BIOFILMS FROM A FOOD MICROBIOLOGY PERSPECTIVE: STRUCTURES, FUNCTIONS AND CONTROL STRATEGIES

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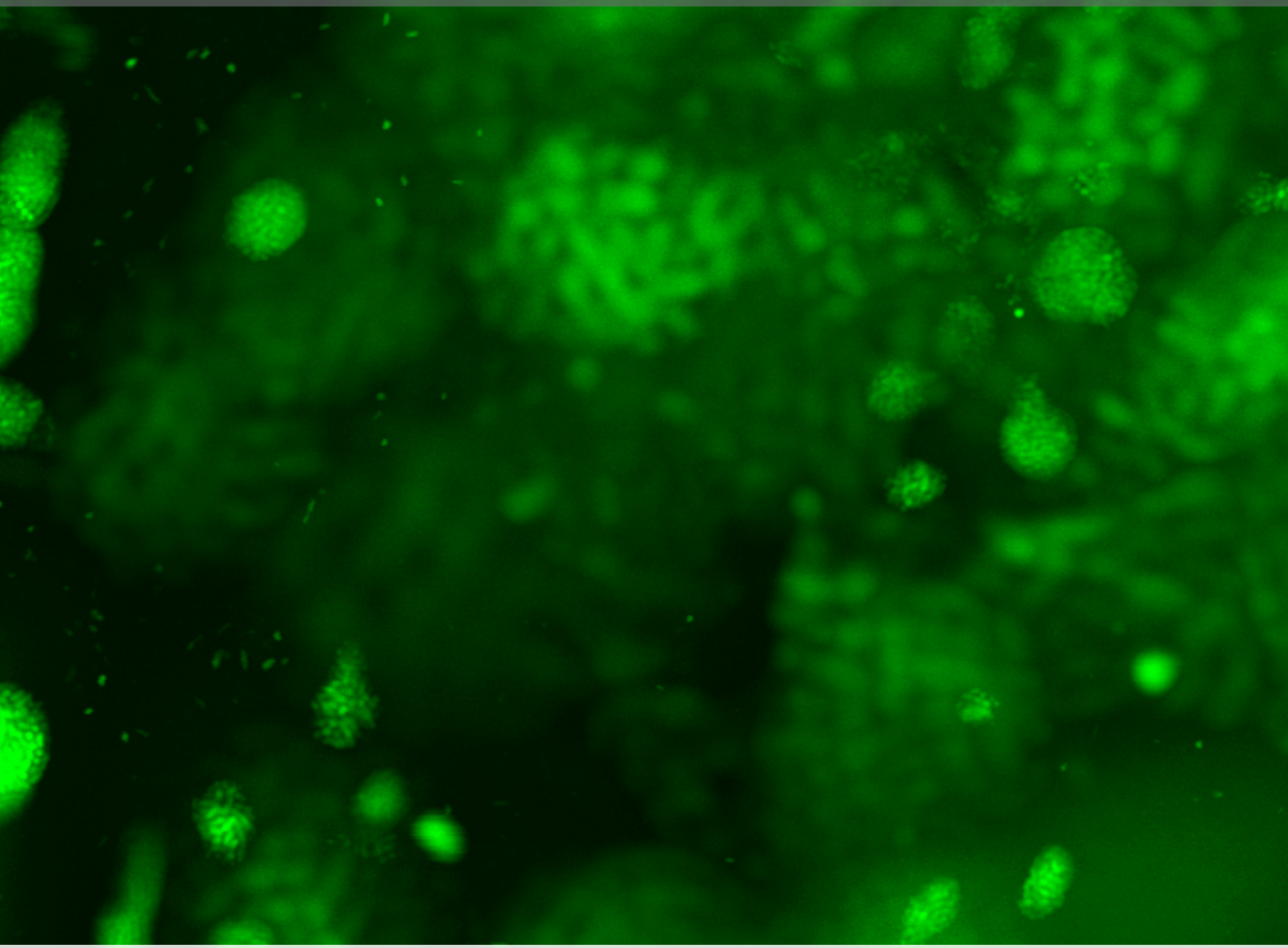

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BIOFILMS FROM A FOOD MICROBIOLOGY PERSPECTIVE: STRUCTURES, FUNCTIONS AND CONTROL STRATEGIES

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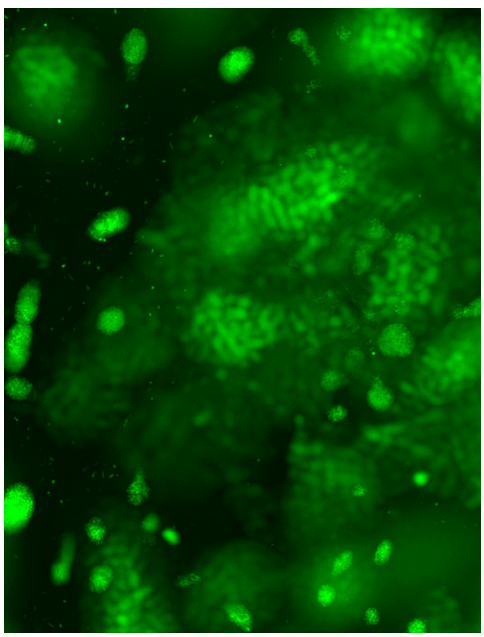

Confocal images of *Pantoea agglomerans* biofilms grown in microplates (30°C, 24 h , Tryptic Soy Broth). By Julien Deschamps, INRA

Materials and equipment in food processing industries are colonized by surface-associated microbial communities called biofilms. In these biostructures microorganisms are embedded in a complex organic matrix composed essentially of polysaccharides, nucleic acids and proteins. This organic shield contributes to the mechanical biofilm cohesion and triggers tolerance to environmental stresses such as dehydratation or nutrient deprivation. Notably, cells within a biofilm are more tolerant to sanitation processes and the action of antimicrobial agents than their free living (or planktonic) counterparts. Such properties make conventional cleaning and disinfection protocols normally not effective in eradicating these biocontaminants. Biofilms are thus a continuous source of persistent microorganisms, including spoilage and pathogenic

microorganisms, leading to repeated contamination of processed food with important economic and safety impact. Alternatively, in some particular settings, biofilm formation by resident or technological microorganisms can be desirable, due to possible enhancement of food fermentations or as a means of bioprotection against the settlement of pathogenic microorganisms.

In the last decades substantial research efforts have been devoted to unravelling mechanisms of biofilm formation, deciphering biofilm architecture and understanding microbial interactions within those ecosystems. However, biofilms present a high level of complexity and many aspects remain yet to be fully understood. A lot of attention has been also paid to the development of novel strategies for preventing or controlling biofilm formation in industrial settings. Further research needs to be focused on the identification of new biocides effective against biofilm-associated microorganisms, the development of control strategies based on the inhibition of cell-to-cell communication, and the potential use of bacteriocins, bacteriocin-producing bacteria, phage, and natural antimicrobials as anti-biofilm agents, among others.

This Research Topic aims to provide an avenue for dissemination of recent advances within the "biofilms" field, from novel knowledge on mechanisms of biofilm formation and biofilm architecture to novel strategies for biofilm control in food industrial settings.

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EDITORIAL
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Editorial: Biofilms from a Food Microbiology Perspective: Structures, Functions, and Control Strategies

Avelino Álvarez-Ordóñez[1]* *and Romain Briandet*[2]

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Keywords: biofilms, structure, control, food safety, food quality, bioprotection

Editorial on the Research Topic

Biofilms from a Food Microbiology Perspective: Structures, Functions, and Control Strategies

Materials and equipment in food processing industries are colonized by surface-associated microbial communities called biofilms. In these biostructures microorganisms are embedded in a complex organic matrix composed essentially of polysaccharides, nucleic acids, and proteins. This organic shield contributes to the mechanical biofilm cohesion and triggers tolerance to environmental stresses such as dehydration or nutrient deprivation. Notably, cells within a biofilm are more tolerant to sanitation processes and the action of antimicrobial agents than their free living (or planktonic) counterparts. Such properties make conventional cleaning and disinfection protocols normally not effective in eradicating these biocontaminants. Biofilms are thus a continuous source of persistent microorganisms, including spoilage and pathogenic microorganisms, leading to repeated contamination of processed food with important economic and safety impact. Alternatively, in some particular settings, biofilm formation by resident or technological microorganisms can be desirable, due to possible enhancement of food fermentations or as a means of bioprotection against the settlement of pathogenic microorganisms.

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This research topic aims to provide an avenue for dissemination of recent advances within the "biofilms" field, from novel knowledge on mechanisms of biofilm formation and biofilm architecture to novel strategies for biofilm control in food industrial settings.

The research topic comprises three review articles, one perspective and 11 original research articles. Most of the contributions cover the most recent investigations on aspects related to the structures, architecture, and strategies for the control of biofilms formed by pathogenic or spoilage microorganisms on food processing surfaces, while two contributions are focused on the evaluation of biofilm formation by resident, technologically important or desirable microorganisms.

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Various contributions deal with biofilms formed by strains of *Bacillus* spp. The review article by Majed and co-authors discusses the state-of-the-art on biofilms produced by *Bacillus cereus*, and by the two closely related pathogens, *Bacillus thuringensis* and *Bacillus anthracis* (Majed et al.). The review summarizes economic issues caused by *B. cereus* biofilms, the ecological and functional impact of biofilms in their lifecycle and management strategies implemented to control them. The research article by Hayrapeytan and co-authors shows the existence of intraspecies variability in the genome-encoded repertoire of iron-transporting systems and in the ability to grow and form biofilms in the presence of complex iron sources within *B. cereus*, which may influence *B. cereus* survival and persistence in food-related niches (Hayrapetyan et al.). Duanis-Assaf and co-authors report in their research article that lactose may induce biofilm formation by *Bacillus subtilis* through a quorum sensing dependent (LuxS) pathway (Duanis-Assaf et al.). In particular, they demonstrate that lactose induces formation of biofilm bundles, an increase in autoinducer-2 production in response to lactose, and an up-regulation of two gene operons responsible for extracellular matrix synthesis (e.g. *eps* and *tapA*).

In relation to *Campylobacter jejuni* biofilms, Brown and co-authors show in their contribution that extracellular DNA (eDNA) is an important component of *C. jejuni* biofilms formed on stainless steel surfaces (Brown et al.). The authors also evidence that eDNA may also contribute to the spread of antimicrobial resistance in *C. jejuni*. Finally, they report that degradation of eDNA by DNase I leads to rapid biofilm detachment, which shows promise for the control of *C. jejuni* biofilms in food industries. The research article by Turonova and co-authors reports that acclimation of two *C. jejuni* strains to oxygen-enriched conditions leads to a significant enhancement of biofilm formation during the early stages of the process, indicating that oxygen demand for biofilm formation is higher than for planktonic growth (Turonova et al.). The authors also identify the regulator CosR as a key protein in the maturation of *C. jejuni* biofilms. The research article by Bronnec and co-authors is aimed at evaluating the adhesion capacity and the ability to develop a biofilm of *C. jejuni* Bf, an atypical clinical isolate able to survive and grow under aerobic conditions (Bronnec et al.). The authors show that *C. jejuni* Bf can adhere to abiotic surfaces and human epithelial cells and can develop biofilms under both microaerobiosis and aerobiosis. They also conclude, from whole genome sequencing and transcriptomic analyses, that the behavior of this strain under aerobic atmosphere may result from the combination of different insertions and mutations and the modification of regulatory processes.

Two contributions are related to biofilms formed by strains of *Staphylococcus* spp. The perspective article by Oniciuc and co-authors shows that protein-based matrices are of relevance for the architecture of biofilms produced by *Staphylococcus aureus* strains isolated from food samples, as opposed to studies existing in the literature mentioning the predominance of exopolysaccharide-based matrices in biofilms formed by clinical and environmental isolates (Oniciuc et al.). Fagerlund and co-authors describe in their research article that the biofilm matrix composition has a significant impact on the efficacy of cleaning and disinfection agents against food associated Staphylococci (Fagerlund et al.). The authors show that some strains of *Staphylococcus* spp., able to form biofilms with a polysaccharide matrix, are resistant to benzalkonium chloride disinfectants, which are on the contrary effective for the removal of biofilms with a proteinaceous matrix.

Regarding biofilms formed by the foodborne pathogen *Listeria monocytogenes*, Zetzmann and co-authors report in their contribution that biofilms of *L. monocytogenes* are DNase-sensitive at low ionic strength conditions, which might induce bacterial lysis and chromosomal DNA release (Zetzmann et al.). This suggests that DNase I treatment is an attractive option to prevent or remove *L. monocytogenes* biofilms in food processing environments, where low nutrient concentrations and increased osmotic pressures are frequently found conditions. Puga and co-authors evaluate by confocal laser scanning microscopy changes in spatial organization, biovolume, viable cell content and substratum surface coverage of biofilms produced on glass by *L. monocytogenes* in co-culture with *Pseudomonas fluorescens* (Puga et al.). The authors conclude: "when this dual-species consortium develop biofilms on a solid surface, species interactions, cold stress and aging contribute to a more compact structure than the one built by *P. fluorescens* in single species biofilms."

Two review articles are related to strategies for the control of biofilms formed by pathogenic or spoilage microorganisms. Gutiérrez and co-authors examine environmental factors determining biofilm development in food processing equipment and discuss available information and future prospects on the use of bacteriophage-derived tools as successful disinfectants for the removal of biofilms (Gutiérrez et al.). On the other hand, Coughlan and co-authors discuss the problems associated with bacterial biofilms in the food industry and summarize recent strategies explored to inhibit biofilm formation, with special focus on those targeting quorum sensing (Coughlan et al.).

Two original research articles deal with biofilm formation by desirable microorganisms. The research article by Bastard and co-authors shows that *Oenococcus oeni* produces biofilms capable of efficient malolactic fermentation during winemaking and that *O. oeni* biofilms attached to oak can modulate wood-wine transfer of volatile aromatic compounds during wine fermentation and aging (Bastard et al.). Gómez and co-authors report in their contribution that probiotic strains can be good alternatives for the control of biofilm production by pathogenic bacteria in food-related environments (Gómez et al.). The authors evaluate the probiotic properties of several bacteriocinogenic and non-bacteriocinogenic lactic acid bacteria (LAB) and develop protective biofilms with some good probiotic candidates and test them for exclusion of *L. monocytogenes*, *Escherichia coli* O157:H7 and *Salmonella* Typhimurium, obtaining promising results, with more than 6-log reductions in viable counts being achieved with some of the LAB strains.

Finally, in the last contribution, Ostrov and co-authors develop a method (Cleaning-In-Place model system) to evaluate the effectiveness of cleaning agents in removal of biofilm derived

spores from the surfaces of stainless steel in milking equipment in dairy farms (Ostrov et al.).

This editorial summarizes the articles published in this Research Topic, in the confidence that readers will find this information useful with the most recent research on microbial biofilms from a food microbiology perspective. We sincerely hope that this collection of papers will prompt further research and contribute to advance the knowledge on food-related biofilms and to develop novel or improved strategies of food safety and quality management.

AUTHOR CONTRIBUTIONS

AÁ and RB designed and wrote the Editorial.

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REVIEW
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Bacillus cereus Biofilms—Same, Only Different

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Bacillus cereus displays a high diversity of lifestyles and ecological niches and include beneficial as well as pathogenic strains. These strains are widespread in the environment, are found on inert as well as on living surfaces and contaminate persistently the production lines of the food industry. Biofilms are suspected to play a key role in this ubiquitous distribution and in this persistency. Indeed, *B. cereus* produces a variety of biofilms which differ in their architecture and mechanism of formation, possibly reflecting an adaptation to various environments. Depending on the strain, *B. cereus* has the ability to grow as immersed or floating biofilms, and to secrete within the biofilm a vast array of metabolites, surfactants, bacteriocins, enzymes, and toxins, all compounds susceptible to act on the biofilm itself and/or on its environment. Within the biofilm, *B. cereus* exists in different physiological states and is able to generate highly resistant and adhesive spores, which themselves will increase the resistance of the bacterium to antimicrobials or to cleaning procedures. Current researches show that, despite similarities with the regulation processes and effector molecules involved in the initiation and maturation of the extensively studied *Bacillus subtilis* biofilm, important differences exists between the two species. The present review summarizes the up to date knowledge on biofilms produced by *B. cereus* and by two closely related pathogens, *Bacillus thuringiensis* and *Bacillus anthracis*. Economic issues caused by *B. cereus* biofilms and management strategies implemented to control these biofilms are included in this review, which also discuss the ecological and functional roles of biofilms in the lifecycle of these bacterial species and explore future developments in this important research area.

Keywords: ***Bacillus, cereus*, *thuringiensis*, *anthracis*, biofilm, ecology, regulation, food**

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INTRODUCTION

Bacillus cereus is a large, Gram-positive bacterium which produces spores and displays a peritrichous flagellation. Soil has long been considered to be the natural habitat of this species, although its spores can be isolated from various materials, such as invertebrates, plants, or food (Sneath, 1986). Recently, the ecological niches of *B. cereus* were suggested to include insects and nematodes guts (Jensen et al., 2003; Ruan et al., 2015), or plant roots (Ehling-Schulz et al., 2015). The high diversity of *B. cereus* habitats is reflected by the genetic polymorphism of this species (Helgason et al., 2004), and is illustrated by the existence of probiotic (Cutting, 2011) as well as pathogenic strains. *B. cereus* is indeed one of the most frequent agent of food poisoning

outbreaks, which symptoms can be either emetic or diarrheal. Emetic strains of *B. cereus* can secrete in the food a highly toxic and heat-stable Non-ribosomal cyclic peptide which can withstand cooking temperatures and induce, when ingested, vomitic symptoms (Ehling-Schulz et al., 2015). For diarrheal strains, according to the current model of *B. cereus*-induced diarrheal gastroenteritis, spores contained in the food are ingested by the host and germinate within the intestine, where vegetative cells can grow and produce enterotoxins. Three enterotoxins (Hbl, Nhe, and CytK) can be secreted by *B. cereus* (Stenfors Arnesen et al., 2008). In addition to enterotoxins, *B. cereus* can produce several other toxins (hemolysins HlyI and HlyII) and degradative enzymes (phospholipases and proteases), which are either secreted or directed to the cell-surface, and which are controlled, for most of them, by the PlcR transcriptional activator (Gohar et al., 2008). PlcR is one of the numerous *B. cereus* quorum-sensing systems, which, together with a great number of chromosomally-encoded sensors and regulators (De Been et al., 2006), make the bacterium highly responsive to environmental changes and give it the ability to adapt to diverse conditions. The adaptative properties of *B. cereus* is also a consequence of the presence, within the bacterium, of a number of plasmids, which size is in the 2–500 kb range. *Bacillus thuringiensis* and *Bacillus anthracis*, for instance, are two species of the *B. cereus* group *sensu lato* which differ from *B. cereus sensu stricto* mainly by the presence of megaplasmids carrying genes encoding toxins specifically active against, respectively, invertebrates or mammals.

B. cereus, *B. thuringiensis,* and *B. anthracis* (called hereafter *B. cereus sensu lato*) are all able to produce biofilms. In most isolates of these species, biofilms are found as floating pellicles, but can also stick on immerged abiotic surfaces or even be present on living tissues. These complex communities are likely to be a key element in the ability of *B. cereus* to colonize different environments. Together with spores, they confer to the bacterium a high resistance to various stresses and a high adhesive capacity on various substrates, including stainless steel, a material widely used in the food processing lines. In these facilities, *B. cereus* can persist for long durations and can even withstand sanitization procedures. The exponential increase in the number of articles published on *B. cereus* biofilms (**Figure 1**) illustrates the rising interest of the scientific community for this subject. Indeed not only are biofilms a key issue in *B. cereus* life, they also display interesting specificities. Although some of the molecular mechanisms involved in biofilm formation and in its regulation are shared with *Bacillus subtilis*—a saprophytic bacterium extensively studied for biofilm formation—striking differences exists between the two species regarding the biofilm structure, the effectors of matrix formation and the regulation pathways controlling them.

In the last decade, a considerable knowledge has been accumulated in a wide area of research regarding biofilm formation in *B. cereus sensu lato*. The aim of this review is to stress a panoramic view of the current knowledge, from the molecular mechanisms involved in biofilms formation in the three species to the functions and roles of these multicellular structures in the bacterium life, including pathogenesis and food industry contamination. From this panoramic view, we expect to draw the most promising incoming research developments and to address some intriguing questions, such as why has *B. anthracis*, a lethal and capsulated pathogen, kept the ability to produce biofilms. This review will also highlight the variety and prevalence of biofilm formation in the three species and will point, when necessary, to similarities and differences with *B. subtilis*.

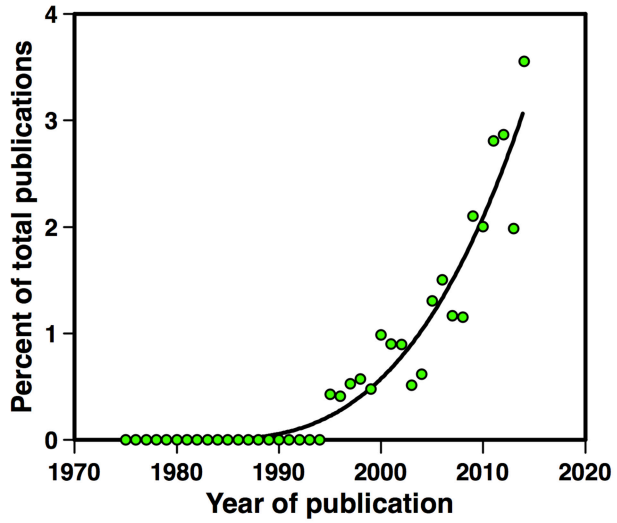

FIGURE 1 | Number of articles published between 1975 and 2015 on *B. cereus* biofilms. Articles published on *B. cereus*, *B. thuringiensis,* or *B. anthracis* biofilms, in percent of the total number of articles published on the same species.

MOLECULAR AND PHYSIOLOGICAL ASPECT

The molecular and physiological aspects of biofilm formation discussed here include the various extracellular macromolecules produced by the bacterium and specifically required for the biofilm matrix, cellular elements involved in biofilm formation such as flagella or cell-surface proteins, and the complex regulation network controlling biofilm formation and connecting it to other cellular functions. Also included in this part of the review is phenotypic heterogeneity within the biofilm, a field of growing interest since it is strongly involved in the bacterial survival in changing environments, and the role of mobile genetic elements in biofilm formation.

The Biofilm Matrix

Biofilms are usually embedded in a self-produced matrix whose structural elements are exopolysaccharides, proteins and DNA (Flemming and Wingender, 2010). *B. cereus* is no exception to this rule and its matrix contains the three components. In *B. subtilis,* most of the structural exopolysaccharides required for biofilm formation are synthetized by the products of the *epsA-O*

operon (Branda et al., 2001; Kearns et al., 2005). Deletion of *epsA-O* leads to a Non-structured and fragile biofilm pellicle (Lemon et al., 2008). An *eps* locus similar to *epsA-O* is found in bacteria of the *cereus* group (Ivanova et al., 2003; Gao et al., 2015). This similarity is supported by the presence, within the locus, of an anti-termination RNA element named EAR, found only in *epsA-O* and in the *eps* locus of the *cereus* group (Irnov and Winkler, 2010). However, deletion of the *B. cereus eps* locus does not affect biofilm formation (Gao et al., 2015), despite the presence of polysaccharides in the *B. cereus* biofilm matrix (Houry et al., 2012), whose origin therefore remains unknown.

The *B. subtilis* biofilm matrix also contains the three structural proteins TasA, TapA, and BslA (Vlamakis et al., 2013). BslA (Biofilm surface layer) forms a hydrophobic envelope surrounding the biofilm (Hobley et al., 2013) while TasA assembles into amyloid-like fibers attached to the cell wall by TapA, resulting in a fiber network strengthening the biofilm (Romero et al., 2011). In *B. subtilis*, *tapA,* and *tasA* are included in the *tapA-sipW-tasA* operon, where *sipW* codes for a signal peptidase, which releases the two proteins TapA and TasA into the extracellular milieu. There is no paralog of *bslA* or *tapA* in the *B. cereus* genome, but *tasA* have two paralogs. One is *tasA*, included in the *sipW-tasA* operon, and the other is *calY*, which is located next to *sipW-tasA* (Caro-Astorga et al., 2015). TasA and CalY are both involved in the production of fibers which can be observed by electron microscopy, and the deletion of their genes or of *sipW* leads to biofilm defects similar to the ones reported in *B. subtilis* (Caro-Astorga et al., 2015).

The extracellular DNA (eDNA) contained in the *B. cereus* biofilm matrix was shown to be produced specifically in biofilms and was reported to be required for adhesion on polystyrene or glass surfaces (Vilain et al., 2009). Its origin remains unknown but might be related to programmed cell death (Abee et al., 2011). However, in planktonic cultures of *B. subtilis*, the production of eDNA is not a consequence of cell-lysis but requires both competence genes and the Opp oligopeptide permease, and is involved in horizontal gene transfer (Zafra et al., 2012). Other bacterial species, including the Gram-positive bacteria *Staphylococcus aureus* and *Streptococcus pneumonia*, also require eDNA for biofilm formation (Whitchurch et al., 2002; Moscoso et al., 2006; Izano et al., 2008). Possible interactions between the eDNA and other consituents of the biofilm matrix have not yet been investigated, neither has the mechanism or the regulation of eDNA production in biofilms.

Role of Flagella

Flagella are cell-surface structures extending far away the bacterial cell. In *B. cereus*, they are not required for adhesion to glass (Houry et al., 2010), but flagellar motility is involved in biofilm formation through 4 mechanisms. First, motility is a key element of biofilm formation when the bacterium must reach by its own (in static conditions) suitable places for biofilm formation (Houry et al., 2010), at the air-liquid interface. The suppression of motility in a strain which forms biofilms at the air-liquid interface resulted in the formation of submerged biofilms (Hayrapetyan et al., 2015b). Secondly, motile bacteria within the biofilm create channels in the matrix, leading to an increase in nutrients exchange and, conversely, favoring the penetration of toxic substances (Houry et al., 2012). Thirdly, motile planktonic bacteria can enter the biofilm and increase its biomass (Houry et al., 2010, 2012). Fourthly, motile bacteria located at the edge of the growing biofilm extend the surface covered by this structure, resulting in colony spreading (Houry et al., 2010). Although flagellin transcription decreases continuously with biofilm age (Houry et al., 2010), the biofilm bacterial population is heterogeneous and includes a fraction of motile bacteria (Houry et al., 2012) which, in *B. subtilis*, is located at the edge of the colony (Vlamakis et al., 2008).

Cell-Surface Properties

B. cereus cells in biofilm differ from their planktonic counterparts regarding their cell-surface properties. For example, the structure of the secondary cell wall polymer (SCWP), a polysaccharide linked to the peptidoglycan by phospho-diester linkages, was shown to vary during biofilm aging in *B. cereus* (Candela et al., 2011). Since SLH (S-layer homology) domain-containing proteins bind to the SCWP, changes in the SCWP structure might result in changes in the proteins displayed on the cell-surface, and possibly involved in the adaptation of the bacterium to its environment. Within these SLH-proteins are autolysins, whose variation during biofilm growth might lead to changes in the bacterial chain length. Similarly, a cell-surface peptidase (CwpFM) involved in autolysis was shown to play a role in biofilm formation, possibly because this autolysin can modulate the length of bacterial chains and consequently act on the motility of the bacterium (Tran et al., 2010).

Regulation Networks

The regulation network controlling *B. cereus* biofilm formation shows a combination of similarities and differences with *B. subtilis*. In *B. cereus sensu lato*, *sipW*, *tasA*, and *calY* transcriptions are repressed by the SinR regulator (Pflughoeft et al., 2011), which controls biofilm formation (Fagerlund et al., 2014) as for *B. subtilis*. SinR is antagonized by SinI and, in both species, deletion of SinI leads to the absence of biofilm and to hypermotility while the reverse phenotype (biofilm overproduction, no motility) is obtained upon deletion of SinR (Kearns et al., 2005; Fagerlund et al., 2014; **Figure 2**). Consequently, the SinI/SinR anti-repressor/repressor pair is likely to act as a switch between biofilm formation and swimming motility in *B. cereus* or *B. thuringiensis* as it does in *B. subtilis*. In addition, Spo0A is required for biofilm formation in *B. thuringiensis* and in *B. subtilis*, and AbrB represses biofilm formation in both species (Hamon and Lazazzera, 2001; Fagerlund et al., 2014).

However, the SinR regulon also displays important differences in the two species: the *B. subtilis epsA-O*, but not the *B. thuringiensis eps*, is included in this regulon. Conversely, the production of kurstakin, a lipopeptide biosurfactant, is controlled by SinR in *B. thuringiensis* while surfactin, a *B. subtilis* lipopeptide, is not in the SinR regulon. Kurstakin is also included in the NprR necrotrophic regulon required for survival in the insect cadaver (Dubois et al., 2012), and the hemolysin Hbl, controlled by SinR in *B. thuringiensis* (Fagerlund et al., 2014),

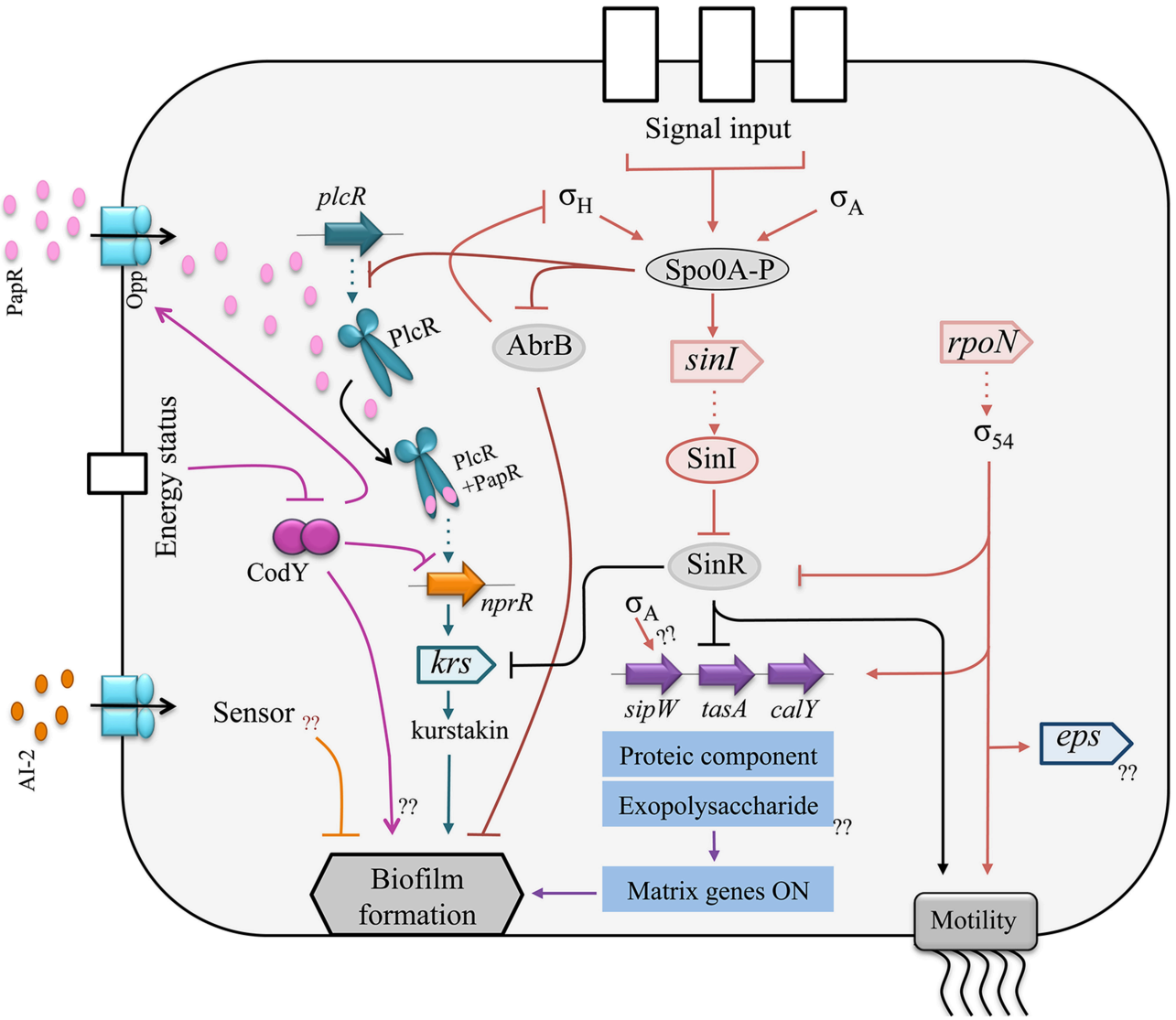

FIGURE 2 | Schematic representation of the regulatory network controlling biofilm formation in *B. cereus*. *Circles symbolize proteins, triangles symbolize open reading frames (ORFs)*. Arrows indicate activation and blunt lines indicate repression. Dotted arrows represent transcription. The protein component of the matrix is encoded by the *sipW-tasA* operon and by *calY* which promoters are activated by σ54 and repressed by SinR. SinR is antagonized by SinI. The transcription of *sinI* is activated by the master regulator of sporulation Spo0A. Furthermore, Spo0A downregulates the regulator AbrB, resulting in biofilm formation. Several quorum sensing systems are involved in biofilm formation. The regulator PlcR activates the transcription of *nprR*. NprR promotes kurstakin production, which itself promotes biofilm formation. The autoinducer AI-2 plays an inhibitory effect on biofilm formation.

is included in the PlcR virulence regulon of this species (Gohar et al., 2008). Other differences, in addition to the SinR regulon, exist between *B. subtilis* and *B. cereus sensu lato* for the regulation of biofilm formation. The AI2 autoinducer represses biofilm formation in *B. cereus* (Auger et al., 2006), but induces biofilm formation in *B. subtilis* (Duanis-Assaf et al., 2015), and the DegU regulator, which controls biofilm formation in *B. subtilis* (Kobayashi, 2007b; Cairns et al., 2014), has no homolog in *B. cereus*.

In *B. thuringiensis*, there is an interaction between biofilm formation, virulence and necrotrophism in insects (**Figure 3**), since PlcR promotes NprR transcription (Dubois et al., 2013), which positively controls kurstakin transcription (Dubois et al., 2012), which, in turn, promotes biofilm formation (Gélis-Jeanvoine et al., 2016). In *B. cereus* strain ATCC14579, PlcR was reported to repress biofilm formation (Hsueh et al., 2006), which is in disagreement with these observations. The disruption of *nprR* by a transposon in strain ATCC14579, and therefore the shutdown of the necrotrophic regulon, can explain this discrepancy. For the same reason, the regulator CodY was reported, either to repress biofilm formation in the *B. cereus* ATCC14579 strain (Lindbäck et al., 2012), or to promote biofilm

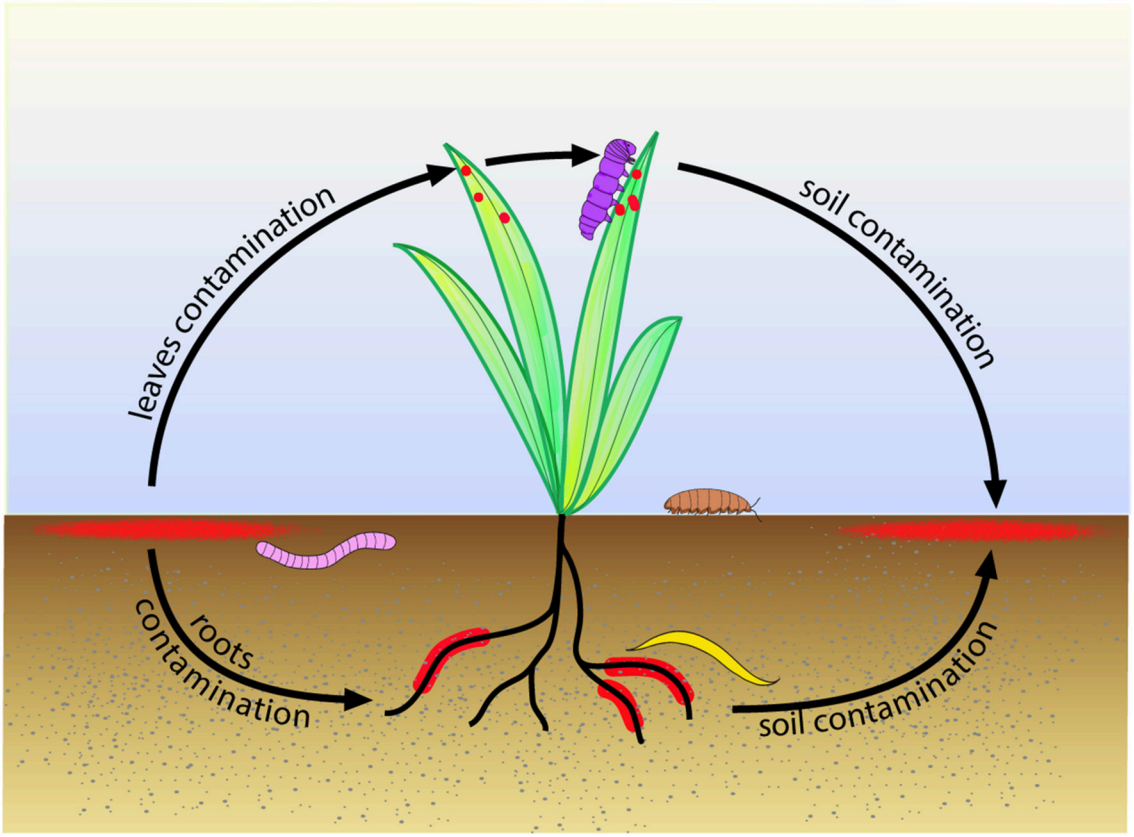

FIGURE 3 | Suggested model for biofilm role in the life cycle of *B. cereus* and *B. thuringiensis* in the environment. Biofilms (in red) growing in the topsoil contaminate the roots and leaves of plants. Earthworm (in pink) feeding on soil organic matter, nematodes (in yellow) feeding on plant roots, caterpillar (in purple) feeding on plant leaves, or isopodes (in brown) feeding on plant debris, ingest bacteria, which can then grow as biofilms in their gut. The invertebrates move further in the environment and, upon death, contaminate back the topsoil, giving birth to a new cycle.

formation in the *B. cereus* UW101C strain (Hsueh et al., 2008). CodY is a regulator sensing the energy and the nutrient state of the bacterial cell (Sonenshein, 2005). It promotes PlcR transcription in stationary phase (Frenzel et al., 2012; Lindbäck et al., 2012) by inducing the production of a transporter required for the import of the PlcR-activating peptide PapR (Slamti et al., 2015), and represses NprR transcription in exponential phase (Dubois et al., 2013). Therefore, the expected effect of CodY on biofilm formation, if this phenotype is induced in early stationary phase, should rather be positive. The connection between biofilm formation and virulence is mediated by another regulator in *B. cereus*. In this species, Sigma 54 (RpoN) promotes the transcription of virulence factors, *eps* genes and flagellins (Hayrapetyan et al., 2015b). These interconnections are an indication that biofilms could be involved in the pathogenic, commensal or necrotrophic lifestyles of *B. cereus sensu lato*.

Heterogeneity in the Biofilm

The limited diffusion of nutrients and signal molecules within the biofilm matrix creates micro-environments and local quorum-sensing states, resulting in a heterogeneous spatial distribution of bacteria in different physiological states. This heterogeneity has been described in several species, including *B. subtilis*, where vegetative cells, sporulating cells, and matrix-producing cells co-exist with different spatial localizations (Vlamakis et al., 2008). In *B. thuringiensis*, motile vegetative cells make from 0.1 to 1% of the total biofilm population and could be beneficial to the whole community by creating channels within the biofilm matrix (Houry et al., 2012). In the same species, in a 48 h-aged biofilm, about 15% of the cells express the enterotoxin Hbl (Fagerlund et al., 2014) which, if it accumulates within the matrix, could make the biofilm a toxic patch-like structure when formed on host tissues. Actually, the biofilm matrix of strains ATCC14579 and ATCC10987 contains the enterotoxins Hbl and Nhe, a collagenase, the phospholipases PI-PLC and sphingomyelinase, and the immune inhibitor protease InhA1, all being virulence factors (Karunakaran and Biggs, 2011). Genes expression heterogeneity within the *B. thuringiensis* biofilm evolves with time, from 24 to 72 h, and shows a decrease in the proportion of bacteria expressing virulence genes, an increase in the proportion of bacteria expressing necrotrophic genes, and a constant proportion of sporulating cells (about 15%; Verplaetse et al., 2015). Interestingly, necrotrophic bacteria arouse mainly from cells which have previously expressed virulence genes. In a

sporulating medium, only necrotrophic and sporulating bacteria were observed in the biofilm (Verplaetse et al., 2016).

Mobile Genetic Elements

Plasmids were shown to be involved in biofilm formation in a variety of Gram-negative and Gram-positive bacterial species (Cook and Dunny, 2014), through conjugative (Ghigo, 2001) as well as Non-conjugative mechanisms, and, conversely, biofilms were reported to favor plasmids transfer, resulting in an increase of genetic exchange between bacteria, including antibiotic resistance genes (Van Meervenne et al., 2014). Plasmids are present in all *B. cereus*, *B. thuringiensis* and *B. anthracis* strains, in number, not including copies, ranging from 1 to 13, and in size ranging from 2 to almost 500 kb (Rasko et al., 2005; Reyes-Ramirez and Ibarra, 2008). Strains of these species also harbor integrated or Non-integrated temperate prophages (Rasko et al., 2005). While mobile genetic elements play a key role in the adaptation of *B. cereus* and related species to their specific environment, data on their involvement in biofilm formation or on the role of biofilms in their transfer are scarce for this group of bacteria. The role of plasmids in biofilm formation have not been considered until now, although there are indications that large pXO1-like plasmids contained in periodontitis or emetic strains might be involved in the specific behavior of these strains regarding this phenotype. Indeed, addition to the culture medium of cereulide, the product of the *ces* locus located on the pCER270 emetic strains pXO1-like plasmid, promotes the formation of biofilm (Ekman et al., 2012). Conversely, phages were shown to act on biofilm formation. The GIL01 and GIL16 prophages of the *tectiviridae* family, present as linear plasmids in *B. thuringiensis* strains, negatively affect biofilm formation and sporulation, and enhance swarming motility (Gillis and Mahillon, 2014). In *B. anthracis*, prophages of different families (*siphoviridae*, *myoviridae,* or *tectiviridae*) could either inhibit sporulation (Wip4, Wip5, Frp1), or induce this phenotype (Wip1, Wip2, Frp2) in culture conditions where spore formation does not usually occur—for example absence of aeration (Schuch and Fischetti, 2009). The lysogenic strains containing one of these phages displayed an increased production of cell-surface exopolysaccharides and an enhanced production of biofilms at the air-liquid interface in BHI culture medium (Schuch and Fischetti, 2009). The phages effect on the ability to produce exopolysaccharides or biofilms was the result of a prophage-chromosome dialog mediated by a sigma-factor-like regulator encoded in the prophage sequence (Schuch and Fischetti, 2009).

STRUCTURE AND PROPERTIES

Data related to the biofilm structure are scarcely available in *B. cereus*. Although the *B. cereus* biofilm macrostructure has been described, the distribution in the biofilm of the different bacterial subpopulations or its morphogenesis are unknown, even more in the case of multispecies biofilms. Biofilm properties include adhesion to surfaces (which is dealt with in the part 5- Biofilm control in the food environment, of this review) and resistance to stresses. They also include the ability of the biofilm to produce spores, a property which add to the problems induced by the biofilm persistence.

Structure

The *B. cereus sensu lato* floating pellicle displays differences in its architecture with the one produced by *B. subtilis*. The *B. subtilis* floating pellicle exhibits a high number of folds and do not bind to the recipient wall (Kobayashi, 2007a). In contrast, *B. cereus* biofilm, when formed at the air-liquid interface, includes a ring strongly sticking to the recipient wall, and the pellicle itself which displays protrusions instead of folds (Fagerlund et al., 2014). Wrinkles in the *B. subtilis* pellicle were shown to be a consequence of biomass extension, confined space, and elasticity of the pellicle, which is dependent from the extracellular matrix (Trejo et al., 2013). In *B. subtilis* colonies on agar plates, wrinkles forms preferentially where cell death occurs (Asally et al., 2012). The difference in the pellicle architecture between *B. cereus* and *B. subtilis* might be a consequence of the strong adhesion of the biofilm to the vessel walls in the former, and of the different polymers present in the matrix produced by the two species.

On immersed surfaces, *B. subtilis* and some *B. cereus* strains (see Section Ecological Aspects) are able to produce submerged biofilms. In the *B. subtilis* immerged biofilm, cells are organized in bundles which can, for some strains, protrude over the biofilm and form aerial structures at heights greater than 100 μm (Bridier et al., 2013). Few data are available on the structure of *B. cereus* immerged biofilm. The amount of biofilm formed in this condition was variable according to the strain, but a strain isolated from a food processing line produced, on stainless steel coupon, a thick and uneven biofilm with an aerial structure (Faille et al., 2014).

Properties: Sporulation and Resistance to Stresses

The limited diffusion of nutrients and signal molecules within the matrix creates microenvironments in the biofilm, resulting in a heterogeneity of the bacterial population, which might include cells in the motile, virulent, necrotrophic, or sporulating states, as discussed in the Section Molecular and Physiological Aspects of this review. Sporulation rates in biofilms were highly variable and were dependent from the strain, the culture medium or the device used to form the biofilm (**Table 1**). Highest rates were obtained with strains isolated from the food environment and grown in poor media, with rates as high as 90%. Sporulation could occur in immerged biofilms although the rate of sporulation was increased when the biofilm was exposed to air or was let to dry (Ryu and Beuchat, 2005; Hayrapetyan et al., 2016), and was greater in the biofilm comparatively to the coexisting planktonic population (Hayrapetyan et al., 2015a). Stainless steel was more favorable to sporulation within the biofilm than polystyrene (**Table 1**). It was hypothesized that this result could be due to an increased iron availability on stainless steel coupons, as a consequence of corrosion (Hayrapetyan et al., 2015a). In addition to be suitable for sporulation, the biofilm confers to bacteria a protection against stresses. In biofilm, *B. anthracis* was from 40 (doxycycline) to 150 (ciprofloxacine) times more resistant to antibiotics than planktonic cells (Lee et al., 2007), and a

TABLE 1 | Sporulation rates in biofilms after 48 h of incubation.

Strain	Subs[a]	Biofilm[b]	Device	Medium[c]	%Spore[d]	References
Bc 98/4	SS	imm	Petri dish	TSB 1/10	87	Faille et al., 2014
Bc 5832	SS	imm	Petri dish	TSB 1/10	61	Faille et al., 2014
Bc D22	SS	imm	Petri dish	TSB 1/10	55	Faille et al., 2014
Ba Sterne	PS	air	96 wells plate	BHI	5	Lee et al., 2007
Bt 407	Glass	air	Glass tube	LBP	15*	Verplaetse et al., 2015
Bt 407	Glass	air	Glass tube	HCT	25*	Verplaetse et al., 2016
PAL25	PS	air	24 wells plate	Y1	91	Wijman et al., 2007
PAL25	PS	air	24 wells plate	LB	22	Wijman et al., 2007
ATCC10987	PS	air	24 wells plate	Y1	39	Wijman et al., 2007
ATCC10987	PS	air	24 wells plate	LB	10	Wijman et al., 2007
BC15	SS	air	12 wells plate	BHI	8	Hayrapetyan et al., 2015a
BC15	PS	air	12 wells plate	BHI	4	Hayrapetyan et al., 2015a
ATCC10987	SS	air	12 wells plate	BHI	2.5	Hayrapetyan et al., 2015a
ATCC10987	PS	air	12 wells plate	BHI	1	Hayrapetyan et al., 2015a
NIZO 4080	SS	air	12 wells plate	Y1	51	Hayrapetyan et al., 2016
NIZO 4080	PS	air	12 wells plate	Y1	38	Hayrapetyan et al., 2016
ATCC10987	SS	air	12 wells plate	Y1	13	Hayrapetyan et al., 2016
ATCC10987	PS	air	12 wells plate	Y1	3	Hayrapetyan et al., 2016

Experiments were done at 30°C except for B. anthracis (37°) or for strains 98/4, 5832, and D22 of B. cereus (25°C).

[a] *Subs, substrate; SS, stainless steel; PS, polystyrene.*

[b] *Imm, immerged biofilm; air: biofilm at the air-liquid interface.*

[c] *Y1: defined culture medium.*

[d] *Percentage of spores relatively to the total number of colony forming units.*

**These values represent the percentage of cells committed to sporulation instead of the actual percentage of spores.*

multispecies biofilms containing *B. cereus* and *Pseudomonas fluorescens* was more resistant to antimicrobials than the biofilm of each species alone (Simoes et al., 2009).

ECOLOGICAL ASPECTS

In nature, bacteria live predominantly in biofilms rather than in a planktonic state (Costerton et al., 1995), and this observation is likely to stand also for *B. cereus* or *B. thuringiensis*. Consequently, biofilms are expected to be a key element for the adaptation of these species to their biotopes and to their biocenosis. However, *B. cereus* and its close relatives are found in a high diversity of biotopes, which questions the role that biofilm formation, in addition to other physiological properties, would play for their fitness to specific environments.

Biofilm Formation among *B. Cereus* Strains

Although biofilms are suspected to be involved in strains adaptation to their specific environment, there is a considerable variation in the ability to produce biofilms among isolates of *B. cereus* and *B. thuringiensis*, and no correlation was found between this ability and the origin (food poisoning, clinical, or environmental) of the strain (Wijman et al., 2007; Auger et al., 2009; Kuroki et al., 2009; Kamar et al., 2013; Hayrapetyan et al., 2015a). However, strains isolated from a specific niche, the oral cavity of periodontitis-diseased patients, were all unable to form biofilms (Auger et al., 2009), although these strains were isolated from dental plaques—which are biofilms. While unexpected, this result looks coherent since periodontal strains of *B. cereus,* as secondary colonizers of the dental plaque, do not need to initiate biofilms. Another interesting finding from prevalence studies is the observation that about 50% of *B. cereus* strains isolated from various food preparations produced less biofilms after 48 h than after 24 h of incubation (Hayrapetyan et al., 2015a), a proportion also found in emetic strains (Auger et al., 2009), which are frequent food contaminants (Ehling-Schulz et al., 2015). In contrast, only a minor proportion (less than 15%) of *B. cereus* strains isolated from blood samples (Kuroki et al., 2009), from the environment, or of *B. thuringiensis* strains (Auger et al., 2009) showed a drop in the biofilm biomass after 24 h of culture. This decrease can be explained by a massive emigration of biofilm cells. When back to the planktonic state, reverting cells will be able to create new biofilms and to spread the colonized area. Therefore, combined with their resistance to cleaning procedures (see the "*Bacillus* biofilms and their control in the food environment" section below), this property would confer food isolates the ability to persist and thrive in the food production lines.

Prevalence studies also revealed that the biomass of biofilms produced on stainless steel by *B. cereus* in LB or in a defined medium (Y1) is greater when they are formed at the air-liquid-solid interface than on submerged surfaces (Wijman et al., 2007). In BHI medium, only one strain, out of 23 isolates from food products, was able to form a submerged biofilm on

polystyrene or on stainless steel coupons (Hayrapetyan et al., 2015a). Consequently, the property to form submerged biofilms appear to be rare among *B. cereus* strains. In the food industry production units, air-liquid interfaces are found in tanks while pipes are mostly in a flooded state. One would expect that the proportion of strains able to produce submerged biofilms would increase in isolates sampled from pipes when compared to isolates from tanks or to other isolates—although we have no data to support this expectation. It would be interesting to proceed to this comparison, since the ability to produce submerged biofilms affect *B. cereus* persistence within the food processing lines.

B. cereus Role in Multispecies Biofilms

Most biofilms found in natural environments include several bacterial species. *B. cereus* or *B. thuringiensis* make no exception to this observation and are found, when in biofilms, in association to other microorganisms. Multispecies biofilms are often described as cooperative consortiums where each partner contributes to the community resilience and development (Davey and O'toole, 2000). For example, periodontitis strains of *B. cereus* are found in the dental plaque (Rasko et al., 2007), which is one of the best studied multispecies biofilms. The dental plaquee is located at the tooth-gum interface and is a severe illness leading, ultimately, to gum bleeding, ligaments digestion and loosening and loss of teeth. Bacteria build the dental plaquee in a precise sequence, where pioneer species such as *Streptococcus mutants* bind first to the teeth enamel, followed by secondary colonizer species which bind to pioneer species or to themselves through a co-aggregation process (Kolenbrander et al., 2006). Secondary colonizers benefit from biofilm settlement by primary colonizers and, in turn, might contribute to the biofilm survival and growth. Indeed, *B. cereus* is able to shift the pH of a *Streptococcus mutants* biofilm from acidic to neutral values and in this way contributes to the biofilm pH balance (Sissons et al., 1998). It can also strongly participate to host tissues digestion owing to the numerous degradation enzymes which it secretes (Gohar et al., 2002) and which are present in the biofilm matrix (Karunakaran and Biggs, 2011). Likewise, *B. cereus* strains isolated from multispecies biofilms settled in paper machines were strong producers of exopolysaccharides (Ratto et al., 2005) and could therefore contribute actively to the biofilm development.

The integration of *B. cereus* vegetative cells can also occur in the depth of a Pre-existing biofilm, thanks to the high motility of these cells, which are able to create channels in the matrix and reach deep areas in the biofilm (Houry et al., 2010). Interestingly, *B. cereus* and *B. thuringiensis* secrete a number of bacteriocins (Ahern et al., 2003; Risoen et al., 2004; Oscariz et al., 2006), which, when produced within the integrated biofilm, could lead to drastic changes in the balance of bacterial biofilm populations. For example, a *B. thuringiensis* strain engineered to produce lysostaphin could invade and replace a *Staphylococcus aureus* biofilm native population (Houry et al., 2012), which clearly indicate that inter-species competition could occur within biofilms. Another example of competition between bacterial species within a natural biofilm is found in the pretreatment filters of water reclamation systems. These filters contain zeolite stones on which multispecies biofilms can grow. The *B. cereus* strains found in these biofilms are able to degrade the Gram-negative bacteria quorum sensing signal AHL (acylhomoserine lactone; Hu et al., 2003), interrupting the communication of their cohabitants and thus conferring a competitive advantage to *B. cereus*.

Biofilms in Soil, Plants, and Invertebrates

The environment is likely to be a major source of food contamination by microorganisms which can live in biofilms on plants or in the soil. *B. cereus* or *B. thuringiensis* are often described as saprophytic species whose natural habitat would be the soil (Vilain et al., 2006), from which they can easily be sampled (Vilas-Boas et al., 2002; Anjum and Krakat, 2016) and in which they can persist for long periods (Hendriksen and Carstensen, 2013). Interestingly, a number of *B. cereus* strains could multiply and form biofilm-like structures when cultivated in a liquid topsoil extract—but not in LB (Vilain et al., 2006), suggesting that some soil components are required to induce the formation of biofilm by *B. cereus* in the culture conditions used. However, not all soils can support *B. cereus* or *B. thuringiensis* growth, since an asporogenic strain of *B. thuringiensis* could not survive in a sterilized soil (Vilas-Boas et al., 2000), and it was speculated that the invertebrate gut rather than the soil might be the main ecological niche of these species (Jensen et al., 2003). *B. cereus* and *B. thuringiensis* were found in the gut of insects (Visotto et al., 2009), earthworms (Hendriksen and Hansen, 2002), nematodes (Schulte et al., 2010; Ruan et al., 2015), and isopods—which are terrestrial crustaceans (Swiecicka and Mahillon, 2006). In the intestine of insects and isopods, *B. cereus* forms filamentous structures described as "Arthromitus," which proved to be chains of dividing bacteria (Margulis et al., 1998). Long chains of *B. cereus* or *B. thuringiensis* vegetative cells are typically found in biofilms, which suggests that these species can form biofilms in the gut of insects or isopods—and probably in the gut of other invertebrates as well.

In addition to the invertebrates gut, *B. cereus* is found in the rhizosphere and in the mycorrhiza of plants. When present in these subterranean structures, *B. cereus* can protect the plant from fungal attacks. For example, *B. cereus* UW85 produces zwittermicin A and kanosamine, both fungistatic molecules being suspected to contribute to the suppression of damping-off disease of alfalfa caused by *Phytophthora medicaginis* (Silo-Suh et al., 1994). Another strain of *B. cereus* (strain 0–9) isolated from roots of wheat cultures, was able to induce a reduction of 31% of the disease caused by the fungal pathogen *Rhizoctonia cerealis*, the agent of wheat sharp eyespot (Xu et al., 2014). A mutant of this strain obtained by random mutagenesis and selected for defective biofilm formation was unable to colonize wheat roots and to control the fungal disease (Xu et al., 2014). *B. cereus* is therefore likely to colonize plant roots through biofilm formation. This hypothesis is supported by the finding that, in *B. subtilis*, *tasA*, a gene required for biofilm formation which paralog is also required for biofilm formation in *B. cereus* (Caro-Astorga et al., 2015), is needed for the colonization of *Arabidopsis thaliana* roots (Lakshmanan et al., 2012). *B. cereus* can also be associated with plants through the mycorrhiza. It was, for example, isolated

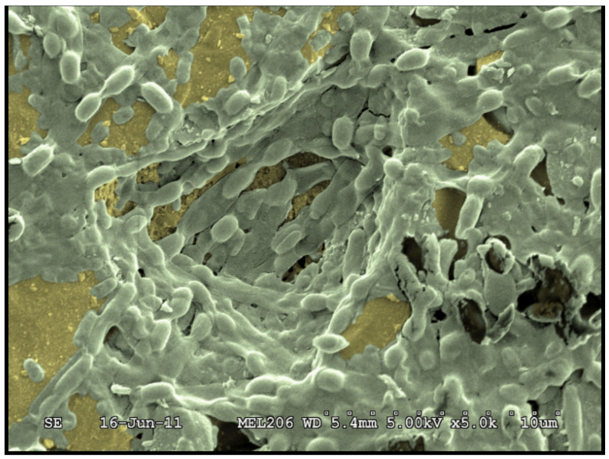

FIGURE 4 | Observation by scanning electron microscopy of a mixed biofilm formed by two strains: *B. cereus* 98/4 and *Comamonas testosteroni* CCL24 (Faille et al., 2014).

from *Glomus irregulare* spores sampled from the rhizosphere of *Agrotis stolonifera* growing in a natural stand (Lecomte et al., 2011) and was shown to form biofilms on the hyphae of *Glomus* sp. (Toljander et al., 2006). The arbuscular myccorhizal fungi are plant roots symbionts which mycelial network can explore soil volumes much larger than the roots themselves (Lecomte et al., 2011).

These data are summarized in the model depicted **Figure 3**, in which *B. cereus* and *B. thuringiensis* growing as biofilms in the topsoil would contaminate germinating plants, leading to biofilms on the rhizosphere and to spores on the phylloplane. Invertebrates feeding on roots (nematodes), soil organic matter (earthworms), vegetal debris (isopods), or leaves (caterpillars) would be infected by these bacteria, which could behave as commensals or as pathogens and settle as biofilms in their host gut. Invertebrates, through their mobility, could disseminate the bacteria in the environment and, upon death, contaminate back the topsoil, thus initiating a new cycle. Biofilms of *B. cereus* settled in soils and on plants could then contaminate raw food materials.

The Case of *B. Anthracis*

Formation of biofilms by *B. anthracis* in the environment is controversial. *B. anthracis* does not need to produce biofilms for its infective cycle in mammals. Its spore is the infective agent, its toxins are extremely efficient and it is protected against the host immune defenses by a capsule. After the host death, *B. anthracis* multiply within the host, sporulate, and the spores are finally released into the environment at the host death spot. It is believed that the spores can survive in the soil for a long time, keeping their full infective properties, until their uptake by a new host. Yet, it has been argued that a multiplication step would be required to explain how slow the spore decay in soil is. Indeed, multiplication was observed in soil on plant roots, where *B. antthracis* formed long chains reminiscent of the bacterial chains found in biofilms (Saile and Koehler, 2006). *B. anthracis* can also produce biofilms in static and in flow conditions (Lee et al., 2007; Schuch and Fischetti, 2009). It expresses the regulators required for biofilm formation and at least a part the proteic components of the biofilm matrix (Pflughoeft et al., 2011), and can sporulate in biofilms (Lee et al., 2007). In addition, *B. anthracis* can colonize the earthworm gut for long periods (Schuch and Fischetti, 2009) and is found in flies and mosquitoes (Turell and Knudson, 1987), although only short-term colonization of flies gut was observed (Fasanella et al., 2010). While these data support a multiplication of *B. anthracis* outside its mammal host, further observations and experiments are required to determine if the model displayed **Figure 5** apply to this bacterium.

BIOFILMS CONTROL IN THE FOOD ENVIRONMENT

Bacillus strains, including strains from the *B. cereus* group, can be isolated from endemic biofilms in various environments such as paperboard production or hospitals (Kolari et al., 2001; Ohsaki et al., 2007; Kuroki et al., 2009), but also food and beverage industries (Evans et al., 2004; Gunduz and Tuncel, 2006; Storgards et al., 2006; Marchand et al., 2012). The presence of biofilms containing *B. cereus* is a great concern for food industry settings such as fresh products, poultry, dairy, and red meat processing, and they are a potential source of recurrent cross-contamination and Post-processing contamination of finished products, sometimes resulting in food spoilage or foodborne illness (Rajkovic et al., 2008). The contamination of food processing lines by *B. cereus* biofilms could therefore be a serious public health risk, especially in foods that undergo mild processing such as minimally heat–treated foods (Tauveron et al., 2006). This risk must be given full attention since the total annual cost caused by *B. cereus* and *Staphylococcus aureus* in food illness is estimated at $523 million in the United States (Bennett et al., 2013).

B. Cereus, a Food Spoilage Agent

As underlined above, the presence of biofilms in the food industry can result in food spoilage. Indeed, *B. cereus* strains produce extracellular proteases and lipases resulting in food degradation and spoilage, like sweet curdling and bitterness of milk sour taste, decreasing the shelf life of the product and therefore resulting in significant economic loss to food producers (Fromm and Boor, 2004; Flach et al., 2014). Even if present in raw milk at low concentration, *Bacillus* sp. become dominant after long periods of storage at a temperature of 10°C (which is often the case in shops), or when produced in improved technological conditions (Samarzija et al., 2012). Consequently, *Bacillus* spp. are today considered the main microbial causes for the spoilage of milk and milk products, and the main reason for significant economic losses in the dairy industry (Meer et al., 1991; Brown, 2000). It is estimated that the dairy industry has losses of up to 30 % due to spoilage and reduced product quality caused by psychrotrophic bacteria, including *Bacillus* sp. (Samarzija et al., 2012).

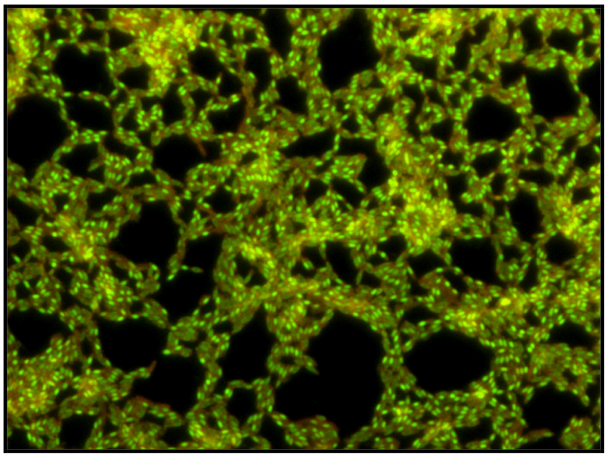

FIGURE 5 | Microscopic images of a *B. cereus* biofilm grown for 48 h in TSB 1/10. Observation by epifluorescence after staining with the Live/Dead stain (magnification × 400). Endospores produced within the biofilm are stained in green, cells are stained in orange-green.

Biofilms in Food Environments

In food environments, *Bacillus* biofilms are found on every food contact surfaces of open or closed equipment, such as conveyor belts, pasteurizers, evaporators, filling machines, storage tanks, but also on cleaning and handling tools (Christison et al., 2007). Depending on the species or the strain, surfaces of cold rooms and equipment of processes lines where elevated temperatures prevail could be contaminated by *Bacillus* biofilms (Sharma and Anand, 2002a; Kolari et al., 2003; Evans et al., 2004; Gunduz and Tuncel, 2006; Kumari and Sarkar, 2014). In fact, *Bacillus* spores or biofilms are capable of contaminating every surface commonly found in food-industry plants, including inert surfaces such as stainless steel surfaces (Faille et al., 2014), plastics or rubber (Mettler and Carpentier, 1997), but also surface of vegetables (Elhariry, 2011). Moreover, *Bacillus* strains are able to form biofilms both under static and flow conditions, and thick biofilms of *B. cereus* would particularly develop at the air-liquid interface (Wijman et al., 2007). Along food processing lines, *B. cereus* is often found in association with other bacterial species to form mixed biofilms (**Figure 4**) where high levels of *Bacillus* isolates have sometimes been reported (Mattila et al., 1990). For example, percentages as high as 25% of *Bacillus* sp. isolates (including *B. cereus* isolates) have been found in dairy processing industries (Sharma and Anand, 2002c). In addition, sporulation occurs within biofilms (**Figure 5**) on food contact surfaces (Storgards et al., 2006), sometimes at very high levels (De Vries et al., 2004; Faille et al., 2014), suggesting a potentially significant role for biofilm-derived spores in contamination of food with *Bacillus* spp. (Scott et al., 2007).

Biofilms Control

In food plants, disinfection of processing lines (e.g., pipes, heat-exchangers, valves tanks) is preceded by a cleaning step, involving alkali or other cleaning agents. Cleaning and sanitation procedures are set up to guarantee the detachment of organic and inorganic contaminations, disinfection of the cleaned surface and elimination of the residues of the sanitation agents (Vlkova et al., 2008). Unfortunately, the detachment of spores and biofilms but also of food residues in the food processing environment is critical since they often accumulate in areas which are difficult to clean, e.g., crevices, valve, gaskets, and dead ends (Czechowski, 1990; Austin and Bergeron, 1995; Sharma and Anand, 2002b). Of particular concern is the increased resistance of biofilms, compared with bacteria in a free-living environment, to disinfection processes. For example, two widely-used sanitizers, a quaternary ammonium compound and sodium hypochlorite, did not effectively inactivate the adherent single cells and biofilms of *B. cereus* at concentrations able to induce a reduction in CFU/ml of more than 5.0 log of their planktonic counterparts. Furthermore, the efficacy of both disinfectant was even lower when biofilms were formed on milk Pre-soiled stainless steel (Peng et al., 2002). Adherent *Bacillus* spores also exhibit a greater resistance to high temperature and disinfectant than spores in suspension (Sagripanti and Bonifacino, 1999; Faille et al., 2001; Kreske et al., 2006a). Indeed, residual *Bacillus* contamination of equipment surfaces after cleaning and/or sanitizing procedures was detected at different points on milk pasteurization lines and on the surface of the packaging machine (Mattila et al., 1990; Sharma and Anand, 2002b; Salustiano et al., 2009). Hence, considering the difficulty in inactivating adherent *Bacillus* spores and biofilms, cleaning the biomass from the surfaces is fundamental for controlling biofilm development.

Cleaning-in-Place Protocols

The cleaning-in-place (CIP) protocols used to clean processing lines without dismantling or opening of the equipment, vary according to industries or the food chain and the residues that need to be cleaned, although caustic and acid cleaning has remained the standard method used in many food processing industries. Both chemical (cleaning agents) and mechanical (shear stresses) actions are supposed to play a major role on soil removal. However, the effectiveness of CIP regimes against *B. cereus* biofilm has not been extensively reported. In the food industries, CIP regimes frequently involve a 60°C cleaning alkali wash (mainly sodium hydroxide), followed by an acid (mainly nitric acid) wash disinfection step (Bremer et al., 2006), but a reduction of viable spores by only 40% has been reported (Andersson et al., 1995). In the case of *Bacillus* biofilms, relatively low efficiency of the reference CIP regime (1% NaOH at 65°C for 10 min—water rinse—1% HNO_3 at 65°C for 10 min—water rinse) has been reported, but the removal would be improved by increasing the concentration of NaOH or the duration of the cleaning procedure (Flint et al., 1997; Bremer et al., 2006; Kumari and Sarkar, 2014).

Mechanical and Chemical Cleaning

In order to better understand the mechanism of spore and biofilm detachment during CIP, the respective role of rinsing vs. cleaning (mechanical and chemical forces) in the detachment of *Bacillus* biofilms and spores was investigated. When the *B. cereus* biofilm was formed on milk Pre-soiled stainless chips (Peng et al.,

2002) or at different shear stresses (Lemos et al., 2015), a rapid population decrease occurred during the first 5 min whatever the detachment conditions, and no further removal was observed for longer times, either in terms of vegetative cells or spores, even if the amount of detached biofilm was significantly higher in the presence of cleaning agents. Similar observations have been reported when *B. cereus* biofilm was formed on milk Pre-soiled stainless chips (Peng et al., 2002) or at different shear stresses (Lemos et al., 2015). Further works, performed on spores from the *B. cereus* group, demonstrated that during a CIP, chemical action plays a major role in the detachment of adherent spores, while mechanical action is poorly effective (less than 90% decrease in the number of adherent spores at wall shear stresses of 500 Pa, whatever the strain; Faille et al., 2013). Spores produced in biofilms showed greater resistance to detachment than the complete biofilms on inert surfaces (Faille et al., 2014) and on vegetables (Elhariry, 2011).

If the contaminated areas are allowed to dry before cleaning, e.g., in half-filled tanks or pipes or on open surfaces, the sporulation level would increase within *Bacillus* biofilms (Hayrapetyan et al., 2016) and the resistance to shear of attached spores increase concomitantly (Nanasaki et al., 2010). The increase in resistance to detachment is particularly noteworthy for long times and/or high temperature of drying (Faille et al., 2016).

In order to improve the efficiency of cleaning procedures, some industrialists opted to develop enzymatic cocktails effective against biofilms found in food processing plants, which are known to poorly respond to traditional cleaning procedures. The enzymes offer major advantages over traditional cleaning solutions, e.g., low toxicological risk and ecological risk, ease of rinsing external residues and compatibility with different surface material. Many products are nowadays commercially available, essentially for medical use. Some of the commercialized cocktails have proven their efficiency against biofilms produced by *B. cereus, B. mycoides* or *B. flavothermodurans*, and also against *B. cereus* adherent spores (Langsrud et al., 2000; Parkar et al., 2004; Lequette et al., 2010). These enzymatic "detergents" being more expensive than conventional products, their use is proposed as a complementary solution to current cleaning procedures.

Spores and, to a lesser extent, vegetative cells embedded in a *B. cereus* biofilm are protected against inactivation by the sanitizers commonly used to control foodborne pathogens, such as chlorine and hydrogen peroxide, which are easy to handle, inexpensive, and are soluble in water and relatively stable over a long storage time. For example, hydrogen peroxide or peracetic acid show little activity on adherent *B. subtilis* and *B. cereus* spores (Faille et al., 2001; Dequeiroz and Day, 2008). At higher temperatures and longer exposures, a significant reduction in *B. cereus* viable counts would be observed, but it is not suitable for practical disinfection due to corrosion and toxicity (Langsrud et al., 2000; Dequeiroz and Day, 2008). However, although the peroxygen-based disinfectants are not sporicidal alone, the use of NaOH 1% (typically used at 0.5–2% in the food and beverage industries) or of an enzymatic cocktail would sensitize *Bacillus* spores to the action of these oxidative disinfectants (Langsrud et al., 2000). The activity of sodium hypochlorite on *B. cereus* spores on surfaces and in field trials is also limited (Te Giffel et al., 1995). Indeed, although hypochlorite solutions are more stable above pH 9.5, they are only efficient at neutral or acidic pH (Sagripanti and Bonifacino, 1999). However, a marked synergistic effect between both was described on the efficacy to reduce spore counts on contaminated surfaces (Dequeiroz and Day, 2008). The same phenomenon was observed with biofilms produced in immersed conditions or exposed to air (Ryu and Beuchat, 2005). Furthermore, chlorine dioxide was less effective than chlorine in killing *Bacillus* spores on stainless steel, mainly in the presence of organic soil (Kreske et al., 2006a) and injured *B. cereus* cells were sometimes seen to recover overnight (Lindsay et al., 2002). Within biofilms, spores were more resistant to chlorine and chlorine dioxide than the vegetative cells (Kreske et al., 2006b).

Control of Multispecies Biofilms Including *B. cereus*

The control of mixed species biofilms including *B. cereus* and other *Bacillus* species has also been investigated. For example, the efficiency of sodium hypochlorite and iodophor, commonly used in the beverage and dairy industries, has been studied in different segments of pasteurization lines (Sharma and Anand, 2002b). Results from this study suggest that sodium iodophors were in some cases more efficient than sodium hypochlorite in inactivating biofilms and that the latter treatment was affected by the constitutive microflora or by spatial heterogeneity of biofilms. However, biofilms were still detected on the different areas even after CIP and iodophor treatment. Since iodophors are much less active against spores than hypochlorite, one can hypothesize that the residual biofilms following treatment with iodophors would be largely composed of *Bacillus* spores. A laboratory work on dual biofilms (*B. cereus* and *P. fluorescens*) showed that dual biofilms are characterized by an increased stability to shear stress and are more resistant to a quaternary ammonium compound (QAC), cetyltrimethylammonium bromide, and glutaraldehyde solutions (sanitizers commonly used in the medical field) than each single species biofilm (Simoes et al., 2009). Once more, a significant proportion of the population of both bacteria remain in a viable state after exposure to antimicrobials. The presence of residual bacterial population after treatment by QACs, also frequently used in food-processing industries, could encourage the development of resistance among food-associated bacteria, as already observed in Gram-negative bacteria and *Enterococcus* spp. (Sidhu et al., 2002).

CONCLUDING REMARKS

In the last decade, a number of studies have shown that although *B. cereus sensu lato* biofilms looked the same as the *B. subtilis* ones, there are quite different in several aspects. These studies brought a huge improvement to our understanding of how *B. cereus* biofilms are built, what is their contribution to the bacterium lifestyle, or how to get rid of them when required. Still, a number of issues stay unresolved or has been brought to light by recent findings. While the role of the TasA-like proteins in the biofilm matrix has been confirmed, the duplication of their

genes asks the question of their role in the biofilm formation and in the adaptation of the bacterium to its environment or to its host. Similarly, the genetic determinants required for the building of the polysaccharidic part of the matrix remains a mystery, as well as the regulation of their production and the role of the large *epsA-O* -like polysaccharidic locus, since this locus does not seems to be involved in biofilm formation. The mechanisms through which eDNA, which was found in high quantities in the *B. cereus* biofilm matrix, is released remains unknown. The possible involvement of programmed cell death (PCD) in this release as well as in the shaping of the biofilm architecture, and the connection of its regulation to the regulation of biofilm formation represent other exciting issues in the forthcoming work on *B. cereus* biofilm formation. The impact of plasmids, which are known to play a major role in *B. cereus sensu lato* pathogenesis, on biofilm formation, and the mechanism through which plasmids act on this phenotype is still to be determined. Regarding pathogenesis, the presence and the evolution of biofilms *in vivo* has not been yet established, nor has been their exact contribution to the bacterium virulence. Another important issue is relative to the role of biofilms in the *B. cereus sensu lato*, including *B. anthracis*, survival and growth in the soil environment. Finally, the traditional hygiene procedures used in the food industry have revealed their limit in the control of surface contamination with *Bacillus* spores and biofilms. If we consider that *B. cereus* and other species can act as spoilage organisms and pathogens, these surface contaminations are still of concern in the food industry. This problem is thus far from being resolved and there are many questions that remain to be addressed concerning the different approaches to manage the surface hygiene and limit the risks to consumers.

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All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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Comparative Genomics of Iron-Transporting Systems in *Bacillus cereus* Strains and Impact of Iron Sources on Growth and Biofilm Formation

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Iron is an important element for bacterial viability, however it is not readily available in most environments. We studied the ability of 20 undomesticated food isolates of *Bacillus cereus* and two reference strains for capacity to use different (complex) iron sources for growth and biofilm formation. Studies were performed in media containing the iron scavenger 2,2-Bipyridine. Transcriptome analysis using *B. cereus* ATCC 10987 indeed showed upregulation of predicted iron transporters in the presence of 2,2-Bipyridine, confirming that iron was depleted upon its addition. Next, the impact of iron sources on growth performance of the 22 strains was assessed and correlations between growth stimulation and presence of putative iron transporter systems in the genome sequences were analyzed. All 22 strains effectively used Fe citrate and $FeCl_3$ for growth, and possessed genes for biosynthesis of the siderophore bacillibactin, whereas seven strains lacked genes for synthesis of petrobactin. Hemoglobin could be used by all strains with the exception of one strain that lacked functional petrobactin and IlsA systems. Hemin could be used by the majority of the tested strains (19 of 22). Notably, transferrin, ferritin, and lactoferrin were not commonly used by *B. cereus* for growth, as these iron sources could be used by 6, 3, and 2 strains, respectively. Furthermore, biofilm formation was found to be affected by the type of iron source used, including stimulation of biofilms at liquid-air interphase ($FeCl_3$ and Fe citrate) and formation of submerged type biofilms (hemin and lactoferrin). Our results show strain variability in the genome-encoded repertoire of iron-transporting systems and differences in efficacy to use complex iron sources for growth and biofilm formation. These features may affect *B. cereus* survival and persistence in specific niches.

Keywords: *Bacillus cereus*, iron transport, genotypes, growth, biofilm formation, complex iron sources

INTRODUCTION

Iron is one of the essential elements required for growth and metabolism of the majority of microorganisms. Despite its important role in microbial cells, the availability of free iron in the environment is limited due to oxidation of ferrous iron to ferric ions which precipitate near neutral pH (Ratledge and Dover, 2000). Free ferrous iron can be toxic to mammals due to formation of oxygen radicals, consequently the majority of host iron is bound to transport molecules such as hemoglobin (red blood cells), transferrin (serum), and lactoferrin (milk and mucosal secretions), or to ferritin-like proteins for intracellular iron storage (Ratledge and Dover, 2000). The storage of iron in complexed form also reduces its availability for invading pathogenic microorganisms. However, many pathogens developed mechanisms to overcome iron scarcity by the expression of scavenging systems specific to complex and non-complex iron sources. Two main scavenging mechanisms for iron have been described. Bacteria may secrete specific molecules with high affinity to iron named siderophores (Ratledge and Dover, 2000; Zawadzka et al., 2009) that facilitate iron transport into the microbial cell. These siderophores sequester iron from different sources such as transferrin (Abergel et al., 2008). The second mechanism involves specific ABC-type transporters encompassing high-affinity surface receptors specific for either complex iron compounds or free iron (Brown and Holden, 2002; Daou et al., 2009). *B. cereus* genomes encode several putative ABC transporters for complexed iron including ferric citrate (Harvie and Ellar, 2005; Fukushima et al., 2012) and ferrichrome, and several others of unknown substrate specificity (Hotta et al., 2010). Furthermore, a possible interplay between different molecules has been suggested. For example the heme-binding surface protein IlsA in *B. cereus* also serves as ferritin receptor and assists in ferritin-iron sequestration by bacillibactin siderophore (Segond et al., 2014). IlsA has also been shown to transfer bound hemin to another surface iron transporting molecule of the IlsA system IsdC (Abi-Khalil et al., 2015).

For *B. cereus, two* different siderophores, bacillibactin (BB), and petrobactin (PB) (Wilson et al., 2006) have been identified. PB is the main siderophore for *B. anthracis* (Koppisch et al., 2005) and important for its virulence since it is not recognized by the innate immune system (Abergel et al., 2006). In *B. cereus,* BB seems to be of higher importance in virulence compared to PB based on experiments in an insect model (Segond et al., 2014).

B. cereus has been reported to use various iron sources for growth that are typically present in red blood cells such as hemoglobin (Hb), hemin, and other hemoproteins (Sato et al., 1998, 1999a,b). For *B. cereus* ATCC 14579, the use of ferritin as an iron source has been described (Daou et al., 2009). Concerning the use of transferrin by different *B. cereus* strains, contradictory reports have been published that conceivably links to strain variability (Sato et al., 1998; Park et al., 2005; Daou et al., 2009) and pointing to the importance to take strain diversity into account in studies on iron metabolism. Lactoferrin, an iron source typically present in milk, cannot be used by *B. cereus* and inhibits its growth when present in high concentrations (Sato et al., 1999b; Daou et al., 2009). Ferric citrate, an iron source formed from citric acid which is commonly present in milk and citrus fruits, can also be used by *B. cereus* (Fukushima et al., 2012). These iron sources can be encountered in different environments including soil, food and processing environments, and mammals or insects. The ability to use these sources largely determines the fitness of bacteria and capacity to adapt to specific niches.

Besides its important role as essential element for bacterial growth and virulence (Cendrowski et al., 2004; Harvie et al., 2005; Porcheron and Dozois, 2015), iron has also been reported to affect biofilm formation (Porcheron and Dozois, 2015). It was recently shown that air-liquid biofilm formation by a selection of *B. cereus* food isolates was stimulated by addition of $FeCl_3$ (Hayrapetyan et al., 2015a). Biofilm formation may serve as survival mechanism in different environments and can be an important factor contributing to host colonization. To our knowledge, the impact of different (complex) iron sources on biofilm formation capacity and type of biofilms formed including submerged or surface-attached liquid-air biofilms, has not been reported for this species.

In this study we investigated the use of different iron sources by 22 *B. cereus* strains in relation to their genome content. Expression of the iron transporters in iron deplete and replete conditions was studied in the reference strain ATCC 10987. Since the ability of *B. cereus* to form biofilms contributes to its persistence in environment and free iron availability is important for biofilm formation of *B. cereus* (Hayrapetyan et al., 2015a), we also studied the effect of iron sources encountered in different environments on biofilm formation.

RESULTS

Iron Transporting Systems Presence and Expression

Genomes of 20 food isolates and 2 reference *B. cereus* strains ATCC 14579 and ATCC 10987 were analyzed for genes with predicted function in iron transport (**Figure 1A** and **Table 1**). Genes encoding for synthesis of siderophore BB structural components (*dhbACBEF)* and transporters were present in all strains, while PB biosynthesis genes (*asbABCDEF*) were absent in seven of the 22 strains analyzed. For five strains, PB biosynthesis genes were present but a functional *fpuA/fhuB* gene cluster necessary for PB uptake was lacking. However, another permease (*fatCD)* with a redundant function with *fhuB* (Dixon et al., 2012), was identified in all the strains in a cluster together with ATP- and substrate-binding proteins (BC5103–5106). Interestingly BC4416, a *fhuD*-like putative iron compound binding protein with unknown specificity (Hotta et al., 2010) was absent in the strains that also lacked PB siderophore biosynthesis genes, which could indicate a role for this protein in PB transport.

The IlsA-system acts as a hemophore, and is encoded by the *ilsA* gene (BC1331) and an *isd*-like operon consisting of the ABC-transporter (BC4544-4546), sortase (BC4543), heme degrading monooxygenase (BC4542), and heme transport associated proteins BC4547, BC4548, and BC4549 (IsdC) in *B. cereus* ATCC 14579 (Daou et al., 2009). Genes encoding the IlsA system are present in all strains. In B4079 the IlsA protein appears to be

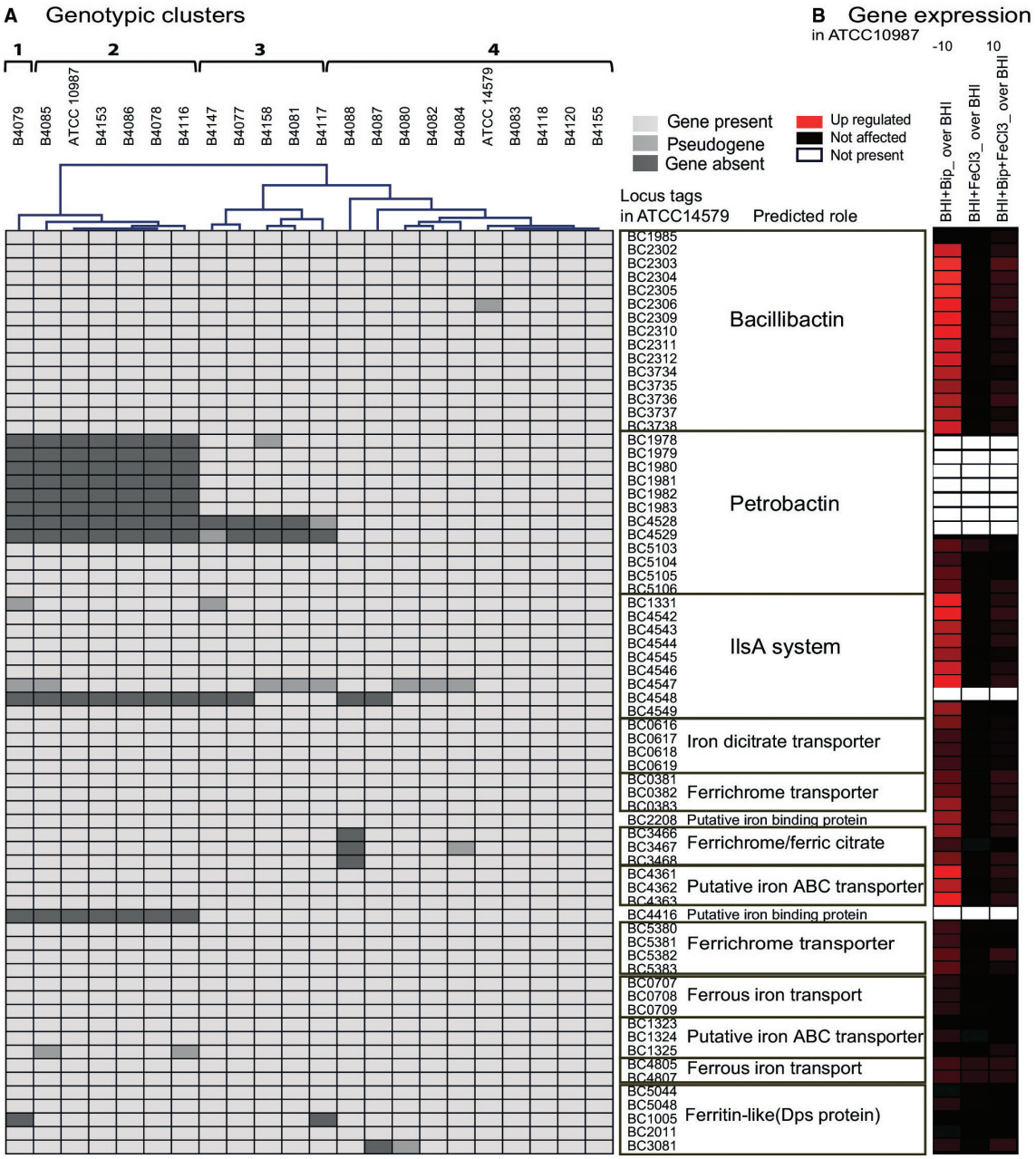

FIGURE 1 | (A) Hierarchical clustering of 22 *B. cereus* strains based on gene repertoire encoding iron transporters. Clustering was performed using Genesis software (Sturn et al., 2002). **(B)** Expression of genes encoding iron transporters in *B. cereus* ATCC 10987 in BHI, BHI+Bip, BHI+$FeCl_3$, and BHI+Bip+$FeCl_3$ at exponential growth phase ($t = 5$ h). Up-regulated genes are presented in red, down-regulated genes in green and unaffected genes in black. The scale −10 to 10 is based on $\log_2$ values of expression ratios compared to BHI.

truncated and non-functional due to a point mutation in the encoding gene that creates a premature stop codon. In B4147 the IlsA also appears to be ineffective due to a large internal deletion identified in the encoding gene (both verified with PCR and sequencing). The transport associated protein (BC4547) was identified as a pseudogene in eight strains. Interestingly, the other transport associated secreted component of this system BC4548, which may function as a hemophore that captures heme from Hb and has 98% identity to *isdX1* of *B. anthracis* (Daou et al., 2009), was absent in 11 strains.

Several other known iron ABC-transporters, such as an iron (III) dicitrate-binding complex (*fhuD, fecCDE*), a ferrichrome-binding complex (*feuA/fhuGB*), a *fepC/fhuGD* complex and a *fepBC/fhuGB* complex, were present in all strains. The *feuA/fhuGB* complex, known to bind ferric citrate in *B. cereus* (Fukushima et al., 2012), was only absent in strain B4088.

TABLE 1 | Genes and their predicted function in iron transport in *B. cereus*.

Locus tag in *B. cereus* ATCC 14579	Locus tag in *B. cereus* ATCC 10987	Predicted role	Name	Predicted function
BC1985	BCE2066	Bacillibactin	ymfD	Hypothetical protein
BC2302	BCE2398		dhbA/entA	3,3-dihydro-3,3-dihydroxybenzoate dehydrogenase
BC2303	BCE2399		dhbC	Isochorismate synthase
BC2304	BCE2400		dhbE	3,3-dihydroxybenzoate-AMP ligase
BC2305	BCE2401		dhbB	Isochorismatase
BC2306	BCE2402		dhbF	Non-ribosomal surfactin synthetase SrfAA
BC2309	BCE2403		mbtH	Hypothetical protein
BC2310	BCE2404			Drug resistance transporter, EmrB/QacA family
BC2311	BCE2405		sfp	Putative 3′-phosphopantetheinyl transferase
BC2312	BCE2406			Hypothetical protein
BC3734	BCE3767		yuiI	Trilactone hydrolase
BC3735	BCE3768		feuD/yusV	Siderophores ABC-transporter, ATP-binding protein FeuC
BC3736	BCE3769		feuC	Siderophores ABC-transporter, permease FeuC
BC3737	BCE3770		feuB	Siderophores ABC-transporter, permease FeuB
BC3738	BCE3771		feuA	Siderophores ABC-transporter, siderophore-binding protein FeuA
BC1978		Petrobactin	asbA	Petrobactin biosynthesis protein AsbA
BC1979	–		asbB	Petrobactin biosynthesis protein AsbB
BC1980	–		asbC	Acyl-CoA synthetase
BC1981	–		asbD	Acyl carrier protein
BC1982	–		asbE	Petrobactin biosynthesis protein AsbE
BC1983	–		asbF	Hypothetical protein
BC4528	–		fpuA	Iron compound ABC transporter substrate-binding protein
BC4529	–		fhuB	Iron-hydroxamate transporter permease subunit
BC5103	BCE5223		fhuC	Iron-siderophore ABC transporter ATP-binding protein
BC5104	BCE5224		fatC	Iron-siderophore ABC transporter permease
BC5105	BCE5225		fatD	Iron-siderophore ABC transporter permease
BC5106	BCE5226		fatB	Iron-siderophore ABC transporter binding lipoprotein
BC1331	BCE144	IlsA	ilsA	Iron-regulated Leu-rich surface protein A
BC4542	BCE4666			Heme-degrading monooxygenase IsdG
BC4543	BCE4667			Sortase B
BC4544	BCE4668			Iron compound ABC transporter, ATP-binding protein
BC4545	BCE4669			Iron compound ABC transporter, permease protein
BC4546	BCE4670			Iron compound ABC transporter, iron compound-binding protein
BC4547	BCE4671			Iron transport-associated protein
BC4548	–			Iron transport-associated protein, NEAT domain
BC4549	BCE4672		isdC	Iron transport associated protein
BC0616	BCE0683	Iron dicitrate transporter	fhuD	Iron (III) dicitrate ABC transporter, iron compound-binding protein
BC0617	BCE0684		fecD	Iron (III) dicitrate ABC transporter, permease protein
BC0618	BCE0685		fecC	Iron (III) dicitrate ABC transporter, permease protein
BC0619	BCE0686		fecE	Iron (III) dicitrate ABC transporter, ATP binding protein
BC0381	BCE0449	Ferrichrome transporter	fhuG	Ferrichrome ABC transporter, permease protein
BC0382	BCE0450		fhuB	Ferrichrome ABC transporter, permease protein
BC0383	BCE0451		feuA	Ferrichrome ABC transporter, ferrichrome-binding lipoprotein
BC2208	BCE2283	Putative iron binding protein	yfiY	Putative iron compound-binding protein
BC3466	BCE3485	Ferrichrome/ferric citrate	feuA-like	Iron compound ABC transporter substrate-bindingprotein FeuA

(Continued)

TABLE 1 | Continued

Locus tag in *B. cereus* ATCC 14579	Locus tag in *B. cereus* ATCC 10987	Predicted role	Name	Predicted function
BC3467	BCE3486		fhuG-like	Ferrichrome transport system permease fhuG
BC3468	BCE3487		fhuB-like	Ferrichrome transport system permease fhuB
BC4361	BCE4448	Putative iron ABC transporter	fepC-lik	Iron compound ABC transporter, ATP-bindingprotein
BC4362	BCE4449		fhuG-like	Iron compound ABC transporter, permease protein
BC4363	BCE4450		fhuD-like	Lipoprotein binding vitamin B13
BC4416	–	Putative iron binding protein	fhuD-like	Putative iron compound-binding protein
BC5380	BCE5509	Ferrichrome transporter	fepB-like	Iron compound ABC transporter, iron compound-binding protein
BC5381	BCE5510		fepC-like	Ferrichrome ABC transporter ATP-binding protein
BC5382	BCE5511		fhuG-like	Ferrichrome ABC transporter permease
BC5383	BCE5512		fhuB-like	Ferrichrome ABC transporter permease
BC0707	BCE0782	Ferrous iron transport	feoB-C	Ferrous iron transport protein FeoB, C-terminal domain
BC0708	BCE0782		feoB-N	Ferrous iron transport protein FeoB, N-terminal region
BC0709	BCE0783		feoA	Ferrous iron transport protein FeoA
BC1323	BCE1436	Putative iron ABC transporter		Putative iron compound ABC transporter, ironcompound-binding protein
BC1324	BCE1437			ABC transporter ATP-binding protein
BC1325	BCE1438			Iron compound ABC transporter permease
BC4805	BCE4965	Ferrous iron transport	feoB	Ferrous iron transport protein B
BC4807	BCE4966		feoA	Ferrous iron transport protein A
BC5044	BCE5191	Ferritin-like (Dps protein)		Ferritin-like diiron-binding protein, Dps family
BC5048	BCE5196			Ferritin-like diiron-binding protein, Dps family
BC1005	BCE1087			Ferritin-like diiron-binding protein, Dps family
BC2011	BCE2092			Ferritin-like diiron-binding protein, Dps family
BC3081	BCE3134			Ferritin-like diiron-binding protein, Dps family

The putative iron-binding protein *yfiY* (BC2208) was identified in all strains. Three additional systems, two of which encode ferrous iron transport FeoB-FeoA proteins (Kim et al., 2012), were identified in all *B. cereus* strains. Besides iron uptake genes, proteins involved in iron storage in bacteria, as for example the ferritin-like di-iron-binding proteins of the Dps family (DNA protection during starvation; Tu et al., 2012) were considered. Five genes with putative function in iron storage were identified and were present in most strains with a few exceptions (**Figure 1A**). The global regulator of iron uptake Fur (Harvie et al., 2005) was also present in all strains.

Transcriptome analysis of ATCC 10987 in iron replete (BHI+$FeCl_3$; BHI+Bip+$FeCl_3$) and deplete (BHI+Bip) conditions showed significant upregulation of most of the above mentioned genes encoding iron transporters under iron starvation evoked by addition of the scavenger (**Figure 1B**).

Iron transport genes were upregulated from 8 up to 900 fold (Supplementary Table 1), which was most prominent for the BB biosynthesis genes. Ferritin-like proteins for storage of intracellular iron were not significantly affected. The second ferrous iron transport cluster FeoA/B (BCE4965-4966) was significantly up regulated during iron starvation, indicating that the so called "living fossil" (Hantke, 2003) might still be functional in atmospheric conditions. Upon supplementation with $FeCl_3$, none of these genes were significantly affected, with exception of BCE3769. This was the case also in the presence of Bip together with $FeCl_3$ (with BCE2399 as an exception), showing that addition of iron reversed the iron starvation effect of Bip and support a role in iron transport and metabolism for these genes. These results indicate that iron scavenger Bip can be used to assess the efficacy of alternative (complex) iron sources to support growth of the selected 22 strains.

