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Influence of interspecies interactions on biomass and extracellular polymeric substances of bacterial biofilms

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ABSTRACT

BACKGROUND

Studies with emphasis on pure and mixed-species biofilms are of significant importance in understanding biofilm formation mechanisms during microbial infections. This research aims to evaluate pure- and dual-species biofilms of *Escherichia coli* (code A), *Staphylococcus aureus* (code B), *Klebsiella pneumoniae* (code C) and *Pseudomonas aeruginosa* (code D) pathogenic bacterial species and their production of biofilm exopolysaccharides at laboratory scale.

METHODS

Biofilm biomass (A595) of pure- and dual-species cultures was determined by means of a microtiter plate assay in triplicate using a microplate photometer (Fisher Scientific, type-357). Extracellular polymeric substances (EPS) and soluble microbial products (SMP) were extracted from the biofilm cells (pure- and dual-species cultures) using the alkaline-heat extraction method. Dry weights (g/L) of EPS and SMP were determined by drying the samples at 105 °C for 8 hours.

RESULTS

Klebsiella pneumoniae biofilm biomass accounted for a 28-72% greater biofilm biomass than the other bacteria. Experimental values of dual-species biofilm biomasses were in the range of 6% to 30% over theoretical values. The experimental value of one dual-species (bacteria B + D) biofilm biomass was 30% higher than its expected value. Decrease or increase in the dual-species biofilm biomass of either bacteria A+C or bacteria B+C combinations was totally dependent on the cell density of bacteria C.

CONCLUSIONS

Biofilm biomass of pure-species cultures was totally species-dependent, and the biofilm biomass of four species was in the following order: bacteria C > D > A > B. Relation between biofilm biomasses and SMP or EPS was inconsistent.

Keywords: Biofilms, bacteria, polysaccharides, pure biofilms, dual-species biofilms, EPS

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INTRODUCTION

Biofilms are self-organized communities of one or more types of microorganisms embedded in an extracellular matrix. They collectively represent the largest biomass and activity center on the planet, playing a major role in the biology and chemistry of the environment (both natural and engineered) and in maintaining public health.[1,2] Over 500 bacterial species have been identified in typical dental plaque biofilms. Biofilms are held together by molecular strands of nucleic acids, collectively termed extracellular polymeric substances or exopolymeric substances (EPS), which are highly hydrated polymers that are mainly composed of polysaccharides, proteins, and DNA. The bacterial cells produce EPS and are held together by these EPS strands, allowing them to develop complex three-dimensional, resilient, attached communities. Biofilms can be as thin as a few cell layers or many inches thick, depending on environmental conditions.[3,4] "Pathogenic biofilms" is one of the hot topics in the world of biomedical research. Several methodologies such as in vitro assays, in vitro catheter-infection assays, and in vivo mouse catheter infection models have been used to assess biofilm formation. The capacity for biofilm formation was found in 59% to 100% of different bacterial and fungal species isolated from intravenous catheters, medical devices, and clinical sites.[5] *Pseudomonas aeruginosa* is an opportunistic human pathogen and has been used as a model organism for the study of bacterial biofilm formation. The bacterium produces at least three exopolysaccharides (alginate, Psl and Pel) for biofilm formation. The polysaccharide Psl is rich in mannose and galactose, while Pel is a glucose-rich, cellulose-like polymer. Mutants deficient in the production of these polysaccharides have been constructed to investigate the interactive contribution of alginate, Psl and Pel to the formation of biofilms.[6] The common opportunistic pathogens *Candida albic*ans and *Staphylococcus aureus* frequently co-exist as dual-species biofilms that cause nosocomial infections associated with medical devices and are more resistant to antibiotics and host immune responses than mono-species biofilms.[7] In a study on dual-species biofilm formation by *B. cereus* and *P. fluorescens*, the concentrations of which were determined by real-time PCR, it was found that in comparison to their respective mono-species biofilms, the dual-species biofilms produced a significantly higher protein content in the EPS and were more resistant to the antibacterial effects of grapefruit seed extract (GSE).[8] In dual-species biofilms of *Pseudomonas fragi* and *E. coli* growing on meat, competition for limited adhesion surface was the principal interplay between the two species, but the competition did not affect the spatial structure and microbial composition of the mature dual-species biofilms. The study also found that *E. coli* was the more aggressive species in regard with surface colonization.[9]

Bacterial exopolysaccharides are an essential group of compounds secreted by bacteria, and these versatile EPS are used individually or in combination with different materials for a wide range of functions in the biomedical field, being commonly used for their biosynthesis pathways and characteristics.[10] Microbial exopolysaccharides have found outstanding medical applications since the mid-twentieth century, with the first clinical trials on dextran solutions as plasma expanders, or other exopolysaccharides entering medicine first as conventional pharmaceutical excipients (e.g., xanthan, as a suspension stabilizer, or pullulan, in capsules and oral care products).

