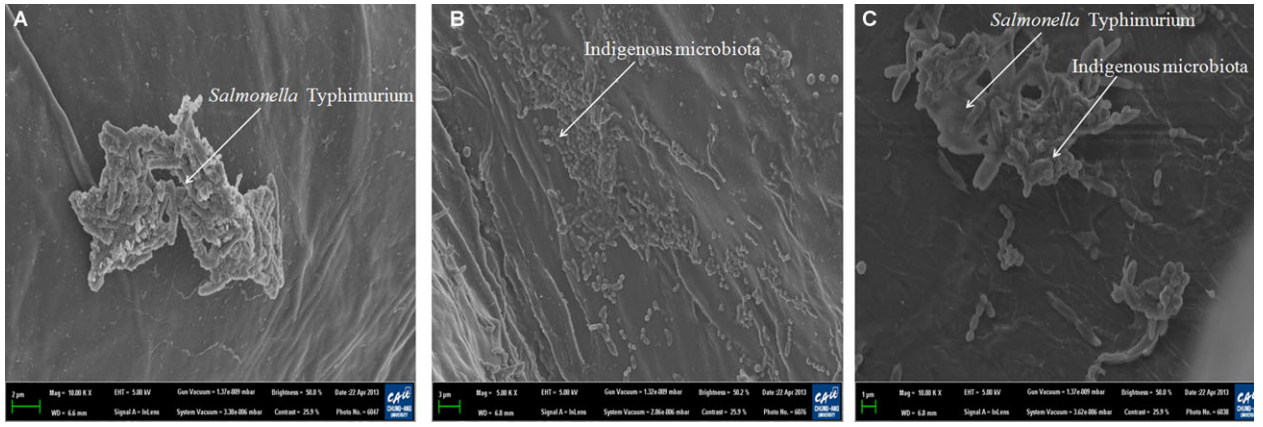

Figure 3–Field emission scanning electron microscopy images of biofilm formation in chicken skin, colonized by *S. typhimurium* (ST) and IM monocultures and mixed cultures. The white arrows indicate biofilm formation. (A) Single colonies of ST on chicken skin. (B) Single colonies of CIM on chicken skin. (C) Mixed colonies of ST and CIM on chicken skin.

Detection Methods of Mixed-Species Biofilms

Various culture-independent and culture-dependent methods have been used for studying mixed-species biofilms structure, physiology, and competitive interactions of different species in various foods and food contact surfaces (Table 1). The most useful technique to differentiate known strains from mixed-species biofilms is a culture-based approach using selective media for specific bacteria. For studies that include cultivable cells, cells forming mixed-biofilms can be differentiated using selective media. The interaction between mono- and mixed species can be identified from the specific counts of selective media (Guillier and others 2008; Alavi and Hansen 2013; Giaouris and others 2013b; Schwering and others 2013). In our laboratory, we used selective media for *Salmonella* with resistance to nalixic acid and novobiocin to differentiate *S. typhimurium* from cultivable natural bacteria from lettuce (Jahid and others 2014b). Alternately, Kay and others (2011) used antibiotic resistance for selectivity. Although culture-based methods are easy methods for differentiating between microorganisms, their disadvantage is that not all types of microorganisms grow in artificial laboratory media. Biofilm-forming microorganisms can change their phenotype and genotype, and a viable but nonculturable state of microorganisms can lead to misinterpretation of results (reviewed in Trevors 2011). To solve this problem, metagenomics of pyrosequencing and Illumina-based sequencing form better alternatives to study the mixed-species present in food niches and their fitness in unknown samples using culture-independent methods (reviewed in McLean and Kakirde 2013). Quantitative PCR based on SYBR Green I fluorescence along with microorganism-specific primers is another alternative method to differentiate between the known mixed-species biofilms population (Ren and others 2013). To visualize and observe the biofilms structure, microscopy techniques have been routinely used for a long time. Mixed-species biofilms can be visualized by light microscopy (Shen and others 2012), SEM (Figure 1, 2, and 3) (Morris and others 1998; Fett 2000; Rayner and others 2004; Jahid and others 2014b), and confocal laser scanning microscopy (CLSM) (Shen and others 2012). CLSM is one of the best alternatives to differentiate between mixed-species biofilms in conjunction with either green fluorescent protein (GFP) (Habimana and others 2010a) or peptide nucleic acid fluorescence *in situ* hybridization (PNA-FISH) (Almeida and others 2011; Cerqueira and others 2013). Strains with integrated GFP may behave differently compared to natural strains, potentially changing experimental results. Hence, it may be better to perform confocal microscopy using 16s rRNA probes or PNA-FISH (Almeida and others 2011). Continuous-optimizing confocal reflection microscopy may also be used to monitor the time course of mixed-species biofilms formations since this procedure does not necessitate a staining procedure (Inaba and others 2013). Recently developed molecular technologies such as DNA microarray (Manuzon and Wang 2007; Dai and others 2011; Pammi and others 2013), denaturing high-performance liquid chromatography (Manuzon and Wang 2007), proteomics (reviewed in Di Cagno and others 2011; Klein and others 2012; Sánchez and others 2013), and *in vivo* studies in mice (Pammi and others 2013) and/or other animal models could also help to identify the gene regulation and virulence of mixed-species biofilms in specific food niches (reviewed in Sauer 2003). The combination of cDNA microarray with an immunomagnetic separation technique has been useful for mixed-species biofilms analysis (Dai and others 2011). A DNA array was successfully employed to demonstrate significant differences in gene expression in mixed-species biofilms, compared to monospecies biofilms (Redanz and others 2011). Thus, combination of new techniques may prove beneficial in understanding the mechanism, of fitness of specific populations, and resistance pattern of mixed-species biofilms.

Formation Stages of Mixed-Species Biofilms

From their planktonic state, microorganisms form biofilms in a stepwise manner, although the individual stages are not always clear since the process is multifaceted and dynamic in nature. However, the cumulative efforts of numerous microbiologists and molecular biologists over the last 30 y have established that biofilms are formed via 5 distinct consecutive stages: (1) initial reversible attachment, (2) irreversible attachment by QS and EPS secretion, (3) microcolony formation, (4) maturation, and (5) dispersal of biofilms communities back to their planktonic counterparts (Figure 4; reviewed in Van Houdt and Michiels 2010; Kostakioti and others 2013). Factors required for biofilms formations vary depending on the growth conditions, surface properties, and environment; the average difference of protein expression has been estimated at 35% for each of the 5 stages (Sauer and others 2002).

