Computational and Structural Biotechnology Journal 17 (2019) 1234–1244

COMPUTATIONAL AND STRUCTURAL BIOTECHNOLOGY JOURNAL

journal homepage: www.elsevier.com/locate/csbj

Recent progress in experimental and human disease-associated multi-species biofilms

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ARTICLE INFO

Article history:
Received 13 June 2019
Received in revised form 18 September 2019
Accepted 21 September 2019
Available online 25 October 2019

Keywords:
Multi-species biofilm
Chronic infections
Microbiota

ABSTRACT

Human bodies are colonized by trillions of microorganisms, which are often referred to as human microbiota and play important roles in human health. Next generation sequencing studies have established links between the genetic content of human microbiota and various human diseases. However, it remains largely unknown about the spatial organizations and interspecies interactions of individual species within the human microbiota. Bacterial cells tend to form surface-attached biofilms in many natural environments, which enable intercellular communications and interactions in a microbial ecosystem. In this review, we summarize the recent progresses on the experimental and human disease-associated multi-species biofilm studies. We hypothesize that engineering biofilm structures and interspecies interactions might provide a tool for manipulating the composition and function of human microbiota.

Contents

1. Introduction

Human bodies are well known to be colonized by trillions of microorganisms, which are part of the human ecosystem and often referred to as human microbiota [1]. The human microbiota consists of bacteria, archaea, viruses and eukaryotes, and its composition varies among the different body sites and individuals. The collection of all the genes in the human microbiota is called human micro-

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https://doi.org/10.1016/j.csbj.2019.09.010
2001-0370/

biome [2]. Recent advances in next generation sequencing and its analysis tool packages have dramatically boosted human microbiome investigations. The implement of the large-scale human microbiome project (HMP) has vastly improved our understanding of the roles of human microbiota on human health and diseases [3].

Besides DNA sequencing, transcriptomics, proteomics, metabolomics approaches have been developed to investigate human microbiome, establishing links between human microbiome with various diseases, such as obesity, inflammatory bowel disease, arthritis and cancers [2]. Animal models and *in vitro* models have been developed to study the structures and functions of the human microbiome. For example, fecal microbiota transplantation from human patients into germ-free mice can result in the appearance of signs and symptoms of human diseases [4,5]. Furthermore, *in vitro* bioreactors have been built to study complex interactions between members of human fecal microbiota for an extended period of time under controlled conditions [6].

Dysbiosis of human microbiome is associated with the development of many human diseases [7]. There are many factors that can influence the human microbiome, including age, diet, infections, inheritance and antibiotic usage. Enormous efforts have been put into the development of strategies for restoration of disbalanced human microbiome back to that of healthy individuals [8]. Interestingly, evidence has been provided that microbiome of healthy adults has stability that can withstand gross perturbation. Subramanian and colleagues have conducted a time-series metagenomic study of fecal microbiota collected during the acute diarrheal and recovery phases of cholera in a cohort of Bangladeshi adults. Their study showed that recovery from cholera is characterized by accumulation of bacterial taxa with similarity to the gut microbiota in healthy Bangladeshi children [9], which suggests that human microbiome has properties of resilience and understanding the microbiota assembly and interactions will facilitate prediction of future dysbiosis, disease as well as managing the human microbiome.

Biofilm is the prevalent lifestyle of microorganisms in nature, where individual microbial cells form aggregates or clusters embedded in their self-generated extracellular polymeric substances (EPS) [10]. Contrary to the free living planktonic cultures, biofilm provides a physical scaffold for maintaining the organized 3D structures of microbial communities, which further allows specific interspecies crosstalk and synergistic interactions. Even though biofilm formation mechanism is thoroughly investigated in certain model mono-species and multi-species microbial systems, there is lack of investigation on structural organizations and interspecies interactions in biofilms associated with human diseases. In this review, we will first describe experimental biofilm investigation approaches for probing the interspecies interactions and functions of model multi-species biofilm communities. Then, we will summarize recent studies on human disease-associated biofilms which might have an important implications on microbiome, including oral, gut and medical device-associated biofilms.

2. Experimental multi-species biofilm models

A variety of *in vitro* and *in vivo* experimental cultivation models have been employed to investigate multi-species biofilms, which have provided novel insights into interspecies interactions of complex microbial communities. The microfluidics-based flow cell biofilm cultivation system is widely used by both medical and environmental microbiologists to observe the biofilm development process of many microbial species. Pioneering works from Tim Tolker-Nielsen's group demonstrated that *Pseudomonas aeruginosa* cultures could rapidly differentiate into motile and non-motile subpopulations in the flow cell biofilm cultivation system, where the non-motile subpopulation initiate biofilm microcolony formation and form a "stalk" structure. After which, the motile subpopulation interact with the non-motile subpopulation through type IV pili and extracellular DNA (eDNA) to eventually form "cap" structures on top of the "stalk" structures [11,12]. Follow-up studies from the same group showed that quorum sensing and iron signaling were involved in the interactions between motile and non-motile subpopulations [13]. Using the flow cell biofilm cultivation system, Yang et al. demonstrated that *P. aeruginosa* also used its type IV pili to interact with eDNA from *Staphylococcus aureus* and formed dual-species macrocolony structures in biofilm co-cultures [14]. With the help of the multifluorescent protein tagging strategy, Lee KWK et al. established and examined a reproducible multi-species biofilm comprising P. aeruginosa (tagged by YFP), P. protegens (tagged by CFP) and Klebsiella pneumoniae (tagged by RFP). They found that the multi-species biofilm exhibited distinct structures that were not present in their single-species biofilms. Most interestingly, they showed that the multi-species biofilm was more resistant to the sodium dodecyl sulfate and tobramycin than their single-species biofilms [15]. Samarian et al. described a microfluidic system that used sterilized natural human saliva as the nutrient source, instead of artificial media, to develop oral multi-species biofilms [16]. This system mimics the *in vivo* communities and facilitates the investigation of impact of host-based effects, such as drinking, eating and antibiotic therapy, on oral biofilm-specific properties. *In vitro* biofilm model systems usually use a confocal laser scanning microscope in conjunction with image analysis tools to study biofilms. Recently, an image analysis software program, called BAIT (Biofilm Architecture Inference Tool), was applied to quantify the architecture of oral multi-species biofilms using a microfluidic biofilm system. BAIT was shown to be able to measure the changes in multi-species biofilm architecture and detect possible antimicrobial and anti-biofilm effects of candidate agents [17].