Iron Sources and Growth

The ability of *B. cereus* strains to use different iron sources for growth was tested in LB+Bip medium (**Figures 2**–**4**). The capacity to cope with iron starvation varied highly among the different strains (**Figures 3**, **4**). Notably, growth of all strains was restored in the presence of either Fe citrate or $FeCl_3$ by 80–135% according to growth index (GI) values. All strains, except B4079, could grow with Hb as sole iron source and restored growth to

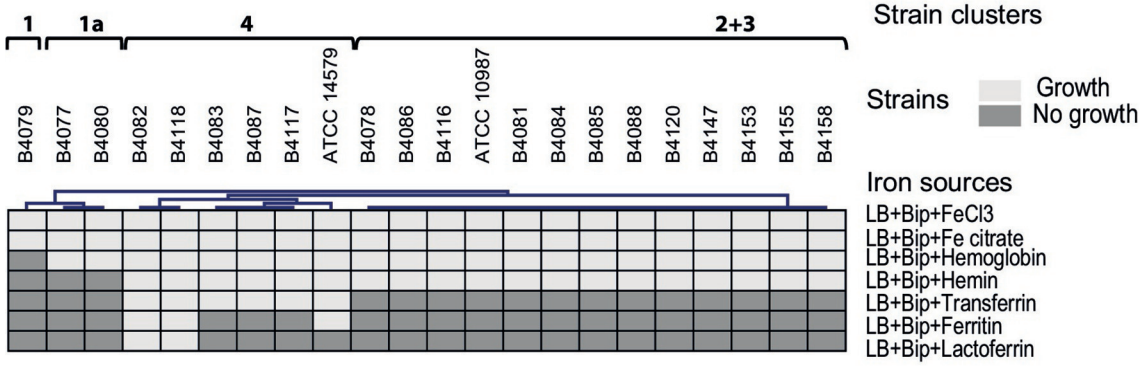

FIGURE 2 | Phenotypic hierarchical clustering of 22 *B. cereus* strains based on ability to grow on different iron sources. Strains were clustered using the Genesis software (Sturn et al., 2002).

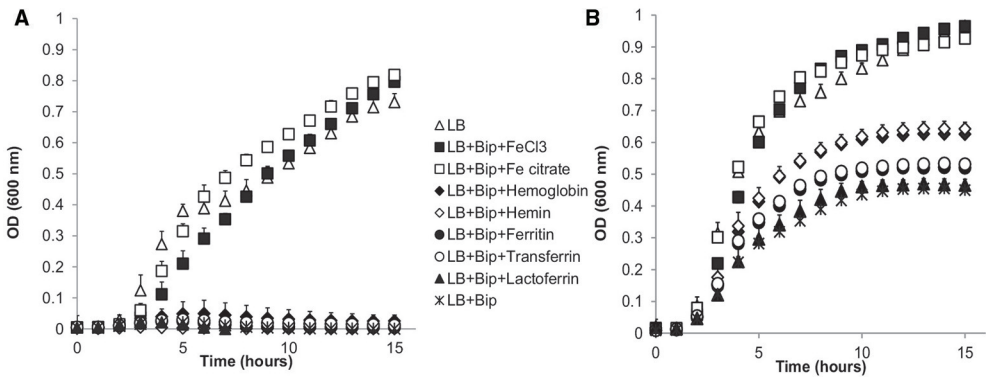

FIGURE 3 | Growth of strains B4079 (A) and ATCC 14579 (B) in LB and LB supplemented with iron scavenger (LB+Bip) with and without addition of different iron sources. Presented values are averages of 3 independent experiments with standard deviation. Data points for LB for both strains are very close to $FeCl_3$ and Fe citrate. For B4079 **(A)** data points of LB+Bip are very close to LB+Bip+Lactoferrin, LB+Bip+Transferrin, LB+Bip+Ferritin and LB+Bip+Hemin. For ATCC 14579 **(B)** LB+Bip data points are very close to LB+Bip+Lactoferrin; LB+Bip+Ferritin is close to LB+Bip+Transferrin; LB+Bip+Hb is close to LB+Bip+Hemin.

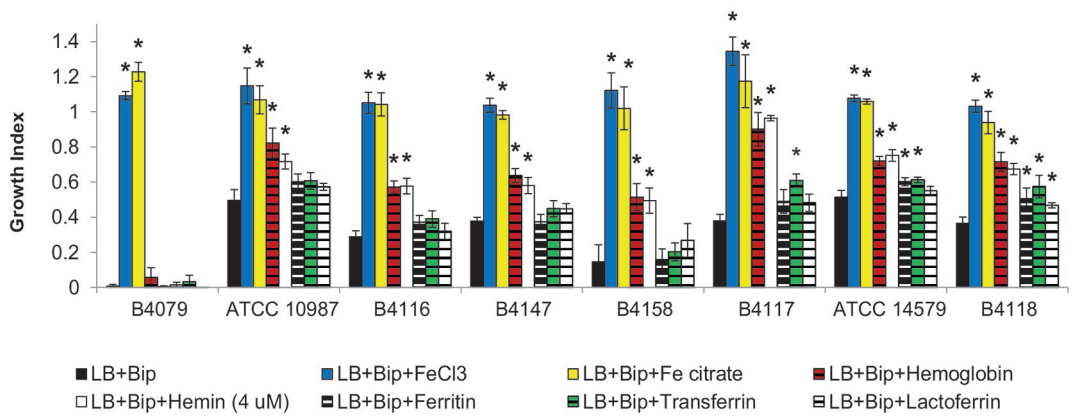

FIGURE 4 | Growth indexes for selected strains in LB, LB supplemented with iron scavenger (LB+Bip) with and without supplementation with different iron sources. Growth indexes represent the ratio of OD(600 nm) reached after 10 h of growth with the corresponding iron source relative to the OD reached in LB. Asterix (*) indicates significant difference ($p < 0.01$) from iron depleted condition (LB+Bip) for each strain, indicating that the strain could grow with the supplemented iron source.

levels ranging from 43% for strain B4078, up to 90% for strain B4117, compared to control conditions (LB medium).

Hemin could be used by all except three strains (B4077, B4079, B4080). Notably, bacteria that use heme as an iron source also have to cope with its toxicity. This is achieved by a tight control of heme transport, biosynthesis, and degradation. All strains harbored genes to synthesize protoheme and heme, as well as genes encoding the heme efflux ABC transporter HrtA-HrtB, and the associated two-component system HssS-HssR (Stauff and Skaar, 2009; not shown). Only in strain B4158 the latter gene cluster appeared impaired due to an internal deletion, and this strain was among those most sensitive to hemin, along with B4118 and B4147 that were inhibited at higher hemin concentrations (**Figure 5**).

Transferrin and ferritin could be used by six and three strains, respectively (**Figure 2**), and both compounds restored growth to a maximum of 60% of the control. Lactoferrin was a poor iron source for most strains and could only be used by strains B4082 and B4118 (**Figure 2**) albeit that growth was restored to a maximum of 47% of the controls (not shown).

Linking Genotypes with Growth Phenotypes

The growth performance data on different iron sources and genome contents were clustered (**Figures 2**, **1A**). Four main clusters could be distinguished but phenotypes did not match fully with predicted capacity based on gene content. B4079 showed poorest growth in iron-depleted condition and with complex iron sources. In line with this observation, B4079 lacks most functional transporters. B4079 clusters separately from the other strains (cluster 1, **Figures 1A,B**) and based on gene content it is most similar to the subgroup of strains lacking PB encoding genes (cluster 2, **Figure 1A**). The strains of cluster 2 (**Figure 1A**), along with the strains missing *fpuA/fhuB* genes for PB import (cluster 3, **Figure 1A**), belong to one large phenotypic cluster (cluster 2+3, **Figure 2**) of strains which can use $FeCl_3$, Fe citrate, Hb, and hemin, but not transferrin, ferritin or lactoferrin. The exceptions are B4077 (no growth on hemin) and B4117 (can use transferrin) which fall out of the phenotypic cluster 2+3. The other five strains that could use more than three of the above mentioned complex iron sources group together based on phenotypes (cluster 4, **Figure 2**) and they harbor all or most iron transporter genes considered (genotypic cluster 4, **Figure 1A**). Notably, the other five strains with all the genes present did not match the expected use of complex iron sources. On the other hand, the *feuA/fhuGB* complex is lacking in strain B4088 which nevertheless can grow on Fe citrate. Overall, the phenotypes for 15 out of 22 strains (70%) corresponded to that predicted based on genome content.

Iron Sources and Biofilm Formation

The ability of the different strains to form biofilms with different types of iron sources was tested on polystyrene microtiter plates. 10 out of 22 tested strains formed a biofilm in LB medium without supplementation (control; **Table 2**). Removal of free iron with Bip eliminated the biofilm forming capacity of nine of these strains, leaving only strain B4155 positive for biofilm formation. For two strains (B4080 and B4120), biofilm formation was promoted under iron deplete condition (**Table 2**), even though the growth was reduced. Supplementation with Fe citrate and $FeCl_3$ not only restored but even increased biofilm forming capacity of the above mentioned 10 strains, and additionally triggered biofilm formation by B4087 (**Table 2**). Hb allowed biofilm formation by 16 strains, among them 6 strains that did not form biofilm in the control condition, albeit the amount of formed biofilm was lower than that formed in presence of $FeCl_3$ or Fe citrate for most of the strains. In the presence of hemin, six strains were able to form biofilm, similar to lactoferrin. These biofilms were completely submerged on the bottom of the well, in contrast to the air-liquid interface biofilm formed in LB, LB+Bip+$FeCl_3$, and LB+Bip+Fe citrate (**Figure 6**).

DISCUSSION

In this study we present data showing the impact of different iron sources on growth and biofilm formation capacity and type of biofilms formed for 20 *Bacillus cereus* food isolates and two reference strains.

Bacillibactin (BB) and petrobactin (PB) are iron-transporting siderophores produced by *Bacillus cereus* group members. The relevance of PB in *B. anthracis* growth and virulence was shown, however for *B. cereus* BB was suggested to be of more importance (Segond et al., 2014). Notably, BB is present in all the strains in this study, while PB is absent in seven strains.

Limitation of free iron impaired the growth of all tested *B. cereus* strains in LB+Bip but was most prominent for B4079, lacking both PB siderophore and functional IlsA. This also prevented efficient use of Hb and hemin by this strain, in contrast to strains missing only one of the mentioned systems. Interestingly, strains able to use ferritin or transferrin as iron source encompass the whole repertoire of iron transporters, with only minor exceptions. This is in agreement with the previously suggested cooperation between different systems such as IlsA and petrobactin siderophore in iron uptake from ferritin (Segond et al., 2014).

The ability of *B. cereus* strains to grow on complex iron sources does not always correspond to the presence of relevant genes. For example, B4120 and B4155 contain the full repertoire of iron transporters, however these strains could not use transferrin, ferritin or lactoferrin as iron sources. This may be explained either by differences in regulation of expression of these genes in the selected conditions, presence of transcriptional activators such as specific iron starvation ECF factors (Visca et al., 2002), or factors that affect translation or activity of the synthesized proteins.

Contradictory data have been reported previously concerning the use of transferrin by *B. cereus*. According to one report, *B. cereus* could use human transferrin as an iron source, albeit with lower efficiency compared to *Staphylococcus aureus, Escherichia coli*, and *Pseudomonas aeruginosa* (Park et al., 2005). Two other studies report inability of *B. cereus* to grow on transferrin (Sato et al., 1998; Daou et al., 2009), or growth inhibition of *B. cereus* and *B. anthracis* by human transferrin

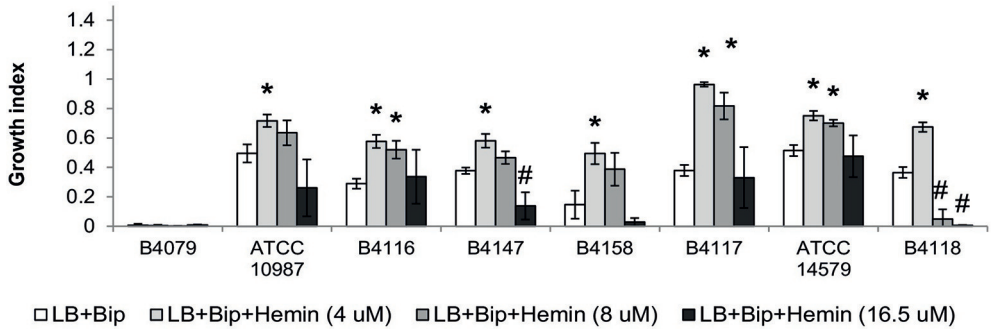

FIGURE 5 | Growth of selected *B. cereus* strains on different concentrations of hemin. Growth is expressed as the growth indexes. Asterix (*) indicates significant difference ($p < 0.01$) from growth index in LB+Bip for each strain showing that the strain could grow on the specified concentration of hemin, while the hash (#) shows that the growth was significantly inhibited. For all of the presented strains, 4 uM hemin was the optimal concentration for growth, with the exception of strain B4079 which did not grow on this iron source with any concentration.

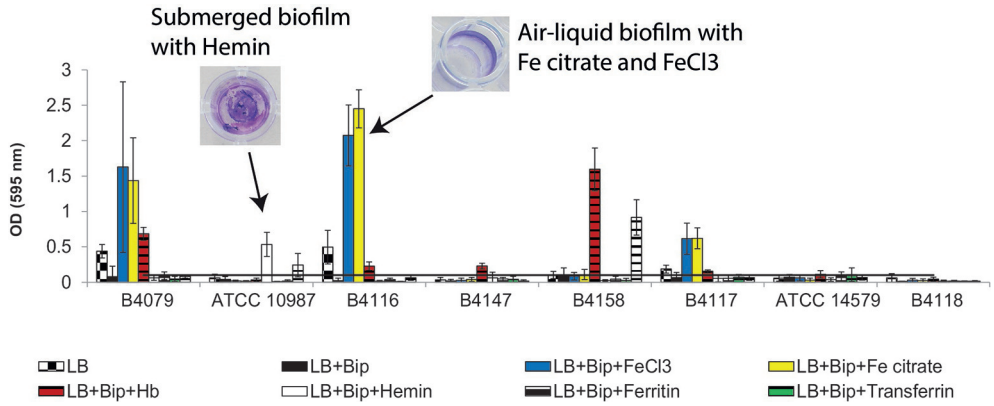

FIGURE 6 | Biofilm formation for selected *B. cereus* strains. The biofilm was formed in polystyrene 96-well-plate in LB and LB supplemented with Bip, with or without addition of different iron sources. The biofilm was measured with the CV assay after 24 h incubation at 30°C.

(Sato et al., 1998) due to iron deprivation (Rooijakkers et al., 2010). Our data show that the ability to use human transferrin is strain and concentration dependent, concentrations exceeding 2 uM displayed a bacteriostatic effect on several strains (not shown), while 1.5 uM transferrin was the optimal concentration that could be used by 6 out of 22 strains. Besides, the source of transferrin seems of importance since the *S. aureus* transferrin receptor was shown to bind preferentially human and rodent transferrin but not that of bovine and porcine origin (Modun and Williams, 1999). Aerobic or anaerobic growth conditions could also play a role since oxygen availability has for example been shown to affect the relative abundance of petrobactin and bacillibactin in *B. anthracis* (Lee et al., 2011). Furthermore, all the strains used in this study, with the exception of ATCC 14579 (isolated from air in a cow shed) were food isolates. Systemic infections caused by *B. cereus* (Bottone, 2010; Uchino et al., 2012) are caused by more clinically relevant strains, that likely differ in their ability to use and tolerate high levels of transferrin compared to food isolates. To test this, further studies including clinical isolates should be performed.

Lactoferrin is abundant in milk, but also in blood and secreted fluids such as tears and displays antimicrobial properties (Oram and Reiter, 1968; Sato et al., 1999b; Orsi, 2004). Lactoferrin can be used as an iron source by *Pseudomonas* ssp. (Xiao and Kisaalita, 1997) and several other microorganisms (Morgenthau et al., 2013), but not by *B. cereus* as reported previously (Sato et al., 1999b; Daou et al., 2009). The latter study used 1.5 uM of lactoferrin, which in our study also did not restore the growth of any of the 22 strains and inhibited the growth for strain B4086 (not shown). However, a concentration of 0.7 uM lactoferrin slightly restored the growth of two strains (B4082 and B4118), which could also use all other tested iron sources, indicating that these strains were in general better equipped for use of complex iron sources, in line with the full repertoire of iron transporting systems present in these strains. The low number of strains able to use lactoferrin is unexpected given the fact that *B. cereus* is a common contaminant in dairy products.

The capacity to use different complex iron sources could not be linked to the isolation source of the strains. However, clustering of the strains used in this study according to Guinebretière et al. (2008), revealed that all strains lacking

petrobactin encoding genes belong to the phylogenetic group III (Warda et al., in press). A common habitat for strains of group III are dehydrated/starchy foods (Guinebretière et al., 2008). Interestingly, all group III strains in the current study were isolated from a starch or dairy containing food product as reported previously (Hayrapetyan et al., 2015a).

Iron Sources and Biofilm Formation

Previously, we reported that addition of free iron ($FeCl_3$) promoted formation of air-liquid interface biofilms by *B. cereus* strains. In this study we show that apart from $FeCl_3$ also Fe citrate promoted biofilm formation. Hb triggered biofilm formation for a subset of strains for which the growth was also restored and resulting in partial submerged and air-liquid biofilms. Even strain B4079, which did not show significant growth recovery with Hb, was able to form biofilm upon its addition. It showed very limited growth in the presence of Hb (to $OD = 0.05$, compared to LB+Bip $OD = 0.01$, **Figure 3**), which may have caused stress conceivably linked to biofilm formation as a response. Hb was previously identified as a component in nasal secretions that promoted colonization by *S. aureus* via repression of the *agr* quorum sensing system resulting in reduced production of proteases with concomitant reduction in biofilm dispersal (Pynnonen et al., 2011). Interestingly this effect was found to be exerted by the Hb protein independently of its iron content. The mechanism of Hb-induced biofilm formation in *B. cereus* remains to be elucidated.

Ferritin and transferrin only slightly supported biofilm formation, mostly for strains already able to form biofilm in iron limited conditions (B4080, B4120, and B4155, **Table 2**). A role for the surface protein IsdC in cell-cell attachment and biofilm formation under iron deplete conditions was shown for *Staphylococcus lugdunensis* (Missineo et al., 2014). Interestingly, this protein is a homolog of BC4549, encoding a component of the IlsA iron transporting system. Since iron starvation most likely triggers the upregulation of such proteins this may be linked to biofilm-promoting effect of iron depletion for strains B4080 and B4120 (**Table 2**).

The iron-chelating properties combined with a direct bactericidal effect of lactoferrin has led to its proposed role as potential anti-biofilm compound (Ammons and Copié, 2013). In our study, lactoferrin triggered submerged biofilm formation by *B. cereus* strains B4158 and ATCC 10987, even though growth was not restored. The underlying mechanism remains to be elucidated.

This study shows that ferric citrate and $FeCl_3$ could be used by all *B. cereus* strains and were preferred iron sources. Hemoglobin, hemin, transferrin, ferritin and lactoferrin could also act as iron sources but their use appeared to be highly strain-dependent. The ability of *B. cereus* strains to grow on complex iron sources correlated largely with the genome content, but could not always be linked to specific iron transporter genes present. The ability to use complex iron sources seems to be dictated by the combined presence or absence of more than one functional iron transporting system, rather than one single system. Furthermore, biofilm formation was found to be affected by the type of iron source used, including stimulation of biofilms at liquid-air interphase ($FeCl_3$ and Fe citrate) and formation of submerged type biofilms (hemin and lactoferrin). Notably, generation of submerged biofilms was in some cases linked to lack of growth stimulation by the complex iron source tested. To conclude, our results show strain variability in the genome repertoire of iron-transporting systems and differences in efficacy to use complex iron sources for growth and biofilm formation. These features may affect *B. cereus* survival and persistence in specific niches including food processing environments and the human host.

MATERIALS AND METHODS

Strains and Culturing Conditions

Twenty *Bacillus cereus* food isolates from the NIZO culture collection were used in this study (Hayrapetyan et al., 2015a) along with two reference strains *B. cereus* ATCC 10987 and ATCC 14579. To obtain overnight cultures, a loop full with stock cultures stored at −80°C was inoculated into 10 ml LB broth (Miller, MERCK), supplemented with 100 μM 2,2-Bipyridine (Bip) (MERCK) to induce iron starvation, and incubated for 18 h at 30°C with shaking at 200 rpm.

The twenty *B. cereus* food isolates were sequenced by next-generation whole genome sequencing. For eight strains (B4077, B4078, B4080, B4086, B4087, B4147, B4153, B4158), total DNA isolation and sequencing details are described elsewhere (Krawczyk et al., 2015), for the remaining 12 isolates (B4081, B4082, B4083, B4084, B4085, B4088, B4116, B4117 [recently re-classified by NCBI as *Bacillus mycoides* based on ANI typing (Federhen et al., 2016)], B4118, B4120, B4155, B4079) draft genomes were obtained and deposited as described in Hayrapetyan et al. (2016).

Searching for Iron-Transporting Systems in *B. cereus* Genomes

Orthologous groups (OGs; i.e., gene families) were determined using OrthoMCL (Enright et al., 2002). This program uses all-against-all protein BLAST where it groups proteins with more homology within the species than homology with proteins outside the species. In this way orthologs (genes in different species that evolved from a common ancestral gene by speciation) are separated from paralogs (genes related by duplication within a genome). In addition to the 20 newly sequenced genomes of food isolates (Krawczyk et al., 2015; Hayrapetyan et al., 2016), the circular genomes of the two reference strains *B. cereus* ATCC 14579 and ATCC 10987 obtained from the NCBI database, were included. Contigs of the 20 newly sequenced genomes were scaffolded into their presumed correct order using the circular reference genomes as templates.

A database (in MS Excel) was built encompassing information about the location and length of orthologous proteins. Multiple sequence alignment files (MSA) were made (MUSCLE, version 3.8; Edgar, 2004), where the protein sequences within ortholog groups were aligned, to facilitate identification of pseudogenes (encoding incomplete proteins).

TABLE 2 | Biofilm formation in the presence of different iron sources.

Strains	LB	LB+Bip	LB+Bip+ $FeCl_3$ (250 uM)	LB+Bip+Fe citrate (250 uM)	LB+Bip+ Hemoglobin (2.5 uM)	LB+Bip+ Hemin (4 uM)	LB+Bip+ Ferritin (0.9 uM)	LB+Bip+ Transferrin (1.5 uM)	LB+Bip+ Lactoferrin (0.7 uM)
B4078	+	–	++*	++*	+*	–*	–	–	–
B4079	+	–	++*	++*	+	–	–	–	–
B4082	–	–	–*	–*	–*	–*	–*	–*	–*
B4083	++	–	++*	++*	++*	+*	–	+*	–
B4086	+	–	++*	++*	+*	–*	–	–	–
B4087	–	–	+*	+*	+*	–*	–	–*	–
B4116	+	–	++*	++*	+*	–*	–	–	–
B4117	+	–	+*	+*	+*	–*	–	–*	–
B4118	–	–	–*	–*	–*	–*	–*	–*	–*
ATCC 14579	–	–	–*	–*	+*	–*	–*	+*	–
ATCC 10987	–	–	–*	–*	–*	+*	–	–	+
B4077	–	–	–*	–*	+*	–	–	–	+
B4080	–	+	–*	–*	+*	+	+	+	+
B4081	–	–	–*	–*	+*	–*	–	–	–
B4084	–	–	–*	–*	–*	–*	–	–	–
B4085	+	–	++*	++*	+*	–*	–	–	–
B4088	–	–	–*	–*	–*	+*	–	–	–
B4120	–	+	–*	–*	–*	+*	+	+	+
B4147	–	–	–*	–*	+*	–*	–	–	–
B4153	+	–	++*	++*	+*	–*	–	–	–
B4155	+	+	+*	+*	+*	+*	+	+	+
B4158	+	–	–*	+*	++*	–*	–	–	+
Total number of biofilm forming strains	10	3	10	11	16	6	3	5	6

The biofilm was formed in polystyrene 96-well-plates in LB medium, and LB supplemented with Bip with or without addition of indicated iron sources. The biofilm was measured with CV assay after 24 h incubation at 30°C.
+, OD (Crystal violet assay) > 0.1.
++, OD (Crystal violet assay) > 1.
–, OD (Crystal violet assay) < 0.1.
**growth was significantly restored compared to LB+Bip.*
All the positives are highlighted in gray.

A literature search was performed to find known iron-uptake systems for *B. cereus* (Daou et al., 2009; Zawadzka et al., 2009; Hotta et al., 2010). Orthologous groups (OGs) containing the locus tags of these known genes were searched for in the OG table. Furthermore, a key word search was done to find additional iron uptake and storage systems, by searching in the annotation of all genomes for keywords: iron, ferric, ferrous, ferritin.

For relevant identified OGs containing pseudogenes, which are fragments of genes (i.e. truncated, frame-shifted or at the end of contigs), which had been classified by OrthoMCL into separate OGs adjacent on the chromosome, were combined into single OGs representing all the fragments of a single pseudogene.

The RAST automatic annotation of the encoded proteins was manually improved using InterproScan (http://www.ebi.ac.uk/Tools/pfa/iprscan/), NCBI-BLAST (http://blast.ncbi.nlm.nih.gov/http://blast.ncbi.nlm.nih.gov/) and NCBI/Genbank database for the comparison of genes with other species (http://www.ncbi.nlm.nih.gov/).

Growth and Biofilm Formation

The growth and biofilm formation on different iron sources was tested in LB (as control), LB supplemented with 600 μM 2,2-Bipyridine (LB+Bip) as iron depleted condition, and in iron-replete conditions using LB+Bip with addition of the following iron sources in final concentrations: $FeCl_3$ (250 μM; LB+Bip+$FeCl_3$), ferric citrate (250 μM; LB+Bip+Fe citrate), hemoglobin (human, 2.5 μM; LB+Bip+Hb), hemin (4, 8, and 16.5 μM; LB+Bip+Hemin), ferritin (from equine spleen, 0.9 μM; LB+Bip+Ferritin), transferrin (human, partially saturated, 1.5 μM; LB+Bip+Transferrin), and lactoferrin (bovine milk, 0.7 μM; LB+Bip+Lactoferrin). 2,2-Bipyridine, $FeCl_3$ and ferric citrate were from MERCK and the remaining iron sources

used were obtained from SIGMA. Selected concentrations were adapted from previously reported concentrations used for *B. cereus* (Daou et al., 2009), (Segond et al., 2014), with some optimization for the culturing conditions and strains of this study.

The strains were grown in a 96-well-plate filled with 200 µl LB with or without supplements inoculated with 1% overnight culture. The growth was monitored by measuring the *OD* at 600 nm in SPECTRAmax (model PLUS384) at 30°C, with shaking for 60 s every 5 min. The growth index (GI) for each iron source was calculated as described elsewhere (Daou et al., 2009), by dividing the *OD* at 600 nm reached in LB after 10 h of growth by *OD* reached when grown with the specific iron source.

The biofilms formed in 96-wells-plates inoculated as described above, were measured after 24 h of static incubation at 30°C using the Crystal Violet (CV) assay as described previously (Hayrapetyan et al., 2015a). Washing, staining and de-staining steps were performed using 250 µl of de-mineralized water, 0.1% crystal violet and 70% ethanol, respectively. After de-staining the *OD* was measured at 595 nm. The strain was considered to form a biofilm if in a given condition the *OD* value was higher than 0.1, a threshold value as defined in (Hayrapetyan et al., 2015a).

Transcriptome Analysis to Identify Iron-Responsive Genes

For transcriptome analysis RNA was isolated from static liquid cultures of *B. cereus* ATCC 10987 grown in BHI (control), BHI supplemented with 450 µM Bip (BHI+Bip) for iron deplete condition, BHI supplemented with 250 µM $FeCl_3$ (BHI+$FeCl_3$) and BHI with both Bip and $FeCl_3$ (BHI+Bip+$FeCl_3$) for iron replete conditions, and the latter to test whether iron supplementation could restore effects evoked by iron starvation induced by Bip. These conditions were based on a previous study in our laboratory showing the role of free iron in biofilm formation (Hayrapetyan et al., 2015a). The samples were taken at exponential growth phase (5 h). RNA was isolated as previously described (Hayrapetyan et al., 2015b). Labeling and hybridization were performed as described elsewhere (Mols et al., 2013). Two independent biological replicates were hybridized on the arrays, each sample was used three times and was labeled with the swapped dyes Cy3 and Cy5.

Custom-made array design for *B. cereus* ATCC 10987 developed by Agilent Technologies (GEO accession number GPL7681; Mols et al., 2010) was used in this study. Microarray scanning and data normalization were performed as previously described (Hayrapetyan et al., 2015b). Genes with more than two fold change in expression and $p < 0.05$ were considered significantly affected. The processed and raw microarray data is deposited in GEO database under accession number GSE74045.

Statistical Analysis

Presented values are averages of at least three independent experiments with standard deviations. The growth was considered recovered if the growth index of the strain on a specific iron source was significantly different from the growth index of the same strain when grown in LB+Bip without iron supplementation. Significance of the growth differences was concluded based on a two-sided student's *t*-test, assuming equal variances and a $P < 0.01$.

AUTHOR CONTRIBUTIONS

Conceived and designed experiments: TA, MG, and HH. Performed the experiments: HH. Analyzed the data: HH. Performed genomic comparisons: RS. Wrote the paper: HH. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.00842

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The LuxS Based Quorum Sensing Governs Lactose Induced Biofilm Formation by *Bacillus subtilis*

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Bacillus species present a major concern in the dairy industry as they can form biofilms in pipelines and on surfaces of equipment and machinery used in the entire line of production. These biofilms represent a continuous hygienic problem and can lead to serious economic losses due to food spoilage and equipment impairment. Biofilm formation by *Bacillus subtilis* is apparently dependent on LuxS quorum sensing (QS) by Autoinducer-2 (AI-2). However, the link between sensing environmental cues and AI-2 induced biofilm formation remains largely unknown. The aim of this study is to investigate the role of lactose, the primary sugar in milk, on biofilm formation by *B. subtilis* and its possible link to QS processes. Our phenotypic analysis shows that lactose induces formation of biofilm bundles as well as formation of colony type biofilm. Furthermore, using reporter strain assays, we observed an increase in AI-2 production by *B. subtilis* in response to lactose in a dose dependent manner. Moreover, we found that expression of *eps* and *tapA* operons, responsible for extracellular matrix synthesis in *B. subtilis*, were notably up-regulated in response to lactose. Importantly, we also observed that LuxS is essential for *B. subtilis* biofilm formation in the presence of lactose. Overall, our results suggest that lactose may induce biofilm formation by *B. subtilis* through the LuxS pathway.

Keywords: *B. subtilis*, biofilm, lactose, quorum sensing, AI-2 LuxS system

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INTRODUCTION

Bacteria often use quorum sensing (QS) as cell–cell communication mechanism to control expression of genes that affect a variety of cellular processes (Fuqua et al., 1994; Miller and Bassler, 2001; Bai and Rai, 2011). QS is based on production, secretion and response to small signaling molecules, termed autoinducers (AI; Bai and Rai, 2011). AI-2, a furanosyl-borate-diester (Chen et al., 2002) is referred as a "universal autoinducer" as it is found in numerous Gram positive and Gram negative bacteria (Schauder and Bassler, 2001; Xavier and Bassler, 2003). AI-2 is synthesized by LuxS through steps involving conversion of ribose-homocysteine into homocysteine and 4,5-dihydroxy-2,3pentanedione (DPD), a compound that cyclizes into several furanones in the presence of water (Schauder et al., 2001). QS modulates various cellular processes involved mainly in the regulation of virulence factors, sporulation, motility, toxin production (Hammer and Bassler, 2003; Henke and Bassler, 2004; Smith et al., 2004; Waters and Bassler, 2006) and

formation of a structured multicellular community of bacterial cells, also termed biofilm (Hall-Stoodley et al., 2004; Kolter and Greenberg, 2006). It appears that biofilm formation is the most successful strategy for bacteria to survive unfavorable environmental conditions (Stewart and Costerton, 2001; Hall-Stoodley et al., 2004). Bacteria in biofilms are highly resistant to disinfection and antibiotic treatments, therefore biofilm formation is considered as a major problem in the industrial fields and in medicine (Simoes et al., 2010).

Bacillus subtilis is a Gram-positive non-pathogenic bacterium, which is a facile model microorganism for biofilm research. *B. subtilis* possesses the ability to form different types of biofilms in different environmental conditions: colony type biofilm at solid-air interface, pellicle at liquid–air interface as well as submerged biofilm at solid-liquid interface (Vlamakis et al., 2013). *B. subtilis* cells can sense different environmental and physiological signals, which may activate one of its histidine sensor kinases. Those kinases are responsible for phosphorylation of Spo0A, a master regulator in the cell. Phosphorylated Spo0A leads to down-regulation of the transcriptional repressors AbrB and SinR, which keeps expression of genes for production of extracellular matrix turned off when conditions are not propitious for biofilm growth (Branda et al., 2006; Vlamakis et al., 2013). When a signal is introduced for biofilm formation, *B. subtilis* cells are shifted from motile bacteria to bacterial chains that stick together by producing an extracellular matrix (Branda et al., 2001; Kobayashi, 2007). The matrix has an important role during the biofilm formation. It provides an attaching source for other bacteria in the surrounding environment and therefore plays a crucial step in biofilm progression (Branda et al., 2001; Kobayashi, 2007). The matrix consisted of two main components, an extracellular polysaccharide (EPS) synthesized by the products of the *epsA-O* operon, and amyloid fibers encoded by *tasA* located in the *tapA-sipW-tasA* operon (Branda et al., 2006; Vlamakis et al., 2013).

Biofilms formed by *Bacillus* species are vastly found throughout dairy processing plants (Oosthuizen et al., 2001). Moreover, the major source of contamination of dairy products is often associated with members of the *Bacillus* genus (Sharma and Anand, 2002; Simoes et al., 2010). Recently, it was found that certain milk components enhance biofilm formation by *Bacillus* species (Pasvolsky et al., 2014). Lactose, a β1,4-linked disaccharide, is the main carbohydrate in milk and numerous dairy products. Our previous study showed that lactose increases biofilm formation by the Gram-positive bacteria *Streptococcus mutans* (Assaf et al., 2015). Since lactose is an abundant disaccharide sugar in milk and its products, it might serve as an environmental trigger for biofilm formation by other bacteria too, for instance *B. subtilis*. Interestingly, it has been shown that *B. subtilis* might use QS to regulate motility and biofilm formation (Lombardía et al., 2006). However, the link between sensing environmental cues and the QS induced biofilm formation by *B. subtilis* is poorly known. Therefore, the aim of this study was to investigate the role of lactose, the primary sugar in milk, on biofilm formation by *B. subtilis* and its possible link to QS process.

MATERIALS AND METHODS

Strains and Growth Media

Strains used in this study are listed in **Table 1**. For routine growth, all bacterial strains were grown in Lysogeny broth (LB; 10 g of tryptone (Neogen, Lansing, Michigan, USA), 5 g of yeast extract (Neogen, Lansing, MI, USA) and 5 g of NaCl per liter) and incubated at 37°C at 150 rpm for 5 h. The LB medium was solidified by addition of 1.5% agar (Neogen, Lansing, MI, USA) (Pasvolsky et al., 2014). Although, LB is suitable for bundle formation experiments, it was found to be less favorable for colony type biofilm or pellicle formation (Branda et al., 2001; Shemesh and Chai, 2013). Therefore, we studied colony biofilm and pellicle formation using chemically defined medium (CDM). CDM was prepared as previously described with slight modifications (Branda et al., 2001). Briefly, CDM contained 5mM potassium phosphate (pH 7), 100 mM 3-[*N*-Morpholino] propane sulfonic acid (MOPS) (pH 7), 2 mM $MgCl_2$, 700 μM $CaCl_2$, 50 μM $MnCl_2$, 50 μM $FeSO_4$, 1 μM $ZnCl_2$, 2 μM thiamine (Sigma–Aldrich, St. Louise, MI, USA), 0.5% glycerol, 0.5% glutamate, 50 μg/ml tryptophan (Sigma–Aldrich, St. Louise, MI, USA), and 50 μg/ml phenylalanine. (Sigma–Aldrich, St. Louise, MI, USA). For CDA, 1.5% agar (Neogen, Lansing, MI, USA) was added. Medium and plates were freshly prepared and used the following day.

LBGM media was prepared as described previously by supplementing LB with 1% (v/v) glycerol and 0.1 mM $MnSO_4$ (Shemesh and Chai, 2013).

Lactose Preparation

A stock 50% lactose (w/v) (J. T. Baker, London, UK) solution was prepared in distilled deionized water and sterilized using a 0.2 μm filter (Whatman, Dassel, Germany). The stock solution of lactose was diluted in LB to final concentrations of 0–5% (w/v) or CDA to final concentration of 3% (w/v) (Assaf et al., 2015).

Biofilm Formation

Colony biofilms are produced when cells are placed on a solid agar surface. Importantly, one of the major characteristics of biofilm colony is the production of extracellular matrix which harbors the biofilm bacteria (Vlamakis et al., 2013). For colony type biofilm formation (Branda et al., 2001), starter cultures were prepared as describe above. 2.5 μl (around 3×10^5 CFU) from the starter culture was dropped on CDA with or without 3% lactose. The plates were incubated at 30°C for 24 h. Images were taken using a Zeiss Stemi 2000-C microscope with an axiocamERc 5s camera.

For bundle formation, an overnight culture of cells was diluted 1:100 (to obtain $O.D._{(600)}$ of 0.07) into LB supplemented with or without 3% lactose (w/v). The samples were incubated at 37°C at 50 rpm for 5 h ($O.D._{(600)}$ of 1). One milliliter of each sample was collected and centrifuged at 5000 rpm for 2 min. The supernatant was discarded, the pellet was re-suspended and 3 μl of the suspension placed on a glass slide was visualized in a transmitted light microscope using Nomarski differential interference contrast (DIC), at 40× magnification

TABLE 1 | Strains used in this study.

Strain	Genotype	Characteristic description	Reference
Bacillus subtilis			
NCIB3610	wild type	Undomesticated WT strain	Branda et al., 2001
YC161	P_{spank}-*gfp*	Produces GFP constantly	Chai et al., 2011
YC164	P_{eps}-*gfp*	Produces GFP under the control of promoter for *eps*	Chai et al., 2008
YC189	P_{tapA}-*cfp* at the *amy*E locus in 3610, $Spec^R$	Produces CFP under the control of promoter for *tapA*	Chai et al., 2008, Pasvolsky et al., 2014
	Δ*luxS*	Mutant in *luxS* gene Which does not produce AI-2	Chai lab collection
RL3852	Δ*epsH* in 3610, Tet^R	Mutant in production of EPS	Kearns et al., 2005
SB505	Δ*tasA* in 3610, $Spec^R$	Mutant in production of amyloid fibers	A gift of Branda S.
V. harveyi			
MM77	Δ*luxLM*, Tn5, Δ*luxS*, Cm^R	Mutant in both systems of quorum sensing (QS) which does not produce AI-1 and AI-2	Surette et al., 1999

(Pasvolsky et al., 2014; Oknin et al., 2015). Furthermore, a confocal laser scanning microscope (CLSM) was used to visualize cyan fluorescent protein (CFP) or green fluorescent protein (GFP) expression. CFP expression of strain YC189 was observed using 458-nm argon laser, while GFP expression of strains YC161 and YC164 was observed using 488-nm argon laser (Zeiss LSM510 CLS microscope, Carl Zeiss, Oberkochen, Germany).

For pellicle formation, bacteria were inoculated from the agar plates into LB broth and incubated for 5 h at 37°C at 150 rpm. Next, 5 μl of the culture was seeded in a 12 wells plate (Nunc, Roskild, Denmark) containing 4 ml of CDM per well. The plates were incubated at 30°C. Pictures were taken after 24 h using SAMSUNG Galaxy camera.

AI-2 Production Assay

To determine the effect of lactose on AI-2 production, we used a bioluminescence assay as described before (Aharoni et al., 2008; Shemesh et al., 2010). Briefly, *B. subtilis* cells were grown in conditions inducing bundle formation as described above. One milliliter of each sample was collected and centrifuged at 5000 rpm for 2 min. Supernatant was collected and neutralized to pH 7 using 1 M NaOH. An overnight culture of *Vibrio harveyi* MM77, a mutant strain which does not produce either AI-1 nor AI-2, was diluted 1:5,000 in a mixture of 90% (v/v) fresh AB medium and 10% (v/v) neutralized supernatant to a total volume of 200 μl per well. The negative control contained bacteria in fresh AB medium alone, while the positive control contained the bacteria, fresh AB medium and 10% (v/v) supernatant medium containing AI-2 of *V. harveyi* BB152 (AI-1–, AI-2+). The luminescence readings were performed in triplicate in white 96-well plates with an optic bottom (Nunc, Roskild, Denmark) using a plate reader (GENiosTECAN, NEOTEC Scientific Instrumentation Ltd. Camspec, Cambridge, UK) at 30°C. Luminescence measurements were recorded every 30 min in parallel with optical density (595 nm) readings. To avoid dissimilarities caused by differences in growth rates, the relative luminescence (RLU) was calculated. Briefly, the value of each reading was divided by the optical density values to normalize the luminescence value of each sample to its cell density. Fold induction above the non-specific luminescence background of the negative control was determined at the end of bacterial growth, after approximately 20 h of growth. The area under the curve (AUC) was calculated to better demonstrate the differential expression in RLU values by means of the sum of: the average of Y values/the average of X values (Aharoni et al., 2008; Soni et al., 2015).

AI-2 Effect on Biofilm Formation

To determine the effect of AI-2 on bundle formation as well as *tapA* expression, we used (S)-4,5-Dihydroxy-2,3-pentandione (DPD) (Omm Scientific, Inc, Dallas, TX, USA) which is the precursor for AI-2. Bacterial cells prepared as described above and were incubated in the presence of DPD in LB at 37°C at 50 rpm for 5 h. The cells were collected and visualized in a transmitted light microscope using DIC. Furthermore, a CLSM was used to visualize CFP expression using 458-nm argon laser (Oknin et al., 2015). For complementation tests, DPD was supplemented in LB medium to final concentration of 200 μM as an exogenous precursor for AI-2.

Statistical Analysis

The data obtained were analyzed statistically by means of ANOVA following *post hoc t*-test with Bonferroni correction using Microsoft Excel software. *P*-values less than 0.01 were considered significant.

RESULTS

Lactose Induces Biofilm Formation by *B. subtilis*

Initially, we found that addition of lactose to growth media such as LB or chemical defined agar (CDA) enhances biofilm

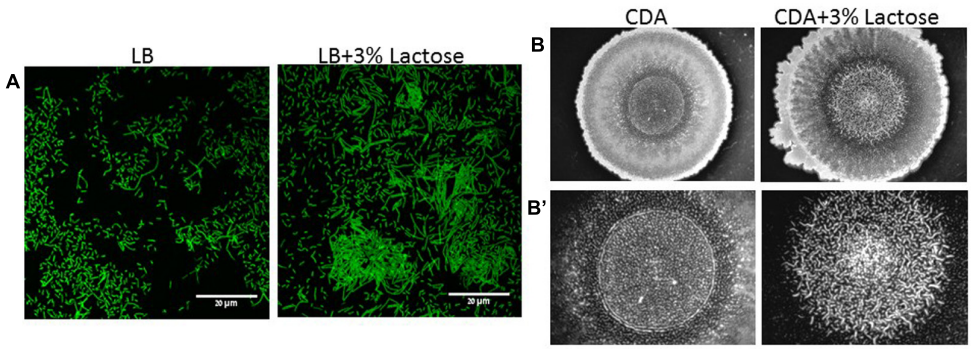

FIGURE 1 | Lactose induces biofilm formation by *B. subtilis*. **(A)** CLSM images of bundles formation. Overnight cultures of *B. subtilis* (YC161) were diluted into LB or LB supplemented with 3% lactose. Cultures were then incubated for 5 h at 37°C and 50 rpm. A sample from each culture was then analyzed using a confocal microscope. Images are representative of three biological repeats. **(B)** Colony biofilm was generated on chemical defined agar (CDA) and CDA supplemented with 3% lactose. **(B')** Zoomed images of the center of generated biofilm. The pictures were taken using a Zeiss Stemi 2000-C microscope with an axiocamERc 5s camera. Images are representative of four biological repeats.

formation by *B. subtilis*. As it can be seen in **Figure 1A**, a majority of *B. subtilis* (YC161) cells preferably generated long chains of cells attaching to each other to form a biofilm-related structure (bundle) in the presence of lactose. Similarly, lactose also induced colony type biofilm formation on CDA, as seen in the center of the colony (**Figure 1B**). The structure of the biofilm formed on the CDA with addition of lactose has higher structure complexity. Accordingly, the morphology of the biofilm in the presence of lactose is more developed and structured as seen in the center of the colony (**Figure 1B**). Subsequently, we tested whether the increase in biofilm formation in the presence of lactose is due to the increase in bacterial growth rate. The bacterial growth of *B. subtilis* was not affected by addition of lactose (Supplementary Figure S1). Therefore, we assume that the effect of lactose is specific to the biofilm formation.

Lactose Up-Regulates Expression of Genes Associated with Extracellular Matrix Production

In order to confirm our above findings and to determine if the bundles induced by lactose are biofilm related, we used genetically modified *B. subtilis* strains, which express fluorescent proteins under the control of important extracellular matrix related promoters. To examine the expression of *tapA* operon, we used the strain (YC189) which produces CFP under the control of the *tapA* promoter, whereas, the expression of *eps* operon was determined using strain (YC164) which produces GFP under the control of *eps* promoter (Chai et al., 2008). The amounts of the fluorescent proteins as well as their intensity represent the expression of the tested promoter in the different samples. As it is demonstrated in **Figure 2**, the expression of both operons was increased as a result of lactose introduction into the growth medium. Moreover, mutant strains which are defected in production of extracellular matrix showed deficiency in bundles formation in the presence of lactose (**Figure 3**).

Lactose Triggers AI-2 Production

Next, we decided to test whether lactose affects AI-2 production. Using *V. harveyi* MM77 as a reporter strain enables us to examine the effect of lactose on QS via the LuxS dependent pathway. Supernatants from *B. subtilis*, grown with or without lactose, were used for evaluating the amount of AI-2 secreted to the media. The RLU indicates the relative amount of AI-2 in the suspension; a peak of the relative bioluminescence was detected following 14 h in all tested samples which was found to be remarkably higher in the presence of lactose (in dose dependent manners; **Figure 4A**). Indeed, there was a significantly increase in the production of AI-2 by *B. subtilis* cells in the presence of all tested lactose concentrations especially in the presence of 3% of lactose (**Figure 4B**).

luxS is Essential for Biofilm Formation in the Presence of Lactose

We further investigated the connection between LuxS dependent QS and induction in biofilm formation. Thus, we used the YC189 strain (harboring the P_{tapA}-*cfp* transcriptional fusion) which was grown in the presence of different concentrations of DPD (precursor for AI-2). Interestingly, increasing concentrations of DPD stimulated the biofilm bundles formation as well as *tapA* expression (**Figure 5**). The induction in bundle formation and *tapA* expression seems to be in linear correlation with the concentration of DPD.

To further investigate a possible role of LuxS on biofilm formation in the presence of lactose, we tested the ability of *B. subtilis luxS* mutant to form bundle as well as pellicle and colony biofilm with or without lactose. As seen in **Figure 6**, the Δ*luxS* mutant is somehow defected in generating developed and structured pellicle and colony biofilm in the presence of lactose compared to the WT. Furthermore, Δ*luxS* mutant could not form biofilm bundles in the presence of lactose (**Figure 7**). Interestingly, addition of DPD restored at least partially the bundling phenotype of the Δ*luxS* mutant (**Figure 7**).

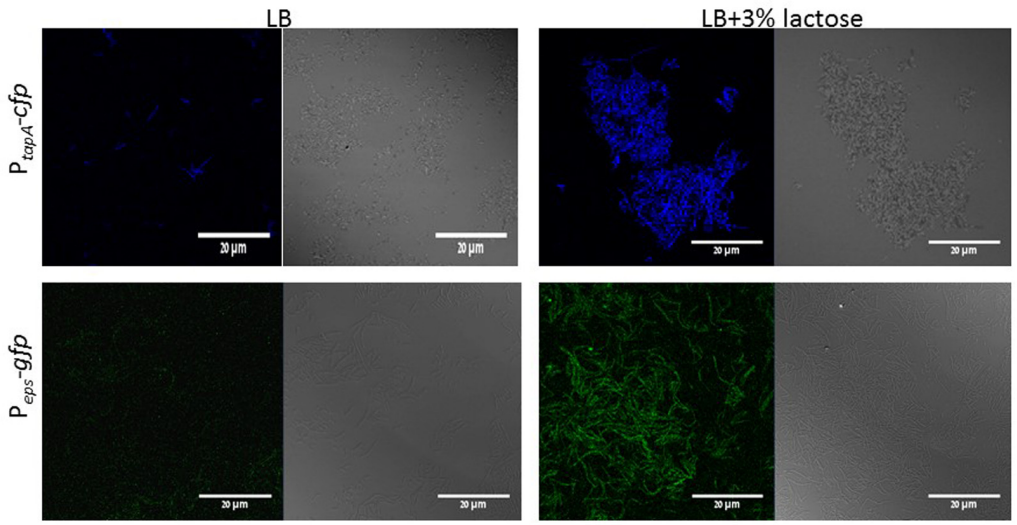

FIGURE 2 | Lactose triggers expression of *tapA* and *eps* operons in *B. subtilis*. WT cells harboring P_{tapA}-*cfp* (YC189) or P_{eps}-*gfp* (YC164) were grown in LB or LB supplemented with 3% lactose. Cultures were then incubated for 5 h at 37°C and 50 rpm. A sample from each culture was then analyzed using a confocal microscope. The right picture are the bacteria taken using Nomarski differential interference contrast (DIC), at 40× magnification and the left pictures are the fluorescent bacteria. The top panel shows the expression of CFP and the bottom panel expression of GFP. Images are representative of five biological repeats.

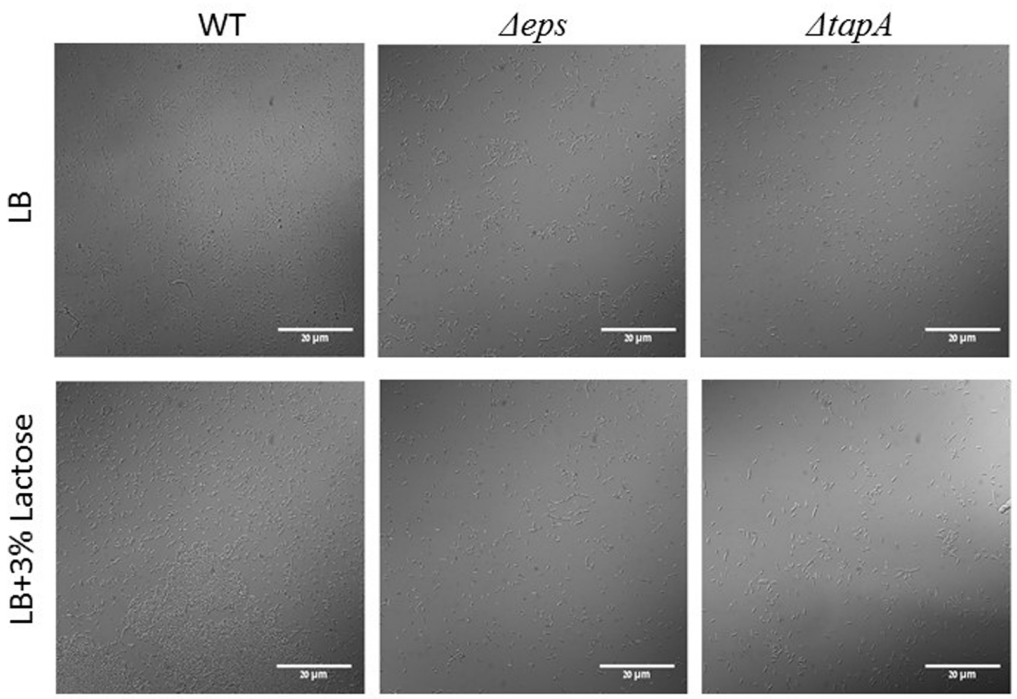

FIGURE 3 | The *epsA-O* and *tapA-sipW-tasA* operons are essential for bundle formation in the presence of lactose. The WT, Δ*epsH* and Δ*tasA* cells of *B. subtilis* were diluted into LB or LB supplemented with lactose. Cultures were incubated for 5 h at 37°C and 50 rpm. A sample from each culture was analyzed using a confocal microscope. Images are representative of two biological repeats.

DISCUSSION

Our results show that lactose triggers bundle formation as well as formation of colony type biofilm by *B. subtilis*. This result falls in line with our previous study which showed that lactose enhances biofilm formation by *Streptococcus mutans* (Assaf et al., 2015). Expression of *epsA-O* and *tapA* operons, which are responsible for biofilm matrix

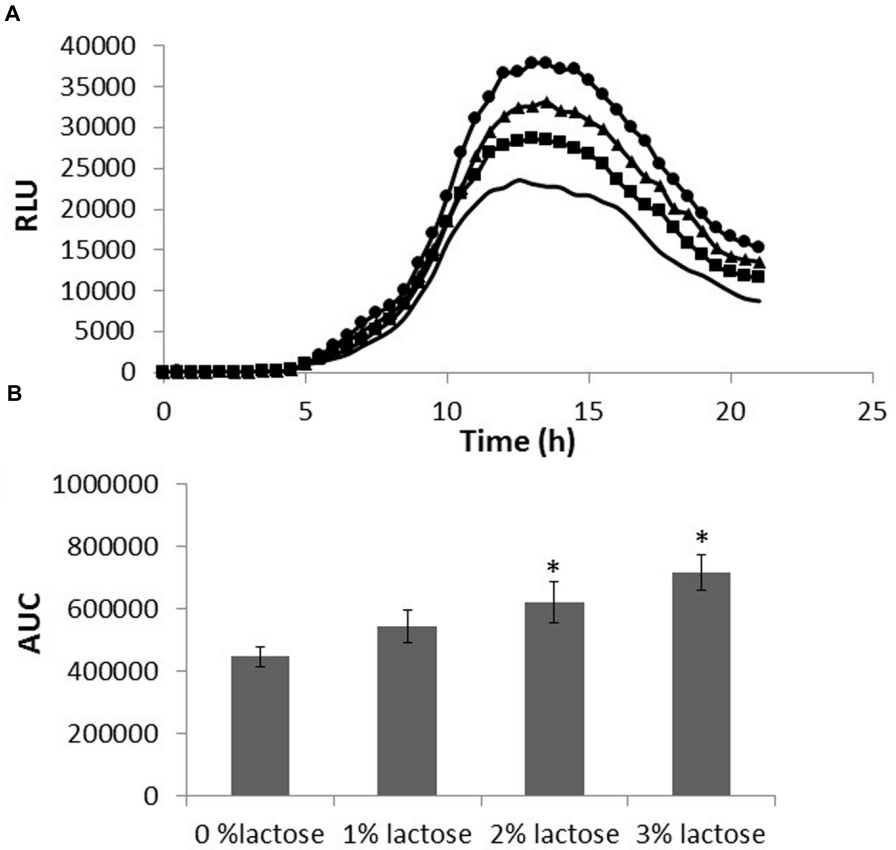

FIGURE 4 | Lactose induces AI-2 secretion in *B. subtilis*. *B. subtilis* cells were grown in LB supplemented with 0–3% lactose. Cultures were then incubated for 5 h at 37°C and 50 rpm. A supernatant sample from each culture was taken and incubated with *Vibrio harveyi* MM77. Optical density and luminescence data were recorded every 30 min. **(A)** Bioluminescence kinetics using relative luminescence (RLU). The data are displayed as a mean value of results from two biological repeats each performed as triplicate. (-LB, ■ 1% lactose, ▲2% lactose, ●3% lactose). **(B)** The area under the curve (AUC). The data were analyzed by means of ANOVA following *post hoc t*-test with Bonferroni correction. **P*-value < 0.01 compared to control.

production, were notably increased when lactose was added to the LB medium (**Figure 2**). Interestingly, induction in expression of both operons is correlated with biofilm bundles formation by *B. subtilis* cells. Bundle formation is one of the first stages in biofilm development (Branda et al., 2001). Moreover, investigation of the mutant strains for these operons shows absence of the bundling phenotype as a response to lactose (**Figure 3**). This result indicates that lactose induce biofilm formation depends on *tapA* and *epsA-O* expression.

In recent years, there has been an increasing interest in the quorum-sensing signaling molecules related to food spoilage. Various signaling compounds associated with QS, such as AI-2, have been detected in different food systems such as milk (Pinto et al., 2007). Furthermore, studies have shown that QS is important for social behavior of *B. subtilis* and other bacteria (Lombardía et al., 2006). Using *V. harveyi* as a reporter strain for bioluminescence, we were able to track the level of produced AI-2 molecules. We observed an increase in the AI-2 production as a response to lactose in dose dependent manners (**Figure 4**). It has been shown previously that simple dietary sugars can affect QS, specifically production of AI-2 by *Klebsiella pneumoniae* (Zhu et al., 2012). In our study, the cell density of all tested samples was the same at the sampling time, consequently, changes in the AI-2 production is apparently not related to the cell density but to the metabolic state of the bacteria. Thus, our results support previous studies that showed that AI-2-dependent signaling is a reflection of metabolic state of the cell and environmental factors and not cell density (Bassler, 1999; Beeston and Surette, 2002). Previous studies also suggested that activation of QS through LuxS can be regulated in response to sugar metabolism by cyclic AMP receptor protein molecules (Lyell et al., 2013). In *B. subtilis* cells, lactose may affect the energetic metabolic balance in the cell, and through second messengers such as cyclic AMP, or CCP can lead to expression of QS genes such as *luxS*.

The main finding of this study is the apparent link between lactose induced biofilm formation and activation of QS system through increased production of AI-2 molecules in *B. subtilis*. Addition of synthetic precursor for AI-2, DPD, to the media resulted in enhanced bundle formation as well as up-regulation

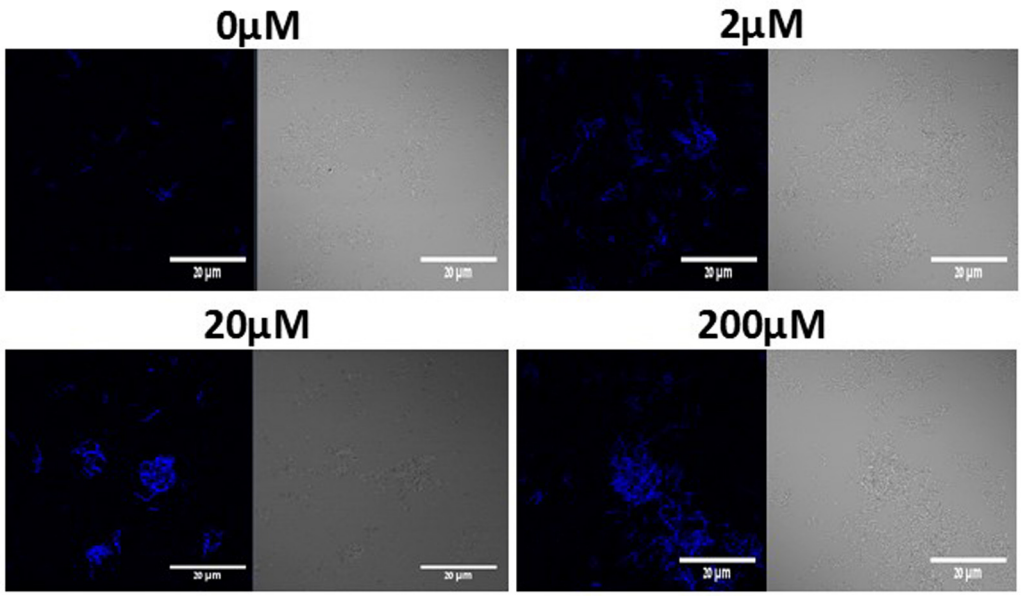

FIGURE 5 | DPD triggers expression of *tapA* operon and bundle formation in *B. subtilis.* WT cells harboring P_{tapA}-*cfp* were grown in LB or LB supplemented with 0–200 μM DPD. Cultures were then incubated for 5 h at 37°C and 50 rpm. A sample from each culture was then analyzed using a confocal microscope. The right pictures are the bacteria taken using DIC, at 40× magnification and the left pictures are the fluorescent bacteria. Images are representative of three biological repeats.

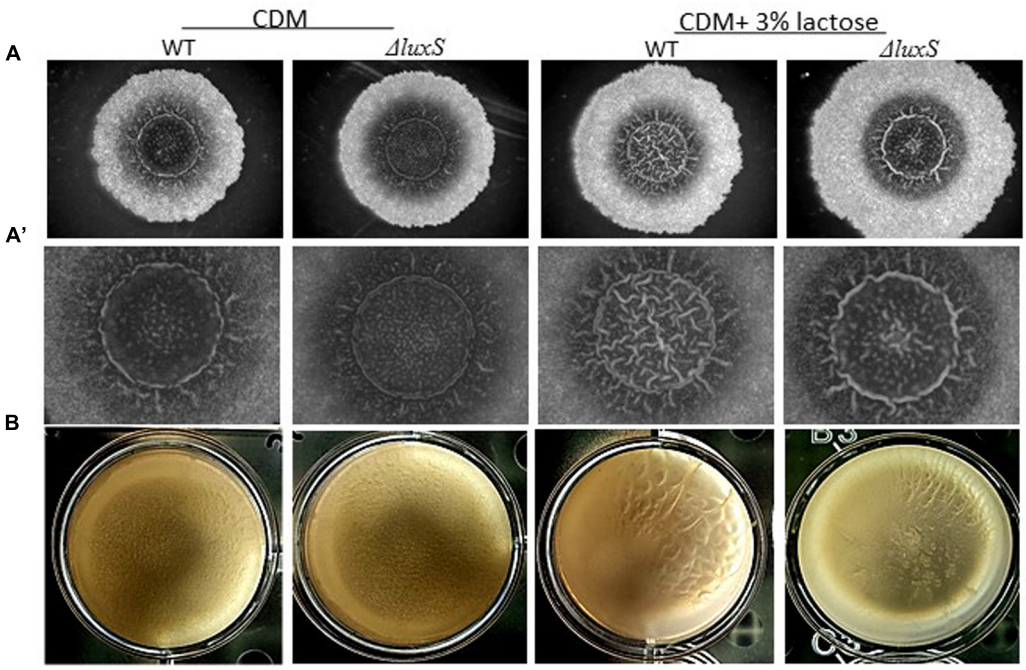

FIGURE 6 | *luxS* is essential for colony biofilm formation in the presence of lactose. (A) WT and Δ*luxS* cells were used for colony biofilm formation. Biofilms were generated on chemically defined agar (CDA) and CDA supplemented with 3% lactose. **(A')** are zoomed images of the center of generated biofilm. The pictures were taken using a Zeiss Stemi 2000-C microscope with an axiocamERc 5s camera. Images are representative of four biological repeats. **(B)** WT and Δ*luxS* cells were used for pellicle biofilm formation. Biofilms were generated in chemical defined medium (CDM) and CDM supplemented with 3% lactose. Pictures were taken using Sumsung galaxy digital camera. Images are representative of two biological repeats.

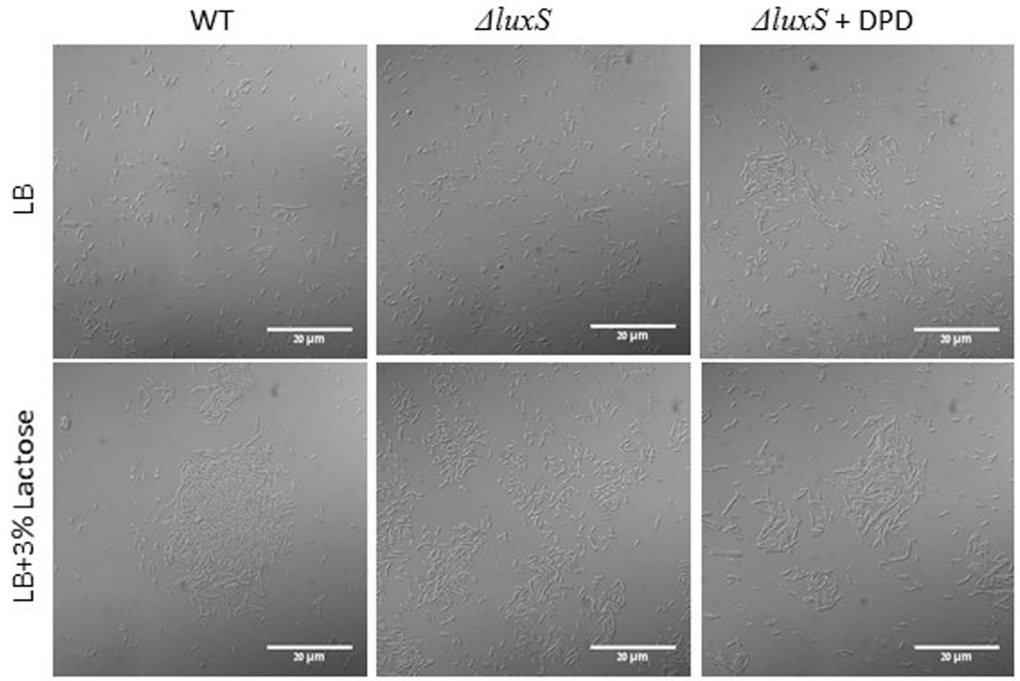

FIGURE 7 | LuxS is essential for biofilm bundle formation in the presence of lactose. WT and Δ*luxS* cells were diluted into LB or LB supplemented with lactose. For complementation tests, 200 μM DPD was added to suspensions containing mutant cells. Cultures were then incubated for 5 h at 37°C at 50 rpm. Samples of each culture were then analyzed using a confocal microscope. Images are representative of four biological repeats.

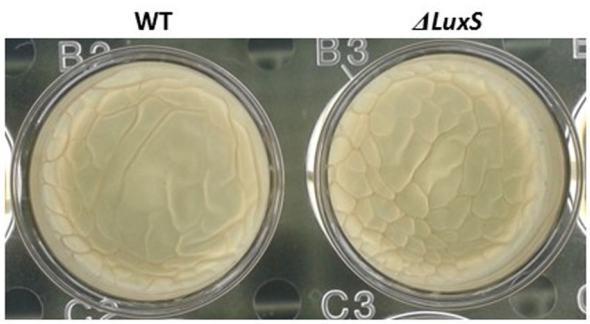

FIGURE 8 | Pellicle formation by *B. subtilis* in LBGM is not LuxS dependent. WT and Δ*luxS* cells were used for pellicle formation in LBGM. The pictures were taken using a Zeiss Stemi 2000-C microscope with an axiocamERc 5s camera. Images are representative of two biological repeats.

of *tapA* expression (**Figure 5**). Similarly, the direct effect of AI-2 molecules on EPS biosynthesis has been observed previously in *Vibrio cholera*e where the AI-2 molecules up-regulated expression of the EPS biosynthesis genes (Hammer and Bassler, 2003). According to our results, examination of biofilm formation in CDM of the *B. subtilis* Δ*luxS* mutant resulted in deficiency of biofilm formation (bundle, and colony types) (**Figures 6A** and **7**). These results suggested that QS via LuxS cascade plays an important role in biofilm formation in the presence of lactose. This is consistent with previous research which showed that LuxS is important for *B. subtilis* social behavior (motility and biofilm formation) (Lombardía et al., 2006). Another study showed that blocking the AI-2 pathway, using an AI-2 analog, inhibited biofilm formation by *B. subtilis* (Ren et al., 2002). Similar results were found for *Hafnia alvei*, a food-related bacterium that can be found in dairy products. QS in *H. alvei* is required for differentiation of individual cells into a complex multicellular structure of biofilm (Souza Viana et al., 2009).

Interestingly, we observed that the *luxS* mutant strain could form pellicle in biofilm promoting medium LBGM (**Figure 8**). Although, a pellicle formation in LBGM appears to be not LuxS dependent, it seems that in CDM there is a slight induction in pellicle formation in response to lactose (**Figure 6B**). As it was shown recently (Shemesh and Chai, 2013), glycerol and manganese activate KinD-Spo0A pathway for matrix production. In case of lactose, it seems that enhanced production of AI-2 affects not directly on the biofilm formation cascade. This may explain the differences found between phenotypes in CDM supplemented with lactose and in LBGM. Activation of biofilm formation via QS system might be an additional regulatory mechanism which enables fine tuning of the biofilm formation pathway that has been previously described (Shemesh and Chai, 2013).

The LuxS system possesses an inherent metabolic function in the activated methyl cycle; phenotypic defects in *luxS* mutants may not strictly be attributed to AI-2 signaling but possibly to metabolic disturbances. For instance, biofilm defects in a

Lactobacillus rhamnosus luxS mutant are not restored by AI-2 molecules but rather by the addition of cysteine, indicating a sole metabolic role of LuxS (Lebeer et al., 2007). In order to test whether the deficiency of biofilm formation in the presence of lactose in the mutant strain is due to AI-2 signal molecules or due to metabolic reason, we used DPD for complementation tests. It was shown previously that the synthetic AI-2 precursor (DPD) has the ability for specific AI-2 complementation during biofilm formation by *Streptococcus intermedius* (Ahmed et al., 2008). In the complementation test, we observed restoration of the biofilm phenotype. The Δ*luxS* mutant showed ability for increased bundle formation in media supplemented with lactose and 200 μM of DPD (**Figure 7**), indicating that the abolished biofilm formation is mostly connected to AI-2 and not to LuxS enzyme function.

In overall, results of the present study suggest that QS via LuxS system plays an important role in biofilm formation induced by lactose in *B. subtilis*. As lactose affects activation of LuxS system, it is likely related to activation of Spo0A which leads to biofilm formation through a known pathway of up-regulation of the extracellular matrix operons. Moreover, Spo0A has been shown to be a negative regulator of LuxS system (Lombardía et al., 2006). Additional research on lactose in association with QS will further elucidate the role of QS in biofilm formation of *Bacilli* and the effect of this dairy component on biofilm related gene expression.

AUTHOR CONTRIBUTIONS

DD-A together with MS planned the experiments and wrote the original manuscript. DD-A performed the experiments described in the manuscript. DS and YC assisted in planning biofilm experiments as well as revised the manuscript critically for important intellectual content. DD-A, DS, and MS integrated all of the data throughout the study and crafted the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.01517

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Campylobacter jejuni biofilms contain extracellular DNA and are sensitive to DNase I treatment

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Biofilms make an important contribution to survival and transmission of bacterial pathogens in the food chain. The human pathogen *Campylobacter jejuni* is known to form biofilms *in vitro* in food chain-relevant conditions, but the exact roles and composition of the extracellular matrix are still not clear. Extracellular DNA has been found in many bacterial biofilms and can be a major component of the extracellular matrix. Here we show that extracellular DNA is also an important component of the *C. jejuni* biofilm when attached to stainless steel surfaces, in aerobic conditions and on conditioned surfaces. Degradation of extracellular DNA by exogenous addition of DNase I led to rapid biofilm removal, without loss of *C. jejuni* viability. Following treatment of a surface with DNase I, *C. jejuni* was unable to re-establish a biofilm population within 48 h. Similar results were obtained by digesting extracellular DNA with restriction enzymes, suggesting the need for high molecular weight DNA. Addition of *C. jejuni* genomic DNA containing an antibiotic resistance marker resulted in transfer of the antibiotic resistance marker to susceptible cells in the biofilm, presumably by natural transformation. Taken together, this suggest that eDNA is not only an important component of *C. jejuni* biofilms and subsequent food chain survival of *C. jejuni*, but may also contribute to the spread of antimicrobial resistance in *C. jejuni*. The degradation of extracellular DNA with enzymes such as DNase I is a rapid method to remove *C. jejuni* biofilms, and is likely to potentiate the activity of antimicrobial treatments and thus synergistically aid disinfection treatments.

Keywords: ***Campylobacter jejuni*, biofilm, food safety, extracellular matrix, extracellular DNA, antibiotic resistance**

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Introduction

Campylobacter jejuni is the most common cause of bacterial foodborne infection within the UK (Nichols et al., 2012). Its success as foodborne pathogen contrasts with its fastidious nature, as it requires specific atmospheric conditions, nutrient-rich growth medium and has a narrow temperature range (between 35 and 45°C) for growth. Several mechanisms for survival in the food chain have been proposed, including the ability of *C. jejuni* to enter a viable but none culturable (VBNC) state (Rollins and Colwell, 1986), as well as formation of *de novo* biofilms or integration into existing (multispecies) biofilms (Teh et al., 2014). Biofilms are defined as surface attached populations, either single or multiple species, which are surrounded by a self-produced extracellular matrix (Donlan, 2002). The extracellular matrix differs depending on the species within the biofilm but typically comprises of DNA, proteins and polysaccharides (Branda et al., 2005).

The extracellular matrix is an essential component of bacterial biofilms, and usually accounts for more than 90% of the dry mass of a biofilm (Flemming and Wingender, 2010). It allows cells to remain hydrated and metabolically active by trapping nutrients and liquid near the bacterial cells. It also reduces access of larger molecules such as antimicrobials (Mulcahy et al., 2008; Billings et al., 2013), leading to increased bacterial persistence, and is structurally important, maintaining the shape of the biofilm and ensuring the cohesion of the biofilm (Sutherland, 2001). Extracellular DNA (eDNA) appears to have a structural role in the biofilms of many different species, including *Pseudomonas aeruginosa* (Chiang et al., 2013), *Staphylococcus aureus* (Mann et al., 2009), *Listeria monocytogenes* (Harmsen et al., 2010), and *Escherichia coli* (Zhao et al., 2013).

Recent studies have shown that eDNA is important for biofilm establishment and maintenance by *C. jejuni* strain 81–176 in laboratory conditions (Bae et al., 2014; Svensson et al., 2014), but this has not yet been studied in the context of the conditions encountered by *C. jejuni* in the processing environment. Previous studies have shown that food chain relevant conditions such as atmospheric oxygen levels (Reuter et al., 2010), reduced temperatures (Buswell et al., 1998) and surface soiling (Brown et al., 2014) all increase *C. jejuni* biofilm formation, and as such may also influence the composition of the *C. jejuni* biofilm, necessitating the study of *C. jejuni* biofilms in these conditions.

The aim of this study was to further investigate the role of eDNA within the *C. jejuni* biofilm, with particular reference to its role in food chain relevant environments. Here we present evidence that eDNA is also present in biofilms of *C. jejuni* reference strains NCTC 11168 and 81116 when incubated in aerobic conditions and on food chain relevant surfaces such as stainless steel. Degradation of eDNA by DNase I leads to a rapid loss of biofilm structure, releasing cells into the planktonic phase. Treatment of surfaces with DNase I also inhibits *de novo* biofilm formation, either due to re-growth from single, attached, cells or from *de novo* attachment of *C. jejuni* cells. Addition of *C. jejuni* DNA to biofilms results in the transfer of genetic markers, which can contribute to spread of antimicrobial resistance in *C. jejuni* populations.

Materials and Methods

C. jejuni Strains and Growth Conditions

C. jejuni strains NCTC 11168 (Parkhill et al., 2000), its derivative expressing a green fluorescent protein and chloramphenicol resistance marker (*C. jejuni* NCTC 11168 *cj0046::gfp*-Cm^R) (Brown et al., 2015), strain 81116 (Pearson et al., 2007) and all microaerobic biofilm incubations were routinely cultured in a MACS-MG-1000 controlled atmosphere cabinet (Don Whitley Scientific) under microaerobic conditions (85% N_2, 5% O_2, 10% CO_2) at 37°C. For growth on plates, strains were either grown on Brucella agar or BAB with Skirrow antibiotic supplement (10 μg/ml vancomycin, 5 μg/ml trimethoprim, 2.5 IU polymyxin-B). Broth culture was carried out in Brucella broth (Becton & Dickinson).

Campylobacter Growth for Biofilm Assay

Frozen 50 μl single-use glycerol stocks were thawed, inoculated onto Skirrow plates and grown overnight at 37°C in microaerobic conditions (5% O_2, 10% CO_2 and 85% N_2). Cells from the Skirrow plate were used to inoculate Brucella broth then grown overnight as a shaking culture (37°C, microaerobic conditions). Following overnight growth, cell cultures were adjusted to an A_{600} of 0.05 in Brucella medium or Brucella medium supplemented with 5% v/v chicken juice. To allow biofilm formation, 1 ml of this solution was added to either a sterile borosilicate glass test tube (Corning) or 3 ml to a six-well polystyrene tissue culture plate (Corning) containing a sterile stainless steel coupon (Stainless steel type 1.4301 according to EN 10088-1, with a Type 2B finish according to EN 10088-2). In each biofilm assay a test tube containing sterile Brucella medium was incubated alongside the *C. jejuni* containing tubes to ensure sterility was maintained and, following crystal violet staining, to quantification of staining levels where biofilm was not present. Tubes were incubated at 37°C in atmospheric air conditions using an Innova 4230 (New Brunswick Scientific) incubator at 37°C. Unless otherwise stated all biofilms were formed in aerobic conditions at 37°C for 48 h before staining procedures were carried out. For each assay a microaerobic biofilm control was also undertaken, to ensure that oxygen availability does not have a major effect on results and to allow comparison with previous studies (Reuter et al., 2010; Brown et al., 2013, 2014, 2015) . This sample was prepared in exactly the same way as the aerobic biofilm cultures but test tubes were placed back in the 37°C microaerobic incubator for all static incubations.

Preparation of Chicken Juice

Chicken juice was prepared as described previously (Brown et al., 2013, 2014). Briefly, frozen commercially available whole chickens were purchased from UK supermarkets before thawing at room temperature. Exudate was collected, centrifuged to remove debris and sterilized by using a 0.2 μm sterile polyethersulfone (PES) syringe filter (Millipore) before aliquotting and storage at −20°C until use. Chicken juice was diluted v/v in Brucella medium for use in biofilm assays.

Enzyme Treatment of *C. jejuni* Biofilms

For DNase I treatments, unless otherwise stated, a volume of 4 μl DNase I enzyme (Fermentas), giving a final concentration within the biofilm of 4 U/ml v/v and 4 μl of DNase I buffer (Fermentas) were added to each test tube, along with 1 ml of diluted cell suspension at either the start of the static incubation or after 12, 24, 36, or 48 h of static incubation. Following treatment, static cultures were placed back in 37°C, aerobic conditions to complete the 48 h incubation before staining with crystal violet to allow biofilm quantification. For restriction enzyme digest of biofilms 4 μl of 10 U/μl *Bam*HI, *Blp*I, *Hae*III, *Hin*dIII, *Msc*I or *Rsa*I, (NEB), or DNase I (Fermentas), or RNase (QIAGEN) were added to test tubes containing diluted *C. jejuni* suspension prior to static incubation and then incubated at 37°C for 48 h in aerobic conditions. Equal volumes (4 μl) of the buffers and bovine serum albumin were also added if recommended by the manufacturers. For the assays assessing the time required for DNase I activity,

biofilms were allowed to form for 48 h before addition of 4 U/ml v/v DNase I enzyme (1 U/μl, Fermentas) and 4 μl of DNase I buffer to the samples, followed by incubation for up to 2 h. During the incubation with enzyme the samples were placed in 37°C, aerobic conditions. All samples were subsequently stained with crystal violet.

For assessment of biofilm regrowth, biofilms were allowed to establish for 48 h followed by a 15 min incubation with DNase I. Tubes were then washed twice with sterile PBS followed by addition of either an equal volume of bacterial culture with an A_{600} of 0.05, or sterile Brucella medium, followed by a further 48 h incubation at 37°C in aerobic conditions. All samples were subsequently stained with crystal violet. In order to ensure consistency between control and treatment samples all tubes were manipulated in exactly the same way, being removed and placed back in the same incubation conditions during each enzyme addition. Heat inactivated DNase I was prepared by incubating an aliquot of DNase I and its buffer at 95°C for 10 min and allowing to cool before addition to the biofilm cultures.

Visualization of Extracellular DNA from Shaking Cultures and Biofilms

Following incubation to allow biofilm formation in both aerobic and microaerobic conditions, the supernatant was removed and the tubes were rinsed once with sterile PBS to remove loosely attached bacterial populations. After rinsing and removal of the rinse suspension a second 1 ml volume of sterile PBS was added to each test tube and a sterile cotton wool swab was used to gently swab to walls of the test tube, releasing the biofilm from the walls of the test tube and in to suspension. The resuspended biofilm (PBS containing the loosened biofilm cells) and supernatant (liquid initially contained within the test tube) from several biofilm cultures were collected and pooled before diluting to a A_{600} of 0.3. Aliquots were mixed with gel loading buffer (NEB) and added to a 0.9% agarose gel and run at 100 V for 45 min in 0.5% TBE buffer. A 1 kb ladder (NEB) was used for size comparison. Following electrophoresis, nucleic acids were stained using ethidium bromide, and DNA was visualized using a GelVue UV light and documented using a U:Genius gel documentation system (Syngene). The amount of eDNA in planktonic and biofilm fractions was quantified by comparing the intensity of the DNA bands after UV illumination and comparison with the 3 kb marker fragment (125 ng), using ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997–2014). Quantification was based on three independent experiments.

Restriction Digest of *C. jejuni* Genomic DNA

A 1 μl volume of restriction enzyme (*Bam*HI, *Blp*I, *Hae*III, *Hin*dIII, *Msc*I, or *Rsa*I, all supplied by NEB), or DNase I (1U/μl, Fermentas), or RNase (QIAGEN) was added to a mixture containing ∼500 ng of *C. jejuni* NCTC 11168 or 81116 genomic DNA, prepared using a commercial kit (QIAGEN) following manufactures guidelines, 1 μl of 10× enzyme buffer (if required), 1 μl of 1 mg/ml BSA (if required) and molecular grade water to a final volume of 10 μl. Samples were incubated for 60 min in a 37°C water bath to allow digestion of the genomic DNA. DNA was visualized using a GelVue UV light and documented using a U:Genius gel documentation system (Syngene).

Assessment of Natural Transformation within the Biofilm

Genomic DNA was extracted from the *C. jejuni* NCTC 11168 *cj0046::gfp*−Cm^R mutant (Brown et al., 2015) using a commercial kit (QIAGEN), following manufacturers guidelines. DNA concentration was calculated after the final elution and stored at −20°C until use. The standard 48 h static biofilm incubation was carried out, using duplicate test tubes for all conditions. A total of 2 μg genomic DNA was added to test tubes either prior to the start of biofilm incubation, or following 24 h of static incubation. Following a total of 48 h of incubation one test tube of each condition was stained using crystal violet and the second tube washed once with 1 ml PBS and the biofilm population released by swabbing with a sterile cotton bud. Both the supernatant and released biofilm population were retained for viability assessment.

Crystal Violet Staining

Cell suspensions were removed from the test tubes before washing with distilled water before drying at 60°C for 30 min. A 1 ml of 1% w/v crystal violet solution was added and tubes were further incubated on a rocker at room temperature for 30 min. After this incubation, the non-bound dye was removed from the tubes by thorough washing in water followed by drying at 37°C. Bound crystal violet was dissolved by adding 20% acetone/80% ethanol and incubating on a rocking platform for 15 min at room temperature. The resulting dissolved dye was measured at a wavelength of 590 nm using a Biomate 5 spectrophotometer (Thermo Scientific).

2,3,5 Triphenyltetrazolium Chloride (TTC) Staining

This method was carried out as previously described (Brown et al., 2013, 2014). Following a 48 h incubation to allow biofilm formation, cell suspensions were removed and test tubes were washed twice with 1 ml of sterile PBS. A 1.2 ml volume of Brucella broth supplemented with 0.05% w/v TTC was then added to each test tube before further incubation at 37°C in microaerobic conditions for 72 h. Following secondary incubation, the TTC solution was removed and the test tubes were air dried. Bound TTC dye was dissolved as above using 20% acetone/80% ethanol and the A_{500} of the solution measured.

Assessment of Cell Viability by Culture

To determine the number of viable cells, the planktonic fraction, or released biofilm population was 10-fold serially diluted eight times in PBS and 5 μl of each dilution spotted on Brucella agar plates or (for assessment of natural transformation) Brucella agar containing 10 μl/ml chloramphenicol. After 2 days of growth at 37°C in microaerobic conditions, the dilution resulting in two or more colonies was recorded. Cell viability in biofilm assays was assessed upon initial addition of cultures into static culture and following static incubation, prior to crystal violet staining and, where necessary, following the 72 h TTC incubation.

Statistics

Statistical analysis was carried out using GraphPad Prism software. At least three biological replicates (each with three technical replicates unless otherwise stated) were used to calculate mean and standard deviation. Significance was measured using either a Mann–Whitney test (biofilm formation) or ANOVA (DNA yield).

Results

Extracellular DNA Is Present within the *C. jejuni* Biofilm and during Both Aerobic and Microaerobic Incubation

Biofilms of *C. jejuni* NCTC 11168 and 81116 were generated and used for the investigation of eDNA. Separation of nucleic acids on agarose gels showed the presence of extracellular DNA in both the biofilm and planktonic fractions, independent of whether the biofilm samples were incubated in aerobic or microaerobic conditions (**Figure 1**). Within the biofilm samples, there was no distinguishable difference between the eDNA bands produced by *C. jejuni* NCTC 11168 and 81116, although in the supernatant, *C. jejuni* NCTC 11168 cultures contained less DNA than *C. jejuni* 81116. The atmospheric conditions used for the incubation did not seem to affect eDNA levels, although as previously reported, total biofilm mass increased during aerobic biofilm incubation (Reuter et al., 2010; Brown et al., 2014).

Addition of DNase I Leads to Rapid Reduction of Biofilm Levels and Prevents Formation of New Biofilms

To assess whether the role of eDNA differs between different stages of biofilm maturity in *C. jejuni*, DNase I was added at 12 h intervals over the total of a 48 h incubation in aerobic conditions. There was no detectable *C. jejuni* biofilm after incubation with 4 U/ml DNase I, regardless of the age of the biofilm (**Figure 2A**), indicating that eDNA is an important extracellular matrix component during both initial attachment and maturation. We next assessed how rapidly degradation occurs by treating biofilms grown for 48 h with DNase I followed by detection with crystal violet at timed intervals over a two h period. Following only a 5 min incubation with DNase I, there was no detectable staining on the glass surface, and A_{590} values were indistinguishable from the negative control containing Brucella medium only (**Figure 2B**). Levels of staining did not reduce further at later time points, suggesting that a 5 min treatment can achieve degradation of the eDNA in the *C. jejuni* biofilm and results in a reduction of biofilm levels below the detection limit (Tresse et al., 2006).

Finally, the concentration of DNase I required to degrade the biofilm was also investigated. Addition of DNase I at concentrations ranging from 0.01 to 5 U/ml were significantly reduced crystal violet staining, and there was no statistically significant difference between DNase I treated test tubes and the negative control tube containing Brucella medium only (**Figure 2C**). It is interesting to note that DNase I treatment had no impact on cell viability, and most likely only degrades the biofilm matrix, resulting in the release of attached cells into suspension. Biofilms incubated with DNase I in microaerobic conditions also showed the same pattern, confirming that the effects observed were not a response to atmospheric condition, but DNase I treatment. Inactivation of DNase I by heat treatment removed its ability to affect C. *jejuni* biofilms (Figure S1), but did not inhibit growth of *C. jejuni*.

The long-term effects of DNase I-mediated degradation of *C. jejuni* biofilms from abiotic surfaces was assessed by adding fresh *C. jejuni* NCTC 11168 culture to DNase I-treated and washed borosilicate test tubes previously containing a *C. jejuni* biofilm. There was no detectable *C. jejuni* biofilm in either the tubes with added Brucella medium or the tubes with added

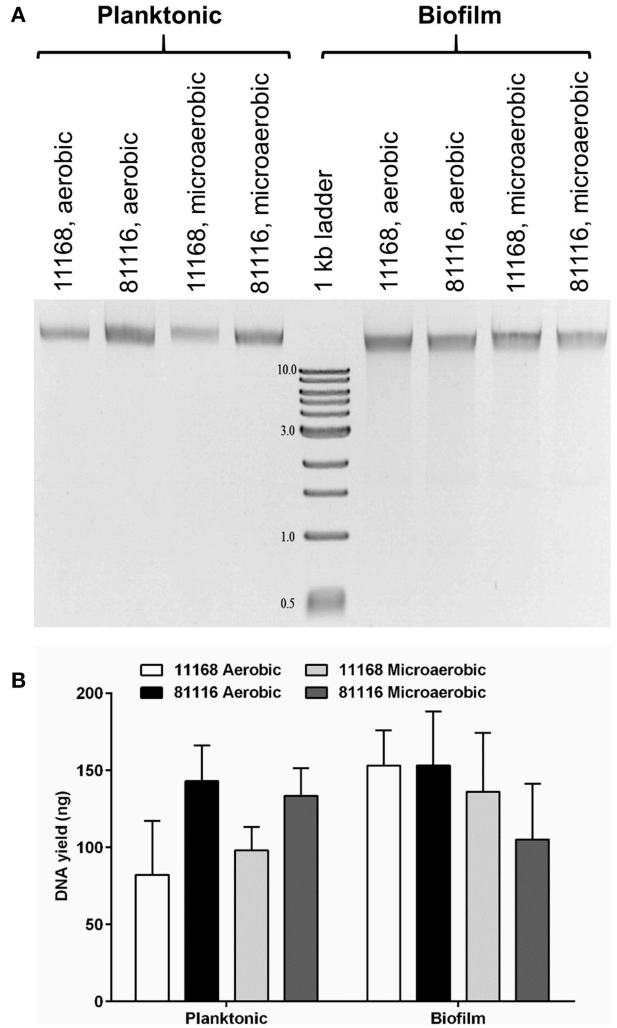

FIGURE 1 | Extracellular DNA is present in *C. jejuni* biofilms incubated in either aerobic and microaerobic conditions. (A) A representative agarose gel showing both planktonic and adhered cells recovered from 48 h static cultures and loaded directly on a 0.9% agarose gel. Following gel electrophoresis, DNA was detected using ethidium bromide and UV light. Numbers next to 1 kb marker bands indicate the size of the fragments in kilobases. **(B)** DNA was quantified ($n = 3$) by comparing the intensity of the DNA bands with the 3 kb fragment (125 ng) using ImageJ. Error bars show standard deviation. There is no statistically significant difference in DNA yield between the strains or culture conditions as determined using ANOVA.

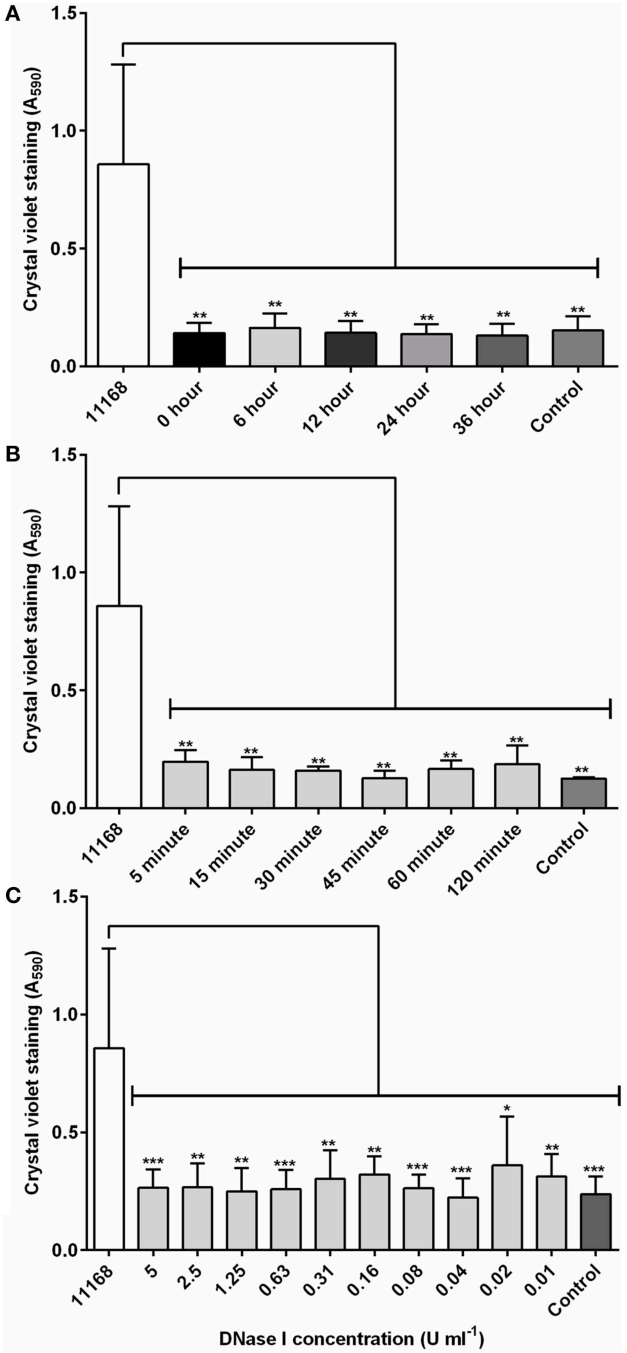

FIGURE 2 | DNase I is able to rapidly degrade *C. jejuni* NCTC 11168 biofilms. (A) DNase I (4 units/ml) was added at defined intervals to aerobically incubated NCTC 11168 cultures over a 48 h static incubation and biofilm degradation assessed by crystal violet staining. **(B)** Following a 48 h static incubation to allow biofilm formation, DNase I was added to biofilms for between 5 and 120 min before biofilm degradation was assessed. **(C)** The concentration of DNase I required for biofilm control was also assessed using DNase I concentrations of between 0.01 and 5 U/ml. In each graph, "11168" represents an untreated biofilm culture of *C. jejuni* NCTC 11168 and "control" represents a tube containing sterile Brucella medium only. Error bars show standard deviation. Statistically significant results, as determined using the Mann–Whitney U test, are indicated using an asterisk (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

C. jejuni in either aerobic or microaerobic conditions (**Figure 3**). This suggests that DNase I treatment is not only a rapid method of degrading *C. jejuni* NCTC 1168 biofilms but also prevents biofilm regrowth.

Restriction Digestion of eDNA Leads to Reduced Levels of *C. jejuni* Biofilm

The eDNA found within the *C. jejuni* NCTC 11168 and 81116 biofilms is of high molecular weight (**Figure 1**), and we speculated that biofilm formation requires high molecular weight nucleic acids, rather than simply the presence of any nucleic acids. Six restriction enzymes were selected, which are predicted to digest *C. jejuni* genomic DNA to a range of fragment sizes (**Figures 4C,D**), and these enzymes were assessed for their ability to degrade 48 h old *C. jejuni* biofilms. With *C. jejuni* NCTC 11168 there was a significant reduction in crystal violet staining for all six restriction enzymes tested, with little variation between enzyme treatment and the negative control (**Figure 4A**). Although the same trend was observed with *C. jejuni* 81116 biofilms, this was not statistically significant except for DNase I treatment (**Figure 4B**). This was consistent with the reduced digestion observed with *C. jejuni* 81116 genomic DNA, producing fragments of higher molecular weight than those obtained by digestion of *C. jejuni* NCTC 11168 genomic DNA (**Figure 4D**).

DNase I Treatment is Also Effective on Food Chain Relevant Surfaces

The effect of DNase I treatment on *C. jejuni* biofilms formed on food-relevant surfaces such as stainless steel (Somers et al., 1994; Thormar and Hilmarsson, 2010), and on heavily soiled surfaces (De Cesare et al., 2003; Brown et al., 2014) was assessed using *C. jejuni* NCTC 11168 biofilms formed on sterile stainless steel coupons. There was a significant reduction of crystal violet staining following DNase I treatment (**Figure 5A**). Crystal violet staining of the coupons showed no detectable biofilm following static aerobic incubation in the presence of DNase I, however significant levels of biofilm formation were observed when DNase I was not present (**Figure 5A**). In order to mimic environments where heavy soiling occurs, *C. jejuni* NCTC 11168 cultures were incubated statically in Brucella medium containing 5% v/v chicken juice. Chicken juice is a complex, undefined exudate obtained from defrosted whole chickens (Birk et al., 2004, 2006) and has a high protein and lipid content, and its presence results in increased biofilm formation due to its ability to condition abiotic surfaces (Brown et al., 2014). DNase I treatment of biofilms formed in the presence of 5% v/v chicken juice did result in a significant reduction of staining compared to untreated biofilms, although there was some residual staining, suggesting that on heavily soiled surfaces DNase I treatment does not provide the same level of biofilm degradation as observed in culture medium only (**Figure 5B**).