While pathogenic biofilms contribute to states of chronic inflammation, probiotic *Lactobacillus* biofilms cause a negligible immune response and, in states of inflammation, exhibit robust anti-inflammatory properties. These probiotic biofilms colonize and protect the gut and vagina and have been implicated in improved healing of damaged skin. Overall, biofilms stimulate a unique immune response that we are only beginning to understand.[11] Most EPS are exopolysaccharides i.e. extracellular carbohydrate

polymers produced and secreted by microorganisms which accumulate outside cells and are capable of being released into the surrounding environment.[12] Exopolysaccharides are carbohydrate macromolecules, usually together with proteins, which are synthesized by bacterial enzymes and excreted and accumulated extracellularly giving the culture a mucilaginous appearance. The main monosaccharides present in exopolysaccharides are: rhamnose: 28-45%, glucosamine: 12-20%, glucose: 20-30%, galactose: 15-25%.[13] Bacterial EPS also have extensive commercial applications in the pharmaceutical and food industries. Owing to the structural and functional diversity, genetic and metabolic engineering strategies are currently employed to increase EPS production.[14]

In regard to the biofilm formation potentials of different pathogenic bacteria, strains of *Staphylococcus aureus, Pseudomonas aeruginosa,* and *Acinetobacter baumannii* were found to produce strong biofilms, whereas moderately strong biofilms were reportedly produced by strains of *Staphylococcus saprophyticus* and *Staphylococcus epidermidis*.[15] Biofilm formation allows otherwise unicellular organisms to assume a temporary multicellular communal lifestyle, which affords protection from harsh environmental conditions and provision of centralized and concentrated resources, such as nutrients and genetic exchange opportunities.[16] During biofilm formation, several species of bacteria communicate with one another, employing quorum sensing (QS) by monitoring the number of small molecular autoinducers.[17] In pathogens, this QS circuit allows the expression of specific virulence factors, with consequent physiological changes associated with the infection. It is noteworthy that in *E. coli* the QS circuit makes it possible to coordinate the expression of genes that participate in some important metabolic pathways, and this is probably one of the reasons why the carbon storage regulator A (CsrA) protein is essential in this bacterium.[18] *S. aureus* secretes various toxins that harm the host, one of them being the pore-forming protein, alpha toxin. Based on the lytic activity of alpha toxin in red blood cells, a complex mechanism of action has been suggested for the intoxication of nucleated cells. Previously, it has been proposed that alpha toxin plays an essential role in the pathogenesis of pneumonia caused by *S. aureus*.[19] The influence of the cell density of one strain on the dual-species biofilm biomass is demonstrated by the fact that cooperative interactions in dual-species biofilms lead to higher biomass and higher tolerance to disinfectants. However, in real food processing environments, the presence of many other microbial species clearly adds additional complexity to the behavior of mixed-species biofilms; all incorporated microorganisms may compete, cooperate, and communicate with each other. One problem that has been poorly addressed with respect to pathogenic bacterial species, is whether biofilm biomasses are influenced by soluble microbial products (SMPs) and EPS.[20] A wide range of microorganisms produce EPS that are fundamental for microbial life and provide an ideal environment for chemical reactions, nutrient entrapment, and protection against environmental stresses such as salinity and drought.[21]

Investigating dual-species biofilms containing different densities of partner strains is vital in understanding pathogenic biofilms and their disease mechanism. Most investigations have been focused on the pure-species biofilms; but such results are far from complete for understanding of biofilms of natural environments. To the best of our knowledge, investigations that are based on the analysis and characterization of biofilm biomasses in interactions of two species with each other are very few in number. The present investigation focused on an interactive approach of four bacterial species. Therefore, in the present investigation, we aimed to address fundamental questions in order to understand more about the pure- and duals-species biofilms of four different clinically important bacterial species, i.e. *Escherichia coli, Staphylococcus aureus*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*.