The microwell static biofilm cultivation system is another widely used tool for biofilm investigation. This type of biofilm cultivation system is easy to set up and suitable for high-throughput screening. Using 8-well microtiter slides, Li et al. showed that the human skin commensal *Malassezia globosa* is able to secrete aspartyl protease 1 (MgSAP1), which can hydrolyze *S. aureus* protein A and attenuate its biofilm formation [18]. Montelongo-Jauregui et al. built an *in vitro* dual-species oral biofilms of *Candida albicans* and *Streptococcus gordonii* using 96-well microtiter plates [19]. They found a clear synergistic effect in the formation of biofilms when both microorganisms were seeded together and further evaluated the structural and architectural features of the resulting biofilms. Importantly, they showed that dual-species biofilms of *C. albicans* and *S. gordonii* displayed higher levels of resistance against antimicrobial treatments under several tested conditions, as compared to single-species biofilms. Using the 96-well microtiter plates as a high-throughput screening system, Reisner et al. examined the stimulatory effects of cocultivation of natural E. coli isolates with E. coli K-12 on biofilm formation and found that 189 out of 403 strains (47%) exhibited significantly sturdier biofilm formation in cocultures compared to the monocultures of these isolates [20]. Interestingly, they also found that 56 *E. coli* isolates out of these 189 exhibited the strongest effects and it was linked to conjugative transmission of natural plasmids carried by these *E. coli* isolates. Thus, the microfluidics-based flow cell biofilm cultivation system and microwell-based static biofilm cultivation system complement with each other and are often combined in usage for multi-species biofilm investigations.

3. Interspecies interactions in coculture biofilm models

Microorganisms use both contact based and non-contact based strategies to interact with each other in biofilms and differential

species can form microbial consortia with specific functions. Bacterial cell surface appendages such as pili (fimbriae) and flagella are employed for attachment, motility and invasion. The abundance of bacterial surface appendages and their movement are regulated by chemotaxis systems as well as signaling mechanisms such as cAMP and c-di-GMP. In an early study, An et al. showed that *P. aeruginosa* can dominate in cocultured biofilms with *Agrobacterium tumefaciens* by "blanketing" or burying immature *A. tumefaciens* microcolonies using its flagellar and type IV pili [21]. In a dual-species biofilm formed by *P. aeruginosa* and *S. aureus*, Yang et al. demonstrated that *P. aeruginosa* type IV pili can interact with the eDNA from *S. aureus* and form mixed-species microcolonies, which can be impaired by DNase treatment [14]. Unlike eDNA which can be a shared resource by different microbial species, other biofilm matrix components could be exclusively used only by the producers and thus bring them a competitive advantage in the mixed-species biofilms. In an individual-based simulation model, Xavier and Foster showed that biofilm EPS polymer producer pushes its later generations up and out into better oxygen condition in biofilms while suffocating neighbouring nonpolymer producers [22]. This modelling result was supported by a later experimental biofilm study by Yang et al., in which it was shown that *P. aeruginosa* wild-type strain can outcompete with its Psl exopolysaccharide-negative mutant in biofilm co-cultures [23]. In a *P. aeruginosa-S. aureus* dual-species biofilm model, Chew et al. found that *P. aeruginosa* Psl exopolysaccharide production is associated with increased *P. aeruginosa* abundance and reduced *S. aureus* aggregation in early stage biofilm formation (Fig. 1), which is corelated with the activation of the *P. aeruginosa* diguanylate cyclase SiaD [24]. Interestingly, Periasamy et al. showed that even though the *P. aeruginosa* Psl exopolysaccharide can mainly increase its own fitness over other species in mixed-species biofilms, it can increase the communal stress resistance of the three species biofilms formed by *P. aeruginosa*, *P. protegens*, and *K. pneumoniae* [25]. This study suggests that it is not the absolute abundance of a microbial species but its physiology that determines the functions of microbial communities.

Type VI Secretion Systems (T6SS) is widely distributed among bacterial species and multi T6SS can be evolved in a single species for those who are living in diverse environments. Bacterial cells employ T6SS to translocate effector proteins into target cells using a contractile nanomachine composed of several subcomplexes. It has been extensively shown that T6SS is an efficient weapon to outcompete rival bacteria in polymicrobial environments [26]. In a recent study, Cheng et al. cultivated *P. aeruginosa* in 18 species planktonic and biofilm microbial communities to understand its physiology under complex microbial condition. They reported that *P. aeruginosa* became the most dominant species only under biofilm coculture while not in planktonic coculture. Both type IV pili and Psl exopolysaccharide, the biofilm determining factors in mono-species *P. aeruginosa* cultures, were found to also contribute to the fitness of *P. aeruginosa* over other species in biofilms. In addition, Cheng et al. has performed transcriptomic analysis to compare *P. aeruginosa* physiology in 18-species biofilms vs. its mono-species biofilms and found that *P. aeruginosa* T6SS genes were highly induced in the 18-species biofilms compared to its mono-species biofilms. The *P. aeruginosa* T6SS deficient mutants significantly reduced fitness over other species in the 18-species biofilms and were not able to impair the macrocolonies formed by other species (Fig. 2) [27]. Interestingly, besides *P. aeruginosa*, *K. pneumoniae* was found to be the second dominant species in the 18-species biofilms and seem to be resistant to the *P. aeruginosa* T6SS. This might due to the fact that *K. pneumoniae* produces large amount of exopolysaccharide which has previously been shown to confer resistance towards T6SS attack by other bacterial species [28].

Besides contact-based strategies, a variety of small molecules have been employed for mediating interspecies interactions in biofilms, including quorum sensing molecules, iron siderophores and antibiotic-like molecules. N-acylhomoserine lactone (AHL) molecules are the most studied quorum sensing signaling molecules in Gram-negative species and have been reported to convey interspecies communications in mixed-species biofilms that might existing in the lungs of cystic fibrosis (CF) patients [29]. *P. aeruginosa* often coexists with *Stenotrophomonas maltophilia* in CF patients and the diffusible quorum sensing signal factor (DSF) by *S. maltophilia* can interact with the *P. aeruginosa* PA1396 sensor kinase, which leads to the formation of extended filaments in *P. aeruginosa* biofilms and increased tolerance to polymyxins [30]. In a recent study, Keogh et al. demonstrated that *Enterococcus faecalis*, a frequent biofilm forming pathogen that causes infections of the urinary tract, indwelling catheters, and surgical wound sites, could significantly boost the growth of *E. coli* biofilms under iron-restrict *in vitro* and *in vivo* conditions by exporting L-ornithine. The L-ornithine was further found to stimulate biosynthesis of the enterobactin siderophore of *E. coli* and increase its efficacy of iron uptake [31].