Biofilms Allow Genetic Transfer of Antibiotic Resistance to *C. jejuni*

Given the presence and structural importance of the eDNA we hypothesized that addition of exogenous DNA may further

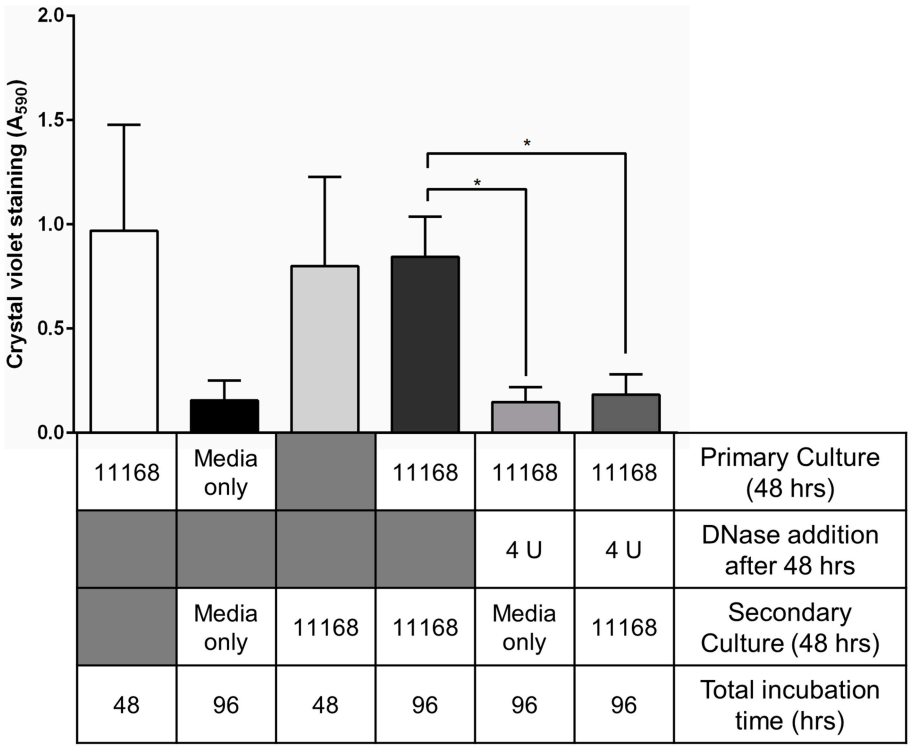

FIGURE 3 | Treatment of pre-existing biofilms with DNase I leads to inhibition of biofilm regrowth. *C. jejuni* NCTC 11168 biofilms were allowed to form for 48 h in sterile borosilicate glass test tubes. To assess biofilm re-growth following DNase I treatment, two sets of tubes were treated with 4 U/ml DNase I for 15 min then washed with sterile PBS. Tubes were then supplemented with either fresh Brucella media (fifth bar) or fresh *C. jejuni* NCTC 11168 culture (sixth bar) and incubated for a further 48 h. The following controls were also prepared: *C. jejuni* NCTC 11168 biofilm formation following primary culture (first bar, white), tubes supplemented with sterile Brucella media (second bar, black), *C. jejuni* NCTC 11168 biofilm formation following only secondary culture (third bar, light gray), and 48 h-old *C. jejuni* NCTC 11168 biofilm, washed with PBS, then supplemented with fresh *C. jejuni* NCTC 11168 culture (fourth bar, dark gray). Error bars show standard deviation. Statistically significant results, as determined using the Mann–Whitney U test, are indicated using an asterisk (*$P < 0.05$).

increase biofilm formation. This was tested by the addition of 2 μg of genomic DNA, isolated from a *C. jejuni* NCTC 11168 strain expressing a GFP protein and containing an antibiotic resistance marker. Addition of genomic DNA did not lead to significant differences in the levels of crystal violet staining (**Figure 6A**). This indicates that although eDNA is essential for biofilm formation and structural stability, in contrast to previous research on *C. jejuni* 81–176 biofilms (Svensson et al., 2009, 2014), exogenous DNA does not act synergistically with eDNA within the *C. jejuni* NCTC 11168 and 81116 biofilms.

While exogenous genomic DNA was not able to increase biofilm formation, genetic transfer of the antibiotic resistance marker was detected in both the planktonic and biofilm-associated cells (**Figures 6B,C**). Chloramphenicol-resistant colonies were recovered from both planktonic and biofilm phases following addition of *C. jejuni* NCTC 11168 *cj0046::gfp*−Cm^R genomic DNA to static cultures of the wild-type NCTC 11168 and 81116 strains. No resistance was observed in cultures not containing *C. jejuni* NCTC 11168 *cj0046::gfp*−Cm^R genomic DNA, suggesting that neither planktonic or biofilm cultures of strains NCTC 11168 or 81116 are naturally resistant to chloramphenicol at the levels used in these assays (10 μg/ml). Where genomic DNA had been added to the suspension at the start of static incubation, resistant cells were present in both planktonic (**Figure 6B**) and biofilm (**Figure 6C**) cultures. In cultures where genomic DNA had been added at a later (24 h) time point, lower levels of chloramphenicol-resistance were observed (**Figures 6B,C**).

Discussion

Microbial biofilms constitute an important problem for the food industry. There is an increasing body of evidence that biofilms can aid survival of *C. jejuni* in the food chain. *C. jejuni* has previously been shown to form both single (Joshua et al., 2006) and multispecies (Sanders et al., 2007) biofilms, and biofilm formation has also been demonstrated on food chain relevant materials such as stainless steel (Peyrat et al., 2008; Sanders et al., 2008; Brown et al., 2014) and in food chain relevant environmental conditions (Reuter et al., 2010; Brown et al., 2014). While the phenomenon of biofilm formation is well established for *C. jejuni*, there is less information available on the composition and role of the extracellular matrix in the processing environment. Biofouling of surfaces is

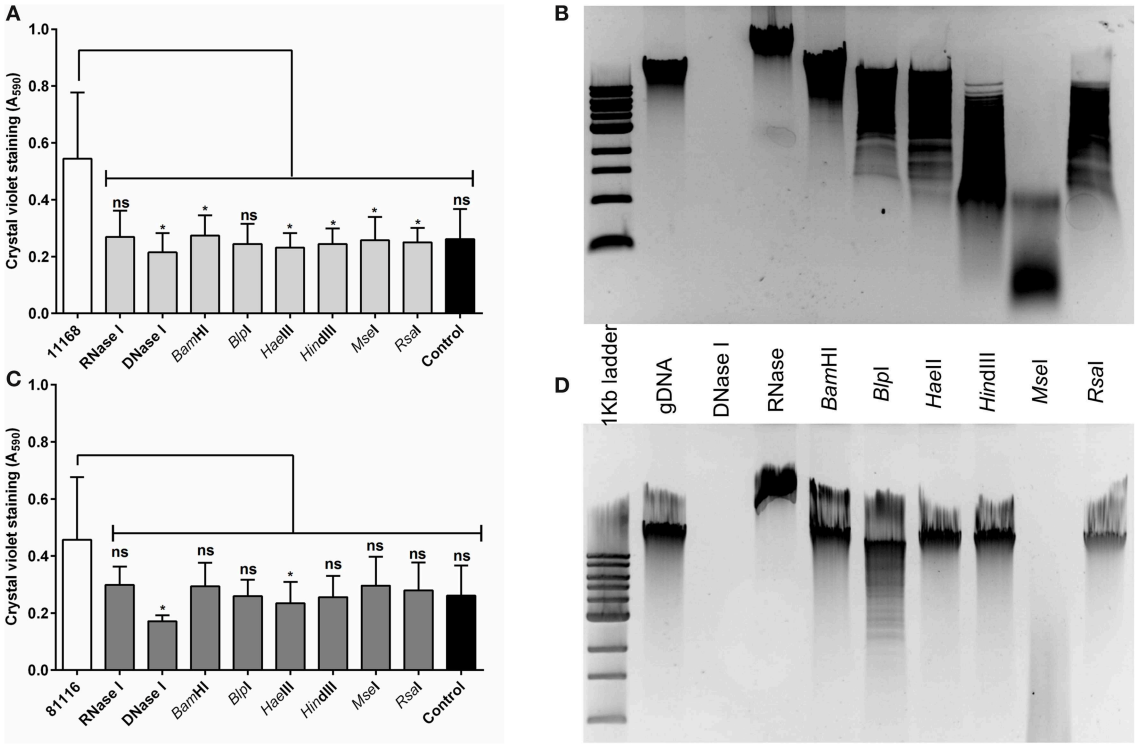

FIGURE 4 | Restriction endonuclease treatment of *C. jejuni* biofilms reduces biofilm formation. Static cultures of *C. jejuni* NCTC 11168 **(A,B)** and 81116 **(C,D)** were prepared then supplemented with either DNase I, RNase, or a single restriction endonuclease. Cultures were incubated for 48 h at 37°C in aerobic conditions. A range of restriction enzymes was selected, based on varying levels of DNA fragmentation following digestion of *C. jejuni* NCTC 11168 **(B)** and 81116 **(D)** genomic DNA. Restriction enzyme and DNase I treatment of NCTC 11168 biofilms lead to a reduction in biofilm formation. The same trend was observed for *C. jejuni* 81116, although only DNase I and *Hae*III digestion were significantly different from the control. Error bars show standard deviation. Statistically significant results, as determined using the Mann–Whitney U test, are indicated using an asterisk (*$P < 0.05$).

a problem within the food industry, where organic materials are present, and areas of attention have not only been on antimicrobial treatment, but also on biofilm dispersal and prevention. Combination treatment including various enzymatic treatments, surfactants and chelating agents may provide a suitable alternative to the chemical treatments currently in use for biofilm degradation within food processing areas (Lequette et al., 2010). The use of DNase I is an example of one such enzymatic treatment.

Treatment of biofilm-based bacterial infections with DNases has increased in recent years, and the human recombinant DNase dornase alpha (Pulmozyme) is now frequently used in the treatment of cystic fibrosis (Konstan and Ratjen, 2012). DNase I is expensive to produce, and hence the use of DNase I on biofilms has been limited to medical applications, for example inner ear infections (Thornton et al., 2013) and wound biofilm control (Swartjes et al., 2013). More recently investigations have also been carried out into the activity of enzyme treatments with foodborne bacterial pathogens such as *Listeria monocytogenes*. For example, *L. monocytogenes* biofilms formed on stainless steel can be removed by both DNase I and Proteinase K treatments (Nguyen and Burrows, 2014), similar to reported here for *C. jejuni* biofilms.

The eDNA within the extracellular matrix appears to have multiple functions, depending on the bacterial species investigated. Previous research in *P. aeruginosa* biofilms has shown that eDNA can not only provide structural stability at early stages of biofilm formation (Whitchurch et al., 2002) but is also found to be localized to specific areas of the biofilm as it matures (Ma et al., 2009), again suggesting a structural role for eDNA in *P. aeruginosa* biofilm organization and expansion (Gloag et al., 2013), with DNase I treatment of developing biofilms leading to significant decreases in biofilm levels. DNA can be used as nutrient source by *E. coli*, *Shewanella*, and *P. aeruginosa* when exposed to phosphate and carbon deficient environments (Palchevskiy and Finkel, 2006; Pinchuk et al., 2008; Mulcahy et al., 2010). Since bacteria within the biofilm are typically immobilized, DNA could provide an easily obtainable food source. Finally, for naturally competent bacteria such as *C. jejuni*, the eDNA can contribute to the spread of genetic traits within populations, both in the biofilm and in the planktonic populations. Genetic material can be transferred within the biofilm either by direct cell to cell transmission or uptake of exogenous DNA. Conjugation within biofilms is a well reported phenomenon, with examples reported in mixed species oral biofilm models (Hannan et al., 2010), drinking water

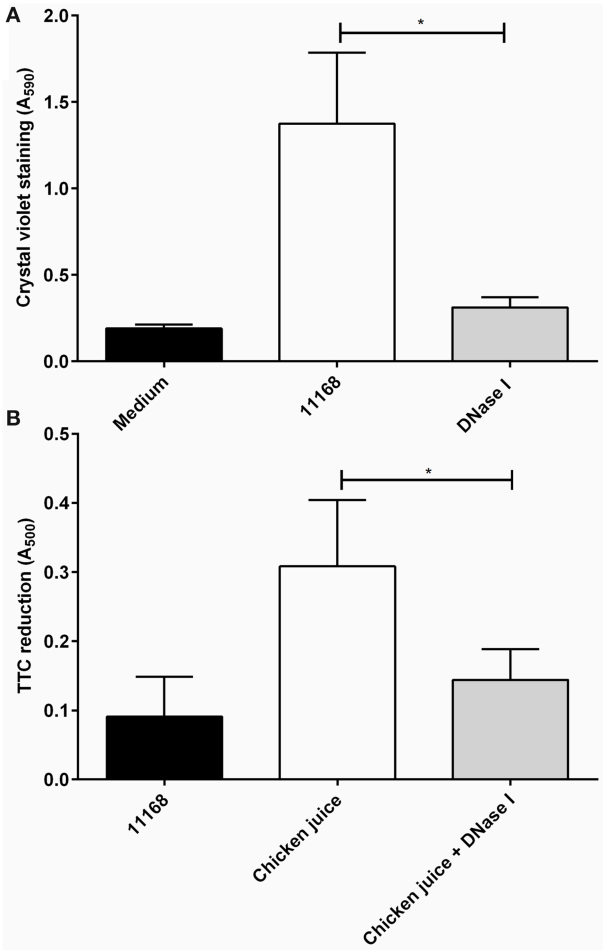

FIGURE 5 | DNase I treatment is effective against *C. jejuni* biofilms on stainless steel surfaces and in the presence of organic materials in aerobic conditions. The ability of DNase I to inhibit biofilm formation of *C. jejuni* NCTC 11168 on sterile, stainless steel coupons **(A)** or in the presence of chicken juice, mimicking a conditioned surface **(B)**. TTC staining was used to measure biofilm formation in the presence of chicken juice **(B)**. DNase I is able to significantly decrease biofilm formation in both conditions. Error bars show standard deviation. Statistically significant results, as determined using the Mann–Whitney U test, are indicated using an asterisk (*$P < 0.05$).

systems (Lisle and Rose, 1995) and within bacterial populations colonizing the nasopharynx (Marks et al., 2012). Recent work has shown that *C. jejuni* strains NCTC 11168 and 81–176 in microaerobic cultures are able to transfer genetic material between bacterial cells both within biofilms and planktonic suspension (Bae et al., 2014; Svensson et al., 2014). The work presented here shows that *C. jejuni* is also able to utilize exogenously added DNA for acquisition of genetic traits. This transfer is also able to occur in aerobic conditions, more closely resembling the conditions *C. jejuni* encounters while in the food chain.

We demonstrate here that eDNA is an important component of the *C. jejuni* extracellular matrix at all stages of maturation. This is in contrast to *P. aeruginosa*, which become less susceptible to DNase I treatment as the biofilm matures (Whitchurch et al.,

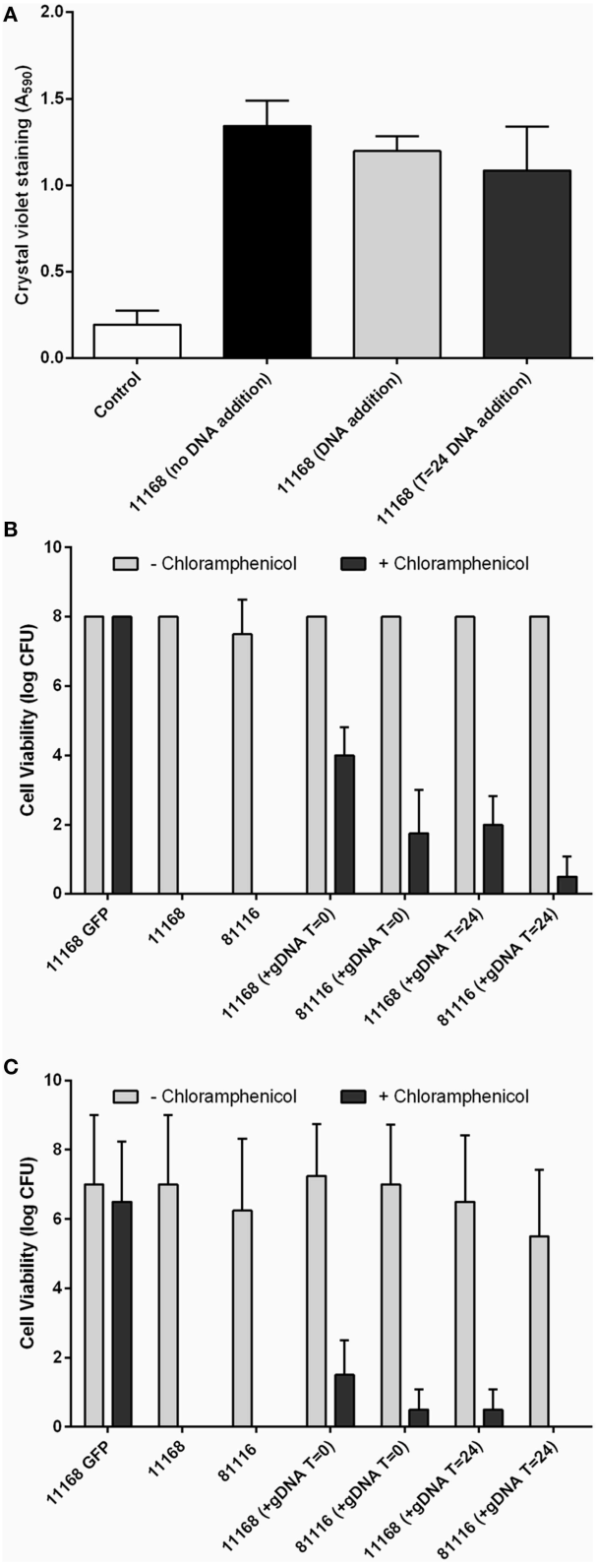

FIGURE 6 | *C. jejuni* NCTC 11168 and 81116 can acquire genetic traits from exogenous DNA during static incubation. *C. jejuni* NCTC 11168 and *(Continued)*

FIGURE 6 | Continued
81116 biofilms were allowed to develop for 48 h in the presence of 2 μg *C. jejuni* NCTC 11168 *cj0046*::*gfp*+−CmR genomic DNA. Supplementation with eDNA did not lead to changes in biofilm formation **(A)**. Plating both planktonic **(B)** and biofilm **(C)** cells on both Brucella media and Brucella media supplemented with 10 μg/ml chloramphenicol shows emerging chloramphenicol resistant cells suggesting integration of the chloramphenicol resistance gene, via natural transformation, into the genomes of both planktonic and biofilms cells. Error bars show standard deviation.

2002). Some outer membrane and flagella proteins have been identified as been important in *C. jejuni* biofilm formation, but to date there has been little investigation of the extracellular matrix components themselves. *C. jejuni* produces a polysaccharide containing β1-3 and/or β1-4 linkages which is reactive to calcofluor white (McLennan et al., 2008), and hence further studies are required to distinguish between the roles of eDNA and other polysaccharides in *C. jejuni* biofilms.

Although eDNA has been shown to be present within the biofilms of many different bacteria, the mechanism of its release into the extracellular milieu is still under investigation. There are two main mechanisms of DNA release; secretion and cell lysis. Secretion of eDNA has been shown in several species, including *Neisseria gonorrhoeae* (Hamilton et al., 2005) and *P. aeruginosa* (Renelli et al., 2004). Although secretion of eDNA has been observed in some bacteria, it is widely accepted that lysis is a more common method of eDNA release (Wu and Xi, 2009). For instance, *Staphylococcus aureus* eDNA can be released via co-ordinated lysis of a subset of the population, controlled by quorum sensing (Mann et al., 2009). To date quorum sensing mechanisms have not been described in *C. jejuni* (He et al., 2008; Adler et al., 2014), and although it is possible that a yet unknown quorum sensing system controls co-ordinated eDNA release in *C. jejuni*, this will require further investigation. *P. aeruginosa* biofilms showed higher concentrations of eDNA within the biofilm when cultures were supplemented with salmon sperm DNA (Chiang et al., 2013), suggesting that some biofilm-forming bacteria are able to utilize eDNA from several sources. Our results suggest that although *C. jejuni* NCTC 11168 and 81116 are able to utilize exogenous DNA, this does not lead to a net increase in biofilm formation. In contrast, addition of eDNA to *C. jejuni* 81–176 biofilm cultures led to increased biofilm biomass (Svensson et al., 2014).

Another problem frequently encountered within food processing environments is the presence of food product debris. This presence of this debris on surfaces can lead to surface conditioning and increased bacterial attachment, as observed with chicken juice and *C. jejuni* (Brown et al., 2014). The attachment of *L. monocytogenes* to stainless steel surfaces is enhanced by surface pre-conditioning with fish and meat emulsions (Gram et al., 2007), and surface conditioning by chicken juice has been shown to enhance *C. jejuni* biofilm formation (Brown et al., 2014). Surface conditioning can also decrease the effectiveness of chemical cleaning products, leading to reduced killing or biofilm degradation (Gram et al., 2007). In heavily soiled environments broad spectrum enzymatic treatments may provide a useful and effective addition to current cleaning regimes, as they are able to degrade not only biofilm extracellular matrix, but potentially also the conditioning layer. Our results show that DNase I treatment is able to significantly reduce *C. jejuni* biofilms formed on surfaces conditioned with chicken juice, suggesting that DNase I treatment could provide a useful addition to current treatment regimens.

It should be noted that we found DNase I treatment had no effect on cell viability, only biofilm shedding. This is as expected since DNase I is only in contact with the DNA of the extracellular matrix, reducing the structural integrity of the colonies forming the biofilm, but is not able to cause a loss of viability in bacterial cells with intact membranes. This means that although the DNase I treatment provides a rapid and effective method of biofilm dispersal it would best be used in combination with antimicrobial treatments, ensuring effective biofilm degradation and bacterial inactivation.

In conclusion, eDNA is an essential component of the *C. jejuni* biofilm and its degradation results in a reduction of biofilm levels below detection levels (Tresse et al., 2006). Treatment of abiotic surfaces containing *C. jejuni* biofilms with DNase I also prevents re-establishment of biofilms, possibly allowing more efficient antimicrobial treatment. DNase I treatment is effective on food chain relevant surfaces and hence could provide a useful addition to current food chain cleaning regimes.

Author Contributions

HB, MR, RB, and AV designed the study. HB, KH, and MR performed the experimental work and analyzed the data. HB prepared the manuscript, and KH, MR, RB, and AV contributed to the final manuscript.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00699

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Biofilm spatial organization by the emerging pathogen *Campylobacter jejuni*: comparison between NCTC 11168 and 81-176 strains under microaerobic and oxygen-enriched conditions

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During the last years, *Campylobacter* has emerged as the leading cause of bacterial foodborne infections in developed countries. Described as an obligate microaerophile, *Campylobacter* has puzzled scientists by surviving a wide range of environmental oxidative stresses on foods farm to retail, and thereafter intestinal transit and oxidative damage from macrophages to cause human infection. In this study, confocal laser scanning microscopy (CLSM) was used to explore the biofilm development of two well-described *Campylobacter jejuni* strains (NCTC 11168 and 81-176) prior to or during cultivation under oxygen-enriched conditions. Quantitative and qualitative appraisal indicated that *C. jejuni* formed finger-like biofilm structures with an open ultrastructure for 81-176 and a multilayer-like structure for NCTC 11168 under microaerobic conditions (MAC). The presence of motile cells within the biofilm confirmed the maturation of the *C. jejuni* 81-176 biofilm. Acclimation of cells to oxygen-enriched conditions led to significant enhancement of biofilm formation during the early stages of the process. Exposure to these conditions during biofilm cultivation induced an even greater biofilm development for both strains, indicating that oxygen demand for biofilm formation is higher than for planktonic growth counterparts. Overexpression of cosR in the poorer biofilm-forming strain, NCTC 11168, enhanced biofilm development dramatically by promoting an open ultrastructure similar to that observed for 81-176. Consequently, the regulator CosR is likely to be a key protein in the maturation of *C. jejuni* biofilm, although it is not linked to oxygen stimulation. These unexpected data advocate challenging studies by reconsidering the paradigm of fastidious requirements for *C. jejuni* growth when various subpopulations (from quiescent to motile cells) coexist in biofilms. These findings constitute a clear example of a survival strategy used by this emerging human pathogen.

Keywords: ***Campylobacter jejuni*, biofilm, CLSM, oxidative stress, CosR**

Introduction

Campylobacter has emerged as the leading cause of bacterial foodborne infections in developed countries (Epps et al., 2013; Golz et al., 2014). The resulting disease in humans, campylobacteriosis, is characterized by acute enteritis with the presence of blood and leukocytes in a stool, abdominal pain, and fever (Cameron et al., 2012; Lu et al., 2012). It is also associated with late onset complications such as Guillain-Barré syndrome, its variant Miller-Fisher syndrome (Salloway et al., 1996; Nachamkin et al., 1998; Kudirkiene et al., 2012), and inflammatory bowel diseases (Kaakoush et al., 2014). The underlying molecular mechanisms responsible for its pathogenesis, persistence, and survival seem to be unique to *Campylobacter* as compared to other invasive foodborne bacterial pathogens (*Listeria monocytogenes*, *Salmonella enterica,* and *Staphylococcus aureus*). These features might result from high level of genomic polymorphism, restricted catabolic capacity, self-regulation and deregulation of genes, and other undefined survival routes.

The main reservoir of *Campylobacter* is the intestinal tract of birds and other endothermic animals, especially livestock. It is primarily isolated from poultry and, to a lesser extent, pork and beef. The infection of the human host is generally caused by the consumption of undercooked and mishandled poultry or by cross-contamination of cooking tools and fresh vegetables (Butzler, 2004; Guyard-Nicodeme et al., 2013). A significant increase in the prevalence of campylobacteriosis cases has been observed over the past 5 years in the EU, based on quantitative epidemiological analyses from farms to retail outlets (EFSA, 2012, 2013). A baseline survey, conducted in 28 European countries in 2010, indicated that 71.2% of broiler batches and 75.8% of broiler carcasses were contaminated by *Campylobacter* (EFSA, 2010). These data were reinforced by an in-depth analysis over a 3-year period at the UK-wide level showing that in over 37 abattoirs (representing almost 90% of the total UK slaughter throughput), 79.2% of the slaughter batches were positive for *Campylobacter* (Lawes et al., 2012). In addition, 87.3% of the broiler carcasses were contaminated by *Campylobacter* with 27.3% of them showing a load over 1000 cfu.g^{-1} (Powell et al., 2012). In the USA, 168 pathogen-food combinations of 14 major pathogens across 12 food categories were compared (Batz et al., 2012). The combination "*Campylobacter*-poultry" reached the first rank in terms of annual disease burden including illnesses, hospitalizations, deaths, and costs. Overall, these exhaustive data on *Campylobacter* contamination indicate that this microorganism can survive outside of its reservoir through breeding farms, slaughterhouses and food processing, defying environmental conditions, and human defense mechanisms.

The main pathogenic species, *Campylobacter jejuni*, has been isolated in more than 80% of the campylobacteriosis cases (Moore et al., 2005). Being an obligate microaerophilic bacterium, *Campylobacter* has to develop adaptation strategies to survive oxidative conditions from food environments and macrophage attacks. It has been suggested that adhesion to surfaces and formation of biofilms could be one of the strategies used to maintain *C. jejuni* survival (Nguyen et al., 2012). Moreover, the bacterium can be sheltered in mixed species biofilms (Sanders et al., 2007; Ica et al., 2011). *C. jejuni* can form three different types of biofilm: (i) a structure attached to an abiotic surface, (ii) aggregates floating in the liquid culture, or (iii) a pellicle formed at the gas/liquid interface (Joshua et al., 2006). Biofilm formation occurs within 48 h of cultivation with cell detachment becoming prominent after a prolonged cultivation time (Sanders et al., 2007; Ica et al., 2011). In line with other biofilm-producing foodborne bacteria, the substratum composition and its physicochemical properties influence the biofilm formation of *C. jejuni* (Nguyen et al., 2011). These properties could play an important role in the early stages of biofilm formation when cells adhere to the surface. This assumption is supported by a variation in adhesion rates to inert surfaces, such as nitrocellulose membrane, glass, and stainless steel (Joshua et al., 2006; Kalmokoff et al., 2006; Gunther and Chen, 2009). The wide range of adhesion capability in *Campylobacter* spp. also raises the question of biological fitness among strains, in regards to their ability to attach irreversibly to a surface and initiate biofilm formation (Joshua et al., 2006; Sulaeman et al., 2010; Teh et al., 2010).

Molecular mechanisms regulating biofilm formation of *C. jejuni* are still poorly understood. So far, genes described to be involved in the process include those responsible for cell motility (*flaA, flaB, flaC, flaG, fliA, fliS,* and *flhA*; Joshua et al., 2006; Kalmokoff et al., 2006; Reeser et al., 2007; Reuter et al., 2010), cell surface modifications (*peb4*, *pgp1*, and *waaF*; Asakura et al., 2007; Naito et al., 2010; Frirdich et al., 2014), quorum sensing (*luxS*; Reeser et al., 2007), and stress response (*ppk1*, *spoT*, *cj1556*, *csrA*, *cosR*, and *cprS*; Candon et al., 2007; Fields and Thompson, 2008; McLennan et al., 2008; Svensson et al., 2009; Gundogdu et al., 2011; Oh and Jeon, 2014). It was found that biofilm formation is flagellum-mediated as the first step of the process—cellular adhesion—requires presence of flagella, although its functionality is not crucial for the biofilm initiation (Svensson et al., 2014). Other components essential for development of biofilm structure are extracellular DNA (eDNA) and DNA-binding protein Dps, whose presence is required for proper formation of microcolonies and structuralization of biofilm (Svensson et al., 2014; Brown et al., 2015). Genes regulating biofilm formation were not fully identified so far. Experiments using knock-out and knock-down mutants of various regulators revealed several genes influencing the process of biofilm formation. Except of aforementioned motility apparatus regulated by *flhA* (Kalmokoff et al., 2006), and functional quorum sensing *luxS* (Reeser et al., 2007), other regulators involved mostly in stress response were found to be critical for biofilm formation. Interestingly, while mutants lacking genes responsible for oxidative stress response such as *cj1556* and *csrA* were defective in biofilm formation (Fields and Thompson, 2008; Gundogdu et al., 2011), knock-out/down of genes responsible for general stress response (*spoT*, *ppk1*, and *cprS*) resulted in increased biofilm formation suggesting that the process represents alternative pathway of

Abbreviations: BFI, Biofilm index; CLSM, Confocal laser scanning microscopy; MAC, Microaerobic conditions; OEC, Oxygen-enriched conditions; OEC_C, Cultivation in OEC; OEC_A, Acclimation to OEC; ROS, Reactive oxygen species; TCS, Two-component system.

stress defense in *Campylobacter* (Candon et al., 2007; McLennan et al., 2008; Svensson et al., 2009). Another regulator possibly involved in biofilm formation is gene *cosR*. This orphan two-component system (TCS) was recently discovered to be involved in the regulation pathway of ROS detoxification in *C. jejuni* (Hwang et al., 2011, 2012). It was previously reported that CosR regulates transcription of 93 different genes in *C. jejuni* (Hwang et al., 2012), it is overexpressed in sessile cells (Kalmokoff et al., 2006) and was already shown to influence biofilm formation by regulation of alkyl hydroperoxide reductase *ahpC* (Oh and Jeon, 2014). All these facts suggest that CosR might play significant role in biofilm formation of *C. jejuni*.

So far, analyses of pure cultures have mostly been carried out in an optimal growth atmosphere and were focused on the strain NCTC 11168 (Kalmokoff et al., 2006; Ica et al., 2011). Using colorimetric assessment methods (Crystal violet and Congo red assays) for biofilm detection in glass tubes, Reuter et al. (2010) showed that aerobic cultivation enhanced *C. jejuni* NCTC 11168 biofilm. In a previous study, we have shown that the strain 81-176, grown under controlled oxygen-enriched conditions (19% O_2, 10% CO_2, and 71% N_2), is able to overexpress membrane proteins involved in biofilm initiation and virulence process (Sulaeman et al., 2012). In this study, we compared the biofilm development of two *C. jejuni* strains responsible for human outbreaks (NCTC 11168 and 81-176), and the effect of dioxygen (O_2) on biofilm development. The usage of controlled atmosphere eliminated other factors possibly affecting biofilm formation. It was therefore possible to explore whether the increase of biofilm formation in aerobic conditions could be attributed solely to the level of oxygen and if the trend of enhanced biofilm formation is present in other strain of *C. jejuni*. This was evaluated, for the first time, using specific biofilm parameters (maximum height, biomass volume, and ultrastructure) from confocal laser scanning microscopy (CLSM) analyses. This non-invasive sensitive technique has been used previously to examine *Campylobacter* cell morphology and viability (Chantarapanont et al., 2003; Lee et al., 2004; Jang et al., 2007; Ica et al., 2011) and bacterial interactions with live tissues (Mooney et al., 2003). The CLSM has also been used for the detection of *C. jejuni* in mixed species biofilms (Sanders et al., 2007; Ica et al., 2011). In the present work, the impact of pretreatment and cultivation of cells in oxygen-enriched conditions (OEC) on *C. jejuni* biofilm formation and its ultrastructural organization was investigated in comparison with cells cultivated in microaerobic conditions (MAC). In addition, analyses using an overexpressing *cosR* transformant were performed to determine the role of this regulator in *C. jejuni* biofilm development.

Results

Biofilm Development and Architecture

Two *C. jejuni* strains, NCTC 11168 and 81-176, were chosen in order to explore their biofilm formation capacities using CLSM with Syto 9 staining. This cell-permeable dye emits fluorescence after binding to nucleic acids and therefore allows the visualization of cells and any extracellular DNA present in the biofilm matrix. Both strains were able to form biofilm within 24 h of cultivation (**Figure 1**). At the initial stages of biofilm formation, cells gathered in clusters partially attached to the surface, forming finger-like structures. After 48 h, most of the biofilm mass remained attached to the bottom of the well. The biofilm structure evolved during the time of cultivation, increasing in both maximum height and biomass volume for both strains. However, 81-176 formed more biofilm than the NCTC 11168 strain (after 48 h: 233.33 ± 64.63 and 130.67 ± 14.70 μm, respectively, for the maximum height; $42.3 \times 10^5 \pm 5.7 \times 10^5$ and $0.4 \times 10^5 \pm 0.09 \times 10^5$ μm^3, respectively, for the biomass volume; $n = 3$). In addition, unlike NCTC 11168, the biofilm of the 81-176 strain exhibited a pronounced open ultrastructure full of voids and channels, even after 96 h of incubation (data not shown). As growth rates of both strains were similar ($\mu_{max} = 0.69\,h^{-1}$ for NCTC 11168 and $\mu_{max} = 0.67\,h^{-1}$ for 81-176), these differences in biofilm formation cannot be explained by different growth abilities. During the experiment, no formation of pellicle or floating aggregates was observed probably due to the cultivation in static conditions.

Cell Motility in Biofilm

Motile cells, tracked using CLSM, were observed around or inside the biofilm structure after 48 h of cultivation (Supplementary Videos). However, the motility of cells differed according to their position in the biofilm structure. The highest number of motile cells was detected at the bottom of the well (Supplementary Videos 1, 3) moving more or less freely through the structure, while the motility and the number of motile cells decreased in the middle part of the biofilm (Supplementary Videos 2, 4). Furthermore, high number of motile cells was detected within the biofilm structure of 81-176 (Supplementary Videos 1, 2), whereas for NCTC 11168 the motile cells were detected mostly outside the biofilm (Supplementary Videos 3, 4).

Effect of Oxygen on Biofilm Formation of NCTC 11168 and 81-176 *C. jejuni* Strains

Two different approaches were used to evaluate the effect of subinhibitory oxygen concentration on biofilm formation of

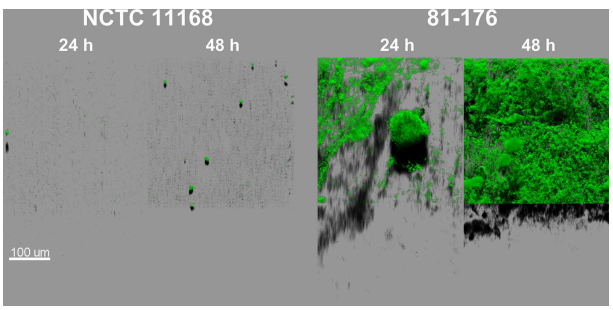

FIGURE 1 | *C. jejuni* NCTC 11168 and 81-176 biofilm architecture and development are different after incubation for 24 and 48 h in MAC (5% O_2, 10% CO_2, 85% N_2). The CLSM images represent an aerial view of biofilm structures with the shadow projection at the bottom. The structures were visualized using Syto 9, an intercalating agent staining the nucleic acids.

two strains with different biofilm forming ability (NCTC 11168 and 81-176). Firstly, biofilms were cultivated under controlled oxygen-enriched conditions (OEC_c) as described previously by Sulaeman et al. (2012). In OEC, the same concentration of CO_2 (10%) as in MAC was maintained, while the O_2 concentration was increased to a sublethal level (19% O_2 in OEC vs. 5% in MAC). This enabled the evaluation of the effect of increased O_2 concentration on biofilm development of *C. jejuni* regardless of its capnophilic nature requiring increased concentration of CO_2. Biofilm volume of both strains was significantly increased ($P < 0.01$) when cultivated in OEC_c (**Figure 2** and Supplementary Table 1). Incubation time and O_2 concentration had a significant effect ($P < 0.01$) on increased biomass production in OEC_c when compared to MAC_c. Interestingly, some significant differences in both maximum height and biomass volume ($P < 0.01$) remained between the two strains even after cultivation in OEC, with a greater biofilm development for 81-176 than for NCTC 11168, indicating that strain biology impacts biofilm formation (Supplementary Table 1). This was confirmed by formation of a denser compact biomass for NCTC 11168 biofilm while 81-176 induced more voids and open water channels across the biofilm.

In the second approach, both strains were acclimatized to OEC (OEC_a) prior to biofilm formation in MAC. Acclimatized cells of both strains formed significantly larger biofilms than non-acclimatized ones after 24 h of cultivation, as expressed by the fold changes in maximum height and biomass volume values (**Figure 3A**). Conversely, the acclimatization of cells to OEC was no longer an advantage for biofilm formation after 48 h, as demonstrated by reduction of biofilm formation for both strains. This was also confirmed by statistical analyses, with the highest *F*-ratios of the interaction effect between "Incubation time" and "O_2 pretreatment," showing higher variation in maximum height and biomass volume, than for the other factors (Supplementary Table 2).

In order to distinguish the effect of OEC prior to or during *C. jejuni* biofilm formation, biofilm development was compared between the cells acclimatized to OEC and the cells subjected to OEC during biofilm formation (OEC_a and OEC_c, respectively; **Figure 3B**). Although the fold change (OEC_c/OEC_a) was not in favor of NCTC 11168 biofilm formation during the first 24 h, after 48 h both strains cultivated in OEC_c showed enhanced biofilm formation with a marked difference in biomass volume for 81-176. This was confirmed statistically with a significant effect of OEC treatment ($P < 0.0001$) for O_2 treatment, and the interaction between "Incubation time" and "O_2 treatment" with the highest *F*-ratios (Supplementary Table 3).

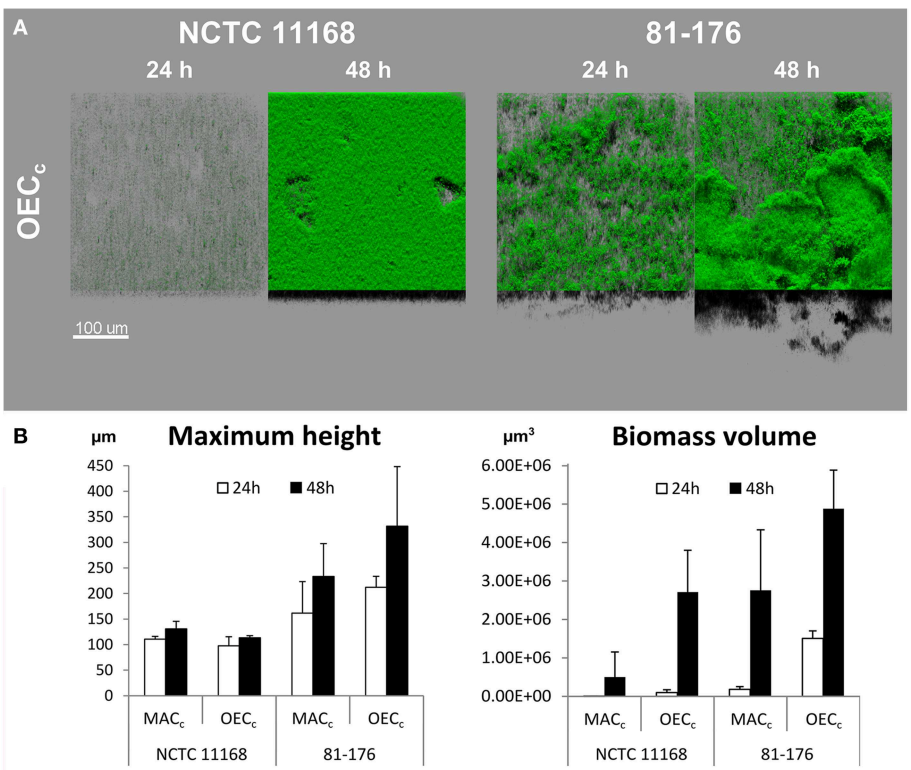

FIGURE 2 | Oxygen enhances biofilm development of *C. jejuni* NCTC 11168 and 81-176 after incubation for 24 and 48 h. (A) The CLSM images represent an aerial view of the biofilm structures in OEC (19% O_2, 10% CO_2, 71% N_2) with the shadow projection at the bottom. **(B)** The effect of cultivation time (24 h white bars, 48 h black bars) and OEC on biofilm formation of the two *C. jejuni* strains as expressed by maximum height and biomass volume. Results show the means and standard deviations of three replicates. Statistical data are presented in Supplementary Table 1.

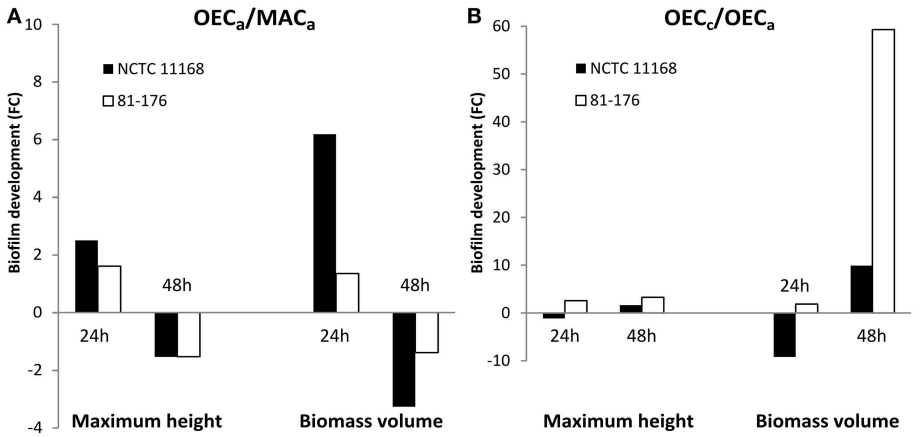

FIGURE 3 | Oxygen-enriched conditions enhanced biofilm development prior to and during biofilm formation of *C. jejuni*. Fold changes (FC) of biofilm development of *C. jejuni* strains NCTC 11168 (black bars) and 81-176 (white bars) as expressed by maximum height and biomass volume. **(A)** FC representing biofilm formation of cells acclimatized to OEC (OEC$_a$: 19% O_2, 10% CO_2, 71% N_2) and to MAC (MAC$_a$: 5% O_2, 10% CO_2, 85% N_2) prior to biofilm formation in MAC. **(B)** FC of biofilm development of cells submitted to OEC (OEC$_c$) during biofilm cultivation and cells submitted to OEC (OEC$_a$) prior to biofilm cultivation in MAC. Statistical data are presented in Supplementary Tables 2, 3.

Role of *cosR* in Biofilm Development

A second copy of *C. jejuni* gene *cosR* and its promoter were inserted into the poorer biofilm-forming strain NCTC 11168 to determine its role in *C. jejuni* biofilm formation. This construction, with an ectopic copy of the *cosR* gene and its promoter, enabled to double the expression of the transcript level of *cosR* in the cells (Supplementary Figure 1) in a same manner as in the *cosR*-overexpressing strain obtained by Hwang et al. (2011) and used by Oh and Jeon (2014). Then, the parental NCTC 11168 strain and the *cosR* overexpressing strain, namely transformant (Trf*cosR*), were compared for their ability to adhere to an inert surface and to develop a biofilm (**Figure 4**). Using the BioFilm Control Ring Test®, a significantly higher ΔBFI was obtained ($P = 0.0007$) for the transformed strain, indicating its greater ability to adhere to inert surfaces (**Figure 4A**). In addition, using the crystal violet assay, the transformant showed enhanced biofilm formation after 24 and 48 h ($P = 0.0006$ and 0.02, respectively) but not after 72 h ($P > 0.05$) when compared with its parental strain (**Figure 4B**). The CLSM observations and biofilm analyses indicated that the transformant formed significantly more ($P < 0.01$) biofilm than its parental strain (**Figure 5**, Supplementary Table 4). In addition, the maximum height and biomass volume reached by the transformant was not significantly different from those obtained with the strongest biofilm-forming strain 81-176 (Supplementary Table 5). These data showed that the presence of two genes encoding *cosR* significantly enhanced biofilm development in MAC (592.7-times higher biomass volume after 24 h). Interestingly, this was correlated with the formation of an open biofilm ultrastructure with voids and water channels similar to the one described for 81-176 (**Figures 1**, **5A**). Comparison of genomic sequences using xBASE2 (Chaudhuri et al., 2008) showed that the *cosR* gene (*cj0335c* and *cjj0379c,* respectively) and its flanking regions are 100% identical in NCTC 11168 and 81-176. Both strains carry the exact same form of the gene. Therefore, some other mechanisms, related to the *cosR* sequence and its flanking regions, for regulating the *C. jejuni* biofilm formation, should exist. Moreover, unlike the two wild strains, an increased O_2 concentration during cultivation did not promote biofilm formation of the transformant (**Figure 5**). These data indicate that a second ectopic copy of *cosR* enhanced biofilm development by promoting a complex architecture of *C. jejuni* biofilm irrespective of O_2 demand. Nevertheless, further experiments should be performed to evaluate *cosR* transcript level and CosR expression throughout all phases of biofilm development.

Discussion

As the leading cause of bacterial foodborne diseases, whose incidence has been significantly increasing during the recent years in Europe (EFSA, 2010, 2012, 2013), this pathogen has to adapt and survive environmental conditions outside and inside its main hosts, particularly oxidative stress. In this study, we have shown that *C. jejuni* can form biofilm in static conditions with a clearly defined finger-like structure. Our observation is consistent with previous studies indicating that *C. jejuni* could develop monospecies biofilms (Kalmokoff et al., 2006; Asakura et al., 2007; Corcoran and Moran, 2007; Reeser et al., 2007; Fields and Thompson, 2008; Hanning et al., 2008; McLennan et al., 2008; Sanders et al., 2008; Gunther and Chen, 2009). Both examined strains were able to produce a biofilm, although their maximum height, biomass volume, and ultrastructure differed significantly between the two strains. In previous studies, stronger adhesion to an inert surface was observed for 81-176 than for NCTC 11168 (Gunther and Chen, 2009; Sulaeman et al., 2010; Teh et al., 2010). Although the adhesion strength could not be fully correlated to the capability of bacterial species to form biofilms, biofilm initiation is crucial to anchor the embryonic core of the biofilm. Our qualitative and quantitative data indicated that

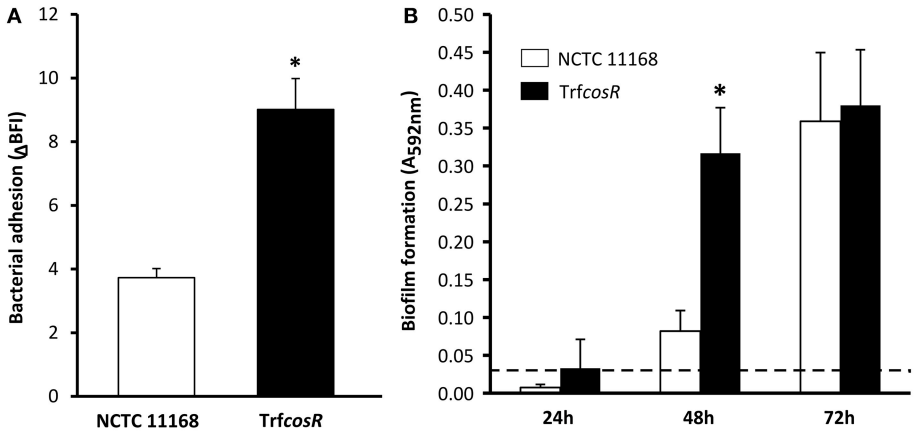

FIGURE 4 | The NCTC 11168 *cosR* overexpressing transformant enhanced cell adhesion to inert surface and biofilm formation in comparison with its parental strain. Adhesion to an inert surface **(A)** and biofilm formation **(B)** of *C. jejuni* NCTC 11168 (white bars) and the *cosR* overexpressing transformant (Trf*cosR*) (black bars) strains. Bacterial adhesion was determined after 2 h by calculating the BioFilm Index (BFI) using the BioFilm Ring Test®. Biofilm formation was measured in 24-well microtitre plates at 24, 48, and 72 h using the crystal violet assay. Error bars represent the standard deviation of three independent experiments. Asterisks indicate significant differences ($P < 0.05$) between the parental strain and the transformant. A dashed line represents detection limit of the crystal violet assay.

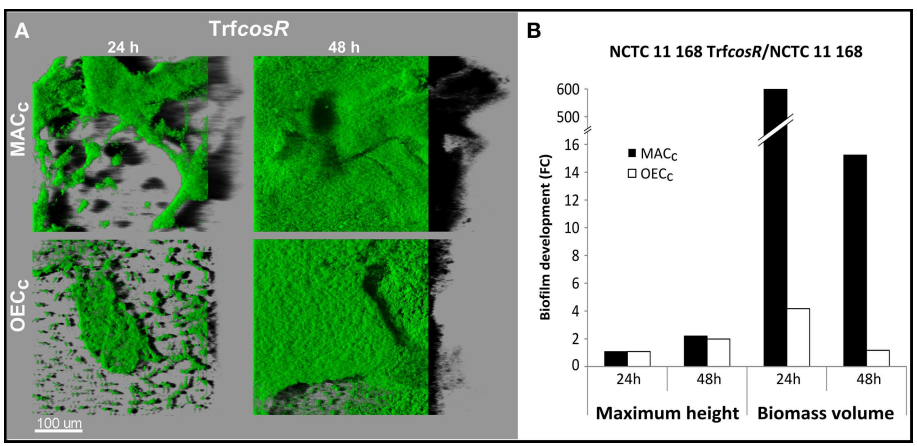

FIGURE 5 | The CosR is responsible for biofilm maturation in *C. jejuni*. Biofilm structure of *C. jejuni* NCTC 11168 and the *cosR* overexpressing transformant (Trf*cosR*) after incubation for 24 and 48 h in MAC (black bars) or OEC (white bars). **(A)** The CLSM images representing an aerial view of biofilm structures with the shadow projection on the right. **(B)** Trf*cosR* biofilm development in comparison to the parental strain expressed as a fold changes of maximum height and biomass volume. Statistical data are presented in Supplementary Tables 4, 5.

NCTC 11168 formed a thin but compact multilayered biofilm without achieving a more complex organization during the time of incubation. In contrast, the 81-176 strain was able to form a thick biofilm with an open ultrastructure composed of voids and channels. This kind of heterogeneous structure is considered to be the signature of a mature biofilm. It enhances the formation of convective flows bringing nutrients to cell aggregates and draining metabolic waste from cells in these aggregates (Donlan and Costerton, 2002). The heterogeneity of the 81-176 biofilm was confirmed by tracking the motile cells within the *C. jejuni* 81-176 biofilm. In contrast to many other bacteria, *C. jejuni* is able to maintain the expression level of genes responsible for cell motility and flagella biosynthesis when grown in biofilms (Joshua et al., 2006; Kalmokoff et al., 2006; Asakura et al., 2007; Reeser et al., 2007). In our study, we observed the presence of motile, less motile and sessile cells, indicating that the biofilm is composed of cells in different physiological states. Due to the biofilm organization, different cell phenotypes coexist in the structure and therefore a wide range of cells can be found in the biofilm, from dormant to motile cells. As in nature (*ex vivo* or *in vivo*) *C. jejuni* cells may encompass various physiological states, biofilm could be considered as a model of mixed subpopulations of *C. jejuni* which could be found in food products, food-processing plants, in poultry gut, or human digestive tract.

Although *C. jejuni* is sensitive to increased concentrations of oxygen, absence of oxygen in anaerobic conditions induces

cell death. *C. jejuni* requires a basal amount of available oxygen to maintain the processes essential for respiration and multiplication (Kelly, 2008). The availability of dissolved oxygen is therefore one of the main environmental parameters for the survival of *C. jejuni*. Previous studies showed that aerobic conditions enhanced biofilm formation of the strain NCTC 11168 (Asakura et al., 2007; Reuter et al., 2010). In those studies, biofilms grown in glass tubes or in 24 well plates were detected by crystal violet or Congo red after exposition to air and air supplemented with CO_2. These colorimetric assays showed enhancement of biofilm formation under the oxidative stress, but could not predict whether and how the biofilm structure would change. The controlled O_2 gaseous conditions, respecting the capnophilic nature of *C. jejuni*, and the use of CLSM allowed us not only to quantify the amount of biofilm, but also to evaluate any structural changes caused by increased oxygen concentration. In accordance to our expectations, we did observe an increased biofilm formation for both strains under OEC. Moreover, the data obtained using CLSM suggest that the response to an increased O_2 level is strain-dependent. Although the biofilm formation for both strains was enhanced, the ultrastructures were remarkably different. The poorer biofilm forming NCTC 11168 produced more voluminous biofilm without increasing its thickness and without switching to a maturation phase as observed for 81-176. As the physiological state of cells may correspond to their close environment, the cell response to environmental conditions could differ according to its location in the biofilm structure. The formation of voluminous flat biofilm may be beneficial for NCTC 11168 under OEC, as a smaller area, and therefore a smaller number of cells, is exposed to the malignant effect of oxygen. On the other hand, the 81-176 strain increased in both biofilm volume and height, keeping the porous ultrastructure of the biofilms produced under MAC. It seems like the strain disregards the negative effects of an increased oxygen level and is supported to multiply and form a mature biofilm composed of mixed subpopulations of cells. The biofilm organization may therefore offer a favorable oxygen tuning niche for *C. jejuni*. These findings indicate that oxygen growth requirements of *C. jejuni* are not as fastidious when cells are organized in biofilm. Consequently, the paradigm of fastidious requirements for *C. jejuni* growth (Jones, 2001; Park, 2002) should be reconsidered according to the cell physiological state and cell population cooperation.

Unlike well-studied aerobes, *C. jejuni* lacks specific and global regulators involved in oxidative stress resistance, such as SoxRS, OxyR, or RpoS (Garenaux et al., 2008). *C. jejuni* carries two Fur homologs, Fur, and PerR (peroxide stress regulator), which regulate iron homeostasis and contribute to the oxidative stress response (Van Vliet et al., 2002). Recently, Hwang et al. (2011, 2012) have suggested that the orphan TCS Cj0355c could be involved in the oxidative stress response and named it CosR. The protein product of *cosR* shares 60% amino acid identity with Hp1043, a TCS response regulator element from the close relative *Helicobacter pylori*. Deletion of *hp1043* induced death of *H. pylori* in the same way as it has been observed for *cosR* and *C. jejuni* (Stahl and Stintzi, 2011). However, the *hp1043* gene has been successfully substituted by *C. jejuni cosR* (Muller et al., 2007) suggesting that CosR exhibits similar biological functions to Hp1043.

In this study, the essential gene *cosR* was overexpressed in the poorer biofilm-forming strain, NCTC 11168, in order to investigate the role of this TCS in biofilm formation and structuring. In our study, the adhesion of cells to inert surfaces was correlated to the biofilm formation detected by Crystal violet and analyzed by CLSM. All three different detection techniques led to the same conclusion. The significantly greater adhesion to an inert surface and the increased biofilm formation of the transformant (Trf*cosR*) revealed that the expression of this gene is connected to biofilm formation. This was also confirmed by the CLSM experiments, which showed a much greater thickness and volume of the transformant's biofilm under MAC than those observed for the parental strain. This result is in accordance with the previously published work describing increased expression of CosR in *C. jejuni* NCTC 11168 biofilm as compared to planktonic counterparts (Kalmokoff et al., 2006), although Oh and Jeon (2014) observed decrease of biofilm formation in strain overexpressing *cosR*. This discrepancy might be explained by looking at the structure of biofilms of parental strain and the transformant. Interestingly, the ultrastructure of the Trf*cosR* biofilm was found to be more similar to the one described for 81-176 than the parental strain, showing an open organization with pores and channels across the structure. Unexpectedly, when the transformant was cultivated under OEC, the maximum height and biomass volume of the biofilm were not higher than when the biofilm was produced under MAC. Nevertheless, the values still remained higher than those of the parental strain. These data indicate that cells overexpressing *cosR* were not stimulated by the higher O_2 concentrations to enhance the biofilm formation. Thus, CosR seems to be crucial for initiation of the maturation phase of *C. jejuni* biofilm development. This might be the reason of arisen discrepancy between our work and the one published by Oh and Jeon (2014). The authors used Mueller Hinton broth and higher temperature of cultivation. These factors were previously found to increase the biofilm formation of *C. jejuni* (Reeser et al., 2007). The usage of supportive cultivation conditions in combination with enhanced initiation of biofilm maturation caused by overexpression of CosR might result in earlier dispersal of cells and microcolonies from mature biofilm. Such acceleration of dispersal would result in reduction of biofilm mass attached to the surface of the well and *cosR* transformant would therefore seem to be less biofilm forming. This is in accordance with our observation of the dramatic biovolume decrease after 48 h of cultivation (from 600- to 16-times more biofilm mass than the parental strain) observed for the transformant. Nevertheless, further experiments should be performed in order to confirm or refuse this hypothesis.

The regulator CosR was initially identified as a potential regulator of ROS scavengers by promoting or repressing genes encoding KatA, AhpC, and SodB in *C. jejuni* (Hwang et al., 2011, 2012). It was also differently expressed after a superoxide stress induced by paraquat (Garenaux et al., 2008). Binding to the promoter of *luxS*, CosR might also be contributing to a quorum sensing system (Hwang et al., 2011). Recently, it was also demonstrated that CosR is involved in the expression of the

antibiotic efflux pump CmeABC in *C. jejuni* (Hwang et al., 2012). In this study, role of CosR in the maturation of *C. jejuni* biofilm, independently of oxidative stress, adds a new element in favor of its pleiotropic function in the main metabolic processes allowing the survival of *C. jejuni* in response to environmental stresses.

In contrast to its highly restricted catabolic capacity, *C. jejuni* is able to develop strategies to survive environmental oxidative stress using O_2 as an advantage for biofilm development. As *C. jejuni* is equipped to withstand oxidative stress through cooperation of subpopulations within a biofilm, further analyses are required to assess if this feature could explain the survival of this emerging pathogen in slaughterhouses, after evisceration, during food processing, or during macrophage attack. In addition, these findings advocate further studies to analyze quiescent, dormant and sessile *C. jejuni* cells and cell cooperation in response to environmental stresses, to identify the underlying cellular and molecular mechanisms supporting the persistence and resistance of this mysterious pathogen.

Materials and Methods

Bacterial Strains and Culture Conditions

All experiments were performed using three strains of *C. jejuni*: two well-documented clinical isolates 81-176 and NCTC 11168 purchased from general collections, and a *cosR* overexpressing transformant built for this study as described below. All strains were subcultured from the stock stored at −80°C by cultivation on Karmali agar plates (Oxoid, UK) at 42°C for 48 h in MAC (5% O_2, 10% CO_2, and 85% N_2, namely MAC). Grown colonies were inoculated onto Karmali agar plates and incubated either for 24 h at 42°C in MAC, or for 42 h in oxygen-enriched conditions (19% O_2, 10% CO_2, and 71% N_2, namely OEC) to allow acclimation of cells to oxidative stress. The OEC were previously described as a sublethal atmosphere not repressing growth of *C. jejuni* NCTC 11168 and 81-176 (Sulaeman et al., 2012). The gas conditions were maintained using hermetic stainless steel jars vacuum flushed and then filled with commercially purchased gas mixture. The process was repeated two times to minimize air residua in the cultivation atmospheres. The growth rates of all tested strains were determined from cultivation in BHI (Merck, Germany) in MAC using plate counts in triplicates with the appropriated decimal dilution.

Construction of the *cosR* Overexpressing Strain

For the construction of the *cosR* overexpressing strain, the cj0355c (*cosR*) gene was amplified from the strain NCTC 11168 using PCR primers Cj0355c F and Cj0355c R (Supplementary Table 6). The positions of the forward and reverse primers were chosen upstream and downstream of *cosR* within the *folB* (start position at 325186) and *fdxB* (end position at 323902) genes, respectively, to ensure that *cosR* was under the control of its own promoter. The PCR product was purified using the Qiagen PCR purification kit (Toronto, ON, Canada) and then cloned into the pRRK-1 plasmid (Reid et al., 2008). The cloning step was achieved using the Clontech In-Fusion™ PCR cloning kit (Mountain View, CA, USA). Briefly, the primers were designed with 15 bp extensions that allow recombination with the nucleotides flanking the XbaI restriction site on the pRRK vector. The recombinant pRRK + *cosR* plasmid was transformed into Fusion-Blue competent cells and positive transformants were selected on LB agar plates supplemented with Km. The cloned plasmid with the *cosR* gene was extracted from the grown transformants, purified, and sequenced to confirm the absence of point mutations. The plasmid was then naturally transformed into *C. jejuni* NCTC 11168 grown to mid-log phase. Following incorporation of the *cosR* into the chromosome was achieved by heterologous recombination. The location of the inserted gene was determined by amplifying three possible insertion sites on the chromosome using the ak233, ak234, ak235, and AR56 primers (Supplementary Table 6). The expected PCR product size was detected using the ak234 and AR56 primers indicating that *cosR* was inserted downstream of *cj0431*. The NCTC 11168 + *cosR* + Km^R strain is henceforth referred to as the *cosR* overexpression transformant or, for simplicity, the "transformant" or "Trf*cosR*." The growth rates of the parental NCTC11168 strain and the transformant were similar (μ_{max} = 0.69 and 0.72 h^{-1}, respectively). The overexpression of *cosR* was validated using quantitative RT-PCR after RNA extraction according to Sulaeman et al. (2012) with the following modifications. The quantity of total RNA was assessed using a Nanodrop 2000 (Thermo Fisher Scientific, Courtaboeuf, France), and the integrity of the RNA was verified with an Experion™ Automated Electrophoresis Station (BioRad) using the Experion RNA StdSens Analysis Kit (BioRad) according to the manufacturer's guidelines. Absence of DNA in the samples was confirmed by PCR with primers targeting *flaA* (Supplementary Table 6). Only high quality RNA samples without DNA contamination were used in qRT-PCR assays.

Adhesion to an Inert Surface

The adhesion capability of *C. jejuni* strains was determined using the BioFilm Ring Test® (BioFilm Control, France) as described previously by Sulaeman et al. (2010). Briefly, each culture was pelleted and resuspended in buffered peptone water, the OD_{600nm} was adjusted to 1 and suspension was used for inoculation of plate wells. After 2 h of cultivation under MAC at 42°C, adhesion was determined by measuring BFI (Biofilm Index) using the BioFilm Control developed software. The BFI correlates to the number of magnetic microbeads detected after well magnetization. The ΔBFI was calculated by subtracting the BFI of blank control from the BFI of the sample. The assay was repeated three times with three technical replicates for each independent culture.

Biofilm Formation

The crystal violet biofilm assay was used to determine the amount of biofilm produced by *C. jejuni*. The protocol was adapted from that described by Djordjevic et al. (2002). Briefly, 2 ml of *C. jejuni* suspension was inoculated in 24-well sterile microtitre plates. Each plate was incubated statically for 24, 48, or 72 h at 42°C in MAC. After cultivation, planktonic cells were washed out and biofilm was stained with 1% crystal violet solution. The crystal violet bound to the biofilm was then eluted using 99% ethanol and the absorbance of the eluate was measured at 595 nm.

Qualitative (ultrastructural) and quantitative data (maximum thickness and biomass volume) of *C. jejuni* biofilm were measured on biofilm produced in 96-well polystyrene microtitre plates with a μ clear® base (thickness of 190 ± 5 μm; Greiner Bio-one, Germany). Prior to the inoculation of microtitre plates, grown cells were transferred from Karmali plates into BHI, washed once and resuspended in sterile BHI to final OD_{600nm} = 0.8 ± 0.05. The suspension was then loaded onto the microtitre plate in triplicates for each strain (250 μl per well). The plates were incubated in MAC or OEC for 4–5 h at 37°C allowing *C. jejuni* cells to adhere to the substratum. After that, the bacterial suspension was carefully replaced with 250 μl of sterile BHI and microtitre plates were incubated for the next 24 and 48 h at 37°C in MAC or OEC, depending on the experiment. The μ clear® base material allows diffusion of gas molecules into the liquid media and therefore ensures formation of biofilm that is attached to the bottom of the well and not floating on the air-liquid interphase. Finally, wells containing biofilm were stained using 50 μl of Syto 9 solution (Invitrogen, USA) diluted in BHI to the final concentration of 2 μl/ml. The Syto 9 is cell-permeable dye intercalating with DNA and therefore staining the cells and the eDNA of biofilm matrix. All biofilms were observed using confocal laser scanning microscope (CLSM) as described below. For each condition, three independent replicates were analyzed.

Confocal Laser Scanning Microscopy (CLSM) Image Acquisition

Each well of the microtitre plates was scanned using the inverted Leica SP2 AOBS confocal laser scanning microscope (LEICA Microsystems, Germany) at 400 Hz with a 40x/0.8 water immersion objective lens Leica HCX Apo. The fluorophore Syto 9 was excited with a 488-nm argon laser. The whole well area was inspected to verify the presence of biofilm, then the most representative place was scanned providing a stack of horizontal planar images (512 × 512 pixels representing an area of 375 × 375 μm) with a z-step of 1 μm. At least one stack of horizontal planar images was acquired for each replicate.

Image Analysis

The stacks obtained from the microscopic observations were processed using Imaris 7.6.4 software (Bitplane, Switzerland). Images representing an aerial view of biofilm structure were rendered using the Easy 3D view with the auto-adjustment function to correct pixel intensities. Numerical data and 3D models of the biofilm structures were generated using the surface generator function of the Measurement Pro module with the minimal threshold set at 40 for the green channel (Syto 9). Only objects bigger than 10 voxels were included in the analysis. Biofilm development was normalized according to height (thickness determined from z-stacks as the last image showing consecutive signal from biofilm structures) and biomass volume (cell abundance).

Statistical Analyses

The numerical data obtained from Imaris were processed with STATGRAPHICS Centurion 16.1.11 software (StatPoint, Inc., Herndon, VA, USA) with the maximum height (biofilm thickness) and the biomass volume (cell abundance) as explanatory values. For all variance analyses, ANOVAs were performed to determine the individual effect of each factor and potential interacting effects with the confirmation of a normal distribution for each data set.

Assay variations were excluded from interacting effects, as they were not significantly different at the first order. The significance level was selected at 99%, consequently an effect was considered significant if its *P*-value was lower than 0.01. All *F-ratios* were based on the average residual squared error. When the transformant (Trf*cosR*) was used, a multiple comparison using the Scheffé method was implemented in ANOVAs to classify the significant variations (at 95% confidence) according to the strains.

For cell adhesion to an inert surface and crystal violet biofilm assays, significant differences were determined using two-sided Student's *t*-test comparisons at a 95% significance level with the confirmation of a normal distribution for each data set.

Author Contributions

HT, RB, and OT conceived and designed the study. NH, MH, and AS built and validated the transformant. HT, RR, and RB performed the experimental work. HT prepared the manuscript and RB and OT contributed to the final manuscript.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00709

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Adhesion, Biofilm Formation, and Genomic Features of *Campylobacter jejuni* Bf, an Atypical Strain Able to Grow under Aerobic Conditions

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Campylobacter jejuni is the leading cause of bacterial enteritis in Europe. Human campylobacteriosis cases are frequently associated to the consumption of contaminated poultry meat. To survive under environmental conditions encountered along the food chain, i.e., from poultry digestive tract its natural reservoir to the consumer's plate, this pathogen has developed adaptation mechanisms. Among those, biofilm lifestyle has been suggested as a strategy to survive in the food environment and under atmospheric conditions. Recently, the clinical isolate *C. jejuni* Bf has been shown to survive and grow under aerobic conditions, a property that may help this strain to better survive along the food chain. The aim of this study was to evaluate the adhesion capacity of *C. jejuni* Bf and its ability to develop a biofilm. *C. jejuni* Bf can adhere to abiotic surfaces and to human epithelial cells, and can develop biofilm under both microaerobiosis and aerobiosis. These two conditions have no influence on this strain, unlike results obtained with the reference strain *C. jejuni* 81-176, which harbors only planktonic cells under aerobic conditions. Compared to 81-176, the biofilm of *C. jejuni* Bf is more homogenous and cell motility at the bottom of biofilm was not modified whatever the atmosphere used. *C. jejuni* Bf whole genome sequence did not reveal any gene unique to this strain, suggesting that its unusual property does not result from acquisition of new genetic material. Nevertheless some genetic particularities seem to be shared only between Bf and few others strains. Among the main features of *C. jejuni* Bf genome we noticed (i) a complete type VI secretion system important in pathogenicity and environmental adaptation; (ii) a mutation in the *oorD* gene involved in oxygen metabolism; and (iii) the presence of an uncommon insertion of a 72 amino acid coding sequence upstream from *dnaK*, which is involved in stress resistance. Therefore, the atypical behavior of this strain under aerobic atmosphere may result from the combination of insertions and mutations. In addition, the comparison of mRNA transcript levels of several genes targeted through genome analysis suggests the modification of regulatory processes in this strain.

Keywords: food borne pathogen, biofilm, confocal microscopy, oxidative stress, genome sequence

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INTRODUCTION

Campylobacter is a Gram-negative bacterium, spiral-shaped and motile. This human pathogen lives as commensal of the gastrointestinal tract of most warm-blooded animals, especially poultry but also mammals (Park, 2002). Human infection by *Campylobacter* is commonly associated to the consumption of contaminated poultry meat. The genus *Campylobacter* includes very heterogeneous species that are present in a variety of environments but more than 80% of confirmed cases of campylobacteriosis were reported to be associated to *Campylobacter jejuni* (EFSA and ECDC, 2016).

The clinical manifestation of campylobacteriosis is severe gastro enteritis. However, *Campylobacter* infection is occasionally a precursor of serious post-infectious illness, including immune-reactive complications such as Guillain Barré and Miller Fisher Syndromes, two chronic and potentially fatal forms of paralysis (WHO, 2013). Since 2005, *Campylobacter* has been the most commonly reported human gastrointestinal bacterial pathogen in the European Union (EFSA and ECDC, 2016). In 2014, 236,851 cases of human campylobacteriosis were reported in EU. This zoonosis represents an incidence rate of 71 per 100,000 population exceeding the number of salmonellosis, which has a notification rate of 23.4 cases per 100,000 population. In addition, the cost of campylobacteriosis to public health systems and the loss of individual health and productivity were evaluated around 2.4 billion Euros per year in Europe (EFSA and ECDC, 2016) and between 1.2 and 4 billion $ for the US (Eberle and Kiess, 2012; Batz et al., 2014). The need for controlling this pathogen along the food chain explains the numerous studies reported in the literature that aimed at understanding its metabolism and virulence.

Campylobacter jejuni presents specific growth requirements, as it is thermotolerant with an optimal growth temperature of 40–42°C, microaerophilic (optimal O_2 concentration of 5%), and capnophilic requiring 10% CO_2 for an optimal growth. However, *C. jejuni* is able to persist in different environmental stress conditions explaining its high prevalence around the world. This food-borne pathogen has indeed developed adaptation mechanisms to survive under various harsh conditions it can encounter, from poultry gastrointestinal tract to the consumer's plate. One of the most important characteristics of this bacterium is its ability to survive in aerobic environments despite its microaerophilic nature. This suggests an ability to cope with oxidative stress mediated by environmental oxygen tension and reactive oxygen species. To survive against such stresses, biofilm formation has been suggested to be one of the strategies used by this pathogen to persist in the environment (Buswell et al., 1998; Nguyen et al., 2012; Turonova et al., 2015). Commonly, biofilms are defined as multicellular layers of bacteria embedded within a matrix of extracellular polymeric substances (EPSs; Costerton, 1995; Costerton et al., 1995; Donlan, 2002; Donlan and Costerton, 2002). *C. jejuni* strains have been reported to be able to form different types of biofilm characterized as a structure attached to a surface, a pellicle formed at the surface of the liquid, or aggregates floating in the liquid culture (Joshua et al., 2006). Recently, we have reported the atypical property of *C. jejuni* Bf, a strain able to grow on plates under aerobic atmosphere, thus with a very low concentration of CO_2 (0.035%), but with 21% O_2 (Rodrigues et al., 2015). The possible growth of *C. jejuni* strains under aerobiosis and after various oxidative stresses was previously reported (Chynoweth et al., 1998; Garénaux et al., 2008b; Hinton, 2016). The aim of this study was to investigate the ability of *C. jejuni* Bf to adhere to biotic and abiotic surfaces and to form biofilm. We compared the behavior of this strain under both microaerobiosis and aerobiosis to determine a possible increased capacity to resist to the presence of high level of O_2, which can be encountered during meat products processing and storage. Finally, genome comparison was also performed in order to detect genetic elements putatively involved in the phenotype of this strain. For that purpose, the draft genome (Bronnec et al., 2016) was completed and the gene and metabolic repertoires of *C. jejuni* Bf were compared to those of other complete or draft genomes.

MATERIALS AND METHODS

Bacterial Strain and Culture Conditions

Stains used in this study are presented **Table 1**. *C. jejuni* strains were stored at −80°C in Brain Heart Infusion broth (BHI) containing 20% (vol/vol) glycerol. Prior to each experiment frozen cells were streaked on Karmali agar plates (Oxoid Limited, UK), incubated at 42°C for 24 h under microaerobic conditions in CampyGen sachet (Oxoid Limited, UK): 5% oxygen, 10% carbon dioxide, and 85% nitrogen.

As described previously by Rodrigues et al. (2015), *C. jejuni* Bf cells can be acclimated to aerobic conditions (namely AAC cells for aerobically acclimated cells). This was performed by sub-culturing three times (once for 48 h and then twice 24 h) on Karmali agar plates under aerobiosis (air; Rodrigues et al., 2015). In order to maintain the same conditions for all samples, cultures under microaerobiosis were identically performed three times under microaerobiosis (MAC cells for microaerobic conditions).

Adhesion to Inert Surfaces

The adhesion capability was evaluated using BioFilm Ring Test® (BioFilm Control, France) as described by Sulaeman et al. (2010), with several modifications. Briefly, the experiments were performed using the kit commercialized by BioFilm Control (KITC004) including polystyrene Costar plates with flat bottom (Corning, USA), magnetic beads solution (TON004) and contrast liquid (LIC0001). Two conditions were tested for adhesion assay, microaerobiosis and aerobiosis. Grown cells were recovered from Karmali agar plates and suspended at 10^8 CFU/mL ($OD_{610\ nm} = 0.5 \pm 0.1$) in filtered BHI (provided with the kit). *C. jejuni* suspensions (200 µL), containing magnetic beads at 1% (vol/vol), were inoculated in Costar plate wells. After 2 h of incubation at 42°C, the adhesion capability of strains was evaluated by measuring a biofilm formation index (BFI) with the BFC Element 3 software (BioFilm Control, France). Assays were repeated at least three times with three technical replicates.

TABLE 1 | *Campylobacter jejuni* strains experimentally used this study.

Origin	Name*	Source	Reference (published genome)
Clinical	*Cjj* NCTC 11168; ATCC 700819	Diarrheic patient	Parkhill et al., 2000; Gundogdu et al., 2007
	Cjj 81-176	Outbreak	Fouts et al., 2006, Unpublished
	Cjd 269.97	Bacteremia	Fouts et al., 2007, Unpublished
	Cjj 81116; NCTC 11828	Outbreak	Pearson et al., 2007
	Cjj 00-2538	Outbreak	Clark et al., 2014, Unpublished
	Cjj 00-2544	Outbreak	Clark et al., 2014, Unpublished
	Cjj 00-2426	Outbreak	Clark et al., 2014, Unpublished
	Cjj 00-2425	Outbreak	Clark et al., 2014, Unpublished
	Cj Bf	Campylobacteriosis	Bronnec et al., 2016
Meat	*Cj* RM1221	Skin of a retail chicken	Fouts et al., 2005
Poultry	*Cjj* 327	Turkey slaughterhouse	Takamiya et al., 2011
	Cjj 305	Turkey slaughterhouse	Takamiya et al., 2011
	Cjj DFVF1099	Chicken isolate	Takamiya et al., 2011
Cattle	*Cjj* ATCC 33560	Bovine feces	Zeng et al., 2013a

**Cjj: Campylobacter jejuni subsp. jejuni; Cjd: Campylobacter jejuni subsp. doylei; Cj: Campylobacter jejuni.*

Confocal Laser Scanning Microscopy (CLSM)

Static Biofilm Formation Assay

Campylobacter jejuni Bf and *C. jejuni* 81-176 cells were recovered from Karmali agar plates and suspended in BHI at 10^8 CFU/mL ($OD_{610\,nm} = 0.5 \pm 0.1$). Two hundred microliters of bacterial suspension were inoculated in sterile 96-well polystyrene microtiter plates with a micro-clear® bottom 190 ± 5 μm (Greiner Bio One, Germany). Several incubation times (30 min, 1, 2, 4 h) at 42°C were tested to evaluate the minimum time required for adhesion of the cells at the bottom of the well. Adhesion was performed under microaerobiosis (with bacteria first grown under microaerobiosis) and under aerobiosis (with *C. jejuni* Bf grown under aerobiosis and *C. jejuni* 81-176 grown under microaerobiosis). Then, the bacterial suspension in the microtiter plate was carefully replaced with 200 μl of sterile BHI. Plates were then incubated at 42°C for 24 and 48 h under microaerobic or aerobic conditions. At least 1 h before the biofilm observation, the cells were stained by adding Syto 9 at 0.01 mM final concentration (LIVE/DEAD® Kit, Life Technologies, USA) directly into the wells, following the method of Turonova et al. (2015). Experiments were performed using three biological replicates. For each condition, three technical replicates were performed, and two acquisitions in each of them.

Confocal Laser Scanning Microscopy

After staining, image acquisition was performed using a spinning disk confocal microscope (Andor, UK; Olympus, Japan). The entire wells were first inspected to see biofilm formation and its global structure. Two different locations of each well were scanned using a 10X objective lens with the signal recorded in the green channel (excitation 488 nm, emission 500–525 nm). The chosen place for the acquisition was representative of the whole structure and a stack of horizontal planar images with a size of x = 670.8 μm and y = 897.84 μm (e.g., 1040 × 1392 pixels) was scanned with a z-step of 1 μm. Video acquisitions were performed in a selected layer of the same size as described before using a 40X NA 1.4 oil immersion objective lens with an exposure time of 100 ms. Acquisitions were achieved in three distinct positions in the biofilm structure: the bottom, middle and top of the biofilm.

Image Processing

confocal laser scanning microscopy (CLSM) images from top to bottom were processed using IMARIS software (v 7.6, Bitplane AG, Switzerland). For visualization of the biofilm, shadow projections and three-dimensional structures were generated. Beside the biofilm appearance, quantitative structural parameters of biofilms were calculated. Biofilm volume and thickness were the selected parameters used to compare the architectural differences of the biofilms formed. The bio-volume corresponds to the total volume of cells in the acquired field ($x \times y \times z = \mu m^3$) and the thickness is the maximum height reached by the biofilm (μm).

Adhesion Assay to Epithelial Intestinal Cells *In vitro*

Human intestinal cell lines HT29 and HT29-MTX were used to compare adhesion abilities of *C. jejuni* NCTC 11168, *C. jejuni* 81-176 and *C. jejuni* Bf under microaerobic conditions. In addition, adhesion capabilities of *C. jejuni* Bf acclimated to ambient air were also assessed. Maintenance of cells and adhesion assays were performed according to Haddad et al. (2010). Briefly, intestinal cells were grown in Dulbecco's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), containing 200 mM L-glutamine, 250 μg/mL gentamicin (Sigma-Aldrich, USA) and 2.5 μg/mL amphotericin B (Sigma-Aldrich, USA). The cells were grown routinely in tissue culture flasks at 37°C in a 5% CO_2-humidified atmosphere.

For experimental assays, cultured cells were dissociated from plastic flasks using trypsin-EDTA solution (Invitrogen, USA)

and approximately 10^5 eukaryotic cells were seeded into each well of 24-well tissues culture tray and incubated for 5 days at 37°C in humidified atmosphere at 5% of CO_2. The cells were washed with DMEM and each well was inoculated with a suspension of approximately 10^7 CFU of bacteria. To evaluate the number of adhered bacterial cells, the infected monolayers were incubated for 1 h at 37°C in a humidified 5% CO_2 incubator and rinsed five times with phosphate buffered-saline (PBS, Eurobio, France). The cell monolayer was lysed by addition of 0.5 mL of Triton X-100 0.1% (Labo-Si, France) at room temperature for 30 min. *C. jejuni* cells were enumerated from the lysate on Karmali agar plates after 48 h incubation at 42°C under microaerobic condition. Experiments were performed using three biological replicates, and for each two technical replicates.

Genome Sequence Completion and Comparative Genomic Analysis

To complete the draft genome sequence of *C. jejuni* Bf (Bronnec et al., 2016), PCR amplifications were performed on regions presenting uncertainties and for gap-filling purpose on contig extremities with primers designed in the flanking regions of each gap and PCR products were sequenced (Biofidal, France). As genome comparison showed that *C. jejuni* Bf was closer to other genomes than that of the reference genome of *C. jejuni* NCTC 11168 a new mapping was performed on the closest complete genome available (*C. jejuni* ATCC 32488 SRZ049709). Automatic annotation was performed on the MicroScope platform (MaGe; Vallenet et al., 2006, 2013) and manually checked.

Nucleotide sequence accession number: this whole genome project has been deposited in ENA under the accession no. FCEZ01000001-FCEZ01000095. The version described in this paper is the second version, FCEZ01000001-FCEZ01000095.

Using the tools available on the MicroScope platform, genomic comparisons were conducted between *C. jejuni* Bf genome and other *C. jejuni* genomes listed in Supplementary Table S1. A total of 33 complete and 19 draft *C. jejuni* genomes were used. "PkGDB Synteny Statistics" tool was used to perform similarity analysis between *C. jejuni* Bf and all *C. jejuni* genomes available to date on the PkGDB database. "Gene phyloprofile" tool has enabled the genomic comparison by searching specific genes of *C. jejuni* Bf in comparison with the other genomes, with the following homology constraints: minLrap $\geq$ 0.8, maxLrap $\geq$ 0 and identity $\geq$ 30%.

RNA Isolation and Reverse Transcription

After growth AAC or MAC *C. jejuni* cells were recovered from Karmali plates and suspended in BHI at 10^8 CFU/mL ($OD_{610\,nm} = 0.5 \pm 0.1$). RNA isolation, control and reverse transcription were performed according to Haddad et al. (2012) with some modifications. Briefly, one milliliter of this suspension was centrifuged at 3,300 *g* for 6 min at 4°C, and then resuspended in 1 mL of Extract-All (Eurobio, France) and mixed with 0.2 mL of chloroform. After a centrifugation at 12,000 *g* during 15 min at 4°C, RNAs from the aqueous phase were precipitated with isopropanol, washed twice in cold 75% ethanol and then solubilized in 50 μL of RNase-free water. Samples were then treated with TurboDNase (Life Technologies, France) to remove potential DNA contamination. The integrity of RNA was verified using 1% agarose gel and quantified using a NanoDrop spectrophotometer (Thermo Scientific, France). Absence of DNA contamination was validated by PCR. RNA was isolated from three biological replicates. Reverse transcription was performed on 100 ng of RNA using the RevertAid H Minus First-Strand cDNA synthesis kit (Euromedex, France) using random hexamer primers according to the manufacturer's instructions.

Quantitative Real-Time PCR

The quantitative real-time PCR assay was performed using SYBR Green I (Applied Biosystems, USA) and MJ Research PTC-200 Thermal Cycler (GMI, USA). The chosen internal control was *rrs* (Hyytiäinen et al., 2012) with primers rrs_F AAGGGCCATGATGACTTGACG and rrs_R AGCGCAACCCACGTATTTAG. The studied genes were *cosR* (with primers cosR_F TTTGAAAGCTGGAGCTGATG and cosR_R GGTTCCGCCAAGTCTTAGTC) and *dnaK* (DnaK)_F AAACGCCAAGCGGTAACTAA and DnaK_R TTCTTTAGCCGCGTCTTCAT). The operon *oorDABC* (with primers oorD2_F TGCGGTTTTAGGACAAATGA and oorD2_R TTCATCTCTTTTTGCCACCA, oorA2_F GCGGCAATGAGTGGAGTAAA and oorA2_R TTGGAAGA CCTGTTGAAGGA, oorB2_F TGGTAAGTGGAGATGGGG ATA and oorB2_R GTTGGGCTTGTTTGGGAAT, oorC_F GTGGTGGCCCTACTAAGGTG and oorC_R AACCCTTATC TGCAGTCGAAA) was also studied. Finally, a CDS of unknown function (u30002_F TTCAGAACCTACGAGGATGGA and u30002_R TTCAATCCTCCAAGCACACA) located upstream from *dnaK* was also investigated. The PCR mix was prepared as follows: 100 ng to 1 μg of cDNA (for *cosR* expression or *oorDABC*, *dnaK,* and *u30002_F*), 1 μM of each primers and 12.5 μL of SYBR Green I Master Mix. The amplification program included an initial denaturing step of 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. A negative control was included in each run. Relative quantification of gene expression was calculated according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Results were normalized to the gene transcription of the reference strain *C. jejuni* 81-176 in microaerobic conditions. The experiments were performed in triplicate from three independent cultures. For each experiment, at least three technical replicates were realized.

Statistical Analysis

Adhesion results from Biofilm Ring Test were analyzed using Statgraphics Centurion software version 17.1.06 (Statpoint Technologies, USA). An analysis of variance (ANOVA) was assessed to determine the individual effect of each variable (species and atmosphere). Statistical data were completed using the Fisher LSD (least significant difference) technique for multiple comparisons with a significance level at 95%.

Numerical data on biofilm formation obtained from IMARIS were also assessed for an ANOVA. The two variables identified

were the maximum height of biofilm and the biomass volume. The two factors considered were the time of biofilm formation (24 or 48 h) and the combination strain/atmosphere, e.g., *C. jejuni* 81-176 grown under microaerobiosis (81-176$_{\mu O2}$), *C. jejuni* Bf under microaerobiosis (Bf$_{\mu O2}$) and *C. jejuni* Bf under aerobiosis (Bf$_{O2}$). This procedure allows the analysis of variance at several factors for each variable. Significant effects were considered when p-value < 0.05.

Results obtained for the adhesion assay to epithelial intestinal cells *in vitro* and from RT-qPCR were analyzed using Student's t-test. p-value < 0.05 were considered statistically significant.

RESULTS

Adhesion Capability and Biofilm Ultrastructure to Abiotic Surfaces

Ability to Adhere to Abiotic Surface

Adhesion assays using BioFilm Ring Test® method were conducted under microaerobic and aerobic conditions with an initial bacterial concentration of 5×10^6 CFU/well. According to the biofilm formation index measured with the BFC Element 3 software all strains showed adhesion capacity and could be classified into four groups: strains with strong ($0 \leq$ BFI < 4), delayed ($4 \leq$ BFI < 7), or weak adhesion ($7 \leq$ BFI < 16), and those showing no adhesion capacity (BFI ≥ 16; **Figure 1**).

Among the 13 strains tested the ability to adhere to polystyrene varied independently from their clinical, animal, or food origin. Three strains were considered as strongly adherent (*C. jejuni* subsp. *jejuni* 81116, 327 and *C. jejuni* subsp. *doylei* 269.97), six showed a delayed adhesion (*C. jejuni* Bf, NCTC 11168, RM1221, 00-2544, 00-2425, and 305), and three presented a weak adhesion (*C. jejuni* 00-2538, 00-2426, 81-176). *C. jejuni* DFVF1099 appeared non-adherent under microaerobiosis. Although, the BFI values did not significantly differ between microaerobiosis and aerobiosis. Aerobiosis improved adhesion of *C. jejuni* NCTC 11168, 81-176, 00-2425 and DFVF ($p < 0.05$), and only a statistically non-significant tendency to better adhere was observed for the other strains. As among these strains, *C. jejuni* Bf is the only one able to grow on plate under aerobic condition (Rodrigues et al., 2015), the adhesion capability of cells grown under aerobiosis was also tested. As shown **Figure 1** *C. jejuni* Bf grown aerobically was able to adhere to inert surface as well as cells grown microaerobically, and the BFI did not statistically differed between these two conditions. Although, our adhesion results seemed contradictory with previous studies (Gunther and Chen, 2009; Sulaeman et al., 2010; Turonova et al., 2015), we chose to explore the capacity of biofilm formation of *C. jejuni* Bf in comparison to *C. jejuni* 81-176 because this virulent strain is consistently capable of producing mature biofilm (Gunther and Chen, 2009) and often considered as the reference. In addition, this strain could be used as a positive control for biofilm formation by CLSM and its well annotated genome was available.

Biofilm Development and Three-Dimensional Structure

We determined that a period of 2 h of adhesion to the polystyrene resulted in optimal initiation of biofilm formation for the two strains (data not shown).

After 24 h at 42°C under microaerobiosis, *C. jejuni* 81-176 developed a compact and highly structured biofilm strongly condensed at well center (**Figure 2A**, Supplementary Figure S1A). After 48 h of incubation the biofilm observed was quite similar with thick and dense structures (data not shown). Under the same conditions, *C. jejuni* Bf was also capable of forming biofilm but its structure seemed more expanded in the well and more flat in comparison with that of *C. jejuni* 81-176 (**Figure 2B**, Supplementary Figure S1B). The structure was less compact with a patchy coverage of the surface and composed by few large and compact structures and several microcolonies (**Figure 2B**, Supplementary Figure S1B).

During incubation under aerobiosis *C. jejuni* 81-176 did not develop any biofilm but rather, harbored microcolonies of surface attached cells (**Figure 2C**). In contrast *C. jejuni* Bf biofilm appeared more compact and structured under aerobic condition, as compared to the one formed in microaerobiosis (**Figure 2D**, Supplementary Figure S1C). After 48 h of cultivation at 42°C, biofilm formed by *C. jejuni* Bf was more compact with micro colonies less spread around the surface of the well (data not shown).

Quantification and Comparison of Biofilm Structures

The quantity of biofilm was characterized using two variables: bio-volume and maximum thickness. The individual effect of different factors (duration of cultivation, strain, atmosphere) on the two variables were considered (**Figure 3**). For each variable, the period of biofilm cultivation (24 or 48 h) had no significant effect. Multiple-comparison procedure was used to determine the significantly different means (Supplementary Table S2). For maximum thickness the Fisher's LSD method revealed two significantly different groups T1 and T2. The first group (T1) encompasses biofilm structure formed by *C. jejuni* 81-176 and the second group (T2) is composed by biofilms formed by *C. jejuni* Bf under both microaerobic and aerobic conditions. Conversely, a unique homogeneous group (V) was obtained when considering biofilm volume, independently from the strain or the conditions tested.

Cell Motility Observation

As reported previously (Turonova et al., 2015), we observed motile *C. jejuni* 81-176 cells at different locations of the biofilm structure (e.g., at the bottom, middle, and top) after 24 and 48 h of biofilm formation. Similarly, a subpopulation of *C. jejuni* Bf also showed the capacity to move within the biofilm structure in the two conditions tested (Supplementary files S1 and S2). A better motility was detected at the bottom of the biofilm where the structure is more dispersed. No obvious difference was observed in the motility of *C. jejuni* Bf under microaerobiosis or aerobiosis.

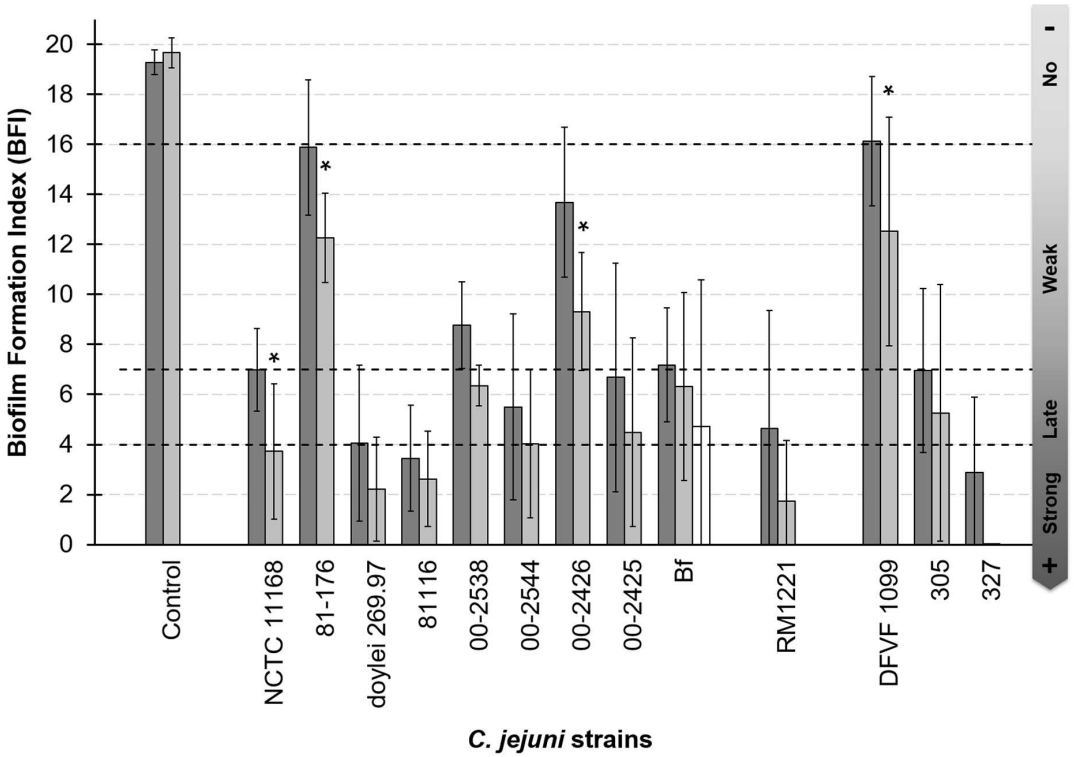

FIGURE 1 | Adhesion capability to polystyrene of *Campylobacter jejuni* is strain dependent. Adhesion capability was measured after 2 h of incubation at 42°C under microaerobiosis with MAC grown cells (dark bars) and under aerobiosis with MAC grown cells (gray bars). White bar indicate biofilm formation index under aerobiosis of *C. jejuni* Bf AAC cells. The nine first strains were clinical isolates, the 10th was isolated from meat and three last strains were isolated from poultry. Four biological replicates were performed in microaerobiosis and three in aerobiosis. Three technical replicate were realized each time. For the control and each strain, the mean values of BFI between O_2 and μO_2 conditions were compared. Asterisks show when these values were statistically different between the two conditions (p-value < 0.05).

C. jejuni Bf Adhesion to Epithelial Intestinal Cells *In vitro*

In addition to interaction with abiotic surfaces, we also determined the ability of *C. jejuni* Bf to adhere to biotic surfaces. For that purpose, the adhesion of *C. jejuni* Bf to HT29 and HT29-MTX cells was compared to those of *C. jejuni* 81-176 and NCTC 11168. The presence or absence of mucus did not significantly affect the adhesion of *C. jejuni* Bf and *C. jejuni* NCTC 11168 strains to intestinal cells (p-value < 0.05), whereas *C. jejuni* 81-176 adhered better to mucus producing cells (**Figure 4**).

Under microaerobic conditions, *C. jejuni* Bf exhibited a significantly (p-value < 0.05) higher adhesion capability than the two reference strains, independently on the cell line used for experiment (**Figure 4**). In addition, after growth under ambient atmosphere *C. jejuni* Bf showed the same adhesion properties than after growth under microaerobiosis (**Figure 4**).

Genome Analysis

The analysis of the draft genome of *C. jejuni* Bf did not reveal any clear gene acquisition or deletion which could explain its ability to grow under aerobiosis (Bronnec et al., 2016). In the present study we completed the genome sequence and a deeper analysis of the gene repertoire of this strain was conducted. We first searched in the genome of *C. jejuni* Bf for functions that could potentially be involved in the singular phenotype of this strain: ability to grow, to adhere and to form biofilm independently from aeration conditions. A list of 165 *C. jejuni* genes reported in the literature as important for biofilm formation, adhesion, and oxygen metabolism was established (Supplementary Table S3) and their presence was searched in *C. jejuni* Bf genome. Some of these genes were putatively involved in several functions, also involved in adhesion to eukaryotic cells, or were reported to be affected by oxidative stress. Therefore, we considered them as significant for our study. Most of the literature dedicated to stress resistance and biofilm formation by *C. jejuni* focused on reference strains such as NCTC 11168, 81-176, and 81116. However, this species presents an important genomic diversity (Jeon et al., 2010; Zeng et al., 2013a). Therefore, we also compared the *C. jejuni* Bf genome sequence to 52 (complete or draft) *C. jejuni* genomes to search for genes that could be mutated or specific of *C. jejuni* Bf.