METHODS

Bacterial cultures

Cultures of four pure species of bacteria (Table 1) and their combinations were investigated for their respective biofilm interactions. The biofilms of the same cultures were used to extract their EPS.

Quantification of pure-species biofilm biomass

Prior to developing biofilms, 4 bacterial species were sub-cultured separately (5 ml each in 10 ml test tubes) using nutrient broth sterilized at 121°C for 20 minutes and incubated at 37°C. Aliquots of these suspensions were stored at 4°C in the refrigerator for regular use and as glycerol stocks at –20°C for future use.

To each of four wells in a 96-well microtiter plate, 90 µL of nutrient broth and 10 µL of a pure bacterial suspension were added to develop pure-species biofilms. This step was repeated with the 3 other bacterial suspensions. In addition, 4 wells were kept separate for water and medium control. Plates were incubated for 48 h at 37°C in an incubator without agitation. After 48 h, the wells were washed twice with 200 µL of sterile distilled water. The biofilms were then fixed with 200 µl of 99% methanol per well. After 15 min, excess methanol was removed from each well and the biofilms were completely air-dried. Finally, 200 µL of 0.1% crystal violet was added to each well and the microtiter plates were incubated at room temperature (~28°C) for 20 min. The biofilm layer was then dissolved by adding 150 µL of 33% acetic acid to each well. The acetic acid samples were then diluted 50-fold in 33% acetic acid 96-well microtiter plates, and absorbance was measured in triplicate at 590 nm using a microplate reader (Fisher Scientific Thermo Scientific Multiskan FC Microplate Photometer Type 357).[22]

Table 1. Bacterial strains used in this study and assigned codes

Strain name	Code
Escherichia coli	A
Staphylococcus aureus	B
Klebsiella pneumoniae	C
Pseudomonas aeruginosa	D

Dual-species biofilms

Dual-species biofilms were prepared in triplicate by using different combinations of the four bacterial species at 3 different cell densities (inoculum sizes) i.e. 1:1, 1:9 and 9:1. For the development of 1:1 dual-species biofilms, 5 µL of culture-1 + 5 µL of culture-2 + 90 µL of nutrient broth were placed in a microtiter plate well. To grow 1:9 dual-species biofilm, 1 µL of culture-1 + 9 µL of culture-2 + 90 µL of nutrient broth were placed in the well, whereas 9:1 dual-species biofilm was cultured by placing 9 µL of culture-1 + 1 µL of culture-2 + 90 µL of nutrient broth in the well. Culturing conditions, harvesting, staining, and measuring of dual-species biofilms were similar to that of pure-species biofilms described in the preceding subsection.

Characterization of biofilm exopolysaccharides

The bound EPS was obtained by removing the SMP by centrifugation of the culture broth (25 ml of the medium stationary phase, i.e., 48 h) at 3500 rpm and 4°C for 15 min. The cell sediment with intact bound EPS was then dissolved in 25 ml of 0.5% NaCl solution and placed in a water bath at 60°C for 30 min. The suspension was then centrifuged again at 12,000 rpm and 4°C for 15 min, and the supernatant was considered to be bound EPS. The dry weight of the gross EPS (in grams per liter) was measured by drying at 105°C for 8 h in an oven.[23]

Statistical analysis

Percent coefficient of variation (%CV) was calculated for the data of pure species biofilm biomasses as well as for theoretical and experimental biofilm biomasses. The One-Sample t-test (95% CI, alpha = 0.05) was used to calculate the column means (experimental and theoretical biofilm biomasses) which were

significantly different from the hypothetical value. The above statistical analyses were done by using the GraphPad Prism 7.00 program.

Ethical considerations

The project was authorized by the Bioethics Committee of the Technical University of Manabí, Portoviejo, Ecuador (Volume: 021-10 Folio: 21-10-017).