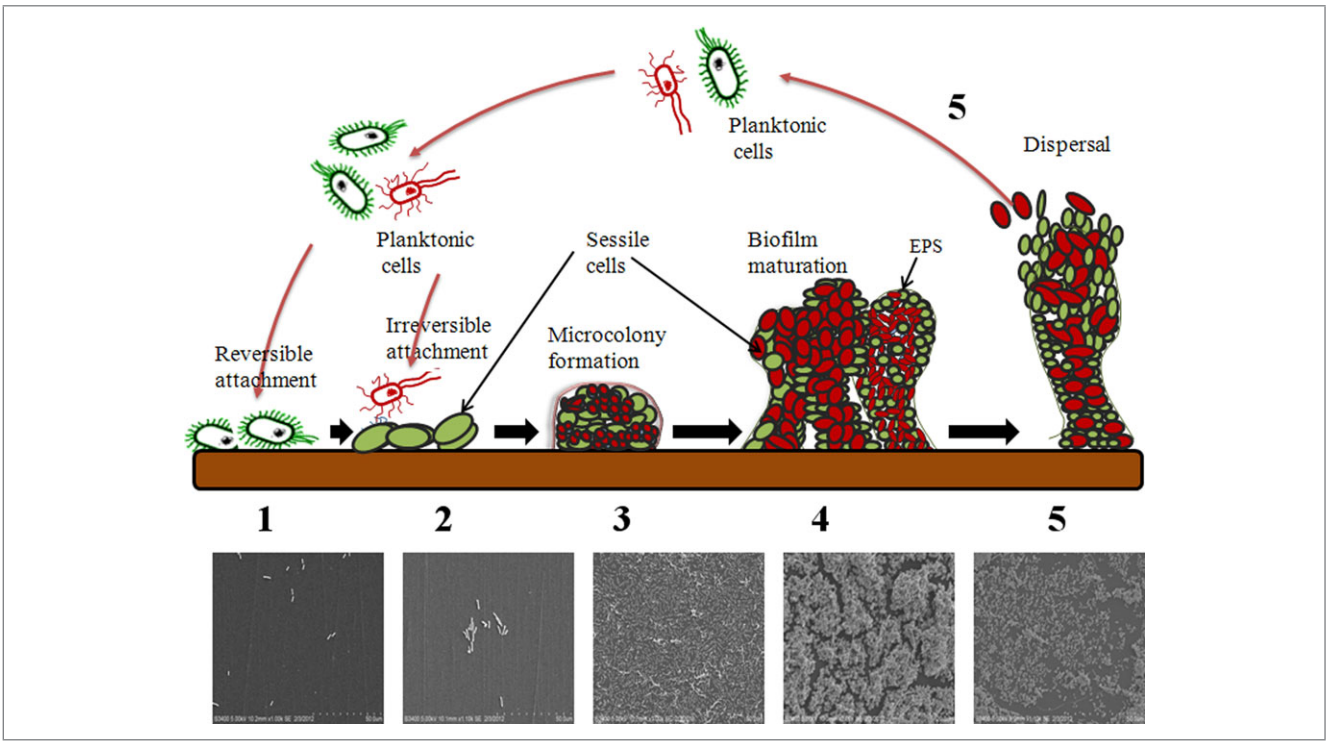

Figure 4–Representation of a hypothetical developmental model of a mixed-species biofilm. Mixed-species biofilm formation involves 5 distinct stages identified as: (1) reversible attachment, (2) irreversible attachment, (3) microcolony formation, (4) mature biofilms, and (5) dispersal. The bottom panels show each of the 5 stages of development represented by a photomicrograph of *A. hydrophila* when grown in microtiter plate.

The general steps for mixed-species biofilms formation are represented in Figure 4, and the schematic representation of the mixed-species biofilms paradigm of *L. monocytogenes, E. coli,* and *Salmonella enterica* is outlined in Figure 5. The formation of mixed-species biofilms is highly dynamic, changes with time, and depends on the interactions of many components such as the microorganisms involved in biofilms formation, the food and food contact niches, and the surrounding external and internal environmental signals (Davey and O'Toole 2000; Donlan 2002; Dunne 2002; Stoodley and others 2002). Below, we summarize the individual stages of the mixed-species biofilms formation process.

Reversible Attachment

Reversible attachment is the 1st step in the formation of biofilms sessile cells from planktonic mobile cells (Figure 4). The initial attachment for monospecies varies due to bacterial cell surface properties such as pili, flagella, fimbriae, outer membrane proteins, cell hydrophobicity, and the ability to coaggregate and autoaggregate (Donlan 2002). The regulating factors for initial attachment are bacterial cell surfaces, flagella, curli fimbriae, surface appendages, surface polysaccharides, temperature, nutrient availability, and pH of the surrounding medium (Figure 5; Van Houdt and Michiels 2010). However, curli fimbriae have been shown to play nonfunctional roles in the initial attachment of *S. enterica* on *Aspergillus niger* hyphae (Brandl and others 2011). In addition, biotic and abiotic surface properties such as roughness, cleanability, disinfectability, wettability, and vulnerability are additional factors contributing to the formation of mixed-species biofilms (Figure 5; Van Houdt and Michiels 2010). As QS in mixed-species are different compared to monospecies, surfaces appendage and motility contribute differently to the initial attachment as these properties are controlled by QS (Daniels and others 2004; Van Houdt and others 2007; Jahid and others 2013). In mixed-species biofilms, initial aggregation is associated with the diverse microorganisms present in the niche. Compared to monospecies, coaggregation was higher in mixed-species biofilms in which *L. monocytogenes* first attached onto the surface followed by the overlay of *Myroides odoratus* (Jacobs and Chenia 2009). In mixed-species biofilms of *L. monocytogenes* serotypes 1/2a and 4b strains, no relationship was observed among flagellae, initial attachment, and biofilms (Pan and others 2009). The authors discovered that even if the initial attachment is similar in both the strains, they may compete with each other, and serotype 1/2a may form biofilms more efficiently than serotype 4b. The initial attachment and subsequent biofilms formation of *E. coli* O157:H7 was found to increase in the presence of the indigenous population present in meat (Dourou and others 2011b). This attachment was independent of abiotic surfaces, such as stainless steel or high-density polyethylene surfaces, but was dependent on the incubation temperature (Dourou and others 2011b). The deduced mechanism of enhanced biofilms formation by *E. coli* on mixed-species is deduced as co-adhesion with adherence-proficient bacteria (Castonguay and others 2006). In mixed-species biofilms, the initial attachment always depends on partners, whereas co-colonization is important for maturation and final biofilms formation (Klayman and others 2009). The planktonic cells of 1 species can either compete or cooperate to pre-colonize other species to form mixed-species biofilms (reviewed in Monds and O'Toole 2009; Wang and others 2012). It has been hypothesized that in mixed-species biofilms, one microorganism initially attaches to a biotic or abiotic surface and begins to grow by supporting or competing with other microorganisms, and that the final biofilms formed depends on the fitness of the microorganisms, their nutrient utilization ability, and other genetic factors (Monds and O'Toole 2009). Monds and O'Toole (2009) also

