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Biofilm formation by *Salmonella* Typhimurium and *Staphylococcus aureus* on stainless steel under either mono- or dual-species multi-strain conditions and resistance of sessile communities to sub-lethal chemical disinfection

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ABSTRACT

Intercellular interactions encountered within and between different bacterial species are believed to play key roles in both biofilm formation and antimicrobial resistance. In this study, *Salmonella* Typhimurium and *Staphylococcus aureus* (3 strains per species) were left to form biofilms on stainless steel coupons incubated at 20 °C for 144 h (i.e. 6 days), in periodically renewable growth medium, under either mono- or dual-species conditions. Subsequently, the developed sessile communities were exposed for 6 min to sub-lethal concentrations of: (i) benzalkonium chloride (BC, 50 ppm), (ii) sodium hypochlorite (NaClO, 10 ppm), or (iii) peroxyacetic acid (PAA, 10 ppm). The dominance of each strain in the mono- and dual-species biofilm communities, both before and after disinfection, was monitored by pulsed field gel electrophoresis (PFGE). Results revealed that dual-species conditions led to a significant (*ca.* 10-fold) reduction in the number of sessile cells for both species, compared to mono-species ones, with inter-species interactions however found to not exert any significant effect on the disinfection resistance of each species as a whole. However, PFGE analysis revealed that the different strains here employed behaved differently with regard to biofilm formation and disinfection resistance, with this effect to be also strongly dependent on the culture conditions (mono-/dual-species) and the disinfectant applied. Such results expand our knowledge on multi-species biofilms formed by foodborne pathogenic bacteria and could hopefully be helpful in our efforts to develop effective elimination strategies and thus improve food safety.

1. Introduction

Salmonella spp. are significant zoonotic Gram-negative enterobacteria, which are also commonly associated with a variety of foods, provoking thus outbreaks of foodborne diseases in developing, as well as industrialized countries (Sánchez-Vargas, Abu-El-Haija, & Gómez-Duarte, 2011). Extra-animal survival is an important parameter for the environmental dissemination of salmonellae, with the ability of these bacteria to survive in the food chain to be largely due to their tremendous ability to sense and adapt to a diverse range of adverse environmental conditions (Runkel, Wells, & Rowley, 2013). Numerous previous studies aiming to explain stress adaptation and survival mechanisms of *Salmonella* have further linked these processes to the ability of these bacteria to attach to various both abiotic and biotic surfaces and create biofilms (Giaouris & Nesse, 2015). These are assemblages of microorganisms adherent to each other and usually to a surface and embedded in a matrix of self-produced extracellular polymeric substances (EPS)

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(Hall-Stoodley, Costerton, & Stoodley, 2004).

Staphylococcus aureus is a Gram-positive, ubiquitous bacterial species commonly found on the skin and hair, as well as in the noses and throats of people and animals (Castro, Santos, Meireles, Silva, & Teixeira, 2015). It is the causative agent of a wide spectrum of human infections (Otto, 2013) and is also often responsible for foodborne intoxications through the production of heat stable enterotoxins in a variety of food products (Hennekinne, De Buyser, & Dragacci, 2012). At the same time, methicillin resistance *S. aureus* (MRSA) has been primarily considered as a health care-associated pathogen, causing potential serious invasive disease, in which multidrug resistance poses a substantial challenge to successful treatments. Nevertheless, MRSA has also been isolated from meat and other food products (Wendlandt, Schwarz, & Silley, 2013) and has also been implicated in foodborne outbreaks as well (Jones, Kellum, Porter, Bell, & Schaffner, 2002). Like for other bacteria, the ability of *S. aureus* to attach to surfaces and create biofilms can greatly allow its survival in hostile environments, such as those of food processing. In particular, these bacteria are known to produce a multilayered biofilm embedded within a slime layer with heterogeneous protein expression throughout (Laverty, Gorman, & Gilmore, 2013).

The last decades, biofilm formation by bacterial pathogens has attracted much attention, mainly in the medical and food processing fields, due to its potential risks, including stress resistance, persistence and virulence factor production (Bridier et al., 2015). It is also now well accepted that biofilms represent a microbial phenotype, characterized by an explicit organization level, wherein microorganisms may be involved in complex intercellular interactions, that occur both within and between species and can be competitive, cooperative or even neutral, depending on the microbial species involved and the prevailing environmental conditions in the specific niche (Giaouris et al., 2015). These cell-to-cell interactions surely affect both the temporal and spatial formation of a highly organized community architecture (Moons, Michiels, & Aertsen, 2009). For instance, interactions encountered at the stage of microbial adhesion determine the initial community structure, while as biofilm development subsequently proceeds, stabilizing interactions between species can lead to increased biofilm thickness and stability, influencing thereby biofilm architecture and physiology. To this direction, several studies have further demonstrated that interactions between biofilm bacteria can influence their relative resistance (Burmølle et al., 2006; Giaouris, Chorianopoulos, Doulgeraki, & Nychas, 2013; Lee et al., 2014; Simões, Simões, & Vieira, 2009).