RESULTS

Out of four pure species bacterial cultures (i.e. Bacteria A, B, C and D), Bacteria C showed the highest amount of biofilm biomass (A_{590}= 0.1015) (Figure 1), followed by bacteria D (A_{590} =0.0793), bacteria A (A_{590}=0.0735), and bacteria B (A_{590}=0.059). The increment in the biofilm biomass of bacteria C was 28%, 38%, and 72% higher than bacteria D, bacteria A and bacteria B, respectively. Highest (72%↑) and lowest (8%↑) biofilm biomass differences were found between bacteria B versus bacteria C and bacteria A versus bacteria D, respectively. With respect to pure-species biofilm biomasses, nevertheless, 8-72% of variation was found among the 4 bacterial species studied (Figure 1). The coefficient variation percent (CV%) values of bacteria B, A, D, and C biofilm biomasses were 1.857, 3.726, 1.029, and 22.125, respectively.

Dual-species (1:1) biofilm biomasses (A_{590}) of 6 different combinations (bacteria A+B, A+C, A+D, B+C, B+D, and C+D) of four studied species were in the range of 0.0665-0.0905 (Figure 2), where the combinations of bacteria A + bacteria B and bacteria C + bacteria D showed the lowest and highest amount of biofilm biomasses, respectively. Based on the data presented in the Figure. 1, the expected theoretical values of the biofilm biomasses were calculated for those 6 combinations of dual-species cultures and compared with the experimental biofilm biomass values (Figure 2). In four out of six dual-species cultures, experimental biofilm biomass values were higher

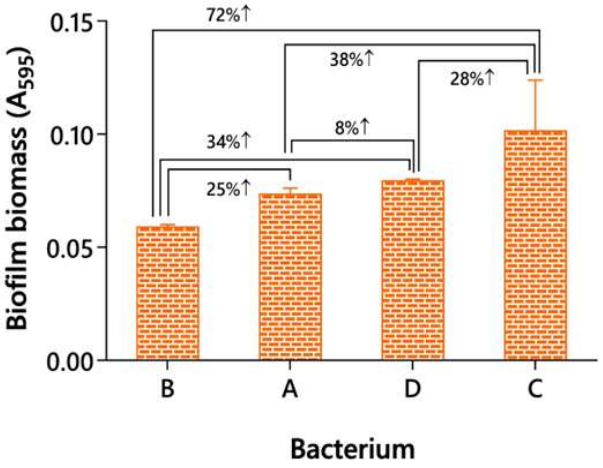

Figure 1. Pure species biofilm biomasses (A_{595}) of four different bacterial strains [*Escherichia coli* (code A), *Staphylococcus aureus* (code B), *Klebsiella pneumoniae* (code C) and *Pseudomonas aeruginosa* (code D)] as determined on 96-well microtiter plates.

(1-30%) than the theoretical values, but were lower (3-6%) than the theoretical values in the remaining two dual-species cultures (i.e. bacteria A+C and C+D) (Figure 2). Nonetheless, the highest difference between experimental and theoretical values was shown by the dual-species culture B+D, where the experimental value was 30% higher than the expected theoretical value. The lowest and highest %CV values were found to be 0.5297 and 18.6783 for the combinations A+B and B+D, respectively. In conclusion, a significant difference ($p<0.0001$) was found between the experimental and theoretical values of biofilm biomasses of different combinations.

In a dual-species biofilm biomass, bacterial cell density or inoculum size (i.e. 10% (1:9), 50% (1:1), and 90% (9:1)) played a significant role in influencing the final biofilm biomass. This was investigated with dual-species biofilms, either of two gram-negative bacteria (A+C) (Figure 3) or of a mixed culture of gram-negative and gram-positive bacteria (B+C) (Figure 4). Experimental values of biofilm biomasses of the three different cell densities (1:9, 1:1, 9:1) were compared with the theoretical values calculated for the respective cultures based on the data in Fig.1. When bacteria A density was 10% in a dual-species culture