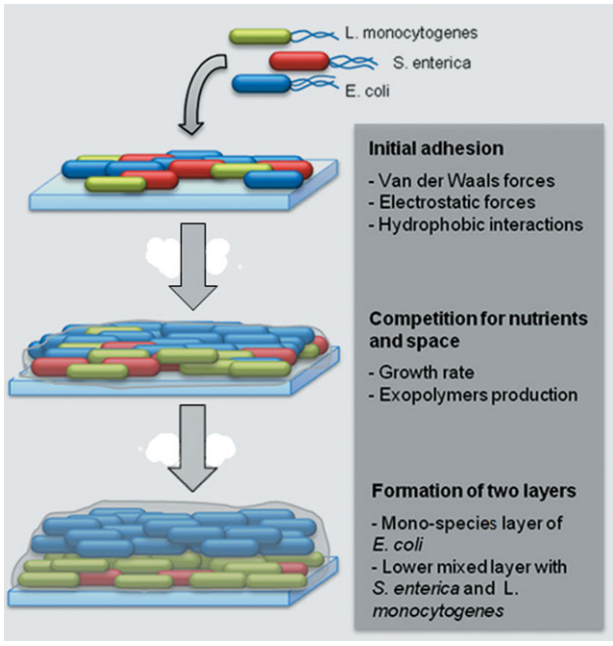

Figure 5–Hypothetical representation of trispecies biofilm formation by *L. monocytogenes, S. enterica, and E. coli*, showing the main steps and the key factors involved on the 2 layers shown. The image was adapted from Almeida and others (2011), discriminating the multispecies populations in biofilms using peptide nucleic acid fluorescence *in situ* hybridization (PNA FISH). PLoS ONE 6(3): e14786.

speculate that the microenvironment and mixed-species adaptations may help to form mature biofilms. Thus, the initial attachment depends on the species involved, their interactions, and extrinsic environmental conditions, which are generally different from those in monospecies biofilms. Even in the presence of all favorable factors, initial attachment is a reversible and dynamic process, and can revert back to the planktonic state due to hydrodynamic and repulsive forces (Dunne 2002) as well as nutrient availability (Anderson and others 2008; Wu and Outten 2009).

Irreversible attachment

After the initial reversible attachment to biotic or abiotic surfaces, microorganisms secrete QS molecules and EPS, which then leads to irreversible attachment (Figure 4), initially controlled by type 1 pili, curli fibers, and Antigen 43 (Figure 5; Kjærgaard and others 2000; Kostakioti and others 2013). Antigen 43 is known to enhance the intraspecific and interspecific cell aggregation between *E. coli* and *P. fluorescens* in a flow chamber (Kjærgaard and others 2000). The process of reversible to irreversible attachment also involves several genetic elements such as the *sad* gene encoding type IV pili, the lapBCE-encoded ABC transporter, and the secreted LapA protein (Hinsa and others 2003) and QS and EPS secretions (Davey and O'Toole 2002; Caiazza and O'Toole 2004). For centuries, bacteria were thought to be single cells that could not communicate with each other, like eukaryotes. However, it is now accepted that prokaryotes can communicate with other prokaryotes and even with eukaryotes using the so-called microbial language (Miller and Bassler 2001; Schauder and Bassler 2001), which has led to the development of socio-microbiology, the study of microbial coordinations and multicellular behavior (reviewed in Parsek and Greenberg 2005; Dunny and other 2008; Nadell and others 2009). Although bacteria and yeasts are unicellular, QS studies have shown that they can behave like multicellular organisms for the production of antibiotics and bacteriocins, biofilms formations, and for causing diseases in plants and animals (Dunny and other 2008; Nadell and others 2009). QS is widely used by both Gram-positive and Gram-negative bacteria to communicate with each other (Miller and Bassler 2001; Schauder and Bassler 2001) and with eukaryotes (Tait and others 2009; Venturi and Fuqua 2013). In most food niches, bacteria primarily reside as biofilms where microorganisms "talk and listen" to each other using QS-based extracellular cell–cell-signaling systems (Schauder and Bassler 2001). There are 4 kinds of QS molecules discovered so far. In general, Gram-negative bacterial genomes encode for autoinducer-1 (AI-1) that secretes *N*-acylhomoserine lactones (AHLs) and autoinducer-3 (AI-3), while Gram-positive bacterial genomes encode autoinducing peptide (AIP) that functions in signaling pathways for intraspecific communication (Miller and Bassler 2001; Schauder and Bassler 2001; Bai and Rai 2011). In addition, all types of bacteria have autoinducer-2 (AI-2) for interspecific communication (Miller and Bassler 2001; Schauder and Bassler 2001; Bai and Rai 2011). Although the study of QS in mixed species is in its infancy, QS has been historically important in terms of general microbiology and food safety. Many studies (Table 1) and reviews (Smith and others 2004; Bai and Rai 2011; Skandamis and Nychas 2012) have been published on QS in foods and food contact surfaces. Various intrinsic factors such as glucose (Jahid and others 2013) and extrinsic factors such as temperature (Blana and Nychas 2014; Jahid and others 2014a), pH, and NaCl control QS. QS secretions control the enzymes responsible to spoilage of foods by food-spoilage organisms (Wevers and others 2009). We have previously reported the glucose-mediated inhibition of QS and biofilms of *Aeromonas hydrophila* (Jahid and others 2013), and have also demonstrated that temperature modulates QS, biofilms formation, internalization, and resistance of *A. hydrophila* to cold oxygen plasma (Jahid and others 2014a). However, mixed-species biofilms of different genera in minced beef induced microbial growth of genera of the family Enterobacteriaceae, *Pseudomonas* spp., and LAB, as well as AHL molecules with the temperature increasing from 0- to15 °C (Blana and Nychas 2014). The authors also reported that QS production was induced as the time of incubation increased. Lau and others (2013) isolated and characterized Enterobacteriaceae from lettuce and found *Enterobacter asburiae* to be positive for the production of N-butanoyl homoserine lactone (C4-HSL). However, some studies did not find a correlation between the heat and acid resistance of AI-2 based on QS of the foodborne pathogens *Salmonella* spp. and *E. coli* O157:H7 (Yoon and Sofos 2008, 2010). The pathogenic, resistant, and biofilms properties of *Salmonella* spp. and *E. coli* may be attributed to the AHL signal received from other bacteria present in food systems using the receptor sdiA and not by AI-2. Production of secondary metabolites like pyocyanin has been identified in mixed species of *Enterobacter* sp. and *Pseudomonas aeruginosa* by QS, but was absent in pure culture (Angell and others 2006). Despite the above evidence, the importance of QS in biofilms formation is still debated, and some authors (Kjelleberg and Molin 2002) argue that hydrodynamics, nutrient load, and intracellular carbon influx may actually have a major impact on biofilms rather than QS.