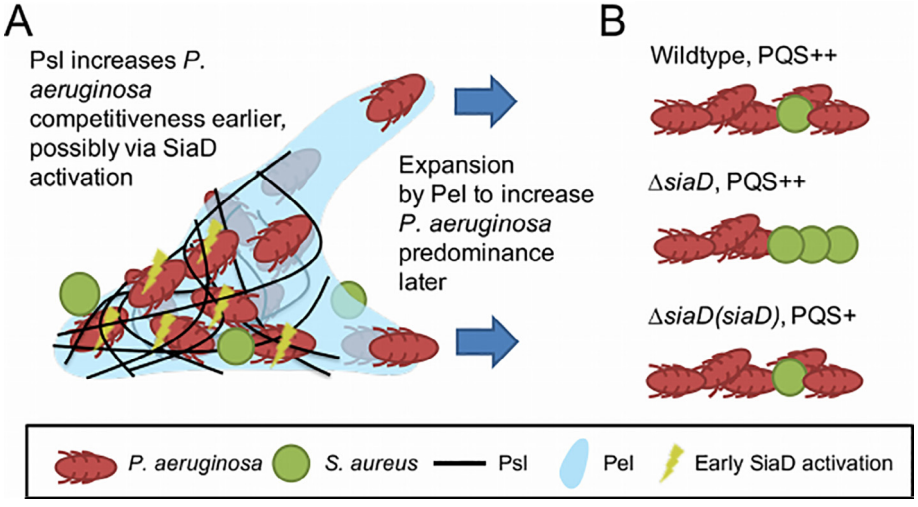

Fig. 1. *P. aeruginosa* matrix polysaccharides Pel and Psl and SiaD diguanylate cyclase contribute to its predominance in dual-species biofilms with *S. aureus*. Psl enhances *P. aeruginosa* competitiveness in early stages, possibly via SiaD activation, whereas Pel enables biofim expansion to increase *P. aeruginosa* predominance in the later stages. Figure was adapted from [24] with permission.

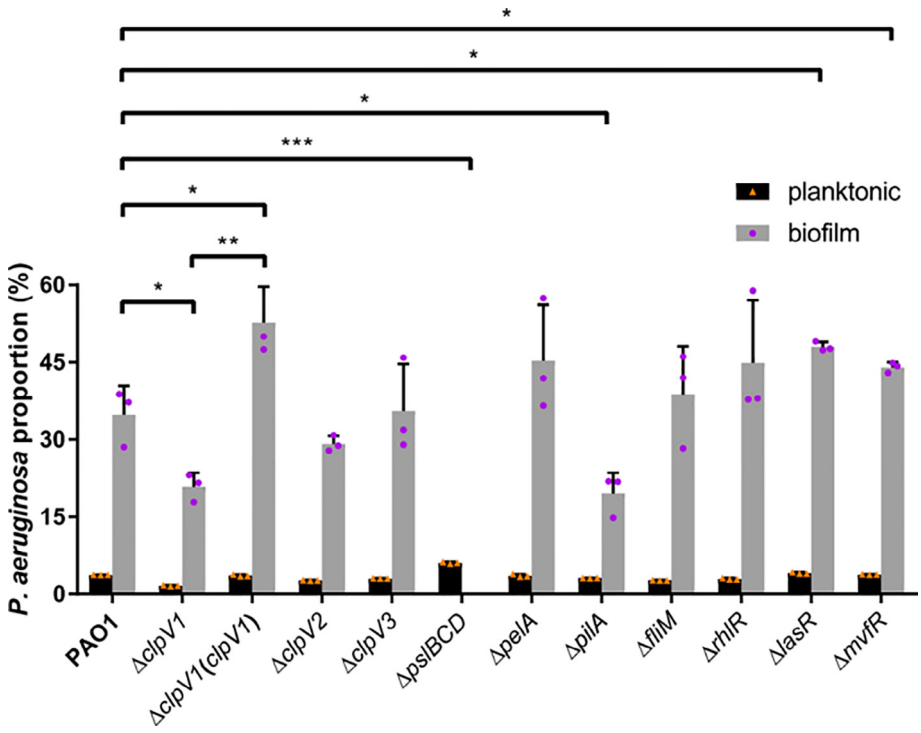

Fig. 2. *P. aeruginosa* population dynamics in the multi-species biofilm community is affected by H1-T6SS (*clpV1*) and biofilm formation determinants such as Psl exopolysaccharide (*pslBCD*), type IV pili (*pilA*) and quorum sensing (*lasR* and *mvfR*). Grey bars: biofilm microbial community; Black bars: planktonic microbial community. Figure was adapted from [27] with permission.

The above model studies have clearly showed that interspecies interactions happen frequently in mixed-species biofilms, which drive biofilm structure development and facilitate special functionalization. Next, we will review interspecies interactions in several human diseases-associated biofilms. We choose oral biofilms, gut biofilms as well as biofilms related to ventilator-associated pneumonia (VAP) and catheter-associated urinary tract infection (CAUTI) as examples. Several other biofilm-associated infections such as chronic wounds and cystic fibrosis lung infections have been reviewed regularly [32–35] for their microbiome and biofilm composition and thus not included in this review.

4. Multi-species oral biofilms

Human gastrointestinal (GI) tract extends from oral cavity to the rectum, passing esophagus, stomach and intestines. Organs along the GI tract are colonized by microorganisms to different extend. Among these microbe-bearing organs along the GI tract, oral cavity and gut are massively colonized by various microorganisms. Since the first observation of oral microbiota by Anton van Leeuwenhoek, more than seven hundreds of bacterial species have been identified to inhabit in the human oral cavity, among which 100–200 species are found in any individual with extensive diversity among different individuals [36]. Bacteria residing at the areas with solid substrates, such as the tongue, the teeth, periodontal area and the gingival sulcus, remain in the oral cavity after personal hygiene process and form antibiotic-resistant biofilms around their habitats. Epithelial cells shed and thus control biofilm formation of soft tissues. Biofilms on hard tissues persist and cause diseases after prolonged growth. Lack of regular oral cleaning allows maturation of biofilms on teeth and formation of dental plaques. The spatial arrangement of microbes, inter-species interactions among the microbes and changes in microbial composition in the biofilms are associated with the development of oral diseases such as dental caries, gingivitis and periodontitis.