Gene Repertoire of *C. jejuni* Bf Related to Biofilm Formation and Adhesion

Many genes have been reported as directly or indirectly related to the biofilm development although the molecular mechanisms

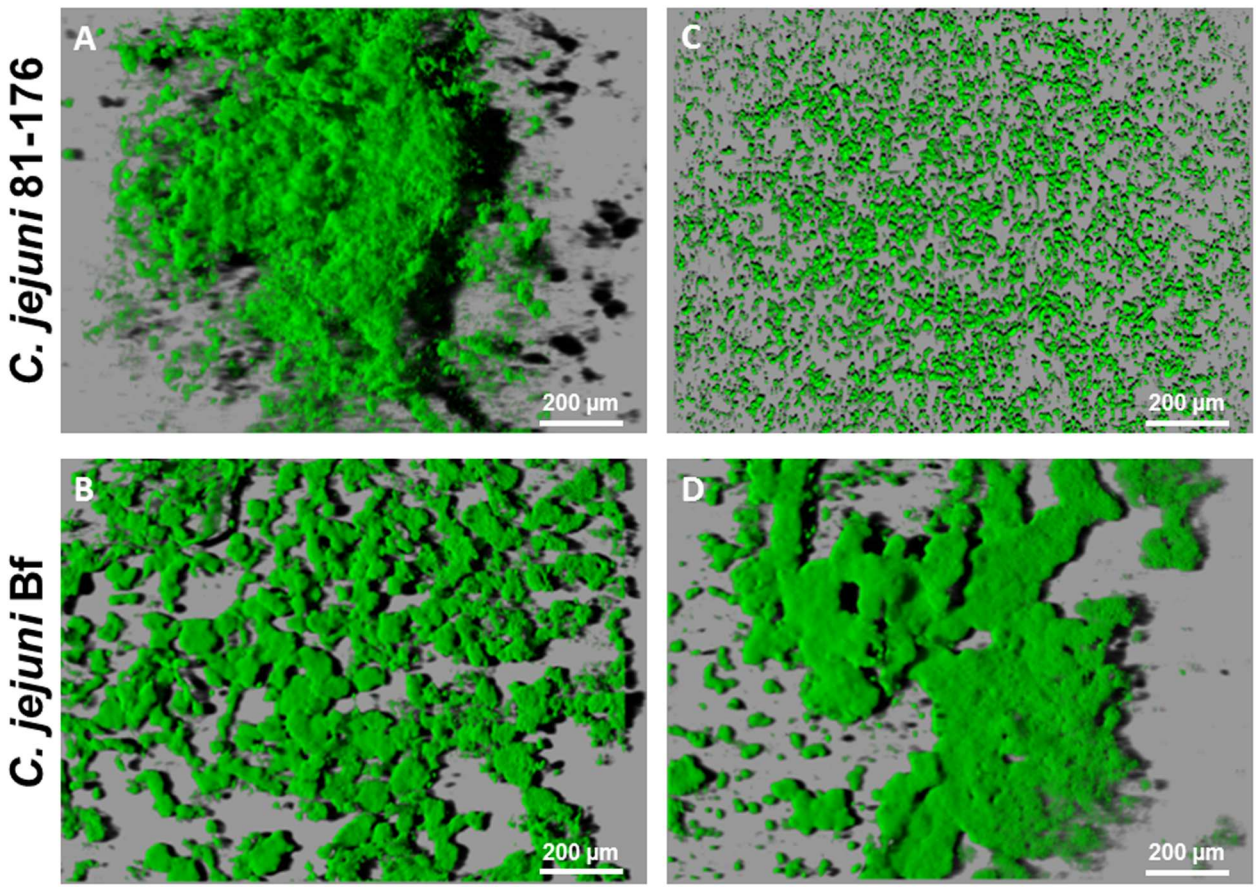

FIGURE 2 | *Campylobacter jejuni* biofilm architecture after IMARIS processing of CLSM images from top to bottom. Shadows projections were acquired for the biofilm developed after 24 h incubation at 42°C. Under microaerobiosis *C. jejuni* 81-176 **(A)** and *C. jejuni* Bf **(B)** established a biofilm. Under aerobiosis *C. jejuni* Bf **(D)** was able to develop a biofilm and *C. jejuni* 81-176 **(C)** persisted in its planktonic state.

of their involvement are not clearly understood in *C. jejuni*. From various studies on *C. jejuni* we have selected 64 genes potentially required for strong biofilm formation and searched for their presence/absence in the genome of *C. jejuni* Bf. The results are presented Supplementary Table S3. Only four out of the 64 genes were missing in *C. jejuni* Bf. These correspond to CDS tagged as *cj0628*, *cj0755*, *cj1564,* and *cj1725* in *C. jejuni* NCTC 11168. The gene *cj0628* encodes CapA (*Campylobacter* adhesion protein A) an auto-transporter which was considered as an adhesin necessary for adhesion to Caco-2 cells and chicken colonization (Ashgar et al., 2007). The gene *cj0755* encodes the ferric enterobactin receptor CfrA and is overexpressed in *C. jejuni* NCTC 11168 biofilm cells but its absence has already been reported in other *C. jejuni* strains (Kalmokoff et al., 2006; Zeng et al., 2013a,b; Sung and Khan, 2015). Tlp3, a transducer-like protein recently renamed CcmL (Rahman et al., 2014) for *Campylobacter* chemoreceptor for multiple ligands is encoded by *cj1564*. A mutation of *ccmL* reduce motility and enhance biofilm formation in *C. jejuni* 11168-O (Rahman et al., 2014). These three genes and the putative periplasmic protein *cj1725*; also overexpressed in *C. jejuni* NCTC 11168 biofilm cells (Kalmokoff et al., 2006); are absent from *C. jejuni* Bf as previously reported for other *C. jejuni* genomes (Pearson et al., 2007; Hepworth et al., 2011).

A number of *Campylobacter* genes have been previously described as mediating *in vitro* adhesion to human cells. Most of these genes were present in *C. jejuni* Bf genome (Supplementary Table S3). Among those, genes encoding the fibronectin binding proteins CadF (Konkel et al., 1997; Ziprin et al., 1999; Monteville et al., 2003) and FlpA (Flanagan et al., 2009; Konkel et al., 2010), the adhesins PEB 1, PEB 4 (Kervella et al., 1993; Pei et al., 1998; Del Rocio Leon-Kempis et al., 2006; Asakura et al., 2007), and JlpA (Jin et al., 2001) were recorded in *C. jejuni* Bf. Moreover, the membrane proteins known to be involved in adhesion step, such as the major outer membrane protein MOMP, a porin (Moser et al., 1997), and KpsE involved in the export of the capsular polysaccharide (Bachtiar et al., 2007) were found on *C. jejuni* Bf genome. As well, the lipooligosaccharide (LOS) biosynthesis gene cluster composed of 14 genes flanked by *waaC-htrB* and *waaV-waaF* was also present. Moreover, the genes *cstII* and

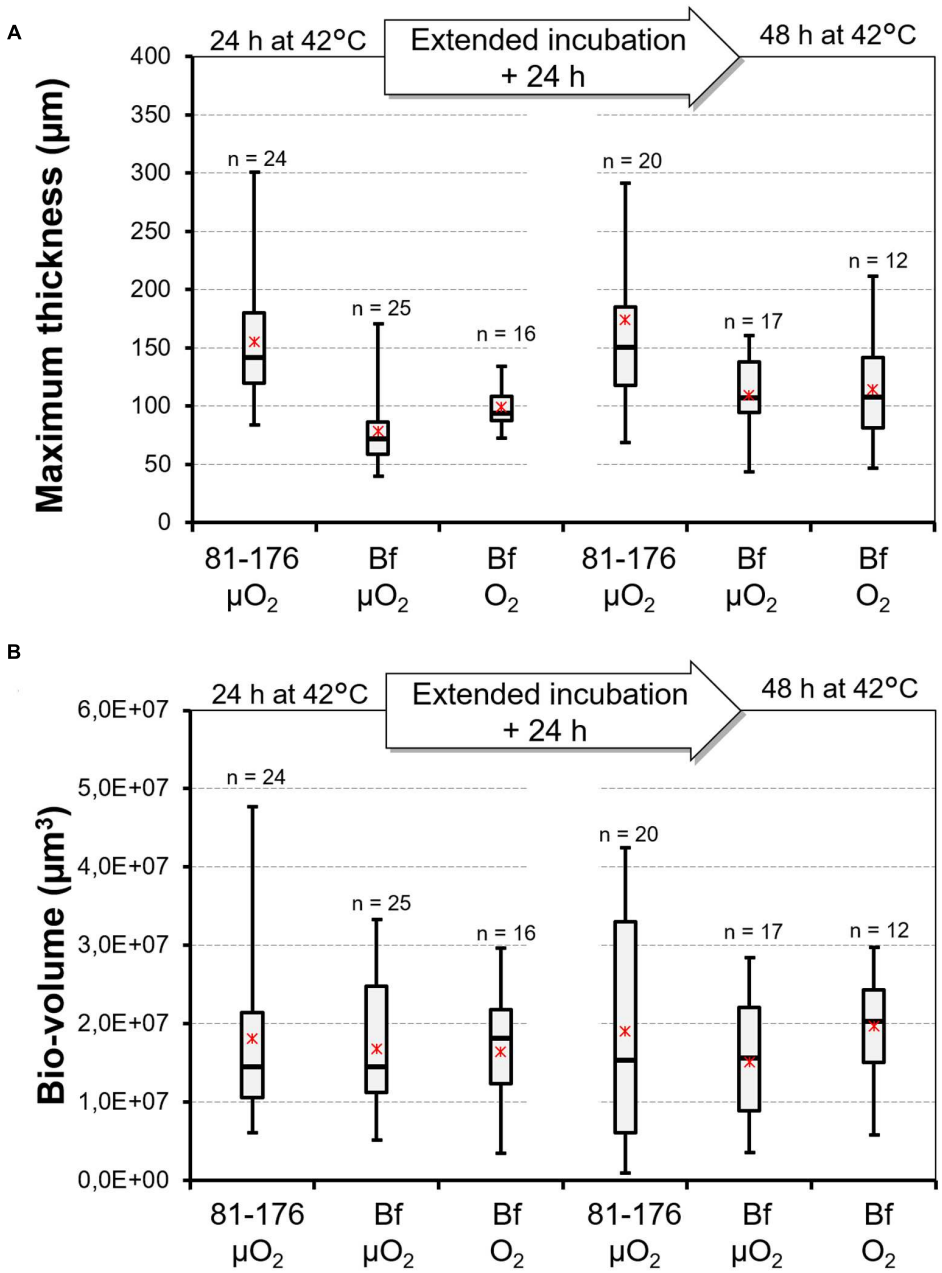

FIGURE 3 | Distribution of data describing the biofilm architecture of *C. jejuni* 81-176 and *C. jejuni* Bf. Box plot representing the distribution of maximum thickness **(A)** and bio-volume **(B)** values observed for the biofilms developed by *C. jejuni* 81-176 and *C. jejuni* Bf under microaerobiosis (μO_2) and under aerobiosis (O_2) after 24 and 48 h of incubation at 42°C. Minimum and maximum values are reported. Asterisks indicate the mean and dashes the median. The number of repeats was added above each box plot.

neuBCA responsible for the sialylation of LOS (Parker et al., 2005, 2008) were observed in the genome of *C. jejuni* Bf. Interestingly, *C. jejuni* Bf possesses the 13 genes encoding an entire type VI secretion system (T6SS; Bleumink-Pluym et al., 2013) firstly described in *C. jejuni* by Lertpiriyapong et al. (2012), including *hcp* and *icmF1* genes.

Although *C. jejuni* Bf possesses a large repertoire for adhesion and biofilm formation, some genes previously described as related to adhesion were absent from its genome. As mentioned above, the gene encoding the autotransporter protein CapA (Ashgar et al., 2007) is absent from *C. jejuni* Bf genome. In addition, the γ-glutamyltranspeptidase (GGT) involved in colonization of chicken is also absent from this strain. These genes are also absent in many *C. jejuni* isolates (Flanagan et al., 2009; Floch et al., 2014), for which the biofilm forming ability is yet unknown.

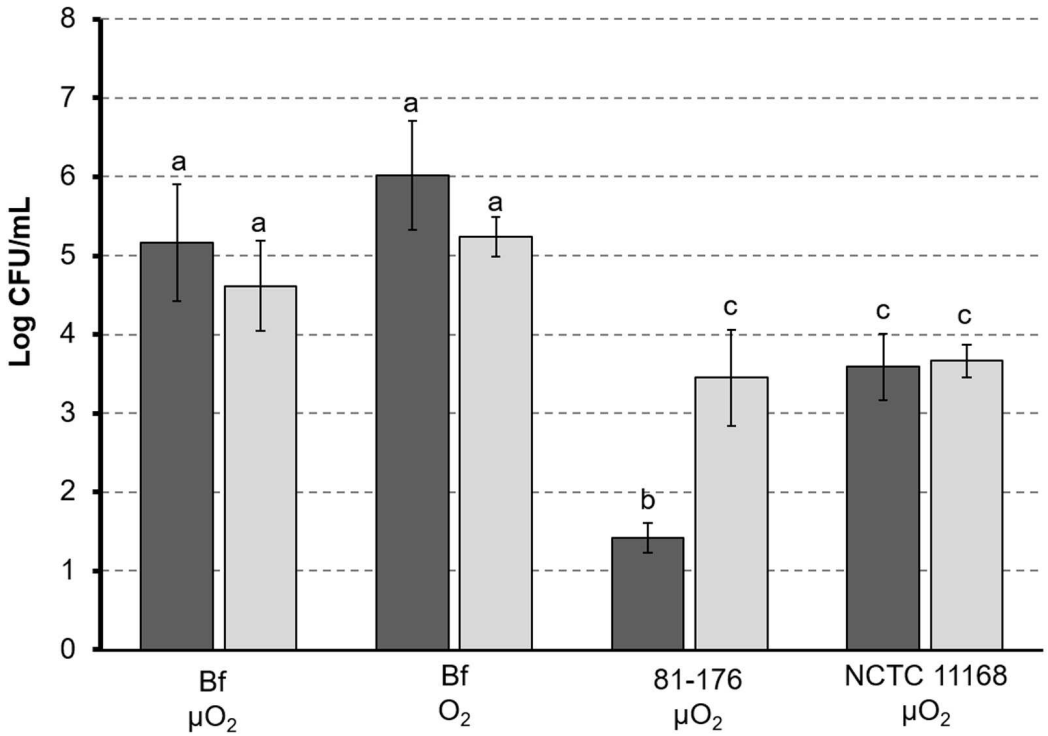

FIGURE 4 | Adhesion of *C. jejuni* to intestinal cells. *Campylobacter jejuni* 81-176 and *C. jejuni* NCTC 11168 were grown under microaerobic conditions (μO_2) and *C. jejuni* was cultured under microaerobic conditions (μO_2) or ambient atmosphere (O_2). Cells lines HT29 (black) and HT29-MTX producing mucus (gray) were used for adhesion tests. Adhesion of *C. jejuni* is expressed as amount of bacterial cells (expressed as CFU/mL) released from lysed eukaryotic cells after 1 h adhesion. Letters indicate values statistically similar (p-value < 0.05).

Gene Repertoire to Cope with Oxygen

Various enzymes and proteins are thought or known to protect bacteria against oxidative stress. Among them seven main enzymes/proteins and few regulators are well-documented in *C. jejuni* (Pesci et al., 1994; Grant and Park, 1995; Baillon et al., 1999; Ishikawa et al., 2003; Atack et al., 2008; Butcher et al., 2010; Hwang et al., 2011; Flint et al., 2014; Kim et al., 2015). These proteins involved in peroxide or superoxide detoxification include the alkyl hydroxyperoxide reductase (AhpC), the superoxide dismutase (SodB), the catalase (KatA) and Cj1386, the thiol peroxydase (Tpx), the bacterioferritin co-migratory protein (Bcp), and the bacterioferritin (Dps). The regulators Fur, PerR, and CosR have been reported to be involved in oxidative stress response. All the genes encoding enzymes or regulators involved in oxidative stress response are present in the genome of *C. jejuni* Bf (Supplementary Table S3).

A complete aerobic respiration pathway was detected with *ccoNOQP*, *petABC*, *cydAB nuoABCDEFGHIJKLMN*, and *sdhBC* gene clusters encoding cytochrome c oxidase, cytochrome bc and cytochrome bd complexes, NADH quinone oxidoreductase, and succinate dehydrogenase, respectively. As previously reported (Bronnec et al., 2016) the gene *oorD,* from the gene cluster *oorDABC* encoding 2-oxoglutarate oxidoreductase – a component of tricarboxylic acid (TCA) cycle – harbors a point mutation that may affect its activity. Since TCA cycle serves as electron donor for oxidative phosphorylation, we also search for genes involved in this metabolic route in *C. jejuni* Bf genome but did not notice any difference with other *C. jejuni* genomes (data not shown).

Comparative Genomics of *C. jejuni* Bf vs. Other Genomes

Comparing the gene repertoire of *C. jejuni* Bf with that of other strains, on the basis of the functions putatively involved in oxygen metabolism, biofilm formation and adhesion did not reveal any obvious missing gene in this strain. Therefore, we performed genome comparison without focusing on functions but rather to detect which strains were the closest, to narrow our analysis.

The genome similarity analysis was based on the number and percentage of identity of genes and on synteny groups. The comparison was realized using 52 genomes available (32 complete and 19 draft). We observed that *C. jejuni* Bf was divergent from the well-studied reference genomes (*C. jejuni* NCTC 11168 and *C. jejuni* 81-176). Among the other genomes included in our genomic comparison, *C. jejuni* ATCC 33560 draft genome was the closest. Interestingly, both strains belong to the same MLST group (Rodrigues et al., 2015; MLST database http://pubmlst.org/campylobacter). More than 98% of the CDS of *C. jejuni* Bf were in bidirectional best hits (BBHs) with the CDS of *C. jejuni* ATCC 33560 draft genome (34 contigs). Such a similarity between the two strains prompted us to compare their

phenotype. *C. jejuni* ATCC 33560 was not aerotolerant (data not shown). Consequently, we focused on the differences between the genome sequences of these two strains. Thirty eight CDS were unique to the two strains compared to the 51 others strains, most of them considered as encoding peptides of unknown function (Supplementary Table S4). Among those we noticed a small CDS inserted in the cluster *hcrA*/*grpE*/*dnaK,* directly upstream of *dnaK*. This gene, of unknown function, encodes a protein of 72 amino acids that may potentially affect the expression of *dnaK*. Among the 37 remaining unique CDS, many were of small size and could be considered as false or doubtful CDS or resulting from fragmented genes. None could be associated to functions related to oxygen metabolism.

Comparison of Gene Transcription in *C. jejuni* Bf under Different Atmospheres

The phenotype of *C. jejuni* Bf regarding growth, adhesion to biotic and abiotic surfaces and biofilm formation suggested that this strain behaves similarly under air or under atmosphere conditions described as optimal (low O_2 concentration and high CO_2 concentration). Since only few genome features specific to this strain were observed, we hypothesized that a subtle change in gene expression may be involved. According to the literature, CosR is involved in oxidative stress response but also in biofilm maturation in *C. jejuni* (Hwang et al., 2011, 2012, 2014; Oh and Jeon, 2014; Turonova et al., 2015). The expression of *cos*R from cells grown under microaerobic or aerobic condition was measured. As well we determined the expression of several genes that were pointed out during genome analysis: *oorDABC* genes, *dnaK* and its upstream CDS. *C. jejuni* 81-176 grown was used as a control. Under microaerobiosis, *cosR* and *oorDABC* gene expression levels in *C. jejuni* Bf were not statistically different from those of *C. jejuni* 81-176 whereas we noticed an 8-fold increase of *dnaK* expression in *C. jejuni* Bf.

After aerobic growth of *C. jejuni* Bf, the relative expression of *cosR* and *oorDABC* were strongly increased in comparison with *C. jejuni* Bf grown in microaerobiosis. Indeed, *cosR* expression level was 12-times higher in aerobiosis. As well, *oorD*, *oorA*, *oorB*, and *oorC* were expressed 22, 19, 18, and 12 times more, respectively. The expression of *dnaK* and its upstream CDS were constitutive in *C. jejuni* Bf whatever the conditions tested.

We searched for the presence of the CosR box previously reported in *C. jejuni* NCTC 11168 by Hwang et al. (2011, 2012) upstream from these genes. We observed a motif similar to the CosR box upstream from *oorD* with only 14 out of the 21 bp consensus sequence conserved. Interestingly, a similar box was also present upstream from *dnaK* due to the insertion of a small CDS. Although, the motif was moderately conserved (14 out of 21 bp) we cannot exclude that such an insertion in *C. jejuni* Bf may modify *dnaK* expression or regulation by comparison to *C. jejuni* 81-176.

DISCUSSION

During the last decade, *C. jejuni* has been regularly reported as the leading cause of bacterial foodborne infection in Europe. Given the public health significance of this zoonosis it is relevant to understand the survival mechanisms adopted by this pathogen. Indeed, passage through the food chain exposes this microaerophilic pathogen to various harsh environmental conditions including oxidative stress. Among the strategies to resist, biofilm is a life-style known to protect bacteria from various environmental stresses, antimicrobial agents and also increased bacterial resistance to host immune response (Gilbert et al., 1993; Donlan and Costerton, 2002; Chmielewski and Frank, 2003). Recently described, *C. jejuni* Bf presents a higher ability to survive against oxidative stress and this clinical strain also presents the particularity to grow under aerobic conditions (Rodrigues et al., 2015). In this report, we studied the ability of this strain to adhere and develop biofilms. We also evaluated the influence of aerobiosis on adhesion properties. Finally, we searched for genomic features that may explain the atypical phenotype of the strain.

Biofilm formation is a succession of several steps beginning with initial attachment. Therefore, we have investigated the capacity of *C. jejuni* to adhere to an inert surface in order to evaluate subsequently its ability to initiate and develop a biofilm. The adhesion capability was variable between the 13 strains we tested. *C. jejuni* Bf showed a delayed adhesion, suggesting that a longer contact period with the polystyrene may lead to a stronger adhesion. Surprisingly, *C. jejuni* 81-176 strain showed a low adhesion capacity, even after several verification tests, although, this strain was previously reported to adhere and develop biofilm (Gunther and Chen, 2009; Sulaeman et al., 2010; Turonova et al., 2015). The main differences between the current study and previous ones rely on the experimental design, especially the media used for growth. These have been already reported to influence *C. jejuni* adhesion to inert surface (Reeser et al., 2007).

We have also investigated the capacity of *C. jejuni* to adhere and form biofilm under aerobiosis. Interestingly, cultivation of *C. jejuni* Bf under aerobiosis enhanced its adhesion to polystyrene. Few studies have been conducted to evaluate the ability of *C. jejuni* to form biofilm aerobically (Asakura et al., 2007; Reuter et al., 2010; Turonova et al., 2015). As raised by Turonova et al. (2015, 2016) the use of CLSM allows observation of structural changes in the biofilm formed by *C. jejuni*. Subsequently to our adhesion assay, the capacity of *C. jejuni* Bf and *C. jejuni* 81-176 to produce biofilm under aerobiosis were also evaluated and observed using CLSM. The ultrastructure of the biofilm formed by *C. jejuni* 81-176 being well-characterized (Gunther and Chen, 2009; Turonova et al., 2015), we chose this stain as a reference. In optimal growth conditions (e.g., under microaerobiosis and at 42°C), *C. jejuni* Bf is also able to develop a structured biofilm as previously described for several *C. jejuni* strains (Asakura et al., 2007; Gunther and Chen, 2009; Reuter et al., 2010; Turonova et al., 2015). Comparison of bio-volume and thickness of the biofilm formed by the two strains cultivated in microaerobiosis revealed structural differences. Indeed, the biofilm developed by *C. jejuni* 81-176 appeared thick with heterogeneous structures, whereas the one formed by *C. jejuni* Bf was more homogeneous, flatter and

spread in the well. Statistical analysis confirmed that *C. jejuni* 81-176 developed a biofilm 1.7 fold higher than *C. jejuni* Bf but with a non-significant difference in volume level. The microaerophilic strain *C. jejuni* 81-176 was unable to develop a biofilm in ambient atmosphere at 42°C even after 48 h of incubation. This apparent contradiction with other studies reporting that aerobiosis enhances biofilm formation may rely on differences in experimental conditions and on the strain that were used. Indeed most studies focused on *C. jejuni* NCTC 11168. These were performed under different growth conditions with the use of Brucella (Reuter et al., 2010) or Muller-Hinton broths (Asakura et al., 2007) and an incubation temperature of 37°C. The study including *C. jejuni* 81-176 was performed to compare only the influence of oxygen using O_2 and CO_2-enriched conditions, e.g., 19% O_2, 10% CO_2, 71% N_2 (Turonova et al., 2015) which are different from the gaseous conditions we used (ambiant air). In addition, incubation temperature was 37°C and adhesion duration was longer (4–5 h; Turonova et al., 2015) vs. 42°C and 2 h in the present study.

Campylobacter jejuni Bf is able to develop biofilms under both microaerobiosis and aerobiosis, with no significant modification in terms of bio-volume and thickness. We can hypothesize that under aerobiosis *C. jejuni* Bf develops a more structured biofilm resulting in a microaerobic local environment more adequate for its growth, as was proposed for NCTC 11168 (Stewart and Franklin, 2008; Reuter et al., 2010; Turonova et al., 2015). Nevertheless, this study is the first report on the capacity of a *C. jejuni* strain to form biofilm after growth under aerobiosis.

Adhesion to surface is clearly a preliminary step to biofilm formation and some proteins involved in adhesion to inert surfaces are also important for interaction with epithelial cells. Compared to *C. jejuni* 81-176 and NCTC 11168, *C. jejuni* Bf presents a higher ability to adhere to human intestinal cells after growth in either microaerobiosis or aerobiosis. Mucus production did not modify adhesion capability of *C. jejuni* Bf and NCTC 11168, but enhanced that of *C. jejuni* 81-176. The better ability of the clinical strain *C. jejuni* Bf to adhere to human intestinal cells might be explained by the presence of a complete T6SS as reported in few other strains (Lertpiriyapong et al., 2012; Harrison et al., 2014; Corcionivoschi et al., 2015). This structure is absent from *C. jejuni* NCTC 11168 and 81-176.

Once the phenotype characterization performed, we focused on comparative genomics to point out genes specific of *C. jejuni* Bf. The genome analysis revealed that this strain possesses the genes necessary to develop a biofilm. Among all of the genes identified in the literature related to biofilm formation only four were absent, which is not particularly relevant since these genes are also absent from several *C. jejuni* genomes (Hofreuter et al., 2006; Rahman et al., 2014). In addition, we cannot totally exclude that their absence could result from sequencing errors or sequence misassembly. The gene repertoire of *C. jejuni* Bf necessary to resist to oxidative stress revealed no difference with that of other strains. In *C. jejuni* the CosR regulator has been reported as responsible for the regulation of genes participating to oxidative stress response but also to biofilm formation (Kalmokoff et al., 2006; Svensson et al., 2009; Garénaux et al., 2008a; Hwang et al., 2011, 2012; Oh and Jeon, 2014; Turonova et al., 2015). We have shown that *C. jejuni* Bf *cosR* was 12-fold over-expressed in aerobiosis, suggesting that the regulation of genes involved in oxidative stress response and biofilm formation might be modified in this strain. We highlighted two genetic modifications in *C. jejuni* Bf that may rely on its behavior: a point mutation in *oorD* (Bronnec et al., 2016) and an insertion upstream from *dnaK*. The *oorD* mutation may result in a different phenotype toward oxygen metabolism since in *Helicobacter pylori*, the 2-oxoglutarate oxidoreductase encoded by *oorDABC*, was reported as important for the microaerophilic phenotype of this species (Hughes et al., 1998). In addition, we showed that *C. jejuni* Bf *oorDABC* operon is up-regulated under aerobiosis. Conversely, *dnaK* transcription was constitutive in *C. jejuni* Bf regarding atmosphere used for growth. However, this gene is up-regulated in *C. jejuni* Bf, in comparison with 81-176. This may be the consequence of the insertion just upstream from *dnaK* which may result in a modification of its transcription. Furthermore, DnaK belongs to a protein family involved in general stress response. Its high level of expression in *C. jejuni* Bf might explain a better resistance to oxidative stress of this stain compared to that of *C. jejuni* 81-176. Comparing the resistance of the two strains to other stresses would be necessary to confirm this hypothesis. In addition, DnaK has also been described as moonlighting in several bacteria, i.e., harboring a different function when expressed on the cell surface (Amblee and Jeffery, 2015 and references therein). Indeed, DnaK from several Gram+ and Gram- species has been shown to bind plasminogen or eukaryotic cell surfaces when present on bacterial surface. We have no evidence of such a location in *C. jejuni* Bf, but this should be considered to search for a potential role of this protein, which gene is highly express in a clinical strain capable of adhering to surfaces and developing biofilm.

CONCLUSION

The ability of *C. jejuni* to develop a structured biofilm is highly variable depending on the surface, the environmental conditions but also the strain. *C. jejuni* Bf has the particularity to multiply under aerobiosis, but we also have shown that this strain is able to form a structured biofilm when cultured in aerobic condition. Further experiments could be conducted at environmental temperatures (vs. optimal one, 42°C) to investigate *C. jejuni* Bf ability to form biofilm under aerobiosis. Genome analysis did not highlight any obvious acquisition of functions in this strain. Its atypical behavior apparently results from a modification in the regulation of several genes involved in oxidative stress response, oxygen metabolism, adhesion, and biofilm formation.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: VB, NH, OT and MZ; performed the experiments: VB, HT, RR, AB and SC; analyzed the

data: VB, MZ, NH, OT and SC; wrote the paper: VB, NH and MZ; corrected the paper: VB, NH, HT, OT, SC and MZ.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.01002

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PERSPECTIVE
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Compositional Analysis of Biofilms Formed by *Staphylococcus aureus* Isolated from Food Sources

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Sixteen *Staphylococcus aureus* isolates originating from foods (eight from dairy products, five from fish and fish products and three from meat and meat products) were evaluated regarding their biofilms formation ability. Six strains (E2, E6, E8, E10, E16, and E23) distinguished as strong biofilm formers, either in standard Tryptic Soy Broth or in Tryptic Soy Broth supplemented with 0.4% glucose or with 4% NaCl. The composition of the biofilms formed by these *S. aureus* strains on polystyrene surfaces was first inferred using enzymatic and chemical treatments. Later on, biofilms were characterized by confocal laser scanning microscope (CLSM). Our experiments proved that protein-based matrices are of prime importance for the structure of biofilms formed by *S. aureus* strains isolated from food sources. These biofilm matrix compositions are similar to those put into evidence for coagulase negative staphylococci. This is a new finding having in view that scientific literature mentions exopolysaccharide abundance in biofilms produced by clinical isolates and food processing environment isolates of *S. aureus*.

Keywords: *Staphylococcus aureus*, biofilm, food, CLSM, exopolysaccharide, protein

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INTRODUCTION

Few studies have been reported so far regarding the biofilm formation by *Staphylococcus aureus* isolated from foods (Di Ciccio et al., 2015) and the impact of the environmental factors encountered in food processing plants on the adherence and biofilm formation (Vázquez-Sánchez et al., 2013; Santos et al., 2014).

In food industry it is important to know the conditions under which *S. aureus* is able to survive, adhere to surfaces and form biofilms (Futagawa-Saito et al., 2006), leading to contamination of food products. In planktonic form, *S. aureus* does not appear resistant to disinfectants, compared to other bacteria, but it may be among the most resistant ones when is attached to a surface (Fratamico et al., 2009). *S. aureus* can produce a multilayered biofilm embedded within a glycocalix with heterogeneous protein expression throughout, forming at least two types of biofilms: *ica*-dependent, mediated by polysaccharide intercellular adhesin (PIA)/poly-N-acetyl-1,6-β-glucosamine (PNAG), and *ica*-independent, mediated by proteins (Beloin and Ghico, 2005). Biofilm-associated protein (Bap), which shows global organizational similarities to surface proteins of Gram-negative (*Pseudomonas aeruginosa* and *Salmonella enterica* serovar Typhi) and Gram-positive (*Enteroccocus faecalis*) bacteria (Cucarella et al., 2001), was the first protein that has been found to be involved in biofilm formation by staphylococcal strains isolated from mammary glands in ruminants suffering from mastitis (Speziale et al., 2014). Meanwhile, Foulston et al. (2014) discovered

that the extracellular matrix of clinical *S. aureus* biofilms comprises cytoplasmic proteins that associate with the cell surface in response to decreasing pH. Regarding the capacity to form biofilms, Bridier et al. (2010) demonstrated that *S. aureus* strains from different sources (five clinical, two originating from water, two unknown, and one milk isolate from ewes with mastitis) produce biofilms with high bio volumes and high substratum coverage.

Having in view the significant damages caused by biofilms in food industry in general, more studies should be conducted to elucidate formation of such biofilms and to develop countermeasures for their removal from food contact surfaces (Marques et al., 2007). This study was carried out to evaluate the ability of *S. aureus* strains isolated from food products to form biofilms on hydrophobic surfaces at 37°C, followed by biofilm matrix characterization. The composition of the biofilms formed by *S. aureus* strains on polystyrene surfaces was first inferred using enzymatic and chemical treatments and later confirmed by confocal laser scanning microscope (CLSM).

MATERIALS AND METHODS

Bacterial Strains

Sixteen *S. aureus* strains isolated from food products of animal origin (8 from dairy products, 5 from fish and fish products and 3 from meat and meat products) (Oniciuc et al., 2015) were tested to show their ability to form biofilms. Prior to inoculation, all strains were transferred from the stock cultures (preserved in 25% glycerol at −80°C) to Baird Parker (BP) (Biolife Italiana srl., Milano, Italy) and incubated aerobically at 37°C for 24 h. For biofilm assays we used overnight precultures in Tryptic Soy Broth (TSB) (Liofilchem srl., Roseto degli Abruzzi, Italy) incubated aerobically at 37°C, with shaking.

Media Screening and Biofilm Formation Overtime

Media screening consisting in TSB with/ without addition of 0.4% glucose (TSBG) or 4% NaCl (TSBN) (Liofilchem srl.) for supporting 24 h biofilm formation was performed. Glucose (B. Braun Melsungen AG, Melsungen, Germany) sterilized by filtration (0.22 μm) was added after autoclaving. Prolonged incubation time (48, 72 h) was also performed (Peeters et al., 2007).

Biofilms were grown in 96-well plates tissue cultured (Orange Scientific, Braine-l'Alleud, Belgium) with a total volume of 200 μL of TSB, TSBG and TSBN per well and a starting inoculum approximately equal to 10^6 CFU/mL. Only broth media were introduced in the assay as negative controls, and *S. aureus* ATCC 25923 as positive control (clinical isolate). The plates were incubated aerobically at 37°C, on an orbital shaker (ES-20/60 Environmental Shaker BIOSAN) set at 120 rpm. Biofilm quantification was performed according to the procedure developed by Stepanović et al. (2000), by using 1% crystal violet (CV) (Merck KGaA, Darmstadt, Germany). Biofilm formation in the microplates was measured in an ELISA reader set at 570 nm, and values were expressed in optical density (OD) values.

Matrix Characterization

Biofilm detachment assays were carried out as described by Kogan et al. (2006) and Fredheim et al. (2009) with slight modifications, for six strains capable to form strong biofilms with an OD>4 × OD_{NC}. Biofilms were washed twice with 200 μL of 0.9% NaCl and then treated for 2 h at 37°C without shaking, with 200 μL of 40 mM of sodium periodate ($NaIO_4$), or 200 μL proteinase K (0.1 mg/mL in 20 mM Tris-HCl:1 mM $CaCl_2$). Control wells were filled with 0.9% NaCl. After treatment, the biofilms were washed once with 200 μL of 0.9% NaCl, and then resuspended into 200 μL of 0.9% NaCl and dislodged by scraping followed by sonication using a cycle of 5 s and an amplitude of 22%. Biomass quantification was performed by measuring the OD at 600 nm of each sonicated cell suspension. Measuring the OD of sonicated cell suspensions was preferred for this assay as we observed that $NaIO_4$ used to assess polysaccharides reacts unspecific with CV therefore yielding false positive results.

Biofilm Composition by CLSM

The composition of 48 h biofilms was observed by CLSM, exposed to three types of dyes: (i) SYTO dye that stains nucleic acids; (ii) FilmTracer SYPRO Ruby Biofilm Matrix stain (Invitrogen, Paisley, UK), which labels most classes of proteins (Berggren et al., 2000); (iii) wheat germ agglutinin (WGA) conjugated with Oregon Green (Invitrogen), which stains *N*-acetyl-D-glucosamine residues (Wright, 1984). The fluorescence of dyes was detected using the following combination of laser excitation and emission band-pass wavelengths: 476 nm/500–520 nm for SYTO, 405 nm/655–755 nm for SYPRO and 459 nm/505–540 for WGA. After each staining step, the biofilms were gently rinsed with sterile water. The biofilm images were acquired in an Olympus^{TM} FluoView FV1000 confocal laser microscope and biofilms were observed using 40x water-immersion objective. The images were analyzed sequentially using two virtual channels. Three stacks of horizontal images (640 × 640 pixels) were acquired for each biofilm at different areas in the well. Two surfaces of two independent replicates were observed in each CLSM experiment.

RESULTS AND DISCUSSIONS

Glucose and NaCl have been previously shown to induce biofilm formation in clinical strains of *S. aureus* (Fratamico et al., 2009). Measuring the effect of 0.4% glucose and 4% NaCl on biofilm formation enabled us to determine the conditions necessary for *S. aureus* strains isolated from food to form biofilms. For most strains, there was not a significant difference within the media used showing a small degree of variability regarding the amount of biomass produced, but overall, six strains (E2, E6, E8, E10, E16, E23; 37.5%) with OD > 0.4 were distinguished for higher biofilm formation with TSBG (**Supplementary Figure 1**, left graphic). As the determination of the total biomass over a specific period of time is a common practice for the characterization of biofilms and *S. aureus* biofilms are growing slowly, prolonged incubation times were used in our experiment too. Not surprisingly, quantification of biofilm proved a progressive accumulation of biomass during the

TABLE 1 | Biomass reduction of *S. aureus* biofilms when using metaperiodate or proteinase K.

S. aureus strains	Biomass reduction, %	
	With NaIO4	With proteinase K
E2	23 ± 10.34	71 ± 4.1
E6	34 ± 2.74	71 ± 0.74
E8	46 ± 11.07	69 ± 0.63
E10	20 ± 6.51	66 ± 3.5
E16	25 ± 0.71	64 ± 1.75
E23	49 ± 3.71	67 ± 6.05
ATCC 25923	28 ± 5.25	9 ± 1.9

Preformed biofilms were treated with NaIO4 or proteinase K for 2 h at 37°C. Control wells were filled with 0.9% NaCl. Average results ± SD of eight wells for each strain are shown. The experiments were performed in triplicate. Values of negative controls have been subtracted from the shown values.

analyzed time course (**Supplementary Figure 1**, right graphic). Based on these findings we further characterized *S. aureus* biofilms after 48 h of incubation.

In order to reveal the molecules behind biofilm accumulation, the biofilm chemical compositions were assessed by measuring the ability of $NaIO_4$ or proteinase K to disperse *S. aureus* biofilms. Although both ATCC and food isolates have PNAG and proteins in the matrix, proteins prevail on PNAG, thus having a relevant role in maintaining biofilm structure. In this sense, biomass formed by *S. aureus* strains isolated from foods was reduced by 60–70% when anti-protein agents were used, while a reduction of 20–49% was obtained in the presence of the anti-polysaccharide agent (**Table 1**). Proteinase K treatment enhanced dispersion of Bap-positive *S. aureus* biofilms as demonstrated by Shukla and Rao (2013). The disruption effects observed on 48 h biofilms were similar for all isolates originating from food sources.

Differences were observed in the biofilm disruption pattern when comparing results obtained for biofilms formed by *S. aureus* isolated from food sources with those developed by the clinical isolate *S. aureus* ATCC 25923, presenting a high density of cell clusters embedded in polysaccharides. At present, there are no references to composition of biofilms formed by *S. aureus* isolated from food sources. Literature mentions only biofilms produced by strains of *Staphylococcus* spp. isolated from a poultry processing plant, which have been described by Ferreira et al. (2014), as containing a significant amount of exopolysaccharides (EPS).

CLSM in conjugation with three different fluorescent dyes was used to differentiate bacterial cells from PNAG and proteins within the biofilm matrix. Qualitative approach was preferred as biofilms obtained were heterogeneous and more than three sections per each biofilm were needed for a meaningful quantification. Biofilm matrices of E8 and E10 formed by *S. aureus* strains isolated from food are represented in **Figure 1** in comparison with those formed by the reference strain. These experiments confirmed that proteins are of prime importance for the structure of biofilms formed by *S. aureus* strains isolated from food sources as revealed by the quantitative approach from biofilm disruption assays.

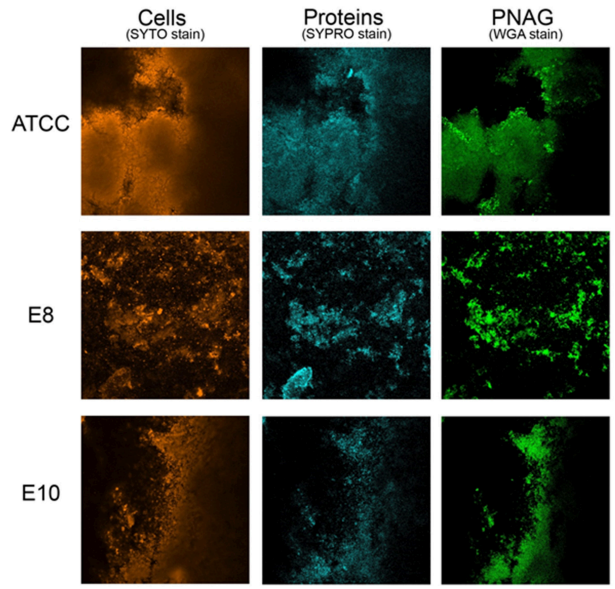

FIGURE 1 | Biofilm matrix structure obtained from confocal microscopy observations of *S. aureus* ATCC 25923, E8 isolated from poultry, and E10 isolated from artificial red caviar. One z-stack is represented for each biofilm.

CONCLUSIONS AND PERSPECTIVES

Phenotypic production of EPS by *S. aureus* strains used in the present study suggests that staphylococcal biofilm development may have occurred *via* an *ica*-independent pathway. Clearly, in our population of bacteria, PIA independent biofilm formation was more prevalent. Nevertheless, to determine if this characteristic is in fact a key difference between food-borne *S. aureus* and clinical isolates or food processing environment isolates, future research is needed to include a broader range of food-borne isolates.

Presence of biofilm forming strains of *S. aureus* in food and food processing environments is equally important as for the medical sector. Besides causing serious engineering problems as described by Garrett et al. (2008), biofilms are involved in cross contamination events. The proteic extracellular matrix developed by *S. aureus* isolates of food origin can behave in a similar way that the one developed by clinical isolates of *S. aureus* allowing enhanced flexibility and adaptability for this bacterium in forming biofilms and supporting the formation of mixed-species biofilms either with spoilage or pathogenic bacteria as demonstrated by Foulston et al. (2014). Composition of biofilms has to be known to provide a basis for the development of better strategies for cleaning surfaces and cross contamination avoidance.

AUTHOR CONTRIBUTIONS

All three authors contributed equally to the following sections: Introduction, Results and Discussions and Conclusions

and Perspectives. EO wrote Materials and Method section together with NC and prepared the graphs shown in **Supplementary Figure 1**. NC prepared **Figure 1**. AN wrote the abstract and prepared the table. Several versions of the manuscript circulated between the authors until they all agreed on the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.00390

Supplementary Figure 1 | *S. aureus* biofilm development. Biomass accumulation when using 0.4% glucose and 4% NaCl to the standard TSB (**left**). Biofilm formation overtime using TSBG (**right**). Bars represent the means of the OD value ± standard deviation (SD) evaluated in three independent measures obtained upon different treatments tested, as indicated. Values of negative controls have been subtracted from the shown values.

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ORIGINAL RESEARCH
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Biofilm Matrix Composition Affects the Susceptibility of Food Associated Staphylococci to Cleaning and Disinfection Agents

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Staphylococci are frequently isolated from food processing environments, and it has been speculated whether survival after cleaning and disinfection with benzalkonium chloride (BC)-containing disinfectants is due to biofilm formation, matrix composition, or BC efflux mechanisms. Out of 35 food associated staphylococci, eight produced biofilm in a microtiter plate assay and were identified as *Staphylococcus capitis* (2), *S. cohnii*, *S. epidermidis*, *S. lentus* (2), and *S. saprophyticus* (2). The eight biofilm producing strains were characterized using whole genome sequencing. Three of these strains contained the *ica* operon responsible for production of a polysaccharide matrix, and formed a biofilm which was detached upon exposure to the polysaccharide degrading enzyme Dispersin B, but not Proteinase K or trypsin. These strains were more tolerant to the lethal effect of BC both in suspension and biofilm than the remaining five biofilm producing strains. The five BC susceptible strains were characterized by lack of the *ica* operon, and their biofilms were detached by Proteinase K or trypsin, but not Dispersin B, indicating that proteins were major structural components of their biofilm matrix. Several novel cell wall anchored repeat domain proteins with domain structures similar to that of MSCRAMM adhesins were identified in the genomes of these strains, potentially representing novel mechanisms of *ica*-independent biofilm accumulation. Biofilms from all strains showed similar levels of detachment after exposure to alkaline chlorine, which is used for cleaning in the food industry. Strains with *qac* genes encoding BC efflux pumps could grow at higher concentrations of BC than strains without these genes, but no differences were observed at biocidal concentrations. In conclusion, the biofilm matrix of food associated staphylococci varies with respect to protein or polysaccharide nature, and this may affect the sensitivity toward a commonly used disinfectant.

Keywords: ***Staphylococcus*, biofilm, matrix, ica, MSCRAMM, quaternary ammonium compound, benzalkonium chloride**

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INTRODUCTION

Despite daily cleaning and disinfection, staphylococci are frequently isolated from machines and surfaces in food processing plants (Sundheim et al., 1992; Møretrø et al., 2003; Marino et al., 2011). Coagulase negative staphylococci (CNS) dominate, but also the food borne pathogen *Staphylococcus aureus* that may cause intoxications in humans and mastitis in cows has been

isolated from food processing environments (Langsrud et al., 2006; Marino et al., 2011). Survival of staphylococci in the harsh conditions may be linked biofilm formation protecting them from detachment by cleaning agents and killing by disinfectants and specific resistance mechanisms such as efflux pumps (Campanac et al., 2002; Luppens et al., 2002; Wassenaar et al., 2015).

Biofilms of staphylococci are common sources of infections on medical implants in the human body (Arciola et al., 2015), and the mechanisms of biofilm formation have been studied in detail for clinical *S. aureus* and *S. epidermidis*. The most common mechanism of biofilm formation in these species depends on production of the polysaccharide intercellular adhesin (PIA) as the most important component of the biofilm matrix. PIA is produced by the proteins encoded by the *ica* operon comprising the *icaADBC* genes and the regulatory gene *icaR* (Arciola et al., 2015). Extracellular DNA (eDNA) and cell wall associated teichoic acids are also believed to have structural roles in *S. aureus* and *S. epidermidis* biofilms, while unspecific electrostatic and hydrophobic interactions mediated by teichoic acids, eDNA, and hydrophobic surface proteins can contribute to primary adhesion to abiotic surfaces (Izano et al., 2008; Jabbouri and Sadovskaya, 2010; Becker et al., 2014; Büttner et al., 2015).

Staphylococcus aureus and *S. epidermidis* strains that can produce biofilms without PIA exopolysaccharide are dependent on protein-mediated intercellular adhesion. It is recognized that several staphylococcal cell wall anchored (CWA) surface proteins may promote not only surface adhesion to biotic and abiotic surfaces, but also the accumulation phase of biofilm formation through mediating cell–cell adhesion (Foster et al., 2014; Speziale et al., 2014; Arciola et al., 2015). These include SdrC, ClfB, FnBPA, and FnBPB, which belong to the class of CWA proteins originally termed microbial surface components recognizing adhesive matrix molecules (MSCRAMM) based on their ability to mediate specific interaction with components of human extracellular matrix (ECM; Abraham and Jefferson, 2012; Geoghegan et al., 2013; Barbu et al., 2014). MSCRAMMs are characterized by having a non-repetitive N-terminal adhesion domain composed of two or three immunoglobulin (IgG)-like folds, followed by a region of tandem repeat domains and a C-terminal LPxTG peptidoglycan sorting signal. Serine-rich repeat glycoproteins (SRRP), like the *S. aureus* SraP protein, are another family of CWA adhesins that can mediate biofilm formations *via* intercellular adhesion (Sanchez et al., 2010; Lizcano et al., 2012). Other types of CWA proteins which have been shown to be involved in mediating biofilm formation in staphylococci include the Biofilm associated protein (Bap; Cucarella et al., 2001), the G5-E repeat family protein termed Accumulation-associated protein (Aap) in *S. epidermidis* (SasG in *S. aureus*; Rohde et al., 2005; Geoghegan et al., 2010), the *S. aureus* proteins SdrC, SasC, and Protein A (Merino et al., 2009; Schroeder et al., 2009; Barbu et al., 2014), the *S. epidermidis* protein SesC (Khodaparast et al., 2016), and the NEAT motif family protein IsdC (Missineo et al., 2014). Also non-covalently attached cell surface proteins, like the bifunctional autolysin/adhesins AtlE and Aae (Heilmann et al., 1997, 2003) and the giant (1 MDa) protein termed Extracellular matrix binding protein (Embp) in *S. epidermidis* (Ebh in *S. aureus*; Christner et al., 2010), have been shown to mediate staphylococcal biofilm formation. It has been shown that the sensitivity of biofilms to enzymes, can indirectly be used as a method to find the nature of the matrix of the biofilm (Chaignon et al., 2007; Fredheim et al., 2009).

Food associated *Staphylococcus* spp. can form both *ica*-dependent and *ica*-independent biofilms (Møretrø et al., 2003; Rode et al., 2007). It has been suggested that *ica*-independent biofilm formation of staphylococci from mastitis was connected to the presence of the gene encoding the Bap, but this mechanism seems to be less frequent in staphylococci from other sources (Cucarella et al., 2001; Vautor et al., 2008).

Benzalkonium chloride (BC), a quaternary ammonium compound (QAC), is widely used in disinfectants in the food industry and in healthcare facilities (Tezel and Pavlostathis, 2015). A number of bacteria have been reported to harbor genes encoding membrane protein efflux pumps that can export and provide increased tolerance to BC. In staphylococci six different efflux proteins (QacA, QacB, QacC, QacG, QacH, and QacJ) have been reported and shown to be widely spread in strains of both clinical and food origin (Wassenaar et al., 2015).

All Qac efflux proteins provide staphylococci with low-level tolerance to BC and other QACs (Furi et al., 2013; Wassenaar et al., 2015). Typical minimal inhibitory concentrations (MIC) of staphylococci expressing Qac proteins are in the range 4–12 ppm compared to MIC-values $\leq$2 ppm for sensitive strains (Heir et al., 1995). These tolerance levels are much lower than the lowest concentration of QAC used in the food industry, which is typically above 200 ppm (Tezel and Pavlostathis, 2015). It has been shown that staphylococci in biofilms have higher tolerance to QAC compared to planktonic phase staphylococci (Campanac et al., 2002). However, whether the presence of *qac* genes may be advantageous for staphylococci in biofilms and under food industry relevant conditions and concentrations when exposed to QAC, has to our knowledge not been reported.

In the present study, the biofilm matrix composition of *Staphylococcus* spp. isolated from the food industry was determined using enzymes targeting specific matrix components. Genetic determinants for biofilm associated and cell-wall anchored (CWA) proteins were investigated by whole genome sequencing. Furthermore the effect of the composition of the biofilm matrix as well as the presence of *qac* resistance genes on the efficacy of the disinfectant BC was studied.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

A collection of 35 staphylococci, from food (eight strains) or food processing environments (27 strains) from the Nofima strain collection were used in initial screening for biofilm formation. The eight strains identified as capable of forming biofilms and subjected to further characterization are listed in **Table 1** along with the reference strains used. Unless stated otherwise the bacteria were stored at −80°C and cultured at 30°C on tryptic soy agar (TSA) or TSB with shaking. For *S. aureus* RN4220/pSK265 and RN4220/*qacC*, chloramphenicol (6 ppm, final concentration)

TABLE 1 | *Staphylococcus* strains used in this study.

Strain no.[1] (species)	Origin	Other designations and characteristics	Reference
Food and food industry strains			
MF1767 (*Staphylococcus lentus*)	Poultry processing equipment		Sundheim et al., 1992
MF1862 (*S. lentus*)	Poultry		Sundheim et al., 1992
MF1844 (*S. cohnii*)	Poultry processing equipment		Sundheim et al., 1992
MF4371 (*S. saprophyticus*)	Salmon processing plant		Schirmer et al., 2012
MF6029 (*S. saprophyticus*)	Meat processing equipment	Isolate 12; *qacC*[2]	Sundheim et al., 1992; Heir et al., 1995
MF1871 (*S. capitis*)	Bakery industry product	Isolate 6; *qacA*[2]	Heir et al., 1995
MF1872 (*S. capitis*)	Poultry	Isolate 7; *qacA*[2]	Sundheim et al., 1992; Heir et al., 1995
MF1789 (*S. epidermidis*)	Poultry processing equipment		Sundheim et al., 1992
Control strains			
ATCC35984 (*S. epidermidis*)		RP62A	Christensen, 1982
RN4220 (*S. aureus*)			Kreiswirth et al., 1983
RN4220/pSK265 (*S. aureus*)		Strain RN4220 with plasmid cloning vector pSK265	Jones and Khan, 1986; Heir et al., 1998
RN4220/*qacC* (*S. aureus*)		Strain RN4220 with *qacC* cloned into pSK265	Heir et al., 1998

[1]*MF numbers refer to Nofima's strain collection.* [2]*Previous designation and strain characteristics according to Heir et al. (1995).*

was included in the growth medium of overnight cultures used in the experiments.

Biofilm Assay

Biofilm formation was assayed by cultivation in microtiter plates (Falcon) in 200 µl TSBNG [Tryptic Soy Broth (Oxoid) + 0.33 % glucose + 0.26 % NaCl; modified from Schwartz et al., 2012] at 30°C for 48 h. The suspensions were poured off and the plate was washed with dH_2O with a plate washer (Wellwash AC, Thermo Electron Corporation). After the washing 200 µl 0.1 % crystal violet (Merck) was added and after 4 min the plates were washed again to remove non-binding crystal violet. Two hundred microliters of ethanol added 0.2% HCl (37%) was added to release crystal violet, incubated for 2 min with shaking, before 100 µl was transferred to a new microtiter plate, and $OD_{600\ nm}$ was measured (SpectroStar Nano, BMG Labtec) as an indicator for biofilm formation.

Effect of Enzymes and Chlorine on Biofilm Detachment

Biofilms were grown in microtiter plates in TSBNG for 48 h as described above. The suspension was poured off and the plate washed with dH_2O with a plate washer. For each strain 200 µl enzyme or chlorine solution was added to three parallel wells. The following enzymes were tested (final concentrations in parentheses). Dispersin B (50 µg/ml, Kane Biotec Inc), DNAse I (100 µg/ml, Sigma–Aldrich), Proteinase K (100 µg/ml, Sigma–Aldrich) and Trypsin (100 µg/ml, Sigma–Aldrich). Dispersin B, a glycoside hydrolase, is known to degrade polysaccharide matrix (Itoh et al., 2005), DNase I degrades eDNA (Qin et al., 2007) and Proteinase K and trypsin are able to degrade protein-based biofilm matrix (Chaignon et al., 2007). Concentrations were chosen based on previous studies (Itoh et al., 2005; Kogan et al., 2006; Chaignon et al., 2007; Harmsen et al., 2010). A solution of 0.03% chlorine, pH 12 was made by dilution from hypochlorite (Klorin, Lilleborg, Oslo, Norway) and by addition of NaOH). Alkaline chlorine based cleaning agents are among the most commonly used in the food industry (Fukuzaki, 2006). For controls, 200 µl phosphate buffered saline (PBS) were added to five parallel wells. The biofilms were exposed for 2 h at 30°C on a rolling table. The suspensions were poured off and the plates were washed and stained with crystal violet and treated as described above before measurement of the remaining biofilm as OD_{600nm}. The degree of detachment was calculated by comparing enzyme treated and PBS (control) treated biofilms.

Genome Sequencing and Assembly

DNA isolation, genome sequencing and *de novo* genome assembly was performed as previously described (Fagerlund et al., 2016), with paired-end 2 × 300 bp reads on a MiSeq instrument (Illumina). Contigs with size < 200 bp and with coverage < 15 were removed from the assemblies. The sequences were annotated using the NCBI Prokaryotic Genomes Automatic Annotation Pipeline (PGAAP) server[1]. All sequence data associated with this project have been deposited at NCBI under the BioProject ID PRJNA311173.

Sequence Analysis

Identification at the species level was confirmed by RDP search of the 16S rRNA genes from the whole genome assemblies[2].

The publicly available genome sequences of *S. epidermidis* ATCC 35984 (GenBank accession CP000029), the ATCC 35984 pSERP plasmid (CP000028), the complete genome sequence of *S. aureus* NCTC 8325 (CP000253), from which *S. aureus* RN4220 is derived (Herbert et al., 2010), in addition to the draft genome of *S. aureus* RN4220 (AFGU01000000), were included in the analyses. The genome sequences were downloaded from the GenBank database[3].

The genomes were analyzed for the presence of genes of interest using BLAST+ v2.2.30 (Camacho et al., 2009). Proteins

[1]http://www.ncbi.nlm.nih.gov/genome/annotation_prok/

[2]https://rdp.cme.msu.edu/seqmatch/

[3]http://www.ncbi.nlm.nih.gov/

selected for use as query sequences fitted one of three criteria: (i) QAC efflux pump proteins, (ii) proteins known to be associated with biofilm formation in staphylococci, including proteins known to function as intercellular adhesins or (iii) surface bound proteins possessing LPxTG anchoring motifs (LPxTG). The last criterium was included since it is known that CWA proteins may promote biofilm formation in *S. aureus* and *S. epidermidis*, and the presently analyzed genomes belonging to other *Staphylococcus* spp. may potentially employ novel CWA adhesin proteins during biofilm formation. The list of proteins used as queries in BLAST search is listed in Supplementary Materials. The annotated proteins in each genome assembly were subjected to a Pfam domain search to identify proteins with YSIRK type signal peptide (PF04650) and the Gram positive anchor (PF00746) domains.

Predicted protein function was assessed using the InterProScan tool[4]. Alignments were created using CLC Main Workbench 7.5 (CLCbio). Protein structure prediction was performed using homology modeling methods based on sequence profiles generated by iterative BLAST searches, using the Phyre2 prediction server (Kelley and Sternberg, 2009).

Assembly of genome sequences from Illumina reads often results in gaps in the genome assembly at repetitive sites, like, e.g., the sequences of genes encoding large proteins with tandemly repeated domains. When loci containing partial genes next to gaps in the assembly were investigated, the initial partial genes (and subsequently identified matching sequences) were used as queries in Blastn searches against the genome assembly sequences. Obtained search hits were aligned to assess whether they were likely to represent segments of the same gene. When more than one locus next to different assembly gaps encoded identical repeat domains the loci were considered likely to belong to the same gene.

Minimal Inhibitory Concentration of Benzalkonium Chloride

An overnight culture in TSB was diluted 1:100 in TSBNG and 20 μl was added to the wells of 100-well plates (Oy Growth Curves Ab Ltd) with 180 μl of BC (Sigma–Aldrich) diluted in TSBNG, resulting in final concentrations of BC of 1, 2, 4, 6 and 8 ppm. The plates were incubated at 30°C for 20 h and the optical density measured automatically every 10 min (with 10 s shaking before each measurement) using a Bioscreen FP-1100-C (Oy Growth Curves Ab Ltd). The MIC was calculated using a cut-off value for detectable growth of $OD_{600\ nm}$ 0.1 after 20 h.

Lethal Effect of Benzalkonium Chloride on Biofilms

The lethal effect of user-concentrations of BC (200 ppm) was determined against biofilms grown on stainless steel. A stainless steel coupon (AISI 304 2B) of 2 cm × 2 cm was placed in each well of a six wells tissue culture plate. The well was added 5 ml overnight culture diluted in TSBNG to approximately 10^7 cfu/ml. After an attachment phase of 3 h at 30°C, the suspension was removed and the coupons rinsed gently with sterile distilled water. The rinsed coupons were placed in new wells, 3 ml TSBNG added, and the biofilms grown at 30°C for 48 h. After incubation, the suspensions were pipetted off and the coupons rinsed gently with dH_2O. The biofilms were exposed to 6 ml 200 ppm BC. Controls were added 6 ml dH_2O. After 5 min exposure at room temperature the coupon was transferred to a glass tube with 6 ml Dey Engley Neutralizing broth (Difco). The tube with the coupon was sonicated (40 Hz) for 10 min to dislodge the bacteria, then 34 ml Dey Engley neutralizing broth was added and the number of cfu determined after serial dilution and plating to TSA.

Disinfection Suspension Test of Benzalkonium Chloride

The effect of BC was tested in a modified European suspension test (CEN, 1997). An overnight culture in TSBNG was diluted 10 times with peptone water and 0.5 ml of the resulting suspension was transferred to 4.5 ml with 10 ppm benzalkonium chloride or sterile dH_2O (control). After 5 min exposure to BC at room temperature, 0.5 ml of the suspensions were transferred to new tubes with 4.5 ml Dey/Engley Neutralizing broth. Dilution series were made in peptone water and the number of surviving bacteria determined by plating to TSA. Log reductions were calculated by comparing BC treated suspensions with controls.

Statistical Analysis

Minitab®(v16.1.1, 2010[5]) was used to calculate statistical significance of differences between groups (2-sample-*t*-test). The mean values of technical replicates were calculated and statistical tests based on the variation between the biological replicates. Standard errors were calculated in Microsoft Excel.

RESULTS

Detachment by Enzymes Targeting Specific Matrix Components

Eight strains (**Table 1**) formed biofilms ($OD_{600\ nm} > 0.2$) out of a collection of 35 staphylococci isolated from food and food processing environments. The effect of the enzymes Dispersin B, DNase I, Proteinase K, and trypsin on the detachment of preformed biofilms was tested for these eight strains and for the reference strains *S. epidermidis* ATCC 35984 and *S. aureus* RN4220, known as strong biofilm formers harboring *ica*-genes (Ziebuhr et al., 1999; Møretrø et al., 2003; You et al., 2014) (**Figure 1**). Based on the detachment pattern after exposure to enzymes, these ten strains could be clustered into two groups. Biofilms of five strains (*S. lentus* MF1767 and MF1862, *S. cohnii* MF1844, and *S. saprophyticus* MF4371 and MF6029) were strongly disrupted upon treatment with Proteinase K and trypsin, while little effect was observed upon treatment with the glycoside hydrolase Dispersin B. For simplicity, this group was termed "protein biofilm group" based on literature showing that this phenotype is associated with strains that produce a biofilm

[4]http://www.ebi.ac.uk/interpro

[5]www.minitab.com

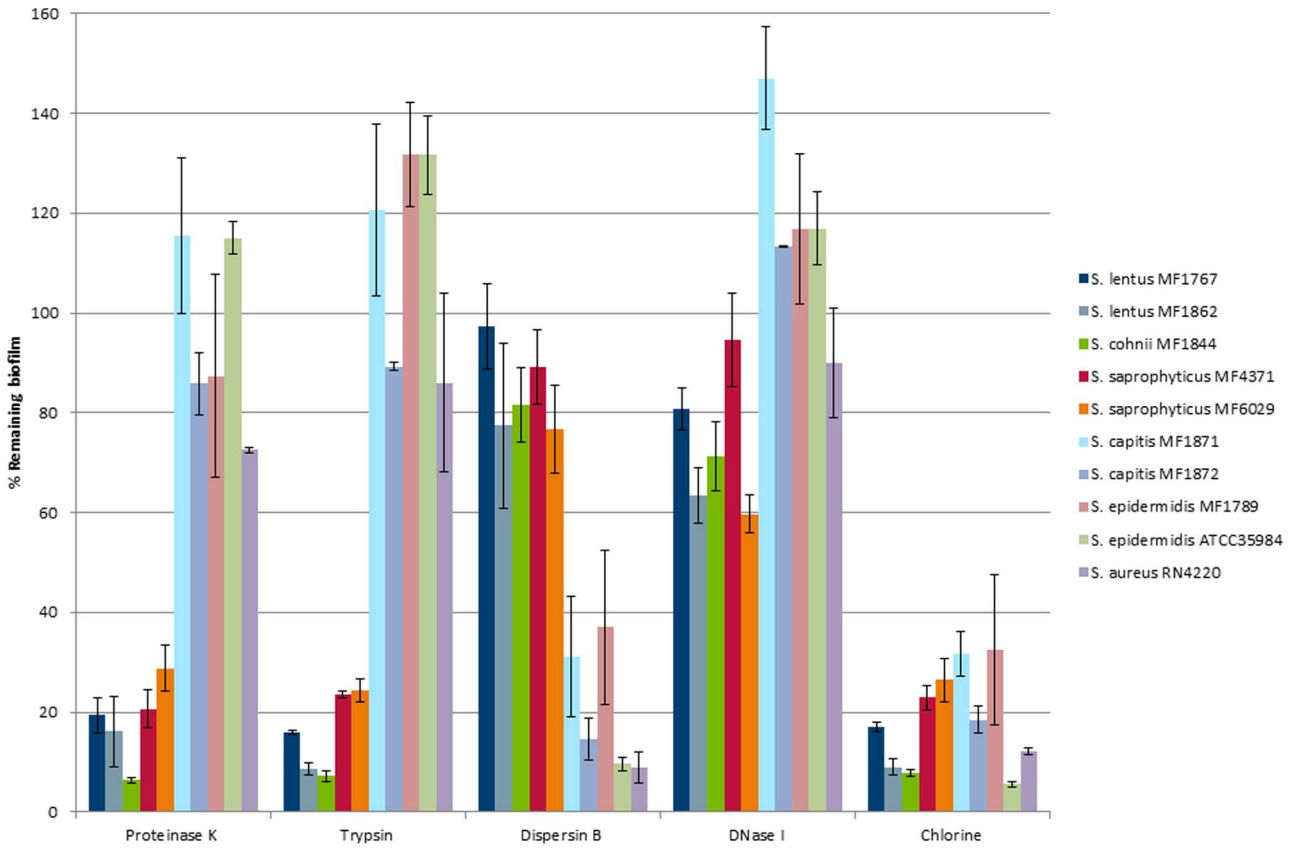

FIGURE 1 | Remaining biofilm (%) after treatment with enzymes and chlorine for ten *Staphylococcus* spp. strains. Data presented are means and standard errors of percent remaining biofilm after treatment compared to control (PBS treatment) for triplicate experiments.

matrix primarily consisting of proteins and not polysaccharides (Jabbouri and Sadovskaya, 2010). In contrast, biofilms made by the strains *S. capitis* MF1871 and MF1872, *S. epidermidis* MF1789 and ATCC 35984, and *S. aureus* RN4220 detached upon treatment with Dispersin B, but not upon treatment with Proteinase K or trypsin (**Figure 1**). For simplicity, these strains were termed as belonging to "PIA biofilm group." A detachment effect ($p = 0.014$) of DNase I was observed for the strains belonging to the protein biofilm group (26% mean detachment), while no effect ($p = 0.14$) was observed for the strains in the PIA biofilm group. Chlorine had a strong detachment effect on biofilms of all strains and there were no significant differences in effect of chlorine on biofilm detachment between the two groups (**Figure 1**; $p = 0.61$).

Genome Sequencing and Analysis

The genomes of the eight biofilm producing staphylococci (**Table 1**) were sequenced to examine the presence of specific biofilm- or matrix-associated genes and BC resistance determinants (see below). The main general features of all eight genome assemblies are shown in Supplementary Table S1. The genome sizes ranged from 2.5 to 2.7 Mb and the GC content ranged from 31.8 to 33.1%, which is in the range typically found in *Staphylococcus* spp. genomes (Suzuki et al., 2012).

Genome sequence analyses showed that all five strains of the PIA biofilm group contained the complete *icaR-icaADBC* locus required for production of PIA. The *ica* genes were not found in any strains from the protein biofilm group. Genes encoding putative additional Baps are summarized in **Table 2**, with additional information detailed in Supplementary Table S2 and further described below.

A gene encoding homologs to the Small basic protein (Sbp) reported to be critical for biofilm formation in *S. epidermidis* (Decker et al., 2015), and genes encoding homologs to the two reported autolysin/adhesins AtlE and Aae were found to be conserved across all analyzed genomes.

Putative Biofilm Associated Genes Present in the Protein Biofilm Group

The two *S. lentus* strains MF1767 and MF1862, which belonged to the protein biofilm group, each encoded homologs to ClfB and IsdC, known to mediate biofilm formation under specific conditions (Abraham and Jefferson, 2012; Missineo et al., 2014). In both *S. lentus* genomes, we also found evidence of a large CWA protein, encoded on several different contigs, which we will refer to as *Staphylococcus lentus* surface protein A (SlsA). (**Table 2** and **Figure 2A**). The N-terminal parts of SlsA containing YSIRK signal peptide domains were encoded by genes AXY34_13120

TABLE 2 | Presence of genes potentially associated with biofilm formation, including cell wall anchored (CWA) proteins[a].

	Protein biofilm group					PIA biofilm group				
Species	***S. lentus***		***S. cohnii***	***S. saprophyticus***		***S. capitis***		***S. epidermidis***		***S. aureus***
Strain	**MF1767**	**MF1862**	**MF1844**	**MF4371**	**MF6029**	**MF1871**	**MF1872**	**MF1789**	**ATCC 35984**	**RN4220[b]**
Locus tag prefix	**AXY34_**	**AXY37_**	**AXY36_**	**AXY40_**	**AXY41_**	**AXY38_**	**AXY39_**	**AXY35_**	**SERP**	**SAOUHSC_[b]**
PIA	–	–	–	–	–	*icaR icaADBC*	*icaR icaADBC*	*icaR icaADBC*	*icaR icaADBC*	*icaR icaADBC*
Embp/Ebh	–	–	–	–	–	*embp*[cd]	*embp*[d]	*embp*[d]	*embp*	*ebh*
SasC/Mrp/FmtB domain proteins	–	–	–	–	–	*sesA sesG*	*sesA sesG*[c]	*sesA*	*sesA sesG*	*sasB sasC*
Bap family	–	–	*bap*[d]	–	–	–	–	–	*bhp*	–
Serine-aspartate repeat proteins	*clfB*	*clfB*	–	–	–	*sdrX*[c] *sdrZL*[c]	*sdrX*[c] *sdrZL*[c]	*sdrE*[c] *sdrF sdrG sdrH*	*sdrF*[d] *sdrG sdrH*	*clfA clfB sdrC sdrD*
FnBPA, FnBPB	–	–	–	–	–	–	–	–	–	*fnbA fnbB*
S. saprophyticus surface protein G	–	–	–	*sssG*[c]	–	–	–	–	–	–
Uro-adherence factor A	–	–	–	*uafA*[c]	*uafA*[c]	–	–	–	–	–
S. lentus surface protein A	*slsA*[c]	*slsA*[c]	–	–	–	–	–	–	–	–
S. cohnii surface protein E	–	–	*scsE*[c]	–	–	–	–	–	–	–
S. epidermidis surface protein C	–	–	–	–	–	*sesC*	*sesC*	*sesC*	*sesC*	–
G5-E repeat family protein	–	–	–	–	–	–	–	–	*aap*	*sasG*
Three-helical bundle protein	–	–	–	–	–	*spa*[d]	*spa*[d]	–	–	*spa spi*
SRRP proteins	–	12885[c]	–	–	–	*sraP*[c]	*sraP*[c]	*sraP*	*sraP*	*sraP*
S. saprophyticus surface protein F	–	–	*sssF*	*sssF*	*sssF*	–	–	–	–	–
NEAT domain proteins	*isdC* 10605 12645	*isdC* 06145 05890	–	–	–	*isdC* 05010 04985 04990	*isdC* 11495 04030 04025	–	–	*isdC isdA isdB isdH*
Other LPxTG domain proteins	–	–	09850 09225 11360[c]	–	12070	*sesB sesE*-like *sesH*-like 10330	*sesB sesE*-like *sesH*-like 10295	*sesB sesE sesH*	*sesB sesE sesH sesI*	*sasD*[d] *sasF sasH*
Small basic protein (Sbp)	09055	01990	08170	06645	04110	11465	05970	05425	*sbp*	00617
Bifunctional autolysin AtlE	06865[c]	08990[c]	03795[c]	*aas*	*aas*[c]	*atlE*	*atlE*	*atlE*	*atlE*	*atl*
Multifunctional autolysin Aae	00485	01440	11620	10120	09065	07140	07640	*aae*	*aae*	*aaa*
Eap/Emp	–	–	–	–	–	–	–	–	–	*eap emp*

[a]*For each identified gene, the gene name or locus tag is listed. The locus tag prefixes for each strain is listed below the strain names in the table header. Further details are found in Supplementary Table S2.* [b]*Locus tags are obtained from the genome of S. aureus NCTC 8325 (CP000253), from which S. aureus RN4220 is derived (Herbert et al., 2010), since the publicly available genome sequence of RN4220 (AFGU01000000) is not annotated. All listed genes were also present in RN4220.* [c]*The predicted protein is encoded on multiple contigs. For further details, see Supplementary Table S2.* [d]*The gene encodes a truncated protein and/or contains an internal stop codon.*

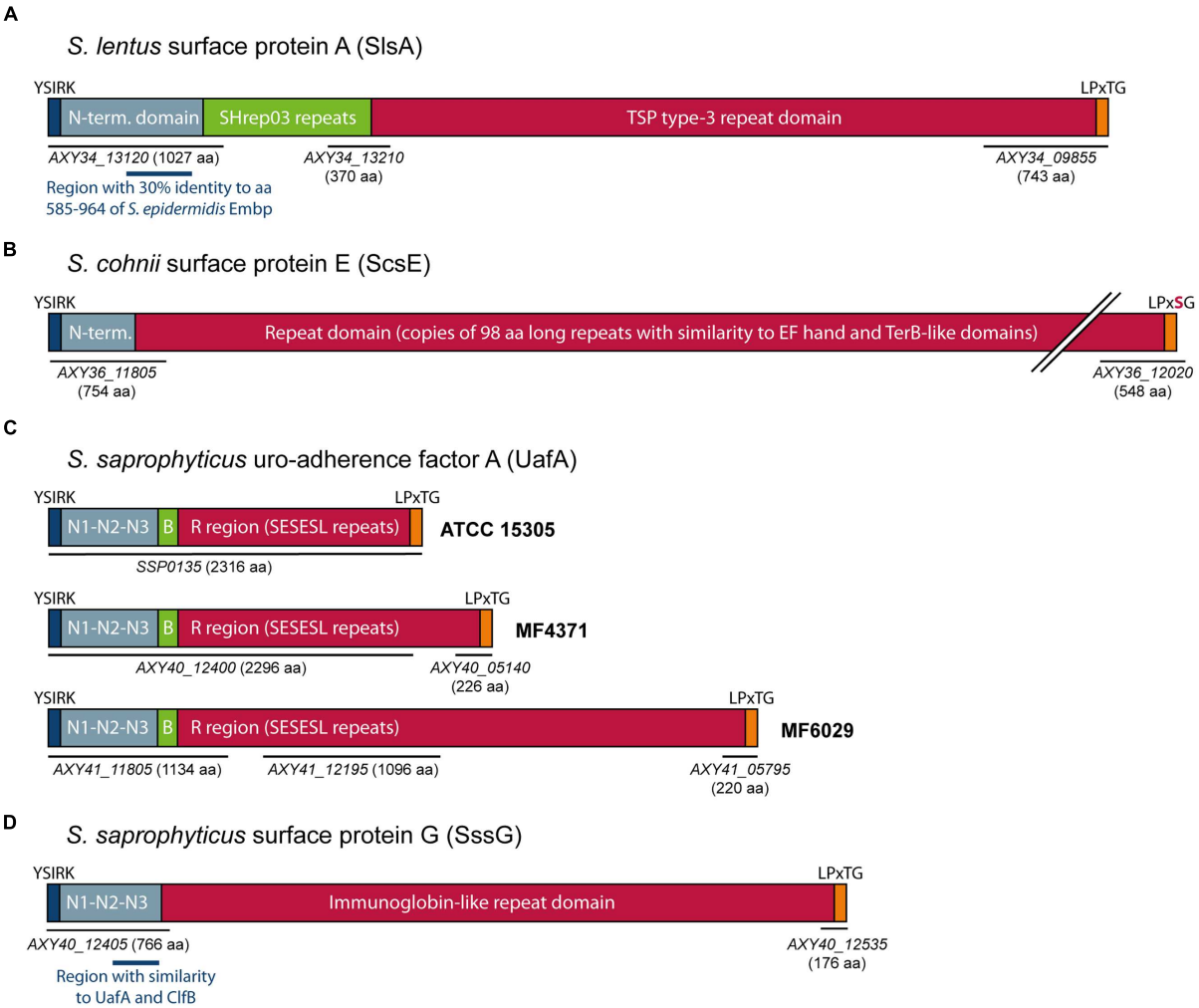

FIGURE 2 | Cell-wall anchored proteins identified in food associated *Staphylococcus* spp. strains producing proteinaceous biofilms (A–D). The diagrams show the predicted organization of protein subdomains. All proteins except UafA from strain ATCC 15305 (in panel **C**) were encoded on multiple contigs in the genome assemblies. Selected loci encoding specific protein sections are indicated by black bars below each diagram, and selected regions of similarity with known biofilm associated proteins are shown with blue bars. The relative length of each diagram is meant to illustrate the size of the proteins estimated from the length and read coverage of the contigs encoding each protein. However, in **(B)**, for clarity, the repeat domain of ScsE is shortened relative to the estimated length.

and AXY37_12645. These partial proteins showed about 30% identity at the amino acid level to the N-terminal domain of *S. epidermidis* Embp protein in an alignment covering ~400 amino acids (aa). They also contained tandem copies of a 90 aa long repeat sequence similar to those referred to as SHrep03 repeats in the protein encoded at locus SH1471 in *Staphylococcus haemolyticus* strains JCSC1435 (accession AP006716). In both strains, ORFs containing copies of the SHrep03 repeat and tandem TSP type 3 repeat domains (IPR028974) were encoded on short contigs (AXY34_13210 and AXY37_12875), predicted to represent the central part of *slsA*. The putative C-terminal of each protein, with tandem TSP type 3 repeat domains and a Gram positive anchor domain containing a LPxTG motif was identified next to a gap in each assembly (AXY34_09855 and AXY37_10540). In addition, several short contigs containing ORFs harboring the SHrep03 and TSP type 3 repeat domains were identified. When considering the length and assembly coverage for the identified contig fragments covering this putative gene, the length of a putative intact gene was estimated to be about 20 Kbp, which would correspond to a protein almost 7000 amino acids in length.

One of the *S. lentus* strains; MF1862, additionally contained a second partial gene, AXY37_10705, located next to an assembly gap and which encoded a protein with an LPxTG anchor motif. This protein contained repeats similar to those found in SRRPs such as SraP of *S. aureus*, which have been shown to promote biofilm formation in microtiter plates (Sanchez et al., 2010). The MF1862 genome additionally contained four short contigs encoding single ORFs harboring serine-rich repeats similar to those found in AXY37_10705. Located downstream of AXY37_10705 were two genes encoding glycosyltransferases GtfA and GtfB, which are involved in the first step of SRRP

glycosylation, however, the MF1862 genome did not encode the accessory Sec proteins usually associated with SRRP genes in other species (Lizcano et al., 2012).

The third strain belonging to the protein biofilm group, *S. cohnii* MF1844 (**Figure 1**), contained several genes encoding putative CWA proteins (**Table 2**). One of these genes, AXY36_09850, encodes a 1123 aa long LPxTG protein containing four MucBP (MUCin-Binding Protein) domains (PF06458). A second locus contained two neighboring genes (AXY36_12050 and AXY36_12055) which encode protein fragments with around 60% identity toward regions 1–937 and 1467–2164, respectively, of the 2276 aa long Bap from *S. aureus* V329 (AAK38834; Cucarella et al., 2001). However, the segment aligning to *S. aureus bap* contains an internal stop codon in the region encoding the spacer fragment separating the N-terminal B region of Bap from the C repeat domain. This presumably renders the *bap* gene non-functional in MF1844.

Staphylococcus cohnii MF1844 also harbored sequence fragments strongly indicating the presence of a large CWA protein with a large central domain containing tandem repeats, flanked by a non-repetitive N-terminal domain and a C-terminal anchor domain (**Table 2** and **Figure 2B**). We will refer to this protein as *Staphylococcus cohnii* surface protein E (ScsE). The C-terminal of ScsE was encoded at locus AXY36_12020, located about 5 Kb upstream of the locus showing homology to *bap*, and contained a non-canonical LPxSG cell-wall sorting domain. The N-terminal domain containing an YSIRK type signal peptide sequence was encoded by the partial gene at locus AXY36_11805. These partial protein sequences have lenghts of 754 and 549 aa, respectively, and align with 99 and 97% identity toward the corresponding parts of a 3192 aa long uncharacterized protein encoded at locus XA21_08340 in *S. cohnii* strain 532 (accession LATV01000000). Two additional homologs were found in *S. cohnii* strain 57 (LATU01000000) and *S. cohnii* strain hu-01 (AYOS02000000). The central region of these proteins harbor various numbers of a tandem repeat of length 98 aa, which show similarity to the protein domains named ≪EF-hand domain pair≫ (IPR011992) and ≪TerB-like≫ (IPR029024). In *S. cohnii* MF1844, 23 additional short contigs encoding single ORFs aligning to this repeat were identified. The combined lengths of these ORFs were 4717 aa, indicating that the MF1844 homolog would have a length of at least 6000 aa. However, since the 23 short contigs on average have levels of coverage over fivefold higher than the overall average assembly coverage for the MF1844 genome, a putative functional homolog in MF1844 could potentially be significantly larger than this.

The final two strains belonging to the protein biofilm group were *S. saprophyticus* MF4371 and MF6029 (**Figure 1**). Three genes encoding CWA proteins were identified in each genome (**Table 2**). Both strains encoded the MSCRAMM adhesin named uro-adherence factor A (UafA) previously described in *S. saprophyticus* ATCC 15305 (Kuroda et al., 2005; Matsuoka et al., 2011); (**Figure 2C**). The N-terminal parts of the UafA proteins, containing the YSIRK signal peptide domain, the A-region which consists of the three subdomains N1, N2, and N3, the B-region, and the first part of the low complexity Ser-Glu-rich R region (composed of SESESL-like repeats) were encoded next to assembly gaps on loci AXY40_12400 and AXY41_11805 in the genomes of MF4371 and MF6029, respectively. These ORFs showed 99% amino acid sequence identity toward UafA of *S. saprophyticus* ATCC 15305. The C-terminal regions were encoded at loci AXY40_05140 and AXY41_05795, and contained the last part of the R region and the wall-membrane-spanning regions containing LPxTG motifs, which was identical in the three strains MF4371, MF6029 and ATCC 15305. In MF4371, one additional short contig encoding the R region SESESL-like repeats was identified (AXY40_12580), while in MF6029, six such contigs were identified. The assembly coverage for these short contigs were significantly higher than the average assembly coverage for the MF4371 and MF6029 genomes, indicating that the R region of UafA in these strains were expanded compared to in UafA from ATCC 15305, in particular in MF6029 (**Figure 2C**).

Staphylococcus saprophyticus MF4371 appears to also encode a second MSCRAMM protein, which we will refer to as *Staphylococcus saprophyticus* surface protein G (SssG; **Figure 2D**). Fragments of the *sssG* gene were identified on four different contigs in the genome assembly. The N-terminal region of SssG (AXY40_12405) contained two adhesion domains (IPR008966) similar to those found in the N-terminal A domains of MSCRAMM proteins such as UafA, FnBPA, and ClfA. While alignments show only around 20–24% amino acid sequence identity between SssG and these MSCRAMMs, analysis using protein structure prediction methods indicates that this region of SssG adopts a fold similar to that of the ligand-binding N2-N3 domains of MSCRAMM proteins such as ClfA (PDB: 1N67), Bbp (PDB: 5CF3), and UafA (PDB: 3IRP). AXY40_12405 also contains part of the central repeat domain of SssG. Sections of the central repeat domain were also present in the locus encoding the C-terminal fragment harboring the LPxTG motif (AXY40_12535), and on two additional short contigs (AXY40_12590, AXY40_12620). Alignments of fragments encoding the central repeat domain revealed a 89 aa long repeat unit which was 62% identical and 78% similar to the immunoglobin (Ig)-like B repeats found in the central region of the *S. epidermidis* Bap family protein Bhp (Tormo et al., 2005). The two short contigs had read coverage about 20x higher than the average MF4371 assembly coverage, suggesting that SssG harbors multiple, highly identical tandemly repeated Ig-like domains. A transposase gene was located downstream of the locus encoding the C-terminal of SssG, suggesting that *sssG* is found on a mobile genetic element.

Repertoire of Surface Proteins in the Strains of the PIA Biofilm Group

The PIA biofilm group is composed of two *S. capitis* strains and two *S. epidermidis* strains, in addition to the reference strain *S. aureus* R4220. All five strains are members of the Epidermidis–Aureus species group, and thus relatively closely related compared with the strains in the protein-biofilm group (Lamers et al., 2012). The close relationship between these strains was reflected in a similar content of cell-wall associated proteins encoded in their genomes (**Table 2**). The close relationship was

particularly evident for the two *S. capitis* strains, for which the majority of analyzed proteins showed 100% identity between the two strains.

Overall, we identified 10–19 CWA proteins encoded in the genomes of the PIA-biofilm strains, which is a significantly higher number than that found in the strains of the protein-biofilm group (3–6 CWA proteins). Homologs to several CWA proteins which have previously been shown to be involved in mediating biofilm formation in microtiter plate assays, namely Aap/SasG, SdrC, SasC, SesC, and SraP, were encoded in more than one of the strains in the PIA biofilm group (Rohde et al., 2005; Geoghegan et al., 2010; Sanchez et al., 2010; Barbu et al., 2014; Khodaparast et al., 2016). Furthermore, all strains harbored homologs to genes encoding the giant protein Ebh/Embp (Christner et al., 2010). However, in the three food-associated strains in this group, the *ebh*/*embp* genes contained multiple internal stop codons separating the gene into several open reading frames, similar to what has previously been observed for *S. aureus* N325 and Mu50 (Clarke et al., 2002).

Presence of QAC-Tolerance Associated Genes

The *S. capitis* strains MF1871 and MF1872 were previously known to contain the *qacA* gene encoding the QacA MFS multidrug efflux pump known to increase tolerance to multiple substrates including the biocides QAC and chlorhexidine (Heir et al., 1995). The presence of genes encoding QacA and the QacR transcriptional repressor was confirmed by WGS of both strains. The *qacR-qacA* genes (locus tags AXY38_11220/AXY38_11225 and AXY39_11475/AXY39_11470) were present on contigs which showed sequence similarity toward several *Staphylococcus* spp. plasmids, suggesting that the *qacA* genes in MF1871 and MF1872 were plasmid-borne. None of the other analyzed genomes contained genes encoding QacA or the highly similar QacB proteins (Wassenaar et al., 2015).

Sequence analysis furthermore showed that three of the analyzed strains contained genes encoding QacC/Smr family SMR multidrug efflux pumps. The two *S. saprophyticus* strains MF4371 and MF6029 encode QacJ (AXY40_12555) and QacC (AXY41_12200), respectively. Their respective *qac* genes were found on short (∼3 Kbp) contigs, having higher read coverage than the average read depth for the whole genome assemblies, and which contained genes encoding a plasmid replication protein. This suggests that the *qacJ* and *qacC* genes in MF4371 and MF6029 reside on small, multicopy plasmids. In contrast, *S. cohnii* MF1844 encodes a protein (AXY36_07250) with 94% identity toward QacH (WP_019467894) which appeared to be chromosomally encoded. The *qacH* gene was present on a 404 Kbp long contig with read coverage similar to the average assembly coverage for MF1844 and which encoded typical chromosomal genes.

Tolerance to Benzalkonium Chloride

The two food-associated *S. saprophyticus* isolates MF4371 and MF6029 harboring plasmid-encoded *qacJ* and *qacC* genes, respectively, as well *S. aureus* RN4220/*qacC* (control strain expressing *qacC*; **Table 1**), had MICs of 6–8 ppm BC. The two *qacA*-positive *S. capitis* isolates MF1871 and MF1872 and the *qac*-negative isolate *S. lentus* MF1862 had MICs of 4 ppm. The remaining isolates, including the *qacH*-positive *S. cohnii* MF1844, had MICs below 4 ppm BC.

The strains that formed protein-dependent biofilms (biofilms degraded by proteinase) were more susceptible to the lethal action of BC in biocidal tests than strains producing biofilms degraded by the glycoside hydrolase Dispersin B. This difference was significant both in biofilms ($p = 0.04$; **Figure 3A**) and in suspensions ($p = 0.014$; **Figure 3B**). There were no differences ($p = 0.89$ for biofilm, $p = 0.73$ for suspension) in susceptibility toward BC between strain containing *qac* genes and the other strains. The strains were more susceptible to BC in suspension than in biofilm.

DISCUSSION

Biofilm Formation in Food Associated Staphylococci

As has also been shown in other studies, the frequency of food associated staphylococci showing strong biofilm formation *in vitro* was low compared to what has been reported for clinical/human strains, even when using methodology that is optimized with high salt and sugar concentrations and temperatures allowing growth (Møretrø et al., 2003; Jaglic et al., 2010). Also, in a survey of attached microbiota from dairies, it was concluded that since only one out of eight staphylococci isolated were strong biofilm formers, biofilm formation was unlikely an explanation for survival on milk contact surfaces (Cherif-Antar et al., 2016). In the present study, five out of nine poultry associated CNS belonging to four different species were strong biofilm formers, suggesting an association between biofilm formation abilities and poultry origin. However, a larger collection of strains would be necessary to confirm this.

Resistance toward BC

Staphylococcus, especially coagulase-negative species are among the most frequently isolated bacteria from food processing surfaces and survival after both cleaning and disinfection has been explained by specific resistance mechanisms and formation of a protective biofilm matrix (Langsrud, 2009). As also shown by others (Campanac et al., 2002; Luppens et al., 2002) biofilm formation can protect cells from disinfection, illustrated by a similar range of bactericidal effect at 10 ppm BC in suspension tests and 200 ppm (user-concentration) in biofilm tests. One explanation for the observed protection is that the bactericidal agent does not reach the target cells because of reduced diffusion and/or neutralization of the compounds by the matrix (Bridier et al., 2011). Interestingly, not only biofilm in itself, but the matrix composition appeared to affect bacterial resistance as strains belonging to the protein biofilm group were generally more susceptible than those belonging to the PIA biofilm group. This suggests that a biofilm matrix dominated by polysaccharides protects staphylococci against BC better than a matrix dominated

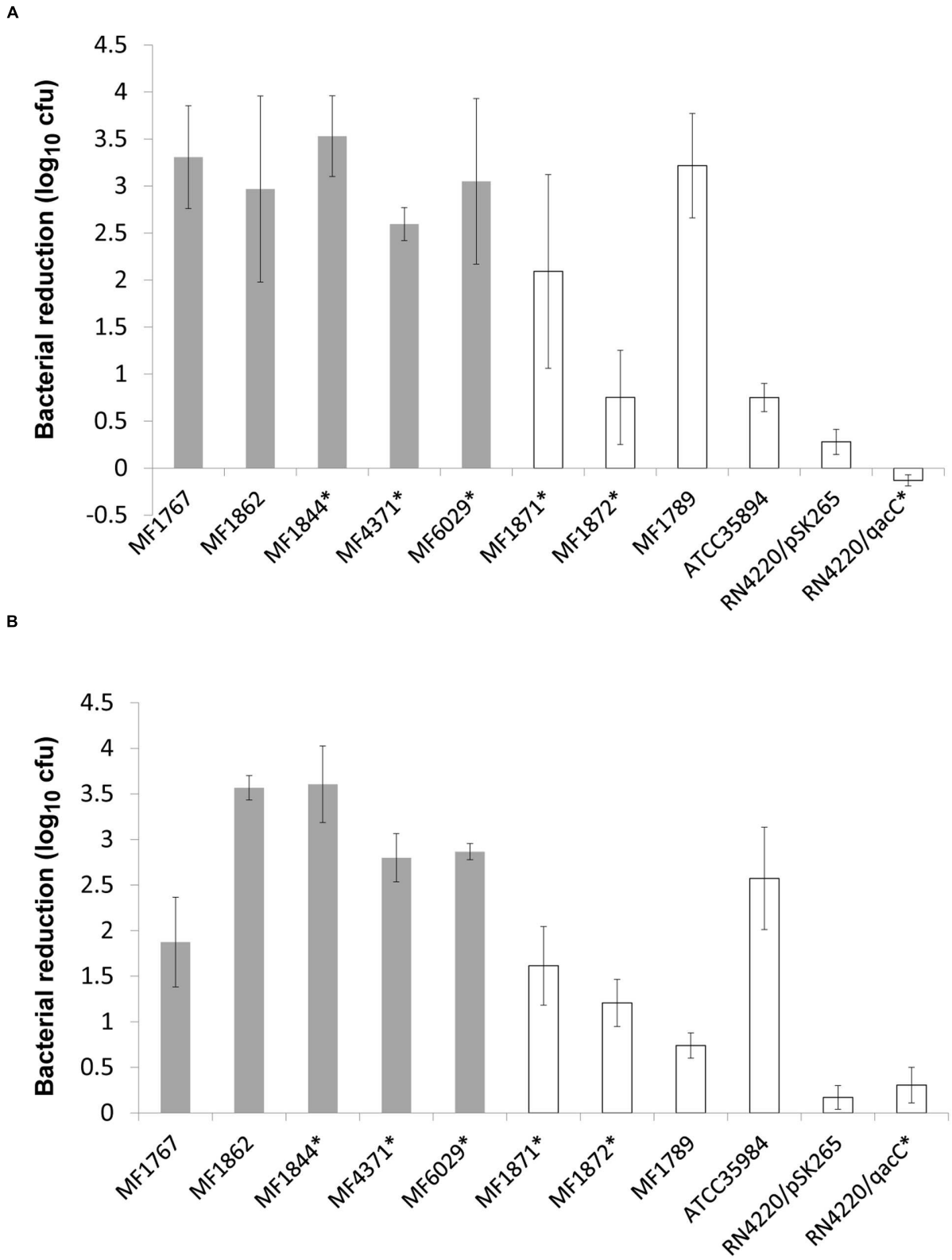

FIGURE 3 | Effect of benzalkonium chloride (BC) against *Staphylococcus* spp. strains in (A) biofilm (200 ppm BC) and (B) suspension (10 ppm BC). Strains belonging to "protein biofilm group" in gray and "PIA biofilm group" in white. Presence of *qac* genes is indicated with asterisk after strain name. Means and standard errors are shown.

by proteins. One possible explanation is reduced diffusion of the positively charged BC in a biofilm in which the negatively charged PIA is a major matrix component, a resistance mechanism that has been suggested also by others (Ganeshnarayan et al., 2009). It should be pointed out that the difference in BC susceptibility between the two groups were not restricted to biofilms, but also appeared in suspension. This indicated that other, intrinsic mechanisms could be involved, or that PIA to a certain extent is also produced in suspension (Vandecasteele et al., 2003). Also, one of the PIA biofilm strains showed an equal level of sensitivity to BC as the protein biofilm group strains. Together, the large variances in phenotypic resistance patterns observed reflected the profound genomic differences between strains (see below).