Microcolony formation

When microorganisms attach to biotic or abiotic surfaces, they communicate with each other by QS and secreted EPS, and then multiply inside the EPS to form a microcolony (Figure 4 and 5). The basic building block of biofilms is the microcolony; thus, the

basic biofilms processes such as QS, disinfectant resistance, EPS production, and fitness of species on surfaces can be elucidated from the microcolony structure of biofilms (Donlan and Costerton 2002). Limited research has been conducted on microcolonies of mixed-species biofilms and their roles in antimicrobial resistance. For microcolony formation, several factors such as motility (flagella, pili, and curli fimbriae), QS, and EPS production are required (Figure 5; Hung and others 2013). Previous reports have implicated microcolony formation and different biofilms structures in mixed-species biofilms under different circumstances (An and others 2006; Dominiak and others 2011; Almeida and others 2011; Lee and others 2013). In mixed-species biofilms, eDNA plays a vital role in strengthening microcolony formation (Dominiak and others 2011). The authors also found 300 mg of eDNA per g of organic matter in each microcolony. When dual-species cocultivation of *P. aeruginosa* and *Agrobacterium tumefaciens* was performed, *P. aeruginosa* was found to dominate the coculture biofilms and cover the microcolony of *A. tumefaciens* (An and others 2006). In mixed-species biofilms, microcolony formation is mostly dependent on the types of species present. *L. monocytogenes* is known to gather outside the microcolony formed by *Kocuria varians* and *Staphylococcus capitis* but forms a separate microcolony in dual-species biofilms with *Comamonas testosteroni* (Carpentier and Chassaing 2004). Using confocal microscopy image analysis, Lee and others (2013) observed the formation of biofilms in which *Klebsiella pneumoniae* microcolonies were surrounded by the randomly distributed *P. aeruginosa* and *Pseudomonas protegens*.

Maturation

Maturation is the most stable stage in biofilms formation where the sessile populations can exist as aggregates of monolayers or multilayers, and cell clusters have different shapes such as mushroom, tulip-like structures with channels for liquid and gases that can outflow the waste products surrounded by EPS (Figure 4 and 5; Donlan 2002; Stoodley and others 2002; Kostakioti and others 2013). However, other researchers refrain from differentiating the processes of microcolony formation and biofilms maturation (Monds and O'Toole 2009). The structure of biofilms varies depending on bacterial types (Bridier and others 2010), age of biofilms (Doiron and others 2012; Xiao and others 2012), nutrients present on biotic or abiotic surfaces or the surrounding liquid media (Xiao and others 2012; Jahid and other 2013), and the species present in mixed-species biofilms (Figure 5; Stoodley and others 2001; Bridier and others 2012). The proportion of species present varies according to their propensity to form biofilms and, generally, the contribution of different species is unequal (Stoodley and others 2001). The authors demonstrated the presence of *K. pneumoniae* (81.6%), with *P. aeruginosa* (11.5%), *S. maltophilia* (5.0%), and *P. fluorescens* (1.9%) in the final mature stage of biofilms formation. We also demonstrated that without glucose, monospecies *A. hydrophila* film formed 3-dimensional structures, whereas at 1% glucose, monolayer biofilms were observed by SEM (Jahid and others 2013). In mixed-species biofilms, media nutrients such as glucose and sucrose were found to modulate EPS secretion and, ultimately, biofilms maturation (Xiao and others 2012). The authors also reported that higher EPS production results in longer times of biofilms maturation, and that EPS regulates biofilms structures as well as their virulence properties (Xiao and others 2012). Several studies have demonstrated higher EPS production in mixed-species biofilms compared to monospecies (Cowan and others 2000; Bridier and others 2012; Alavi and Hansen 2013; Wang and others 2013b; Jahid and others 2014b). Additional research has found that mixed-species biofilms can induce the formation of compact 3-dimensional structures (Møller and others 1998; Ibusquiza and others 2012; Lee and others 2013; Schwering and others 2013), differential gene expression (Da Re and others 2013), as well as increased biomass compared to monospecies biofilms (Burmølle and others 2006; Kuznetsova and others 2013).

Biofilms are composed of cells and EPS, which contain 50% to 90% of total organic carbon content (Donlan 2002). The microorganisms secrete EPS, or so-called "public goods," for protection from stress conditions such as nutrient deprivation, desiccation (Alavi and Hansen 2013), oxidative stress, antimicrobial stress (Wang and others 2013b; Jahid and others 2014b), biofilms structure (Xiao and others 2012; Alavi and Hansen 2013), and virulence (Xiao and others 2012). The composition of EPS, especially in mixed-species biofilms, is very complex and depends greatly on the component species, growth conditions, external cues, and QS. The main components of EPS are often categorized into various polysaccharides, lipids, proteins (Diggle and others 2006), pili, flagella, other adhesive fibers (Pinkner and others 2006; Cegelski and others 2009), and eDNA (Watnick and Kolter 2002). In general, it has been documented that the production of EPS may be enhanced by the interactions of mixed species in such biofilms (Cowan and others 2000; Xiao and others 2012; Jahid and others 2014b). Xiao and others (2012) noted that the high EPS secretion by *Streptococcus mutans gtfB/gtfC* genes was further enhanced by *Actinomyces naeslundii* and *Streptococcus oralis*. In mixed-species biofilms of *S. typhimurium* and *E. coli*, EPS-negative strains were more resistant compared to EPS-producing strains, indicating that the "public goods" are used by nonproducers without losing energy and increasing resistance to sanitizers (Wang and others 2013b). Higher eDNA has been reported by several authors in mixed-species biofilms due to autolysis (Dominiak and others 2011; Pammi and others 2013). By contrast, EPS has been found to reduce the biofilms formation of pathogens such as enterohemorrhagic *E. coli* (EHEC) (Kim and others 2009).