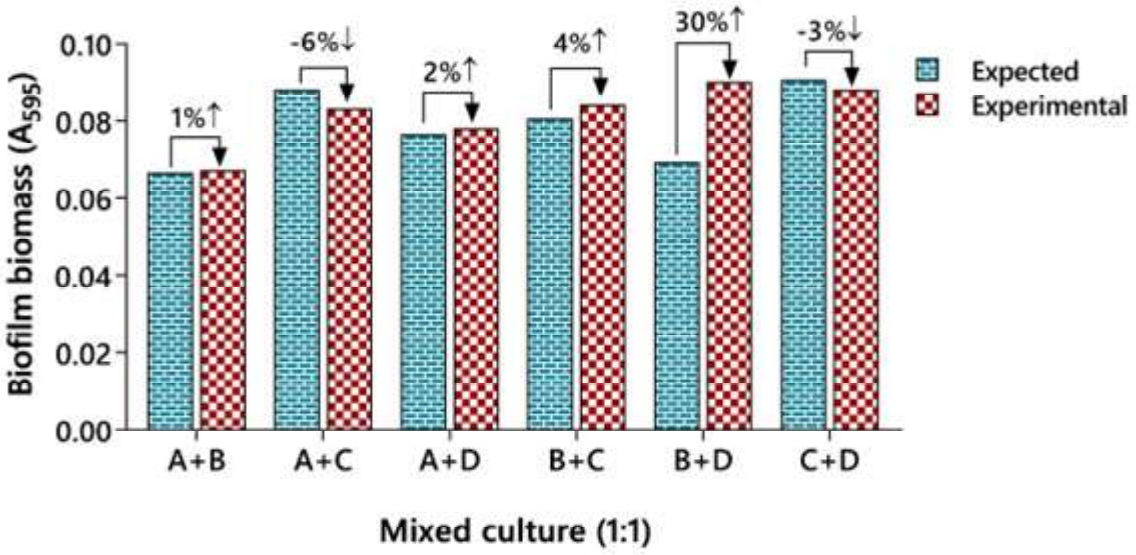

Figure 2. Theoretical and experimental values of biofilm biomasses (A595) of mixed culture bacterial species. Stimulatory and inhibitory experimental values in relation to expected values were in the range of 1-30% and 3-6%, respectively. Only bacteria B (*Staphylococcus aureus*) showed stimulatory interaction with either bacteria A (*Escherichia coli*), C (*Klebsiella pneumoniae*) or D (*Pseudomonas aeruginosa*) during biofilm formation.

containing bacteria A+C, the experimental biofilm biomass was 12.5% higher than the theoretical value. However, when the density of bacteria A was increased to higher values (i.e. 50% and 90%), the experimental biofilm biomasses declined significantly, and this decline was dose-dependent on bacteria A culture. For instance, the decrements in the experimental biofilm biomass were ~6% and ~22% when the densities of bacteria A were 50% and 90%, respectively (Figure 3). Similarly, by increasing the percentage of bacteria B in a dual-species culture containing bacteria B + C, the experimental dual-species biofilm biomass was significantly reduced (Figure 4). For instance, when bacteria B densities were 10% (1:9), 50% (1:1), and 90% (9:1), the experimental biofilm biomasses of the dual-species culture of bacteria B + C were reduced by 19% and 4%, for bacteria B densities of 50% and 90%, respectively, and increased by 11% for the bacteria B density of 10%, over the theoretical biofilm biomass values for the same combinations (Figure 1).

Finally, SMPs and EPSs were extracted from the pure- and dual-species biofilms and their dry weights were determined (Table 2), and the different concentrations were determined in mixed species: 1:1 (culture 1 = 50% and culture 2 = 50% in the mixture); 1:9 (culture 1 = 10% and culture 2 = 90% in the mixture), and 9:1 (culture 1 = 90% and culture 2 = 10%).

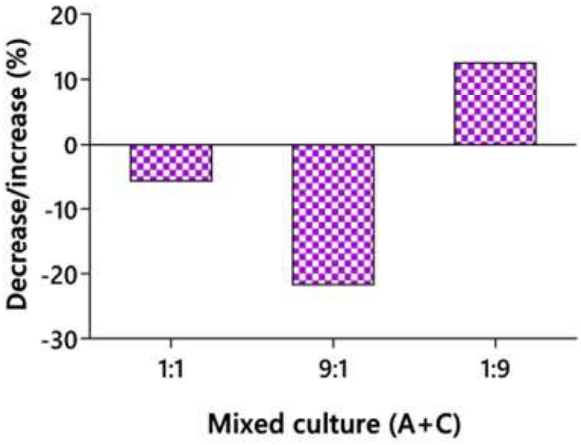

Figure 3. Deviation (per cent of decrease or increase) of experimental biofilm biomass of a dual-species (A+C at 1:1, 1:9 and 9:1 ratio) culture from theoretical value (A+C at 1:1). When bacteria A cell density was increased from 50% (1:1) to 90% (9:1), dual-species biofilm biomass was decreased by 28%, but biofilm biomass was increased when bacteria A cell density was decreased to 10% (1:9). (bacteria A = *Escherichia coli,* bacteria C = *Klebsiella pneumoniae*)