Differences in tolerance to BC in staphylococci have traditionally been explained by the presence of *qac* genes encoding efflux pumps. Apparently, biofilm growth is a much more powerful resistance mechanism than these efflux mechanisms. In accordance with recent results obtained by Furi et al. (2013), we observed no protective effect of *qac*-genes in bactericidal tests against BC in biofilms or in suspension. Nevertheless, our results supported earlier reports about the role of *qac* genes for the ability to grow in the presence of low concentrations of BC (Furi et al., 2013; Skovgaard et al., 2013; Marchi et al., 2015). *S. cohnii* MF1844 was susceptible to BC, despite harboring a *qacH*-like gene. This could be due to a lower gene copy number, low gene expression or less effective efflux mechanism compared to similar pumps. The intermediate susceptibility of the *qac*-negative *S. lentus* may be due to unknown efflux mechanisms or resistance acquired, e.g., from adaptation. The biofilms of all strains were equally removed by user-concentrations of alkaline chlorine, which is a frequently used cleaning agent in the food industry. Chlorine has broad activity, can dissolve and remove proteins, polysaccharide, DNA, and lipids (Fukuzaki, 2006), and has been shown to eradicate biofilms of MRSA (Lee et al., 2009). Whether the hypochlorite treatment can level out differences in susceptibility to disinfectants should be further studied.

Strains Showing PIA-Dependent Biofilm Formation

Phylogenetically, the species of the genus *Staphylococcus* may be divided into 15 cluster groups and six species groups (Lamers et al., 2012). The four CNS strains in the PIA biofilm group were all members of the Epidermidis cluster group, belonging to the Epidermidis–Aureus species group. The *ica* locus has been found in several different staphylococcal species (Cramton et al., 1999; Møretrø et al., 2003) but its presence does not necessarily lead to PIA production since expression is regulated in response to environmental conditions (Arciola et al., 2015). In the current study, the growth medium was supplemented with glucose and sodium chloride to promote PIA production (Ammendolia et al., 1999; Rode et al., 2007) and all *ica*-positive biofilm forming strains produced a biofilm matrix that was detached by Dispersin B (**Figure 1**). This suggested that PIA was a main structural component of the biofilm matrix in these strains. For the *ica*-positive control strain *S. epidermidis* ATCC 35984, this result was in accordance with previous findings (Chaignon et al., 2007).

Homologs to a number of genes encoding proteins that have been associated with staphylococcal biofilm formation under conditions similar to those used in the current study, including *aap*/*sasG*, *sdrC*, *sasC*, *sesC*, *spa*, *sraP*, and *embp* (**Table 2**) were found in strains belonging to the PIA biofilm group. However, since the biofilms formed by this group of strains were almost completely eradicated upon treatment with Dispersin B (**Figure 1**), these proteins did not appear to be able to compensate for the loss of structural stability seen upon degradation of PIA in the biofilm matrix. Further examination would be required to determine whether any of these proteins nevertheless does contribute to one or more of the stages during biofilm development in these strains.

CNS Strains Producing Proteinaceous Biofilm Matrix

Due to their relevance as human pathogens, biofilm formation has been extensively investigated in *S. epidermidis* and *S. aureus* (Arciola et al., 2015), while in contrast, much less is presently known about the mechanisms of biofilm formation in more distantly related CNS strains. However, proteinaceous biofilms have earlier been reported for several CNS strains outside of the Epidermidis cluster group, including *S. lugdunensis*, *S. haemolyticus,* and *S. cohnii* (Chaignon et al., 2007; Fredheim et al., 2009; Potter et al., 2009). The five CNS strains from the current study producing *ica*-independent biofilms were identified as *S. cohnii* and *S. saprophyticus*, belonging to the Saprophyticus species group, and *S. lentus*, which belongs to the Sciuri species group (Lamers et al., 2012). With the exception of homologs to the three biofilm associated genes encoding Sbp and the autolysin/adhesins AtlE and Aae, present in all ten examined strains (regardless of their sensitivity to Dispersin B and proteinases), and the genes encoding ClfB and IsdC, found in the two examined *S. lentus* strains, no genes encoding known Baps were identified in the genomes of the strains in the protein biofilm group in the current study (**Table 2**). The presence of *sbp* and *atlE*/*aae* is probably required, but not sufficient, for biofilm formation. Furthermore, ClfB and IsdC only appears to mediate biofilm formation in the absence of calcium and under low-iron growth conditions, respectively (Abraham and Jefferson, 2012; Missineo et al., 2014), which are conditions not encountered in the current study. It therefore seems likely that yet undescribed mechanisms may account for the observed ability of these strains to build a biofilm.

Search for Putative Novel Biofilm Associated Proteins

In *S. aureus* and *S. epidermidis*, proteins able to mediate biofilm formation in the absence of PIA are generally found to be large CWA proteins. Of these, several MSCRAMM proteins appear to play dual roles, able to act both as adhesins binding to human ECM proteins and as mediators of biofilm formation on abiotic surfaces by promoting bacterial intercellular interactions (Abraham and Jefferson, 2012; Geoghegan et al., 2013;

Barbu et al., 2014). In order to identify potential proteins involved in biofilm formation in the *ica*-negative isolates examined in the current study, the genomes were screened for the presence of proteins with cell wall anchor domains, in addition to searching for homologs to genes encoding known Baps. Overall, we identified a much lower number of CWA proteins encoded in the genomes of the five *S. lentus*, *S. cohnii* and *S. saprophyticus* strains (3–6 proteins), compared with the numbers found in the five examined strains belonging to the Epidermidis–Aureus species group (10–19 proteins; **Table 2**). It should be noted that the method of WGS employed in the current study, in which *de novo* genome assemblies were generated from relatively short-read sequencing data, is known to result in gaps in the genome assembly at sites of sequence repeats. Therefore we were not surprised to find that most of the genes encoding the highly repeat-rich proteins identified in the current study were encoded on more than one contig in the genome assembly.

Both *S. lentus* strains encoded what appeared to be a large CWA protein with a C-terminal LPxTG motif, which we have referred to as SlsA (**Figure 2A**). The N-terminal domain of SlsA is similar in sequence to that of *S. epidermidis* Embp, and the central and C-terminal domains of SlsA harbor two types of repeat sequences: SHrep03 repeats and TSP type 3 repeats. To our knowledge, a protein with this domain organization has not been previously described. However, the modular domain structure composed of an N-terminal non-repetitive region followed by various repeat domains is similar to that found in several staphylococcal biofilm-associated CWA proteins. Therefore, we consider SlsA as a candidate for a specific protein responsible for the observed biofilm phenotype in the examined *S. lentus* strains. One of the *S. lentus* strains, MF1862, additionally encoded a SRRP. This protein could possibly contribute to protein-dependent biofilm formation in this strain as SRRPs are known to mediate adhesion, bacterial aggregation, and biofilm formation (Lizcano et al., 2012).

Five CWA proteins were identified in *S. cohnii* MF1844 (**Table 2**). Of these, the protein encoded at locus AXY36_09850 contained four MucBP domains, and may potentially be involved in primary attachment. Proteins containing MucBP domains have been suggested to play a role during intestinal adhesion in *Lactobacillus* spp. (Kleerebezem et al., 2010), and contribute to biofilm formation in *Streptococcus thermophilus*, (Couvigny et al., 2015). Also, a gene encoding what appears to be a very large CWA protein, which we have named ScsE (**Figure 2B**) was identified as a candidate for a novel protein capable of mediating protein-dependent biofilm formation in *S. cohnii* strains. The predicted protein contained a ∼550 aa long non-repetitive N-terminal region, and multiple copies of a 98 aa long repeat showing similarity to EF-hand domain pair and TerB-like domains. Homologs to *scsE* from MF1844, encoding proteins with variable numbers of repeat domains, were found in three publicly available *S. cohnii* genome sequences. Neither the N-terminal domains nor the repeat domains from these proteins show any homology to any domains found in characterized CWA proteins known to be involved in adhesion or biofilm formation. However, as for the *S. lentus* SlsA protein, their overall domain organization is similar to that found in many staphylococcal MSCRAMM adhesins and known biofilm-associated CWA proteins. ScsE is therefore a candidate for a novel protein capable of contributing to protein-dependent biofilm formation in *S. cohnii* strains.

Staphylococcus saprophyticus, being a frequent cause of urinary tract infections in humans, has a repertoire of cell wall associated proteins which is slightly better described in the literature compared with that of the generally non-pathogenic *S. lentus* and *S. cohnii* (Becker et al., 2014). The CWA proteins UafA, UafB, and SdrI have been associated with adhesion in this species (Kuroda et al., 2005; Sakinc et al., 2006; King et al., 2011). Of these, only UafA was encoded in the genomes of *S. saprophyticus* strains MF4371 and MF6029. UafA is an hemagglutinin and an adhesin associated with adherence to uroepithelial cells (Kuroda et al., 2005) and has a domain structure typical of MSCRAMM adhesins, with a characteristic A region composed of subdomains N1, N2, and N3, a B region and a C-terminal Ser-Glu rich region of low complexity (**Figure 2C**) (Kuroda et al., 2005; Matsuoka et al., 2011). To our knowledge, the ability of UafA to mediate PIA-independent biofilm formation on abiotic surfaces has not been assessed. One report does, however, indicate an association between increased expression of UafA and increased biofilm formation in a microtiter plate based assay (Goneau et al., 2015), potentially suggesting that UafA may be a member of the growing list of MSCRAMMs that have been shown to be able to promote biofilm formation on abiotic surfaces through mediating intercellular adhesion. It has been suggested that the C-terminal Ser-Glu rich region of UafA may act as a stalk to present the ligand-binding A and B regions away from the bacterial cell surface (Matsuoka et al., 2011). If so, it is possible that elongated Ser-Glu rich region in the UafA homologs of MF4371 and MF6029 (**Figure 2C**) may enhance the accessibility of UafA for adhesion in these strains.

Staphylococcus saprophyticus MF4371 also encodes a previously undescribed CWA protein which we have referred to as SssG (**Figure 2D**). SssG has a highly interesting domain structure, containing what appears to be a N-terminal A-domain typical of those found in MSCRAMM family proteins, fused to a central domain composed of tandem repeats highly similar to those of the B-repeat region of the Bap family protein Bhp (Tormo et al., 2005). Potentially, both UafA and SssG may contribute to proteinaceous biofilm formation in strain MF4371.

Further work will be required to reveal whether any of the identified proteins described above, including SlsA, ScsE, UafA, and SssG, represent novel mechanisms of protein-mediated biofilm in CNS strains.

DNase I Treatment Had Limited Effect on Biofilm Detachment

DNase I had a slightly adverse effect on biofilm formation for four of the five *ica*-negative strains, but no effect on the *ica*-positive strains. The reason for this difference is not clear. Possibly, eDNA is more important for the structure of the protein dominated matrices, maybe by binding to proteins and stabilizing the matrix, while the PIA dominated matrix could be more stable in absence of eDNA. PIA-dependent biofilms have a more pronounced

mechanical robustness compared to protein-dependent biofilms and are significantly more stable against washing procedures (Büttner et al., 2015). For *S. epidermidis* it has been shown that eDNA is especially important in the early phases of biofilm formation (Qin et al., 2007), and this may explain why DNase I only had limited detachment effect on the mature biofilms in the present study.

CONCLUSION

In the present study differences in composition of biofilm matrix of food associated staphylococci was found, and strains with a protein biofilm were more susceptible to the disinfectant BC than strains with a PIA biofilm. Several putative novel mediators of proteinaceous biofilm formation in CNS strains were identified. Genes encoding staphylococcal QAC efflux proteins provide increased MIC-values to BC, but their presence was not associated with increased tolerance of staphylococci to biocidal concentrations.

AUTHOR CONTRIBUTIONS

AF, SL, EH, MM, and TM planned and designed experiments, interpreted and discussed results. AF, SL, EH, and TM wrote manuscript. AF performed the genome analyses. MM performed biofilm studies and disinfection experiments.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.00856

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DNase-Sensitive and -Resistant Modes of Biofilm Formation by *Listeria monocytogenes*

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Listeria monocytogenes is able to form biofilms on various surfaces and this ability is thought to contribute to persistence in the environment and on contact surfaces in the food industry. Extracellular DNA (eDNA) is a component of the biofilm matrix of many bacterial species and was shown to play a role in biofilm establishment of *L. monocytogenes*. In the present study, the effect of DNaseI treatment on biofilm formation of *L. monocytogenes* EGD-e was investigated under static and dynamic conditions in normal or diluted complex medium at different temperatures. Biofilm formation was quantified by crystal violet staining or visualized by confocal laser scanning microscopy. Biomass of surface-attached *L. monocytogenes* varies depending on temperature and dilution of media. Interestingly, *L. monocytogenes* EGD-e forms DNase-sensitive biofilms in diluted medium whereas in full strength medium DNaseI treatment had no effect. In line with these observations, eDNA is present in the matrix of biofilms grown in diluted but not full strength medium and supernatants of biofilms grown in diluted medium contain chromosomal DNA. The DNase-sensitive phenotype could be clearly linked to reduced ionic strength in the environment since dilution of medium in PBS or saline abolished DNase sensitivity. Several other but not all species of the genus *Listeria* display DNase-sensitive and -resistant modes of biofilm formation. These results indicate that *L. monocytogenes* biofilms are DNase-sensitive especially at low ionic strength, which might favor bacterial lysis and release of chromosomal DNA. Since low nutrient concentrations with increased osmotic pressure are conditions frequently found in food processing environments, DNaseI treatment represents an option to prevent or remove *Listeria* biofilms in industrial settings.

Keywords: biofilm, *Listeria monocytogenes*, extracellular DNA, osmotic pressure, DNase

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INTRODUCTION

Listeria monocytogenes (*Lm*) is a ubiquitous saprophytic soil bacterium and an opportunistic food-born human pathogen with a well characterized intracellular life-cycle (Vázquez-Boland et al., 2001; Hamon et al., 2006; Freitag et al., 2009). Severity of *Lm* infections and the symptoms of the associated disease (i.e., listeriosis) are dependent on the immune status of the patient

(Hamon et al., 2006; Freitag et al., 2009). Healthy people infected with *Lm* develop only mild gastrointestinal symptoms or remain totally asymptomatic. By contrast, *Lm* may cause severe systemic infections in at-risk individuals including pregnant women, newborns, elderly people and immunocompromised patients with mortality rates of up to 30% in these groups (Hamon et al., 2006; Freitag et al., 2009). All outbreaks reported in recent years have been associated with consumption of contaminated food. In 2009–2010, a listeriosis outbreak caused by acid curd cheese was reported in Austria and Germany with a total of 34 cases, eight of which were fatal. Subsequent genotyping revealed that these cases of listeriosis were actually the result of two independent outbreaks caused by distinct strains (Rychli et al., 2014). A recent outbreak in Denmark caused by a traditional meat product has claimed 13 deaths amongst 28 cases (Ethelberg, 2014) and a nation-wide outbreak in the USA in 2011 with 147 patients and 33 deaths could be traced back to contaminated cantaloupe (McCollum et al., 2013). Since then several smaller food-related outbreaks have been recorded in the USA (http://www.cdc.gov/listeria/outbreaks/).

As a saprophytic soil organism and intracellular pathogen that causes infections via the gastrointestinal route, *Lm* is able to survive and grow under a wide range of temperatures and stressful environmental conditions including acid and osmotic stress (Milillo et al., 2012; Gahan and Hill, 2014). Inside host cells nutrients are abundantly available and temperature is at constant 37°C. By contrast, in soil and food processing environments temperature is variable, nutrients are usually scarce, and osmotic conditions are suboptimal. It is not surprising that growth of *Lm* under host-conditions differs markedly from growth under environmental conditions (Freitag et al., 2009). Important features including biofilm formation, flagellar motility, and expression of virulence genes are subject to complex regulation by several mechanisms that depend on temperature, PrfA and σB (Johansson et al., 2002; Kamp and Higgins, 2009; Toledo-Arana et al., 2009; Lemon et al., 2010; Garmyn et al., 2012). Another system involved in the switch from saprophytism to virulence is the *agr* peptide sensing system. Mutants in one of the components of the *agr* system are attenuated for virulence *in vitro* and *in vivo* (Autret et al., 2003; Riedel et al., 2009) but also show defective biofilm formation and survival in soil (Rieu et al., 2007, 2008; Riedel et al., 2009; Vivant et al., 2015).

The ability to withstand (or even grow under) harsh environmental conditions or treatments usually applied to preserve fresh and ready-to-eat food products make *Lm* a serious problem for the food industries (Valderrama and Cutter, 2013). *Lm* has been shown to form biofilms on various surfaces and in different media (Harvey et al., 2007; Di Bonaventura et al., 2008; Rieu et al., 2008; Lemon et al., 2010; Renier et al., 2011). This feature greatly facilitates survival of *Lm* in this wide spectrum of habitats and, more importantly, in food processing environments. Moreover, biofilm formation not only provides protection against harmful environmental conditions but also increases resistance to sanitizing agents (Robbins et al., 2005; Pan et al., 2006; Berrang et al., 2008).

Biofilms are single- or multispecies microbial communities, which are embedded in a self-produced matrix of extracellular polymeric substances (EPS; Hall-Stoodley et al., 2004). Depending on the microorganism (or the community), EPS is composed of proteins, polysaccharides and/or extracellular DNA (eDNA; Flemming and Wingender, 2010). eDNA was shown to be an important structural component of the EPS matrix of a wide range of Gram-positive and -negative bacteria (Okshevsky and Meyer, 2015). For *Lm*, it was shown that stationary phase cultures grown in BHI medium contained DNA (Harmsen et al., 2010). Removal of DNA from the supernatants by DNaseI treatment inhibited initial attachment of bacteria in cultures diluted in phosphate-buffered saline (PBS) to glass and markedly delayed biofilm formation of bacteria grown in minimal medium in polystyrene microtiter plates (Harmsen et al., 2010).

With the present study, the role of eDNA during biofilm formation of *Lm* was investigated at different temperatures in normal and diluted complex medium. A wide range of different media (complex and defined, full strength and diluted) and temperatures are used by different groups to study biofilm formation of *Lm* (Monk et al., 2004; Folsom et al., 2006; Pan et al., 2006; Lemon et al., 2007; Riedel et al., 2009; Harmsen et al., 2010; Garmyn et al., 2011; Guilbaud et al., 2015). For the sake of simplicity, conditions were selected that represent normal and reduced nutrient concentrations with increased osmotic pressure (normal vs. diluted complex medium) as well as flagellated or non-motile bacteria (25 vs. 37°C). The results suggest that, irrespective of the temperature, *Lm* is able to form DNase-sensitive and -resistant biofilms depending on the osmotic conditions.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

All *Listeria* sp. strains used in this study are listed in **Table 1**. Bacteria were cultivated in brain heart infusion broth (BHI, Oxoid) or 10-fold diluted BHI (0.1BHI) at 25 or 37°C. Where indicated PBS (2.7 mM KCl, 137 mM NaCl, 1.5 mM KH_2PO_4, 7 mM Na_2HPO_4, pH 7.4) was used instead of demineralized water to prepare diluted medium for biofilm assays. Phosphate

TABLE 1 | Bacterial strains used in the present study.

Species/Strain	Lineage	Serotype	Reference/Source
L. monocytogenes EGD-e	II	1/2a	Bécavin et al., 2014[a]
L. monocytogenes LO28	II	1/2c	[a]
L. monocytogenes 10403S	II	1/2a	Bécavin et al., 2014[a]
L. monocytogenes F2365	I	4b	Nelson et al., 2004[a]
L. monocytogenes 33032	I	1/2b	Ducey et al., 2007[a]
L. innocua CIP10775	–	–	[b]
L. ivanovii CIP78.42	–	–	[b]
L. grayi CIP68.18	–	–	[b]
L. seeligeri SLCC3954	–	–	Steinweg et al., 2010[b]

[a]*Strains were kindly provided by Pat Casey and Colin Hill, University College Cork, Ireland.* [b]*Strains were kindly provided by Frederic Borges, Université de Lorraine, France.*

buffer and saline were prepared by omitting KCl and NaCl or KH_2PO_4 and Na_2HPO_4, respectively. To prepare an inoculum for biofilm assays, 10 ml BHI were inoculated with a single colony from a fresh agar plate and incubated aerobically at 37°C over night (o/N).

Microtiter Plate Biofilm Assays

Static biofilm assays were performed using a standard microtiter plate assay as described previously (Riedel et al., 2009). An o/N culture was diluted to an optical density (OD_{600}) of 0.05 in fresh BHI or 0.1BHI medium. Aliquots of 200 µl were distributed in polystyrene 96-well plates (Sarstedt) with four technical replicates per strain and condition. Where indicated, 1 unit (U) of DNaseI (Thermo Scientific) was added to the wells directly after inoculation. Plates were incubated at 25 or 37°C for 24 h. For analysis, biofilms were washed gently twice with PBS followed by staining with 0.1% (v/v) crystal violet solution (Merck) for 30 min. After three further washings with PBS crystal violet was released from biofilms by addition of 100 µl 96% (v/v) ethanol and incubated for 10 min. Biofilm biomass was quantified by measuring absorption at 562 nm ($Abs_{562\ nm}$) with background correction, i.e., crystal violet staining in wells incubated with sterile media under the same conditions. Background levels were $Abs_{562\ nm} = 0.10 \pm 0.02$ depending on the medium. In all cases, stained biomass of untreated biofilms was at least twofold above background.

Preparation and Detection of DNA in Biofilm Supernatants

For isolation of DNA, biofilms were prepared as described above. Supernatants from at least 12 wells per sample were collected and sterilized with 0.22 µm filters (Sarstedt). Sodium chloride was added to 1 ml supernatant to a final concentration of 250 mM. DNA was precipitated with 2.5 volumes of 96% (v/v) ethanol at −20°C o/N and harvested by centrifugation. DNA was washed once with 70% (v/v) ethanol, air-dried, and then dissolved in 50 µl demineralized water. To confirm the source of the isolated DNA, PCR was performed on the following genes: prfA, secA, lmo0849 and lmo1215. The primers used are listed in Supplementary Table S1. Taq polymerase S (Genaxxon BioScience GmbH) was used for amplification and annealing temperatures and extension times were optimized for each amplicon/primer pair. *Lm* EGD-e chromosomal DNA was used as control. DNA was analyzed by electrophoresis on 0.8% agarose gels in 1x TAE buffer and 1 kb or 50 bp ladders (Fermentas) were used as markers.

Analysis of Biofilms Grown Under Flow Conditions

For flow chamber biofilms, an o/N culture was diluted in fresh BHI or 0.1BHI medium to an OD_{600} of 0.05 and 200 µl of this inoculum was injected into the chamber of an IBIDI® µ-slide $VI^{0.4}$ Uncoated, which had previously been flushed with media. This inoculum was incubated for 1 h without flow in a horizontal position to allow for initial attachment of bacteria to the surface. The chamber was moved to a vertical position and flow of medium was started at a rate of 3.3 ml/h. Biofilms were incubated for 24 h at either 25 or 37°C prior to imaging. For DNaseI treatments, medium flow was turned off. Channels containing biofilms to be treated were flooded with 250 µl of a 100 µg/ml of DNaseI (247 Keunitz units/ml, Sigma) solution in PBS and incubated without flow for 1 h at room temperature prior to imaging.

Confocal Microscopy of Biofilms

Biofilms were grown under the conditions described above. For static biofilms ibidi® µ-Plate 96 Well Uncoated plates were used instead of polystyrene microtiter plates. After 24 h, medium was removed gently by aspiration, and biofilms washed three times with PBS. Biofilms were stained as described previously (Okshevsky and Meyer, 2014) in PBS containing 10 µM Syto 60® (Thermo Scientific), a red-fluorescent, membrane permeable dye staining live bacteria and 2 µM TOTO-1® (Thermo Scientific), i.e., a green-fluorescent dye staining eDNA or DNA of bacteria with a compromised membrane. Imaging was performed on a Zeiss LSM700 confocal laser scanning microscope (CLSM) equipped with 555 and 635 nm lasers and a variable dichroic beam splitter for simultaneous recording of the emitted light from the two fluorophores by separate photomultipliers. All images were captured with a 63× objective and analyzed using Zen 2012 software (Zeiss).

Statistical Analysis

All experiments were performed with at least three independent bacterial cultures (biological replicates). Normal distribution of the sample populations was assumed. Data was analyzed using Student's *t*-test or ANOVA with Bonferroni post-test analysis as indicated in the figure legends and $p < 0.05$ was considered statistically significant.

RESULTS

DNaseI-Sensitive and -Resistant Modes of Biofilm Formation by *Lm*

Initial attachment of *Lm* to glass and plastic surfaces was shown previously to be dependent on eDNA and later stages of biofilm formation are sensitive to DNaseI treatment (Harmsen et al., 2010). To characterize the role of eDNA in biofilm formation of *Lm* in more detail, biofilm assays were performed in polystyrene microtiter plates under static conditions at different temperatures in full strength or 0.1BHI (**Figure 1A**). These conditions were selected to represent normal and reduced nutrient concentrations with increased osmotic pressure (normal vs. diluted complex medium) as well as flagellated or non-motile bacteria (25 vs. 37°C). Moreover, BHI and these temperatures were used in previous studies on transcriptional profiling of *Lm* EGDe (Riedel et al., 2009; Garmyn et al., 2012). After 24 h, a maximum of biomass in *Lm* biofilms was obtained in BHI at 37°C ($A_{562\ nm} = 2.27 \pm 0.13$) and lowest levels of biofilm formation were observed in the same medium at

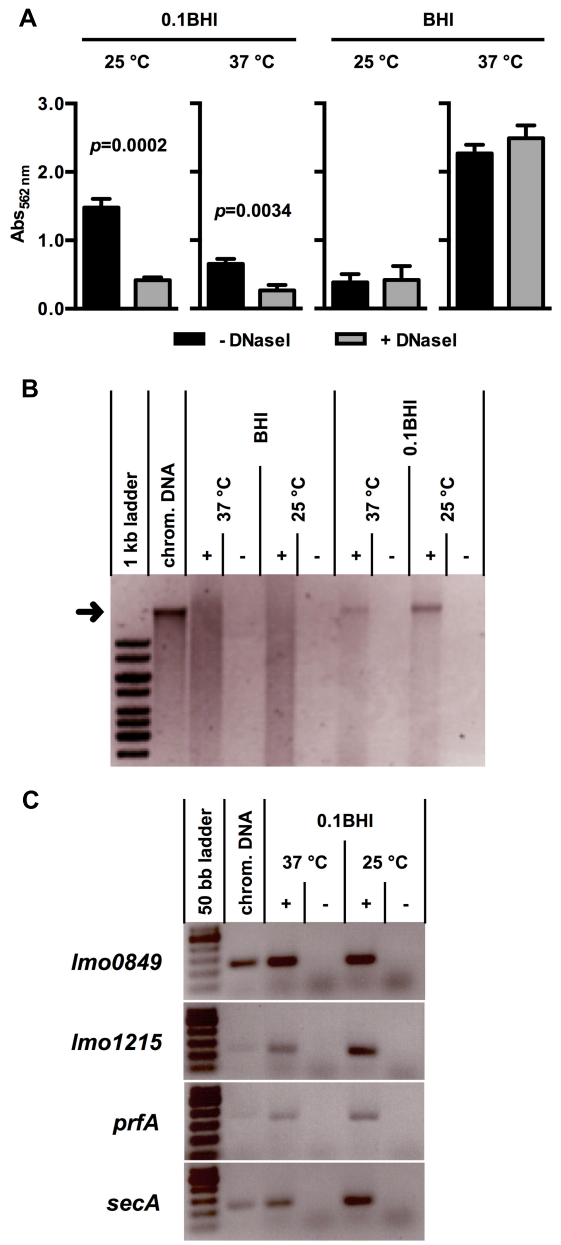

FIGURE 1 | (A) Biofilm formation of *Lm* EGD-e grown at 37 or 25°C in BHI or 0.1BHI in the presence (gray bars) or absence (black bars) of DNaseI. Values are mean ± standard deviation of three independent experiments. Data was analyzed using Student's *t*-test and *p*-values of statistically significant differences are indicated (all other comparisons: not significant, i.e., $p > 0.05$). **(B)** Precipitated DNA in supernatants of biofilms (+) shown in **(A)** (only biofilms without DNaseI) resolved by electrophoresis on a 0.8% agarose gel (size marker: 1 kb ladder). As controls, sterile media (–) were incubated under the same conditions and used for DNA precipitation. The size of isolated *Lm* EGD-e chromosomal DNA, which was run in a separate slot of the gel as control, is indicated by a black arrow. **(C)** PCR products targeting four genes encoded on the *Lm* EGD-e chromosome resolved by electrophoresis on a 2.0% agarose gel (size marker: 50 bp ladder). As template, DNA precipitated from biofilm grown in 0.1 BHI at 25 or 37°C (+) or sterile media controls (–) was used. *Lm* EGD-e chromosomal DNA served as a positive control for PCR reactions. In **(B)** and **(C)**, results of one representative of three independent experiments are shown.

25°C ($A_{562\ nm} = 0.38 \pm 0.12$). In 0.1BHI, biofilm biomass was higher at 25°C ($A_{562\ nm} = 1.48 \pm 0.13$) compared to 37°C ($A_{562\ nm} = 0.65 \pm 0.07$).

All experiments were performed in the presence and absence of DNaseI (**Figure 1A**). Interestingly, presence of DNaseI inhibited biofilm formation only in 0.1BHI at both temperatures and this effect was more pronounced at 25°C (37 °C: $p = 0.0034$; 25°C: $p = 0.0002$). Similar results were obtained when biofilms were grown for up to 48 h in the presence and absence of DNaseI (Supplementary Figure S1) or at 20 and 30°C (data not shown). Under all conditions tested, biofilms grown in 0.1BHI were sensitive to DNaseI but no significant effects of DNaseI treatment were observed in full strength BHI medium. Likewise, treatment of established biofilms for 1 h with DNaseI reduced biofilm biomass in diluted but not full strength medium and heat-inactivated DNaseI had no effect (Supplementary Figure S2). This demonstrates that enzymatic activity rather than presence of the protein is responsible for the observed effect.

Presence of eDNA in *Lm* Biofilms Grown Under Static Conditions

To further investigate presence and source of eDNA, nucleic acids were precipitated from biofilm supernatants. This yielded a distinct band of high molecular weight DNA in supernatants of *Lm* biofilms grown in 0.1BHI but not in full strength medium, which corresponded to the size of isolated chromosomal DNA of *Lm* EGD-e (**Figure 1B**). To further confirm the chromosomal origin of this eDNA, PCR targeting four distinct loci randomly distributed across the *Lm* EGD-e chromosome was performed using DNA isolated from biofilm supernatants as template. For all target genes, specific products were obtained from cultures grown in 0.1BHI (**Figure 1C**) suggesting that the observed bands (**Figure 1B**) are indeed chromosomal DNA.

In further experiments, eDNA in biofilms was visualized by confocal laser scanning microscopy. After 24 h of growth under static conditions large diffuse patches of eDNA were only observed when biofilms were grown in 0.1BHI (**Figures 2A,B**). Biofilms grown in 0.1BHI had a clear three-dimensional architecture with a confluent layer of bacteria at the bottom and large, cloud-like patches of eDNA extending up to 30 μm toward the top of the biofilm (**Figures 2A,B**). In biofilms grown in 0.1BHI at 25°C, a number of bacteria appeared to be in close proximity of these eDNA clouds suggesting they might be attached to these structures. Moreover, biofilms grown in 0.1BHI at 37°C had a more complex structure with hollow domes and channels in which eDNA appeared to serve as a structural component (**Figure 2B**).

In full strength BHI, biofilms were mostly flat and rather featureless (**Figures 2C,D**). In these biofilms, only a few, well defined spots stained positive for DNA. These signals had approximately the size of SYTO-60 positive live bacteria and thus probably represent intracellular DNA of intact, dead cells with a compromised membrane rather than eDNA from lysed bacteria.

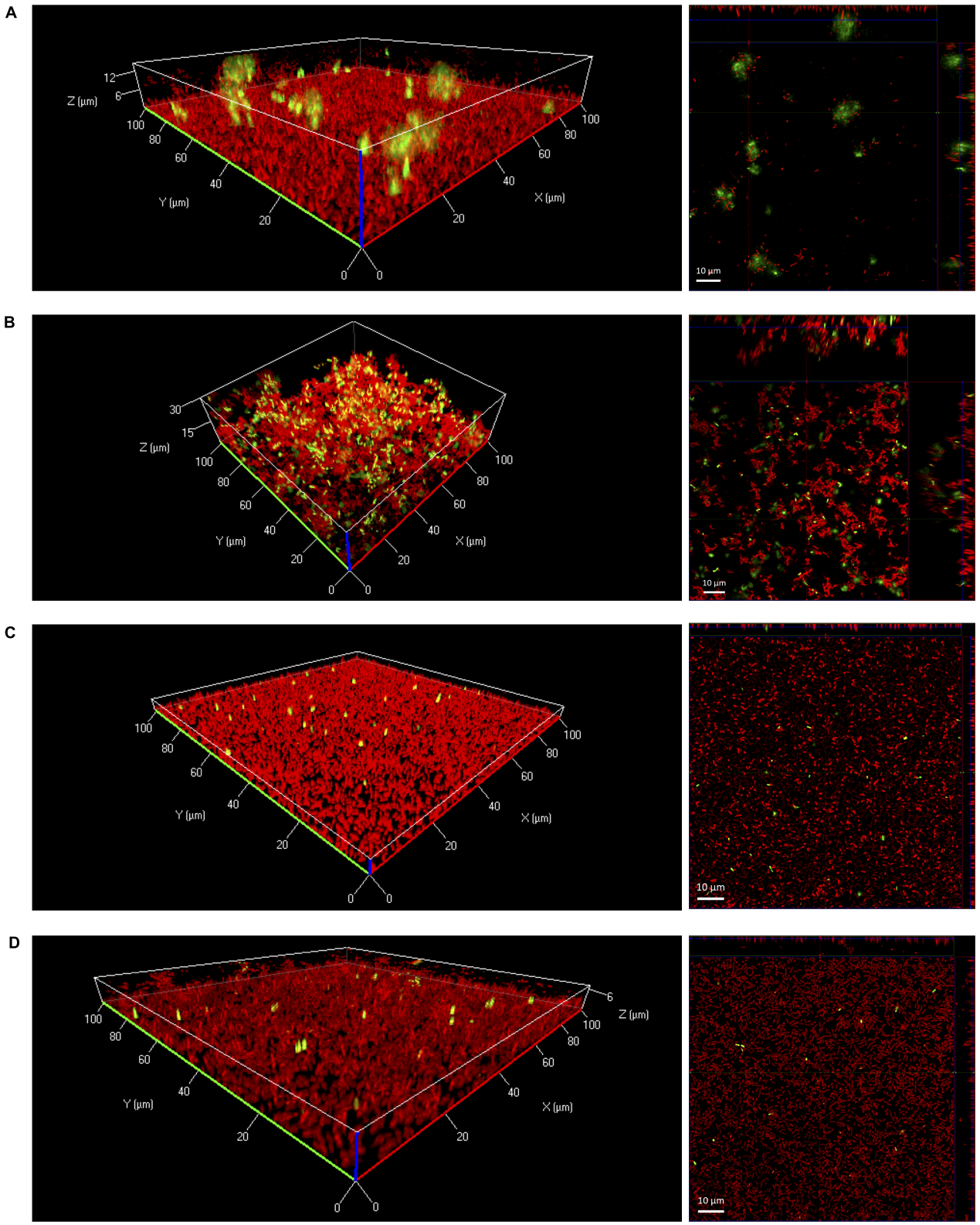

FIGURE 2 | Three-dimensional (left panels) or orthogonal projections (right panels) of CLSM Z-stack images of *Lm* EGD-e biofilms grown for 24 h under static conditions in 96-well microtiter plates. Media and temperatures were: **(A)** 0.1BHI at 25 °C, **(B)** 0.1BHI at 37°C, **(C)** BHI at 25°C, and **(D)** BHI at 37°C. Live bacteria are stained by SYTO-60 (red) and eDNA with TOTO-1 (green). Size bars in orthogonal projections indicate 10 µm.

Presence of eDNA in 0.1BHI Depends on Osmotic Conditions

One factor influencing bacterial lysis is ionic strength of the extracellular environment and, in consequence, intracellular osmotic pressure. Dilution of BHI in demineralized H_2O to obtain 0.1BHI results in a hypotonic solution increasing the osmotic pressure. Thus, further experiments were performed to test if an increase in osmotic pressure in 0.1BHI contributes to DNase sensitivity of biofilms. At 25°C, the use of PBS to dilute BHI instead of demineralized H_2O completely abolished the effect of DNase treatment on biofilm formation (**Figure 3A**). This effect could be attributed to the presence of higher ionic strength in PBS since a similar inhibition of DNase sensitivity was observed with saline but not phosphate buffer (**Figure 3A**). Similar observations were made at 37°C (**Figure 3B**). Again, biofilm formation was reduced by DNaseI treatment in 0.1BHI diluted with H_2O or phosphate buffer but not with PBS or saline. Instead, addition of DNase enhanced biofilm formation in BHI diluted with PBS or saline at 37°C. To exclude any effects on enzymatic activity of DNaseI, control experiments were performed. Under all conditions tested DNaseI retained full activity (Supplementary Figure S3).

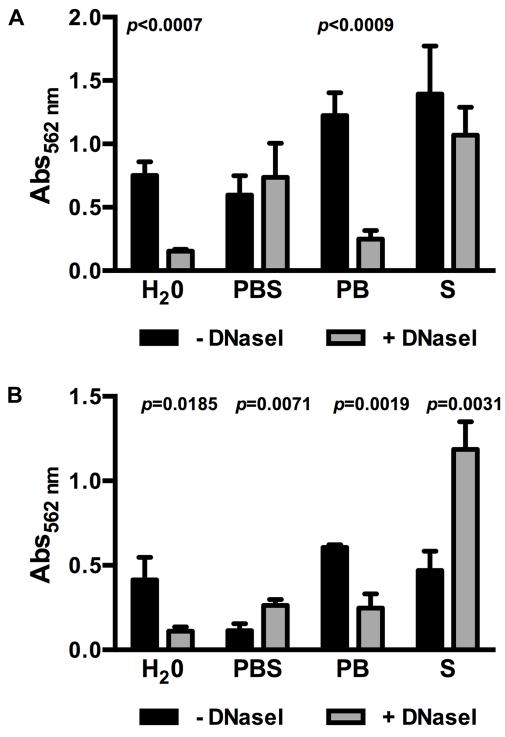

FIGURE 3 | Biofilm formation of *Lm* EGD-e grown in 0.1BHI prepared with either dH_2O, PBS, phosphate buffer (PB), or saline (S) grown at 25°C (A) or 37°C (B) in the presence (gray bars) or absence (black bars) of DNaseI. Values are absorbance at 562 nm and are mean ± standard deviation of three independent experiments. Data was analyzed using Student's *t*-test and *p*-values of statistically significant differences are indicated (all other comparisons: not significant, i.e., $p > 0.05$).

Presence of eDNA in *Lm* Biofilms Grown Under Flow

Further experiments were performed in flow chambers to investigate the role of eDNA in *Lm* biofilms under dynamic conditions. Confocal microscopy analysis of eDNA in biofilms grown in full strength and diluted BHI at 25 or 37°C revealed a similar picture as in static biofilm assays. At 37°C, large amounts of eDNA were present in biofilms grown in 0.1BHI and appeared to be a structural component of the matrix throughout the entire biofilm from the bottom to the top (**Figure 4A**; Supplementary Figure S4). By contrast, only very few dead cells or small patches of eDNA were present in biofilms grown in full strength BHI (**Figure 4B**; Supplementary Figure S4).

At 25°C, flow chamber biofilms differed considerably compared to those formed under static conditions. Under flow, only few isolated microcolonies were observed in 0.1BHI and these microcolonies were mostly found around patches of eDNA (**Figure 4C**; Supplementary Figure S4). Upon higher magnification, the eDNA patches appeared as filamentous structures directly on the slide surface, which had several bacteria attached (**Figure 4D**). In full strength BHI, only a few isolated bacteria were found to be attached to the surface and no eDNA, microcolonies, or biofilm were observed (Supplementary Figure S4).

Based on these results, the potential of DNaseI-treatment to dissolve established biofilms of *Lm* was investigated. After 1 h of incubation, eDNA in biofilms grown in 0.1BHI was efficiently digested by DNaseI (**Figure 5**; Supplementary Figure S5). Moreover, these biofilms were almost completely removed after flow was turned on again. By contrast, biofilms grown in full strength medium were unaffected by DNaseI treatment probably due to the lack of eDNA (**Figure 5**).

DNase-Sensitive and -Resistant Biofilms of Different *Listeria* sp. Strains

Finally, a range of *Lm* strains from different lineages as well as different species of the genus *Listeria* were tested for DNase-sensitive and -insensitive modes of biofilm formation under static conditions. All *Lm* strains as well as *L. innocua* and *L. ivanovii* formed DNase-insensitive biofilms at 37°C in full strength BHI (**Figure 6A**) but biofilm formation was reduced by DNaseI in 0.1BHI at 25°C (**Figure 6B**). Similar DNase-sensitive and insensitive biofilms were observed for these strains grown in 0.1BHI at 37°C or BHI at 25°C (data not shown). No significant biofilm formation was observed for *L. grayi* under all conditions tested and *L. seeligeri* only formed DNase-resistant biofilms in BHI at 37°C.

DISCUSSION

Biofilm Formation Depends on Temperature and Dilution of Media

A number of studies have investigated the impact of nutrients and temperature on biofilm formation of various *Lm* strains (Folsom et al., 2006; Harvey et al., 2007; Di Bonaventura et al.,

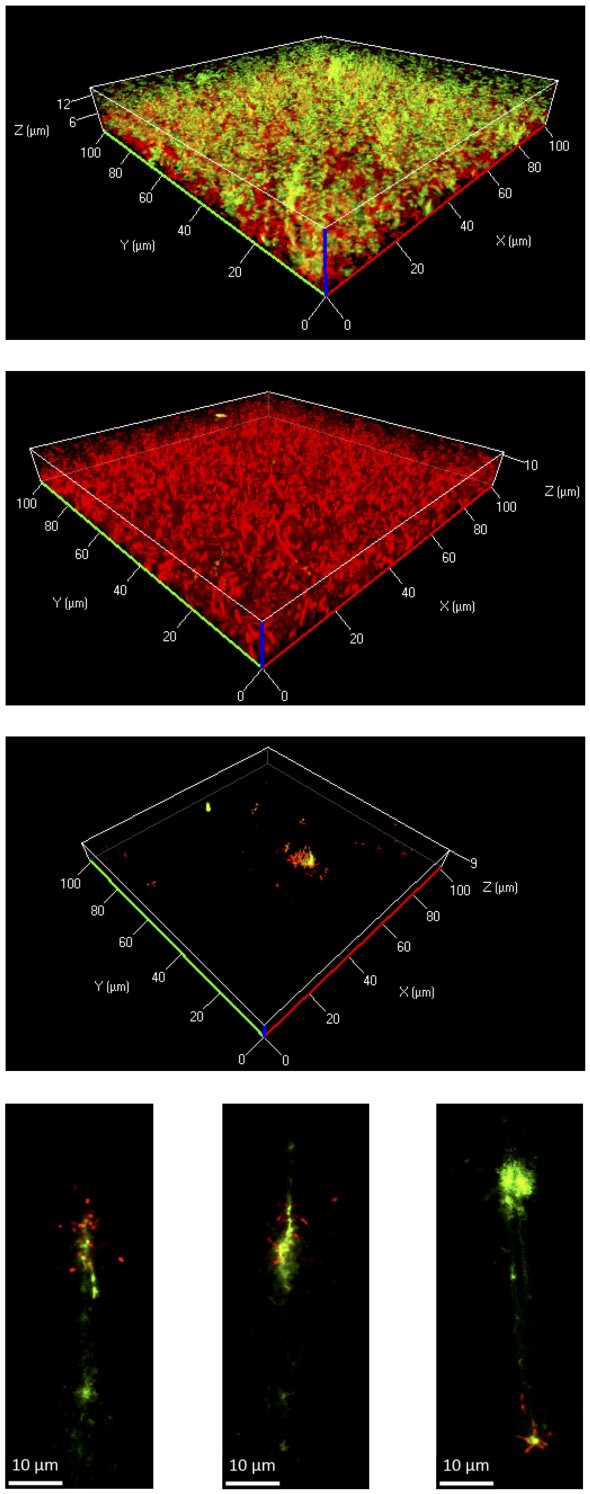

FIGURE 4 | Three-dimensional projections or single layers of CLSM images of *Lm* EGD-e flow chamber biofilms grown for 24 h under hydrodynamic conditions at 37°C in 0.1BHI (A) or BHI (B) or at 25°C in 0.1BHI (C) and (D). Live bacteria are stained by SYTO-60 (red) and eDNA with TOTO-1 (green). **(D)** Digital magnification of eDNA spots identified in the layer of the z-stacks that corresponds to the surface of the slide (size bar indicates 10 µm).

2008). The media and conditions vary from study to study but the authors uniformly report a strain-specific pattern with some strains forming more biofilm in full strength complex media while others form more biofilm in diluted or chemically defined media. In line with previous findings (Riedel et al., 2009), the ability of *Lm* EGD-e to form biofilms varies with temperature and dilution of media. Highest levels of biofilm formation by *Lm* EGD-e were achieved at 37°C in full strength BHI, i.e., high nutrient levels, and least biofilm formation was observed at 25°C in the same medium. By contrast, in 0.1BHI biofilm formation was increased at 25°C compared to 37°C.

eDNA-Dependent and -Independent Biofilm Formation

The importance of eDNA during early phases of biofilm formation has been established for a number of bacteria (Whitchurch et al., 2002; Qin et al., 2007; Gödeke et al., 2011; Barnes et al., 2012). The results of the present study confirm a role of eDNA for biofilm formation in media with low concentrations of osmotically active substances (**Figure 1**). Similar observations were made in a previous study showing that eDNA is important for initial attachment of *Lm* EGD-e, to glass (Harmsen et al., 2010). Moreover, the authors report inhibition of biofilm formation in minimal medium and removal of biofilms established in diluted BHI by DNaseI. In another study, presence of DNaseI markedly reduced biofilm formation on polystyrene of three *Lm* strains including EGD-e in full strength TSB medium at 37°C (Nguyen and Burrows, 2014). By contrast, biofilm formation in full strength BHI was not affected by DNaseI, suggesting that, under these conditions, eDNA is neither involved in initial attachment nor during later stages of biofilm formation.

A smear of nucleic acids could be precipitated from supernatants of biofilm grown under all conditions. This signal was by far more prominent in full strength medium and an additional distinct band was only observed in diluted medium (**Figure 1B**). This nucleic acid is clearly of bacterial origin since it is absent in sterile media. Control PCRs yielded specific products for all genes tested in supernatants of bacteria grown in diluted medium. For some of the genes tested, PCR products were also obtained when PCR was performed on supernatants of full strength BHI cultures at 25°C although the band signals were very faint at the same number of PCR cycles (Supplementary Figure S6) suggesting that the amount of template DNA was significantly lower compared to 0.1BHI supernatants. This indicates that the DNA smear in full strength BHI represents fragmented chromosomal DNA, which is not functional in promoting biofilm formation. Similar observations were made by Harmsen et al. (2010), who could show that, unlike intact chromosomal DNA, shorter DNA fragments do not support initial attachment of *Lm*.

So far, DNA-dependent and -independent modes of biofilm formation have only been described in *Neisseria meningitidis* (Lappann et al., 2010). However, in this organism the two modes of biofilm formation were not shown for the same

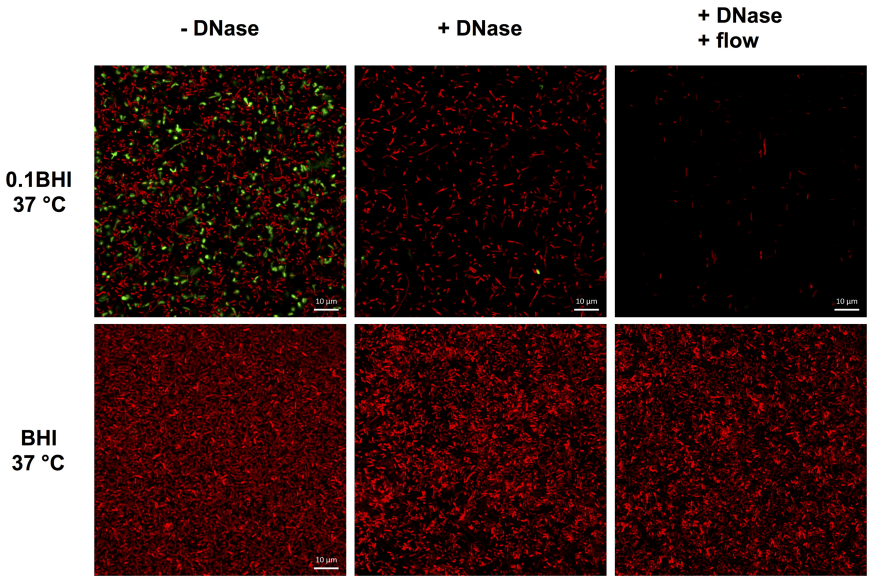

FIGURE 5 | Confocal laser scanning microscope (CLSM) images of *Lm* EGD-e flow chamber biofilms grown for 24 h under hydrodynamic conditions at 37°C in 0.1BHI (upper panels) or BHI (lower panels). Images were captured at the basal layer of bacteria immediately above the slide surface and at the same position before (left, −DNase) and at the end of DNaseI treatment (middle, +DNase) and after medium flow had been turned on again (right, +DNase + flow). Live bacteria are stained by SYTO-60 (red) and eDNA with TOTO-1 (green). Size bars indicate 10 µm.

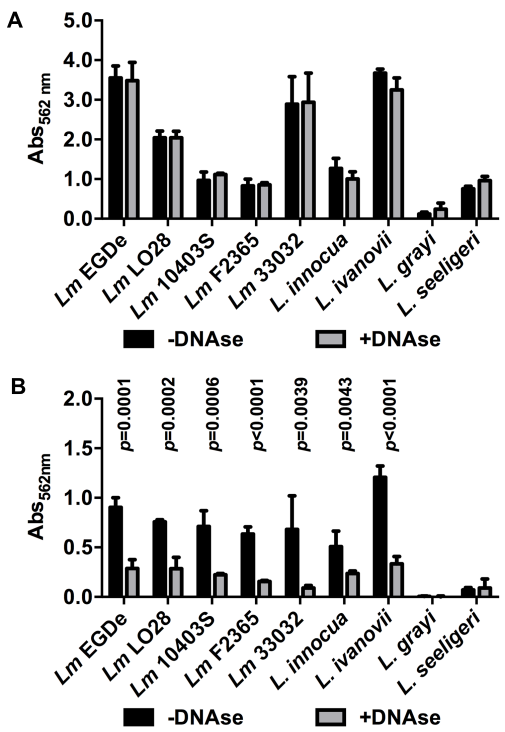

FIGURE 6 | Biofilm formation of different *Lm* strains and other *Listeria* species grown in BHI at 37°C (A) or 0.1BHI at 25°C (B) in the presence (gray bars) or absence (black bars) of DNaseI. Values are mean ± standard deviation of three independent experiments. Data was analyzed using Student's *t*-test and *p*-values of statistically significant differences are indicated (all other comparisons: not significant, i.e., $p > 0.05$).

strains but are distributed amongst different clonal complexes. Pathogenic strains of clonal complexes with high prevalence form eDNA-dependent biofilms that are more resistant to shear forces, possibly leading to a more stable interaction with the host. Strains of other clonal complexes show an eDNA-independent mode of biofilm formation with less stable microcolonies. Our results suggest that, in contrast to *N. meningitidis*, *Lm* EGD-e is able to form biofilms that either contain or lack eDNA in response to different environmental conditions and eDNA promotes biofilm formation specifically under conditions with low concentrations of osmotically active substances. Moreover, DNA-dependent and -independent modes of biofilm formation seem to be conserved in the species *Lm* and was also observed in other but not all species of the genus.

Source of eDNA in *Lm* Biofilms

Several studies have investigated the source of eDNA in bacterial biofilms. Dilution of BHI in PBS or saline but not H_2O or phosphate buffer abolished the effect of DNaseI on biofilm formation (**Figures 3A,B**) arguing for a contribution of the osmotic conditions to DNA release. In other bacteria, eDNA was released upon expression of autolysin genes (Qin et al., 2007; Rice et al., 2007; Mann et al., 2009; Lappann et al., 2010), induction of prophages in a subpopulation of the biofilm bacteria (Carrolo et al., 2010; Gödeke et al., 2011; Petrova et al., 2011; Binnenkade et al., 2014) or formation of vesicles (Allesen-Holm et al., 2006; Liao et al., 2014). Additionally, based on the observation that some bacteria employ type IV secretion systems for conjugational gene transfer, injection of DNA into host cells and active secretion

of chromosomal DNA (Hamilton et al., 2005; Alvarez-Martinez and Christie, 2009), active and lysis-independent export of DNA was proposed as another source of eDNA. Further experiments are required to investigate if these mechanisms contribute to release of eDNA by *Lm*.

eDNA as a Structural Component of *Lm* Biofilms

Microscopic images provide evidence that eDNA not only supports initial attachment but also serves as a structural component of the biofilm matrix of *Lm* EGD-e in diluted media under both static and dynamic conditions (**Figures 2** and **4**). Extracellular DNA was shown to be present in the matrix of mature biofilms of various bacteria (Hall-Stoodley et al., 2008; Izano et al., 2008; Mann et al., 2009; Seper et al., 2011; Liao et al., 2014) cooperating with proteins and polysaccharides to ensure structural integrity of the biofilm (Das et al., 2013; Okshevsky and Meyer, 2015). As a consequence, eDNA is discussed as a target to prevent or disperse biofilm formation of these microorganisms (Okshevsky et al., 2015).

Biofilm Formation by *Lm* Under Static vs. Dynamic Conditions

Under dynamic conditions, significant biofilm formation was only observed when bacteria were grown at 37°C but not at 25°C, i.e., when bacteria express flagella. A possible explanation is that under static conditions in microtiter plates, when bacteria are located in a confined space, motility facilitates multiple contacts with the surface eventually leading to initial attachment. In fact, flagellar motility was shown to be required for efficient biofilm formation by *Lm* under static conditions (Lemon et al., 2007). Also, under these conditions non-motile strains were shown to form less structured, more homogenous biofilms (Guilbaud et al., 2015). By contrast, in flow chambers, motility might actually have the opposite effect on biofilm formation: motile bacteria that do not attach are efficiently washed away. However, once single, attached bacteria lyse under conditions of increased osmotic pressure (i.e., in 0.1BHI), eDNA may serve as attachment site for further bacteria. This is supported by the fact that filamentous eDNA patches were observed in 0.1BHI at 25°C (**Figure 4D**). These eDNA filaments were orientated in the direction of the medium flow and are presumably chromosomal DNA released from lysed bacteria, which was then spread out by medium flow. This sticky DNA may then serve as attachment site or scavenger for further bacteria leading to formation of microcolonies observed at lower magnification (**Figure 4C**). It remains to be investigated if these microcolonies develop into mature biofilm upon longer incubation periods.

CONCLUSION

Based on the presented results a hypothetical model for biofilm formation of *Lm* is proposed. 37°C and high levels of nutrients are conditions encountered by *Lm* in the gastrointestinal tract of the host. On the other hand, 25°C and low levels of nutrients and other osmotically active substances are conditions encountered in the environment and in food production lines. It may thus be hypothesized that efficient and rapid formation of DNA-independent biofilms on, e.g., food particles or host tissue contributes to colonization and prolonged persistence of *Lm* and, in consequence, to sustained exposure to the pathogen. By contrast, under environmental conditions, eDNA released by lysed bacteria (or present in the environment) supports initial attachment to surfaces. Hypotonic conditions may favor increased lysis of bacteria already attached to the surface. The chromosomal DNA released by these lysed bacteria then serves as an anchoring site for dividing cells in growing microcolonies but also a scavenger capturing further planktonic bacteria.

Collectively, the presented results may have practical implications for contact surfaces in food production lines at risk for contamination by *Lm*. Targeting eDNA in the biofilm matrix by DNases or nucleases, as suggested for other bacteria (Nguyen and Burrows, 2014; Okshevsky et al., 2015), may be an effective treatment to limit or prevent initial attachment and disperse already existing *Lm* biofilms.

AUTHOR CONTRIBUTIONS

MD, RM, and CR conceived the study. MZ, MO, JM, AS, NC, MA, and MW carried out experiments. MZ, MO, MD, RM, and CR analyzed data. MZ, MO, RM, and CR drafted the manuscript and all the authors contributed to preparing the final version of the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.01428

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Listeria monocytogenes Impact on Mature or Old *Pseudomonas fluorescens* Biofilms During Growth at 4 and 20°C

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Changes in spatial organization, as observed by confocal laser scanning microscopy (CLSM), viable cell content, biovolume, and substratum surface coverage of the biofilms formed on glass by *Pseudomonas fluorescens* resulting from co-culture with *Listeria monocytogenes*, were examined. Two strains of *L. monocytogenes*, two culture temperatures and two biofilm developmental stages were investigated. Both *L. monocytogenes* strains, a persistently sampled isolate (collected repeatedly along 3 years from a meat factory) and Scott A, induced shrinkage in matrix volume, both at 20°C and 4°C, in mature or old biofilms, without loss of *P. fluorescens* cell count per surface unit. The nearly homogeneous pattern of surface coverage shown by mono-species *P. fluorescens* biofilms, turned into more irregular layouts in co-culture with *L. monocytogenes*. The upper layer of both mono and dual-species biofilms turned to predominantly consist of matrix, with plenty of viable cells underneath, in old biofilms cultured at 20°C, but not in those grown at 4°C. Between 15 and 56% of the substratum area was covered by biofilm, the extent depending on temperature, time and *L. monocytogenes* strain. Real biofilms in food-related surfaces may thus be very heterogeneous regarding their superficial components, i.e., those more accessible to disinfectants. It is therefore a hygienic challenge to choose an adequate agent to disrupt them.

Keywords: ***Listeria monocytogenes, Pseudomonas fluorescens*, biofilms, interspecies interactions, low temperature, CLSM**

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INTRODUCTION

Known previously as an animal pathogen and ubiquitous in nature, *Listeria monocytogenes* emerged as a foodborne human pathogen in the 1980s (Ryser and Marth, 2007; Warriner and Namvar, 2009). That can be attributed to an unprecedented global improvement of hygienic practices in food industry from the 1970s, including both better cleaning and disinfection methods and a more widespread use of refrigeration. This public health progress, reducing the prevalence of most foodborne diseases, had an undesirable side effect. Elimination by low-temperature of the constraint of microbial competitors implied a new chance for *Listeria*, one of the few psychrotrophic bacterial pathogens, to thrive in refrigerated foods. As adequate storage of pasteurized and/or Ready To Eat (RTE) foods requires low temperatures, cold-tolerant,

i.e., psychrotrophic, bacteria tend to be selected in those foods. That is the case of *Pseudomonas* (*fluorescens, putida, fragi*), able to cause important quality defects in protein-rich foods (Andreani et al., 2015).

Coinciding with this trend and the development of microbial ecology approaches to food safety and quality, there has been an increasing interest in biofilms (Costerton et al., 1995; Srey et al., 2013). *Pseudomonas* species were soon characterized as quick and thick biofilm producers, even the non-pathogenic species, often dominant in food spoilage. Their understanding has been driven by the far more abundant clinical and basic information on *Pseudomonas aeruginosa*'s biofilms (Silby et al., 2011; Mann and Wozniak, 2012). Many authors also have studied *L. monocytogenes*'s carrying biofilms (Moretro and Langsrud, 2004; Rieu et al., 2008; Bonsaglia et al., 2014; Guilbaud et al., 2015).

Interactions between *Pseudomonas* and *L. monocytogenes* in biofilms were initially described by Sasahara and Zottola (1993). Their claim on the need of a primary surface colonizer, such as *Pseudomonas* (in that case *P. fragi*) for *L. monocytogenes* attachment, was a very relevant one in its time and not just for the food microbiology field. Multispecies biofilms have attracted attention mostly because their partners can resist harder antimicrobial challenges than single species biofilms (Burmølle et al., 2006; Simões et al., 2009; Sanchez-Vizuete et al., 2015) and because they are now acknowledged to be widely distributed in both natural and industrial environments. Various hypotheses have been used to investigate the specific properties of mixed biofilms and to characterize the interactions between the partners and toward newcomers (Carpentier and Chassaing, 2004; Moons et al., 2009; Yang et al., 2011; Elias and Banin, 2012; Burmølle et al., 2014; Giaouris et al., 2014, 2015; Jahid and Ha, 2014; Bridier et al., 2015) and many attempts have been made to identify the natural biofilm cohabitants at critical sites, including specific food related facilities (Fox et al., 2014; Røder et al., 2015; Rodríguez-López et al., 2015).

New insights on the regulation of biofilm formation are helping to deepen the knowledge about the sort of biofilms that can be found in food industry, where multiple strategies to prevent or delay microbial growth are commonly combined to preserve foods (low temperature, low pH, high osmotic pressure, modified atmospheres, presence of natural antimicrobials, etc.). Food preservation conditions are adverse situations that may activate stress response in some of the present microorganisms, which are thus selected. Certain *Pseudomonas* and *L. monocytogenes* strains belong to those selected at low temperatures (Moretro and Langsrud, 2004; Hemery et al., 2007; Chan and Wiedmann, 2009; Ortiz et al., 2010; Silby et al., 2011; Mann and Wozniak, 2012; Valderrama and Cutter, 2013; Rodríguez-López et al., 2015) and they may jointly form biofilm on raw materials, foods, and inert surfaces at food handling facilities. Though refrigeration tends to be used in food processing and food service facilities during operating hours, higher environmental temperatures tend to occur during pauses or implementation of cleaning and disinfection tasks. Biofilm life may thus switch from 4 to 20°C, or even larger intervals at those sites. *L. monocytogenes* strains that have been found to persist for months or even years (Ortiz et al., 2010; Carpentier and Cerf, 2011) are likely to have often experienced changing culture conditions, apart from partial elimination and repeated sanitizer exposure, by daily but not fully effective cleaning and disinfection cycles. Development of more effective, cheap, and sustainable eradication methods requires more information on the target biofilms where *L. monocytogenes* inhabits.

This study, still in the track of Sasahara and Zottola (1993), tries to follow the formation and aging of *P. fluorescens* and *L. monocytogenes* mixed biofilms in temperature conditions that are realistic for food industry. One *P. fluorescens* and one *L. monocytogenes* strain of food industry origin were used, adding well known *L. monocytogenes* Scott A for comparison. Viability counting was combined with culture-independent evaluations, to get a hint of the heterogeneity in biofilm setups that could be useful for food hygiene purposes. Previous evidence of spatial distribution in these dual-species biofilms has already been reported by the same authors (Puga et al., 2014).

MATERIALS AND METHODS

Bacterial Strains

Pseudomonas fluorescens ATCC 948^{TM} and two strains of *L. monocytogenes* were selected as biofilm former organisms. S1 is a *L. monocytogenes* persistent strain, (serotype 1/2a; lineage II) isolated by Ortiz et al. (2010) from an Iberian pig slaughterhouse and its associated processing plant; the other *L. monocytogenes* was the reference clinical strain Scott A (4b; lineage I). All of them were stored at –20°C in Tryptone Soya Broth (TSB, OXOID) with 15% glycerol. Preinocula were obtained in TSB after 24 h incubation at 20°C while shaking (80 rpm) to reach mid exponential phase. Working cultures were obtained from this as follows: 100 μL of preinocula were transferred into a test tube containing fresh TSB and incubated at 20°C for 24 h. Then, cells were harvested by centrifugation at 4000 × *g* for 10 min, washed twice in sterile TSB and their OD_{600} adjusted (0.12), to be used as inocula, in order to reach 10^4 CFU·mL^{-1} for each bacterial strain at the start of either single or binary cultures.

Experimental System

Biofilms developed on single-use 22 mm × 22 mm thin, borosilicate commercial microscope glass coverslips. These coverslips provide single-use, relatively wide, clean and undamaged smooth surfaces, without scratches or other microtopographic irregularities, moderately more hydrophilic than stainless steel, and allowing for more reproducible biofilms than reusable metal coupons. As described in Orgaz et al. (2011), 16 coverslips were held vertically by marginal insertion into the narrow radial slits of a Teflon carousel platform (6.6 cm diameter). The platform and its lid were assembled by an axial metallic rod for handling and placed into a 600 mL beaker (**Figure 1**) which was heat-sterilized as a unit, before aseptically introducing 60 mL of inoculated TSB. The glass coupons used in this study as substratum surfaces, were immersed in the liquid culture medium, which covered two thirds of the coupon area.

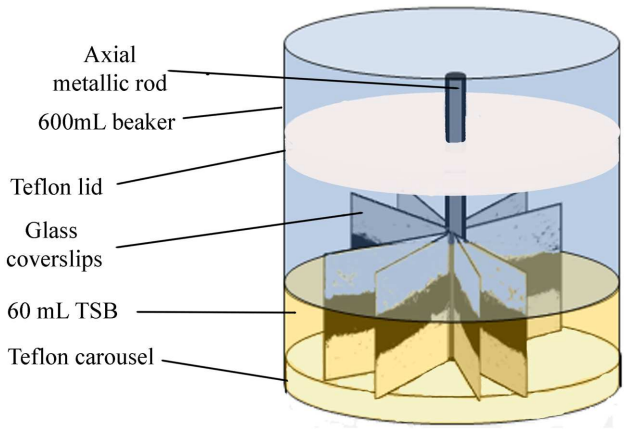

FIGURE 1 | Scheme of the experimental system used for the development of biofilms.

To check whether the covered area was homogeneous in terms of biofilm colonization, the coupon was arbitrarily divided into three equal horizontal bands. The top one, not covered by liquid was the Air-Phase (AP). The intermediate one (ALI), covered and located around the Air-Liquid Interphase, was intensely aerated and exposed to liquid shear during rotation shaking. The Fully Immersed one (FI), less aerated zone, was at the bottom. For multispecies biofilms containing *P. fluorescens* and one of the two *L. monocytogenes* strains afore mentioned, both bacteria were inoculated at the same level (1:1). *P. fluorescens* mono-species biofilms were used as controls. Incubation was carried out at 20°C or 4°C, in a rotating shaker at 80 rpm. Under these conditions, biofilm growth occupied almost 70% of the coverslip's surface. Samples corresponding to "mature biofilm" were taken after 48 h at 20°C, or 10 days at 4°C. Those taken at 20°C/144 h or 4°C/20 days were here called "old biofilm."

Cell Recovery and Counting

For sampling biofilm cells, glass coverslips were withdrawn with tweezers, and were carefully rinsed in sterile 0.9% NaCl to discard weakly attached cells. Then, attached cells of both coverslip faces were removed by swabbing (withdrawing all attached material from both coverslip faces with a cotton swab that was later immersed into an sterile tube containing 1.5 mL peptone water). Tubes were then vigorously stirred in a vortex to break up cell aggregates. Biofilm cells were decimally diluted in peptone water to be plated according to the drop method described by Hoben and Somasegaran (1982). Briefly, three 20 μL drops of each dilution were deposited onto plates of selective media, PALCAM (OXOID) or *Pseudomonas* Agar Base (PAB, OXOID), for counting *Listeria* sp. and *Pseudomonas* sp., respectively, in mono and dual-species biofilms. For purity control, plating on Tryptone Soy Agar (TSA, OXOID) was used to visually detect potential contaminant colonies. Counting was performed after 48 h incubation, at 37°C or 30°C, for *L. monocytogenes* or *P. fluorescens,* respectively. The results presented are the average of two coupons per experiment and three independent experiments ($n = 6$).

Confocal Laser Scanning Microscopy (CLSM)

The structural effects of *L. monocytogenes* on dual-species biofilms structure were examined by CLSM. For observation, the biofilms developed on the glass coverslips were rinsed with sterile 0.9% NaCl and stained with Syto 13 (S7575, Life Technologies) which labels all bacteria in a population, and CalcoFluor White (18909, FLUKA) a non-specific fluorochrome that binds to cellulose and other polysaccharides present in the extracellular polymeric substances (EPS) biofilm matrix. Thus, for quantification, green here corresponds to cells, whereas blue corresponds to EPS. Five representative regions of 0.12 mm × 0.12 mm located at the air-liquid-interphase zone were selected from each coupon. For this, the side of the coupon (22 mm) was divided into five regions (4.4 mm each one) and the center point of each one was later scanned. CLSM images of these locations were obtained with a FLUOVIEW® FV 1200 Laser Scanning Confocal Microscope (OLYMPUS) and an oil immersion objective lens 60X. Three-dimensional projections (Maximun Intensity Projection, MIP) were reconstructed from z-stacks using IMARIS® 8.1 software (BITPLANE AG, Zurich, Switzerland). The parameter here called *biovolume* was calculated using the MeasurementPro module of IMARIS; the whole image was thus segmented into two channels, green and blue, to estimate the volume occupied by either cells or EPS. The total biovolume (μm^3) was the sum of cells and EPS biovolumes, using the five fields. Biovolume reduction measurements were here calculated considering the biovolume occupied by *P. fluorescens* in mono-species biofilms represented 100%. The Matrix/Cell ratio was calculated for every image.

Biomass Determination

To evaluate the surface coverage of the attached biomass (cells plus EPS matrix) five coverslips of each type of biofilms (i.e., young and old biofilms; warm and cold biofilms; *P. fluorescens* mono-species and dual species with *L. monocytogenes*) were dried and stained for 2 min in a 1‰ Coomassie Blue (Brilliant Blue R, SIGMA) solution in acetic acid/methanol/water (1:2.5:6.5) mixture. This step was repeated twice. Once rinsed and dried again, the coverslips were scanned using a 600 dpi resolution (HP Scanjet 300) and analyzed using ImageJ (http://imagej.net). Densitometry allows analyzing the whole area of the stained coupon in comparison with confocal microscopy where fields are much smaller. The aim here was to integrate the biomass results of the whole coupon, segmented in areas with different aeration. A parameter called **% of *covered area*** was estimated for every image. For this, the scanned images were transformed into a binary system (i.e., black and white) and the surface occupied by black was quantified. Each coupon was divided into three zones, as described before. For calculations, the occupation in the air phase was discarded, as that area was scarcely covered, assuming as total biomass coverage the sum of the air-liquid interphase and the fully immersed zone (**Figure 2**).

RESULTS

Effects of *L. monocytogenes* Co-culture on *P. fluorescens*'s Biofilm at 20°C

What is here called "mature" or fully grown biofilm corresponds to the maximum attached population attained in these batch conditions (48 h at 20°C), with around 4×10^7 CFU of *P. fluorescens*/cm^2 (**Table 1**). At that stage, viable *P. fluorescens* cell numbers experienced almost no change if co-cultured with a *L. monocytogenes* strain. Both strains of *L. monocytogenes* grew more slowly than *P. fluorescens* in the binary biofilms at 20°C, particularly S1 (7.7 log versus 5.9 log). CLSM images, which in this study did not discriminate *P. fluorescens* and *L. monocytogenes* cells (**Figure 3A**), showed a rather homogeneous surface coverage in the case of single species *P. fluorescens* biofilms and a patchy, heterogeneous pattern for the binary biofilms, in spite of the low *L. monocytogenes* numbers (**Table 1**). As seen in **Table 2**, displaying cell and matrix biovolumes, and **Table 3**, presenting biomass distribution and substrate surface occupation, co-culture resulted in a decrease in biofilm biovolume and maximal thickness,. Considering that *P. fluorescens* viable cell number did not decrease, the outcome was a rise in density, in compactness. The matrix to cell ratio (**Table 2**) which was 0.7 in the single species *P. fluorescens* biofilms, was not changed by the presence of the food industry-persistent S1 strain of *L. monocytogenes*, but went down to 0.2 when co-cultured with *L. monocytogenes* Scott A. This strain caused a 75–80% matrix loss in binary biofilms (**Table 2**).

Binary old biofilms (144 h at 20°C) were clearly into the dispersal stage, having already lost 1–2 log of its viable *P. fluorescens* cells (**Table 1**). By then, *L. monocytogenes* Scott A counts were 1 log less than those of *P. fluorescens* and the S1 strain, 2 log less, though still representing a substantial population in the binary biofilm (2×10^4 CFU cm^{-2}; **Table 1**). Maximal biofilm thickness (**Table 2**) in both mono and dual-species biofilms had at that stage decreased by approximately 50% with respect to their corresponding mature biofilms (from 37 to 22 μm on average). It is to be noticed a change in accessibility of the biofilm cells, which appeared then covered by matrix (**Figure 3B**). Remaining cells were thus underneath, packed in a deeper, more protected position, in both the mono and dual-species old biofilms.

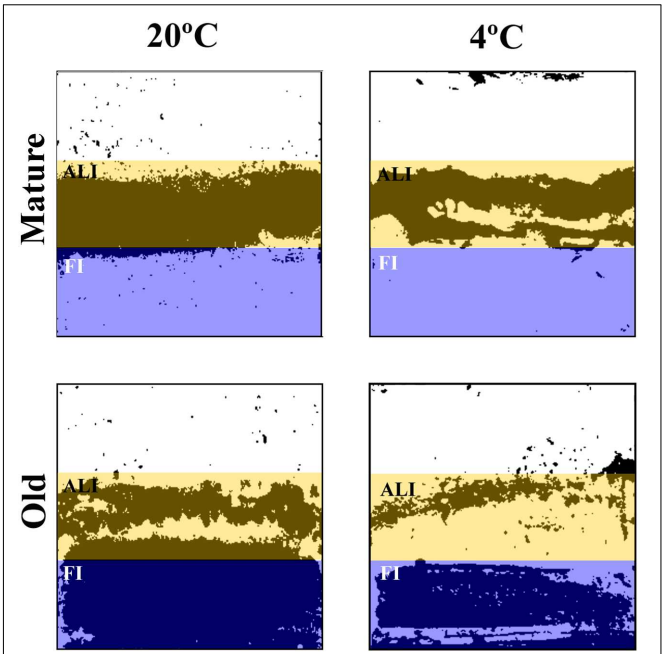

FIGURE 2 | Scanned coupons black and white images of mature and old *P. fluorescens* mono-species biofilms. Colored boxes mark the two different areas analyzed. Mature: 20°C/48 h; 4°C/10 days. Old: 20°C/144 h; 4°C/20 days.

Effects of *L. monocytogenes* Co-culture on *P. fluorescens*'s Biofilm at 4°C

In a previous work of this group (Puga et al., 2014), it was observed that biofilms growing at 4°C for 10 days were approaching the end of the stage featuring a net increase of attached cells per surface unit. Mono-species *P. fluorescens* biofilms matured at low temperature had about 1 log less viable counts/cm^2 than when matured at 20°C (**Table 1**) and presented about half their maximal thickness (**Table 2**). Just as

TABLE 1 | *P. fluorescens* and *L. monocytogenes* viable cells in biofilms.

Sample*	P (Log_{10} CFU cm^{-2})	PI (Log_{10} CFU cm^{-2})		PSc (Log_{10} CPU cm^{-2})	
	P. fluorescens X ± SD	*P. fluorescens* X ± SD	*L. monocytogenes* X ± SD	*P. fluorescens* X ± SD	*L. monocytogenes* X ± SD
20°C/mature	7.6 ± 0.1^{aA}	7.7 ± 0.1^{aA}	5.9 ± 0.6^{aC}	$7.5 \pm 0.3^{aA,E}$	$6.7 \pm 0.3^{aB,C}$
20°C/old	5.7 ± 0.1^{cB}	6.2 ± 0.1^{bA}	4.4 ± 0.2^{bc}	6.3 ± 0.0^{bA}	5.4 ± 0.1^{bB}
4°C/mature	$6.6 \pm 0.8^{b,cA}$	6.7 ± 0.1^{bA}	3.1 ± 0.1^{cC}	6.4 ± 0.0^{bA}	4.4 ± 0.2^{cB}
4°C/old	6.2 ± 0.1^{cA}	5.8 ± 0.7^{bA}	$5.6 \pm 0.2^{aA,B}$	$5.0 \pm 0.2^{cB,C}$	4.6 ± 0.2^{cC}

*Different superscripts in small letters mean important statistical differences in columns. Different superscripts in capital letters mean important statistical differences in rows (n = 6). *P: mono-species P. fluorescens; P1: P. fluorescens and L. monocytogenes S1; PSc: P. fluorescens and L. monocytogenes Scott A. Mature: 20°/48 h and 4°/10 days; Old: 20°/144 h and 4°/20 days.*

TABLE 2 | Structural parameters obtained from CLSM images in Figure 3.

Sample*	20°C Biofilms			4°C Biofilms		
	Max. BF Thickness (μm)	Biovolume reduction (%)	Matrix/ Cells ratio	Max. BF thickness (μm)	Biovolume reduction (%)	Matrix/ Cells ratio
P-mature	43 ± 7		0.7	23 ± 10		0.9
Pl-mature	31 ± 5	27	0.7	29 ± 7	22	0.6
PSc-mature	39 ± 9	55	0.2	13 ± 5	39	0.8
P-old	20 ± 1		1.1	27 ± 13		0.7
Pl-old	21 ± 3	67	1.0	13 ± 4	96	0.1
PSc-old	26 ± 5	73	1.0	26 ± 1	55	0.9

**P: mono-species P. fluorescens; P1: P. fluorescens and L. monocytogenes S1; PSc: P. fluorescens and L. monocytogenes Scott A. Mature: 20°/48 h and 4°/10 days; Old: 20°/144 h and 4°/20 days.*

TABLE 3 | Structural parameters obtained from scanned coupons analyzed by ImageJ of the biofilms in the ALI: Air-Liquid Interphase and FI: Fully Immersed bands of the coupons shown in Figure 2.

Sample*	20°C Biofilms			4°C Biofilms		
	Biomass distribution		Covered area (%)	Biomass distribution		Covered area (%)
	ALI	FT		ALI	FT	
P-mature	90 ± 3	10 ± 4	30 ± 2^{b}	81 –	19 –	26 ± 7^{a}
Pl-mature	84 ± 5	15 ± 5	44 ± 5^{a}	84 ± 9	16 ± 9	19 ± *I*^{a}
PSc-mature	99 ± 0	1 ± 0	28 ± *I*^{b}	94 ± 4	6 ± 4	16 ± *I*^{a}
P-old	35 ± 4	65 ± 4	48 ± 7^{b}	21 ± 8	80 ± 8	32 ± 3^{a}
Pl-old	48 ± 4	52 ± 4	57 ± 3^{a}	22 ± 6	77 ± 6	36 ± 4^{a}
PSc-old	27 ± 7	73 ± 7	37 ± 4^{c}	17 ± 4	83 ± 4	32 ± 3^{a}

*Different superscripts mean important statistical differences between P, P1 and PSc mature biofilms or old biofilms.*P: mono-species P. fluorescens; P1: P. fluorescens; and L. monocytogenes S1; PSc: P. fluorescens and L. monocytogenes Scott A. Mature: 20°/48 h and 4°/10 days; Old: 20°/144 h and 4°/20 days.*

at 20°C, however, in mature binary biofilms obtained at 4°C, *L. monocytogenes* viable counts were 1–2 log inferior to those of *P. fluorescens*, which remained as in the mono-species controls (**Table 1**). Global biovolume reduction due to co-culture was *L. monocytogenes*-strain dependent, being more severe in old than in mature biofilms (**Table 2**). Regarding matrix distribution, whereas in the mono-species biofilms EPS appeared mostly layered on top of the cells, in the binary biofilms there was a considerable amount of matrix material scattered on void substratum spaces, away from cells (**Figure 3**).

Pseudomonas fluorescens population level in the old biofilms (20 days at 4°C) was just slightly lower than in the mature ones (**Table 1**). This could be either due to moderate dispersal or to regrowth, compensating in number the dispersed cells. Global biovolume reduction strongly depended on *L. monocytogenes* strain. It was the food industry-persistent strain S1 that caused more shrinkage in binary biofilms: a 96% loss in biovolume. It also brought about a 50% loss in maximal biofilm thickness and a sharp fall, from 0.6 to 0.1, in matrix to cell ratio (**Table 2**, **Figure 3**).

Aeration in Mono or Dual-Species *P. fluorescens* Biofilm Development

Every experimental system to develop biofilms has its own particularities. The coupons used in this study have areas with different aeration. To find out how could this influence local biofilm formation and affect the significance of temperature, age, and species interaction, the coupon surface was divided into three band zones as described in the "Material and Methods" section and the biomass attached to each of them (**Figure 2**) was quantified (**Table 3**). As it is shown in the whole coupon images of **Figure 2**, at any incubation moment, important zonal differences in biomass coverage did happen. For one thing, the Fully Immersed surface was colonized after the more aerated zone. Indeed, in mature biofilms most of the biomass was located in the more aerated zone (ALI; ranging from 81 to 99%), whereas in old biofilms the percentage of biomass located in the fully immersed zone (FI) increased (ranging from 52 to 83%; **Table 3**). The latter effect was much more intense in cold biofilms. No significant biomass shrinkage was observed as a result of species interaction, independently of the temperature of biofilms development (**Table 3**). Overall surface coverage reached maxima of 57 and 36% in cultures at 20 and 4°C, respectively. These surface coverages were achieved for the old dual-species biofilms between *P. fluorescens* and *L. monocytogenes* strain S1.

DISCUSSION

Biofilms formation in the food industry is a serious concern, especially of those where *L. monocytogenes* can persist. More

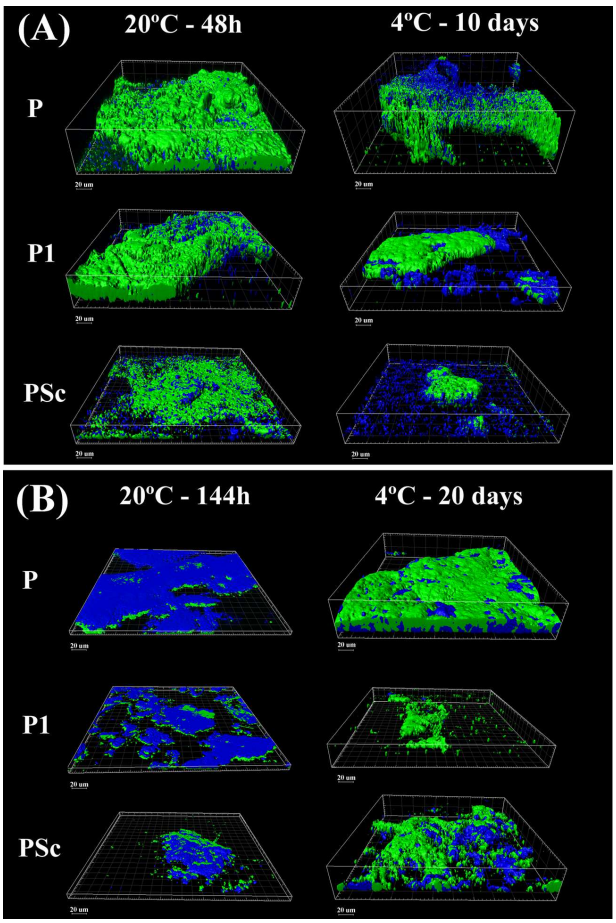

FIGURE 3 | Three-dimensional CLSM reconstruction of mature (A) and old (B) *P. fluorescens*-carrying biofilms obtained at 20°C or 4°C. Green: cells; blue: EPS matrix. P: *P. fluorescens* biofilms; P1: mixed *P. fluorescens* and *L. monocytogenes* S1 biofilms; PSc: mixed *P. fluorescens* and *L. monocytogenes* Scott A biofilms.

information on these biofilms could be helpful to develop strategies to successfully eradicate them. Nevertheless, conditions usually found in food processing plants, such as low temperature, are often disregarded when developing target biofilms. In this work, the impact that low temperature and biofilm aging have on the population and the structure of mixed biofilms has been evaluated. As biofilm forming microorganisms, one *P. fluorescens* and one *L. monocytogenes* strain of food industry origin were used, plus the reference strain Scott A for comparison. Viability counting was combined with imaging techniques, to gain an insight in the features of these biofilms that could serve as starting point for improving the current cleaning and disinfection strategies.

When surface biomass was measured in the more or less aerated zones of the coupons, it was confirmed that oxygen availability determined a different pattern of surface colonization at the different coupon areas (**Figure 2**). Similar situations can be found in food industry; biofilms with heterogeneous age and physiology are to be expected in close proximity in real locations. Physiological heterogeneity is inherent to complex natural communities (Stewart and Franklin, 2008). On the other hand, these coupons with zonal biofilm heterogeneity, are the ones we use as experimental system. That means that viable cell countings, such as those in **Table 1**, are average measurements, integrating physiologically heterogeneous biofilm situations across a coupon (650 mm^2 surface) and where at least six coupons were averaged. By comparison, CLSM fields (0.014 mm^2) show detailed but very localized information (five fields are summed up for volumetric measurements). The two techniques (viable cell counting and CLSM) supply different but complementary information.

The outcome of species interaction on surfaces is assumed to depend on culture conditions, particular species or strains involved, sequence of arrival to the surface (Carpentier and Chassaing, 2004) and the respective population sizes (Mellefont et al., 2008). Regarding population levels, in the present study, those of *L. monocytogenes* were initially as large as those of *P. fluorescens*; this proportion is unrealistic for food industry as a whole, where different species of *Pseudomonas* are far more prevalent. However, high local concentrations of *L. monocytogenes* may occur at particular food industry harborage sites, considering its general endurance (Moretro and Langsrud, 2004; Ryser and Marth, 2007; Valderrama and Cutter, 2013) and a good desiccation survival ability (Alavi and Hansen, 2013). Only two *L. monocytogenes* strains were tested here, but previous studies with different food industry isolates (Puga et al., 2014) support and complement the present results. There, a commensal relationship was found to exist in biofilms between the two species, with a stimulation of *L. monocytogenes* population without an effect on that of *P. fluorescens*, in terms of viable cell numbers. Besides, a stratified species distribution was seen, using specific species labeling, with *L. monocytogenes* occupying the deeper, more anaerobic biofilm layers, in spite of its late incorporation into the biofilm. In the present work, co-culture with *L. monocytogenes* was observed to induce a reduction in *P. fluorescens* biofilm volume (**Figure 3**, **Tables 2** and **3**) without decrease of its cell counts per surface unit (**Table 1**). According to early assumptions on the role of species interaction for joint surface colonization, a good biofilm former species, such as *Pseudomonas*, would play the role of primary colonizer and provide shelter for poor biofilm formers such as *L. monocytogenes* (Sasahara and Zottola, 1993). In this case, *L. monocytogenes* seem to actively redesign the biofilms formed by *P. fluorescens* (Puga et al., 2014) favoring its own proliferation in there and introducing extra compactness in their structure.

Listeria monocytogenes counts in this sort of denser biofilms may be underestimated by experimental systems such as those using crystal violet staining, which do not discriminate between cells and matrix. A denser matrix, on the other hand, may contribute to the mechanisms making mixed biofilms more resistant than mono-species ones against external attack with enzymes, antimicrobials, or other agents (Simões et al., 2009; Burmølle et al., 2014; Sanchez-Vizuete et al., 2015). The shrinkage of the matrix could be possibly caused by the production of an additional extracellular matrix component as a result of the interaction between species, such as amyloid fibers.

These surface-associated proteins, produced by some members of the Enterobacteriaceae family such as *Escherichia coli* and *Salmonella*, have also been described in certain *P. fluorescens* strains (Larsen et al., 2007; Dueholm et al., 2010; Zhou et al., 2012). In addition, other forms of alteration of the original *P. fluorescens* matrix framework may be involved (Steinberg and Kolodkin-Gal, 2015). According to Periasamy et al. (2015), polysaccharide composition of an individual species significantly impacts mixed species biofilm development and the emergent properties of such communities. If an abundant and extracellular matrix such as that produced by *P. fluorescens* can be considered as "public goods" when shared (Nadell et al., 2009), a reinforced, more compact matrix, induced if not produced, by *L. monocytogenes* in binary biofilms with *Pseudomonas*, could perhaps be considered as *L. monocytogenes*'s contribution to enhanced public goods, providing more protection in spite of less growth, to both partners.

Biofilm aging appeared in this work to involve more changes than cell dispersal, such as structural modifications and cell regrowth. For one thing, not all cells seemed to get detached in these rather old biofilms, just part of them. CLSM images of the old biofilms formed at 20°C (**Figure 3B**) showed that dispersal had cleared out cells from the surface, but many viable cells remained underneath, about 10^6 *P. fluorescens* CFU cm^{-2} and 10^4–10^5 CFU cm^{-2} of *L. monocytogenes* (**Table 1**). There is another aspect worth noting. Practically only the matrix was accessible in those old biofilms. This could at least partly explain the fact that aging adds resistance against stress in general (Lee et al., 2014; Serra and Hengge, 2014). On the other hand, it suggests that enzymatic or other matrix-eroding procedures may be a prerequisite to have access to old biofilm dwelling cells. Another issue related to age is regrowth. Here, no discrimination between residual and fresh cells was made, so it is not possible to know how many of the viable cells in old biofilms are in fact starting a new proliferation cycle.

Biofilm development at 4°C was not merely slower than at 20°C, but cold stress had an impact on biofilm structure. The structural contraction or shrinkage observed as a result of co-culture, was intensified by low temperature and culture time. Besides, biofilms grown at 4°C, particularly binary ones, were more irregular in structure, thickness and matrix distribution (**Figure 3** and **Table 2**). Both *P. fluorescens* and *L. monocytogenes* are known to express at low temperatures a wide range of different membrane components and enzymatic activities (Regeard et al., 2000; Hemery et al., 2007; Chan and Wiedmann, 2009; Durack et al., 2013); some of them could be involved in the development of the mentioned biofilm features.

CONCLUSION

When this dual-species consortium develop biofilms on a solid surface, apparently species interaction, cold stress and aging contribute to a more compact structure than the one built by *P. fluorescens* in single species biofilms at 20°C. The actual change in the matrix framework and the mechanism to obtain it, deserves further work, as the pathogen's shelter is thus reinforced. The types of biofilms resulting from the interaction between *P. fluorescens* and *L. monocytogenes,* cold stress and aging could be used as targets for cleaning and disinfection procedures.

AUTHOR CONTRIBUTIONS

CP: conception of the work; analysis and interpretation of the data; drafting the work. BO: design of the work; interpretation of the data; drafting and revising the work; Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. CS: drafting the work and revising it critically; Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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REVIEW
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Bacteriophages as Weapons Against Bacterial Biofilms in the Food Industry

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Microbiological contamination in the food industry is often attributed to the presence of biofilms in processing plants. Bacterial biofilms are complex communities of bacteria attached to a surface and surrounded by an extracellular polymeric material. Their extreme resistance to cleaning and disinfecting processes is related to a unique organization, which implies a differential bacterial growth and gene expression inside the biofilm. The impact of biofilms on health, and the economic consequences, has promoted the development of different approaches to control or remove biofilm formation. Recently, successful results in phage therapy have boosted new research in bacteriophages and phage lytic proteins for biofilm eradication. In this regard, this review examines the environmental factors that determine biofilm development in food-processing equipment. In addition, future perspectives for the use of bacteriophage-derived tools as disinfectants are discussed.

Keywords: biofilm, bacteriophage, phage lytic proteins, food industry, disinfection

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INTRODUCTION

Food safety is an important issue for health authorities and industries due to the health impact and economic losses caused by the contamination of foodstuffs. Despite the implementation of Good Manufacturing Practices (GMP) and Hazard Analysis Critical Control Point (HACCPs) in food industries, in 2014 the European Food Safety Authority (EFSA) reported a total of 5,251 foodborne outbreaks resulting in 6,438 hospitalizations (EFSA and ECDC, 2016). In the United States, 866 foodborne outbreaks were reported in 2014, resulting in 714 hospitalizations: (http://wwwn.cdc.gov/foodborneoutbreaks/; accessed: November 27, 2015).

Food is often contaminated during processing and packaging through contact with equipment surfaces. Of note, contamination with hemolytic bacteria (*Staphylococcus aureus* and *Streptococcus agalactiae*) was detected in hands, hand-contact and food-contact surfaces in foodservice settings (DeVita et al., 2007); the presence of coliforms in washing water and industrial facilities are involved in the low microbiological quality of tomatoes (van Dyk et al., 2016) or the notable incidence of *S. aureus* and other pathogenic bacteria on food industry surfaces in Spain (Gutiérrez et al., 2012a) are some of the great number of reported examples.

In fact, elimination of bacteria in food processing environments is greatly hindered by the presence of biofilms which provide a reservoir of foodborne pathogens. Usually most bacteria are organized in multispecies communities attached to a surface as biofilms, which confer ecological advantages that free-living bacteria in planktonic cultures do not have. Extracellular matrix, composed of a mixture of polymeric compounds such as polysaccharides, proteins, nucleic acids, and lipids, keeps the bacteria in close proximity each other and forms channels to distribute water, nutrients, oxygen, enzymes, and cell debris. This structure provides a microenvironment

with physicochemical gradients, horizontal gene transfer, and inter-cell communication. In addition, biofilm matrix protects the involved bacteria from environmental damages, antimicrobial agents, and host immune defenses (Flemming and Wingender, 2010). The low diffusion of antimicrobial substances through the matrix, together with an altered growth rate of bacteria constitutes the main barrier in the fight against relevant microorganisms living in biofilms (Donlan and Costerton, 2002).

Biofilm formation has notable implications in industrial processes, in particular in food processing, with a negative impact on food safety and the subsequent economic losses (Van Houdt and Michiels, 2010). In this regard, further studies about biofilm development and disassembly have been performed for important pathogenic bacteria such as *S. aureus* (Boles and Horswill, 2011; Periasamy et al., 2012) and *Listeria monocytogenes* (da Silva and De Martinis, 2013). Numerous biofilm control strategies have been proposed but the problem remains unsolved, probably because of the complexity of these structures, which contain both cells and extracellular substances. Ideally, a biofilm removal system should be able to get inside the biofilm structure and eliminate efficiently all the matrix components and the bacteria.

New approaches are focused on preventing biofilm formation by the development of anti-adhesive surfaces (Gao et al., 2011; Kesel et al., 2014; Salwiczek et al., 2014) or by the inhibition or reduction of bacterial adhesion (Cegelski et al., 2009; Pimentel-Filho et al., 2014). Moreover, removal strategies like physical and chemical treatments (Van Houdt and Michiels, 2010), antimicrobial photodynamic therapy (Sharma et al., 2011), induction of biofilm detachment (Cerca et al., 2013), blocking of biofilm regulation (Romling and Balsalobre, 2012), matrix degradation (Ramasubbu et al., 2005; Alkawash et al., 2006), and quorum sensing inhibitors (Hentzer et al., 2003) have been explored.

Another promising approach to control and eradicate biofilms is the use of bacteriophages. These viruses are harmless to humans, animals, and plants because they specifically target and kill bacteria. Virulent phages follow a lytic cycle where they multiply within bacteria to finally release the phage progeny by lysis of the cell. This process confers phages their antimicrobial activity. Phages have been used as treatment against human infections in countries from Eastern Europe, but the increase in antibiotic resistance has boosted new research and a notable interest worldwide for the use of phages to fight against pathogenic bacteria in clinical, veterinary, food safety, and environment (O'Flaherty et al., 2009; García et al., 2010). Phage-encoded lytic proteins such as endolysins and virion-associated peptidoglycan hydrolases (VAPGHs) have also been assessed as antimicrobial agents against pathogens (Schmelcher et al., 2012; Rodríguez-Rubio et al., 2013) and other phage-encoded proteins with polysaccharide depolymerase activity can be used as anti-biofilm agents (Cornelissen et al., 2011; Gutiérrez et al., 2012b, 2015a). Therefore, bacteriophages are not only bacterial killers but also a source of antimicrobial phage-derived proteins that can be exploited to fight against pathogenic bacteria.

Overall, the aim of this review is to assess both bacteriophages and bacteriophage-derived proteins as potential compounds to be applied as part of the cleaning and disinfecting processes of food-contact surfaces in the food industry.

RELEVANCE OF BIOFILMS IN THE FOOD INDUSTRY AND DISINFECTION HURDLES

Biofilm formation is a major concern in industrial settings, since it is one of the causes of operating troubles by decreasing heat transfer, blocking tubes, plugging filters, and causing damage to surfaces (Myszka and Czaczyk, 2011). Specifically in the food industry, the ability of bacteria to attach to food-contact surfaces provides a reservoir of contamination for pathogens with the consequent risks to human health. Analysis of the microbial composition of biofilms formed on food industrial surfaces revealed the presence of mixed biofilms including pathogenic and spoilage bacteria (Gounadaki et al., 2008; Gutiérrez et al., 2012a). These microorganisms can reach the food industry through several sources such as water, raw foods, animals, and can persist in the equipment for long periods of time. Therefore, food products can be contaminated at any stage of the food chain, even though all required cleaning protocols have been applied, because disinfecting and cleaning processes in the food industry are often ineffective. For instance, some microorganisms are able to survive after cleaning-in-place procedures, like in the case of dairy industries (Anand and Singh, 2013).

Biofilms mainly cause problems in the dairy (Latorre et al., 2010), meat (Giaouris et al., 2014), poultry (Silagyi et al., 2009), seafood (Thimothe et al., 2004), and vegetable processing industries (Liu et al., 2013). Depending on the food-processing industry, the type of bacteria and the route of access to foodstuffs differs (**Figure 1**).