Streptococcus spp. and *Actinomyces spp*. interact with salivary pellicles covering tooth surfaces to initialize microbial attachment while *Fusobacterium nucleatum* serves as linker of other bacteria, including *Capnocytophaga spp*., *Prevotella spp*., *Veillonella* spp., *Actinomyces* spp., *Porphyromonas* spp., and *etc*., to those initial colonizers to form cell layers and build up biofilms [37,38]. *Streptococcus* spp., *Actinomyces spp*., *Corynebacterium* spp., and *Veillonella spp*. predominate in healthy oral environment [37,39]. Spatial arrangements and architectures of oral biofilms at different locations, such as tooth surface and subgingival areas, were observed using various methods. Zijinge et al. observed the arrangement of predominating species in different layers of biofilms on supragingival and subgingival areas of teeth of patients with periodontal problems using fluorescent *in situ* hybridization (FISH) approach [40]. Generally, *Streptococcus spp*. and *Actinomyces spp*. locate at basal layer, *F. nucleatum* and *Tannerella* spp. locate at middle layer, while other microbes such as *Lactobacillus*, *Spirochaetes*, *Porphyromonas*, other periodontal pathogens and yeast reside differentially in the oral microbiota depending on biofilm locations [40].

Gram-positive bacteria communicate with surrounding cells using signaling peptides while Gram-negative bacteria communicate with each other by quorum sensing autoinducers in oral biofilms. Studies done by McNab et al. and Benítez-Páez et al. proved that quorum sensing autoinducers play essential roles in the composition and formation of oral biofilms [41,42]. High concentration of autoinducer-II promotes the growth of pathogenic bacteria in oral biofilm community [37]. Moreover, exopolysaccharide also plays important role in the oral biofilm establishment and maturation. Cariogenic pathogen, *Streptococcus mutans,* secretes *gtf*-gene-encoded glucosyltransferases (GtfBCD), which bind to salivary pellicles and catalyze the formation of glucans, the major EPS in oral biofilms, from sucrose. Such EPS plays a key role in the initiation of oral biofilm formation and in maintenance of structural integrity of microcolonies. GtfB is the key regulator of microcolony

formation and also serve as a linker between *S. mutans* and other oral bacteria such as *Streptococcus*, *Lactobacillus*, and *Actinomyces* due to its surface-binding characteristics [43,44]. A later study showed that an amino acid, L-arginine, disrupt the synthesis of GtfB-derived exopolysaccharide and alters the cariogenic competency of the biofilm by preventing the growth of *S. mutans* upon inducing H_2O_2 production by *Streptococcus gordonii* in the multi-species biofilm model with *Actinomyces naeslundii* and *S. gordonii* [45]. Zhu et al. demonstrated that polymicrobial biofilm formation promoted the tolerance of *Porphyromonas gingivalis*, a Gram-negative bacterium which is regarded as one of the keystone pathogens in chronic periodontitis, to oxidative stress under micro-aerobic conditions. The presence of *Streptococcus sanguinis*, a Gram-positive oral commensal bacterium, inhibited the survival of *P. gingivalis* in dual-species biofilms via the secretion of hydrogen peroxide (H_2O_2). Interestingly, this repression could be attenuated by the presence of *Aggregatibacter actinomycetemcomitans*, a Gram-negative facultative anaerobe bacterium that is often found in chronic periodontitis, in a tri-species biofilm. *A. actinomycetemcomitans* has the capacity to grow in both supragingival [46] and subgingival [47] biofilm models, and in the latter case it is shown to cause proteomic shifts within the biofilm, such as increased metabolic rate (including increased protein transport and fatty acid biosynthesis), and ferric iron-binding [47]. These findings reveal that polymicrobial interactions play important roles in shaping bacterial community in biofilms [48].

Taxonomic composition and the abundance of different bacteria in oral biofilms vary extensively from health to disease conditions. A time-course metagenomics and metatranscriptomics study of dental biofilm done by Edlund et al. group revealed that the community composition of dental biofilm, virulence mechanisms and abundance of cariogenic species like *Lactobacillus fermentum* are dramatically influenced by pH change in the oral cavity [49]. Significant increase in the abundance of acid-tolerant species in the dental biofilms including *Streptococcus mutans*, other *Streptococcus spp*., *Actinomyces spp*., *Veillonella spp*., *Lactobacillus fermentum*, *Bifidobacterium spp*,. was observed in the individuals with caries comparing to the healthy individuals [50]. Later, Aas et al. claimed that the abundance of *Streptococcus mutans* is insignificant in dental caries while other bacteria such as other *Streptococcus spp*., *Actinomyces spp*., *Veillonella spp*., *Bifidobacterium spp*., *Lactobacillus spp*., *Propionibacterium spp*, and *Atopobium spp*. predominant different sites of caries and promote the development of caries [51]. Pathogenic species in the biofilms induce changes in other commensal species to outcompete with the pathogens. Antagonistic interactions among *S. mutans* vs *S. gordonii*/*A. naeslundii* and *S. mutans* vs *S. gordonii*/*S. sanguinis* were observed that the growth of the pathogen, *S. mutans*, was restrained upon production of hydrogen peroxide by the other species while mutacins secreted by *S. mutant* have lytic activities on other *Streptococcus* species [45,52]. Such antagonistic interactions have also been observed between *S. mutans* and *Streptococcus oralis* in an oral biofilm environment, in which *S. oralis* as commensal keeper of homeostasis in the biofilm by antagonizing *S. mutans*, thus preventing a caries-favoring dysbiotic state [53]. Contents in hosts' diet and oxygen availability have significant influence on the composition shifts in oral biofilms. Furthermore, *in vitro* study of dual-species biofilms of *S. mutans* and the yeast, *Candida albicans*, indicated that although there was higher production of lactic acid in mixed biofilm, *C. albicans* could impede the formation of dental caries by reverting the acidic pH and release of calcium after 72 h of growth [54]. A multispecies biofilm study of *C. albicans* with saliva samples revealed that *C. albicans* has insignificant influence on lactic acid production and biomass of biofilm but promotes the growth of anaerobic bacteria including *Veillonella*, *Prevotella*, and others [55]. Gingivitis and periodontitis are caused by the establishment and progress of dental plaques with higher biofilm biomass and dynamic microbial composition, especially the anaerobic bacteria, depending on environmental conditions at the gingival and periodontal areas. *Fusobacterium nucleatum*, *Prevotella intermedia* and *Porphyromonas gingivalis* produce high concentration of autoinducer-II and are dominant species in the periodontal biofilm [37,56]. Beside *in situ* infections, oral biofilm bacteria and secreted endotoxins link with various diseases such as cardiovascular diseases [57,58], Alzheimer's disease [59], and colorectal cancers [60]. Such discoveries indicate that biofilm formation may result in chronic inflammations of oral tissues allowing the oral pathogens to invade and persist in different human tissues and cause the onset and progression of various oral pathogen associated diseases.