Dispersal

Bacterial biofilms proceed through a cyclic process, dispersal being the final stage of their life cycle (Figure 4). Dispersal generally involves the movement of single cells to form aggregates with a diameter of approximately 500 μm (Stoodley and others 2001). Dispersal of monospecies biofilms can be governed by several cues such as the species present in biofilms, nutrient fluctuations, changes in oxygen levels, c-di-GMP, cAMP, degradation of the biofilms matrix, induction of motility, nitric acid, and increase in toxic products or metabolites and/or surfactants (Sauer and others 2004; Karatan and Watnick 2009; McDougald and others 2011; Huynh and others 2012; Kostaki and others 2012). The genetic background of the individual species in mixed-species biofilms is also important to the dispersal from biofilms. It has been noted that biofilms dispersal protein BdcA from *E. coli* modulates c-di-GMP, which, in turn, modulates the motility and EPS production leading to the dispersal of mixed-species biofilms of *E. coli* with *P. aeruginosa*, *P. fluorescens*, and *Rhizobium melilot* (Ma and others 2011). Iron regulators (such as fur and pvdS) of *P. aeruginosa* modulate the dispersal of *A. tumefaciens*, whereas the latter species cannot control the dispersal of *P. aeruginosa* (Hibbing and Fuqua 2012). Programmed cell death is thought to play a key role in dispersal (Webb and others 2003). Likewise, glucose starvation is also known to induce cAMP and disperse *P. aeruginosa* biofilms (Huynh and others 2012). Not only the biofilms formation but

also the jamming of QS plays a vital role in the dispersal of biofilms populations from mixed-species biofilms (Hong and others 2012). Thus, it can be concluded that mixed-species biofilms dispersal is completely different from its monospecies counterpart, and interactions between different species play essential roles in dispersal in response to environmental cues.

Jamming of QS of Mixed-Species Biofilms in Food

Since QS is density- and dose-dependent, its regulation in mixed- and monospecies may be different; for example, QS regulates the virulence, biofilms formation, motility, and protease production in mixed species (McNab and others 2003; An and others 2006). The significant difference is due to jamming of QS and regulation of 1 population by another population or its metabolites. It is already established that AHL is an interspecific QS molecule, while Al-2 is an intraspecific QS molecule (Miller and Bassler 2001). In mixed-species biofilms, bacteria communicate with each other and inhibit one another by producing antibacterial, antiadhesive compounds, jamming of QS, and induction of biofilms dispersal (Rendueles and Ghigo 2012). The AHL degrader *Bacillus cereus* showed degradation of *Yersinia enterocolitica* on pork meat (Medina-Martínez and others 2007). Recently, acylated homoserine lactones-containing cultures from chicken breast muscle broth were found to inhibit the biofilms formation of *P. aeruginosa*, indicating potential jamming of QS and reduction of biofilms, which could, in turn, contribute to food safety (Zhang and others 2014). The jamming of QS is species-specific and it has been reported that AHLs and AI-2 signaling compounds present in the cell-free culture supernatants (CFSs) of *P. aeruginosa*, *Y. enterocolitica*, and *Serratia proteamaculans* are reduced, while *P. aeruginosa* accelerates the metabolic activity and growth of *Salmonella enteritidis* and *S. typhimurium* (Dourou and others 2011a). Wang and others (2013a) revealed that CFS of *P. aeruginosa* isolated from chicken meat had the ability to inhibit biofilms formation by *S. enterica* isolates in pork meat, chicken meat, and meat processing surfaces. It has also been observed that CFS of *Hafnia alvei*, but not the artificial AHLs, reduced the early stages of biofilms formation of *S. enteritidis* (Chorianopoulos and others 2010). It is worth noting that both a QS mutant and antimicrobial mutant of *Serratia plymuthica* did not compete with *E. coli* mixed-species biofilms (Moons and others 2006). Chan and others (2011) also discovered the coexistence of both QS and QQ bacteria belonging to the genera *Acinetobacter*, *Burkholderia*, and *Klebsiella* from the ginger rhizosphere. In our laboratory, we also found that mixed-species biofilms of *S. typhimurium* and *Pectobacterium carotovorum* secrete less AHL compared to monospecies (unpublished data). Proteomic analysis revealed that *Lactobacillus acidophilus* cell extract downregulates virulence factors and biofilms formation by jamming the AI-2 activity of *E. coli* O157:H7 (Kim and others 2008). By contrast, QS in mixed-species biofilms has been shown to be unidirectional where *Burkholderia cepacia* receives signals from *P. aeruginosa* but not vice versa (Riedel and others 2001).

From the above discussion, it may be reasoned that both QS and QQ microorganisms and/or only the positive QS-secreting species can form mixed-species biofilms in natural food niches and modify the mixed-species biofilms formation steps with selection of microorganisms. Thus, jamming of QS significantly might control the foodborne pathogens biofilms formation as well as diseases outbreaks.