In seafood industries, the most common bacterial pathogens that form biofilms are *Vibrio* spp., *Aeromonas hydrophila*, *Salmonella* spp., and *L. monocytogenes* (Mizan et al., 2015). *Vibrio parahaemolyticus* can form biofilms on different surfaces including the chitin of oysters, and this process is recognized as vital to the physiology of these microorganisms (Thompson et al., 2010). *Vibrio cholerae* can form biofilms attached to the surface of phytoplankton and zooplankton, from where they can contaminate seafood products after consumption (Mizan et al., 2015). A correlation between the persistence of *Salmonella* spp. in the fish-processing industry and the ability for biofilm formation was also reported (Vestby et al., 2009). It was also demonstrated that other bacteria such as *L. monocytogenes* isolated from seafood industries can form biofilms on stainless steel surfaces (Gudmundsdottir et al., 2006).

In the fresh produce industry, bacteria such as *Salmonella*, *E. coli* O157:H7, *L. monocytogenes*, *Shigella*, *Bacillus cereus*, *Clostridium perfringens*, and *Yersinia* go into the processing facilities adhered to the plant tissues where they can grow forming biofilms (Beuchat, 2002; Da Silva Felicio et al., 2015). The accessibility of sanitizers to these microorganisms is hindered, not only by the presence of biofilms, but also by the intrinsic structure of vegetables, making it necessary to optimize the

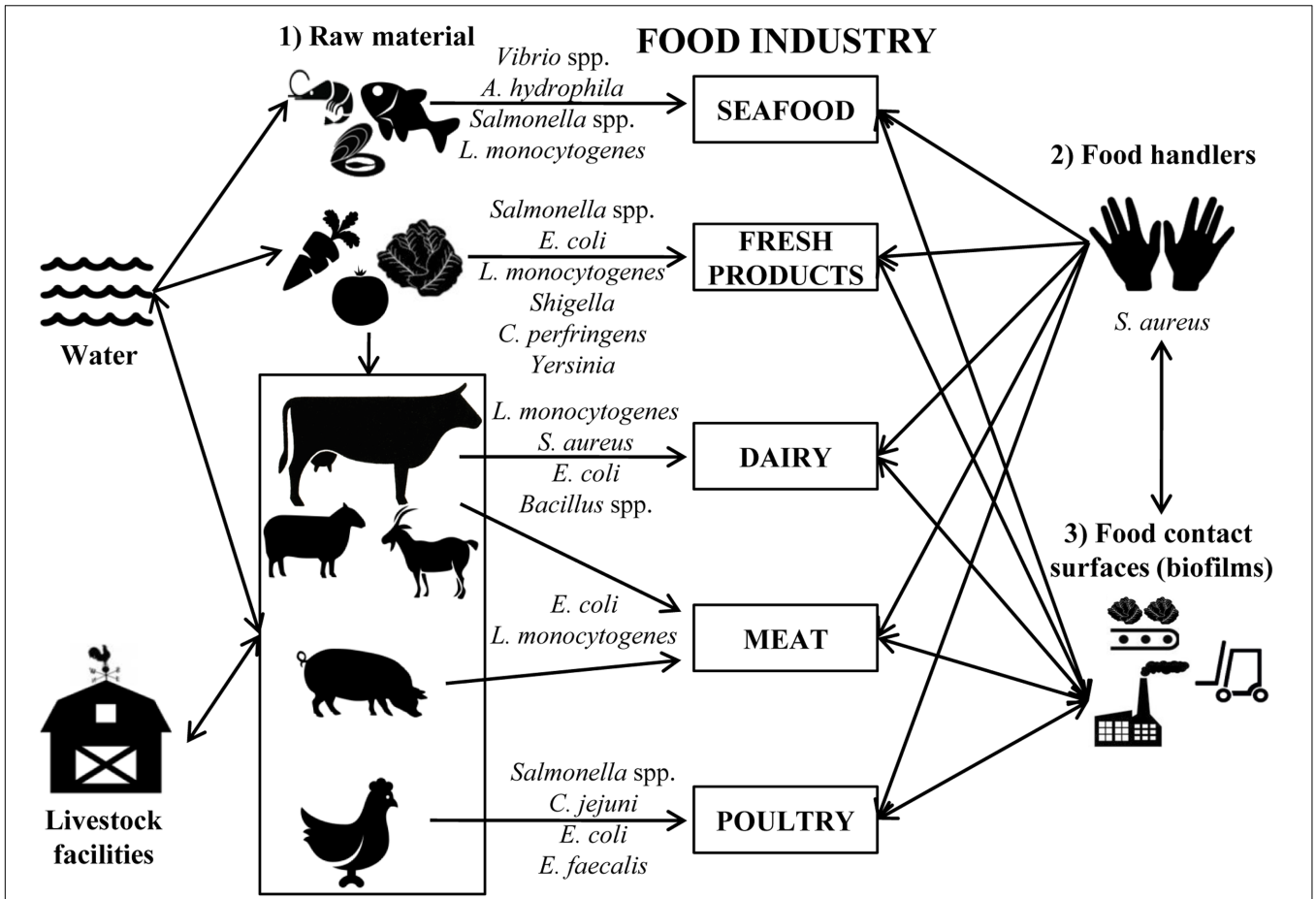

FIGURE 1 | Schematic representation of the main sources of contamination in food industries. Most common bacteria detected in each agri-food industry are indicated.

decontamination methods to extend their shelf-life (López-Gálvez et al., 2010).

In the dairy industry, most contamination comes from inadequate cleaning of the equipment and the presence of pathogenic bacteria; e.g., *L. monocytogenes* in milking equipment was determined to be the cause of contamination of bulk tank milk (Latorre et al., 2010). In addition, biofilm formation by *L. monocytogenes* may be promoted by specific conditions in the dairy industry like those used in cheese manufacturing (low pH values during milk fermentation and increased salt concentration). Thus, some strains increased their adherence to polystyrene after salt adaptation, and the exposure to acid increased the survival of cells adhering to stainless steel (Adriao et al., 2008). Milk proteins are also able to increase the attachment of *E. coli*, *L. monocytogenes*, and *S. aureus* to stainless steel (Barnes et al., 1999). On the other hand, members of the *Bacillus* genus are very common in dairy plants, where biofilm formation is triggered during milk lipolysis (Pasvolsky et al., 2014).

E. coli O157:H7 is a pathogenic bacteria also related with contamination in the meat industry. The ability of this bacterium to attach to meat-contact surfaces is influenced by the type of meat residues and the temperature. In fact, this microorganism significantly increases its counts number during inactivity periods of facilities (15°C) and also during cold storage temperatures (4°C; Dourou et al., 2011). Recently, it has been reported that *E. coli* O157:H7 strains isolated from a "high event period" (period of time during which commercial meat plants undergo a higher rate of contamination with this pathogen than normal) have a significantly higher potential of mature biofilm formation after incubation for 4–6 days, and also exhibit significantly stronger resistance to sanitization (Wang et al., 2014). *L. monocytogenes* was also isolated from bovine carcasses and meat processing facilities (Peccio et al., 2003; von Laer et al., 2009). The ability of this bacterium to colonize materials used in food processing surfaces (Rodriguez et al., 2008; Hingston et al., 2013), and to survive in niches that are difficult to sanitize such as countertops, cutting blades, or joints is well known (Verran et al., 2008).

Salmonella spp. and *Campylobacter* spp. are the most common pathogens found in poultry industries. *Salmonella* adhesion is influenced by different physicochemical properties of surfaces; for instance, *Salmonella* is able to grow at 16°C on stainless steel, while adherence was hindered on glass (De Oliveira et al., 2014). Recent studies have found that chicken meat exudation increases

Campylobacter jejuni biofilm formation on glass, polystyrene, and stainless steel surfaces by covering and conditioning the surface (Brown et al., 2014). In addition, aerobic or stressful conditions (Reuter et al., 2010) and the presence of other bacteria such as *Enterococcus faecalis* and *Staphylococcus simulans*, also found in poultry processing environments, increase the level of biofilm formation allowing the survival of *C. jejuni* under detrimental conditions (Teh et al., 2010).

Overall, the main concern about biofilms is their wide resistance to disinfectants commonly used in food industries, which include quaternary ammonium compounds such as benzalkonium chloride (BAC). The resistance to these compounds shown by several foodborne pathogens results in their reduced efficacy (Saa-Ibusquiza et al., 2011); e.g., *L. monocytogenes* is able to modify the physicochemical properties of the cell surface as a response to low concentrations of BAC resulting in a higher resistance to this compound (Bisbiroulas et al., 2011). Biofilm resistance to antimicrobials is attributed to several intrinsic biofilm properties such as reduced diffusion, physiological changes of cells, reduced growth rates, and the production of enzymes that degrade the antimicrobial compounds (Bridier et al., 2011). In this regard, it has been shown that the extracellular material constitutes a physical barrier for biocides and the chemical interaction with this material reduces the rate of diffusion to the biofilm inside. Besides the physical barrier found by the antimicrobial compounds to penetrate into the biofilm, there is a physiological resistance due to the altered growth rate of cells forming the biofilm, which grow more slowly than planktonic cells and consequently are less affected by the biocide (Evans et al., 1991). The presence of persister cells, which are tolerant to antimicrobials, could also explain the resistance of biofilm to biocides along with an adaptive tolerance (Simoes et al., 2011). Thus, it has been suggested that exposure to sublethal concentrations of biocides allows bacterial adaptation and survival at the level of biocide concentrations used in the food environment (Capita et al., 2014). In many bacteria, such as *S. aureus*, multidrug efflux pumps are responsible for this biocide resistance (Rouch et al., 1990). In fact, prolonged exposure to sublethal concentrations of biocides can lead to the overexpression of these efflux pumps and hence to the increased multidrug resistance in bacteria (Gilbert and McBain, 2003). In this regard, *in vitro* cross-resistance with antibiotics has been described for some biocide-resistant foodborne pathogens (Davin-Regli and Pages, 2012; Gnanadhas et al., 2013) supporting the need for monitoring and regulating the usage of biocides. The maturation stage of biofilms may also enhance resistance to disinfectants, since it has been reported that sodium hypochlorite, sodium hydroxide, and BAC failed to eradicate mature *Salmonella* biofilms (Corcoran et al., 2014).

IMPACT OF FOOD-PROCESSING CONDITIONS ON BIOFILM DEVELOPMENT

In food processing environments, there are a number of variable conditions such as temperature, pH, oxygen and nutrients availability, and surface type, which can modulate biofilm development (**Figure 2**). Surface properties such as electrostatic charges, hydrophobicity, and roughness influence biofilm development in some species. For instance, hydrophilic surfaces are more quickly colonized by *L. monocytogenes* (Chavant et al., 2002), whereas *S. aureus* have not shown any differences between hydrophobic and hydrophilic surfaces (da Silva Meira et al., 2012), and *Salmonella* has a higher ability to adhere to some materials used in food-contact surfaces like Teflon, followed by stainless steel, glass, Buna-N rubber, and polyurethane (Chia et al., 2009). In some cases, biofilm retention is more affected by the surface roughness than by the chemical composition (Tang et al., 2011). Other components of food environments such as NaCl also contribute to increase the adhesion of *L. monocytogenes* to surfaces (Jensen et al., 2007), although it is influenced by temperature and nutrients as well (Moltz and Martin, 2005), and even by the presence of other bacteria in the food-processing environment (Carpentier and Chassaing, 2004).

Food-related environmental factors have a variable impact on biofilm development. Bacteria sense these factors through sophisticated intracellular and extracellular signaling networks resulting in a negative or positive response (Karatan and Watnick, 2009). For instance, nutrient limitation induces *Salmonella enterica* serovar Typhimurium to biofilm formation (Gerstel and Romling, 2001), whereas *V. cholerae* needs a nutrient-rich environment to develop a biofilm structure (Yildiz et al., 2004). Similarly, in *S. aureus* an increase in biofilm formation was observed in a nutrient-rich growth media (Herrera et al., 2007) and at high incubation temperatures (Vázquez-Sánchez et al., 2013). Secondary metabolites such as antibiotics may also induce biofilm formation (Hoffman et al., 2005).

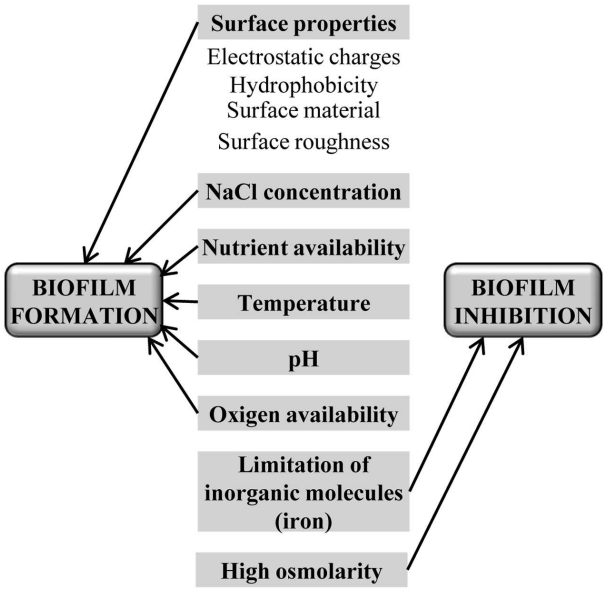

FIGURE 2 | Main food industry's parameters that can influence biofilm development.

Another example was recently reported by Nesse et al. (2014), where potentially human-pathogenic *E. coli* from the ovine reservoir can form biofilms under conditions used in the food production chain [on different surfaces such as stainless steel, glass, and polystyrene and at temperatures relevant for food production and handling (12, 20, and 37°C)]. Of note, for most bacteria, limitation of inorganic molecules such as iron and inorganic phosphate has an inhibitory effect on biofilm formation (Mey et al., 2005; Monds et al., 2007), and high osmolarity inhibits in general, biofilm formation, although this effect is clearly dependent on the osmolyte (Jubelin et al., 2005).

BACTERIOPHAGES PROPERTIES AS ANTIMICROBIALS

Bacteriophages are viruses of prokaryotes widespread in all habitats where their hosts are located. Classifications of bacteriophages are based on their shape, size, and kind of nucleic acid. The most abundant belong to the *Caudovirales* order (tailed-bacteriophages), which is divided into three families (*Myoviridae*, *Podoviridae*, and *Siphoviridae*) according to the microscopic features of the tail morphology. Bacteriophages belonging to the *Siphoviridae* family are the most abundant (57.3%; Ackermann and Prangishvili, 2012).

Bacteriophages can infect bacteria by following two different life cycles, lytic and lysogenic (Kutter et al., 2004). In most phages, the lytic cycle ends with the lysis of host bacteria and the progeny release. Thus, antimicrobial properties of bacteriophages are linked to the lytic cycle (lytic phages) since the infected host is intended to die. On the contrary, the lysogenic cycle followed by temperate bacteriophages implies the survival and establishment of the phage genome into the bacterial chromosome (prophage) until environmental signals trigger a lytic cycle, thereby killing only a part of the infected population. In addition, lysogenic bacteria, carrying a prophage, are resistant to infection for a related phage (superinfection immunity; Kutter et al., 2004).

In nature, most bacteria are living in biofilms (Hall-Stoodley et al., 2004). The interaction between the host bacteria and the lytic phages occurs in six different steps (**Figure 3**). The adsorption of the bacteriophage and release of the new phage progeny play a key role in the bacteriophage infection process. When host bacteria are included in a biofilm, the biofilm matrix can constitute a first physical barrier to the phage. To solve this problem, some phages possess polysaccharide depolymerases which are specific hydrolytic enzymes that can use polysaccharides or polysaccharides derivatives as substrate (Pires et al., 2016; **Figure 4A**). Numerous studies have shown that polysaccharide depolymerase activity is related to tail-spike proteins which are components of the tail of many bacteriophages (Barbirz et al., 2009). The presence of

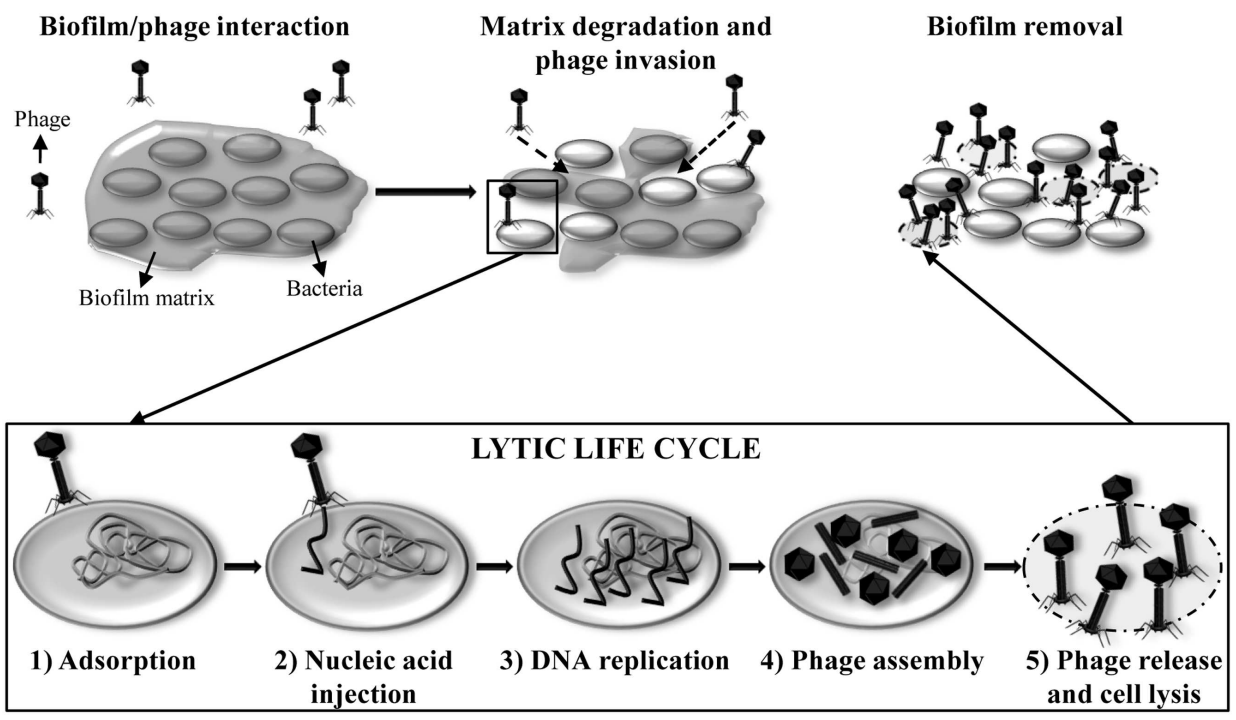

FIGURE 3 | Lytic life cycle of phages inside a biofilm. (1) Adsorption of the phage particle onto the host bacterial cell surface. Tail fibers bind to specific receptors on the cell surface. **(2)** Injection of the nucleic acid into the cytoplasm of the host bacterium. **(3)** Replication of the phage genome in multiple copies. Phage early genes are expressed to regulate the host metabolic machinery to be subjected to phage propagation. **(4)** Formation of new phage particles by expression of the phage late genes and assembly of the phage heads and tails, packaging of the nucleic acid inside the heads and maturation of the virions. **(5)** Lysis of the host bacterium and release of the new phage progeny ready to infect other cells in the biofilm and start a new cycle.

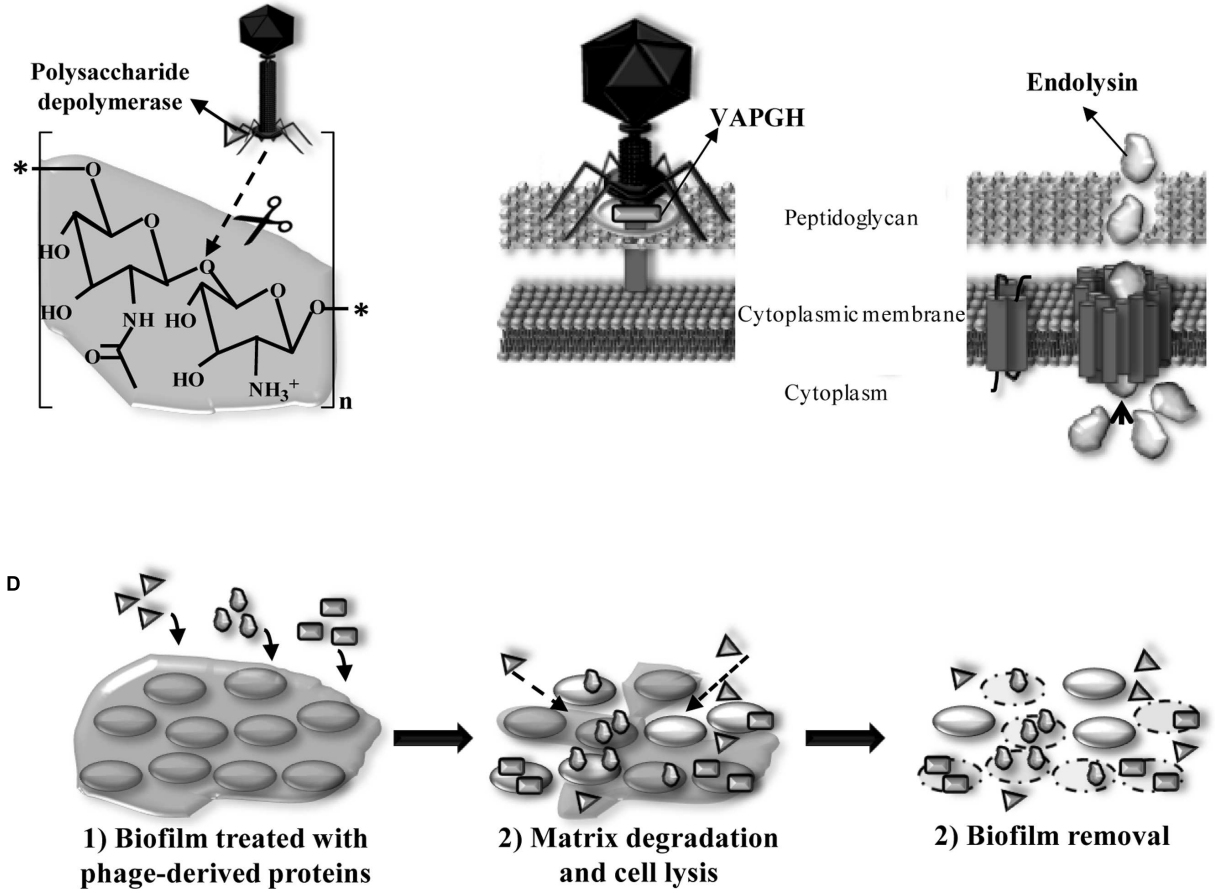

FIGURE 4 | (A) Location of exopolysaccharide depolymerase degrading β-(1,6) bonds of the biofilm extracellular matrix (PIA/PNAG) of staphylococcal species in the phage particle and mode of action. **(B)** Location of virion-associated peptidoglycan hydrolase (VAPGH) at the phage particle and its role in the infection process. **(C)** Structure of Gram-positive bacteria cell wall and role of the endolysin during the bacterial lysis. **(D)** Activity of phage derived proteins when added exogenously and their application as anti-biofilm agents degrading polysaccharidic matrices (polysaccharide depolymerases) and lysing bacteria (VAPGHs and endolysins).

polysaccharide depolymerases confers the phage an important advantage since it enhances the process of invasion and dispersion through the biofilm to start the infection process of new bacteria. Moreover, some phages are provided with lytic enzymes which are named VAPGHs, with a role in the first step of the infection cycle (**Figure 4B**). Their activity produces a small hole in the cell wall through which phage genetic material reaches the cytoplasm, being responsible for the "lysis from without" caused by the adsorption of a high number of phages to the cell at the initial infection step (Moak and Molineux, 2004). Recently, these proteins have also been proposed as new antimicrobials due to their lytic activity (Rodríguez-Rubio et al., 2013).

Double-stranded phages encode lytic proteins, named endolysins, which act along with holins to disrupt the cell wall and lyse the host bacteria at the last step of the lytic infection cycle (**Figure 4C**). Endolysins, which are also peptidoglycan hydrolases, access the periplasmic space through holes formed by holins in the cytoplasmic membrane. Hydrolysis of covalent bonds in the peptidoglycan molecule produces the destabilization of the cell wall structure and lysis by the increase of the osmotic pressure inside the cytoplasm. In Gram-positive bacteria, endolysins are able to degrade the peptidoglycan when they are added from outside the cell, which gives them an antimicrobial activity (enzybiotics; Fischetti, 2008). In Gram-negative bacteria, peptidoglycan is protected by the outer membrane, these bacteria being insensitive to endolysins. Nowadays, research efforts made into endolysin applications against Gram-negative pathogens are changing this rule. This is the case of Artilysins that combine a polycationic peptide able to penetrate the outer membrane with an endolysin, which renders a protein with high bactericidal activity against Gram-negative pathogens (Briers et al., 2014a,b).

The use of phage-encoded proteins as antimicrobials has some advantages over the use of the viral particles; e.g., no resistant bacteria to phage lytic proteins has been described to date (Nelson et al., 2012; Rodríguez-Rubio et al., 2013). Additionally, the spectrum of activity of endolysins is usually broader than the host range of bacteriophages and, there are no risks of transferring virulence genes (Fischetti, 2008).

Due to the above described working mechanisms, bacteriophages and phage-derived proteins could be used in the production of foodstuffs against unwanted bacteria to ensure quality, safety, and good hygienic conditions, covering the entire food chain production ("from farm to fork"). This includes strategies to improve animal health (phage therapy), decontamination of fresh-food and ready-to-eat products, disinfection of food-contact surfaces, as well as their use as biopreservatives to inhibit the development of pathogenic or spoilage bacteria during storage, and also as tools for detecting undesirable bacteria through the different manufacturing steps (García et al., 2010).

CONTROL OF BIOFILMS USING BACTERIOPHAGES AND PHAGE-DERIVED PROTEINS

The recent interest in phage therapy as an alternative to conventional antibiotics has fostered the use of phages in multiple applications, among which their use as anti-biofilm agents should be noticed. Biofilms are the lifestyle of bacteria in nature and therefore, phages have evolved to infect cells at this stage (Hall-Stoodley et al., 2004). There are two putative limitations for phage infection of cells inside the biofilm. First, the accessibility of phages to cells due to the structure of biofilm and the presence of extracellular material. Briandet et al. (2008) demonstrated that phage c2 was able to penetrate inside the *Lactococcus lactis* biofilm structure through water channels and cell clusters; in addition, the infection of *E. coli* surface-attached cells was confirmed by using T4 fluorescently labeled phages (Doolittle et al., 1996). Some phages, provided with exopolysaccharide depolymerases, can degrade the extracellular polymeric material, thus facilitating the entrance of phages into the deeper layers of the biofilms with subsequent lysis of the target bacteria (Parasion et al., 2014). The second limitation in the treatment of biofilms with bacteriophages is the metabolic status of a proportion of the population, persister cells and stationary phase bacteria, which have a slow metabolism. Bacteriophages infect preferably exponentially growing bacteria but recently, it has been demonstrated that persister bacteria can be infected by phages when bacteria switch to normal growth rate (Pearl et al., 2008). Moreover, persister cells can be removed by phage lytic proteins (Gutiérrez et al., 2014) due to these proteins are able to easily penetrate into the biofilms (Shen et al., 2013). Furthermore, bacteriophages can be engineered to express proteins intended to enhance their anti-biofilm properties. For instance, phage T7 was genetically engineered to incorporate the gene *dspB* encoding a polysaccharide depolymerase from *Actinobacillus actinomycetemcomitans*, which was more effective at reducing the bacterial count in *E. coli* biofilms (Lu and Collins, 2007). In addition, it has been demonstrated that engineered bacteriophages overexpressing proteins able to suppress bacterial SOS response network in *E. coli* are more effective against persister cells (Lu and Collins, 2009).

Several studies using biofilms preformed in laboratory conditions confirm the potential of phages in biofilm removal (**Table 1**). For biofilms formed by pathogenic bacteria with relevance in the food industry, there is evidence of effective removal in different conditions and using materials similar to those found in food-contact surfaces. Regarding this, three phages LiMN4L, LiMN4p, and LiMN17 infecting *L. monocytogenes* were assayed, individually and as a cocktail, against 7-day biofilms formed by a mixture of three strains on stainless steel coupons (10^4 cfu/cm^2), previously covered with a fish broth layer that simulated seafood processing facilities. Treatments with the single phages (10^9 pfu/ml) reduced adhered bacterial cells to up 3 log units, whereas treatment with the phage cocktail reduced cell counts to undetectable levels after 75 min (Ganegama-Arachchi et al., 2013). Similarly, a treatment with phage P100 (10^9 pfu/ml) reduced biofilms formed by *L. monocytogenes* strains in 3.5–5.4 log/cm^2, irrespective of the serotype, growth conditions and biofilm level (Soni and Nannapaneni, 2010). Despite the efficacy of bacteriophages to reduce *L. monocytogenes* biofilms, there is evidence that complete removal is not always achieved. By using epifluorescence microscopy, *L. monocytogenes* was monitored after treatment with phage P100 (10^8 pfu/ml) and, although disaggregation of biofilms could be observed after 8 h, viable cells were still present up to 48 h later, indicating that other sanitization methodologies should be used in combination with phages (Montañez-Izquierdo et al., 2012).

Staphylococcus aureus is another important foodborne pathogen with the ability to form biofilms on different surface's materials. Staphylococcal phage K and a mixture of derivative phages with broader host ranges were used to effectively prevent *S. aureus* biofilm formation over incubation periods of 48 h. It was also shown that the removal of bacteria by the phage cocktail (10^9 pfu/ml) was time-dependent, with the highest reduction occurring after 72 h at 37°C (Kelly et al., 2012). A similar result was obtained using phage K combined with another staphylococcal phage, DRA88 (MOI 10), to treat established biofilms produced by three *S. aureus* isolates, which were significantly reduced after 4 h and completely removed after 48 h at 37°C (Alves et al., 2014). Other staphylococcal phages such as ISP, Romulus, and Remus applied individually at 10^9 phages per polystyrene peg were able to degrade by 37.8, 34.4, and 60.4%, respectively, an *S. aureus* PS47 biofilm after 24 h (Vandersteegen et al., 2013). Similar results were obtained after the application of phages phiIPLA-RODI, phiIPLA-C1C, and a mixture of both phages, against biofilms formed by *S. aureus* where a reduction by about 2 log units was achieved after 8 h of treatment at 37°C (Gutiérrez et al., 2015b). In some cases, however, it was also necessary to combine phages with other antimicrobials to increase their effectiveness. Thus, treatment of 1-day-old biofilms formed by *S. aureus* D43 strain with phage SAP-26 reduced live bacteria by about 28% while a synergistic effect with rifampicin allowed a reduction of about 65% (Rahman et al., 2011).

Bacteriophages were also assayed against *C. jejuni* biofilms. Two virulent phages, CP8 and CP30 led to 1–3 log cfu/cm^2 reduction in viable counts after 24 h of treatment. However, a high percentage of bacteriophage-resistant bacteria in biofilms were observed for some *C. jejuni* strains (Siringan et al., 2011).

TABLE 1 | Application of bacteriophages and phage proteins for biofilm removal.

Phage or phage protein	Scope of application	Bacteria	Efficacy of the treatment	Reference
Phages LiMN4L, LiMN4p, and LiMN17	Stainless steel	*L. monocytogenes*	Phage cocktail reduced biofilm cell counts to undetectable levels after 75 min	Ganegama-Arachchi et al., 2013
Phage P100	Stainless steel	*L. monocytogenes*	Reduction in the cell counts from 3.5 to 5.4 log units/cm^2	Soni and Nannapaneni, 2010
Phage P100	Stainless steel	*L. monocytogenes*	Reduction of the biofilm cell counts to undetectable levels after 48 h	Montañez-Izquierdo et al., 2012
Phage K and phage derivatives	Polystyrene	*S. aureus*	Complete elimination of the biomass after 72 h of incubation. Complete inhibition of biofilm formation was achieved when co-culturing phage mixture and bacteria	Kelly et al., 2012
Phage K and DRA88	Polystyrene	*S. aureus*	Complete elimination of the biomass after 48 h of treatment	Alves et al., 2014
Phages ISP, Romulus, and Remus	Polystyrene	*S. aureus*	Biofilm reduction of 37.8, 34.4, and 60.4% after 24 h treatment when using phages ISP, Romulus, and Remus, respectively	Vandersteegen et al., 2013
Phages philPLA-RODI and philPLA-C1C	Polystyrene	*S. aureus*	Reduction by 2 log units/well was achieved after 8 h of treatment	Gutiérrez et al., 2015b
Phage SAP-26	Polystyrene	*S. aureus*	Reduction of bacteria about 28% after phage treatment, while a synergistic effect with rifampicin allows a reduction of about 65%	Rahman et al., 2011
Phage CP8 and CP30	Glass	*C. jejuni*	Reduction in the biofilm cell counts of 1–3 log units/cm^2	Siringan et al., 2011
Phage KH1	Stainless steel	*E. coli* O157:H7	Reduction of 1.2 log units per coupon after 4 days treatment at 4°C	Sharma et al., 2005
BEC8 (phage mixture)	Stainless steel, ceramic tile, and high density polyethylene	*E. coli* O157:H7	Reduction of the biofilm cell counts to undetectable levels after 1 h of treatment at 37, 23, and 12°C	Viazis et al., 2011
Phage mixture	Spinach harvester blade	*E. coli* O157:H7	Reduction of biofilm cell counts by 4.5 log units per blade after 2 h of treatment	Patel et al., 2011
Phage T4	Polystyrene	*E. coli* O157:H7	Complete elimination of the biomass after phage treatment combined with cefotaxime	Ryan et al., 2012
Endolysin from phage phi11	Polystyrene	*S. aureus*	Complete elimination of the biomass after 2 h of treatment at 37°C	Sass and Bierbaum, 2007
Endolysin SAL-2	Polystyrene	*S. aureus*	Reduction of the biomass after 2 h of treatment at 37°C	Son et al., 2010
Endolysin LysH5	Polystyrene	*S. aureus*	Reduction of biofilm cell counts by 1–3 log units after 3 h of treatment	Gutiérrez et al., 2014
Domain $CHAP_K$ derived from endolysin LysK	Polystyrene	*S. aureus*	Complete elimination of the biomass after 4 h of incubation Complete inhibition of biofilm formation was achieved	Fenton et al., 2013
Chimeric lysin ClyH	Polystyrene	*S. aureus*	Reduction of the biomass in more than 60% after 30 min of treatment	Yang et al., 2014
Endolysin Lys68	Polystyrene	*S.* Typhimurium	Reduction of biofilm cell counts by 1 log unit after 2 h of treatment in the presence of outer membrane permeabilizers	Oliveira et al., 2014
Exopolysaccharide depolymerase Dpo7	Polystyrene	*S. aureus*	Degradation of 30% of the polysaccharidic matrix of the biofilm	Gutiérrez et al., 2015a

Sharma et al. (2005) assayed the lytic bacteriophage KH1 (7.7 log pfu/ml) against stainless steel coupons containing *E. coli* O157:H7 biofilms (2.6 log cfu/coupon). These were treated for 4 days at 4°C and a reduction of 1.2 log units per coupon was observed. Better results were obtained when treating *E. coli* O157:H7 biofilms preformed on other materials typically used in food processing surfaces (stainless steel, ceramic tile, and high density polyethylene), since a reduction to undetectable levels was observed after 1 h of treatment at 23°C with a phage mixture named BEC8 (MOI 100; Viazis et al., 2011). The use of a phage mixture to remove biofilms formed on blades used to harvest spinach was also demonstrated, a reduction of 4.5 log units of the viable cells of *E. coli* O157:H7 being achieved after 2 h of phage treatment (Patel et al., 2011). As it was previously reported, a combination of T4 bacteriophage and cefotaxime significantly enhanced the eradication of *E. coli* biofilms when compared to treatment with phage alone (Ryan et al., 2012).

Phage lytic proteins are also an alternative for removing bacterial biofilms in food-related environments (**Figure 4D**; **Table 1**). Endolysin from phage phi11 (10 μg/well) was able to remove biofilms formed by *S. aureus* strains on polystyrene surfaces after 2 h at 37°C (Sass and Bierbaum, 2007). Similarly, endolysin SAL-2 from bacteriophage SAP-2 eliminated *S. aureus* biofilms using 15 μg/well (Son et al., 2010). Recently, Gutiérrez et al. (2014) showed that endolysin LysH5 (0.15 μM) is able to remove staphylococcal biofilms after treatment of 12 h at 37°C and even to lyse persister cells. Engineered endolysins, by deletion or shuffling domains, have also been successfully used as anti-biofilm agents. For instance, peptidase $CHAP_k$ (31.25 μg/ml), derived from the staphylococcal endolysin LysK, was able to completely prevent biofilm formation. This protein also removed staphylococcal biofilms after treatment of 4 h at 37°C (Fenton et al., 2013). In addition, the minimum concentration (6.2–50 mg/l) of ClyH, a staphylococcal chimeric lysin, required for *S. aureus* biofilm eradication was lower than that needed when antibiotics were used (Yang et al., 2014). This protein contains the catalytic domain of endolysin Ply187 and the cell wall binding domain of phiNM3 lysin. Regarding biofilms formed by Gram-negative bacteria, removal of these structures by using endolysins needs an additional component to disestablish the outer membrane. Biofilms formed by *S. enterica* serovar Typhimurium were treated with endolysin Lys68 (2 μM), which reduced by 1 log unit the viable cells in preformed biofilms after 2 h of incubation in the presence of outer membrane permeabilizers (Oliveira et al., 2014).

Regarding phage-encoded exopolysaccharide depolymerases, there is scarce data about the biofilm dispersion mediated by these proteins but they seem to be very promising. Cornelissen et al. (2011) identified an exopolysaccharide-degrading activity associated to a tail spike protein from *Pseudomonas putida* phage Φ15, which is involved in the hydrolysis of extracellular material. However, the addition of the purified tail spike protein did not result in biofilm removal. Since the addition of 10^6 phages yielded a significant biofilm degradation of 37% in 24 h, this seems to require phage amplification (Cornelissen et al., 2012). Recently, an exopolysaccharide depolymerase named Dpo7 was identified in the *S. epidermidis* phage phiIPLA7. Purified protein was used to treat *S. aureus* biofilms, showing its ability to degrade up to 30% of the polysaccharidic matrix formed by *S. aureus* 15981 (Gutiérrez et al., 2015a).

Overall, these results showed a noticeable potential of phages and phage-derived proteins, but undoubtedly additional studies are necessary to transfer this knowledge to the food industry. For instance, application of these anti-biofilm compounds would be feasible as long as their application can be implemented as part of the standard processes of cleaning in the industrial facilities. Therefore, the study of synergy/antagonism with disinfectants and the effectiveness at temperatures commonly used in the industry could be relevant. It should be also noticed the scarce data available about the use of phages and phage lytic proteins against mixed biofilms formed by different strains from several species in food industrial surfaces. This gap should be filled in to go further into the control of bacterial biofilms.

FUTURE PERSPECTIVES FOR PHAGE-BASED DISINFECTANTS

The most important issues to address before the implementation of phages and phage-derived proteins as disinfectants are the following.

Safety

Beyond efficiency, safety of phage-based products must be a priority to take into account. Only phages fully characterized at molecular level and with the complete genome sequenced should be taken into consideration as potential components of disinfectants to avoid the presence of virulence and antibiotic resistance genes. These phages must be lytic, since temperate bacteriophages have the ability to integrate their genomes into their host bacterium's chromosome, and non-transducers, i.e., without the ability to transfer genetic material from host bacteria. One of the most important characteristics of bacteriophages, their high specificity for the host bacteria, could be a potential limitation in their use as disinfectants. A cocktail of different phages with overlapping host ranges or the use of polyvalent phages with a wide host range would solve this problem. Finally, in the selection of phages to be included in the cocktail, the presence of those encoding polysaccharide depolymerase enzymes should be preferred.

Regarding the safety of engineered phages, the main hurdle for their use is the generalized opposition of consumers to genetic manipulation, despite of engineered phages can overcome the limitations of phages as antimicrobials and even specific modifications can eliminate some of their risks such as virulence genes or gene transfer (Nobrega et al., 2015).

Before the extensive use of bacteriophages as disinfectants, the absence of an ecological impact on the environment must be also guaranteed. In this regard, bacteriophages should be inactivated before their release outside the industry settings. Some commercial sanitizers and disinfectants commonly used in the food industry can be effective to inactivate phages, oxidizing agents and quaternary ammonium compounds being the most efficient ones (Campagna et al., 2014). Other treatments such as CO_2, high pressure and UV light could be evaluated for each phage (Guglielmotti et al., 2011; Cheng et al., 2013). On the other hand, development of phage insensitive bacteria could be a cause of concern, since they may hamper the effectiveness of the phage disinfection process. Generally, the rate at which bacteria develop resistance is very low, especially when a cocktail of different phages is used. Moreover, phage-insensitive bacteria are associated with a reduced fitness (Gutiérrez et al., 2015b); therefore, this question is expected to have minor relevance.

In this context, endolysins have some important advantages compared to phages due to their proteinaceous nature, which is easily degraded in the environment. Regarding safety, the most important is their inability to transfer virulence genes.

Large-Scale Production

Implementation of phages as disinfectants in the food industry implies obtaining large volumes of phage suspensions with high

titer using an inexpensive protocol. Therefore, some work is still necessary to optimize propagation and purification processes for each phage (Bourdin et al., 2014). In this regard, phages should be propagated in a non-pathogenic bacterium and then purified in order to remove cell debris or other contaminating substances. For preparations of bacteriophages infecting Gram-negative bacteria some procedures to remove endotoxin have been reported (Boratynski et al., 2004), and several companies also sell kits for endotoxin detection. However, the importance of these contaminating components in medical applications is more crucial than for disinfection. In the latter, undesirable effects of phages could be related with allergy by skin contact or by inhalation of aerosols. At present, nevertheless, there are no reported side effects of the use of bacteriophages in animal models of phage therapy applications (Golkar et al., 2014), which is not surprising as phages are abundant in human microbiota (De Paepe et al., 2014) and in the environment (Díaz-Munoz and Koskella, 2014). Overall, the purification methods used at laboratory-scale are well defined and consist of the precipitation of phages by polyethylene glycol followed by purification of phages in a cesium chloride gradient. However, these procedures are neither easy to scale up, nor cheap for the large-scale production required for the application of phages as disinfectants. New purification alternatives are being studied, e.g., suitable methods designed for purification of bionanoparticles, based on anion-exchange chromatography, with a 60% recovery of viable phages (Adriaenssens et al., 2012). Alternatives to centrifugation such as tangential flow filtration and specific membrane materials could also be explored (Hambsch et al., 2012).

The main drawback in the extensive use of endolysins might be the difficulty of their effective expression in *E. coli* (Rosano and Ceccarelli, 2014). Other bacteria like *L. lactis* have been proposed as suitable cell factories (D'Souza et al., 2012), but even the expression might need to be optimized (Rodríguez-Rubio et al., 2012). Moreover, large-scale production and purification of proteins is a costly process in itself. A similar scenario might be drawn for exopolysaccharide depolymerases due to the requirement of having large amounts of pure protein. In addition, more research is necessary to find out how specific the activity is for its substrate.

Regarding phage-derived proteins, thermostability seems to be a challenge, and a big concern, when applying enzymes for disinfection. However, heat stability seems to be a recurring property of phage structural lysins or VAPGHs (Rodríguez-Rubio et al., 2013). On the other hand, although the thermolabile nature of endolysins is well known (Varea et al., 2004; Obeso et al., 2008; Filatova et al., 2010; Heselpoth and Nelson, 2012), there are exceptions to the rule. In fact, two novel thermostable endolysins have recently been described, Lys68 from *Salmonella* phage phi68 (Oliveira et al., 2014) and Ph2119 from bacteriophage Ph2119 infecting *Thermus scotoductus* strain MAT2119 (Plotka et al., 2014). This thermostability supports the potential use of these phage-derived enzymes as disinfectants.

In addition to propagation and purification of phages and phage-derived proteins, other parameters such as a proper formulation, stability under non-refrigerated conditions, and lytic activity under usual conditions for the food industry should also be studied. No data about phage formulations and storage other than lyophilization (Merabishvili et al., 2013) and spray drying (Vandenheuvel et al., 2013) is available. Survival of phages in both processes is strictly dependent on an appropriate protector, in most cases sucrose being the most effective agent to protect phages (Merabishvili et al., 2013). However, this sugar is not suitable as excipient for disinfection processes. The controlled delivery of phages and their stability in encapsulated microspheres are worth studying. In fact, strategies under development for medical applications include phage encapsulation using different materials suitable for oral delivery or inhalation (Puapermpoonsiri et al., 2009; Dini et al., 2012; Balcao et al., 2014). Furthermore, bacteriophages could be useful to develop specific antimicrobial packaging materials for use in the food industry (Han et al., 2014).

Market and Regulatory

The potential of phages in the food industry is so extensive that several companies have developed phage-based products against important foodborne pathogens that could be used as disinfectants on surfaces and as food-processing aids. OmniLytics Inc. (Sandy, UT, USA) has developed two washing products, BacWash^{TM} against *Salmonella*, and Finalyse^{TM} against *E. coli* O157:H7, marketed by Elanco (Greenfield, IN, USA). Intralytix Inc. (Baltimore, MD, USA) developed three phage products, ListShield^{TM}, EcoShield^{TM}, and SalmoFresh^{TM}, to be used in the food industry against *L. monocytogenes*, *E. coli*, and *Salmonella*, respectively. In Europe, Micreos BV (Wageningen, Netherlands) has commercialized Listex^{TM} (P100) against *L. monocytogenes*, and Salmonelex^{TM} against *Salmonella*. All these products are setting a precedent for future approval of phages as disinfectants. In fact, one of the most important drawbacks in the use of phage-based products might be the specific regulatory framework of each country. The US Department of Agriculture and FDA have already approved the use of several phage-based products, mentioned above, in food production environments, including their application as both food biopreservatives and disinfectants of food-contact surfaces. In Europe, however, the EFSA has argued that it is not clear whether bacteriophages can protect food against a re-contamination in spite of having been reported that bacteriophages are effective in the elimination of pathogens (EFSA, 2012). Finally, it is worth noticing that bacteriophages have also been approved as processing-aids in food processing and handling in several countries, but nothing has been reported about the use of phages as antimicrobial agents for the cleaning of industrial surfaces.

CONCLUDING REMARKS

The development of new disinfection products, non-toxic to humans and friendly to the environment, has good prospects for the future. Bacteriophage-based disinfectants fulfill all the requirements regarding effectiveness and safety. However, two main challenges have to be overcome before the implementation of phages in the food industry: (i) more research is necessary to

solve the technical problems in manufacturing, such as the scaling up of the processes of propagation or expression, and purification of phages and proteins, and (ii) a regulatory framework for phage applications should be established, which would boost investment in these new products.

AUTHOR CONTRIBUTIONS

PG, AR, and BM conceived the revision work. DG and LR-R designed the figures. DG, LR-R, BM, AR, and PG wrote the manuscript.

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REVIEW
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New Weapons to Fight Old Enemies: Novel Strategies for the (Bio)control of Bacterial Biofilms in the Food Industry

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Biofilms are microbial communities characterized by their adhesion to solid surfaces and the production of a matrix of exopolymeric substances, consisting of polysaccharides, proteins, DNA and lipids, which surround the microorganisms lending structural integrity and a unique biochemical profile to the biofilm. Biofilm formation enhances the ability of the producer/s to persist in a given environment. Pathogenic and spoilage bacterial species capable of forming biofilms are a significant problem for the healthcare and food industries, as their biofilm-forming ability protects them from common cleaning processes and allows them to remain in the environment post-sanitation. In the food industry, persistent bacteria colonize the inside of mixing tanks, vats and tubing, compromising food safety and quality. Strategies to overcome bacterial persistence through inhibition of biofilm formation or removal of mature biofilms are therefore necessary. Current biofilm control strategies employed in the food industry (cleaning and disinfection, material selection and surface preconditioning, plasma treatment, ultrasonication, etc.), although effective to a certain point, fall short of biofilm control. Efforts have been explored, mainly with a view to their application in pharmaceutical and healthcare settings, which focus on targeting molecular determinants regulating biofilm formation. Their application to the food industry would greatly aid efforts to eradicate undesirable bacteria from food processing environments and, ultimately, from food products. These approaches, in contrast to bactericidal approaches, exert less selective pressure which in turn would reduce the likelihood of resistance development. A particularly interesting strategy targets quorum sensing systems, which regulate gene expression in response to fluctuations in cell-population density governing essential cellular processes including biofilm formation. This review article discusses the problems associated with bacterial biofilms in the food industry and summarizes the recent strategies explored to inhibit biofilm formation, with special focus on those targeting quorum sensing.

Keywords: biofilm, food, industry, quorum sensing, quorum sensing inhibitors

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INTRODUCTION

Certain bacteria develop a fortress or biofilm in the environments they colonize which provides shelter from antimicrobials and other sanitation procedures. A biofilm is formed when planktonic (or free/stand-alone) cells in an aqueous environment adopt a multicellular lifestyle by attachment to, and colonization of, a solid surface (Claessen et al., 2014). This may occur on a submerged surface or at the air-liquid interface (known as pellicle formation; Wu et al., 2012). Some bacteria begin biofilm formation without surface attachment *via* the aggregation of planktonic cells. Subsequent attachment of pre-formed aggregates to a solid surface results in true biofilm formation (Melaugh et al., 2016). The production of an extracellular matrix of DNA, carbohydrates, protein and lipids reinforces the sessile colony, facilitating the trapping of nutrients and protecting it against sanitation and even manual removal.

Biofilm formation is a serious problem in both the food and healthcare industries. Spoilage and pathogenic bacteria colonize, in the form of biofilms, the inside of mixing tanks, vats and tubing, compromising food safety and quality. In hospital settings, biofilm-forming bacteria persist in catheters, implants and on living tissues of patients suffering from chronic infections, such as those caused by *Staphylococcus epidermis* and *Pseudomonas aeruginosa* (Stewart and William Costerton, 2001). Despite the knowledge that the vast majority (∼80%) of infectious and persistent bacteria are biofilm-formers (National Institutes of Health, 2002) and that in nature microorganisms are actually forming biofilms (Hall-Stoodley et al., 2004), most of the research carried out to date is focused on the properties and control of planktonic bacteria. In this literature review the knowledge available with respect to biofilm formation in the food industry and current biofilm control strategies is compiled and critically discussed with key focus on anti-biofilm approaches targeting the bacterial quorum sensing system.

BACTERIAL BIOFILMS IN THE FOOD INDUSTRY

In the food processing industry, microorganisms indigenous to certain foods generally do not harm the consumer and in some cases convey some benefit (e.g., fermented foods in which bacteria are intentionally introduced in the form of a starter culture). Therefore, efforts are not usually made to rid the processing environment of such microbes unless overgrowth or visible product spoilage occurs. Biofilms formed by pathogenic and spoilage microorganisms, however, serve as a reservoir of problematic microbial cells which may contaminate raw materials and food products during processing, resulting in food spoilage and economical losses for the producers (Winkelströter et al., 2014a). Persistence of unwelcome bacteria in industrial settings has been linked to such capabilities as antimicrobial and disinfectant resistance, tolerance of certain environmental stresses and biofilm formation. Consumers may be affected by reduced shelf life of the contaminated product and possible contraction of foodborne illnesses. Fresh, minimally processed foods are at high risk of bacterial contamination. The produce industry, responsible for providing raw and ready-to-eat fruit, vegetables and derived products, faces repeated contamination of food due to spoilage and pathogenic bacteria forming biofilms on industrial equipment or on the foods themselves (Jahid and Ha, 2012). In the dairy industry, a wide range of thermophilic and psychrophilic bacteria dwell along the different stages of processing and pasteurization. Persistent *Bacillus cereus* spores adhered to industrial surfaces act as a conditioning film promoting the prompt attachment of bacterial cells introduced into the system that would otherwise be removed by methods effective against planktonic cells (Marchand et al., 2012). Other thermophilic bacilli, such as *Geobacillus* spp., can grow at temperatures as high as 65°C and their heat-resistant spores prove problematic for the manufacture of milk powders (Palmer et al., 2010). Psychrotrophic bacteria complicate storage of milk and other dairy products as they can thrive at refrigeration temperatures. *Pseudomonas* are common spoilage psychrophiles which can reach high population numbers and form biofilms at low temperatures on walls of milk cooling tanks and pipelines prior to heat processing and often secrete heat-stable lipolytic and proteolytic enzymes which contribute greatly to milk spoilage (Marchand et al., 2009). In addition, *Pseudomonas* biofilms have been shown to be capable of providing shelter to other pathogenic bacteria (e.g., *Listeria monocytogenes*) in multi-species biofilms (Marchand et al., 2012). *L. monocytogenes* is an important psychrotrophic food pathogen associated with the dairy (as well as the produce and poultry) industry. It is an opportunistic gastrointestinal (GI) foodborne pathogen also capable of causing serious systemic infectious disease (listeriosis) in certain individuals including the very young, the elderly, in pregnant woman and immunocompromised patients (Hamon et al., 2006; Freitag et al., 2009). The seriousness of *L. monocytogenes* occupancy in food related environments and, subsequently, the human host is as a result of the bacteria's ability to multiply at a wide range of temperatures (Walker et al., 1990) and to tolerate and adapt to harsh environmental conditions such as osmotic stress (Dykes and Moorhead, 2000) and bile acid in the human GI tract (Gahan and Hill, 2014). This resistance to harsh conditions and its ability to form biofilms allow *L. monocytogenes* to persist in food processing environments, a serious threat to the food industry. Indeed, the persistence of several specific *L. monocytogenes* strains in food and food processing areas across seven out of 48 processing facilities in the Republic of Ireland over a period of 12 months has recently been demonstrated (Leong et al., 2014). Infections caused by food-associated pathogens capable of forming biofilms, e.g., *L. monocytogenes, Campylobacter* spp., *Salmonella* spp., seriously impact public health on a global scale with the annual health-care costs associated with common food-borne pathogens reaching $15.5 billion in the USA per year (EFSA, 2009; Scallan et al., 2011; Hoffmann et al., 2015). Infection with *Campylobacter* species is the leading cause of food-borne bacterial gastroenteritis worldwide (World Health Organization, 2012) with *Campylobacter jejuni* claiming responsibility for the majority of those cases. Acute infection may lead to serious complications with long term consequences

such as peripheral neuropathy symptoms typical of Guillain–Barre syndrome (GBS) which has long been associated with *Campylobacter* infection (Nachamkin et al., 1998), reactive arthritis (Pope et al., 2007) and post-infectious irritable bowel syndrome (IBS; Schwille-Kiuntke et al., 2011). *C. jejuni* readily forms biofilm on food industry related surfaces (Teh et al., 2014), is frequently associated with poultry, and it has even been demonstrated that chicken juice increases biofilm formation on food industry-related equipment (Brown et al., 2014). Another serious pathogen is *Salmonella enterica* serovar Typhi (*S.* Typhi), the causative agent of typhoid fever, which is responsible for 21.7 million human infections and 217,000 deaths annually (Crump and Mintz, 2010) and is capable of forming biofilms (Kalai Chelvam et al., 2014) and persisting on materials often used in the food industry such as stainless steel, rubber and plastics, as comprehensively reviewed by Steenackers et al. (2012). Additionally, other *Salmonella* serovars able to form biofilm on food-related surfaces, such as *S. enterica* serovar Typhimurium (*S.* Typhimurium), cause a typhoid-like disease which is usually not fatal to healthy individuals but is commonly the source of poultry and meat products-related food poisoning (Jackson et al., 2013).

BIOFILM FORMATION AND REGULATION

Biofilm formation occurs over a series of sequential steps, in short: attachment (reversible and irreversible), cell-to-cell adhesion, expansion, maturation, and dispersal (**Figure 1**). Successful attachment to solid surfaces is governed by a slew of factors concerning both the bacterial cell and the surface of the potential biofilm site (reviewed by Chmielewski and Frank, 2003; Persat et al., 2015). Biofilm-forming bacteria possess motility and anchoring appendages which enable movement through liquid and attachment to an appropriate surface such as flagella are proteinaceous structures protruding from the bacterial cell surface which enable swimming motility (Van Houdt and Michiels, 2010). Other adhesion molecules such as pili (or fimbriae) (Mandlik et al., 2008) and curli (Cookson et al., 2002) contribute to biofilm formation by enabling active attachment. Once attached, the bacteria proceed to colonize the surface through the formation of cellular aggregates known as microcolonies. Under permissive environmental conditions, microcolonies form two-dimensional dynamic structures as cell numbers increase, the first step toward structural organization on the chosen surface. This framework further matures into a defined architecture with cells arranged in simple or elaborate structures suited to thriving in their particular environment (Pilchová et al., 2014). Mature biofilm formations include flat monolayers, three-dimensional structures or mushroom- or tulip-like assemblies with low surface coverage and intervening water channels for nutrient and waste exchange (Karatan and Watnick, 2009; Jahid and Ha, 2012). Exopolymeric substance (EPS) is a gelatinous material encasing the cells of a biofilm which is composed of substances excreted by the cells themselves including proteins, polysaccharides, nucleic acids, lipids, dead bacterial cells, and other polymeric substances hydrated to 85–95% water (Costerton et al., 1981; Sutherland, 1983). EPS functions to anchor to biotic and abiotic surfaces (Characklis and Marshall, 1990), concentrate nutrients from the surrounding environment within the biofilm, limit access of antimicrobial agents (contributing to resistance) and prevent the biofilm from desiccation (Carpentier and Cerf, 1993). The final stage in the biofilm life cycle involves the return of a number of adhered cells to the surrounding environment. In active detachment

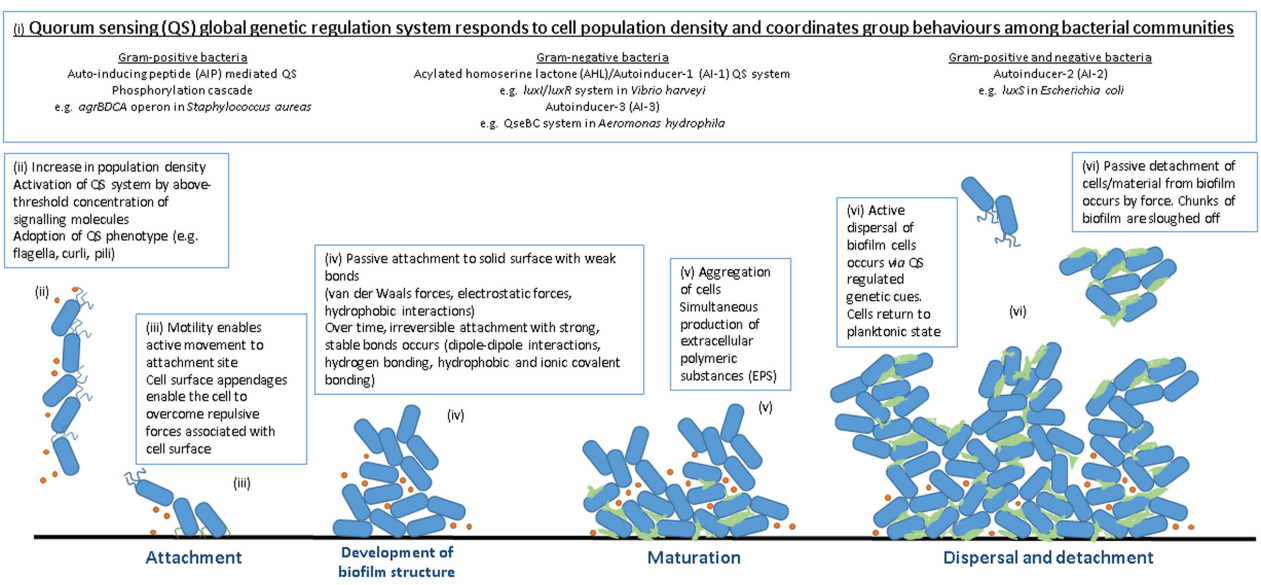

FIGURE 1 | Stages of biofilm formation. (i) QS signaling molecules (ii) high population density, high QS signal (iii) attachment to solid surface (iv) increase in cell numbers, irreversible attachment, development of biofilm structure (v) biofilm maturation and EPS production (vi) dispersal.

cells revert back to their planktonic state and leave the biofilm in response to cellular cues (encouraging them to search for an additional attachment site when conditions are favorable). Passive detachment occurs as a result of environmental changes, such as nutrient availability and movement of surrounding liquid, and involves the sloughing off or erosion of parts of the biofilm by chemical means or force (Kaplan, 2010). Dispersal (reviewed by McDougald et al., 2012) facilitates the spreading of bacterial contaminants and the spoilage of foodstuffs by allowing the biofilm to act as a reservoir releasing cells back into the environment to carry out the cycle elsewhere.

Biofilms in nature and, indeed, in the food industry generally consist of multiple bacterial species as opposed to the mono-species biofilms usually cultured in laboratory studies (Yang et al., 2011). Life in a multispecies biofilm is advantageous, providing increased shelter and resistance to antimicrobials compared to corresponding single species biofilms (Burmølle et al., 2006). A study by van der Veen and Abee (2011) demonstrated that mixed species biofilms containing two *L. monocytogenes* strains and a *Lactobacillus plantarum* strain displayed increased resistance to the commonly used disinfectants benzalkonium chloride and peracetic acid in comparison to disinfection carried out on monospecies biofilms formed by the same strains. Wang et al. (2013) investigated the biocidal effect of the commercial sanitizer Vanquish (a quaternary ammonium compound-based product) and a chlorine solution prepared from Clorox (a germicidal bleach product) on mono- and multispecies biofilms formed by several Shiga toxin-producing *Escherichia coli* O157:H7 and *S. enterica* serovar Typhimurium strains. Increased resistance to sanitizers was observed in multispecies biofilms in which one of the strains was an EPS producer. EPS-producing strains of one species conferred protection to non EPS-producing strains of another, ultimately protecting both (to some degree) from sanitation. The results suggest the importance of the EPS component of bacterial biofilms in conveying resistance to the producer and in this case to the companion strains of mixed biofilms.

Cells that form biofilms have unique properties that enable them to do so, the expression of which is under the control of a global gene regulation system that responds to fluctuations in population density, known as quorum sensing (QS; Fuqua et al., 1994). Specific signaling molecules are produced and detected, governing community behavior. The higher the population density is, the higher the concentration of signaling peptides reached. When a minimal threshold stimulatory concentration of signaling molecules is reached, the QS system is activated and, thus, expression of QS-related genes occurs. QS is responsible for organizing the expression of many genes including those involved in essential cell processes, those encoding various virulence factors and also genes regulating biofilm formation. QS may be organized into three main sub-systems, classified by the type of signaling molecules employed: the acyl homoserine lactone (AHL) or autoinducer-I (AI-I) system is observed in Gram-negative bacteria, the peptide-mediated QS system in Gram-positive bacteria and the autoinducer-2 (AI-2) system present in both Gram-negative and Gram-positive bacteria.

Acyl homoserine lactones were originally discovered in marine bacteria (*Vibrio* spp.) having been found to be responsible for bioluminescence regulation, and have since been identified in numerous Gram-negative bacteria. Synthesis of an AHL signaling molecule involving a LuxI type protein occurs when an acyl-carrier protein-bound fatty acyl derivative is transferred to the amino group of *S*-adenosyl-methionine (SAM; Brackman and Coenye, 2015). AHL-mediated QS is well-described by Waters and Bassler (2005) using the control of the *Vibrio fischeri* luciferase operon as an example. Different bacteria produce different types of AHLs, controlling a range of functions. In addition, the same AHL may be produced by a number of bacteria spanning several genera. All AHLs contain the same homoserine lactone moiety but differ in the length and structure of their acyl groups. The diversity and specificity of AHL molecules, conveyed by the length, backbone and saturation of their fatty acyl side chains suggests their function in intraspecies communication. These N-acylated side chains vary in length from 4 (e.g., C4-HSL) to 18 carbons often with an oxo (e.g., 3-oxo-C6-HSL) or hydroxyl group (e.g., 3-hydroxyl-C6-HSL) on their third carbon atom and may also contain double bonds (Skandamis and Nychas, 2012). A huge variety of AHLs exists and has been reported in a wide range of bacterial species, including microorganisms associated with food and food processing. For example the common milk contaminant *Pseudomonas fluorescens* produces both C4-HSL and 3-oxo-C8-HSL AHL signaling molecules (Liu et al., 2007). *Hafnia alvei*, which is often isolated from cheese, produces the AHL *N*-3-oxohexanoyl HSL (Bruhn et al., 2004). In fact, AHL production by food-dwelling species has been associated with food spoilage. The detection of AHLs in some spoiled foods has led to suggestions that the secretion of certain proteolytic, saccharolytic and lipolytic enzymes, associated with food spoilage, is under the influence of AHL signaling (reviewed by Bai and Rai, 2011; Skandamis and Nychas, 2012).

It has been proposed that the AI-2 signaling system is used for both inter and intraspecies bacterial communication as AI-2 signaling molecules are non-specific. This system was first identified in *Vibrio harveyi*, an AHL-deficient strain which was capable of producing the bacterium's characteristic bioluminescence suggesting that another regulatory system was responsible for controlling its operation (Bassler et al., 1993). AI-2 synthesis involves two major enzymatic steps (Brackman and Coenye, 2015): 5′ methylthioadenosine nucleosidase (MTAN which is encoded by *pfs*) is produced and cleaves adenine from *S*-adenosyl-homocysteine (SAH). This results in the production of *S*-ribosyl-homocysteine (SRH), which is subsequently cleaved by LuxS to form 4, 5-dihydroxy-2, 3-pentanedione (DPD). Spontaneous rearrangements and modifications of DPD yield a combination of molecules collectively referred to as AI-2. The presence of *luxS*, and thus AI-2 mediated QS, has been reported in some foodborne pathogens. Reeser et al. (2007) showed that AI-2 is critical for mature biofilm formation in *C. jejuni* M129 through the construction of a *luxS* deficient mutant. This strain, unable to produce the QS signaling molecule AI-2, was seen to have greatly decreased biofilm formation at the 48 and 72 h time points when compared to the wild type of the same strain, despite both having a similar growth rate.

The group also showed that flagella are important for biofilm formation to the strain at hand by constructing a *flaAb* mutant, which also showed reduced biofilm formation at the 48 and 72 h time points and again no changes in growth rate. AI-2-like activity has also been reported in *L. monocytogenes* and deletion of the *luxS* gene resulted in the bacterium forming thicker than normal biofilm, indicating a strong link between AI-2 signaling and biofilm regulation in *L. monocytogenes* (Sela et al., 2006). More recently, the relationship between *luxS* and biofilm formation was demonstrated in *E. coli* by Niu et al. (2013) by comparing the biofilm forming abilities of a modified set of *E. coli* W3110 (a laboratory strain) with the wild type. The set included a *luxS* deficient mutant, a *luxS* mutant carrying an inducible plasmid containing *luxS* complement and a *luxS* mutant hosting a blank pBAD18 plasmid as a negative control. AI-2 production, quantified by measuring bioluminescence induced in the reporter strain *V. harveyi* BB170, was observed to be higher in the *luxS* complement strain than the wild type and absent in both the *luxS* mutant and the negative control. Following on from this, biofilm formation in a continuous flow cell was assessed by differential interference contrast (DIC) light microscopy and confocal laser scanning microscopy (CLSM) for each strain. While the *luxS* mutant and the negative control were found to form compact clusters, the *luxS* complement formed tall, thick biofilms and the wild type a combination of the observed phenotypes. The results indicate a strong correlation between AI-2 expression and quality of biofilm, suggesting the key role of AI-2 mediated QS in biofilm formation in *E. coli* W3110. As well as the Gram-negative microbes mentioned above, *luxS* has also been studied in Gram-positive bacteria. *Bacillus subtilis*, a spoilage bacterium regularly isolated from dairy products and processing facilities (reviewed by Gopal et al., 2015) was reported to regulate biofilm formation through *luxS*-mediated quorum sensing (Duanis-Assaf et al., 2015).

The presence of a third autoinducing molecule (AI-3) has been reported in Gram-negative bacteria such as *E. coli, Klebsiella pneumoniae, Shigella* spp., *Salmonella* spp., and *Enterobacter cloacae* (Walters et al., 2006). Sperandio et al. (2003) first described AI-3 when studying gene expression of the foodborne pathogen *E. coli* O157:H7 in response to a eukaryotic cell signal. The group found AI-3 (presumed to be LuxS-dependent) to be responsible for the activation of virulence gene expression, including flagella regulation genes, and proposed AI-3 as a possible agent of cross-communication between bacterial and host cells as substitution of either AI-3 or the mammalian hormone epinephrine (Epi) restored the virulence phenotype in a *luxS* deficient mutant, suggesting that AI-3 and Epi employ the same signaling pathway. A later study by Walters et al. (2006) showed that *luxS* mutants were forced to synthesize homocysteine *via* an alternative pathway using oxaloacetate and that culturing the mutants in media supplemented with L-aspartate alleviated the demand for oxaloacetate and restored AI-3 production without affecting AI-2 production. This work demonstrates that AI-3 production is not LuxS-dependent and the true mechanism for synthesis of this molecule is yet unclear (reviewed by Bai and Rai, 2011).

In Gram-positive bacteria, QS communication is mediated by autoinducing peptides (AIPs; Bai and Rai, 2011). Bacteria employing this system do so with unique, species-specific signaling molecules, suggesting that peptide-mediated signaling enables intraspecies communication alone. The biphasic mode of infection employed by *Staphylococcus aureus* is an elegant example of QS signaling in Gram-positive bacteria, reviewed by Waters and Bassler (2005). Examples of bacteria employing QS peptide signaling are the opportunistic foodborne pathogen *Clostridium perfringens*, for the regulation of virulence, sporulation, toxin production (Ma et al., 2015) and biofilm formation (Vidal et al., 2015), and *L. monocytogenes* for virulence, invasion and biofilm regulation (Riedel et al., 2009; Abee et al., 2011).

STRATEGIES UNDERTAKEN TO PREVENT BIOFILM FORMATION AND REMOVE EXISTING BIOFILMS

The best strategy to eradicate bacterial biofilms from food-related environments is to prevent their formation. This can be achieved by preventing the presence of biofilm forming bacteria in critical areas, e.g., sterile manufacture (aseptic processing) or terminal sterilization of parenteral preparations and equipment. In most cases, especially in food production, sterility of the environment is neither possible nor cost-effective and so measures are taken to instead reduce the numbers of harmful and biofilm-forming bacteria in the production area. In food production facilities, detailed hygiene practices are carried out by trained staff in an effort to prevent the introduction of microbes into the processing and finishing areas. Daily sanitation/disinfection processes are carried out in every food manufacturing plant to eliminate microbes that have made it inside and aim to prevent colonization or persistence. The measures involved incorporate mechanical, chemical, and thermal processes to prevent biofilm formation as efficiently as possible.

Cleaning and Disinfection

Measures such as Good Manufacturing Practice (GMP) and Hazard Analysis Critical Control Point (HACCP) schemes (Sharma and Anand, 2002) are active in food processing facilities to ensure that food quality and safety meet high standards. Documented and validated cleaning procedures exist and their implementation is legally enforced *via* inspection by regulatory bodies. A general cleaning procedure for food processing and production areas involves six necessary sequential steps: pre-clean (physical), washing (detergents), rinsing, sanitation, final rinsing, and drying (Safefood, 2012). The first of these is a preparatory measure known as a gross (or dry) clean, the aim of which is to manually remove all bulk soil, packaging materials and tools, essentially all unnecessary equipment and large debris. Equipment to be manually cleaned must also be disassembled and laid out for ease of access during the subsequent steps. In dairy manufacturing plants (DMPs), and others, a control protocol known as Clean-In-Place (CIP) is implemented to reduce biofilm formation and microbial load in general (Bremer et al., 2006).

CIP is a semi- or fully automated programmed cycle of timed rinsing and cleaning stages for the efficient cleaning of equipment interiors that are inaccessible or their manual cleaning ineffectual. Next, a pre-rinse is carried out during which the equipment and area is rinsed with water until surfaces are visibly clear of soils and deposits. Higher water pressure may be used for removal of stubborn soils though care must be taken not to cause cross-contamination through splash-back or migration of aerosolized water onto other surfaces. Following this step, excess water must be removed to avoid pooling around or backing up of drains and to prevent dilution of the cleaning solutions/solvents used in later steps. The next step involves the application of a detergent to remove remaining food deposits such as proteins and grease, layers in which bacteria can survive and re-enter the system post-cleaning. Detergents may be applied in the form of foam or aerosol spray, at an appropriate concentration, and adequate contact time with surfaces must be allowed to ensure efficient action. Alkaline and acidic products are commonly used detergents in the food industry (Simões et al., 2010) with alkalis showing success in the removal of *Pseudomonas putida* biofilms from stainless steel (Antoniouand and Frank, 2005). In the following step of the cleaning protocol, detergent and lifted food deposits are removed from the area through rinsing with water at the lowest effective pressure. The surfaces should be visibly clean and free of layers of soil and any marks or residues left by the detergent. Again, excess water is evacuated. At this stage, disinfection is performed to reduce microbial load. Disinfectants may be applied as a liquid spray directly to surfaces or as a fine mist *via* aerial fogging to target airborne microorganisms, which then also settles on and disinfects surfaces. The ambient temperature and the contact time between the disinfectant solution and the surface should be factored into the procedure to maximize the biocidal effect. Some commonly used disinfectants that have demonstrated competence in reducing biofilms in the food industry include hydrogen peroxide (H_2O_2), sodium hypochlorite (NaClO), which is also an effective sanitizer, ozone, and peracetic acid (Srey et al., 2013). Toté et al. (2010) found H_2O_2 and NaClO to be effective in the removal of *S. aureus* and *P. aeruginosa* biofilm cells and EPS matrix from 96-well assay plates. It has been demonstrated that ozone and especially H_2O_2 are effective at inhibiting *Vibrio* spp. biofilms associated with seawater distribution networks used in fish-processing plants (Shikongo-Nambabi et al., 2010) and also that peracetic acid is active against *L. monocytogenes* biofilms (Cabeça et al., 2012). Although sanitizers, which possess the combined action of both detergents and disinfectants, are used in some cleaning protocols, it is believed that splitting these steps and introducing an intermediate rinsing step is more effective than sanitizing alone. Even so, sanitizers remain in use and sanitizing compounds such as NaClO and Spartec, a quaternary ammonium compound (QAC), have been found to be effective against *B. cereus* biofilms when applied under specific cleaning protocols (Peng et al., 2002). The next stage in the cleaning process is the rinsing away of the disinfectant. Most disinfectants are safe to leave on surfaces that do not have direct contact with food, however water of a high quality is used to rinse food contact surfaces and in some cases non-contact surfaces as well. Finally, the equipment is dried to remove rinsing water. Although regular application of cleaning agents reduces microbial populations (Jahid and Ha, 2012), it is normally not efficient at removing mature biofilms. Cleaning and disinfection can remove unwanted bacteria before they have a chance to attach to a surface and form a biofilm, however, due to the fast rate at which attachment and biofilm formation occurs, they are not completely efficient at preventing contamination of food processing environments. In addition, due to residual soil and previous biofilm matrix present on surfaces, sanitation may not be effective alone and the use of disinfectants may select for resistant bacteria (Simões et al., 2010). Interestingly, bacteria residing in biofilm matrices are remarkably (100–1000 times) more resistant to cleaning and sanitation processes than planktonic cells (Gilbert et al., 2002) and it is noteworthy that the majority of chemical disinfectants that are commonly implemented in food, industrial, clinical and domestic cleaning procedures are based on bactericidal studies performed on planktonic cells (Annonymous, 1997). The reasons for increased resistance of bacteria in biofilms are not yet fully understood but the phenomenon has been well-documented (Nickel et al., 1985; Luppens et al., 2002).

Processing Equipment Materials and Design

Facility design and staff training is highly important for minimizing cross-contamination between high risk and low-risk areas within the plant that can be caused by unchecked foot traffic between stations. Zone establishment segregating exposed product areas from packaging areas, the limiting of access to high-risk areas to authorized personnel and strict garbing and hand-washing requirements on entering restricted areas all play a role in maintaining hygiene standards. Cross departmental knowledge and awareness of potential consequences of contamination ensures compliance and lessens the likelihood of accidental breach of policy. Included in facility design is the selection of appropriate materials for use in the processing areas. Materials for the design of food processing and manufacturing equipment are selected based on a number of factors, most importantly ease of cleaning for reduction of contamination and associated risks. Materials should also be reasonably resistant to chemical and age-related corrosion for maintenance of a smooth and easy-to-clean surface and to prevent contamination risks and downtime associated with frequent replacement of damaged/corroded equipment. Surface topography is important as microorganisms may attach or find shelter in cracks, scratches, and corners of equipment making them extremely difficult to remove (Bremer et al., 2006). Inert metals are commonly used in the food industry, especially stainless steel and aluminum. Stainless steels contain alloys such as chromium to increase resistance to corrosion (rusting). Type 316 steel is especially resistant to chloride environments and is more costly than type 304 steel which is more commonly used due to its versatility and ease of forming. The smooth surface finishes that are achieved by rolling and polishing steel make it a very valuable material for the production of food processing equipment. Another commonly employed metal is

aluminum, a light weight and economical material which is also highly resistant to corrosion, especially from acids. Aluminum, however, is susceptible to scratching and damage due to a low surface hardness and to corrosion by alkalis, traits which allow the smooth surface to be compromised, increasing the risk of contamination. In milk processing facilities equipment is required to be resistant to corrosion in alkaline and/or acidic conditions (Marchand et al., 2012) and so stainless steel is normally used. Non-metal materials are employed for moving and disposable equipment such as conveyor belts, containers and cutting boards and for components and attachments where soft material is required such as for seals, gaskets, membranes, and piping. These materials are most commonly elastomers (rubbers) such as ethylene propylene diene monomer rubber (EPDM), nitril butyl rubber (NBR, aka Buna-N®), silicon rubber or fluoroelastomer (Viton) and plastics such as polypropylene (PP), polycarbonate (PC), high-density polyethylene (HDPE), unplasticized polyvinyl chloride (PVC), and fluoropolymers such as polytetrafluoroethylene (PTFE aka Teflon®; Faille and Carpentier, 2009; Marchand et al., 2012). Unfortunately, certain bacteria are capable of forming biofilms on these food-approved materials. This attachment is aided by improper cleaning of such materials as soil or debris remaining post-sanitation may form a conditioning film for subsequent attachment of planktonic bacteria to this site (Marchand et al., 2012). Surface preconditioning using surfactants that modify the chemical properties of surfaces have been used to prevent bacteria from attaching (Simões et al., 2010). Indeed, more than 90% inhibition of *P. aeruginosa* adhesion to stainless steel and glass was reported by Cloete and Jacobs (2001) upon treating the surfaces with ionic and anionic surfactants. Biosurfactants, microbial compounds that act as surfactants, may also be employed to reduce or prevent adhesion of problematic biofilm-forming bacteria (Banat et al., 2010). Zezzi do Valle Gomes and Nitschke (2012) investigated the efficacy of biosurfactants such as surfactin from *B. subtilis* and rhamnolipids from *P. aeruginosa* in reducing the adhesion and disrupting the pre-formed biofilms of the pathogenic food-associated bacteria *L. monocytogenes*, *S. aureus,* and *Salmonella* Enteritidis. The biosurfactants studied were effective in the disruption of biofilms formed on polystyrene microplates by all species individually and in the disruption of a multispecies biofilm containing all three. The action of the surfactants in preventing bacterial adhesion was effective against pure culture biofilms of each species. However, they were shown to have reduced impact in preventing adhesion of the mixed bacterial culture to the plates, again highlighting the advantages bestowed to bacteria residing in a multispecies habitat. Gu et al. (2016) reported the effective removal of established *P. aeruginosa* PAO1, *S. aureus* ALC2085 and uropathogenic *E. coli* ATCC53505 biofilms formed on an antifouling surface. The group used shape memory polymers (SMPs) -a type of material specially designed to remember a particular shape, manipulated into keeping a temporary shape and then coaxed back into its original form by external activation- as an attachment surface for the microbes to form biofilm and, upon triggering of SMP shape change, the amounts of adhered cells were dramatically reduced (99.9% in the case of *P. aeruginosa*). This type of study takes anti-biofilm surface topography research to a new level, achieving the physical displacement of established biofilms with minimal (if any) effect on the surrounding environment using biocompatible materials and may in time be applicable to equipment and facility design in the food industry.

Processing Conditions

Another approach to prevent biofilm formation of bacteria present in the production environment involves carrying out the process under conditions unfavorable to biofilm formation. Temperature appears to influence bacterial attachment to solid surfaces. Cappello and Guglielmino (2006) studied the adhesion of *P. aeruginosa* ATCC 27853 to polystyrene plates at 15, 30, and 47°C, reporting a dramatic difference in adhesive ability (measured as percentage hydrophobicity) between cells cultured at the higher temperatures of 30 and 47°C and cells cultured at 15°C. Temperature-dependent variation in biofilm formation was also observed among *L. monocytogenes* strains by Di Bonaventura et al. (2008). In addition to temperature, nutrient availability in a given environment has been shown to influence the quality of biofilms formed. In general, studies have demonstrated that biofilms formed under low nutrient availability or starvation conditions are superior to biofilms formed under high nutrient availability, with bacteria in nutrient rich surroundings failing to form biofilms in some cases (Petrova and Sauer, 2012). Zhou et al. (2012) reported enhanced (thicker and more complex) biofilm formation of *L. monocytogenes* in a poor minimal essential medium (MEM) supplemented with glucose compared to the biofilm formed by the same strain in nutrient rich brain heart infusion (BHI) broth. Similar results were seen previously by Dewanti and Wong (1995) who cultured *E. coli* 0157:H7 biofilms on stainless steel chips in broths of varying nutrient availability. The group reported the formation of biofilms with high cell numbers that formed quickly and produced thicker EPS when grown in nutrient-scarce media in comparison to those formed in tryptic soy broth (TSB). Biofilm formation may also be altered by the pH of the surrounding media. Decreased cell attachment was reported (Tresse et al., 2006) for *L. monocytogenes* biofilms grown at pH 5 than for those at pH 7, which was later (Tresse et al., 2009) attributed to pH-dependent flagellation in *L. monocytogenes* observed as a down-regulation of flagellin synthesis in acidic conditions. O'Leary et al. (2015) investigated the effect of low pH on the biofilm forming capacity of four acid-adapted *S.* Typhimurium DT104 strains, only one of which formed biofilms at both pH 5 and 7, with the remaining three strains unable to form stable biofilms at the mildly acidic pH of 5. Gene expression under the distinct pH conditions was also examined showing that genes involved in biofilm formation were expressed at higher levels at pH 5 than at neutral pH for all isolates, despite the lack of biofilm formation observed in three out of four strains. These results propose the existence of a separate set of genes which aid biofilm formation under acidic conditions and which were not present in three of the strains at hand. Despite the successes of biofilm-limiting conditions in laboratory experiments, in most cases, application of these findings to the food industry is not appropriate as altering process conditions is likely to impact product quality.

Physical Approaches

Physical force is also employed in the food industry for the reduction of microbial load and the removal of biofilms. Brushes, water jets, and turbulent flow in pipelines are used to administer force to susceptible surfaces during cleaning protocols (Safefood, 2012). In addition, in recent years, other physical-based novel technologies have been developed to reduce the microbial load on surfaces or remove biofilms. Plasma treatment involves bombarding surfaces with a partially ionized gas and has been used successfully as a disinfectant targeting planktonic microbes (Laroussi, 1996). A study carried out by Vandervoort and Brelles-Mariño (2014) demonstrated the efficacy of plasma-mediated inactivation against a *P. aeruginosa* biofilm grown on borosilicate glass in continuous culture, better to mimic natural and industrial environmental conditions under which problematic biofilms are generally formed. The group reported changes in biofilm structure post-plasma treatment which they associated with decreased adhesion of the biofilm to the colonized surface. Ultrasonication was found to be successful for the removal of biofilms when used in combination with other anti-biofilm agents such as antibiotics (Peterson and Pitt, 2000), ozone (Baumann et al., 2009) and the chelating agent ethylenediaminetetraacetic acid (EDTA) (Oulahal et al., 2007), reviewed by Srey et al. (2013). A greater understanding of the intricacies of a biofilm (species involved, structure, composition of EPS, etc.) leads to improved, more focused efforts to remove existing biofilms and prevention of biofilm formation of studied species. Manual removal of cells from a biofilm and simple analysis by cell plating followed by microscopic analysis of fluorescently labeled or stained lab-grown biofilms (cultured in high throughput matrices such as 96-well plates or glass/stainless steel coupons) provides detailed information on both the microbes involved and on biofilm architecture. Quantification of live cells (e.g., MTT staining) or biofilm formed (crystal violet staining) may be carried out on cultured biofilms to quantify total biomass, assess external factors and environmental conditions affecting biofilm formation and to evaluate the success of biofilm removal and inhibition strategies, as reviewed by Stiefel et al. (2016). Additionally, polymerase chain reaction (PCR)-based methods allow for rapid detection of pathogens and spoilage bacteria from a biofilm sample, as reviewed by Winkelströter et al. (2014b). Dzieciol et al. (2016) used culture independent methods (pyrosequencing of 16S rRNA gene amplicons) to characterize the microbial communities of floor drain water from four sources in a cheese processing facility for the purpose of monitoring *L. monocytogenes* persistence. Other useful technologies include biofilm detectors which are used to monitor biofilm formation on a surface and can enable intervention in the early stages of biofilm formation in an attempt to prevent its progression into a mature biofilm. Pereira et al. (2008) developed a surface sensor capable of detecting early biofilms, and further developed the technology to monitor cleaning-in-place procedures (Pereira et al., 2009). Al-Adawi et al. (2016) employed CLSM and denaturing gradient gel electrophoresis (DGGE) to study mono and dual species biofilm formation of food-related pathogens on stainless steel and raw chicken meat and the transfer of microbial cells from the abiotic to the biotic surface. As biofilms contribute hugely to cross contamination between equipment in the food industry and the products themselves, such studies are critical in developing novel and appropriate techniques for detecting and analyzing biofilms.

The majority of current strategies aim to prevent introduction of microbes into the food processing environment, contributing also to reduce the risk of biofilm formation through removal of soils and food deposits on processing equipment as improperly cleaned surfaces with soil build-up serve as attachment sites for biofilm forming bacteria. However, most of these approaches do little to remove existing biofilms formed by persistent bacteria within production areas, for example biofilms in milk tanks and tubing that are heat tolerant or thermophilic and are resistant to the high temperatures of pasteurization. Periodic cleaning of equipment requires halting production, drainage and cleaning which negatively impacts output and is not ideal in terms of hygiene.

Enzymes

Enzyme-based detergents are used to improve efficacy of disinfectants against bacterial biofilms. Enzymes can target cells in the biofilm matrix and can cause the matrix to become looser and break up. They can also trigger cell release actions in the biofilm enveloped cells, causing an amount of cells to break off from the biofilm. Enzymes have some role in targeting the bacterial cells encased within a biofilm, however the main function of enzymes is to degrade the lipid, carbohydrate and DNA components of the extracellular matrix, severing the links between cells and subsequently separating them, allowing rapid deterioration of the biofilm integrity (see **Figure 2A**). Disinfectants can then act more powerfully to kill cells that were once embedded in the matrix of the biofilm EPS and can also target released cells which have been forced into the planktonic state by the enzymes action. The types of enzymes commonly employed depend on the composition of the biofilm one is attempting to eradicate and include proteases, cellulases, polysaccharide depolymerases, alginate lyases, dispersin B and DNAses (Bridier et al., 2015). As EPS is a heterogenic matrix, a combination of enzymes with different target substrates is used, and even further tweaking of the mixture is required for multispecies biofilms where there exists a variety of substrates. A study by Walker et al. (2007) demonstrated the success of an enzyme mix against a multispecies biofilm formed on brewery dispense equipment. Additional studies have been carried out which highlight the potency of enzyme-based approaches against food related bacterial biofilms. Mimicking a meat processing environment, Wang et al. (2016) induced biofilm formation by a cocktail of seven *Salmonella* spp. strains isolated from meat processing surfaces and poultry grown in meat thawing-loss broth (MTLB) and on stainless steel. They reported the successful removal of said biofilm through treatment with cellulase followed by immersion in cetyltrimethyl ammonium bromide (CTAB). Oulahal-Lagsir et al. (2003) reported a 61–96% removal of *E. coli* biofilms formed on stainless steel in milk when they synergistically exposed the biofilms to both proteolytic and glycolytic enzymes and ultrasonic waves for 10 s. The action of polysaccharidases against *P. fluorescens* biofilms and the efficacy of serine proteases in the removal

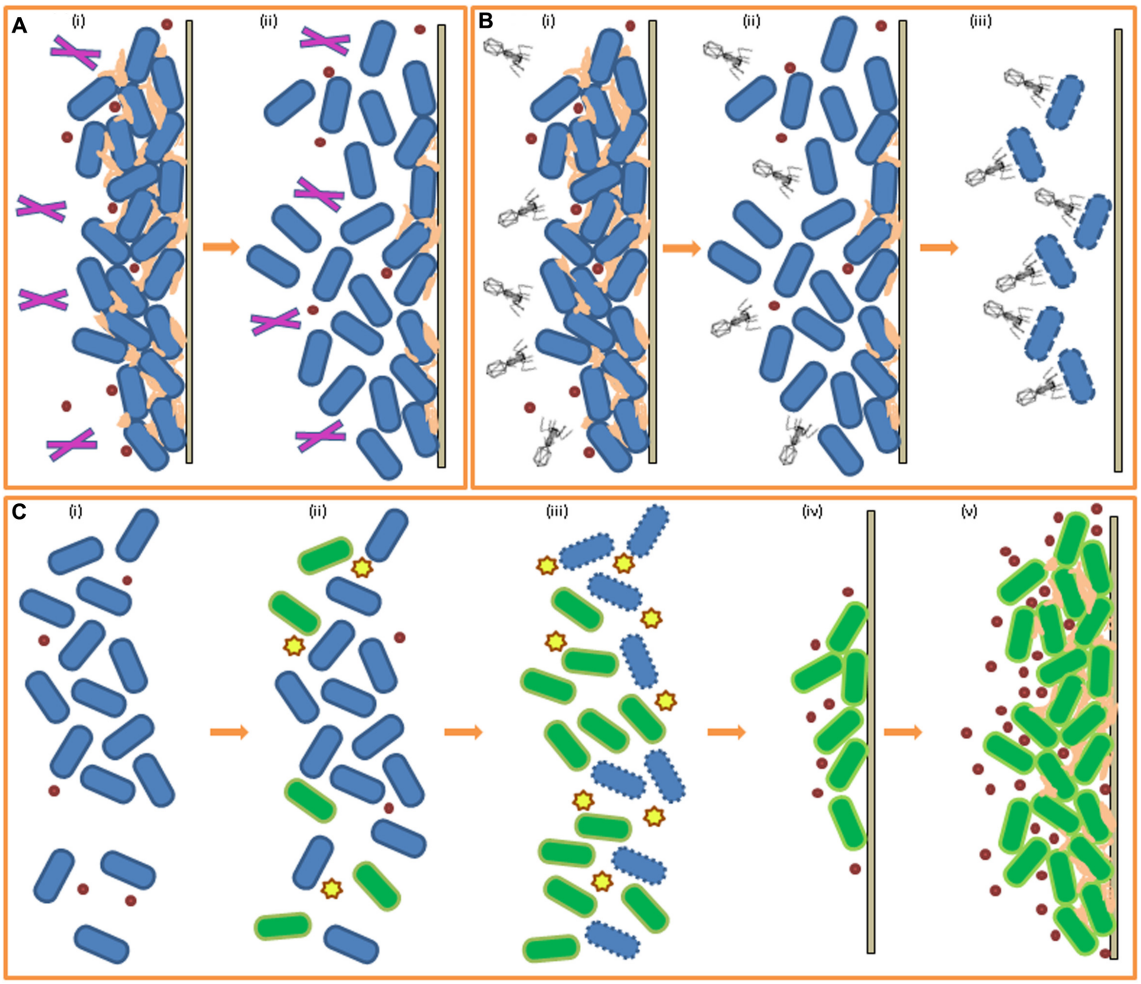

FIGURE 2 | Biofilm control through enzymes, phage, and bacteriocins. (A) Effect of enzymes on pre-existing biofilm (i) biofilm formed, EPS production, addition of enzymes (ii) breakdown of EPS and biofilm reduction by enzymatic action. **(B)** Effect of bacteriophage on pre-existing biofilm (i) biofilm formed, EPS production, addition of phage (ii) degradation of EPS by phage, reduction of biofilm (iii) bacterial cells in biofilm targeted by targeted for infection by phage. **(C)** Effect of bacteriocins and competitive exclusion on biofilm-forming cells (i) planktonic cells of species A (blue) (ii) addition of bacteriocin-producing species B (green) (iii) targeting of species A by bacteriocins, increase in number of species B cells (iv) increase in QS molecule concentration for species B, attachment to solid surface (v) biofilm formation of species B in place of species A.

of *Bacillus* biofilms from stainless steel chips was reported by Lequette et al. (2010). Commercial α-amylases have been found to be effective at both removal and inhibition of *S. aureus* biofilms (Craigen et al., 2011). Another study investigated the potential for commercial proteases and amylases to break down the EPS of biofilms formed by *P. fluorescens* on glass wool (Molobela et al., 2010). The group examined the composition of the EPS and selected appropriate enzymes, which were evaluated as anti-biofilm agents. As the EPS in this case consisted predominantly of proteins, commercial proteases were found to be most effective at biofilm removal in this study. Enzymes sourced from fungal strains were also shown to be successful at removal of biofilms formed by *P. fluorescens* on glass coupons (Orgaz et al., 2006). When employing enzyme-based products, one must consider the reaction of enzymes with food products or ingredients during processing, for example, Augustin et al. (2004) found several commercial enzymes to be useful as cleaning products against biofilms of common dairy-associated spoilage bacterium *P. aeruginosa*. However, the activity of proteinase enzymes is reduced in the presence of milk and so the performance of the enzyme was not sufficient to encourage further development of a product. DNases, which degrade the extracellular DNA component of EPS, have also been studied as enzyme-based formulations for battling biofilms. Extracellular DNA is a crucial component of the bacterially produced EPS constituting the biofilm matrix, with species-dependent roles in cell aggregation and intercellular connection, maintenance of the structure of the biofilm, and as an adhesive with some antimicrobial properties (reviewed by Flemming and Wingender, 2010). Brown et al. (2015) showed that the exogenous addition of DNase I led to rapid degradation of extracellular DNA and removal of a *C. jejuni* biofilm attached

to stainless steel (to mimic a food processing environment). *C. jejuni* is capable of both formation of *de novo* biofilms as well as integration into existing biofilms occupied by other species in food related environments (Teh et al., 2014). The use of DNase I in this study against a *C. jejuni* biofilm was successful in both swift removal of the biofilm from its attached surface and in prevention of reattachment and *de novo* synthesis of a new biofilm for up to 48 h on a DNase I treated surface. Kim et al. (2017) showed that DNase I significantly inhibited the biofilm forming capabilities of one *C. jejuni* and three *Campylobacter coli* strains when added at the beginning of biofilm formation and also disrupted 72 h old mature biofilms of these strains, isolated from commercially bought raw chickens. This study further contributes to the assumption that extracellular DNA plays a key role in *Campylobacter* biofilm formation, highlighting DNase I as a promising candidate for the control of *Campylobacter* biofilms. Zetzmann et al. (2015) reported the formation of DNase I-sensitive biofilms by *L. monocytogenes* EGD-e at low ionic strength, conditions which are commonplace in food processing. DNase I was also found to be effective against biofilm formation in a study carried out by Harmsen et al. (2010) in which its employment inhibited initial attachment of *L. monocytogenes* cultures to glass and delayed biofilm formation in polystyrene microtiter plates.