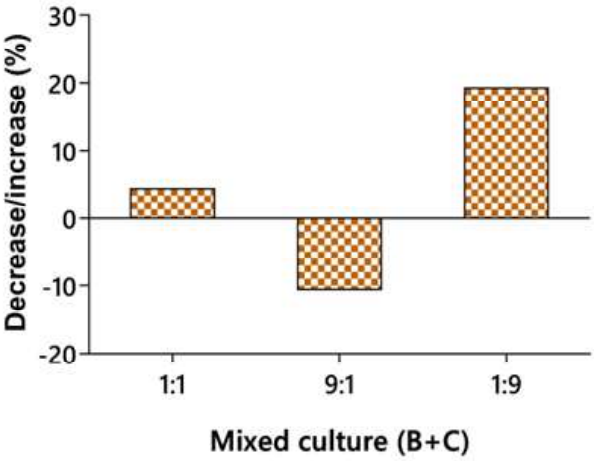

Figure 4. Deviation (per cent of decrease or increase) of experimental biofilm biomass of a dual-species culture (B+C at 1:1, 1:9 and 9:1 ratios) from theoretical value (B+C at 1:1). When bacteria B cell density was either 10% (1:9) or 50% (1:1), dual-species biofilm biomass was increased by 5%-20%, but biofilm biomass was decreased by ~10% when bacteria B cell density was increased to 90% (9:1). [bacteria B = *Staphylococcus aureus*; bacteria C = *Klebsiella pneumoniae*]

DISCUSSION

In the present investigation on the biomass of biofilms of four pure species (Figure 1), it was determined that there was a greater formation of biofilms in the Gram negative bacteria *Escherichia coli* and *Pseudomonas sp.* There was a particularly greater biofilm biomass formation of 40% in *Klebsiella pneumoniae,* while in the biofilm biomass of Gram-positive bacteria the expected results were minimal. In the research directed by Surgers et al.,[24] 57.1% of the bacteria were strong biofilm producers (biofilm formation index [BFI] < 2), while 13.4% lacked biofilm production (BFI >18). Standard biofilm production (BFI <7) was common in *E. coli* isolates (61.9%). Although almost all (90.2%) of *K. pneumoniae* have standard biofilm production, there was a 2.4-fold greater chance of observing biofilm. Cangui-Panchi et al.[25] determined that almost all staphylococcal species (such as *S. epidermidis, S. saprophyticus, S. capitis, S. cohnii, S. hominis, S. aureus,* and *S. haemolyticus*) showed a high prevalence of biofilm-associated infections. Furthermore, all Gram-negative bacteria (*E. coli, P. aeruginosa, K. pneumoniae, and P. mirabilis*) reached 100% of biofilm prevalence except for *A. baumannii* evidencing 77.8% biofilm prevalence obtained in only one study. Finally, *Candida. albicans* and *Corynebacterium striatum* also demonstrated a 100% biofilm prevalence, being the only yeast and Gram-positive bacillus, respectively. [5] In the theoretical and experimental values of biofilm biomass obtained from mixed species (Figure 2), between A and B there is a difference of 1%, while between A and D there is a difference of 2%, these representing minimum percentages. However, it was found that the difference between B and D is 30%, which is greater than in the other mixed species combinations. In the tables analyzed, the biomass species of mixed biofilms determine the relationship between bacterial densities of 50% and 90%, indicating that the interaction and stimulation are greater, that is, the greater the bacterial density, the less will be the formation of biofilms. We characterized the in vitro

Table 2. Dry weight (g/L) of soluble microbial products (SMP) and exopolymeric substances (EPS) of pure- and dual-species biofilms

Pure- or Dual-species bacterial biofilm	SMP (g/L)	EPS (g/L)
A	5.2	13
B	9.2	13
C	13	6.8
D	9.3	7.7
A+B (1:1)	10.6	10.0
A+C (1:1)	5.7	55.3
A+D (1:1)	6.4	7.1
B+C (1:1)	11.4	13.1
B+D (1:1)	12.1	9.2
C+D (1:1)	6.9	7.7
A+C (9:1)	6.2	77.6
B+C (9:1)	6.7	12.3
C+D (9:1)	12.9	8.2
A+C (1:9)	18.1	14.9
B+C (1:9)	7.3	6.0
B+D (1:9)	11.4	11.6