Remarkably, in food processing environments, a variety of different bacteria may attach to surfaces, survive, grow and form multispecies biofilm communities, able in this way to withstand chemical disinfection and other stresses as well (Jahid & Ha, 2014). Moreover, the surface attached cells in such environments may sometimes be exposed to inadequate (sub-lethal) levels of disinfectants, for instance when sanitation is not properly performed and/or equipment and overall factory layout is not properly designed (Langsrud, Moen, Møretrø, Løype, & Heir, 2015). Particularly challenging, in our efforts to combat with biofilms, is the attempt to understand the complexity of inter-bacterial interactions that can be encountered in such sessile consortia, together with their impact on the final outcome of these communities (e.g. maturation, physiology, antimicrobial resistance, virulence, dispersal). Although *S. enterica* and *S. aureus* are likely to coexist in food-processing environments, studies on dual-species biofilm formation by these bacteria are scarce. In such a study evaluating the antimicrobial action of carvacrol at different stages of dual-species biofilm development by *S. aureus* and *S.* Typhimurium in a constant-depth film fermentor, it was shown that this development reached a quasi-steady state in 12 days at 25 °C, with *S. aureus* to constitute ≈99% of the total viable numbers (Knowles & Roller, 2005). In another study (Zhang et al., 2014), have shown the anti-biofilm capabilities of citral, cinnamaldehyde, and tea polyphenols, applied at sub-MIC values, against mixed *S. aureus* and *S.* Enteritidis biofilm formed on stainless steel, together with the enhancement these natural food additives can provoke on the effect of disinfectants (hydrogen peroxide, sodium hypochlorite, and peracetic acid) on the mixed biofilm. However, both these previous studies did not include results of single species biofilm formation and resistance, making it impossible to judge whether there is any effect of interspecies interactions on the ability of each individual species to form biofilm and subsequently resist in the mixed community.

Taking into account all the previous, the objective of this study was to investigate the possible influence of bacterial intra- and inter-species interactions on the ability of *S.* Typhimurium and *S. aureus* to develop mixed culture multi-strain biofilms on a common abiotic substratum, as well as on the subsequent resistance of their sessile cells to sub-lethal chemical disinfection. To do this, 3 strains per species were initially selected and left to form biofilms on stainless steel surfaces, incubated at 20 °C for 144 h, in periodically renewable growth medium, under either mono- or dual-species conditions. Subsequently, the developed sessile communities were exposed for 6 min to sub-lethal concentrations of: (i) benzalkonium chloride (BC, 50 ppm), (ii) sodium hypochlorite (NaClO, 10 ppm), or (iii) peroxyacetic acid (PAA, 10 ppm). The length of disinfectant exposure was chosen to simulate disinfection scenarios encountered in the food industry while enhancing our ability to compare results from different treatments Additionally, the dominance of each strain in the mono- and dual-species biofilm communities, both before and after disinfection, was monitored by pulsed field gel electrophoresis (PFGE). Results obtained highlight the impact of bacterial interactions taking place inside a mixed-culture biofilm community on both its population dynamics and disinfection resistance. These could hopefully serve as a basis for exogenously modulating these interactions according to our wills, resulting in novel approaches for controlling un-wanted biofilms in food areas.

2. Materials and methods

2.1. Bacterial strains and preparation of the inocula

Three *S.* Typhimurium (FMCC B-137, FMCC B-193, FMCC B-415) and three *S. aureus* (FMCC B-134, FMCC B-135, FMCC B-410) strains, isolated from different origins, were used in this study. *S.* Typhimurium strain FMCC B-137 is a human multi-drug resistant isolate (belonging to phage type DT193; Dourou, Ammor, Skandamis, & Nychas, 2011), FMCC B-193 was isolated from calf bowel, while FMCC B-415 (ATCC14028, CDC6516-60) was isolated from pools of heart and liver from 4-week-old chickens (Jarvik, Smillie, Groisman, & Ochman, 2010). *S. aureus* strain FMCC B-410 is the methicillin-resistant (MRSA) strain COL, isolated in the early 1960s from the operating theatre in a hospital in Colindale, England (Dyke, Jevons, & Parker, 1966), while FMCC B-134 (subsp. *aureus* Rosenbach, ATCC6538, FDA209; Junka et al., 2013) and FMCC B-135 (NCBF1499; Drosinos, Paramithiotis, Kolovos, Tsikouras, & Metaxopoulos, 2007) strains were isolated from human lesions.

Before utilization, all the bacteria were stored at −80 °C in bead vials (Protect; Technical Service Consultants, Ltd., Heywood, Lancashire, United Kingdom) and were then resuscitated by adding one bead of each strain to 10 ml of Tryptone Soy Broth (TSB; LAB M; International Diagnostics Group Plc, Bury, Lancashire, UK) and incubating at 37 °C for 24 h (precultures). From each pre-culture, a