Some studies focused on the interactions of biofilm with host tissues. The formation of subgingival biofilms by multi-species, especially the Gram-negative anaerobic species *P. gingivalis*, *Tannerella forsythia* and *Treponema denticola* (also known as the "red complex" species), elicited a large number of transcriptional changes in gingival epithelial cells and gingival fibroblasts, resulting the local release of proinflammatory factors that represent an important initial response for periodontal inflammation [61,62]. A major chemokine produced by the gingival epithelium in response to biofilm challenge is interleukin (IL)-8 and IL-1 family. As part of biofilms, "red complex" species differentially regulate IL-8 in gingival epithelia, potentially affecting the chemotactic responses of the tissue [63]. While the recognition of pathogen-associated molecular patterns that activate IL-1β is predominantly regulated by the nucleotide-binding oligomerization domain-like receptor (NLR) family. Among them, NLRP3 inflammasome is involved in the innate immune responses in periodontal disease. Subgingival biofilms down-regulate NLRP3 and IL-1β expression, partly because of *P. gingivalis*. These dampened host innate immune responses may favor the survival and persistence of the associated biofilm species in the periodontal tissues [64,65]. In addition, inflammatory bone destruction triggered by oral bacteria is a hallmark of chronic and apical periodontitis. Receptor activator of NF-κB ligand (RANKL) activates bone resorption, whereas osteoprotegerin (OPG) blocks its action. By using a *in vitro* supragingival biofilm model, Belibasakis et al. demonstrated that the high responsiveness (RANKL/OPG expression ratio) of dental pulp to the supragingival biofilm challenge could constitute a putative pathogenic mechanism for apical periodontitis [66].

5. Multi-species gut biofilms

Intestines are major organs bearing the most complex and abundant microbial biofilms along the GI tract, consisting mostly anaerobic or facultative anaerobic species. The intestines consist of several compartments extending from small intestine which is composed of duodenum, jejunum, and ileum, to large intestine and rectum. Thousands of culturable as well as non-culturable microorganisms inhabit in variable niches along small and large intestines. Zou et al. have identified more than 1500 gut bacteria via culture and provided sequences of these bacteria as reference genomes for future analysis [67]. Moreover, gut bacteria retain at least 9 million unique genes while Bacteriodetes and Firmicutes are two major phyla found in gut microbiota [68]. The composition of gut microbiomes is highly variable from individual to individual depending on diets, environments, lifestyles *etc*. Formation of microcolonies on the mucosa and spatial arrangement of microorganisms in gut biofilms are observed using different approaches such as microscopic visualization, live/dead staining and FISH [69–71]. Deeper insight into microbial spatial arrangement using Combinatorial Labeling and Spectral Imaging FISH strategy allows further understanding on the microbial interactions in the biofilms

[72,73]. Biofilms play important role on promoting and keeping homeostasis in the gut due to the specific fermentation capability of biofilm bacteria to different substrates [74]. Development and variation of such intestinal microbial biofilms have great impacts on their human host in different ways, extending from digestions of multi compounds to regulation of host immune activities and disease development [75,76].

Human colons contain a dense and highly viscous layer of fast-growing mucus, which is a harsh environment for biofilm development and microbial penetration. Microbial infection may happen and lead to chronic inflammation if this mucus layer is breached. Distinct microbial compositions in the colon were observed from fecal population, mucosal population and lumen population [69,77,78]. Microbial cells evolve different mechanisms for binding to the mucosal layer, such as the formation of mucus-binding pili by *Lactobacillus rhamnosus* [69,79]. Studies have shown that biofilm bacteria in the colon secrete various enzymes including mucin-degrading enzymes for obtaining energy in order to survive and invade mucus layer [80–83].

Many diseases are associated with altered colonic biofilms including immune diseases, digestive diseases and neuronal diseases. Among all, mucus-binding biofilms play essential role in disease pathogenesis and development. *Bacteroides fragilis* is the major species in the gut biofilms of inflammatory bowel disease, *Eubacterium rectale* and *Bacteroides fragilis* are major biofilm components in self-limiting colitis patients, while *Eubacterium rectale-Clostridium coccoides* are dominating in biofilms of patients with irritable bowel syndrome [70]. Microcolonies consisting of *Enterococci*, *Bifidobacteria* and *Bacteroides* were observed on mucus layer. Dominant mucosal bacteria include *Bacteroides* and *Bifidobacterium*, among which *Bacteroides thetaiotaomicron*, *Bacteroides vulgatus*, *Bacteroides fragilis* and *Bifidobacterium adolescentis* are predominating species while *Bifidobacterium angulatum* is the prevalent species, *Peptostreptococci* and *Veillonella* are specific species in patients with ulcerative colitis [71]. Other mucus colonizing bacterial species include *Enterobacteria*, *Clostridia* and *etc.* [84]. Bacteria are able to reach the colonic epithelial cells through the permeable mucus layer in patients with ulcerative colitis [85]. Another common inflammatory bowel disease associated with biofilm is Crohn's disease. *Serratia marcescens*, *Escherichia coli* and the fungus, *Candida tropicalis*, are abundant species in patients with Crohn's disease. Interaction among these three species leads to increased biofilm formation by them and possibly play key role in the development of Crohn's disease [86].