Paradox of Resistance to Antimicrobials within Mixed-Species Biofilms

Compared to monospecies, mixed-species biofilms are known to show higher resistance (Table 1) to common disinfectants such as chlorine (Behnke and Camper 2012; Kostaki and others 2012; Schwering and others 2013; Wang and others 2013b), benzalkonium chloride (van der Veen and Abee 2011; Ibusquiza and others 2012; Kostaki and others 2012; Giaouris and others 2013b), UV-C (Jahid and others 2014b), peracetic acid (van der Veen and Abee 2011; Bridier and others 2012; Kostaki and others 2012), surfactin from *Bacillus subtilis* and rhamnolipids from *P. aeruginosa* (Gomes and Nitschke 2012), essential oils (Millezi and others 2012), and hydrogen peroxide (Burmølle and others 2006; Uhlich and others 2010). The resistance mechanisms of mixed-species biofilms have been hypothesized to include limited diffusion and/or slow penetration due to EPS and 3-dimensional structures, physiological heterogeneity of sessile cells (that is, resistance phenotypes), cross resistance to sanitizers, horizontal gene transfer, altered microenvironment (pH change, waste product accumulation, nutrient depletion), persister cells (that is programmed cell death), and internalization to inaccessible sites such as trichomes and stomata of leaves in fresh produce (Donlan and Costerton 2002; Lewis 2007, 2010; Jahid and Ha 2012; Olaimat and Holley 2012). All these resistance mechanisms are reasoned for monospecies biofilms. The significantly higher resistance of mixed-species biofilms can be attributed to factors such as induction of QS (Vanlint and others 2013), interspecific communication, enhanced eDNA formation (Pammi and others 2013), higher biovolume of biofilms (Burmølle and others 2006; Uhlich and others 2010), higher EPS formation (Wang and others 2013b; Jahid and others 2014b), internalization into foods (Deering and others 2012; Jahid and others 2014b), differential 3-dimensional structures (Lee and others 2013; Schwering and others 2013), dense biofilms structures (Ibusquiza and others 2012), and protection or shielding of 1 species by others (Adam and others 2002). As shown previously (Vanlint and others 2013), loss of cAMP/CRP regulon may potentially enhance the resistance to high hydrostatic pressure due to jamming of QS in mixed-species biofilms (Table 1). Accordingly, every aspect of enhancing activity in mixed-species biofilms and physiology influences the higher resistance of disinfectants, sanitizers, and antibiotics to mixed-species biofilms. In contrast, several studies report that mixed-species biofilms do not differ in their resistance to antimicrobial agents compared to monospecies (Table 1; Lebert and others 2007; Chorianopoulos and others 2008; Kay and others 2011; Kostaki and others 2012). Herein lies why the difference is unknown as to whether mixed-species biofilms are more resistant than monospecies biofilms. It has already been established that biofilms are difficult to eradicate compared to planktonic cells but still there is debate that mixed-species biofilms are more difficult to eradicate than monospecies biofilms or not. Further, since mixed-species biofilms are very common in food, controlling and minimizing their occurrence is essential for food safety. Thus, further research aimed at studying the specific conditions or food niches related to the resistance or sensitivity of mixed-species biofilms is required.

Paradox of Fitness of Different Populations in Mixed-Species Biofilms

Although microorganisms are unicellular, they communicate and cooperate to exhibit many multicellular density-dependent behaviors such as QS, secondary metabolite production, biofilms formation, and virulence properties (West and others 2006). For

these circumstances, the social evolution and behavior of microorganisms closely resemble the multicellular responses attributed to mechanisms of kin selection (West and others 2006). The fitness of mixed-species biofilms of different species can be positive, neutral, and negative on the basis of kin selection or clonality of the populations forming mixed-species biofilms, and they interact using QS and through direct coordination for the productions of antimicrobials, metabolites such as oxygen or iron, and/or toxins, bacteriocins, and secretions of EPS (Figure 1) or eDNA (reviewed in Hibbing and others 2010). As shown in Figure 1, IM and *S. typhimurium* secrete a mesh-like EPS structure and anchor onto the surface of lettuce leaves. Although many studies have been carried out on mixed-species biofilms, their roles in natural food niches are difficult to predict since they contain many types of microorganisms, including eukaryotes. As discussed above, QS could also be used to communicate with eukaryotes, making it difficult to understand the cumulative effects in a natural setting. It is noteworthy that the interactions of mixed-species biofilms vary depending on the microorganisms present in the food environment as well as the type of food (Table 1). Many studies have associated cooperative interactions with higher productivity (Burmølle and others 2006; Ren and others 2013), higher "public goods" (such as EPS or eDNA) production (Cowan and others 2000; Alavi and Hansen 2013), thick biofilms formations (Møller and others 1998; Pan and others 2009; Zameer and others 2010; Kuznetsova and others 2013), special 3-dimensional structures (Cowan and others 2000; Ibusquiza and others 2012; Lee and others 2013), bacterial–fungal nutrient interdependency (Brandl and others 2011), and aerobic–anaerobic interactions (Ica and others 2012). Two alternate studies also suggested that competition could serve to secrete higher amounts of EPS (Wang and others 2013b; Jahid and others 2014b). However, competitive interactions of mixed-species biofilms are more common than positive and neutral interactions since the populations need to contend for food, nutrients, space, and scavenging molecules secreted by individual species (Table 1). In another study, Teh and others (2010) reported a positive effect for mixed-species biofilms in poultry strains of *C. jejuni* with *Enterococcus faecalis* and *Staphylococcus simulans* and a neutral relationship between *Salmonella agona*, *P. aeruginosa*, and *E. coli*. Thus, mixed-species biofilms interactions depend on the microorganism's present, nutrient availability, and environmental cues, demonstrating that the mixed-species biofilms process is far more complex than the monospecies biofilms process. By studying 180 two-species mixtures grown in aquatic microcosms, it has been concluded that mixed-species interactions were in competitive (predator–prey) interactions rather than cooperative or synergistic interactions (Foster and Bell 2012). The most important cause of competition in mixed-species biofilms is jamming of QS, as discussed earlier. Different species can communicate and compete with each other through the secretion of AI-2. In another study, *L. monocytogenes* and/or its culture supernatant induced the AI-2 QS gene expression of *L. acidophilus* while suppressing the growth of its own population as well as causing changes in pH, which may be an example of interspecific communication and cooperative effect on fitness between populations (Moslehi-Jenabian and others 2011). The authors suggested that the "public goods" user, *L. monocytogenes* did not gain any survival advantage. We can thus hypothesize that the fitness of mixed-culture biofilms depends not only on the AHL production or degradation but also on other factors such as pH change or metabolite supply, as antimicrobial agents or toxic warfare secreted extracellularly might function as "public goods" or "public bads," and might contribute to the positive, neutral, or negative effects on fitness of mixed-species biofilms. The combined effect would be the final goal for the selection of species from mixed-species biofilms in food niches. Competitive interactions might regulate biofilms dispersal and the outcome for 1 species from among the population from mixed-species biofilms or reduce the population size (Esteves and others 2005; Almeida and others 2011; Giaouris and others 2013b; Kuznetsova and others 2013). A higher growth rate and stronger adhesive properties of 1 species can cause it to outgrow other populations (Cerqueira and others 2013). Several other studies reveal that the formation of antimicrobial, exometabolite (Kuznetsova and others 2013), and bacteriocin (Tait and Sutherland 2002) producers may have a fitness advantage over other nonproducer populations in mixed-species biofilms. A well-defined microscale spatial structure of mixed-species biofilms and a minimal distance between each species separated by the EPS components could act as "public goods" and barriers to stabilize the mixed-species biofilms (Kim and others 2008). Many foods contain chitin, which functions as a biotic surface for biofilms formation, and many bacteria can secrete chitinases that play important roles in the mixed-species biofilms ecology (Brandl and others 2011; Jagmann and others 2012; Drescher and others 2014). In monospecies biofilms, *S. enterica* produces chitin and attaches to form biofilms on *A. niger*, which contains chitin on its hyphal surfaces; however, chitin nonproducers like *E. coli*, *Pantoea agglomerans*, and *Pseudomonas chlororaphis* are unable to form biofilms (Brandl and others 2011). In mixed-species biofilms of chitin-degrading bacteria, *A. hydrophila* has been outgrown by a chitin-user species of the genus *Flavobacterium* on a chitin surface (Jagmann and others 2012). It has also been suggested that producers of "public goods" (such as AI-2, EPS, and eDNA) lose their energy, while nonproducers benefit from the "public goods" and grow faster than producers, slowly outgrowing the producers in mixed-species biofilms (Chuang and others 2009). An alternate study has also noted that the thick biofilms formation by *Vibrio cholerae* using chitin or fluid flow prohibits access to the nonproducers, thus favoring the growth of the producers (Drescher and others 2014). Even without the QS and EPS production, the change in pH of a local microenvironment in food also plays a role in the competitive interactions in mixed-species biofilms (Moslehi-Jenabian and others 2011; Jahid and others 2014b).