Bacteriophage

Bacteriophage are bacteria's natural enemies and so have potential for use against pathogenic and spoilage bacteria in food (reviewed by Endersen et al., 2014). Phage offer special promise when it comes to eradicating biofilms as they are capable of penetrating the matrix and diffusing through the mature biofilm and, once inside, express their antibacterial properties (Briandet et al., 2008; Donlan, 2009), as illustrated in **Figure 2B**. Work has also been carried out against biofilms with both natural and engineered phage (reviewed in Simões et al., 2010). Phage are extremely specific to their bacterial host and this specificity is important for use in control of undesirable bacterial species in foods as beneficial bacteria are often used in food production, especially starter cultures in fermented foods, in which cases the preservation of the beneficial bacteria is essential for finished product quality (Guenther et al., 2009). Lytic phage are better suited to biocontrol purposes as, unlike lysogenic phage, they engage the lytic pathway to the detriment of the bacterial cell. LISTEX^{TM} is a commercial product developed from the bacteriophage P100 which induces cell lysis and disintegration of the EPS by enzymatic action. It is a natural and non-toxic phage product active against *L. monocytogenes* and is recognized in the USA by the United States Department of Agriculture (USDA) for use in all food products (Listex, 2006). Soni and Nannapaneni (2010) treated 21 *L. monocytogenes* strains, which had formed biofilms on stainless steel coupons, with bacteriophage P100 and reported a significant reduction in cell numbers of the listerial biofilms. Lytic phage φ S1 was shown to be effective against early stage biofilms of *P. fluorescens* (Sillankorva et al., 2004). The biofilms were 5 days old when treated with the bacteriophage φ S1 and this resulted in an 80% removal of the biofilm (under optimal conditions). Another study demonstrated the efficacy of phage K plus six derivatives in the removal and prevention of *S. aureus* biofilms in microtitre plates (Kelly et al., 2012). $CHAP_K$, a peptidase derived from the phage K, successfully disrupted and eliminated staphylococcal biofilms on microtitre assay plates within 4 h (Fenton et al., 2013). In a study by Lu and Collins (2007), *E. coli*-specific bacteriophage T7 was engineered to express intracellularly a biofilm-degrading enzyme, dispersin B, which targets an adhesin required for biofilm formation by *E. coli* and *Staphylococcus* spp. during infection, so that when added to the culture medium the phage was able to simultaneously attack the bacterial cells in the biofilm (as phage do) and also able to penetrate the biofilm matrix through degradation of EPS. The group demonstrated that the approach involving the engineered phage was markedly more efficient at biofilm disruption than the use of a non-engineered phage. Building on this work, enzymatic phage designed with multiple EPS targets could greater improve efficiency of this technique.

Bacteriocins

Ribosomally synthesized antimicrobial peptides secreted by bacteria, known as bacteriocins, or the bacteriocin-producing strains themselves, may be added to culture media to impede initial cell adhesion and biofilm formation of certain susceptible bacteria (da Silva and De Martinis, 2013), as illustrated in **Figure 2C**. Nisin, a bacteriocin secreted by *Lactococcus lactis*, is a safe and effective additive for certain food products (Cotter et al., 2005) and a commercialized form, Nisaplin®, is produced by Dupont (formerly Danisco). Nisin A, produced by a *L. lactis* UQ2 isolated from Mexican style cheese, was investigated for its activity against *L. monocytogenes* biofilm formation on stainless steel coupons (García-Almendárez et al., 2008). Both *L. lactis* UQ2 cells and a spray-dried crude bacteriocin fermentate (CBF) of *L. lactis* UQ2 were assessed using fluorescent *in situ* hybridization (FISH) with specific labeled probes to distinguish between cells of both cultures. The study found that a combination of lactic acid and nisin A, both produced by *L. lactis* UQ2, was successful in the restriction of *L. monocytogenes* biofilm formation by competitive exclusion indicated by the observation of reduced numbers of *L. monocytogenes* cells on the steel chips incubated in co-culture with *L. lactis* UQ2 compared to the *Listeria*-only control. In a study by Field et al. (2015), a modified nisin variant with enhanced antimicrobial and anti-biofilm activity against the canine pathogen *Staphylococcus pseudintermedius* was shown to be more effective than the original peptide from which it was derived. The bioengineered bacteriocin was capable of both impairing biofilm formation and reducing pre-existing biofilms of *S. pseudintermedius*. *Lactobacillus sakei* is a bacteriocin producing lactic acid bacteria commonly used in the preservation and fermentation of meat products (Champomier-Verges et al., 2001). *L. monocytogenes* biofilm formation in the presence of an *L. sakei* strain (*L. sakei* 1) and of the cell-free supernatant (CFS) of *L. sakei* 1 containing bacteriocin, sakacin 1, was assessed on stainless steel coupons (Winkelströter et al., 2011). A non-bacteriocin producing *L. sakei*

strain and its bacteriocin-free CFS were also co-cultured with the *L. monocytogenes* biofilms separately as controls. The bacteriocin-producing strain and its CFS were both efficient in the inhibition of the initial steps of biofilm formation as they were observed to decrease the number of adhered cells present on the stainless steel coupons. However, after 48 h of incubation re-growth of adhered listerial cells was observed in the culture containing the sakacin 1-CFS only and so, inhibitory activity cannot safely be attributed to bacteriocin-production alone. The results are still promising indicating that *L. sakei* and its bacteriocin may be beneficial for the inhibition of early biofilm formation by *L. monocytogenes*. In a similar study, Pérez-Ibarreche et al. (2016) investigated the effect of bacteriocin-producing *L. sakei* strain CRL1862 on biofilms formed by *L. monocytogenes* FBUNT (isolated from artisanal sausages) on industrially relevant stainless steel and polytetrafluoroethylene (PTFE) surfaces. This *L. sakei* strain was found to be effective at biofilm inhibition, leading to the suggestion by the authors of the pre-treatment of food processing equipment with the *Lactobacillus* or its bacteriocin as a potential method of preventing *Listeria* adhesion to the surface concerned.

Many bacteriocins are produced by lactic acid bacteria which are commonly employed as starter cultures for the production of various fermented foods (Buckenhüskes, 1993). In addition to the acclaimed safety profile of LAB for use in food production, their metabolism is known to offer sensory improvements to fermented food products (Leroy and De Vuyst, 2004) and the presence of selected strains may also inhibit the growth of some foodborne spoilage and pathogenic bacteria, making LAB a practical addition to food preparations and processing cycles. In a recent study, recombinant lectin-like proteins that were identified by genome mining of probiotic *Lactobacillus rhamnosus* GG and over-expressed in *E. coli* were found to disrupt biofilms formed by *S.* Typhimurium ATCC14028 on polystyrene pegs (Petrova et al., 2016). Although, the authors carried out this study with clinical applications in mind, employing such proteins or the probiotic strain itself to battle *Salmonella* biofilms in the food industry is a plausible ambition. Woo and Ahn (2013) discussed competitive exclusion in the context of probiotic mediated exclusion and displacement against biofilm formation of *L. monocytogenes* and *S.* Typhimurium. From milk tanks and milking equipment in two traditional Algerian farms, a *Lactobacillus pentosus* strain was isolated that had strong activity against the adhesion of *S. aureus* cells to polystyrene and stainless steel (Ait Ouali et al., 2014). Additionally, this *L. pentosus* LB3F2 (among other LABs isolated) formed biofilms on the industrially relevant surfaces tested, highlighting its potential for use in food processing as a beneficial biofilm former capable of inhibiting *S. aureus* by creating a protective barrier on equipment surfaces and/or *via* competitive exclusion of the pathogen. In the cases of competitive exclusion and beneficial bacteria with barrier functions it must be considered nonetheless that there is potential for the protective strain to develop resistance to the sanitizer/disinfectant used in cleaning protocols and there exists the possibility of transference of the resistant plasmid to the spoilage/pathogenic strain that it is protecting against.

Naturally Sourced

Extracts from aromatic plants are being investigated as natural agents against bacterial biofilms (Bridier et al., 2015). They are generally regarded as safe (GRAS) and so are compatible with current regulations regarding food production. Examples include: oregano oil, thymol and carvacrol effective against *Staphylococcus* biofilms (Nostro et al., 2007). *Thymus vulgare* essential oil caused a 90% reduction in AHL production (measured by quantifying violacein production in the AI-1 QS indicator strain *Chromobacterium violaceum* CV026) of *P. fluorescens* KM121 in a 72 h old culture (Myszka et al., 2016). These results were confirmed by liquid chromatography mass spectrometry (LC–MS). The essential oil also strongly inhibited cell adhesion to stainless steel, viewed by fluorescence microcopy and inhibition of adhesion quantified by the scale described by Le Thi et al. (2001). The results showed *P. fluorescens* KM121 first degree adhesion to be dominant on the stainless steel coupons, meaning that on 50 randomly selected visual fields only 0–5 bacterial cells were present post-washing. Extracted from *Euodia ruticarpa* (a plant in the Rutaceae family), the compounds evodiamine and rutaecarpine and a quinolinone fraction were found to reduce biofilm formation of *C. jejuni* NCTC 11168 on stainless steel after 24 h or more (Bezek et al., 2016). In a recent study, *B. subtilis* biofilms formed on polystyrene microtitre plates and stainless steel coupons were treated with 1 and 2% solutions of organic acids (citric, malic, and gallic) isolated from natural sources and additionally chlorine for comparison. Akbas and Cag (2016) reported citric acid as being as effective at biofilm inhibition and disruption as the chlorine standard, results which may encourage exploration of organic acids as a potential natural alternative to chemical substances for *Bacillus* biofilm control. Maderova et al. (2016) employed an unusual method for the control of *P. aeruginosa* biofilms in a water environment by utilizing food waste materials as QS signaling molecule adsorbents. These authors were successful in reporting reduced biofilm formation (without consequence to cell viability) through the addition of spent grain. Magnetic modification of promising food materials, including the grain, allowed for their separation and removal from the water environment. Following the success of this study, the addition (and subsequent removal afterward) of food materials spoiled by 'safe,' food grade microbes to certain food processing arrangements could be a possible avenue of research for biofilm control in the food industry.

Quorum Sensing Inhibitors

Strategies that target quorum sensing and, therefore, biofilm formation (and other virulence factors) as opposed to bactericidal strategies exert less selection pressure to develop resistance to the inhibitory agent. In these instances, bacteria can be 'controlled' in place of being killed. Many organisms produce quorum quenching (QQ) molecules when competing with neighboring species for nutrients, space, etc. QQ refers to the inhibition of QS through degradation and/or inactivation of the QS signaling molecules (Dong et al., 2001). The inability of the susceptible bacterial cell to sense and respond to its population density interferes with various secondary cell functions, usually

diminishing some aspect of virulence. *P. aeruginosa* metabolizes its own AHL signaling molecules by cleaving QS molecules to form a homoserine or a fatty acid which it consumes as carbon and nitrogen sources (Huang et al., 2003). Signaling molecules are also degraded by the producer to maintain appropriate signal concentration and to prevent improper activation of the QS system. *Agrobacterium tumefaciens* degrades its own QS signaling molecules to terminate QS activities by producing the AHL-lactonase AttM while in its stationary phase of growth (Zhang et al., 2002). The concept of QQ as an anti-biofilm tool lies with the addition of the isolated inhibitory molecule (or the producer itself) as a bioagent in the food industry or its formulation into an antibacterial treatment for clinical use against human pathogens (see **Figure 3**). Strategies employed to prevent biofilm formation targeting the QS system are based on inhibition of cell-to-cell communication, which can be executed in a number of ways, including the inhibition of signaling peptides synthesis or the degradation of the peptides, prevention of signaling peptide–receptor binding or inhibition of the signal transduction cascade further down the line (Brackman and Coenye, 2015). Although a great deal of further study is still required to fully understand the relationship between QS and biofilm formation, it is accepted that QS inhibition is a promising strategy to combat bacterial biofilms. Viana et al. (2009) investigated the role of AHLs in biofilm formation by *H. alvei*, a bacterial food contaminant commonly isolated from raw milk (Ercolini et al., 2009) and cheeses (Coton et al., 2012). Despite *H. alvei* being considered to be an opportunistic human pathogen in some nosocomial infections (Rodríguez-Guardado et al., 2005), the bacterium is often added to certain cheeses to improve taste and aid in ripening and so is considered to be a microorganism with beneficial technological properties for use in food fermentation (Bourdichon et al., 2012). Previous studies (Pinto et al., 2007) have established that *H. alvei* is a producer of AHLs and so the group set out to detect the presence of AHLs in a *H. alvei* biofilm with the objective of establishing a link between QS and biofilm formation. On verifying the presence of AHLs in the biofilm, they also demonstrated the inhibition of biofilm formation by synthetic furanones (previously shown by Manefield et al., 2002). It was also established that *H. alvei halI*, an AHL-synthase gene mutant, was deficient in proper biofilm formation, further strengthening the hypothesis that AHL-mediated QS plays a role in biofilm formation by *H. alvei*. In a study carried out by Van Houdt et al. (2004) *in vitro* biofilm formation was characterized in 68 Gram-negative bacterial strains isolated from a raw vegetable processing line. Accompanying assays using reporter bacteria detected the presence of QS signals produced by each strain. Although, five isolates were determined to produce AHLs and AI-2 signals and a further 26 strains were AI-2 producers, a general correlation between the QS signals detected and measurable biofilm formation was not clear for the strains under investigation. Nevertheless, the authors stipulated that the absence of a link between QS and biofilm formation in their study does not dismiss the influence of signaling molecules in other biofilm formers. Another study highlighted the link between QS and biofilm formation in reporting that *P. aeruginosa lasI* mutant strains that were unable to synthesize the AHL 3-oxo-C12-HSL formed atypical biofilms when cultured in a flow cell (Bjarnsholt et al., 2010). The antibiotic azithromycin was used successfully as a QS blocking agent against the AHLs C4-HSL and 3-oxo-C12-HSL in *P. aeruginosa* and in doing so impacted bacterial biofilm formation by reducing cell adhesion to polystyrene surfaces (Favre-Bonte et al., 2003). Tan et al. (2014) carried out a long term study investigating the role of QS signaling molecules in multi-species microbial communities undergoing granulation through incubation of a mixed bacterial culture in a bioreactor used for water treatment. Simultaneously, they assessed the concentration levels of AHL molecules present at different stages of granule formation. The group found that AHL concentration positively correlated with the behavioral steps involved in granulation and that addition of exogenous AHLs to the culture resulted in increased EPS production, suggesting a role for QS signaling in bacterial granule formation. A later study performed by the same group (Tan et al., 2015) demonstrated

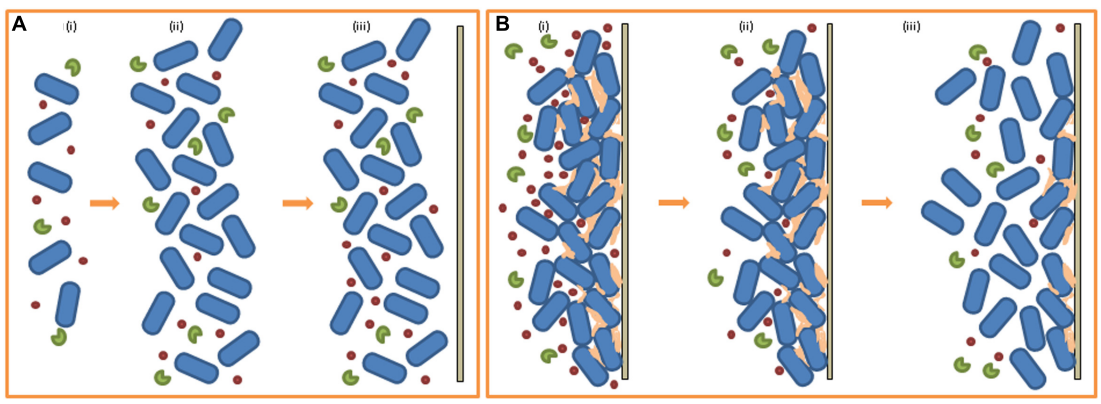

FIGURE 3 | Quorum quenching (QQ) and biofilm formation. (A) Effect of QQ molecules on early stage biofilm formation (i) low population density, low QS signal, addition of QQ molecules (ii) high population density, low QS signal, QS molecules degraded by QQs (iii) absence of attachment to solid surface, biofilm formation does not occur. **(B)** Effect of QQ molecules on early pre-existing biofilm (i) biofilm formed, high QS signal, addition of QQ molecules (ii) QS molecules degraded by QQs, reduction of QS signal (iii) decrease in EPS production, release of cells, return of released cells to planktonic state (i.e., reduced biofilm).

that QQ was the primary mode (as opposed to environmental factors) of QS signal reduction and served as a key player in the regulation of different stages of bacterial granulation formation.

Due to the apparent benefits of inhibiting QS, studies screening large libraries/collections of microorganisms in the search for QQ molecule producers have recently emerged. Christiaen et al. (2011) employed a high-throughput approach to screening environmental samples cultivated in minimal media supplemented with AHLs as their sole sources of carbon and nitrogen. These enriched isolates were screened using the QS inhibition selector biosensor strain *P. aeruginosa* QSIS2 (assay developed by Rasmussen et al., 2005), which revealed 41 isolates with QQ activity (in some cases resistant to heat and proteinase K treatments). Kusari et al. (2014) showed that environmentally derived samples of the endophytic bacteria of the plant *Cannabis satvia* L. were capable of quenching four different AHL molecules of the biosensor strain *C. violaceum* which regulates production of the purple pigment violacein through QS signaling activity. Large numbers of diverse unculturable bacteria from environmental samples may also be efficiently screened for QQ activity through the construction and scanning of metagenomic libraries (Coughlan et al., 2015). For example a functional metagenomic library assembled from soil samples was screened using a QQ biosensor assay employing *A. tumefaciens* NTL4 as an indicator microorganism and in doing so identified three active clones (including two novel lactonases) capable of reducing motility and biofilm formation in *P. aeruginosa* (Schipper et al., 2009). Studies describing the identification of quorum quenching molecules are briefly summarized in **Table 1**.

Quorum quenching activity is predominantly due to the action of certain enzymes that degrade QS molecules such as AHLs. It is thought that there are four potential cleavage sites in AHL QS molecules for cutting by enzymes (Chen et al., 2013). Two microbial enzyme families exist that are capable of cleaving AHL structures. Class I includes lactonases, acylases and paraoxonases. Lactonases or decarboxylases catalyze the degradation of the homoserine lactone ring. Dong et al. (2000) initially reported the AHL-degrading activity of a lactonase encoded by a gene (*aiiA*) cloned from *Bacillus* spp. 240B through cleavage of the lactone ring from the acyl moiety, which inhibited virulent activity of the plant pathogen *Erwinia carotovora*. The AidH AHL-lactonase from *Ochrobactrum* spp., which hydrolyzes the ester bond of the homoserine lactone ring of AHLs, has a very broad range of targets and is effective at reducing biofilm formation of the food spoilage bacterial strain *P. fluorescens* 2P24 (Mei et al., 2010). $AiiA_{B546}$ AHL-lactonase from *Bacillus* spp. B546 displayed a broad range of AHL substrate specificity and showed promise for use in reducing fish mortality by controlling the pathogen *Aeromonas hydrophila* (Chen et al., 2010). Cao et al. (2012) reported the oral administration of a broad-spectrum, thermostable and protease resistant $AiiA_{AI96}$ AHL-lactonase from *Bacillus* spp. AI96 to be successful in the attenuation of *A. hydrophila* infection in zebrafish. In another study, three bacterial strains with QQ activity were isolated from the rhizosphere of ginger (*Zingiber officinale*) from the Malaysian rainforest. The strains belonging to the genera *Acinetobacter* and *Klebsiella* possessed broad spectrum lactonase activity while the *Burkholderia* strain was capable of reduction of 3-oxo-AHLs to 3-hydroxy compounds, thus inactivating the AHL signaling molecules. All three strains were found to attenuate virulence of *P. aeruginosa* and *E. carotovora* in co-culture assays (Chan et al., 2011).

Acylases or deaminases cleave an AHL into a homoserine lactone ring and a free fatty acid moiety through hydrolysis of their amide link (Lin et al., 2003). AHL-acylases generally show higher substrate specificity than lactonases for AHL molecules based on the length of their acyl side chains. AHL-acylase AiiD has a higher affiliation for the degradation of long chain AHLs. Cloning of the *aiiD* gene from *Ralstonia* strain XJ12B into *P. aeruginosa* resulted in inhibition of AHL 3-oxo-C10-HSL accumulation and interference with some QS related traits (Lin et al., 2003). Genes encoding acylases capable of degrading the primary QS signaling molecules of *P. aeruginosa* exist within the *P. aeruginosa* PAO1 genome itself. *quiP* and *pvdQ* encode acylases which specifically degrade 3-oxo-C12-HSL and AHLs with long acyl chains only, excluding those with short acyl chains (Sio et al., 2006). An additional AHL acylase in the *P. aeruginosa* PAO1 genome was reported by Wahjudi et al. (2011). The *pa0305* gene, predicted to encode a penicillin acylase, was cloned and its functional protein PA0305 characterized. The protein was shown to degrade AHLs with acyl side chains of 6–14 carbons in length and its overexpression reduced both accumulation of the QS signaling molecule 3-oxo-C12-HSL and virulence of *P. aeruginosa*. Morohoshi et al. (2008) showed that expression of the *aac* gene from *Shewanella* spp. strain MIB015 in the fish pathogen *Vibrio anguillarum,* which is known to produce three distinct AHL signaling molecules and to regulate biofilm formation through QS (Croxatto et al., 2002), resulted in reduced biofilm formation on a polypropylene plastic surface. An AHL-degrading bacterial strain was isolated from a sea water sample collected in Malacca, Malaysia (Wong et al., 2012a). This strain, which contained genes with high homology to known acylases, was capable of utilizing *N*-(3-oxohexanoyl)-L-homoserine lactone as its sole carbon source and degrading AHLs with and without 3-oxo group substitution at the C3 position in the acyl side chain. The strain was also observed to release AHLs (detected in the supernatant) indicating both QS and QQ activity. This group also isolated a strain with similar activity and phylogenetic roots from tropical wetland water also in Malacca (Wong et al., 2012b).

Another type of QQ enzyme is the lactonase-like paraoxonases isolated from mammalian sera. Enzymes isolated from mammalian sera were reported to be capable of hydrolyzing the lactone ring of AHLs produced by *P. aeruginosa* (Yang et al., 2005). Other examples of anti-QS agents isolated from eukaryotes include two lactonases isolated from a collection of root-associated fungi (Uroz and Heinonsalo, 2008) and various quorum quenchers derived from plants (reviewed by Koh et al., 2013). Class II microbial AHL-targeting enzymes are oxidoreductases which target the acyl side chain

TABLE 1 | Studies describing quorum quenching molecules.

QQ molecule/activity	Producing spp./closest known relatives	Environment sampled	Attenuated virulence of:	Reference
Plant extracts	*Conocarpus erectus, Chamaesyce hypericifolia, Callistemon viminalis, Bucida buceras, Tetrazygia bicolor*, and *Quercus virginiana*	Extracts of six South Florida plants	*Pseudomonas aeruginosa* PAO1 (biofilm reduction)	Adonizio et al., 2008
Novel oxidoreductase	*Acidobacterium* spp. MP5ACTX8	Functional metagenomic library, soil, University of Göttingen, Germany	*P. aeruginosa* PAO1	Bijtenhoorn et al., 2011
Broad spectrum lactonase activity	Genera *Acinetobacter*, *Klebsiella,* and *Burkholderia*	Rhizosphere of ginger (*Zingiber officinale*), Rimba Ilmu, University of Malaya, Malaysia	*P. aeruginosa* PAO1, *Erwinia carotovora* strain GS101 and PNP22	Chan et al., 2011
Lactonase ($AiiA_{B546}$ expressed in *Pichia pastoris* GS115)	*Bacillus* spp. B546	Mud of a fish Pond, Wuqing, Tianjin, China	*Aeromonas hydrophila* ATCC 7966	Chen et al., 2010
41 strains utilizing AHLs as carbon/nitrogen source. 14 with extracellular QQ activity	21 genera, most common *Pseudomonas*, *Arthrobacter*, and *Aeromonas*	16 soil and water samples	N/A	Christiaen et al., 2011
Lactonase (encoded by *aiiA*)	*Bacillus* spp. 240B	Soil	*E. carotovora* strain SCG1	Dong et al., 2000
Furocoumarins, grapefruit juice	N/A	Grapefruit and grapefruit juice	*Escherichia coli* O157:H7, *Salmonella* Typhimurium and *P. aeruginosa* (biofilm reduction in all three)	Girennavar et al., 2008
Acylase Lactonase	*P. aeruginosa* PAO1 *Pseudomonas* strain PAI-A	N/A Soil	Both utilized own AHLs as carbon/nitrogen sources	Huang et al., 2003
Lactonase	*Rhizobium* spp. strain NGR234	N/A	*P. aeruginosa* PAO1 (including biofilm reduction)	Krysciak et al., 2011
Acylase (*aiiD* expressed in *P. aeruginosa)*	*Ralstonia* strain XJ12B	Biofilm in experimental water treatment system, The National University of Singapore	*P. aeruginosa* PAO1	Lin et al., 2003
Lactonase (AidH)	*Ochrobactrum* spp.	Soil, Yunnan Province, China	*Pectobacterium carotovorum* Z3-3, *Pseudomonas fluorescens* 2P24 (biofilm reduction)	Mei et al., 2010
Acylase (*aac* expressed in *E. coli* and *Shewanella oneidensis*)	*Shewanella* spp. strain MIB015	N/A	*Vibrio anguillarum* TB0008 (biofilm reduction)	Morohoshi et al., 2008
Lactonase (*aiiM)*	*Microbacterium testaceum* StLB037	Leaf surface of the potato	N/A (identified through genome sequencing)	Morohoshi et al., 2011
Lactonase (*ahlS*)	*Solibacillus silvestris* StLB046	Leaf surface of the potato	*P. carotovorum* subsp. *carotovorum*	Morohoshi et al., 2012
Essential oils thymol, carvacrol, eugenol	N/A	Sigma-Aldrich Chemicals (St. Louis, MO, USA)	Effective against paper mill-associated biofilms	Neyret et al., 2014
Essential oils oregano (*Origanum vulgare* L.) oil, carvacrol, thymol	*Origanum vulgare* L. N/A N/A	Ocular infections	*Staphylococcus aureus, Staphylococcus epidermidis* (biofilm reduction)	Nostro et al., 2007
Two novel lactonases, one known lactonase	*Nitrobacter* spp. Strain Nb-311A, *P. fluorescens, Xanthomonas campestris*	Soil functional metagenomic library	*P. aeruginosa* (biofilm reduction)	Schipper et al., 2009
Acylase (PA2385)	Purified from *P. aeruginosa* PAO1	Holloway collection	*P. aeruginosa* PAO1	Sio et al., 2006
Two lactonases	*Phialocephala fortinii*, *Ascomycete* isolate, *Meliniomyces variabilis* and a potential mycorrhizal isolate	16 isolates of mycorrhizal and non-mycorrhizal root-associated fungi	N/A	Uroz and Heinonsalo, 2008

(Continued)

TABLE 1 | Continued

QQ molecule/activity	Producing spp./closest known relatives	Environment sampled	Attenuated virulence of:	Reference
Amidolytic activity	*Comamonas* spp. strain D1	Soil	*P. carotovorum* strain GS101	Uroz et al., 2007
Novel lactonase (*qsdA*)	*Rhodococcus erythropolis* strain W2	N/A	N/A	Uroz et al., 2008
Acylase (PA0305 expressed in *E. coli* and *P. aeruginosa)*	Purified from *P. aeruginosa* PAO1	N/A	*P. aeruginosa* PAO1	Wahjudi et al., 2011
Novel lactonase (AiiM protein)	*M. testaceum* StLB037	Leaf surface of the potato	*P. carotovorum* subsp. *carotovorum*	Wang et al., 2010
Acylase activity	*P. aeruginosa* strain MW3A	Subsurface seawater, Malacca, Malaysia	N/A	Wong et al., 2012a
Acylase activity	*P. aeruginosa* strain 2SW8	Tropical wetland water, Malaysia	N/A	Wong et al., 2012b
Lactonase-like paraoxonase	N/A	Serum of six mammalian spp.	Hydrolysis of *P. aeruginosa*-specific AHLS	Yang et al., 2005
Lactonase (*aiiA* expressed in *E. coli*)	*Bacillus amyloliquefaciens* strain PEBA20	Laboratory collection strain	*P. carotovorum* subsp. *carotovorum*	Yin et al., 2010

of AHL molecules and catalyze a modification of the chemical structure of the signal, that is not degraded (Chen et al., 2013). A novel oxidoreductase identified from a metagenomic library reduced pyocyanin production, motility and biofilm formation when expressed in *P. aeruginosa* PAO1 (Bijtenhoorn et al., 2011).

AI-2 QS signaling systems may also be potential anti-biofilm targets. As previously mentioned, *luxS* influences biofilm formation in *L. monocytogenes* (Sela et al., 2006). Potential blockers of AI-2 signal synthesis have been investigated by Zhao et al. (2003) and Alfaro et al. (2004) with the successful design of synthetic AI-2 inhibitors reported by Shen et al. (2006) that act as competitive inhibitors of the LuxS protein interfering with the synthesis of AI-2 precursors. Recently, from a functional metagenomic library, Weiland-Bräuer et al. (2016) reported the identification of a clone originating from a German Salt Marsh to be effective at prevention of biofilm formation in *Klebsiella oxytoca* M5a1 and *K. pneumonia*e isolated from patients with urinary tract infections, species with reported AI-2 mediated QS (Balestrino et al., 2005; Zhu et al., 2011). The purified protein was suspected to possess oxidoreductase activity. To date, the AIP system in Gram-positive bacteria has not been examined as a target for potential biofilm inhibition but it may prove to be a promising route for future study.

As discussed above, the isolation of anti-biofilm agents from nature is an attractive prospect, leading to the search for quorum quenchers from organic sources. Girennavar et al. (2008) reported QS inhibition in *V. harveyi* biosensor strain by grapefruit juice and bioactive extracts from grapefruits. Additionally, they were also found to be capable of inhibition of biofilm formation by *E. coli* O157:H7, *S.* Typhimurium and *P. aeruginosa*, species which often prove troublesome for the food industry. In another study, extracts from six South Florida plants were effective in impacting QS signaling in *P. aeruginosa* with significantly reduced biofilm formation observed in the presence of extracts from three of these plants (Adonizio et al., 2008).

CONCLUSION AND FUTURE PROSPECTS/DIRECTIONS

The majority of bacteria, including those detected in food processing environments, are gifted with the ability to resist standard cleaning measures by their capacity to form biofilms on many of the surfaces approved for use in the food industry. This persistence leads to increased microbial load in both the food processing environments and in the subsequent food products, leading to food spoilage and reduced shelf life and also to increased risk of infectious outbreaks originating from food sources. Food safety is a global concern and increased risk of infection is accompanied by a requirement for more stringent and frequent evaluation of food manufacture and processing plants. Economic losses suffered by food production facilities and health related costs faced during foodborne pathogen epidemics mean that the presence of biofilm-forming bacteria can have a considerable impact on food processing establishments and, so, impeding their ability to persist in these environments is a very attractive objective for both food industry workers and researchers.

Current strategies show promise in laboratory-based experiments, with the successful inhibition of biofilm formation reported in numerous studies. However, there are considerations when applying these approaches to real life situations that limit their value to the food industry. Firstly, it is important that anti-biofilm agents used in food processing facilities meet safety requirements outlined by appropriate regulatory bodies. Agents deemed successful in the lab must also be tested and proven safe for application to food contact surfaces and, especially, if such agents are to be added to the food product itself. Ideally, quorum quenchers derived from food-grade microorganisms, plants and other natural sources would be most suitable. Additionally, researchers developing anti-biofilm strategies must acknowledge that product quality is a top priority for food manufacturers, and so, biofilm inhibitors must not influence the taste, texture or palatability of the food in any way. This is especially relevant

to the dairy industry, where many fermented milk products are developed using specific populations of microorganisms in a carefully refined system that is sensitive to change. Here, strategies that target QS signaling over growth inhibitors or bactericidal agents are useful as they do not threaten the lives of useful bacteria in the process. In such cases, the specificity of the quorum quencher is significant so as not to inhibit QS signals of beneficial bacteria that may regulate certain factors responsible for their fermentation abilities and perhaps the production of particular by-products that lend aromas and textures to the finished food. Searching for quorum quenchers from the food processing environment itself may prove useful here as competition among microbes occupying the same niche leads to the production of compounds, such as bacteriocins and QS inhibitors, specific to their common competitors. This approach may increase the likelihood of discovering quorum quenchers with action specific against the target bacteria. Another necessary factor to consider when introducing a lab-derived method to an industrial setting is the practicality of the biofilm-fighting strategy proposed. Notably with QS inhibitors, being derived from living organisms and often vulnerable to harsh climates, the active bioagents must be capable of withstanding conditions typical of food processing environments. Heat stability as well as activity at low temperatures, a broad pH range of action and resistance to proteases are all attractive qualities in a food-grade quorum quencher, depending on the process in question.

Quorum quenching has been shown to be a promising avenue of anti-biofilm research in food microbiology, with limitations faced in the transferal of laboratory findings to industrial applications. As discussed above, the criteria outlining a suitable QS inhibitor for inhibition of biofilm in the food industry is a detailed and extensive list. The search continues, employing a number of screening techniques on samples from exotic and domestic sources alike.

AUTHOR CONTRIBUTIONS

LC, PC, CH, and AA-O designed the manuscript; LC and AA-O wrote the manuscript; PC and CH critically revised the manuscript.

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Effect of Biofilm Formation by *Oenococcus oeni* on Malolactic Fermentation and the Release of Aromatic Compounds in Wine

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The winemaking process involves the alcoholic fermentation of must, often followed by malolactic fermentation (MLF). The latter, mainly carried out by the lactic acid bacterium *Oenococcus oeni*, is used to improve wine quality when acidity reduction is required. Moreover, it prevents microbial spoilage and improves the wine's organoleptic profile. Prior observations showed that *O. oeni* is able to resist several months in harsh wine conditions when adhered on oak barrels. Since biofilm is a prevailing microbial lifestyle in natural environments, the capacity of *O. oeni* to form biofilms was investigated on winemaking material such as stainless steel and oak chips. Scanning Electron Microscopy and Confocal Laser Scanning Microscopy showed that *O. oeni* was able to adhere to these surfaces and form spatially organized microcolonies embedded in extracellular substances. To assess the competitive advantage of this mode of life in wine, the properties of biofilm and planktonic cells were compared after inoculation in a fermented must (pH 3.5 or 3.2 and 12% ethanol) The results indicated that the biofilm culture of *O. oeni* conferred (i) increased tolerance to wine stress, and (ii) functional performance with effective malolactic activities. Relative gene expression focusing on stress genes and genes involved in EPS synthesis was investigated in a mature biofilm and emphasized the role of the matrix in increased biofilm resistance. As oak is commonly used in wine aging, we focused on the *O. oeni* biofilm on this material and its contribution to the development of wine color and the release of aromatic compounds. Analytical chromatography was used to target the main oak aging compounds such as vanillin, gaiacol, eugenol, whisky-lactones, and furfural. The results reveal that *O. oeni* biofilm developed on oak can modulate the wood-wine transfer of volatile aromatic compounds during MLF and aging by decreasing furfural, gaiacol, and eugenol in particular. This work showed that *O. oeni* forms biofilms consisting of stress-tolerant cells capable of efficient MLF under winemaking conditions. Therefore surface-associated behaviors should be considered in the development of improved strategies for the control of MLF in wine.

Keywords: malolactic fermentation, *Oenococcus oeni*, biofilm, wine, oak

INTRODUCTION

The winemaking process involves the alcoholic fermentation (AF) of must performed by yeast, often followed by malolactic fermentation (MLF) performed by lactic acid bacteria (LAB). MLF is involved in the quality of red, white, and sparkling wines, for which it is necessary to reduce acidity (cool-climate regions). MLF also prevents microbial spoilage through nutrient consumption (sugars, malic acid) and the release of aromatic compounds that improve the organoleptic profile of wine (Bauer and Dicks, 2004). MLF is not in itself a fermentation process but rather the decarboxylation of L-malate (di-acid) into L-lactate (mono-acid) and CO_2 by the malolactic enzyme (MLE). This reaction allows cells to regulate their internal pH and gain energy through the proton gradient across cell membranes (Versari et al., 1999).

Several LAB genera including *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Oenococcus* are able to decarboxylate L-malate. Of the latter *Oenococcus oeni* appears best able to maintain its metabolism in an environment with low pH (ca. 3.5) and the presence of SO_2 (Vuuren and Dicks, 1993; Lonvaud-Funel, 1999). This bacterium can convert malic acid in a one-step reaction (Lonvaud-Funel and Strasser de Saad, 1982; Salou et al., 1994; Kourkoutas et al., 2004). Furthermore, MLF driven by *O. oeni* leads to improving the organoleptic properties and microbiological stability of wine, through residual sugar consumption, the bacterial fermentation of co-products and lactic acid production (Lonvaud-Funel, 1995; Nedovic et al., 2000). However, despite the efficiency of *O. oeni*, spontaneous MLF is difficult to predict. Several physicochemical parameters of wine such as ethanol, low pH, and the presence of sulfite can delay MLF. Winemakers increasingly need to control their production, therefore the use of commercial starter cultures to induce MLF has become common practice. However, because of the rapid loss of cell viability after inoculation, the result is not always successful (Bauer and Dicks, 2004). Other solutions have been sought. For instance, the gene encoding the MLE of *O. oeni* is expressed in genetically modified microorganisms such as *Lactobacillus plantarum* and *Saccharomyces cerevisiae,* but few countries allow GMOs for food processing purposes (Schümann et al., 2012). Likewise, the yeast *Schizosaccharomyces pombe* was studied since it can convert malic acid through malo-ethanolic fermentation. Nevertheless, it increases ethanol levels and provides no beneficial aspects for MLF (Ansanay et al., 1996; Versari et al., 1999). It has been shown that MLF does not necessarily require cell growth: non-proliferating cells of *O. oeni* at 10^6 to 10^7 CFU/ml can decarboxylate malic acid (Lafon-Lafourcade, 1970). These results suggest that, as described in previous works for other alcoholic fermented beverages, surface-associated cells could be used to perform MLF (Nedovic et al., 2000; Kourkoutas et al., 2004; Bányik et al., 2005; Genisheva Z. et al., 2014; Genisheva Z. A. et al., 2014; Nedović et al., 2015).

The capacity of *O. oeni* to compete in a harsh environment such as wine is due to elaborate survival strategies of which we can mention the adjustment of membrane stability by changing the ratio of saturated-unsaturated fatty acids (Grandvalet et al., 2008; Maitre et al., 2014), and the synthesis of stress proteins (Jobin et al., 1997; Guzzo et al., 2000; Beltramo et al., 2006; Maitre et al., 2012). In addition, *O. oeni* can adapt to ethanol stress, especially *via* the synthesis of the small heat shock protein Lo18 (Jobin et al., 1997; Coucheney et al., 2005; Maitre et al., 2012, 2014). Biofilm formation is another way of resisting environmental stresses. This process has been widely described for bacteria, since it represents the dominant mode of microbial existence (Costerton et al., 1995). A biofilm is a community of microorganisms bound together in close proximity within their own protecting exo-polymeric matrix, permitting metabolic cross-feeding, cell–cell interactions and chemical and physical resistance (Davey and O'toole, 2000; Hojo et al., 2009). Due to this specific organization, the biofilm is considered as a whole (Katharios-Lanwermeyer et al., 2014). The biofilm formation of the lactic acid bacterium *Lb. plantarum* biofilm enhances stress resistance to acetic acid (up to 11% v/v) and ethanol (up to 40% v/v). Indeed, the analysis of cell surfaces by scanning electron microscopy (SEM) revealed that that these treatments severely damage planktonic cells whereas biofilm cells were only slightly damaged (Kubota et al., 2008). Many examples of transformation processes using biofilm on the laboratory scale have been documented, such as wastewater treatment and ethanol production, but so far the only industrial application of biofilms for food production purposes known to date is the production of acetic acid by acetic acid bacteria biofilm (Maksimova, 2014).

Up to now, very little attention has been given to *O. oeni* biofilm formation, and only its bacteriocin resistance properties have been reported (Nel et al., 2002). However, a connection has been reported between *O. oeni* EPS production and its increased survival in wine (Dimopoulou et al., 2015).

In a previous experiment, the sampling of oak barrels suggested that microorganisms and particularly LAB were able to withstand wine stress (low pH, ethanol, few nutrients) on this surface. Thus in this context, our study investigated the surface-associated behaviors of *O. oeni* cells and their role in resistance to stresses incurred in wine. We examined the spatial organization of *O. oeni* cells on different contact surfaces, the survival of surface-associated cells, and their ability to perform MLF in wine. Finally, we explored the impact of oak surface-associated *O. oeni* cells on the color and aromatic profile of wine in view of the importance of this material in winemaking and aging.

MATERIALS AND METHODS

Bacteria Strains and Growth Media

This study was conducted using two strains: ATCC-BAA 1163, one of the first strains of *O. oeni* to be sequenced (isolated from red wine, France, Aquitaine) and currently used as a reference (Guzzo et al., 2000; Beltramo et al., 2004; Desroche et al., 2005; Maitre et al., 2012), and Sabo11, an enological strain (isolated from red wine, South Africa) presenting enhanced technological properties and currently used at the *Domaine viticole de l'Université de Bourgogne, Marsannay, France* to perform MLF. Bacteria were grown in MRS modified (MRSm) medium containing: MRS Broth (Laboratorios Conda Spain) 50 g/l; fructose 10 g/l; L-malic acid 4 g/l. The pH was adjusted

to 4.8 (NaOH concentrated solution). For solid MRSm medium, 25 g/l agar was added.

Wine medium was obtained by the fermentation of a commercial white grape juice by commercial yeast (*Saccharomyces cerevisiae* Fermol PB 2023, Spindal AEB Group). The outcome was standardized at 12% ethanol, pH3.2 or 3.5, fermentable sugars 2 g/l and L-malic acid 4 g/l.

Aligoté white wine from the 2014 vintage elaborated at the *Domaine viticole de l'Université de Bourgogne, Marsannay, France,* was used for aroma analysis. This wine finished its alcoholic fermentation with the following enological parameters: 12% ethanol, pH 3.5, and L-malic acid 3.2 g/l.

All the media were sterilized by filtration (0.2 μm cut-off). Cultures were incubated at 28°C with 10% CO_2 in a CO_2 incubator. All the assays were performed in triplicate.

Biofilm Formation Conditions

On Stainless Steel Chips

Each 25 mm × 25 mm stainless-steel chip (Goodfellow) was immersed in 20 ml inoculated MRSm (2×10^7 CFU/ml). After incubation for 3, 7, and 14 days (with a medium turnover every 3.5 days), the plate was rinsed twice with NaCl 150 mM, then placed in 10 ml saline solution with 700 mg of 0.1 mm diameter glass beads. The system was vortexed at maximal power for 2 min to free surface-associated cells. Populations of cells removed from the surface by this procedure were estimated by culturing appropriate dilutions (prepared in NaCl 150 mM) on solid MRSm at 28°C under 10% CO_2. It was previously verified that the bead treatments dislodged surface-associated cells and did not cause cell death, by measurement of viable planktonic cell populations before and after these treatments. The 2-week-old biofilm was detached from the steel plate into the wine to assess biofilm cell viability after 1, 4, and 24 h.

On Oak Chips

The oak wood used in this study was characterized by a previous work (Duval et al., 2013). The 25 mm × 25 mm oak chips were immersed in 20 ml of inoculated MRSm (2×10^7 CFU/ml). The medium was changed every 3 days until the end of incubation (1, 2, or 4 weeks). Surface-associated cell populations were estimated as follows. The chips were rinsed twice with sterile saline solution, placed in 10 ml saline and scrubbed with a toothbrush (2 min per side). Viable cell populations in this solution were determined on solid MRSm medium as described above.

To analyze biofilm survival in wine, the chips were rinsed twice with saline solution, transferred to wine and incubated for 1, 4, 7, 14, or 21 days. Their populations were estimated as described above. All the assays were performed in triplicate.

On a Polystyrene Microplate

Two hundred and fifty micro liter of a mid-exponential phase culture (10^9 CFU/ml) was added to the wells of a polystyrene 96-well microtiter plate (Greiner Bio-one, France) with a μclear® base (Polystyrene, thickness of 190 μm ± 10%) which allowed high resolution confocal imaging. After 1 h of adhesion at 30°C, the wells were refilled with 250 μl MRSm. This preparation was then subjected to Confocal Laser Scanning Microscopy.

Confocal Laser Scanning Microscopy

Surface-associated microorganisms were fluorescently tagged by adding FM4-64 fluorescent membrane marker (Life Technologies, USA) in fresh medium according to the manufacturer's instructions. The plate was incubated for 40 h at 30°C and mounted on the motorized stage of an inverted confocal microscope (Leica SP8 AOBS, LEICA Microsystems, Germany) at the INRA-MIMA2 imaging platform[1]. Observations were performed using a 63X/1.2 N.A. water immersion objective lens (300 μm working distance). Surface-associated microbial agglomerates were scanned using an argon gas laser with a 514 nm line (output power at 30%, AOTF at 10%) and the fluorescence emitted was recorded from 534 to 800 nm using a PMT detector with a gain of 750 V. Single 2D sections of surface-associated agglomerates and 3D acquisitions were acquired at a scan speed of 600 Hz an image definition of 512 × 512 and a z-step of 1 μm between each xy image for a z-stack. Time-lapse automated acquisitions were performed with the LAS X High Content Screening A Matrix Screener module. Three-dimensional projections of agglomerate structure were then reconstructed using the blend mode of the Easy 3D function of the IMARIS 7.7.2 software (Bitplane, Switzerland). Microbial agglomerate biovolumes (μm^3) were extracted from confocal image series using a homemade ICY routine as described previously (Sanchez-Vizuete et al., 2015).

Scanning Electron Microscopy

Cells were fixed on stainless steel by a solution of 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.2 for 1 h at 4°C. The samples were then washed three times with phosphate buffer for 20 min at room temperature. Dehydration was performed by successive immersions in solutions of increasing ethanol content (70, 90, 100%), then three times for 10 min each in successive baths of ethanol-acetone solution (70:30, 50:50, 30:70, 100) and air-dried. Afterward, the samples were coated with a thin carbon layer using a CRESSINGTON 308R and observed with a JEOL JSM 7600F scanning electron microscope (JEOL, Ltd.). SEM was performed at 5 kV and the samples were observed at a working distance of 14.9 mm.

Malolactic Conversion Monitoring

Malolactic fermentation monitoring was performed according to the manufacturer's instructions using the "L-Malic acid Cat No. 020" kit from Biosentec.

Gene Expression Analysis

RNA Extraction and cDNA Preparation

Planktonic cells were sampled in the mid-exponential phase and the surface-associated cells after 2-weeks growth on steel. Cells were centrifuged (8,000 *g*, 10 min) before being resuspended in 1 ml of Tri-reagent (Sigma) and disrupted with glass beads (100 μm) in a Precellys homogenizer (Bertin) for 6 series of 30 s at 6500 rpm. Nucleic acids were extracted in 0.2 volume of chloroform and purified by precipitation in 1 volume of

[1] www.jouy.inra.fr/mima2

isopropanol. RNA pellets were dried and resuspended in 30 μl of RNase-free water. Nucleic acid concentrations were calculated by measuring absorbance at 260 nm using an Infinite 200 PRO spectrophotometer (Tecan). Before reverse transcription (RT), 2 μg of total RNA were treated with 2 U of DNase (Invitrogen), as described by the manufacturer. The absence of chromosomal DNA contamination was checked by real-time PCR. cDNAs were then synthesized by using an iScript cDNA synthesis kit (Bio-Rad) as recommended.

Real-time PCR Experiment

Real-time PCR as described by Desroche et al. (2005) was used to quantify mRNA levels. Gene specific primers (**Table 1**) were designed to amplify the cDNAs of the transcripts of *ldhD, gyrA, hsp18, clpL1, cfa, groEL, levO, wobB, wobO, dsrO, mleA* with the Bio-Rad SYBR green kit in a Bio-Rad I-Cycler. This method was used to analyze their mRNA levels during planktonic growth at mid-exponential phase (10^9 CFU/ml) and 2-weeks of biofilm development on stainless-steel chips (2×10^6 CFU/cm^2) with or without wine stress (pH 3.5; 12% ethanol). The results were analyzed by using a comparative critical threshold method ($\Delta\Delta$CT) in which the amount of targeted mRNA was first normalized using both the specific mRNA standard and then compared to a calibrator condition (Desroche et al., 2005). *ldh* and *gyrA* genes encoding for a glyceraldheyde-3-phosphate dehydrogenase and a gyrase, respectively, were selected as internal standards since their transcript levels were stable under the conditions tested. mRNA quantification was performed in triplicate from the total RNA extracted from three independent cultures.

Measurement of Oak Aroma Compounds Released in Wine by HS-SPME-GC-MS

HS-SPME-GC-MS was carried out using the method of Duval et al. (2013). Five ml of 1-month old wine was placed in a 20 ml sealed headspace vial (Supelco, Bellefonte, PA, USA). Headspace vials were then placed in the agitator/incubator of an automatic headspace sampler (GERSTEL MPS 2, Gerstel Inc., Mülheim an der Ruhr, Germany) and incubated at 70°C for 10 min (incubation time) in order to promote volatile compounds in the headspace. Extractions were performed by immersing a DVB–CAR–PDMS fiber in the headspace for 60 min (extraction time). After each extraction, the extracted compounds were desorbed at 260°C for 7 min in the injection port of an HP 6890GC equipped with an MSD 5973 mass detector (Agilent Technologies, Palo Alto, CA, USA). Calibration solutions were processed in the same way using 5 ml of the wine matrix mixed with target compounds. Volatile compounds (eugenol, guaiacol, furfural, vanillin, cis-, and trans-whisky lactone) were purchased from Sigma–Aldrich and used as received. We used 3,4-dimethylphenol as the internal standard at 10 mg/l in each sample. Using highly aroma-concentrated calibration samples either alone or in mixture, we checked that there were no competition effects for the fiber between aromas. Chromatographic analyses were performed in biological triplicate and technical duplicate.

Chromatographic Conditions

The oven program started at an initial temperature of 40°C for 3 min. The temperature was then increased at a rate of 7°C min^{-1} up to 230°C. A 0.8 mm I.D. liner was used and maintained at 270°C, in splitless injection mode. The carrier gas was helium at 1.0 ml.min^{-1} (99.996%). Ionization was performed by electronic impact (EI), with the electron multiplier set at 1600 eV. The temperatures used were 200°C for the trap, 60°C for the manifold, and 280°C for the transfer line. The compounds were quantified in selected ion storage (SIS) mode, by selecting the appropriate ion masses for each compound: furfural (95 + 96), guaiacol (109 + 124), whisky lactone (99), eugenol (164), 3,4-dimethylphenol (107 + 122), vanillin (151 + 152).

Color Measurements

Color absorbance measurements and data acquisition and analysis were performed with a Konica Minolta CM-5 spectrophotometer using optical glass precision cells with a 50 mm path length (Hellma Analytics) and scanned over the range 740–360 nm (visible range). Black and white calibrations

TABLE 1 | Primers used for gene expression analysis.

Target gene	Function of gene	Forward primer (5′→3′)	Reverse primer (5'→3')	Amplicon length (bp)	Reference
cfa	Cyclopropane fatty acid synthase	GGTATTACATTGAGCGAGGAG	CGTCTTTGAGATCACGATAATCC	113	Beltramo et al., 2006
clpL1	Clp ATPase protein	ATTATAATGACGATCCCTTCGT	GGATCCCTGAACCGTTATTTGCTTGTTG	163	Desroche et al., 2005
dsrO	Glycoside-hydrolase	GGTCGCTGCTGCTTAATTTC	CCGTGGTGTTTTGACATCAG	137	This study
groEL	Heat shock chaperone	TCCCACGAAGTTGAGGATTC	CGATACCTTTGGACTCTTCA	145	This study
gyrA	Gyrase α subunit	CAAGGACTCATAGATTGCCGAA	CGCCCGACAAACCGCATAAA	95	Desroche et al., 2005
hsp18	sHsp Lo18	CGGTATCAGGAGTTTTGAGTTC	CGTAGTAACTGCGGGAGTAATTC	102	Beltramo et al., 2006
ldhD	D-lactate dehydrogenase	GCCGCAGTAAAGAACTTGATG	TGCCGACAACACCAACTGTTT	102	Desroche et al., 2005
mleA	Malolactic enzyme (MLE)	CCGACAATTGCTGATACAATTGAA	GGCATCAGAAACGACCAGCAG	156	Beltramo et al., 2006
levO	Fructansucrase	AATCAAGATACCGCCAGTGC	CCGAACCTGACCATTGTTCT	109	This study
wobB	Rhamnosyl-transferase	TGGTACAAATCGACCGACAA	AAAGTCCGTGATTGGTTTGC	75	This study
wobO	Glycosyltransferase	TGTCGAATGGAACATGAACG	TGATCGTCTCGATGATTGGA	62	This study

were performed using a standard black plate and an empty glass cell, respectively. Color was recorded using the CIE-L* a* b* uniform color space (CIE-Lab), using three dimensions (L*, a*, b*) of the Hunter color scale, where L* ranges from 0 for black to +100 for white, a* ranges from −50 for green to +50 for red, and b* ranges from −50 for blue to +50 for yellow.

Statistical Analysis

Each experiment was carried out in triplicate. Error bars represent standard deviations. Student *t*-test and one-way analysis of variance (ANOVA) followed by a Tukey's HSD (honest significant difference) post hoc test were used to analyze significant differences between groups using XLSTAT Version 2014, Addinsoft; $P = 0.05$. Principal Component Analysis of data was carried out with the same software.

RESULTS

Oenococcus oeni Can Colonize Different Surfaces

Stainless steel tanks and oak barrels are used in winemaking, therefore the development of *O. oeni* was characterized on both surfaces. An *O. oeni* ATCC BAA-1163 population grown on a stainless steel chip was numbered after 3 days, 1 and 2 weeks, respectively (**Figure 1A**). On stainless steel the surface-associated cells reached 4×10^5 CFU/cm^2 in 3 days. At 1 week, they reached a population of almost 10^6 CFU/cm^2 and then exceeded it after 2 weeks (2×10^6 CFU/cm^2) (**Figure 1A**).

On oak, surface-associated cells were around 60-fold more numerous than on steel with a population reaching 2×10^7 CFU/cm^2 and 10^8 CFU/cm^2 at 3 days and 2 weeks, respectively (**Figure 1B**). The growth of these cells slowed down from the 2nd week and the population remained constant.

The difference between the populations studied on steel and oak was confirmed by SEM observation (**Figures 2A,B**). Although it did not cover the entire surface, the tridimensional organization of cells on oak appeared thicker, wider and more mature. The early stages of this tridimensional development were observed at each time on steel (3 days to 2 weeks), showing cell adhesion and microcolonies. The cells adhered, flattened, and produced extracellular material that bonded them to the surface, after which they finally organized themselves in microcolonies (**Figure 2A**). These characteristics observed for the surface-associated cells allowed us to consider that *O. oeni* is able to form a biofilm On oak, there was an observable transition between the 1-week stage and the 2-week growth stage. Indeed, at this point, most of the cells appeared to belong to a larger structure and merged in a matrix (**Figure 2B**).

This matrix was observable as was a polymer that attached the cells to the surface (**Figure 2A**: Steel, 2 weeks, x35 000, and **Figure 2B**: oak, 2 weeks, x35 000), bonded them together (**Figure 2B**: oak, 1 week, x35 000), and coated the surface of the biofilm, so that the cells were indistinguishable (**Figure 2B**: oak, 4 weeks, x35 000). According to these observations, the biofilm appeared mature from 2 weeks on oak.

To gain more insight into *O. oeni* biofilm formation dynamics, we used a Real-Time Confocal Laser Scanning Microscope (RT-CLSM) associated with a fluorescent membrane probe compatible with live *in situ* dynamics to monitor cell growth in 4D over 2 days (**Figure 2A**). Technically, this observation was not possible on wood chips (autofluorescence, non-transparency, interaction with the fluorophore), so measurements were performed in polystyrene microplates. Surface-associated *O. oeni* showed a rapid increase in biovolume, reaching up to 4×10^5 µm^3 after 18 h incubation (**Figure 2C**).

Oenococcus oeni Biofilm, a Mode of Life Allowing Stress Resistance

The survival of planktonic and biofilm cells detached for 2 weeks in wine was compared. Both samples were inoculated in wine

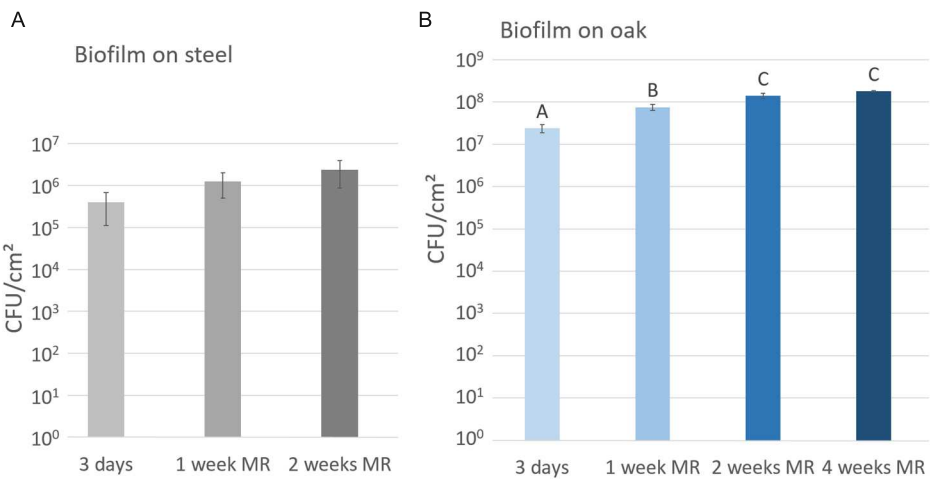

FIGURE 1 | Biofilm growth of *Oenococcus oeni* ATCC BAA-1163 in MRSm renewed twice a week (MR) on (A) stainless steel or (B) oak. Biomass is expressed in CFU/cm^2. Error bars represent the standard deviation of three biological replicates; a different letter means significant difference $P < 0.05$.

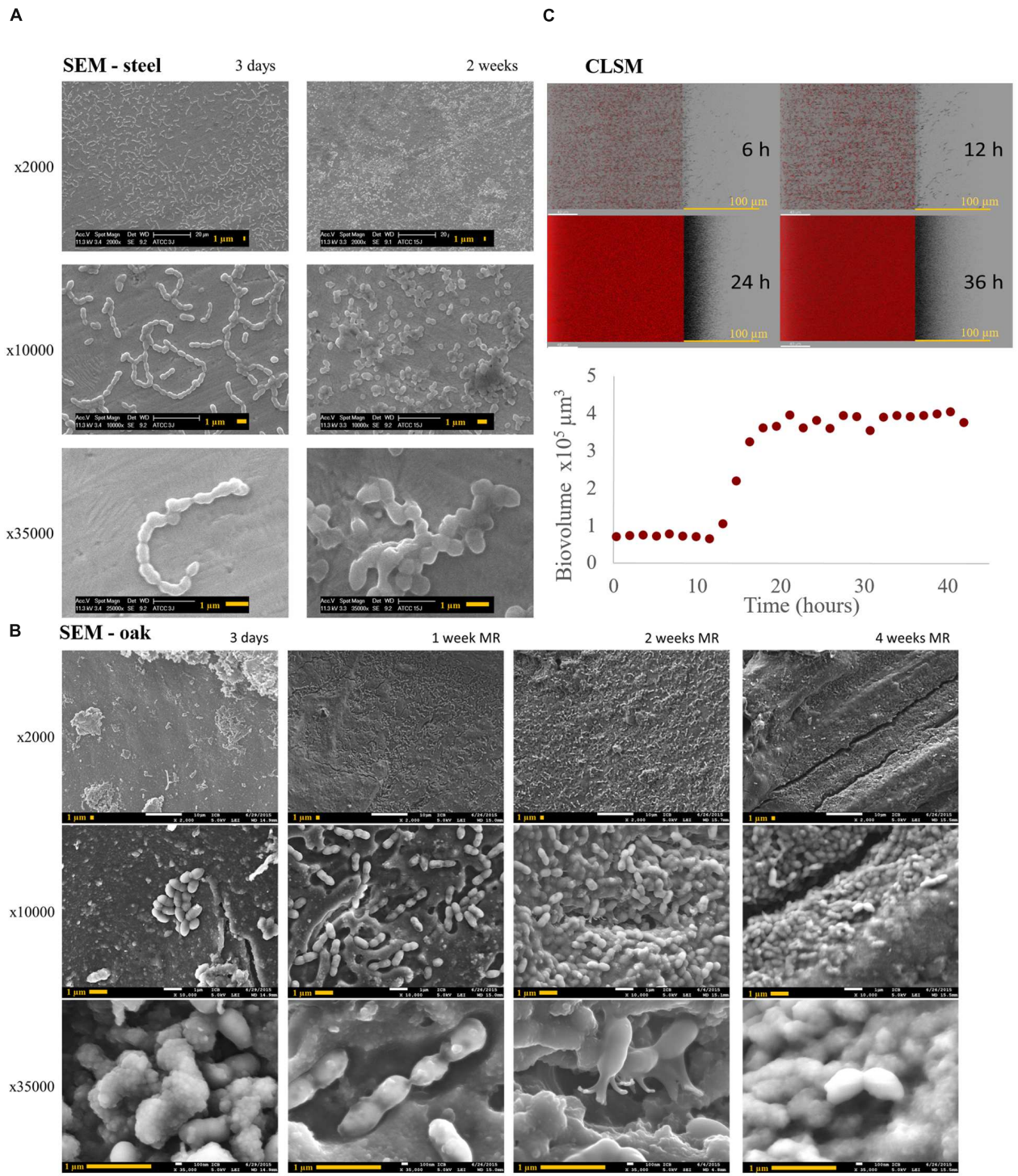

FIGURE 2 | *Oenococcus oeni* ATCC BAA-1163 biofilm microscopy observations. (A,B) Scanning Electron Microscopy (SEM) at x2000 x10,000 x35,000 of biofilm growth showing stages of formation **(A)** on steel at 3 days and 2-weeks' growth, and **(B)** oak at 3 days, 1, 2, and 4 weeks. **(C)** Confocal Laser Scanning Microscopy z-projections for the time lapse of biofilm development at 6, 12, 24, and 36 h on polystyrene microplates. Below, the evolution of the biofilm biovolume.

medium at pH 3.2 with 12% ethanol, which represents severe stress conditions for *O. oeni*. Their survival was monitored for 24 h (**Figure 3**). Planktonic cells inoculated at 10^7 CFU/ml in this medium underwent total mortality within 4 h, while cells detached from the biofilm (inoculated at 3×10^6 CFU/ml) had a loss of 1 log after 4 h incubation. However, viability

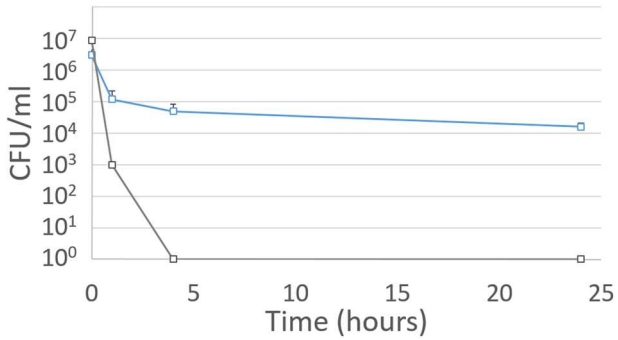

FIGURE 3 | Evolution of *O. oeni* ATCC BAA-1163 population in wine. Gray curve: planktonic cells; blue curve: biofilm cells (2-week growth, medium renewed) detached from the steel. The wine used has a pH of 3.2; 12% ethanol. Error bars represent the standard deviation of three biological replicates.

remained constant over 24 h (**Figure 3**), suggesting that biofilm cells keep their properties even when detached. This made it possible to describe a real biofilm phenotype for the cells in the microcolonies and the cells detached from the biofilm.

The biofilm phenotype increased cell stress resistance, even after detachment from the surface. In order to investigate biofilm tolerance mechanisms, we studied the relative expression of a set of genes encoding for stress proteins (*hsp18, clpL1, cfa, groEL*) (**Figure 4A**) and a set of genes involved in exopolysaccharide production (*levO, wobB, wobO, dsrO*) (**Figure 4B**), during the biofilm development (2-week old biofilm) and the planktonic growth (exponential phase) with or without stress (30 min in wine at pH 3.5 and ethanol 12%). As expected, genes related to stress response were overexpressed in stressed planktonic (PS) cells compared to non-stressed planktonic cells (P) (**Figure 4A**). The *cfa* transcript level was slightly higher and the *groEL* transcript levels were sixfold higher. The highest increases were for *clpL1* and *hsp18* transcript levels, at approximately 70-fold and 150-fold. Regarding biofilm cells (B), all the genes studied in the non-stressed biofilm showed lower expression compared to the non-stressed planktonic cells (P). However, stress genes were over-expressed (except for *groEL*) when biofilm cells were exposed to stress conditions (BS) (**Figure 4A**).

The relative expression levels of four genes involved in EPS production in planktonic and biofilm cells, with or without stress, are described in **Figure 4B**. In stressed planktonic cells (PS), *dsrO* and *levO* exhibited a fourfold decrease in transcription levels compared to the planktonic reference (P). Expressions of the genes studied and involved in the production of EPS were lower in the non-stressed biofilm cells (B) than in the planktonic reference (P) (2.9-fold to 6.7-fold) (**Figure 4B**). In contrast, when biofilm cells were stressed (BS), the expression of these genes increased significantly (10 times the B levels).

Impact of Biofilm and Planktonic Cells of *O. oeni* on the Malolactic Fermentation of Wine

Since the biofilm phenotype provides improved stress resistance, biofilm technological performance was investigated in comparison with planktonic cells. To establish whether

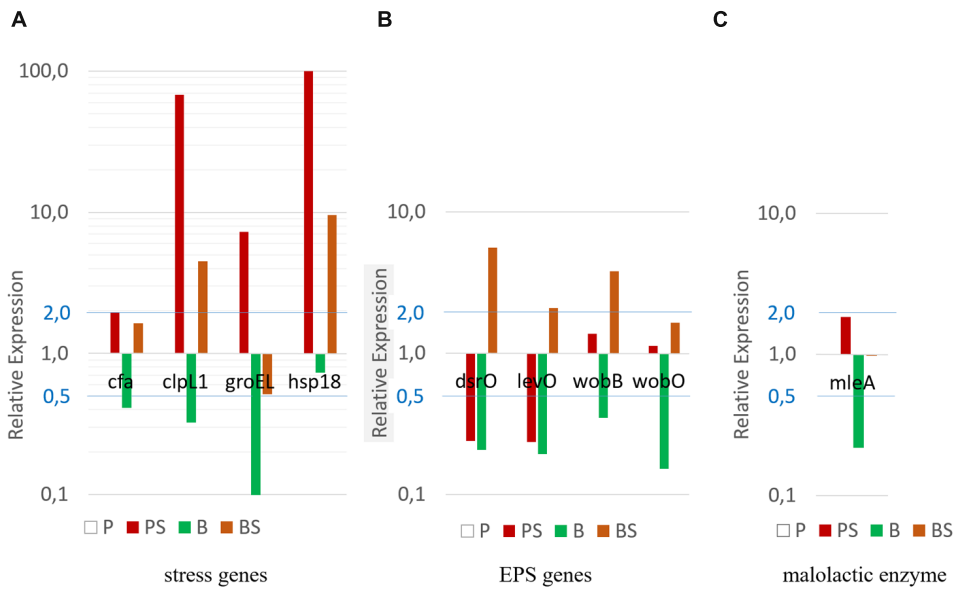

FIGURE 4 | Comparison of relative expression levels of (A) five stress genes, (B) four genes involved in EPS production, and (C) malolactic enzyme (MLE) gene, of *O. oeni* ATCC BAA-1163 in the planktonic exponential phase and biofilm with 2-weeks' growth. Planktonic cell gene expression without stress (P) was used as a calibrator and set at 1; green bars represent biofilm cells without stress **(B)**. Red bars represent planktonic stressed cells (PS), and orange bars stressed biofilm (BS), knowing that each group was exposed to a 30-min wine stress (pH 3.5 ethanol 12%). Gene expression was quantified using RT-qPCR and the comparative critical threshold ($\Delta\Delta C_T$) method. The *ldhD* gene was used as the internal control. One representative repetition of the triplicate is shown.

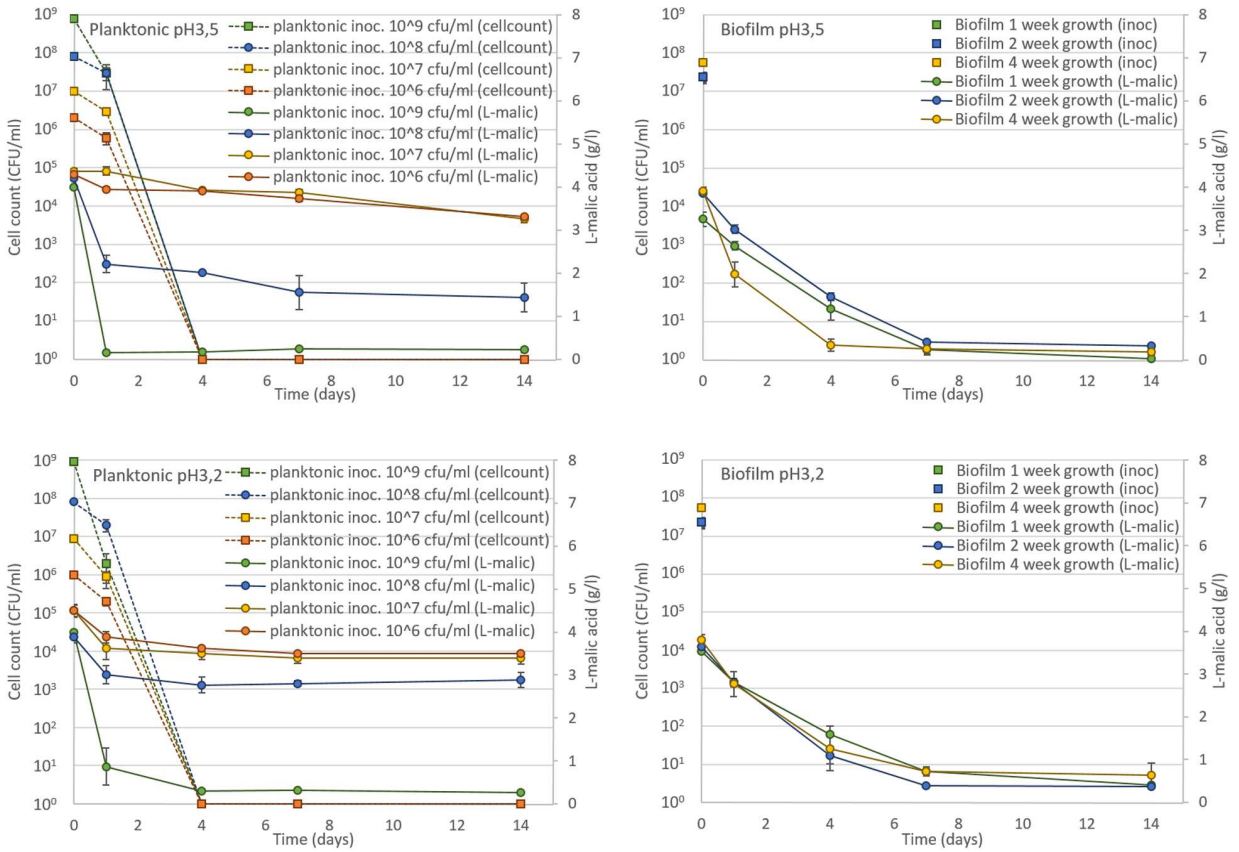

FIGURE 5 | Comparison of L-malic acid conversion by *O. oeni* ATCC BAA-1163 planktonic (left side) and biofilm cells (right side) in wine medium (12% ethanol) at pH3.5 (up), and pH3.2 (bottom). Planktonic cells were inoculated from 10^6 to 10^9 CFU/ml then their mortality was monitored (dashed lines). Biofilms of 1, 2, 4-weeks' growth on oak chip were inoculated at the equivalent of 10^8 CFU/ml. Error bars represent the standard deviation of three biological replicates.

the biofilm of *O. oeni* keeps its enological properties, the consumption of malic acid was monitored simultaneously with the quantitative analysis of transcript levels of the gene encoding for the malolactic enzyme (*mleA*). As shown in **Figure 4C**, *mleA* is less expressed in biofilm cells (B) compared to exponential-planktonic cells (P). However, when biofilm cells were immersed in wine (BS), their *mleA* transcription levels were similar to planktonic cells (P). Indeed, at the time of sampling malic acid was no longer present in the biofilm culture medium contrary to the wine medium, suggesting that *mleA* transcript level is reletad to the acid malic concentration in the medium.

Microvinifications were carried out using a must fermented by *S. cerevisae*, adjusted to pH 3.2 or pH 3.5, 4 g/l L-malic acid and 12% ethanol, inoculated with *O. oeni* ATCC BAA-1163 biofilm on oak at 5×10^7 CFU/ml or planktonic cells as reference (10^6 to 10^9 CFU/ml). After 4 days incubation in this wine (**Figure 5**), the planktonic cells underwent total mortality regardless of the initial concentration inoculated, suggesting that without pre-adaptation they are unable to survive in wine and consequently unable to perform MLF. Despite this mortality, a very large cell population (10^9 CFU/ml) could convert malic acid before dying. In contrast, biofilm cells kept their ability to perform complete MLF, probably due to their enhanced survival in wine (**Figure 5**).

Following this strategy, we made a comparison between an *O. oeni* lab strain ATCC BAA-1163 and Sabo11, a malolactic strain of technological interest (**Figure 6A**). Indeed, Sabo11 completed 100% MLF whereas ATCC BAA-1163 converted 75% of the L-malic acid. This difference was not due to the cell quantity, because both populations exhibited the same viability through time, which decreased from 5×10^7 CFU/ml (beginning) to 10^3 CFU/ml (20 days after). Consequently, Sabo11 was more suitable for performing MLF than the lab strain, ATCC BAA-1163. Therefore this strain was used to perform a winemaking-like experiment involving interaction between bacteria, oak and wine. To this end, a planktonic culture of Sabo11 was adapted to wine stress with the *pied-de-cuve* method (Li et al., 2012). As shown in **Table 2**, we compared five samples in which the presence of oak and the bacteria mode of life vary, in order to test an alternative to traditional wine inoculation through the *pied-de-cuve*. Therefore we used biofilms which were not adapted to wine conditions, unlike the planktonic culture. MLF monitoring in wine is shown in **Figure 6B**. The adapted planktonic cells inoculated at 5×10^7 CFU/ml (P)

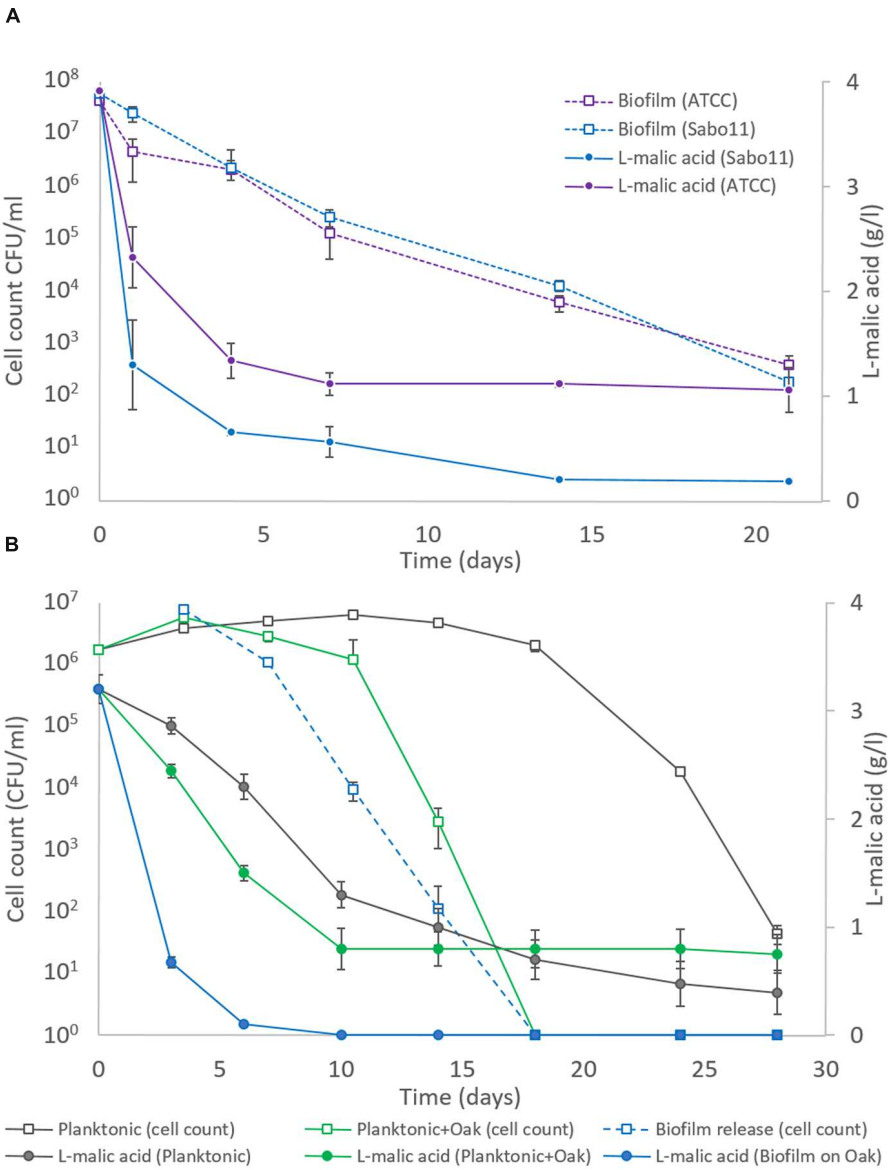

FIGURE 6 | (A) Monitoring of the MLF in *aligoté* wine (pH3.5 ethanol 12%) by two *O. oeni* strains grown in biofilm on oak for 2 weeks. Biofilm inoculum and survival are shown by dashed lines (☐ ATCC BAA-1163; ☐Sabo11). L-malic concentration is shown in straight lines (● ATCC BAA-1163; ●Sabo11). **(B)** Monitoring of MLF (☐) and cell viability (●) in aligoté wine (pH3.5 ethanol 12%) by a adapted planktonic inoculum of *O. oeni* Sabo11 (gray lines), supplemented with oak chip (green lines), and biofilm on oak chip (blue lines). The blue dashed line represents the viable-cultivable cells released by the biofilm in the wine. Error bars represent the standard deviation of three biological replicates.

grew from 2×10^6 to 6×10^6 CFU/ml and converted L-malic acid during the first 10 days and then slowed down. The planktonic cells with oak chip (OP) also converted L-malic acid in 10 days, and then stagnated, due to their decrease in population after 10 days. The biofilm cultivated on oak (BO), inoculated at the equivalent of 5×10^7 CFU/ml, performed complete MLF in 6 days. Interestingly, the biofilm released cells in wine, reaching 10^6 CFU/ml on the 3rd day of MLF.

We continued to monitor MLF under these experimental conditions, and focused on the molecular interactions between *O. oeni*, wine and oak chips. To do this, the concentration of six oak volatile compounds in wine was assessed by HS-SPME-GC-MS analysis (**Figure 7A**). MLF performed by planktonic cells without oak (P) as a control showed that the six compounds did not come from the wine or the bacterial metabolism. Oak chips immersed in wine without cells to perform MLF (O) represented the reference compound transfer without bacterial metabolism. MLF with planktonic cells and oak chips (OP) influenced four compound concentrations, by increasing them (*cis*-whisky lactone, *trans*-whisky lactone, and vanillin) or decreasing them (furfural),

TABLE 2 | Five conditions used to study *Oenococcus oeni*-oak-wine interaction.

Name	Inoculum	Oak
O	–	Untoasted oak chips (120 g/l)
P	Planktonic culture of adapted Sabo11 (5×10^7 CFU/ml)	–
OP	Planktonic culture of adapted Sabo11 (5×10^7 CFU/ml)	Untoasted oak chips (120 g/l)
BO	Biofilm 2-week growth of Sabo11 (5×10^7 CFU/ml)	Untoasted oak chips (120 g/l)

The wine is aligoté wine pH3.5 12% ethanol. The inoculum is absent, or a planktonic or biofilm culture of O. oeni strain Sabo11, the oak chip is immerged or not.

whereas no significant difference was observed for guaiacol or eugenol. The biofilm under the oak chip condition (BO) released fewer oak volatile compounds than the O and OP conditions, except for the whisky lactones. The *cis*-whisky lactone levels of BO were similar to O, whereas the *trans*-whisky lactone level of BO was higher than the others. A principal component analysis was carried out to illustrate these aroma transfers from oak to wine as a function of direct inoculation process (**Figure 7B**). This representation shows that two components, F1 and F2, explain 84% of the variability of aroma concentrations. After only 1 month of micro-vinification, the biofilm lifestyle (BO) could be clearly distinguished from the planktonic lifestyle (OP). The presence of planktonic bacteria increased the vanillin concentration compared to the presence of the oak chip alone in the wine medium. This increase could be due to enzymatic activities, as described previously (de Revel et al., 2005; Bloem et al., 2006).

Wine color, which is another enological parameter, was investigated in these micro-vinifications by measuring the chromatic L*a*b* values (**Figure 8**). Our study showed that MLF did not significantly change the color of wine (W vs. P). Likewise, there was no difference between oak wine with or without MLF (OP vs. O). Nonetheless, as expected, the impact of oak aging (O, OP) versus oak-less conditions (W, P) was an increase in the magenta (a*) and the yellow (b*) colors in the wine and a decrease of lightness (L*). Finally, biofilms on oak chip (BO) reduced wine staining (a*, b*) and preserved lightness (L*) compared to planktonic MLF wine with oak (OP).

DISCUSSION

In this study, culture based investigations and microscopy indicated that *O. oeni* actively colonized both steel and oak surfaces and formed agglomerates displaying the characteristics of biofilms. According to these findings, we investigated the biofilm development of *O. oeni* linked to its ability to perform MLF, a key step of winemaking. The study focused on: (i) the capacity of *O. oeni* to spatially organize in biofilm; (ii) the capacity of this biofilm to withstand the stress found in wine and to perform MLF; and (iii) the modulation of the

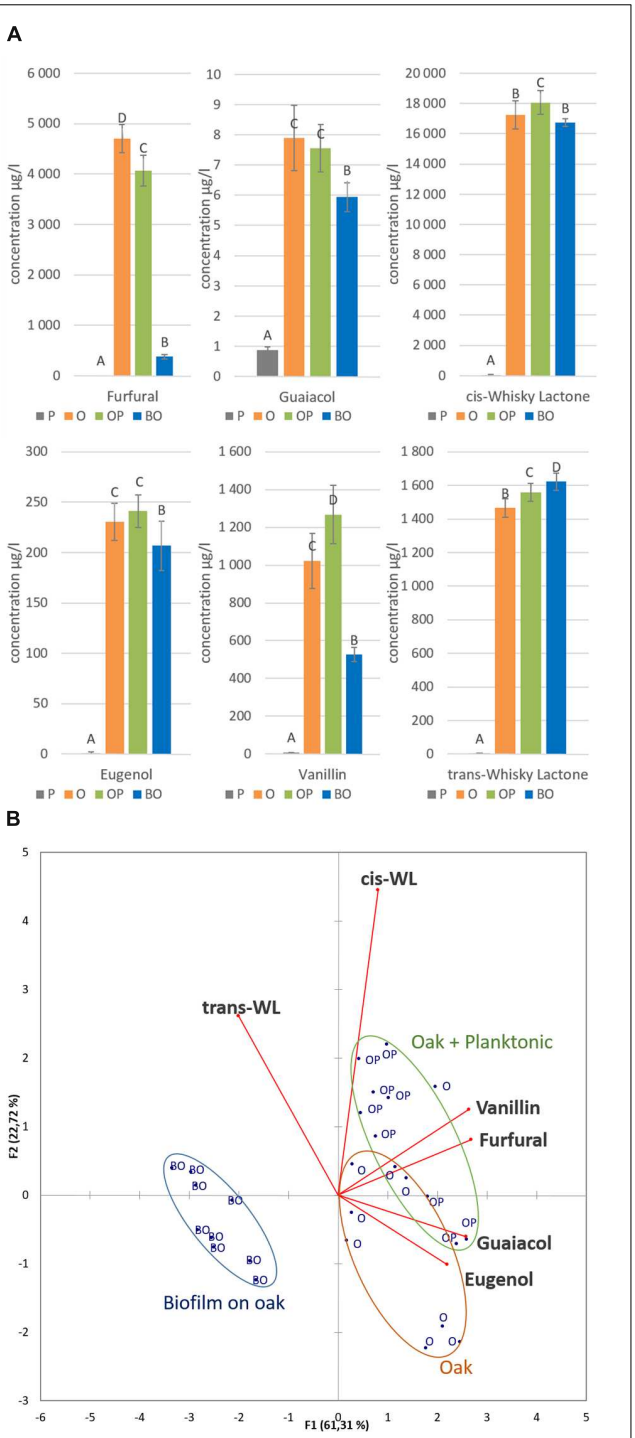

FIGURE 7 | (A) HS-SPME-GC-MS analysis of six oak volatile compounds in wine after 1 month's aging: furfural, guaiacol, *cis* and *trans*-whisky lactones, eugenol and vanillin. Four conditions were experimented, MLF by planktonic cells without oak (P in gray), oak chip immerged in wine without MLF (O in orange), MLF by planktonic cells with oak chip (OP in green), MLF performed by the biofilm on oak chip (BO in blue). Error bars represent the standard deviation of three biological and two technical replicates. **(B)** Projection of compositional data on principal components 1 and 2; the circled dots group the data of the six volatile compounds analyzed: oak alone (orange) oak with MLF (green) and biofilm on oak MLF (blue).

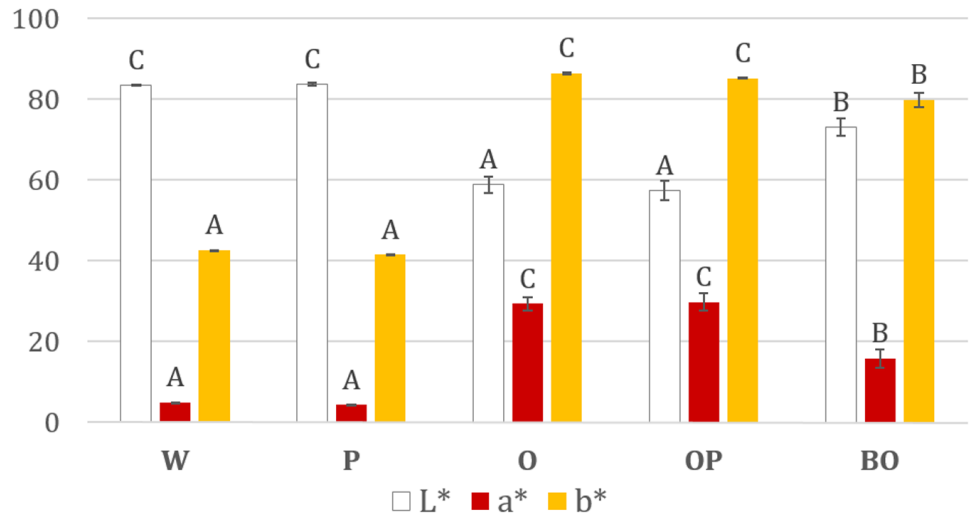

FIGURE 8 | L*a*b* parameters of the five wine conditions after aging for 1 month. W, Wine; P, planktonic MLF; O, Oak; OP, Oak + planktonic MLF; BO, MLF by Biofilm on Oak **(Table 2)**. The error bars represent the standard deviation of three biological and three technical replicates.

organoleptic quality of wine by *O. oeni* biofilms developed on oak.

Investigation of *O. oeni* Biofilm Development and Involvement in Resistance to Drastic Environmental Conditions

First, we highlighted *O. oeni* bacteria adhering to the wine material, which suggested the presence of potential biofilm. For the first time, *O. oeni* biofilm was developed on various materials including stainless steel and oak, which are used in winemaking with pumps, pipes, tanks, and barrels. Biofilm population is higher on oak than steel under the same growth conditions. This was expected, since stainless steel is frequently used in food processing to limit the adhesion of microorganisms (Hilbert et al., 2003), while wood has micro-topographical features and chemical structures that enhance bacteria adhesion (Mariani et al., 2007).

The biovolume of *O. oeni* biofilm assessed with CLSM was 4×10^5 μm^3 from 20-h growth and stayed the same until 40 h. This biovolume was close to those obtained from other LAB such as *Lactobacillus casei, Lb. plantarum*, which are around 2×10^5 μm^3 at 48 h (Rieu et al., 2014), although *O. oeni* has a slower growth rate ($\mu_{max} = 0.11$ to 0.17 h^{-1}) compared to these LAB, e.g., 0.6–0.11 h^{-1} for *L. casei* and *Lb. plantarum*. Therefore, under these confocal microscopy conditions, *O. oeni* biofilm growth reached a level similar to that of other LAB species known to form biofilms.

Biofilm lifestyle is well known to protect bacteria from harsh environmental conditions. In our model, cells from *O. oeni* biofilms were much more resistant than planktonic ones, in agreement with findings on the biofilm cells of *Lb. plantarum* that exhibit improved resistance to ethanol (Kubota et al., 2008, 2009).

In order to understand how biofilm allows cells to withstand environmental stresses, the expression of genes encoding proteins involved in the stress response of *O. oeni*, i.e., Lo18, GroEL and ClpL1 and CFA synthase was investigated (Guzzo et al., 1997; Beltramo et al., 2004, 2006; Grandvalet et al., 2008; Maitre et al., 2014). These studies revealed that stress-related genes are often overexpressed in biofilm *E. coli* populations compared with planktonic cultures, even in the absence of environmental stress (Schembri et al., 2003; Domka et al., 2007). Under our culture conditions, the stress-gene expression observed was lower in biofilm than in planktonic cells. This could be due to the kinetics of these genes' expression as a function of the growth stage in the biofilm. Indeed, stress proteins might have been produced already and fulfilled their protective role. Consequently, the biofilm could preserve its resources and energy (Beloin and Ghigo, 2005). Another explanation is related to the fact that gene expression analysis is generally global, considering the biofilm as a whole. But biofilms are described as heterogeneous populations with local spatiotemporal patterns of gene expression. This overall measure gives us an average picture of the actual gene expressions, which likely smooths out differences between cells (Coenye, 2010; Mielich-Süss and Lopez, 2015). Cells in different metabolic states within the biofilm characterize this heterogeneity. Indeed, a study on *Bacillus subtilis* biofilm cells showed that cells multiply on the surface layer, whereas in the middle of the biofilm cells produce an extracellular matrix to reinforce the biofilm structure (Vlamakis et al., 2008; Mielich-Süss and Lopez, 2015). Despite the low stress gene expression observed, the *O. oeni* cells in biofilm exhibited increasing resistance to stress, suggesting that one or more other mechanisms contribute to this tolerance. We can conclude that this observation favors the involvement of the biofilm EPS matrix, even if *O. oeni* cells in biofilm remain reactive to stress by inducing stress gene expression.

Oenococcus oeni Biofilm is Able to Perform MLF and Modulate the Organoleptic Properties of Wine: an Alternative to Adapt MLF Starters

Our study shows that *O. oeni* cultivated in biofilm kept its malic acid conversion ability under drastic conditions without any prior adaptation, due to the greater survival of biofilm cells and the diffusion of malic acid through the EPS matrix. A previous study using adapted planktonic cells (ATCC –BAA 1163) demonstrated the consumption of malic acid in 16 days (Beltramo et al., 2006). However, comparing different studies is extremely difficult since their conditions also differ. Indeed, a slight change of ethanol concentration (0.5%), pH (0.1 unit), or temperature (5°C) can change the outcome of the study. *O. oeni* biofilm cell resistance and activity seem to be close to those of immobilized cells, which are the subject of intense research. Indeed, several experiments have performed MLF with *O. oeni* immobilized on various surfaces: fibrous cellulose sponge, corn cobs, grape skins and grape stems (Genisheva Z. A. et al., 2014), resulting in varying degrees of success. The common trait between these studies is increased *O. oeni* cell resistance when immobilized, compared to the planktonic reference (Genisheva Z. A. et al., 2014). However, immobilized cells cannot be considered as a proper biofilm since cell growth, cell–cell interaction, and multifunction matrix are highly specific to the biofilm phenotype (Davey and O'toole, 2000; Hojo et al., 2009; Flemming and Wingender, 2010; Coenye, 2010).

Subsequently, our study focused on the modulation of oak flavor compounds in the wine by biofilm grown on oak. *O. oeni* glycosidase activity has been shown to release aromas from oak (Bloem et al., 2008), including vanillin (Bloem et al., 2006). Although oak aroma compounds are sought for increasing wine sensory properties, it is interesting to be able to modulate their concentration in wine (Duval et al., 2013). In our study, white wine whose MLF was carried out by biofilm on oak also exhibited these same differentiations in the aromatic profile, marked by a decrease of oak aromatic compounds (*cis*-whisky lactone, vanillin, eugenol, guaiacol, furfural). Interestingly, in the same wine, *trans*-whisky lactone was present at higher concentrations, suggesting that wood/wine interactions under the action of *O. oeni* biofilm could modulate the aromatic complexity of wine. This could be explained by the matrix covering the oak surface and acting like a filter (Dunne, 2002). These compounds may be bound with the EPS or even be converted by biofilm enzymes. Since the sensory contribution of *trans*-whisky lactone is slight (perception threshold of 110 μg/l), such aroma analyses clearly highlight the potential interest of *O. oeni* biofilms for monitoring the oak aging of wines to obtain the fine-tuned extraction of wood aromas. In the same way, wine color obtained during aging is modulated by the presence of biofilm on oak. As the biofilm modulates the organoleptic profile of wine, we suggest a retention effect of the matrix with possible interaction between EPS and wine molecules, such as macromolecules classified as anthocyanins and tannins (polyphenols).

CONCLUSION

Oenococcus oeni biofilm could be considered as a novel approach for performing MLF, and as an alternative way of adapting MLF starters to wine stress. Moreover, biofilm can modulate the organoleptic profile of the wine. These results were obtained only with unheated wood, and more in-depth investigations are needed to account for the general use of oak aging by winemakers.

AUTHOR CONTRIBUTIONS

AB performed all the experiment. RB and AC contributed to obtain the confocal microscopy data. CC and RG supervised the experiments related to organoleptic profiles. HA took part to wine production. SW and JG conceived the work and supervised the experiments. All the authors contributed to writing the paper.

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Use of Potential Probiotic Lactic Acid Bacteria (LAB) Biofilms for the Control of *Listeria monocytogenes*, *Salmonella* Typhimurium, and *Escherichia coli* O157:H7 Biofilms Formation

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Use of probiotic biofilms can be an alternative approach for reducing the formation of pathogenic biofilms in food industries. The aims of this study were (i) to evaluate the probiotic properties of bacteriocinogenic (*Lactococcus lactis* VB69, *L. lactis* VB94, *Lactobacillus sakei* MBSa1, and *Lactobacillus curvatus* MBSa3) and non-bacteriocinogenic (*L. lactis* 368, *Lactobacillus helveticus* 354, *Lactobacillus casei* 40, and *Weissela viridescens* 113) lactic acid bacteria (LAB) isolated from Brazilian's foods and (ii) to develop protective biofilms with these strains and test them for exclusion of *Listeria monocytogenes*, *Escherichia coli* O157:H7, and *Salmonella* Typhimurium. LAB were tested for survival in acid and bile salt conditions, surface properties, biosurfactant production, β-galactosidase and gelatinase activity, antibiotic resistance and presence of virulence genes. Most strains survived exposure to pH 2 and 4% bile salts. The highest percentages of auto-aggregation were obtained after 24 h of incubation. Sixty-seven percentage auto-aggregation value was observed in *W. viridescens* 113 and *Lactobacillus curvatus* MBSa3 exhibited the highest co-aggregation (69% with *Listeria monocytogenes* and 74.6% with *E. coli* O157:H7), while the lowest co-aggregation was exhibited by *W. viridescens* 113 (53.4% with *Listeria monocytogenes* and 38% with *E. coli* O157:H7). Tests for hemolytic activity, bacterial cell adherence with xylene, and drop collapse confirmed the biosurfactant-producing ability of most strains. Only one strain (*L. lactis* 368) produced β-galactosidase. All strains were negative for virulence genes *cob, ccf, cylLL, cylLs, cyllM, cylB, cylA* and *efaAfs* and gelatinase production. The antibiotic susceptibility tests indicated that the MIC for ciprofloxacin, clindamycin, gentamicin, kanamycin, and streptomycin did not exceed the epidemiological cut-off suggested by the European Food Safety Authority. Some strains were resistant to one or more antibiotics and resistance to antibiotics was species and strain dependent. In the protective biofilm assays, strains *L. lactis* 368 (bac−), *Lactobacillus curvatus* MBSa3 (bac+), and *Lactobacillus sakei* MBSa1 (bac+) resulted in more than six log reductions

in the pathogens counts when compared to the controls. This effect could not be attributed to bacteriocin production. These results suggest that these potential probiotic strains can be used as alternatives for control of biofilm formation by pathogenic bacteria in the food industry, without conferring a risk to the consumers.