Lipopolysaccharides secreted by gut bacteria such as *B. fragilis* and *E. coil* are pro-inflammatory to neurons in the brains of Alzheimer's disease (AD) while the presence of *E. coli* lipopolysaccharides is detected from neocortical and hippocampal parts of AD brain [87,88]. *Helicobacter pylori*, which is normally found in the biofilm in upper GI tract, associates with AD as clearance of this bacterium improves the cognitive state of AD patients [89]. Furthermore, formation gut biofilm and perturbation of microbial composition in such biofilm closely link to the initiation and development of colorectal cancer [76]. Bacterial biofilms had been found from most of right-sided tumors from patients with colorectal cancer (Fig. 3) while the formation of these biofilms leads to lower E-cadherin in crypts and higher permeability of epithelial layer and allow bacterial toxins to reach epithelial cells to activate IL-6 and Stat3 and induce procarcinogenic tissue inflammation [90]. A recent study revealed that *Escherichia coli* encoding polyketide synthase (for colibactin production) and enterotoxigenic *Bacteroides fragilis* co-colonize and dominate in mucosal biofilms in patients with familial adenomatous polyposis and promote tumorgensis in the colon, where *B. fragilis* degrade colonic mucus layer and heightened binding of *E. coli* which promotes DNA damage by colibactin in epithelial cells and interleukin-17 induction [91].

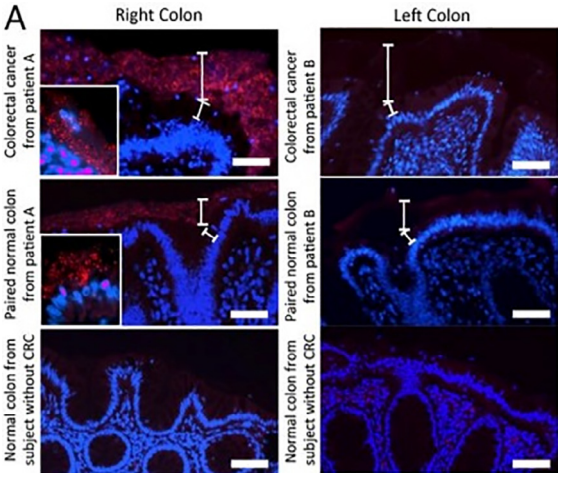

Fig. 3. Biofilms were detected using FISH and DAPI staining on colon tumor of patient with colorectal tumor (upper panel), paired normal colon in same patient (middle panel) and normal colon without colorectal cancer (lower panel). Biofilms detected on right colon (left panel). Biofilms detected from left colon (right panel). Closeup image showing at the lower left corners of top and middle images on left panel indicated the bacteria cells located in close proximity to epithelial cells in patient with colorectal cancer. Bacteria were stained red while nucleus was stained blue. Figure was adapted from [90] with permission.

Gut biofilm formation is regulated by various factors. Hydrogen sulfide is an example of modulating factor of mucus-biofilm interaction in the intestines [92]. Hydrogen sulfide was shown to inhibit growth of planktonic cells, promote biofilm formation and regulate production of mucus in the colon using rodent model and human-originated microbial samples [93]. Another study revealed that human secretory immunoglobulin A is probably a factor mediating and enhancing biofilm formation by gut microbiota [94]. In addition, engineered bacteria could interfere the formation of pathogenic biofilm in the gut and prevent infections. Hwang et al. has proved that probiotic *Escherichia coli* Nissle 1917 expressing biofilm-disrupting enzyme could detect and eradicate *Pseudomonas aeruginosa* biofilm infection in the gut of animal models [95]. Probiotic bacteria, *Lactobacilli*, have inhibitory effect on the biofilm formation and pH-dependent biofilm dispersion effect on intestinal pathogen, *Vibrio cholerae* [96].

6. Multi-species biofilms in ventilator-associated pneumonia

Ventilator-associated pneumonia (VAP) is the most common nosocomial infection in patients admitted to the intensive care unit (ICU) with a prevalence of approximately 15% [97,98] and has considerable mortality of 11–17% [99,100]. Bacteria causing VAP come mostly from the oropharyngeal cavity, although in some cases, especially in intubated patients in the horizontal supine position with a nasogastric tube, they may also come from the stomach or from the sinuses when nasotracheally intubated [97,101]. The accumulation of contaminated oropharyngeal or gastrointestinal secretions in the subglottic space is a critical event in the pathogenesis of VAP. The microorganisms deposited above the endotracheal cuff leak through the folds formed by the cuff in contact with the trachea via microaspiration. Having overcome the obstacle of the endotracheal cuff, micro-organisms can colonize the endotracheal tube (ETT) and the tracheobronchial tree. The ETT used for patient intubation is commonly made of polyvinylchloride. This thermoplastic material is inexpensive, but it also provides an ideal surface to which pathogens can adhere and form biofilms [102]. Biofilms develop rapidly following the intubation, with well-

organized antibiotic-tolerant structures detectable within 24 h [103]. Mounting evidence shows that biofilms on ETTs serve as significant and persistent reservoirs of pathogens to cause VAP [97,104]. Strategies involving modified ETTs to prevent or remove ETT-biofilms were proven to reduce VAP occurrence in adults, including cuffed ETTs and silver or other nanoparticle coated ETTs [105,106]. In addition, Zangirolami et al. [107] reported a photodynamic therapy for eradicating ETT-biofilms. Photodynamic therapy combines light (LED at 450 nm) and a photosensitive molecule (1.25 mg/mL curcumin) for produce reactive oxygen species leading to bacterial death. Two hours of treatment resulted in 70% biofilm reduction. The advantages of this therapy are noninvasive and without the stimulus of microbial resistance.