The genotypes of mixed-species biofilms are difficult to reconcile. Several studies have suggested that mixed-species biofilms enhance EPS production (Jahid and others 2014), eDNA formation (Pammi and others 2013), internalization to foods (Jahid and others 2014), higher heterogeneity of the populations, competitive interactions (Guillier and others 2008), composition and spatial organization of species, that is, resilience (Lee and others 2013), and horizontal gene transfer (Aminov 2011) including conjugal plasmid transfer (Reisner and others 2006; Meervenne and others 2014). Another report documents that the *luxS* (AI-2) mutant strain of *Streptococcus gordonii* is able to form a mixed-species biofilms with the wild-type *Porphyromonas gingivalis* strain but not with the AI-2 mutant strain, suggesting the cooperative usage of AI-2 molecules by both species (McNab and others 2003). Several studies show that both QS and QQ bacteria can be found in the same food niche to contribute to the mixed-species biofilms (Chan and others 2011). High ratios of multiresistance plasmids have been discovered from dual-species biofilms of *E. coli* and *Pseudomonas putida* (Meervenne and others 2014). This kind of horizontal gene transfer in mixed-species biofilms can confer cooperative behavior by a kin selection-like mechanism (Nogueira and others 2009).

The QS within a mixed-species biofilms might also promote horizontal gene transfer among *Vibrios* species including *V. cholerae* (Antonova and Hammer 2011). By contrast, if a sessile cells donor transfers the "public goods" genes to a sessile recipient, the donor species could become a cooperator and the recipient acts as a cheater (Lawrence 2009). It has also been noted that *L. monocytogenes* mixed-species biofilms depend on the genetic background of the resident *Lactococcus lactis* population (Habimana and others 2009). Compared to the wild type, EPS-mutant strains of *L. lactis* inhibit the biofilms formation of *L. monocytogenes* (Habimana and others 2009). These results suggest that "public goods" nonproducers have the ability to compete in *L. monocytogenes* biofilms formation, but producers (wild-type EPS producer strain) cannot outgrow the pathogens *L. monocytogenes*.

Although numerous studies have implicated AHL in biofilms on food and food contact surfaces, the 3 most threatening pathogens, *L. monocytogenes*, *E. coli,* and *Salmonella* spp., do not have AHL and cannot control their virulence and biofilms formation through AHL. However, *E. coli* and *Salmonella* spp. have only the "listening" receptors, sdiA, which can accept the response from AHL secreted by other species in mixed-species biofilms (Michael and others 2001; reviewed in Soares and Ahmer 2011), thus regulating the gene expression for antibiotic resistance, biofilms formation, acid resistance, and virulence (Van Houdt and others 2006; reviewed in Smith and others 2011). The AHL-mediated expression of sdiA in *Salmonella* spp. has been reported in mixed-species biofilms of *P. carotovorum* (Noel and others 2010) and *Y. enterocolitica* (Dyszel and others 2010; Soares and Ahmer 2011). Thus, it can be hypothesized that the IM might play vital roles in virulence, disinfectant resistance, acid resistance, and biofilms on food and food contact surfaces using the sdiA of these 2 pathogens. This is also advantageous to bacteria, since they can use AHL as "public goods" without expending their energy in the production of QS. This suggests that such a community-level resilience of mixed-species of *Salmonella* spp. and *E. coli* may be unique to the most threatening pathogens in foods and on food contact surfaces.

Currently, we have an arsenal of technology to conduct research on the fitness of mixed-species biofilms in food and food contact surfaces using transcriptomics, proteomics, and electron microscopy techniques to solve the conundrum of mixed-species biofilms in terms of food safety.