Keywords: biofilm, probiotic, lactic acid bacteria, exclusion, pathogens, biocontrol

INTRODUCTION

Lactic acid bacteria (LAB) constitute part of the autochthonous microbiota of many types of foods. They are defined as a cluster of lactic-acid-producing, low G + C%, non-spore-forming, Gram-positive rods and cocci and catalase-negative bacteria which share many biochemical, physiological, and genetic properties (Abriouel et al., 2012). This group of bacteria has a particular interest for food industries due to their technological properties, being often used as starter cultures to produce fermented products (Lahtinen et al., 2011). Many reports have shown that traditional fermented foods are rich sources of LAB with probiotic characteristics (Liu et al., 2011; Favaro et al., 2014; Palomino et al., 2015).

According to FAO/WHO (2006), probiotics are live microorganisms which administered in adequate amounts confer a health benefit on the host (FAO/WHO, 2006). The principal functional properties of probiotics include tolerance to acid and bile, adherence to epithelial surfaces, and antagonistic activity toward intestinal pathogens. Probiotics may confer their health benefits by several mechanisms; by contributing to colonization resistance, reinforcing the intestinal barrier (i.e., tight junction expression, secretion of mucus, and antimicrobial peptides), modulating the immune system and instructing the intestinal microbiota composition and activity (Wan et al., 2015). This is based on either direct cell–cell contact, secreting various molecules and/or microbial cross-feeding (Jonkers, 2016). Auto-aggregation of probiotic strains seems to have influence on their adhesion to intestinal epithelial cells, while co-aggregation with pathogens may prevent colonization in the gut and their consumption reduces the viable number of pathogens while strengthening body natural defenses (Savard et al., 2011). Del Re et al. (2000) demonstrated that auto-aggregation is strongly related to adhesion. In addition, adhesion of probiotic bacteria to mucosa is one of the mechanisms by which they can overcome competition with other microorganisms. Nevertheless, production of bacteriocins and other antimicrobial substances by bacteria in biofilms and adhered to mucosal surfaces is considered relevant for the displacement of pathogens, as demonstrated in gastrointestinal tract (GIT) models (Ganzle et al., 1999). Bacteriocin-producing *Lactobacillus curvatus* LTH 1174 provided protection against *E. coli* LTH 1600 and *Listeria innocua* DSM20649 invasion during transit through in a dynamic model of the human stomach and small intestine (GIT model; Ganzle et al., 1999) and bacteriocin-producing *Lactobacillus sakei* 2a protected gnotobiotic mice against experimental challenge with *Listeria monocytogenes* (Bambirra et al., 2007). These data suggest that bacteriocin-producing lactobacilli prevent new strains from invading or maintaining stable populations in the colon. Therefore, bacteriocin production is often considered a probiotic trait in this context.

Studies carried out both in culture media and foods have shown that bacteriocins produced by probiotic or potentially probiotic LAB can act synergistically or have an additive effect in the antimicrobial activity when combined with other antimicrobials (Viedma et al., 2010; Gómez et al., 2012). Interestingly, LAB may simultaneously secrete organic acids, bacteriocins, and biosurfactants (Kanmani et al., 2013). The precise role of these compounds on other bacterial populations present in biofilms is not yet known, but it is well recognized that bacteriocins have stronger antimicrobial activity under acidic conditions (Gálvez et al., 2010).

The presence of biofilms is a relevant risk factor in the food industry due to the potential contamination of food products with pathogenic and spoilage microorganisms. Biofilms can be formed on surfaces becoming permanent reservoirs of bacteria. Most important, biofilms may act as reservoirs of pathogenic and spoilage bacteria, in which these microorganisms can persist against the cleaning and disinfection processes. For example, contamination of equipment with biofilms was a contributing factor to 59% of food-borne disease outbreaks investigated in France (Midelet and Carpentier, 2004). The presence of biofilms is common in food industry and represents a concern because bacteria can adhere to almost any type of surface, such as plastic, metal, glass, soil particles, wood food products (Gandhi and Chikindas, 2007).

Listeria monocytogenes is commonly found in food-processing environment, and it has been isolated from both meat and dairy processing plants (Winkelströter et al., 2013) and Mendonça et al. (2012) also demonstrated that *E. coli* O157:H7 has the potential to form biofilm on different surfaces commonly used in food industry. Common sites for the presence of *Salmonella* spp. in food-processing plants are filling or packaging equipments, floor drains, walls, cooling pipes, conveyors, collators for assembling product for packaging, racks for transporting products, hand tools or gloves, freezers, etc, which are usually made of plastics (Pompermayer and Gaylarde, 2000). In addition, a study of 122 *Salmonella* strains indicated that all had the ability to adhere to plastic microwell plates and that; generally, more biofilm was produced in low nutrient conditions, as can be found in specific food-processing environments, compared to high nutrient conditions (Stepanović, 2004).

The increased resistance of biofilm cells to biocides can be partially due of the exopolymeric matrix interference and this can explains why the disinfectant most effective to planktonic cells is not necessarily the most active against biofilm cells

(Van Houdt and Michiels, 2010). *Listeria monocytogenes* cells residing in so-called refuge sites such as cracks, worn equipment and in hard to reach places such as complex machinery may be subjected to suboptimal disinfection concentrations allowing them to survive and possibly adapt to cleaning and sanitation treatments (Carpentier and Cerf, 2011).

Recent trends in the transmission and emergence of resistant pathogenic bacteria through the food chain reinforce the need to investigate several alternatives for disinfection. For this reason, there is a great interest in the development of novel strategies using natural products to control the persistence of pathogens associated with surfaces or equipment especially in food industry. Therefore, biofilms formed by LAB present in foods, agricultural products or in the GIT of mammals and used as starters in food manufacturing, may offer a promising means to counteract the establishment of pathogenic biofilms (Winkelströter et al., 2013).

A very promising approach for the control of biofilm formation is the use of probiotics to colonize hard surfaces in order to counteract the proliferation of other bacterial species, based on the competitive exclusion principle (Falagas and Makris, 2009; Hibbing et al., 2010). This concept has been designated as biocontrol when the application is antagonistic toward a certain pathogen (Gatesoupe, 1999). LAB successfully reduced *Listeria monocytogenes* in a ready-to-eat poultry processing plant (Zhao et al., 2013) and lactobacilli with biofilm-forming aptitudes were able to control *Listeria monocytogenes* on abiotic surfaces (Pérez-Ibarreche et al., 2014). In addition, several studies have shown that bacteriocin-producing LAB improved the bactericidal effect of biocides on bacterial biofilms (Lobos et al., 2009; Gómez et al., 2012).

Application of bacteriocins and/or their producer strains for inhibition of biofilm formation and/or killing of cells embedded in biofilms is a novel field of research. The objectives of this study were to evaluate the potential probiotic traits of LAB isolated from different fermented Brazilian products and their inhibition effect against *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *S.* Typhimurium biofilm formation. Tolerance to low pH and bile salts, surface properties (aggregation and co-aggregation), biosurfactant production, gelatinase activity, antibiotic resistance and virulence genes absence were evaluated as probiotic properties of the studied LAB.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

The study was conducted with eight LAB strains isolated from foods (**Table 1**): bacteriocin producers *Lactococcus lactis* VB69 and VB94 were isolated from Brazilian charqui (Bíscola et al., 2013) and *Lactobacillus sakei* MBSa1 and *Lactobacillus curvatus* MBSa3 were isolated from salami (Barbosa et al., 2015). Non-bacteriocin producers *Lactococcus lactis* 368, *Lactobacillus helveticus* 354 isolated from goat cheese and *Lactobacillus casei* 40 and *W. viridescens* 113 isolated from ripened cheese (unpublished). The strains were identified by 16S rDNA gene sequencing, according to Cibik et al. (2000), in a CEQ2000 XL DNA Analysis System (Beckman Coulter, Brea, CA, USA). LAB strains were cultivated in De Man et al. (1960) broth (Oxoid, Basingstoke, England) at 30°C for 18 h. *E. coli* O157:H7 ATCC 35150, *Listeria monocytogenes* ATCC 7644 and *S.* Typhimurium ATCC 14028 were cultured in trypticase soy broth (TSB, Oxoid, Basingstoke, England) at 37°C for 20 h. All strains were maintained at −80°C in the appropriate cultivation broth containing 20% (v/v) glycerol.

Auto-Aggregation and Co-Aggregation Assays

Aggregation abilities of LAB strains were studied as described by Collado et al. (2008), with some modifications. Bacterial cells from an overnight culture were harvested by centrifugation (5,000 × *g*, 20 min, 4°C), washed twice with phosphate-buffered saline PBS pH 7.1 (10 mM Na_2HPO_4, 1 mM KH_2PO_4, 140 mM NaCl, 3 mM KCl) and suspended in the same buffer. Absorbance ($A_{600\ nm}$) was adjusted to 0.25 ± 0.05 in order to standardize the number of bacteria (10^7–10^8 CFU/ml). The optical density ($OD_{600\ nm}$) of a homogenized bacterial suspension was first recorded then repeated on the same suspension left to rest for 24 h at 37°C without vortexing. The aggregation percentage was expressed as $[1 - (A_{Time}/A_0) \times 100]$ where A_{Time} represents the absorbance of the mixture at 24 h and A_0, absorbance at time 0.

For the co-aggregation assays, LAB bacterial suspensions prepared as described above were mixed with equal volumes (500 μl) of the cultures of the pathogens listed in Section "Bacterial Strains and Growth Conditions." Mixtures were incubated at 37°C without agitation, and absorbance ($OD_{600\ nm}$) measured after 24 h at 37°C. The percentage of co-aggregation was calculated as $[(A_{pathog} + A_{LAB})/2 - (A_{mix})/(A_{pathog} + A_{LAB})/2] \times 100$ (Handley et al., 1987), where A_{pathog} and A_{LAB} represent the absorbance in the tubes containing only the pathogen or the LAB strain, respectively, and A_{mix} represents the absorbance of the mixture at 24 h (García-Cayuela et al., 2014).

Tolerance to Bile Salts and Acidic pH

The LAB strains were tested for bile salt tolerance (0–10%) and survival at low pH (1.5–3) according to Millette et al. (2008). The bile salt tolerance was ascertained in MRS agar containing a commercial preparation of bile salts normally used to inhibit the growth of Gram-positive bacteria in broth (Sigma–Aldrich, B-3426). The bile salt mixture was added in concentrations varying from 0 to 10% with increments of 1%. Another bile salt preparation (LP 0055; Oxoid, Basingstoke, England) was also evaluated in concentrations varying from 0 to 20% with increments of 4% to avoid differences between the different compounds. The MRS agar containing the bile salts was autoclaved for 15 min at 121°C, cooled, and plated. Aliquots of overnight MRS broth cultures (100 μl of bacteria in the stationary phase obtained after 24 h of growth) were inoculated onto the surface of the bile-salt-containing MRS agar, and incubated at 37°C for 72 h. The plates were examined visually for bacterial growth as a lawn, indicating resistance to bile salts in the tested concentration. For determination of acid tolerance, 1 ml overnight MRS broth cultures were inoculated

TABLE 1 | Bacterial strains used in this study.

Identification code	Strain	Isolation source	Bacteriocine production
MBSa1	*Lactobacillus sakei*	Salami	Sakacine A
MBSa3	*Lactobacillus curvatus*	Salami (Barbosa et al., 2015)	Sakacine P
VB69	*Lactococcus lactis*	Charque (Bíscola et al., 2013)	Nisin Z
VB94	*Lactococcus lactis*	Charque (unpublished)	Nisin Z
40	*Lactobacillus casei*	Ripened cheese (unpublished)	No producer
352	*Lactobacillus helveticus*	Goat cheese (unpublished)	No producer
368	*Lactococcus lactis*	Goat cheese (unpublished)	No producer
113	*Weisella viridescens*	Ripened cheese (unpublished)	No producer

onto 19 ml of simulated gastric fluid (3.2 g/l pepsin and 2 g/l NaCl) adjusted to different pHs (1.5, 2, 2.5, and 3) values with 5 M HCl. After incubation for 30 min at 37°C, 1 ml of the mixture was removed to determine viable counts (expressed as CFU/ml) on MRS agar taking as reference the concentration of bacteria not exposed to simulated gastric fluid. *Lactobacillus rhamnosus* GG (lab collection) was used as a positive control because it is a probiotic bacterium well known for its resistance to gastrointestinal conditions.

β-Galactosidase Activity

The LAB strains were grown in MRS broth at 37°C for 24 h, streaked onto MRS agar and incubated at 37°C for 48 h. One colony was transferred to a tube containing a disk of *O*-nitrophenyl-β-D-galactopyranoside—ONGP (Sigma–Aldrich) and 100 μl sterile saline (0.85% NaCl). A yellow color indicated the release of o-nitrophenol (chromogenic compound) and represented a positive result for the production of β-galactosidase.

Hemolytic Activity

Testing for hemolytic activity was carried out as described by Carrillo et al. (1996). Isolated strains were screened for hemolytic activity on blood agar plates containing 5% (v/v) horse blood and incubated at 30°C for 24–48 h. A clear zone around the colony indicated hemolytic activity, which was probably caused by surfactant production. The zones of clearing were scored as follows: (−) no hemolysis; (+) incomplete hemolysis, when the zone was not totally clear; (++) complete hemolysis with a diameter of lysis < 1 cm; (+++) complete hemolysis with a diameter of lysis between 1 cm and 3 cm; and (++++) complete hemolysis with a diameter of lysis > 3 cm.

Drop Collapse Test

The drop collapse test was carried out as described by Jain et al. (1991). LAB were cultivated in MRS at 37°C for 24 h, centrifuged at 12,000 × *g* for 5 min and 100 μl of the supernatants were added to each well of 96-well microplates (TPP, Switzerland) and then 5 μl of crude motor oil was added to the surface. A result was considered positive for biosurfactant production when the drop diameter was at least 1 mm larger than that produced by deionized water (negative control). Each test was repeated in two separate microtiter plates.

Microbial Adhesion to Hydrocarbon Test (MATH)

Bacterial cell surface hydrophobicity was assessed by measuring adhesion to hydrocarbons (MATH) as described by Kotzamanidis et al. (2010). LAB cultivated in MRS at 37°C for 24 h were washed twice in phosphate-buffered saline (PBS; 10 mM Na_2HPO_4, 1 mM KH_2PO_4, 140 mM NaCl, 3 mM KCl) and re-suspended in 3 mL of 0.1 M KNO_3 to achieve approximately 10^8 CFU/ml ($OD_{600\ nm} = 0.2$). Absorbance of the suspension was measured at 600 nm (A_0). One microliter of xylene was added to the cell suspension to form a two-phase system and after 10 min at room temperature, the two-phase system was mixed by vortexing for 2 min. After 20 min at room temperature (approximately 23°C), the aqueous phase was carefully removed and absorbance at 600 nm (A_1) measured. The percentage of cell surface hydrophobicity (H, %) was calculated using the following formula: $H\ (\%) = (1\ A_1/A_0)\ ^* 100$, where A_1 represents the absorbance of the mixture after 20 min at room temperature and A_0, absorbance at time 0.

Gelatinase Activity

Gelatinase production was verified by spotting 1 μl aliquots of the 24 h cultures onto the surface of five Luria Bertani agar plates (BD, Franklin Lakes, NJ, USA) supplemented with 3% (w/v) gelatin (BD). Plates were incubated at 37°C and 42°C for 48 h, 25°C for 72 h, and 10°C and 15°C for 10 days. After incubation, the plates were maintained at 4°C for 4 h and the hydrolysis of gelatin was recorded by the formation of opaque halos around the colonies (Perin et al., 2014).

Antibiotic Resistance

The resistance to antibiotics was determined by the broth microdilution protocol according to Muñoz et al. (2014) with some modifications. Antibiotics employed in this study were β-lactams (ampicillin: AMP), quinolone (ciprofloxacin: CIP), lincosamide (clindamycin: CLI), aminoglycosides (gentamicin: GEN, kanamycin: KAN and streptomycin: STR), macrolides (erythromycin: ERY), glycopeptides (vancomycin: VAN), chloramphenicol: CMP and tetracycline: TET. These antibiotics were selected based on the European Food Safety Authority recommendations for probiotics strains (European Food Safety Authority [EFSA], 2012). All antibiotics were purchased from Sigma–Aldrich, USA. To prepare the stock antibiotic

solutions, each antibiotic was weighed, dissolved in sterile distilled water (except CMP which was dissolved in sterile distilled water with 0.5% of ethanol), filter-sterilized (0.2 mm) and kept at −20°C until use. The working solutions at specific concentrations were prepared daily. Overnight cultures were adjusted to $OD_{600\ nm}$ of 0.8 (10^9 CFU/ml) with PBS, and used to inoculate (1% v/v) Mueller Hinton broth (Oxoid, Basingstoke, England) containing each antibiotic at tested concentrations (final volume of 100 µl per well of 96 micro-well plates). The plates were incubated at 37°C for 24 h. Resistance rates were calculated according to microbial cut-off values (mg/ml), as recommended by the European Food Safety Authority [EFSA] (2012). The microbiological breakpoints were defined according to Danielsen and Wind (2003), Flórez et al. (2005) and the European Commission (European Commission SCAN, 2007).

Virulence Genes

Total DNA extraction was performed using a Blood and Tissue mini kit Quiagen (German Town, USA). The primers used for the amplification of genes *esp, agg, gelE, efaAfm* and *efaAfs, cylA, cylB* and *cylM* were described by Eaton and Gasson (2001), and primers of *cyl* operon (*cylLL* and *cylLS*) were developed by Semedo et al. (2003). **Table 2** describes the primers used in these tests. All primers were synthesized by Life Technology (Brazil). PCR amplifications were performed in a ThermoCycler AB (Applied Biosystems Veriti, NJ, USA), in 0.2-ml reaction tubes containing 25 µl of GoTaq® Green Master Mix, 2.5 µ1 (10 µM) of each primer, and 1 µl (100 ng) of DNA. Amplification reactions were as follows: initial cycle of 94°C for 1 min, 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, a final extension step of 72°C for 7 min and then cooling to 4°C. Amplification products were submitted to electrophoresis in 1% (w/v) agarose gel at 100 V for 30 min. A 100-bp PCR DNA ladder was used as the molecular weight marker. The gels were photographed on a Gel Doc™ XR+ System (BioRad, Richmond, CA, USA), and image analysis was accomplished using Quantity One software. The positive control was *Enterococcus faecalis* FI 9190 (obtained from Eaton and Gasson, 2001, Institute of Food Research, Norwich Research Park, Norwich, UK). For each PCR, a negative control (sample without template) was included.

TABLE 2 | Primers used to test for the presence of virulence genes.

Target gene*	Primers	Fragment size (pb)
Agg	AAGAAAAAGAAGTAGACCAAC AAACGGCAAGACAAGTAAATA	1,553
GelE	ACCCCGTATCATTGGTTT ACGCATTGCTTTTCCATC	419
esp	TTGCTAATGCTACTCCACGACC GCGTCAACACTTGCATTGCCGAA	933
efaAfs	GACAGACCCTCACGAATA AGTTCATCATGCTGTAGTA	705
efaAfm	AACAGATCCGCATGAATA CATTTCATCATCTGATAGTA	735
cpd	TGGTGGGTTATTTTTCAATTC TACGGCTCTGGCTTACTA	782
Cob	AACATTCAGCAAACAAAGC TTGTCATAAAGAGTGGTCAT	1,405
Ccf	GGGAATTGAGTAGTGAAGAAG AGCCGCTAAAATCGGTAAAAT	543
cylLL	GATGGAGGGTAAGAATTATGG GCTTCACCTCACTAAGTTTTATAG	253
cylLS	GAAGCACAGTGCTAAATAAGG GTATAAGAGGGCTAGTTTCAC	240
cylM	AAAAGGAGTGCTTACATGGAAGAT CATAACCCACACCACTGATTCC	2,940
cylB	AAGTACACTAGTACAACTAAGGGA ACAGTGAACGATATAACTCGCTATT	2,020
CylA	TAGCGAGTTATATCGTTCACTGTA CTCACCTCTTTGTATTTAAGCATG	1,282

**Agg (Aggregation protein), gelE (gelatinase), esp (cell-wall-associated protein) efaAfm and efaAfs (cell wall adhesins), cpd, cob and ccf (sex pheromones, chemotactic for human leukocytes, facilitate conjugation), cylLL and cylLS (Cytolysin precursor), cylM (post-translational modification of cytolysin), cylB (transport of cytolysin) and cylA (activation of cytolysin).*

Biofilm Assay

The quantification of biofilm production was performed as described previously by Borges et al. (2012) with some modifications. The wells of a sterile 12-well polystyrene microtiter plate (TPP, Switzerland) were filled with 2 ml of MRS broth, absorbance ($A_{600\ nm}$) of bacterial suspensions in MRS was adjusted to 0.25 ± 0.05 in order to standardize the number of bacteria (10^7–10^8 CFU/ml) and 200 µl of overnight was added to each well. The plates were incubated aerobically for 48 h at 30°C. To quantify the biofilm formation, the wells were gently washed three times with 2 ml of sterile distilled water. The attached bacteria were fixed with 2 ml of methanol (Romyl, Leics, UK) for 15 min, and then, microplates were emptied and dried at room temperature. Subsequently, 2 ml of a 2% (v/v) crystal violet solution was added to each well and held at ambient temperature for 5 min. Excess stain was then removed by placing the plate under gently running tap water. Stain was released from adherent cells with 2 ml of 33% (v/v) glacial acetic acid. The optical density (OD) of each well was measured at 595 nm using a plate reader (Microplate reader, Bio-Rad, Hercules; CA, USA). Each assay was performed in four replicates and conducted three individual times on different days under the same conditions, and the negative control was performed in uninoculated MRS broth. The cut-off (ODC) was defined as the mean OD value of the negative control. Based on the OD, strains were classified as non-biofilm producers ($OD \leq ODC$), weak ($ODC < OD \leq 2 \times ODC$), moderate ($2 \times ODC < OD \leq 4 \times ODC$) or strong biofilm producers ($4 \times ODC < OD$; Borges et al., 2012).

Inhibition of Biofilm Formation

Lactic acid bacteria strains were inoculated (1% v/v) in 2 ml of MRS broth diluted to one-fifth of the concentration recommended by the manufacturer (55 g/l) and transferred (2 ml/well) to 12-well polystyrene microtiter plates (TPP, Switzerland). The plates were incubated at 30°C for 48 h for attachment of cells to the wells (biofilm formation). The broths were carefully discarded by pipetting and the biofilms visually present on the bottom and sides of the plate were washed with 2 ml PBS pH 7.1 (10 mM Na_2HPO_4, 1 mM KH_2PO_4, 140 mM

NaCl, 3 mM KCl) to remove planktonic and loosely attached cells. Absorbance ($A_{600\ nm}$) of pathogenic bacterial suspensions in TSB was adjusted to 0.25 ± 0.05 in order to standardize the number of bacteria (10^7–10^8 CFU/ml), added to biofilms and incubated at 30°C for 24, 48, and 72h. Every 24 h, half of the broth in the wells was replaced with fresh broth. After incubation, the planktonic cultures were carefully removed and the biofilms were suspended by scrapping and vigorous shaking.. To evaluate the viable count of adherent microorganisms in the biofilm, three wells for each strain were washed three times as previously described and scraped.The obtained suspensions were transferred into sterile tubes and mixed with a vortex mixer for 30 s. Proper dilutions were prepared in saline solution 0.85% (w/v) and plated on xylose lysine deoxycholate agar (XLD) for *S.* Typhimurium, Modified Oxford agar (MOX) for *Listeria monocytogenes* and MacConkey sorbitol agar (SM) for *E. coli* O157:H7. The plates were incubated at 37°C for 24–48 h and bacterial counts were performed.

Listeria monocytogenes, *S.* Typhimurium, and *E. coli* O157:H7 controls were used to monitor the biofilm development of these strains without the presence of LAB biofilms.

Pathogenic planktonic cells counts were performed from the broths discarded by pipetting, following the same procedures used for biofilm cell count (data not shown).

Statistical Analysis

All experiments were carried out three times, with duplicate samples per trial, and results were expressed as average. Standard deviations were determined with Excel programme (Microsoft Corp., USA). A *t*-test was performed at the 95% confidence interval with PASW Statistics—SPSS 17 (IBM Co.), in order to determine the statistical significance of data.

RESULTS

Auto-Aggregation and Co-Aggregation Assays

Aggregation values increased over time in a strain-dependent manner. *W. viridescens* 113 presented the highest auto-aggregation (67%), compared to the other isolates showing only moderate auto-aggregation (**Figure 1**). All LAB strains presented co-aggregation with pathogens (**Figure 2**), in a strain–pathogen combination-dependent manner. *Lactobacillus curvatus* MBSa3 exhibited the highest co-aggregation (69% with *Listeria monocytogenes* and 74.6% with *E. coli* O157:H7), while the lowest co-aggregation was exhibited by *W. viridescens* 113 (53.4% with *Listeria monocytogenes* and 38% with *E. coli* O157:H7).

Tolerance to Bile Salts and Acidic pH

The results showed that tolerance for bile salts mixture from Sigma was 4% for all LAB strains. However, the tolerance to bile salts from Oxoid was 20% for *W. viridescens 113* and *L. lactis* 94 and 8% for *Lactobacillus casei* 40 and *L. lactis* 69 (data not shown) for the rest of studied strains was 4%. The results in **Figure 3** show that all tested strains, including *Lactobacillus rhamnosus* GG, survived to exposure to pH 2.5 for 30 min. No significant difference ($p < 0.05$) between the initial microbial population and the population after 30 min at pH 2.5 was observed for all strains; a reduction of viability was only observed for *W. viridescens* 113, approximately 2 log. However, a significant reduction of viability at pH 2.0 was observed for all tested bacteria except for *L. lactis* 94. In counterpart, complete survival at pH 3 and no survival at pH 1.5 were observed for all strains.

Biosurfactant Production

The three screening tests indicated that all tested LAB strains were capable to produce biosurfactant (**Table 3**). In the hemolysis test, most strains showed zones of clearing in the blood agar with scores corresponding to (++) indicating complete hemolysis with a diameter < 1 cm. The exception was *L. lactis* 94 that was not hemolytic. In the MATH assay, the lowest values observed were 77.2% (*Lactobacillus casei* 40); 76.4% (*Lactobacillus curvatus* MBSa3); 81.2% (*L. lactis* 368) and 88.9% (*Lactobacillus sakei* MBSa1) for the rest of LAB studied the values was over 90% with 91.2% (*Lactobacillus helveticus* 352); 93.9% (*W. viridescens* 113); 95.1% (*L. lactis* 94) and 95.2% (*L. lactis* 69).

All strains resulted positive in the drop collapse test. Flat drops with scoring system ranging from + to ++++ corresponding to partial to complete spreading on the oil surface. The strains studied did not present complete spreading on the oil surface only a partial spreading was observed, varying between + for *L. lactis* 94, *Lactobacillus casei* 40, and *Lactobacillus helveticus* 352 to ++ in the rest of strains studied, *L. lactis* 69, *W. viridescens* 113 *Lactobacillus sakei* MBSa1 and *Lactobacillus curvatus* MBSa3.

Antibiotic Resistance, Presence of Virulence Genes and Gelatinase Activity

The antibiotic susceptibility tests (**Table 4**) indicated that the MIC for ciprofloxacin, clindamycin, gentamicin, kanamycin, and streptomycin did not exceed the epidemiological cut-off suggested by the European Food Safety Authority [EFSA] (2012) for all tested strains. All strains were sensitive to β-lactams (ampicillin: AMP), except *Lactobacillus curvatus* MBSa1. Some strains were resistant to one or more antibiotics: *Lactobacillus casei* 40 and *Lactobacillus curvatus* MBSa1 were resistant to erythromycin, *Lactobacillus sakei* MBSa3 and *Lactobacillus casei* 40 were resistant to chloramphenicol; *Lactobacillus curvatus* MBSa1, *L. lactis* 94 and 368 were resistant to vancomycin. Only *Lactobacillus casei* 40, *Lactobacillus helveticus* 352 and *L. lactis* 69 were sensitive to tetracycline. All strains were sensitive to erythromycin, except *Lactobacillus casei* 40 and *Lactobacillus curvatus* MBSa1.

Table 5 shows the presence of the virulence genes tested in the LAB strains. All strains were negative for *GelE*, *cob*, *ccf*, *cylLL*, *cylLs*, *cyllM*, *cylB*, *cylA* and *efaAfs*, except *W. viridescens* 113 that was positive for *cob* and for *GelE*. Nevertheless, no strain presented gelatinase activity. *Lactobacillus helveticus* 352 was positive for *Agg* and *efaAfm, L. lactis* 368 for *cpd* and *efaAfm* too and the presence of *esp* was observed in *L. lactis* 94 and 69, *Lactobacillus casei* 40, and *Lactobacillus curvatus* MBSa3.

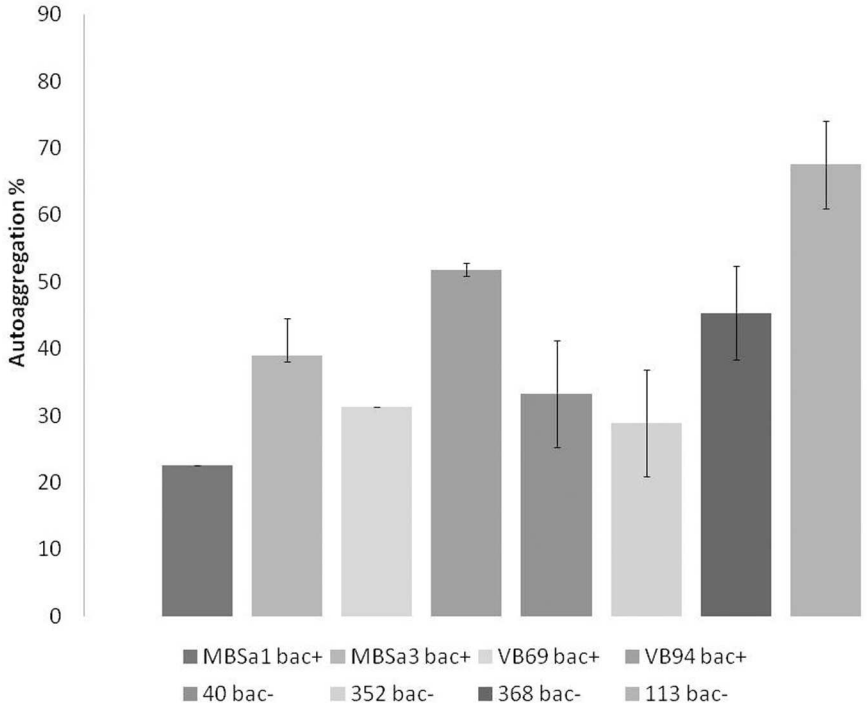

FIGURE 1 | Auto-aggregation of lactic acid bacteria strains cells re-suspended in PBS (pH 7.1) evaluated after 24 h incubation at 37°C. Error bars represent standard deviations of the mean values of results from three replicated experiments (bac^+ = bacteriocin producer).

Biofilm Assay

All the strains studies were biofilm producers in MRS. The biofilm production was strain dependent (**Figure 4**). Based on the OD, all the strains studied were strong producer's except *W. viridescens* 113. The highest values over 1, were observed for *L. lactis* 368 (1.65), *Lactobacillus helveticus* 352 (1.38) and *L. lactis* 94 (1.10). The values for the rest of strains were under 1, but all were strong biofilm producers except *W. viridescens* 113 with moderate biofilm formation.

Inhibition of Biofilm Formation

The total inhibition in pathogens *E. coli* O157:H7, *Listeria monocytogenes* and *S.* Typhimurium biofilm formation, in 24, 48, and 72 h of exposure, was obtained for *L. lactis* 368 (bac−), *Lactobacillus curvatus* MBSa3 (bac+) and *Lactobacillus sakei* MBSa1 (bac+). For the other strains, the inhibition was time-dependent and varied according to the strain and target pathogen (**Figure 5**). The presence of sessile cells of *E. coli* O157:H7, *Listeria monocytogenes* and *S.* Typhimurium in the presence of LAB in 24, 48, and 72 h was significantly reduced in comparison to the pure cultures ($p < 0.05$). *Listeria monocytogenes* was not detected within *L. lactis* 69 (bac+) and 94 (bac+) established biofilms following 24 h and 48h interaction periods. Nevertheless, the presence of *Listeria monocytogenes* biofilms were observed in the cases of *W. viridescens* 113 (bac−), *Lactobacillus casei* 40 (bac−) and *Lactobacillus helveticus* 352 (bac−); 4 log of decrease was observed for 24 h of incubation in presence of *Lactobacillus helveticus* 352 (bac−) biofilm, as well as, 7 log of decrease for *Lactobacillus casei* 40 (bac−) during the same incubation time. After 48 h of incubation 5 log of decrease were detected in the presence of *W. viridescens* 113 (bac−). The presence of *Listeria monocytogenes* biofilms was detected during 72 h of incubation in all cases, varying between 4 log for *W. viridescens* 113 (bac−), *Lactobacillus helveticus* 352 (bac−) and *Lactobacillus casei* 40 (bac−) to 6 log of decrease in the cases of *L. lactis* 94 (bac+) and 69 (bac+). In *S.* Typhimurium experiment, sessile cells were not detected during 24 h of incubation in the presence of most LAB tested, only for *Lactobacillus helveticus* 352 (bac−) 2 log were achieved (6 log of decrease). After 48 and 72 h only in the presence of *Lactobacillus casei* 40 (bac−) sessile cells of *S.* Typhimurium were not detected. For *E. coli* O157:H7 only after 24 h of incubation the presence was not detected, except for *Lactobacillus helveticus* 352 (bac−). During 48 and 72 h approximately 3 log of *E. coli* was detected (5 log of decrease) in the presence of all tested LAB. In most cases, reductions between 5 and 3 log for *E. coli* O157:H7, 4log for *S.* Typhimurium and between 7 and 3 log for *Listeria monocytogenes* were achieved.

In addition, when supernatants were studied, planktonic pathogens cells were not detected, in all studied cases counts of pathogenic cells were below the detection limit (<10 CFU/ml, data not shown).

DISCUSSION

The increased resistance to disinfection processes may be aggravated when bacterial biofilms are formed on surfaces

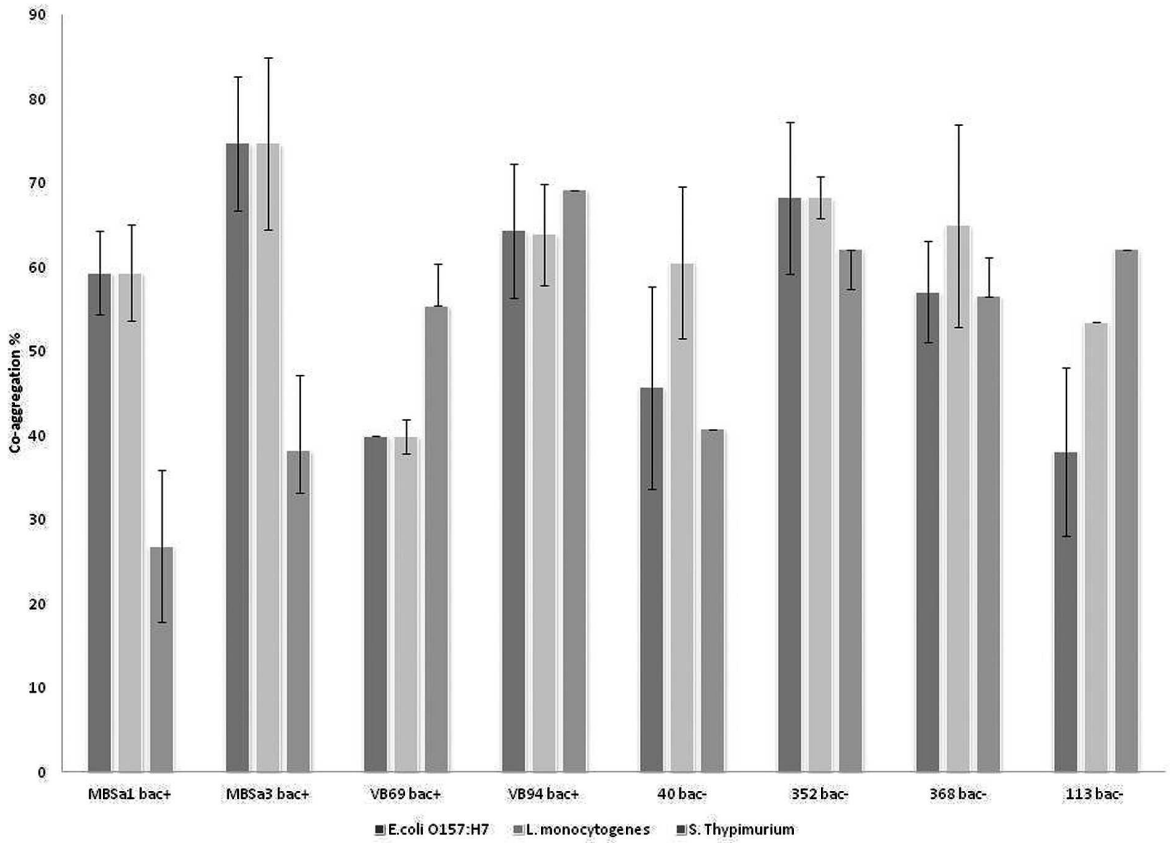

FIGURE 2 | Co-aggregation values recorded for lactic acid bacteria strains with *Listeria monocytogenes* ATCC 7644, *Salmonella* Typhimurium ATCC 14028, and *Escherichia coli* O157:H7 ATCC 35150 after 24 h incubation at 37°C in PBS (pH 7.1). Error bars represent standard deviations of the mean values of results from three replicate experiments (bac^+ = bacteriocin producer).

that are recalcitrant for clean, such as cracks, holes, or tube connections. When planktonic cells are released from these colonization microenvironments, they may enter the food production chain and proliferate if proper conditions for growth occur, compromising the safety, quality, and stability of the final product. The application of the competitive biofilms formed by bacteria that produce natural antimicrobial substances and biosurfactants can provide new opportunities for the control of pathogenic bacteria and avoid food cross contamination.

Aggregation and co-aggregation among bacteria play an important role in prevention of colonization of surfaces by pathogens (García-Cayuela et al., 2014) as it is well known that co-aggregation abilities of LAB strains might interfere with the ability of the pathogenic species to infect the host and can prevent the colonization of food-borne pathogens (García-Cayuela et al., 2014). In this study, the tested LAB, especially the bacteriocin-producing *Lactobacillus* strains, presented high auto-aggregation and co-aggregation results, *Lactobacillus curvatus* MBSa3 exhibited the highest co-aggregation (69% with *Listeria monocytogenes* and 74.6% with *E. coli* O157:H7) and in this case pathogenic biofilms were not detected after three times of incubation tested, 24, 48, and 72; in other side the lowest co-aggregation was exhibited by *W. viridescens* 113 (53.4% with *Listeria monocytogenes* and 38% with *E. coli* O157:H7) and pathogenic cells were detected in 48 and 72 h of incubation in the presence of biofilm from strain. Nevertheless in other strains, there was apparently no relationship between the detection of pathogens and the percentage of co-aggregation with them.

Aggregation can also increase the concentration of excreted inhibitory substances (Kaewnopparat et al., 2013). Thus, these food-associated lactobacilli that co-aggregate numerous pathogens are of special interest with regard to potential applications in food-processing plants. Correlation between adhesion ability and hydrophobicity, as measured by microbial adhesion to hydrocarbons, has been reported for some lactobacilli (Wadström et al., 1987), but also conflicting results have been reported (Vinderola et al., 2004). As a result, adhesion, surface hydrophobicity, autoaggregation, and co-aggregation are phenotypic traits that potentially provide microbial colonization advantages within the intestinal tract. Aggregation abilities and cell surface hydrophobicity may not be the only components responsible for adhesion but these are some of the criteria to bear in mind of a complex mechanism that enables microorganisms to interact with the host and exert its beneficial effect (García-Cayuela et al., 2014).

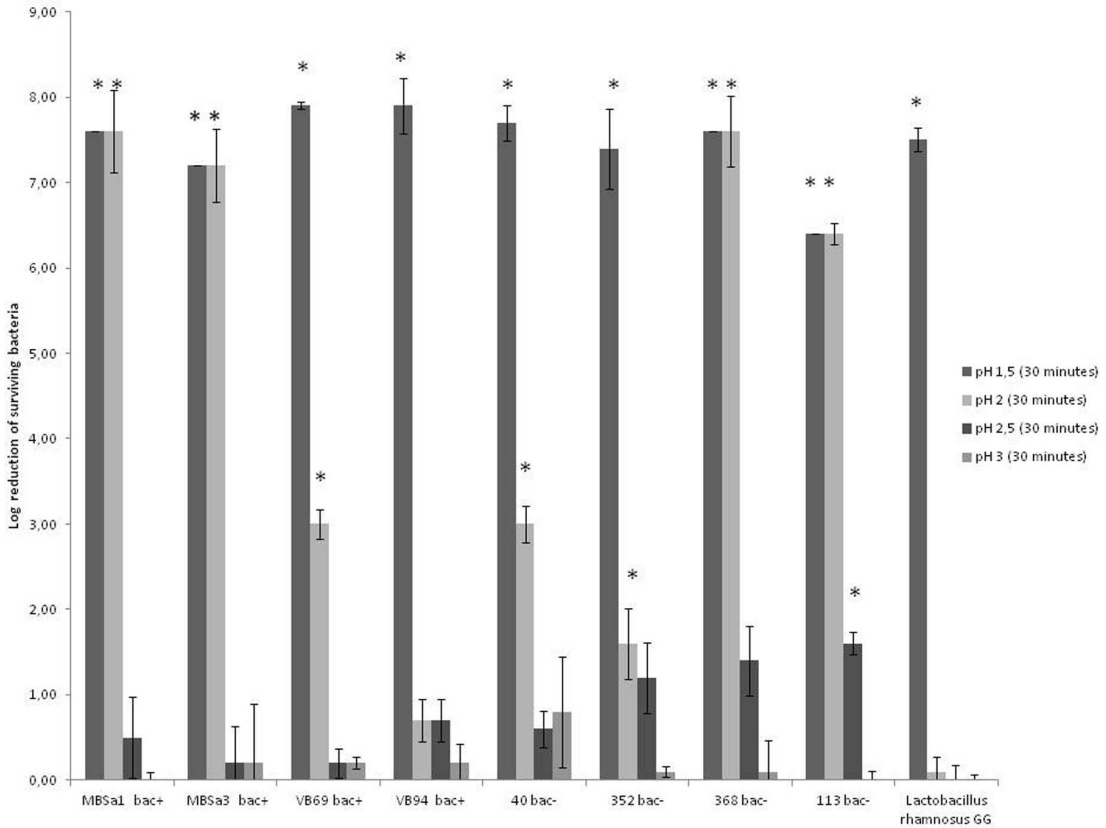

FIGURE 3 | Log reduction of lactic acid bacteria strains after incubation at 37°C for 30 min in (SFG) Simulated Grastric Fluid; 3.2 g/l pepsin and 2 g/l NaCl (pH 1.5–3). Log reductions were estimated by subtracting the log of surviving to the controls (bacteria not exposed to simulated gastric fluid). Data are mean ± standard deviations. Superscript * indicates a significant difference ($p < 0.05$) compared to the control. Detection limit is 10 CFU/ml.

TABLE 3 | Biosurfactant production by the LAB strains.

Strains	Method		
	Hemolysis in blood agar[a]	Drop collapse test[b]	MATH test[c] (%)
Lactobacillus sakei MBSa1 bac+	++	++	88.9 ± 0.14
Lactobacillus curvatus MBSa3 bac+	++	++	76.4 ± 0.23
Lactococcus lactis VB69 bac+	+	++	95.2 ± 0.09
Lactococcus lactis VB94 bac+	–	+	95.1 ± 0.13
Lactobacillus casei 40 bac–	+	+	77.2 ± 0.30
Lactobacillus helveticus 352 bac–	+	+	91.2 ± 0.4
Lactococcus lactis 368 bac–	++	++	81.2 ± 0.11
Weissella virisdescens 113 bac–	++	++	93.9 ± 0.32

[a]*(+) incomplete hemolysis; (++) complete hemolysis with a diameter of lysis < 1 cm; (+++) complete hemolysis with a diameter of lysis > 1 cm but < 3 cm; and (++++) complete hemolysis with a diameter of lysis > 3 cm and green colonies.*
[b]*Flat drops with scoring system ranging from + to ++++ corresponding to partial to complete spreading on the oil Surface. Rounded drops were scored as negative—indicative of the lack of biosurfactant production.*
[c]*Percent of bacterial cell surface hydrophobicity.*

The result obtained in hydrophobicity, aggregation, and co-aggregaton tests correspond with previus works like García-Cayuela et al. (2014). Di Bonaventura et al. (2008) reported a connection between hydrophobicity of cell surface and bacterial attachment, colonization, and biofilm formation. Our results show high values of hydrophobicity as well as a strong biofilm production, for most of the strains studied but there was no apparent correlation between hydrophobicity highest values and the strongest biofilm production. *W. viridescens* 113 shows a moderate biofilm production while displaying one of the highest

TABLE 4 | Determination of minimal inhibitory concentration (MIC) against the LAB strains.

Strains	MICs (μg/ml)									
	CIP	**CMP**	**VAN**	**ERY**	**STR**	**CLI**	**GEN**	**AMP**	**TET**	**KAN**
Lactobacillus sakei MBSa1 bac+	< 0.5	1	**> 20**	**5**	5	< 0.5	1	**5**	**10**	< 0.5
Lactobacillus curvatus MBSa3 bac+	20	**> 20**	< 0.5	10	10	< 0.5	<0.5	< 0.5	**10**	< 0.5
Lactococcus lactis VB69 bac+	1	10	10	5	5	< 0.5	5	1	5	5
Lactobacillus casei 40 bac−	1	**> 20**	>20n.r	**> 20**	20	< 0.5	1	0.5	< 0.5	0.5
Lactobacillus helveticus 352 bac−	< 0.5	<0.5	< 0.5	5	5	< 0.5	1	0.5	< 0.5	<0.5
Lactococcus lactis 368 bac−	1	10	**> 20**	10	10	< 0.5	5	10	**10**	< 0.5
Weissella virisdescens 113 bac−	< 0.5	1	<0.5	1	1	< 0.25	1	0.5	**20**	< 0.5

CIP, ciprofloxacin; CMP, chloramphenicol; VAN, vancomycin; ERY, erythromycin, STR, streptomycin; CLI, clindamycin; GEN, gentamicin; AMP, ampicillin; TET, tetracycline; and KAN, kanamycin.
Resistant strains with an MIC value higher than the breakpoints described in the table are indicated in bold.
nr, not required.

TABLE 5 | Presence of virulence genes in the LAB strains.

Strain	Genes												
	Agg	***GelE***	***esp***	***efaAfs***	***efaAfm***	***cpd***	***cob***	***ccf***	***cylLL***	***cylLS***	***cylM***	***cylB***	***CylA***
Lactobacillus sakei MBSa1 bac+	−	−	−	−	+	−	−	−	−	−	−	−	−
Lactobacillus curvatus MBSa3 bac+	−	−	+	−	+	−	−	−	−	−	−	−	−
Lactococcus lactis VB69 bac+	−	−	+	−	+	−	−	−	−	−	−	−	−
Lactococcus lactis VB94 bac+	−	−	+	-	+	−	−	−	−	−	−	−	−
Lactobacillus casei 40 bac−	−	−	+	−	+	−	−	−	−	−	−	−	−
Lactobacillus helveticus 352 bac−	+	−	−	−	−	+	−	−	−	−	−	−	−
Lactococcus lactis 368 bac−	−	−	−	−	−	+	−	−	−	−	−	−	−
Weissella virisdescens 113 bac−	−	+	−	−	+	−	+	−	−	−	−	−	−

hydrophobicity values. All tested LAB strains were tolerant to bile salts and acidic pH, evidencing their resistance to digestive stress and potential as probiotic agents. For a probiotic microorganism to be of benefit to human health it must survive the passage through the upper GIT and be able to function in the gut environment (Giraffa et al., 2010). Their functional requirements include tolerance to acid and bile, adherence to epithelial surfaces and antagonistic activity toward intestinal pathogens (Ramos et al., 2013; Peres et al., 2014). All LAB strains except *L. lactis* 368 were negative for β-galactosidase production (data not shown). This characteristic is disadvantageous for the probiotic activity of most studied LAB, as strains able to hydrolyze lactose might be useful for minimizing the effects of lactose intolerance (De Vrese et al., 2001).

Resistance of the LAB strains to antibiotics was species and strain dependent. *Lactobacillus helveticus* 352 and *L. lactis* VB69 were susceptible to all tested antibiotics, but *Lactobacillus sakei* MBSa1 was resistant to vancomycin, erythromycin, ampicillin, and tetracycline. Data from various studies on *Lactobacillus* spp. resistance to various antimicrobial agents demonstrate the existence of inter-genus and inter-species differences (Danielsen and Wind, 2003). The natural resistance to multiple classes of antibiotics is probably due to cell wall structure and membrane permeability, complemented in some cases by the efflux mechanisms (Ammor et al., 2007). However, this feature might represent a competitive advantage, especially when a probiotic product is administered with antimicrobials for treatment of an infectious disease thereby reducing the likelihood of disbiosis (microbial imbalance), rapidly rebalancing normal microbiota (Peres et al., 2014). The EFSA requires that bacteria which are to be introduced into the food chain lack acquired antimicrobial resistance determinants to prevent lateral spread of these (van Reenen and Dicks, 2011). Therefore for the cases of strains who presented antibiotic resistances, future genetic studies are needed to confirm if this resistance is due to acquired antimicrobials determinants. The presence of *efaAfm* in some strains seems to have no value as a risk indicator since this gene was also found in starter *E. faecium* strains with a long record of safe use in food (Eaton and Gasson, 2001). High frequencies of positive results were observed for, *esp* and *efaAfm*, in *Lactococcus* and *Lactobacillus* strains (**Table 5**). Furthermore, *efaAfm* and *esp* genes are related to the production of substances enrolled in the microbial colonization and adhesion at biotic and non-biotic surfaces (Valenzuela et al., 2009). *W. viridescens* 113 was positive for *GelE* but did not produce gelatinase, Eaton and Gasson (2001) described that *gelE* expression is highly influenced by the culture conditions, and the laboratory manipulation of the strains can result in the loss of the structural genes, and can explain the loss of gelatinase activity during *in vitro* tests. Moreover, *W. virisdescens* 113 and *L. lactis* 368 were positive for *cob* and *cpd* genes respectively, which are related to sex pheromones, although sex pheromones are not considered *per se* as virulence factors

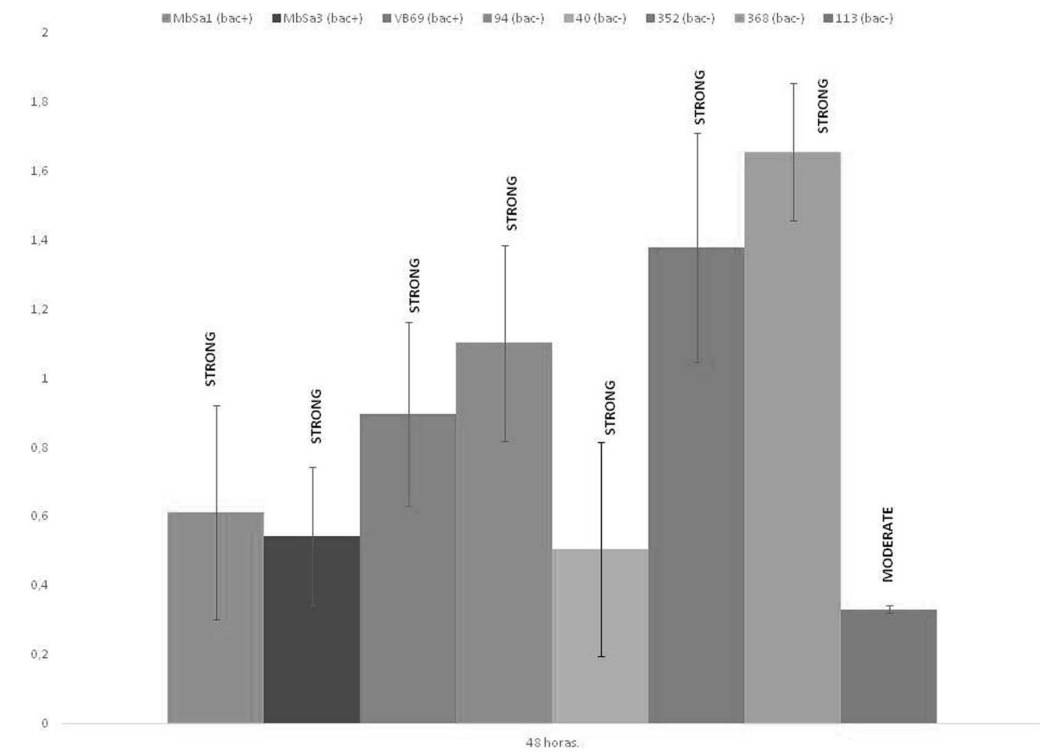

FIGURE 4 | Biofilm formation of LAB using the microtiter plate assay. After incubation at 30°C for 48 h in MRS media. Data are mean ± standard deviations. The cut-off (ODC) was defined as the mean OD value of the negative control. Based on the OD, strains were classified as non-biofilm producers (OD ≤ ODC), weak (ODC < OD ≤ 2 × ODC), moderate (2 × ODC < OD 4 × ODC) or strong biofilm producers (4 × ODC < OD).

(Valenzuela et al., 2008). No strain was found positive for cytolisin family genes, and this confirms that the hemolysis present in blood agar was not related with these virulent genes.

Biosurfactant production is an interesting character, which can be related to the inhibition of the attachment of pathogens. The anti-adhesive and anti-biofilm-forming properties of lactobacilli have been reported in previous studies, such as *Lactobacillus delbrueckii* against *E. coli* (Abedi et al., 2013) and *Lactobacillus brevis* CD2 against *Prevotella melaninogenica* (Vuotto et al., 2013). In addition, *Lactobacillus* species were able to displace adhering uropathogenic *Enterococcus faecalis* from hydrophobic and hydrophilic substrata in a parallel-plate flow chamber (Velraeds et al., 1996). Biosurfactants from LABs have been shown to reduce adhesion of bacterial pathogens to glass, silicone rubber, surgical implants, and voice prostheses (Rodrigues et al., 2004). One xylolipid biosurfactant produced by a *L. lactis* strain with broad antibacterial activity against multidrug resistant *E. coli* and *Staphylococcus aureus* was described (Saravanakumari and Mani, 2010). Biosurfactants also been reported to have strong antifungal and antiviral activity (Singh and Cameotra, 2004). For the screening in biosurfactant production by haemolytic test, all the strains were positive except *L. lactis* 94. The strains showed, complete hemolysis with a diameter of lysis < 1 cm. In addition, drop collapse test was positive for all tested strains corresponding with partial spreading on the oil surface. None of the studies reported in the literature (Johnson and Boese-Marrazzo, 1980; Banat, 1993; Carrillo et al., 1996; Morán et al., 2002) mention the possibility of biosurfactant production without a hemolytic activity. However, in some cases hemolytic assay excluded many good biosurfactant producers (Youssef et al., 2004); hence in the present investigation the MATH assay and drop collapse test with crude oil were also done to confirm biosurfactant production.

The results of this study indicate that the tested LAB was capable to reduce *Listeria monocytogenes*, *Salmonella* and *E. coli* O157:H7 biofilm formation, and present probiotic characteristics and potentially no risk for the consumers. All strains were capable to hinder the development of pathogens in the first 72 h of incubation. Woo and Ahn (2013) obtained similar results of *Listeria monocytogenes* and *Salmonella* inhibition testing probiotic strains. Kim et al. (2013) showed the inactivation of *E. coli* O157:H7 on stainless steel upon exposure to *Paenibacillus polymyxa* biofilms. Zhao et al. (2013) reported the reduction of *Listeria monocytogenes* in a ready-to-eat poultry processing plant by LAB and Pérez-Ibarreche et al. (2014) reported that lactobacilli with biofilm-forming aptitudes were able to control *Listeria monocytogenes* biofilms. In this study inhibition, effect against biofilm adhesion was observed in bacteriocin producers *L. lactis* VB69 and VB94; *Lactobacillus sakei* MBSa1

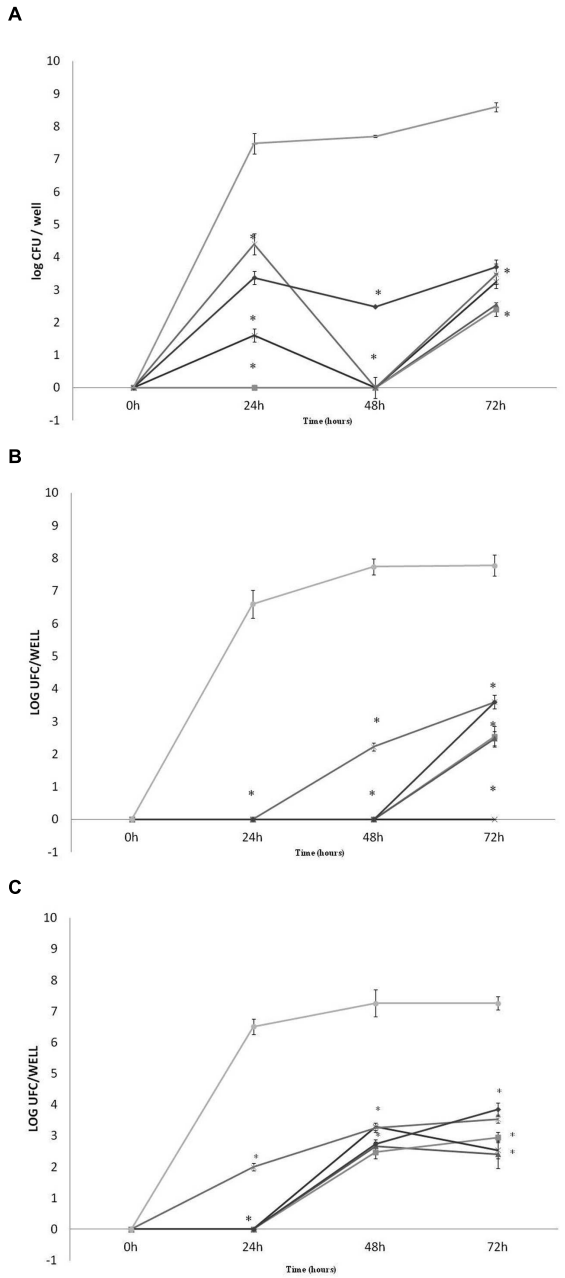

FIGURE 5 | Quantification of pathogen biofilms on microtiter plates in MRS broth (A, *Listeria monocytogenes* ATCC 7644, B, *S.* Typhimurium ATCC 14028, C, *E. coli* O157:H7 ATCC 35150) in the presence of *W. viridescens* 113 bac− (—◆—); *L. lactis* 69 bac+ (—■—); *L. lactis* 94 bac+ (—▲—); *Lactobacillus casei* 40 bac− (—×—), and *Lactobacillus helveticus* 352 bac− (—✳—), biofilms after 24, 48, and 72h at 30°C. *Listeria monocytogenes*, *S.* Typhimurium, and *E. coli* O157:H7 positive control (—●—). No counts of the pathogens biofilms were detected in the presence of *Lactobacillus. sakei* MBSa1 bac+, *Lactobacillus curvatus* MBSa3 bac+ and *L. lactis* 368 bac− biofilms. Results are mean of triplicates and vertical bars show standard deviations. Superscript * indicates a significant difference ($p < 0.05$) compared to the control (pathogens alone).

and *Lactobacillus curvatus* MBSa3 as well as non-bacteriocin producers *Lactococcus lactis—lactis* 368, *Lactobacillus helveticus* 354, *Lactobacillus casei* 40 and *W. viridescens* 113. It seems that inhibition of pathogenic bacteria growth and adhesion is not only due to the bacteriocin production. This outcome can be attributed to a combination of factors like biosurfactant and bacteriocin production as well as mechanisms of pathogens exclusion through their trapping (killing of cells embedded in biofilms). This is in accordance with previous works like Guerrieri et al. (2009) witch suggests the need to apply the bacteriocine-producing microorganism, in biofilms. There may be an influence of EPS (exo-polysaccharide). Kim et al. (2006) found that the EPS of *Lactobacillus acidophilus* A4 had stronger anti-biofilm activity against the growth of entero-hemorrhagic *E. coli* O157: H7, *S. enteritidis*, *S. typhimurium* KKCCM 11806, *Yersinia enterocolitica*, *Pseudomonas aeruginosa* KCCM 11321, *Listeria monocytogenes* Scott A, and *B. cereus*.

CONCLUSION

Our results show that LAB strains from foods can be excellent candidates to form protective biofilms, in accordance with the hypothesis proposed by Falagas and Makris (2009) to use non-pathogenic microorganisms, namely probiotics, as part of daily cleaning products to lower the incidence of pathogenic microorganisms. Evidences on the efficacy of probiotics for the prevention and treatment of infections have been observed both *in vitro* and *in vivo* (Levkovich et al., 2013; Shu et al., 2013). The present study provided new information about the use of potential probiotic LAB biofilms for the control of *Listeria monocytogenes*, *S.* Typhimurium and *E. coli* O157:H7 biofilms formation through exclusion mechanisms. However, more experiments are needed to confirm the ability of these strains to inhibit the pathogen biofilm formation in other environments. Our initial studies are very encouraging and indicate that the LAB that we have tested are promising candidates for controlling the presence of pathogenic biofilms in food-processing facilities. The development of protective biofilms with probiotic LAB present in food could help avoiding problems of contamination into the food chain.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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METHODS
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Development of a Method to Determine the Effectiveness of Cleaning Agents in Removal of Biofilm Derived Spores in Milking System

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Microbial damages caused by biofilm forming bacteria in the dairy industry are a fundamental threat to safety and quality of dairy products. In order to ensure the optimal level of equipment hygiene in the dairy industry, it is necessary to determine the biofilm removal efficiency of cleaning agents used for cleaning-in-place (CIP) procedures. However, currently there is no standard method available for evaluating and comparing cleaning agents for use in CIP procedures in the dairy industry under realistic conditions. The present study aims to establish a CIP model system to evaluate the effectiveness of cleaning agents in removal of biofilm derived spores from the surfaces of stainless steel which is the predominant substrate in milking equipment on dairy farms. The system is based on *Bacillus subtilis* spores surrounded with exopolymeric substances produced by bacteria during biofilm formation. The spores applied on sampling plates were mounted on T-junctions protruding 1.5–11-times the milk pipe diameter from the main loop to resemble different levels of cleaning difficulty. The cleaning tests were conducted using commercial alkaline detergents and caustic soda at conditions which are relevant to actual farm environment. The spores removal effect was evaluated by comparing the number of viable spores (attached to sampling plates) before and after cleaning. Evaluation of the cleaning and disinfecting effect of cleaning agents toward biofilm derived spores was further performed, which indicates whether spores elimination effect of an agent is due to killing the spores or removing them from the surfaces of dairy equipment. Moreover, it was established that the presence of extracellular matrix is an important factor responsible for high level of cleaning difficulty characteristic for surface attached spores. In overall, the results of this study suggest that the developed model system simulates actual farm conditions for quantitative evaluation of the effectiveness of cleaning and disinfecting agents and their cleaning and disinfecting effect on removal of biofilm derived spores.

Keywords: dairy industry, biofilm, *Bacillus subtilis*, biofilm derived spores, spores removal effectiveness, cleaning-in-place

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INTRODUCTION

Bacterial contamination can adversely affect the quality, functionality, and safety of dairy products. It appears that the major source of the contamination of dairy products is often associated with biofilms on the surfaces of dairy processing equipment (Flint et al., 1997; Sharma and Anand, 2002a). Biofilms are highly structured multicellular communities, which allow bacteria to survive in hostile environments (Hall-Stoodley et al., 2004; Kolter and Greenberg, 2006). Bacterial cells in biofilms are characterized by increased resistance to antimicrobial agents and cleaning chemicals (Sharma and Anand, 2002a; Shaheen et al., 2010; Checinska et al., 2015). Biofilms found in the dairy production lines contain significant milk residues, particularly protein, and minerals such as calcium phosphate. The persistence of accumulated microorganisms in the form of biofilms on dairy equipment causes pre- and post-processing contamination, leading to lowered shelf-life of products and possible transmission of diseases (Faille et al., 2002; Sharma and Anand, 2002b; Shaheen et al., 2010). Biofilms are not only a potential source of contamination, but can also increase corrosion rate of metal pipes and equipment often used in the dairy industry, reduce heat transfer and increase fluid frictional resistance (Kumar and Anand, 1998). Thus, it becomes increasingly clear that bacterial biofilms are a major concern to modern dairy industry; especially with current trends toward longer production runs, the use of complex equipment, the automation of plants and increasingly stringent microbiological requirements.

Members of the *Bacillus* genus are of the most common bacteria found in dairy farms and processing plants (Sharma and Anand, 2002a; Simoes et al., 2010). Biofilms of *Bacillus* species may contain both vegetative bacteria and spores. Spore formation occurs preferentially when the biofilm is in direct contact with the oxygen in air and a water-saturated atmosphere (Ryu and Beuchat, 2005; Wijman et al., 2007). This corresponds very well to the situation in a milk line. It was reported that biofilm of *Bacillus* species could consist of up to 90% spores (Wijman et al., 2007; Faille et al., 2014). Since spores are much more resistant to heat and chemicals, they are much more difficult to eliminate than vegetative bacteria (Ryu and Beuchat, 2005). Moreover, the biofilm matrix offers additional protection for imbedded endospores, allowing survival, and colonization of the surrounding environment when conditions are favorable (Branda et al., 2001).

The most common *Bacillus* species found in dairy associated environment are *B. licheniformis*, *B. cereus*, *B. subtilis*, *B. mycoides* and *B. megaterium* (Andersson et al., 1995; Bartoszewicz et al., 2008; De Jonghe et al., 2008; Ledenbach and Marshall, 2009; Ostrov et al., 2015). It was previously shown that the majority of isolates from raw milk from organic and conventional dairy farms belonged to the genus *Bacillus* and showed at least 97% 16S rRNA gene sequence similarity with type strains of *B. licheniformis*, *B. pumilus, B. circulans*, *B. subtilis* and *B. cereus* (Coorevits et al., 2008). Moreover, *B. cereus* and *B. licheniformis* were shown to be predominant species originated from dairy processing environments, raw materials, and processed foods, while *B. subtilis* was among prevalent heat-resistant and highly thermoresistant spore-formers (According to Lücking et al., 2013). *B. licheniformis* was shown to affect the quality of pasteurized milk and cream (Gilmour and Rowe, 1990). *B. cereus* was found to be responsible for sweet curdling (without pH reduction) both in homogenized and non-homogenized low-pasteurized milk (Andersson et al., 1995). *B. subtilis* has been associated with ropiness in raw and pasteurized milk as well as the spoilage of UHT and canned milk products (Heyndrickx and Scheldeman, 2002). Strains belonging to the *B. subtilis and B. cereus* groups were shown to be strongly proteolytic (Lücking et al., 2013). Interestingly, *Bacillus* strains including *B. cereus* (Wijman et al., 2007), *B. licheniformis* (Hoong et al., 2012; Ostrov et al., 2015) and *B. subtilis* (Bridier et al., 2011) are able to form submerged surface-associated biofilm.

Biofilm formation depends on the synthesis of an extracellular matrix that holds the constituent cells together. In *B. subtilis*, the model organism within the *Bacillus* genus, the matrix has two main components, an exopolysaccharide (EPS) synthesized by the products of the *epsA-O* operon, and amyloid fibers encoded by *tasA* located in the *tapA-sipW-tasA* operon (Kearns et al., 2005; Branda et al., 2006; Chu et al., 2006; Vlamakis et al., 2013).

Since biofilm forming microorganisms in the dairy associated environment may hold spoilage and/or health risks, dairy products manufacturing is a subject to extremely stringent regulations (Bremer et al., 2006). The effective cleaning and disinfecting procedures in the dairy industry are a fundamental requirement to ensure the safety and quality of dairy products. Cleaning and disinfection in food manufacturing industries have been incorporated into the cleaning-in-place (CIP) regimes which include regular cleaning of processing equipment, usually with alkaline and acidic liquids at high temperatures (Zottola and Sasahara, 1994; Bremer et al., 2006). However, bacterial contamination and product spoilage due to biofilm formation are recurring problems (Carpentier et al., 1998). The result of the cleaning limitation of CIP procedures is accumulation of microorganisms on the equipment surfaces and formation of biofilm that is very difficult to remove by subsequent cleaning and disinfecting cycles (Peng et al., 2002; Hall-Stoodley et al., 2004). The biofilm formed by thermoresistant bacteria in a milk line can rapidly grow to such an extent that the passing milk is contaminated with cells released from the biofilm (Wirtanen et al., 1996).

Elimination of biofilm and spores is facilitated by a high degree of turbulence of the cleaning solution (Wirtanen et al., 1996) and by the presence of oxidizing substances such as hypochlorite and hydrogen peroxide (Kumar and Anand, 1998). Chlorine-based detergents can therefore facilitate the removal of biofilm. However, rapid recovery of biofilms after chlorine treatment is often observed. This may be due to the rapid regrowth of surviving cells, residual biofilm providing a conditioning layer for enhanced biofilm development, or

selection of resistant microorganisms that survive and thrive after chlorine treatment (Flint et al., 1997). Currently, environmental concerns are driving dairy farms toward the use of chlorine-free detergents, although there is uncertainty about their effectiveness and thus it is difficult to judge whether this change can result in increased milk hygiene problems (Sundberg et al., 2011).

In order to ensure the optimal level of equipment hygiene in the dairy industry it is necessary to determine the removal efficiency of surface attached bacteria by cleaning solutions used for CIP procedures (Parkar et al., 2003; Bremer et al., 2006). However, currently there is no agreed standard method available for evaluating and comparing cleaning agents for use in CIP-procedures in the dairy industry under realistic conditions. Some progress has been made in a study which investigated the cleaning effects of various detergents under controlled realistic temperature and flow conditions (Sundberg et al., 2011). However, this study did not fully simulate the type of hygiene problems common in practice for instance the presence of extracellular matrix. Furthermore, previous studies could not evaluate the cleaning and disinfecting effect of the cleaning agents (whether the elimination effect is due to killing bacteria or removing them from the surfaces of dairy equipment). To this extent the necessity of not only killing bacteria in biofilms, but also removing them together with the extracellular matrix was previously emphasized (Zottola and Sasahara, 1994; Kumar and Anand, 1998; Parkar et al., 2003).

In the present study, we aimed to establish a model system to evaluate the effectiveness of cleaning agents in removal of biofilm derived spores from the surfaces of stainless steel which is predominant surface in milking equipment on dairy farms. Therefore, we developed a system in order to evaluate the cleaning outcome based on *Bacillus subtilis* spores surrounded with exopolymeric substances produced by bacteria during biofilm formation. The developed model system simulates actual farm conditions for quantitative evaluation of effectiveness of cleaning and disinfection agents and their cleaning and disinfecting effect on biofilm derived spores.

TABLE 1 | Strains used in this study.

Strain	Genotype	Reference
B. subtilis NCIB3610	undomesticated WT strain	Branda et al., 2001
B. subtilis YC189	P_{tapA}-*cfp* at the *amy*E locus in 3610, $Spec^R$	Chai et al., 2008
B. subtilis RL3852	Δ*epsH* in 3610, Tet^R	Kearns et al., 2005
B. subtilis YC668	Δ*abrB* in 3610, Kan^R	Pasvolsky et al., 2014

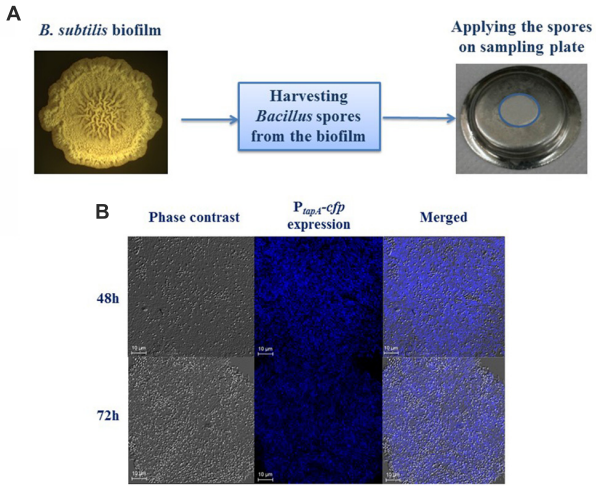

FIGURE 1 | Generation of *Bacillus subtilis* biofilm derived spores. (A) Schematics showing that the spores, harvested from biofilm of *B. subtilis* 3610 (wild type strain) generated on LBGM agar plates, were mounted on the sampling plate. **(B)** Transcription of the *tapA-sipW-tasA* operon responsible for the matrix production in *B. subtilis* is greatly upregulated in LBGM following either 48 or 72 h of biofilm development. Intensity of the *tapA* expression by the cells that bear the P_{tapA} *-cfp* transcriptional fusion (*B. subtilis* YC189) is presented in the middle-hand panel. The biofilm forming cells were obtained as previously reported (Shemesh and Chai, 2013) and analyzed using confocal laser scanning microscope (CSLM, Leica, Germany) visualization. Scale –10 μm.

MATERIALS AND METHODS

Strains and Growth Media

The *B. subtilis* wild type strain NCIB3610 (Branda et al., 2001) and its derivatives: RL3852 (Δ*epsH*::tet), YC668 (Δ*abrB*::kan) and YC189 (P_{tapA} *-cfp* at the *amy*E locus) (**Table 1**) were used in this study. The wild type strain was used for evaluation of effectiveness in removal of biofilm derived spores in the CIP model system as well as evaluation of cleaning/disinfecting effect of the cleaning agents and determining the role of the extracellular matrix in persistence of biofilm derived spores toward cleaning procedures. The RL3852 and YC668 strains were used for determining the role of the extracellular matrix in persistence of biofilm derived spores toward cleaning procedures. The YC189 was used for analysis of the level of the matrix gene expression. For routine growth, the strains were propagated in Lysogeny broth (LB; 10 g of tryptone, 5 g of yeast extract and 5 g of NaCl per liter) or on solid LB medium supplemented with 1.5% agar.

Generation of Biofilm Derived Spores

Biofilm colonies were generated at 30°C in biofilm promoting medium LBGM [LB + 1% (v/v) glycerol + 0.1 mM $MnSO_4$] (Shemesh and Chai, 2013) (**Figure 1A**). The grown colonies were collected and suspended in phosphate buffer saline (PBS; 0.01 M phosphate buffer, 0.0027 M KCl, 0.137 M NaCl per 200 ml, Sigma Aldrich, St. Louis, MO, USA). Chained and bundled cells from the collected biofilm colony were disrupted by mild sonication (amplitude–50%, pulse–10 s, pause–5 s, duration–1.5 min.). Then, heat killing was performed at 80°C for 20 min. Cell numbers after heat killing were quantified by the plating method using LB agar plates.

Preparation and Enumeration of the Spores on the Sampling Plates

Prior to the cleaning tests, 200 μl portions from the suspension of spores (prepared as described above) were applied on each sampling plate and carefully distributed over the sampling area (**Figure 1A**). The goal was to attach approximately two million spores onto each plate. The plates were then placed upright in biological laminar hood to dry for around 1 h. Two or three sampling plates were prepared but not cleaned in the test installation. Otherwise, the subsequent treatment of these control plates was precisely the same as for the cleaned plates. Average spore counts on the control plates were used as the initial value for all plates cleaned on that day when calculating the level of spore reduction.

For enumeration of spores, the sampling area on each plate was carefully swabbed with cotton swabs moistened in PBS buffer. Swabs from each plate were then agitated in PBS in separate test tubes. Serial dilutions from each sample were prepared, followed by spread plating on LB agar for CFU analysis. Plates were incubated for 24 h at 37°C before colonies were counted.

Cleaning Solutions

We choose to use for this study caustic soda (NaOH, pH value–13) and five different commercial alkaline detergents (defined as solutions A–E; pH value between 11–12) which are commonly used in the Israeli dairy farms. All detergents were used at concentration of 0.5% (v/v) in accordance with the manufacturer's recommendations. Caustic soda was used at concentration of 0.5% (m/v). As a control, tap water was used (pH value around 7.7) with a standard level of hardness (∼50 mg/l Ca^{2+}, 50 mg/l Mg^{2+}) without addition of any detergent.

Test Installations

The cleaning tests were carried out in the CIP-model system which was designed to resemble farm conditions as closely as possible. The main components were a 5-m stainless steel milk line (25 mm internal diameter; fitted with a test outfit) for pumping the cleaning agents from the basin (**Figure 2A**), milk releaser, and stainless steel return line to the basin. To generate flushing pulsation of the circulating liquid, air was introduced into the milk line at controlled intervals (every 8 s, Supplementary Video 1). The test outfit had removable sampling plates, attached at the end of T-junctions that protruded either 75 mm or 35 mm from the main loop. In order to reflect different degrees of cleaning difficulty (characteristic for dairy equipment), removable stainless steel nozzles were used to increase the length of T-junctions in certain cleaning tests. The length of each nozzle constituted 50 mm. Thereby, the length of each T-junction could be increased by 50–200 mm (**Figures 2B,C**). The sampling plates were made from stainless steel (304) and the sampling area exposed to the cleaning solutions was about 5.7 cm^2. The temperature of the cleaning agent during the cleaning tests constituted 50°C. The flow rate with air injection was 34.5 l per minute; the flow rate without air injection was 43 liter per minute. The duration of each cleaning cycle was 10 min.

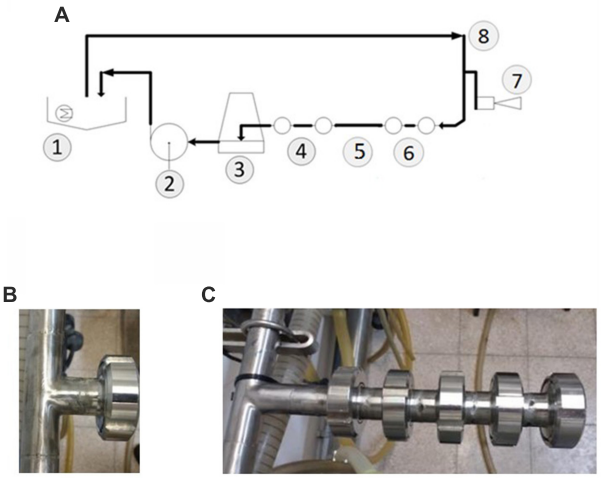

FIGURE 2 | Description of the developed CIP-model system. (A) Schematic design of the model system used in the cleaning tests: (1) sink with the heater, (2) milk pump, (3) releaser, (4) two 35 mm T-junctions, (5) the mane pipeline, Ø25 mm, (6) two 75 mm-T junctions, (7) air injection, (8) pipeline of water supply from the sink and the flow direction of circulating liquid. **(B)** The shortest T-junction (35 mm). **(C)** 75 mm T-junction elongated using removable stainless steel nozzles to the total length of 275 mm.

Evaluation of the Effect of the Cleaning Agents on the Viability of *B. subtilis* Spores

The tested detergents (0.5%, v/v) and caustic soda (0.5%, m/v) were added to spore suspension of *B. subtilis* 3610 containing around 1×10^7 CFU/ml spores. Whereas, the spore suspension within water without addition of detergents was used as control. The samples were incubated in closed tubes at conditions simulating those in CIP-model system (50°C, 200 rpm) for 30 min. The CFU-measurements of the number of viable spores were made every 10 min.

Statistical Analysis

Student's *t* test was used to calculate the significance of the difference between the mean expression of a given experimental samples and the control samples. A P value of < 0.05 was considered significant.

RESULTS

Developing a Model Based on Biofilm Derived Spores

In order to simulate biofilm derived spores, we have developed the system that is based on *B. subtilis* spores surrounded with exopolymeric substances produced by bacteria during biofilm formation. To stimulate the sporulation in biofilm context, we

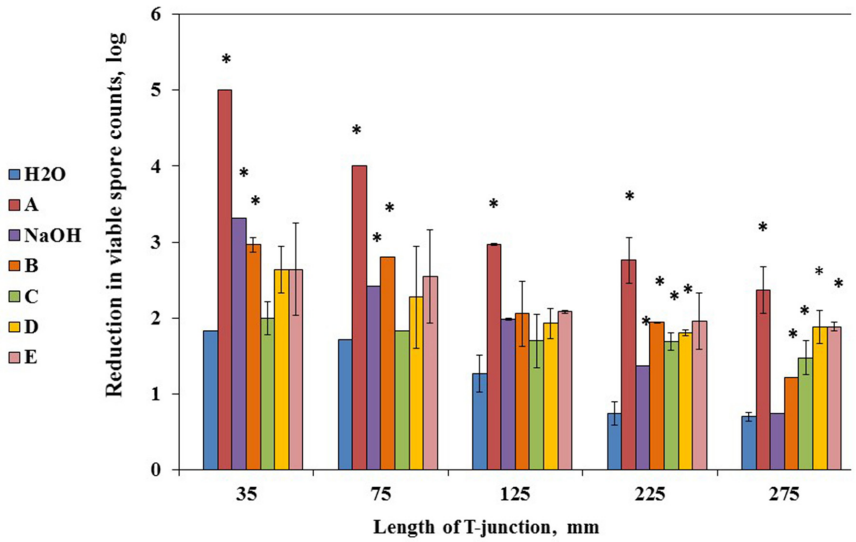

FIGURE 3 | Mechanical effect of water and chemical/biological effect of cleaning agents on removal of biofilm derived spores in the CIP-model system. Sampling plates, each maintaining approximately 2 million spores of *B. subtilis* wild type strain, were mounted on T-junctions protruding 35, 75, 125, 225, and 275 mm from the main loop of the CIP model system, and cleaned in the installation. Caustic soda (NaOH) and five different commercial alkaline detergents (defined as A–E) were used as the cleaning agents. The detergents were dosed as 0.5% (v/v) in accordance with the manufacturer's recommendations. Caustic soda was dosed as 0.5% (m/v). As a control, tap water without addition of any detergent was used. The cleaning effect was evaluated by comparing the numbers of viable spores (attached to sampling plates) before and after cleaning. The results represent the means and standard deviation (SD) of two independent biological experiments performed in duplicates.*statistically significant difference ($P < 0.05$) between reduction in viable spore counts in given sample versus reduction in spore counts after cleaning with water (control).

generated *B. subtilis* colonies in the biofilm promoting medium LBGM (**Figure 1A**). To confirm the high level production of extracellular matrix in the biofilm colonies, we analyzed the level of the matrix gene expression in LBGM using transcriptional fusion of the promoter for *tapA-sipW-tasA* to the *cfp* gene encoding cyan fluorescent protein (Chai et al., 2008) similarly as described previously (Shemesh et al., 2010). We found that the expression of the P_{tapA}-*cfp* was enhanced in a large number of cells both after 48 and 72 h of biofilm development (**Figure 1B**). This finding indicates that *B. subtilis* spores harvested from biofilm colonies could be surrounded with extracellular polymeric substances.

Evaluation of Effectiveness of Biofilm Derived Spores Removal in the CIP Model System

It was suggested that the hydrodynamic effects such as turbulent flow of cleaning agent may facilitate the removal of surface associated bacteria in dairy equipment (Wirtanen et al., 1996; Leliévre et al., 2002, 2003). However, dairy equipment has many so called "dead legs" (milk meters, clusters, etc.) protruding from the main pipelines in which the flow of liquid is much less turbulent. Such "dead legs" might represent higher levels of cleaning difficulty compared to other sites of dairy equipment. To simulate different levels of cleaning difficulty characteristic for dairy equipment in the CIP model system, we used removable stainless steel nozzles to increase the length of T-junctions (**Figures 2B,C**). We hypothesized that the level of efficiency of cleaning agents toward removal of biofilm derived spores is inversely proportional to the length of T-junctions (Supplementary Video 2).

Primarily, we evaluated mechanical effect of water circulation in the CIP system. Cleaning with water alone reflects the mechanical cleaning effect brought about by the flow of liquid in the installation (Sundberg et al., 2011). The difference in cleaning effect between water and a cleaning agent reflects the chemical/biological effect from the substances present in the agent. We found that effectiveness of water in removal of biofilm derived spores was inversely proportional to the length of T-junctions and constituted about 1.8 and 1.7 log reduction in spore counts for 35- and 75-mm T-junctions, respectively; while 1.3 log reduction for 125-mm T-junctions and about 0.7 log reduction for 225- and 275-mm T-junctions, respectively (**Figure 3**). These results confirm that mechanical effect of flow turbulence facilitates the removal of biofilm derived spores.

Next, spores removal efficiency of caustic soda and five different commercial alkaline detergents with chlorine was determined. It was shown that chemical/biological effect of the tested detergents constituted additional 0.5–2 log reduction compared to mechanical effect of water circulation. Among all tested detergents, solution A had the highest removal efficiency leading to additional 2 log reduction in spore counts irrespective of the length of T-junctions (**Figure 3**).

As the water circulates by flushing pulsation in the commercial cleaning units, we tested the effect of air introduction into the CIP system on the spores removal efficiency of the tested agents. Our experiments established that there was no significant difference

($P < 0.26$) in the removal efficiency without the air introduction into the milk line (data not shown). We also tested the effect of temperature on the removal efficiency. We found about 0.5 log improvement in the efficiency of cleaning out biofilm derived spores by elevating the temperature from 35°C to 50°C (data not shown).

Evaluation of the Cleaning and Disinfecting Effect of the Cleaning Agents

Primarily, we determined the ability of the tested agents to reduce the number of viable spores (disinfecting effect). For this, *B. subtilis* spores suspensions were incubated with each of the tested agents in conditions simulating those in the CIP-model system. We found that solution A and caustic soda could not notably reduce the spore counts compared to control (about 0.2 log after 30 min of incubation) (**Figure 4**). At the same time, other tested agents leaded to noticeable reduction (about 0.5 log) in the number of viable spores even after 10 min of incubation.

To determine a correlation between the cleaning and disinfecting effect of the tested detergents we defined the ability of a cleaning agent to reduce the number of viable spores after 10 min of incubation (as the duration of cleaning cycle in the CIP model system is 10 min) as disinfecting effect. We compared the percentage of the disinfecting effect to the total chemical/biological effect of a cleaning agent determined in the CIP system after 10 min of cleaning test conduction (taken as 100%). The difference between the total chemical/biological effect of a cleaning agent and disinfecting effect was defined as cleaning effect. It was established that chemical/biological effect of solution A and caustic soda was mostly due to removal of surface attached spores, solution E was characterized by approximately equal cleaning and disinfecting properties; while chemical/biological effect of B, C, and D was mostly due to disinfecting (**Figure 5**).

Determining the Role of the Extracellular Matrix in Persistence of Biofilm Derived Spores toward Cleaning Procedures

To support the assumption that there is an extracellular matrix around the spores which may provide a protection, we evaluated mechanical effect of water circulation toward the spores produced by Δ*epsH* strain of *B. subtilis* (this mutant strain cannot produce exopolysaccharide component of extracellular matrix). As we hypothesized, there was a notable increase in reduction of viable spore counts for Δ*epsH* compared to wild type strain for the two higher lengths of the T-junctions (**Figure 6**). Furthermore, we evaluated the mechanical effect of water circulation toward the spores produced by the Δ*abrB* of *B. subtilis* (this mutant strain overproduces extracellular matrix). We found that it was far more difficult to remove the spores of Δ*abrB* strain compared to wild type (**Figure 6**). These results indicate that the presence of extracellular matrix is an important factor responsible for high levels of cleaning difficulty.

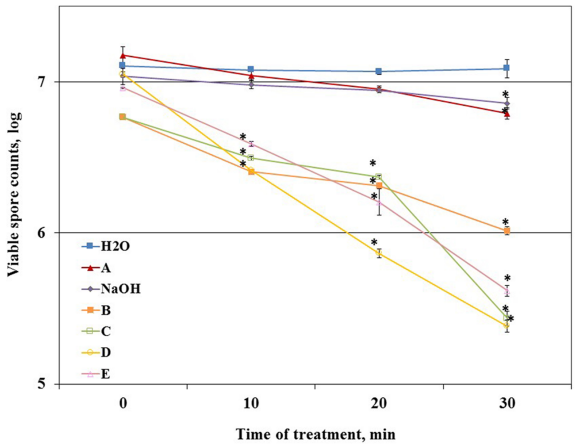

FIGURE 4 | The effect of the tested cleaning agents on the viability of *B. subtilis* spores. Caustic soda (NaOH) and five different commercial alkaline detergents (defined as A–E), were added to the tubes with spore suspension of WT *B. subtilis* within distilled sterile water, containing approximately 10^7 CFU/ml spores. The detergents were dosed as 0.5% (v/v) in accordance with the manufacturer's recommendations. Caustic soda was dosed as 0.5% (m/v). Spore suspension without any detergent was used as control. The samples were incubated at 50°C for 30 min. The ability of detergents to eradicate spores (disinfecting effect) was evaluated by comparing the numbers of viable spores in control and after the treatment with a tested detergent at different time points of incubation. The results represent the means and standard deviation (SD) of two independent biological experiments performed in duplicates.*statistically significant difference ($P < 0.05$) between viable spore counts in given sample versus spore counts after cleaning with water (control).

DISCUSSION

It becomes increasingly clear that the major source of the contamination of dairy products is often associated with attached bacteria on the surfaces of dairy processing equipment (Flint et al., 1997). Thus, there is a need to develop a model system for evaluating and comparing the effectiveness of cleaning agents in removal of attached bacteria from the surfaces of stainless steel under realistic conditions.

This study, investigated the removal efficiency of caustic soda and commercial alkaline detergents toward biofilm derived spores using a developed CIP model system under well-controlled realistic temperature and flow conditions.

We used *Bacillus* spores as a model, because of their high adherence to various materials (Faille et al., 2001) and their resistance to heat and chemicals (Faille et al., 2002). Several previous studies investigated cleaning efficiency during CIP procedures (Leliévre et al., 2003; Sundberg et al., 2011; Faille et al., 2013) using *Bacillus* spores as a model. However, previous models do not fully reflect the type of hygiene problems common in practice such as the presence of extracellular matrix of biofilm origin. The conditions, encountered in the dairy equipment are often propitious for bacterial growth and eventually a biofilm is formed. Previous works have demonstrated that sporulation could occur in biofilms, suggesting that biofilms would be a significant source of food contamination with spores (Wijman

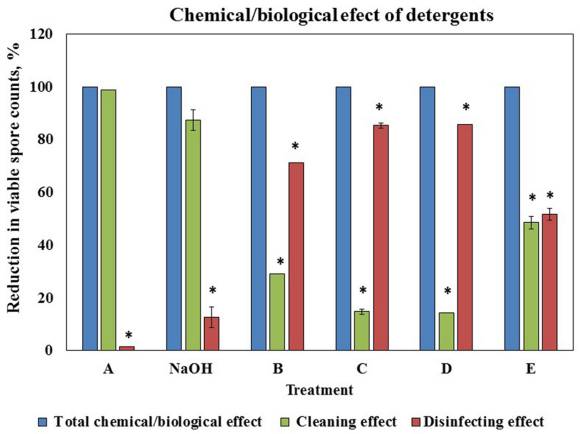

FIGURE 5 | Correlation between cleaning and disinfecting effect of the tested agents. The ability of a cleaning agent to reduce the number of viable spores (*B. subtilis* wild type strain) after 10 min of incubation (as the duration of cleaning cycle in the CIP model system is 10 min) was defined as disinfecting effect. The percentage of the disinfecting effect was compared to the total chemical/biological effect of a cleaning agent determined in the CIP system after 10 min of cleaning test conduction (taken as 100%). The difference between the total chemical/biological effect of a cleaning agent and disinfecting effect was defined as cleaning effect. The results represent the means and standard deviation (SD) of two independent biological experiments performed in duplicates. *statistically significant difference ($P < 0.05$) between reduction in spore counts due to cleaning or disinfecting effect versus total chemical/biological effect of a tested agent.

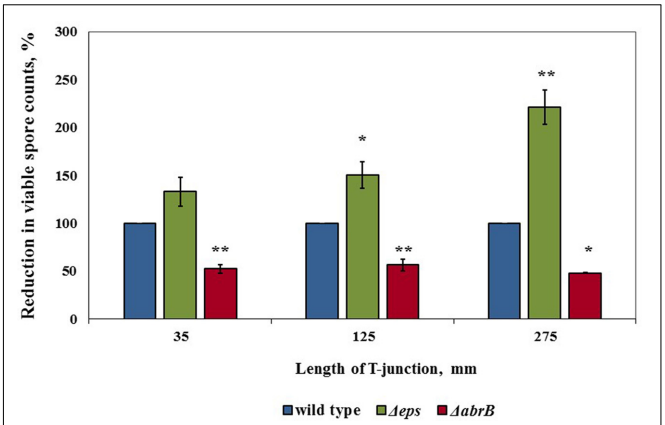

FIGURE 6 | The role of extracellular matrix in persistence of biofilm derived spores against removing by cleaning procedures. Sampling plates, each maintaining approximately 2 million spores of WT *B. subtilis*, *B. subtilis* RL3582 (Δ*epsH*), *B. subtilis* YC668 (Δ*abrB*), were mounted on T-junctions protruding 35, 125, and 275 mm from the main loop of the CIP model system, and cleaned in the installation. Tap water without addition of any detergent was used as a cleaning agent. The cleaning effect was evaluated by comparing the numbers of viable spores (attached to sampling plates) before and after cleaning. The cleaning effect for *B. subtilis* 3610 was taken as 100%. The results represent the means and standard deviation (SD) of two independent biological experiments performed in triplicates.*statistically significant difference ($P < 0.05$); **statistically significant difference ($P < 0.006$) between reduction in viable spore counts in given sample versus reduction in spore counts in control wild type bacteria.

et al., 2007; Faille et al., 2014). Consequently, the spores derived from biofilm represent continuous microbial problem which could be very hard to eliminate partially due to the presence of extracellular matrix that might influence their resistance during cleaning procedures.

Our results show that spores removing efficiency during cleaning procedures was inversely proportional to the length of T-junctions (**Figure 3**). This is in consistence with previous papers suggesting that turbulence may influence removal of surface attached bacteria (Wirtanen et al., 1996; Leliévre et al., 2002, 2003). It is generally considered that a "dead leg" is cleanable when the flow is directed into the "dead leg" and its length does not exceed twice the diameter of the pipeline (Chisti, 1999). In our study, the T-junctions were 35–275 mm that is 1.5–11-times the diameter of the pipeline. Therefore, it is conceivable that we observed notable decrease in effectiveness of spores elimination by the tested agents with the increase of the length of T-junctions. These results confirm that the developed CIP model system simulates different levels of the cleaning difficulty that facilitates proper evaluation of spores elimination effectiveness of cleaning agents at realistic conditions.

Interestingly, our experiments demonstrated that there was no significant difference in spores removal efficiency without the air introduction into the milk line. This could be explained by the relatively low diameter of pipeline which was used in the developed system. Most likely, the differences in flow rate and turbulence were not significant with or without introduction of air.

Moreover, we found around 0.5 log improvement in the spores removal efficiency by elevating the temperature from 35–50°C. This finding is also in consistence with previous studies which demonstrated dependence of the cleaning efficiency on temperature (Peng et al., 2002; Sundberg et al., 2011). Taken together, our results suggest that elevated temperature as well as chemical/biological effect may help to eliminate biofilm derived spores in milking equipment.

The methods of evaluation of cleaning effectiveness described earlier (Parkar et al., 2003; Bremer et al., 2006; Sundberg et al., 2011) do not show if chemical/biological effect of cleaning agents is due to killing bacteria (disinfecting effect) or to removing them from the surfaces of dairy associated equipment (cleaning effect). The necessity of not only killing bacteria in biofilms, but also removing the immobilized bacteria is suggested (Flint et al., 1997; Kumar and Anand, 1998; Parkar et al., 2003) as rapid recovery of biofilms after disinfectant treatment is often observed. Therefore, we developed a method to evaluate the cleaning and disinfecting effect of cleaning agents toward biofilm derived spores. Using this approach it is shown whether chemical/biological effect of a detergent is due to cleaning, disinfecting or both.

In conclusion, a CIP model system was developed and used to evaluate the efficiency of cleaning agents in removing biofilm derived spores from the surfaces of dairy equipment. The developed system simulates actual farm conditions for proper evaluation of the spores elimination effectiveness and cleaning and disinfecting effect of cleaning and disinfection agents.

AUTHOR CONTRIBUTIONS

IO and MS planned the experiments and wrote the original manuscript. IO performed the experiments described in the manuscript. AH and MS designed the CIP-model system described in the manuscript. AH and SB provided technical assistance for conduction of experiments. DS revised the manuscript. IO, DS, and MS integrated all of the data throughout the study and crafted the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.01498

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