New concepts of lung ecology have recently introduced the microbiome variable into the VAP equation. During the past 10 years, the notion of "the normal lung is free from bacteria" has been challenged [108–110]. The healthy lung appears to be populated by multi resident bacterial species, such as *Staphylococcaceae*, *Propionibacteriaceae*, *Corynebacteriaceae*, *Streptococcaceae*, *Veillonellaceae*, *Neisseriaceae* and *Fusobacteriaceae*, among other lineages [111,112], that migrate to the distal airways from the oral cavity [109]. According to the adapted island model, the respiratory microbiome represents a dynamic community, where the equilibrium point is achieved by the balance between immigration and elimination mechanisms [108]. Mechanical ventilation is assumed to imbalance this equilibrium due to several factors [113]. On the one hand, supine positioning, gross aspiration, impaired consciousness and open oropharynx increase the microbial immigration into lower airways. On the other hand, disabled cough reflex, impaired mucociliary clearance and ETT-biofilm decrease the bacterial extinction from the respiratory tract. Potential pathogens may cause pneumonia once a certain bacterial load is achieved as a result of the right growth conditions due to altered lung physiology (decreased pH, the presence of sputum) [114]. Recent studies using 16S rRNA gene amplicon sequencing-based microbial community profiling demonstrated that continued endotracheal intubation and mechanical ventilation caused loss of community diversity and increased relative abundance of well-adapted microbes, which was recognized as potential VAP pathogens such as *Burkholderia*, *Bacillales* (with *Staphylococcus aureus* as most important species) and *Pseudomonadales* [115–117]. Dysbiosis of the respiratory microbiome is more profound in patients who develop VAP than in those that do not develop pneumonia [116]. In addition, dental plaque as a biofilm also impacts VAP. Sands et al evaluated microbial changes that occurred in dental plaque and lower airways of 107 critically ill mechanically ventilated patients. A "microbial shift" occurred in dental plaque, with colonization by potential VAP pathogens *Staphylococcus aureus* and *Pseudomonas aeruginosa* in 70% and 55% of patients, respectively. Respiratory pathogens were also isolated from the lower airways and within the ETT-biofilms. Based on these findings, it was apparent that during mechanical ventilation, dental plaque represents a source of potential VAP pathogens.

P. aeruginosa is a known VAP pathogen, however, it usually cocolonizing with non-pathogenic *Streptococcus* sp., like *S. epidermidis* and *S. mitis*. Recent studies showed that *Streptococcus* sp. not only promotes ETT-biofilm formation of *P. aeruginosa* in neonates but also reduces IL-8, TLR-2 and 4 levels induced by *P. aeruginosa* [118,119]. By determining the composition of ETT biofilm, Hotterbeekx et al. stated that VAP patients with a relative abundance of *Pseudomonadaceae* <4.6% and of *Staphylococcaceae* <70.8% had the highest chance of survival. When *Pseudomonadaceae* were >4.6%, age of the patient (<66.5 years) became the most important predictor of patient survival. These data indicate that the composition of the ETT-biofilm correlates with patient prognosis, and the presence of *P. aeruginosa* is an important predictor of the patient outcome. In addition, *P. aeruginosa* could accumulate genetic mutations in the course of VAP that often lead to its better adaptability to the host environment. With the help of next generation sequencing, for instance, Wang et al. demonstrated that positive selection dominantly shaped *P. aeruginosa* genomes during VAP infections and led to three convergent evolution events, including loss of-function mutations of quorum sensing major regulator LasR, mutational inactivation of Mpl (enzyme responsible for recycling cell wall peptidoglycan), and a pyoverdine-deficient phenotype; suggesting the rapid *in vivo* evolution of *P. aeruginosa* leads to attenuated virulence in VAP patients [120].

7. Multi-species biofilms in catheter-associated urinary tract infections

Urinary catheters are one of the most commonly used medical devices in the world and notoriously prone to infection [121]. The catheter is inserted into the bladder through urethra, in order to measure the urine output and to prevent urine retention or incontinence [122]. The primary challenges in the use of indwelling catheters are biofilm formation [123]. Normally, microorganisms in bladder are present in a planktonic state where they are freely suspended in the urine. In this state, they are unlikely to cause a urinary tract infection (UTI) unless they are present in large numbers that may overwhelm the bladder's innate defenses. When an indwelling urinary catheter is in place, microorganisms can attach to the medical device, forming biofilms [124]. The longer a urinary catheter is in place, the more likely it is for a biofilm to form on its surface and cause catheter-associated urinary tract infections (CAUTIs). Patients who are catheterized for short-term ($\leq$7 days) experience biofilm formation 10–50% of the time; however, practically all patients who are catheterized for long-term (>28 days) are found to present with biofilm formation [125]. It was estimated that approximately $3790 is the minimum amount spent in the treatment and diagnostic of each episode of CAUTI [126], including antimicrobial therapy, increased length of hospitalization, physician visits and morbidity. In addition, it has been reported that patients with CAUTIs might develop numerous complications such as cystitis, bladder stones, prostatitis, epididymitis, pyelonephritis, septicaemia and endotoxic shock [121,126].

CAUTIs can be caused by bacteria or yeasts. The European Center for Disease Prevention and Control (ECDPC, 2015), in an annual epidemiological report from 2014, reported that the most frequently isolated CAUTI microorganisms were *E. coli* (28%), *Candida sp.* (18%), *Enterococcus sp.* (17%), *P. aeruginosa* (14%) and *Klebsiella sp.* (8%) [127]. A study by Chatterjee et al. [128] sampled 150 catheters from patients with no history of UTIs and found that 130 of the catheters had pathogens present both on the catheter and in accompanying urine samples. The most common microorganisms found during the study by Chatterjee et al. included *E. coli, C. albicans*, *P. aeruginosa, K. pneumoniae, S. aureus, S. epidermidis, Proteus mirabilis, Proteus vulgaris, Providentia rettgeri* and *Citrobacter freundii* [128]. Where do these bacteria come from?

The long-held idea that the bladder and urine itself are sterile is a misconception made by early bacteriologists in the 1800s [124]. This idea led many doctors to believe that any UTI or CAUTI was from external contamination only. As the field of microbiology evolving, many culture-independent techniques have been widely used to identify and quantify the microbial diversity from biofilm infections [129]. Today, it is accepted that the prevalence of CAUTIs seems to be caused by a combination of both internal microflora and externally introduced contamination [130]. Thomas-White et al. has isolated and sequenced 149 bacterial strains from catheterized urine of 77 women and found that vaginal microbiota and bladder microbiota have great similarity, with functional

capacities that are distinct from those observed in the gastrointestinal microbiota [131]. Patients who practice intermittent catheterization are most at risk from the microflora of the urethral meatus being pushed up and into the bladder by catheter usage, with *E. coli* being the main species responsible for CAUTIs in intermittent catheter users [132]. With indwelling catheters, the main concern is bacterial biofilms that lead to the creation of crystalline biofilms, with *P. mirabilis* infection being a lead concern to patients [133].