Novel Concepts for the Control of Mixed-Species Biofilms

Since biofilms are resistant to common sanitizers and disinfectants, it is obvious that novel and alternative control measures are essential for food safety and to reduce the mortality and morbidity caused by foodborne pathogens. Studies have already established that mixed-species biofilms are more resistant than monospecies biofilms (Table 1). In addition, current sanitization methods with high product concentrations have additional drawbacks such as the possible toxicity of the disinfectant residues and those that are beyond the proposed guidelines set by different food regulatory agencies. According to Morbidity and Mortality Weekly Report (MMWR) (2013), from 1998 to 2008, no reduction of foodborne pathogens was observed in the U.S.A., although higher outbreaks were noted in 2008 in the case of several foods such as fresh produce, beef, pork, and dairy. Within this time period, many new control techniques were developed, but food safety is still in a critical situation. In these circumstances, the emerging focus is on research for new disinfectants and antibiofilm compounds for the control of mixed-species biofilms. Several review articles have addressed novel and alternative methods for monospecies biofilms control (Xavier and others 2005; Simo˜es and others 2010; Jahid and Ha 2012; Goodburn and Wallace 2013; Srey and others 2013). The novel target for the control of mixed-species biofilms should be a natural product that is able to act at different stages of biofilms formation. Several new promising methods have also been successfully proven to be effective against mixed-species biofilms such as bacteriophages (Kay and others 2011), essential oils (Lebert and others 2007; Chorianopoulos and others 2008), biosurfactants (Valle Gomes and Nitschke 2012), and enzymes (Marcato-Romain and others 2012). Bacteriophages were found to penetrate mixed-species biofilms and successfully kill targeted pathogens within mixed populations (Sillankorva and others 2010; Kay and others 2011). Several commercial companies have already developed a bacteriophage to control foodborne pathogens of *L. monocytogenes*, *Salmonella* sp., and *E. coli* (www.ebifoodsafety.com; http://intralytix.com/index.htm). Poly(ethylene-co-vinyl acetate) copolymer (EVA) films containing essential oil components such as citronellol, eugenol, and linalool have been shown to reduce the mono- and dual-species biofilms of *L. monocytogenes*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *E. coli*, and *P. aeruginosa* (Nostro and others 2013). Furanones isolated from the marine alga *Delisea pulchra* have also been extensively studied as QS inhibitors (de Nys and others 2006; Janssens and others 2008). Dispersal-promoting agents (DPAs) such as polysaccharide depolymerases, esterases, dispersin B, proteases, nucleases, chitinase, and DNase can disrupt the EPS and subsequently control mixed-species biofilms (Xavier and others 2005; McDougald and others 2011). Synergistic effects of 2 or more disinfectants can also be applied to reduce the mixed-species biofilms (Leistner 2000). The combination of modified atmospheres in the presence of oregano essential oil volatile compounds reduced the mixed-species biofilms by IM and QS in meat storage from 0 to 15 °C (Blana and Nychas 2014), while individual effects of essential oils were ineffective in reducing mixed-species biofilms (Millezi and others 2012). Jamming of QS by IM from minced beef, salami, soft cheese, fresh salad, and chicken pâté (for example, LAB, *Brochothrix thermosphacta*, *Pseudomonas* spp., Enterobacteriaceae, and enterococci) could reduce the *L. monocytogenes* biofilms formation (Al-Zeyara and others 2011). Anti-QS strategies have been studied and reported by Priya and others (2013). Phytochemicals are also reported to possess anti-QS and antibiofilm activities in different microorganisms such as *Y. enterocolitica* and *Erwinia carotovora* (Truchado and others 2012), *Chromobacterium violaceum* (Borges and others 2014), and *E. coli* O157:H7 (Lee and others 2013). Rhamnolipids from *P. aeruginosa* and surfactin from *B. subtilis* were also effective against mixed-species biofilms of *S. aureus*, *L. monocytogenes,* and *S. enteritidis* although monospecies were more sensitive than mixed species (Valle Gomes and Nitschke 2012). In addition, lipopeptide biosurfactants from *Paenibacillus polymyxa* have been effective on both monospecies and mixed-species biofilms of *B. subtilis*, *Micrococcus luteus*, *P. aeruginosa*, *S. aureus*, and *Streptococcus bovis* (Quinn and others 2012). EPS from species like the marine bacterium *Vibrio* sp. (Jiang and others 2011) and probiotic bacteria (Kim and others 2009) has also been documented to control mixed-species biofilms. Thus, a distinct green technology might prove to be a novel and alternative control strategy to combat mixed-species biofilms.

Future Research Propescts

In the future, it may be interesting to answer the most relevant questions regarding mixed-species biofilms and food safety

Table 2–Key questions regarding future research on mixed-species biofilms in food and food contact surfaces

How do the indigenous microorganisms influence mixed-species biofilm formation of pathogenic bacteria?
What is the role of sdiA of *Salmonella* spp. and *E. coli* in virulence and pathogenesis?
How is the signaling and coordinating behavior involved in the resistance of pathogens to antimicrobials?
Can novel and alternative techniques minimize mixed-species biofilms?
What is the contribution of "public goods" in mixed-species biofilms?
What are the molecular mechanisms that are involved in food niches?
Does the current research provide sufficient focus on future food safety from mixed-species biofilms?
Which techniques are helpful to differentiate between mixed-species biofilms?
Which sociobiology mechanism is involved in mixed-species biofilms in food systems?
What is the relationship between fitness and resistance to antimicrobials and mixed-species biofilms?

(Table 2). In reference to the current research on mixed-species biofilms and food safety (Table 1), we reasoned that current data undoubtedly support the claim that the fitness of mixed-species is important to food safety and the development of resistance to antimicrobials. The majority of studies have focused on resistance, fitness, and control of mixed-species biofilms by randomly selecting populations without correlation with their food niche. As a result, it is not possible to conclusively support or reject the proposed mechanisms in the context of natural conditions. Metagenomics, which was not possible 10 y ago, is now routinely used in laboratory settings to identify the interactions between IM and pathogens. Culture-dependent and independent methods (such as pyrosequencing) could be useful to identify all the IM present in a food niche and further interaction with foodborne pathogens. We urge researchers to focus more closely on associations with particular food niches using metagenomics, metatranscriptomics, and metaproteomics as well as novel and green technology to combat mixed-species biofilms. By enhancing our knowledge on the interspecific interactions in mixed-species biofilms, we may be able to reduce foodborne diseases and exploit the benefits of food fermentation, probiotics, and other food quality-enhancing effects.

Conclusion

The motivation for writing this review came from a growing concern regarding mixed-species biofilms and their molecular interactions, with special interest in the artificial settings of mixed-species biofilms in food niches rather than natural or IM populations present in specific food niches. Since the discovery of biofilms in 1978 (Costerton and others 1978) and QS in 1994 (Fuqua and Winans 1994), a renaissance of sociomicrobiology (Parsek and Greenberg 2005) has taken place. Mixed-species biofilms have been found to reflect real, true biofilms found in nature. Mixed-species biofilms, their role in food safety, jamming of QS, control processes, novel detection methods, and suggested future essential research in this field by food microbiologists and food specialists could enhance the knowledge in this emerging field.

Finally, as is obvious from this mixed-species review, different novel "green" techniques such as the use of bacteriophages, anti-QS, bacteriocins, antibiofilms, essential oils, surfactants, and enzymes (such as, dispersin B) appear to be better alternatives to wage war against mixed-species biofilms.

Acknowledgments

This research was supported by the Basic Science Research Program through the Natl. Research Foundation of Korea funded by the Ministry of Science, ICT, and Future Planning (2013005051).

List of Abbreviations

Indigenous microbiota (IM), Quorum sensing (QS), Quorum quenching (QQ), Extracellular polymeric substances (EPS), Extracellular DNA (eDNA), Denaturing gradient gel electrophoresis (DGGE), Scanning electron microscopy (SEM), Lactic acid bacteria (LAB), Confocal laser scanning microscopy (CLSM), Green fluoresce protein (GFP), Peptide nucleic acid fluorescence *in situ* hybridization (PNA-FISH), *N*-acylhomoserine lactones (AHLs), Cell-free culture supernatants (CFS)

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