CAUTI biofilms can be characterized as either crystalline or non-crystallized biofilms. Crystalline biofilms generally occur due to infection by urease-producing bacteria, such as *Proteus mirabilis, Proteus vulgaris,* and *Providentia rettgeri* [121]. Among them, *P. mirabilis* is isolated most frequently from patients and produces the most urease, an enzyme that hydrolyses urea, breaking it down into ammonia and carbonate ions [123]. Urease-producing bacteria use the produced ammonia as a source of nitrogen and carbon to support further colony growth [134]. Increasing ammonia levels lead to an increase in the overall pH of the urine in the bladder, and the bacterially produced alkaline environment causes calcium and magnesium to come out of solution and precipitate into crystals. This process of catheter encrustation via crystallization is directly connected to the formation of biofilms and the products produced by the organisms within [135]. There are specific advantages to forming crystallized biofilms. CAUTIs can often persist in patients when a catheter is removed, and several studies believe this could be due to the crystalline biofilm formation [123,136]. As the previously encrusted catheter is removed, crystals can break off, still containing the bacterium that they formed upon. These crystal fragments act as seeds on which newly formed minerals can grow and ultimately form bladder stones. These bladder stones can store pathogens, re-infecting the bladder and allowing the biofilm crystallization of a new catheter, thus perpetuating the cycle [121].

Some urease-producing bacterial species do not form crystallized biofilms as their urease production levels are too low. These include *P. aeruginosa, S. aureus, K. pneumoniae, E. coli, Morganella morganii,* and *Providencia stuarii,* to name a few [137]. While these microbes do have the ability to form biofilms, it will not form crystalline in structure without help from other species as their lower urease output, although able to hydrolyze urea into ammonia, is not high enough to raise the pH of urine to >8.0, which is needed for apatite and struvite to form [136]. Interestingly, *K. pneumoniae* and *P. aeruginosa,* although they cannot produce crystals, can still block catheters and cause the same problems associated with reduced or halted bladder drainage [123]. Broomfield et al. [136] investigated different approaches to controll crystalline biofilms on catheters, and during their testing, they observed that both *K. pneumoniae* and *P. aeruginosa,* while not able to produce a crystalline biofilm, produced large amounts of a mucoid material that did not block the catheter but did greatly reduce urine flow. Lassek et al. combined metaproteomics approach with *in vitro* proteomics analyses to unravel bacterial community structure and function as well as host-pathogen interactions induced by catheter-associated microbial biofilms [138]. Their proteome analysis revealed that the investigated catheter biofilms is mainly colonized by three bacterial species, *P. aeruginosa, Morganella morganii*, and *Bacteroides* sp. Based on the analysis, the authors proposed that N-acylhomoserine lactone- and autoinducer 2-mediated quorum sensing might contribute to the expression of virulence factors by these pathogens and determine the overall biofilm physiology (Fig. 4). *P. aeruginosa* was shown to express secreted and surface-exposed proteases for amino acids usage while *M. morganii* was proposed to take up sugars and degrade urea. What's more, iron limitation was identified as a major challenge in this catheter biofilm and *P. aeruginosa* is suggested to utilize siderophores produced by *M. morganii* and/or *Bacteroides* sp. (Fig. 4).

8. Summary and prospective view

It is evident that biofilms have a huge impact on composition and function of human microbiome. Biofilm formation can be a strategy for structural organization of microbiota, which confers long term stability. The extensive interspecies interactions within biofilms might facilitate division of labour for different species within the microbiota. Numerous questions remain to be answered regarding the fundamental mechanisms of biofilm formation in microbiota: i) which species are the core biofilm formers that initiate biofilm formation? 2) what are the key biofilm physical and chemical properties that determine microbiome composition and function? 3) what are the effective strategies to manipulate the biofilms (e.g. induce biofilm formation or dispersal) within human

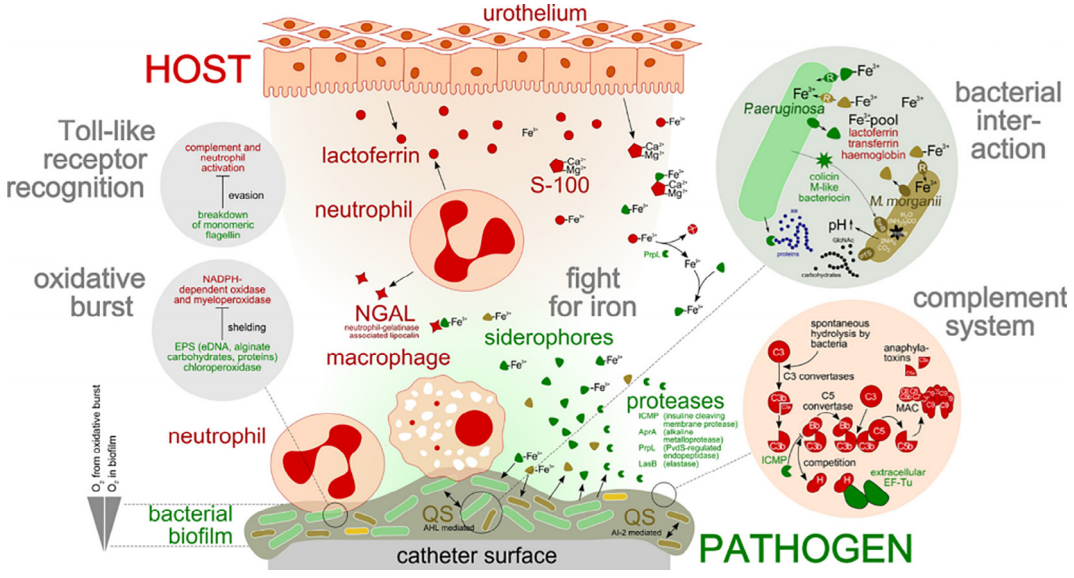

Fig. 4. Host-pathogen interactions model during the catheter-associated urinary tract infection. The left circles describe bacterial strategies to evade host immune response while the right circles depict interspecies interactions and adaptation strategies by *P. aeruginosa* and *M. morganii.* Figure was adapted from [138] with permission.

microbiota? Answering these questions might provide novel insights on the human microbiome and its associated diseases.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by Natural Science Foundation of China (31870130) and Singapore centre for environmental life sciences engineering. L.Y. is supported by the Start-up Grant (Y01416206) from Southern University of Science and Technology (SUSTech).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.csbj.2019.09.010.

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