Notes: A = *Escherichia coli;* B = *Staphylococcus aureus;* C = *Klebsiella pneumoniae*; D = *Pseudomonas aeruginosa*; SMP: soluble microbial products; EPS: exopolymeric substances

polymicrobial biofilm of *C. albicans* and *S. aureus*, as they have been found together in biofilm-related infections. We found the interesting the fact that fetal bovine serum (FBS) affects the adhesion of both microorganisms, as well as the formation of *S. aureus* biofilms and of polymicrobial biofilms. We suggest that plasma proteins, such as albumin and fibrinogen, may act as an interface, binding to biofilm-derived microorganisms on colonized catheters. *C. albicans* contributed the most to the overall biofilm density, in which β-1,6-linked polysaccharides appear to be important components. Finally, *C. albicans* adhered preferentially to all tested surfaces, while *S. aureus* mainly adhered to the surface of *C. albicans*. The nutrient requirements that may be involved in all steps of biofilm formation, such as adhesion, cell growth, extracellular matrix formation, and dispersal, need to be analyzed in the future.[26] During the interactions of *P. fluorescens* and *S. aureus* as multispecies biofilms, as well as their behavior in the presence of carvacrol, it was found that *P. fluorescens* in mono- and dual-species cultures exhibited similar numbers of planktonic cells (free flowing bacteria in suspension) and biofilm cells.[27]

Extracellular polymeric substances are of importance for the functions and characteristics of microbial aggregates in biological wastewater treatment, among which EPS are involved in both beneficial and detrimental characteristics of microbial aggregates. Specifically, EPS are involved in biofilm formation and stability, sludge behaviors as well as SBR granulation, whereas they are also responsible for membrane fouling in membrane bioreactors (MBRs).[28]

Chitosan has the potential to be used in an immobilization technique for retaining biomass of *Caldicellulosiruptor* species at a lower concentration of 1 mg L. This property was associated with natural biofilm produced by *C. owensensis* in the chemostat at higher maximum dilution rate (D).[29] Unlike the present research, 200 μL of 0.1% crystal violet is added to each well and the microtiter plates are incubated at room temperature for 20 min. The biofilm layer is then dissolved by adding 150 μl of 33% acetic acid to each well. Finally, acetic acid samples are diluted 50-fold (plus 33% acetic acid) on 96-well microtiter plates with transparent bottoms and black walls, and absorbance is measured at 590 nm in triplicate in a plate reader. The role of the biofilm three-dimensional structure in this tolerant phenotype has been studied extensively; however, the impact of small molecules released from biofilm bacteria in modulating host immune function is less well understood. A model of mixed-species biofilms composed of *Salmonella enteritidis* (SE) in the presence of the food-processing environmental bacterium *Bacillus paramycoides* B5 responses to bioactive molecules released from either biofilm or planktonic bacteria. [30] The behavior of *Salmonella enteritidis* (SE) in the presence of the food-processing environmental bacterium *Bacillus paramycoides* B5 (Bp5) during dual-species biofilm formation was investigated by means of Illumina RNA-seq transcriptome analysis combined with phenotype validation. The results showed that SE initial adhesion was significantly enhanced with large microcolony formation when cocultured with Bp5.[30]

This research describes how bacteria of mixed species interact with each other, and what leads to the formation of the biofilms that they can produce. In addition, raising awareness that bacteria are currently the main sources of diseases, therefore “one health” implies the interrelationship between public, animal and environmental health actors, taking into account that bacteria have the capacity and ability to adapt to the environment in which they find themselves.[30] To reach this goal it is important that day by day training and innovating technology is being updated to determine how the developmental process of bacteria changes according to the environment and the circumstances in which they live. The major limitation of this study was our studying only four bacterial species. In order understand more about

the biofilm formation potentials of pathogenic bacteria, following future directions of research are greatly warranted – mixed species biofilms containing a wide range of non-model pathogenic bacteria; quantification of each bacterial species in mature mixed-species biofilms by using the standard plate count (SPC) method; and characterization of EPS and SMP by Fourier transform infrared (FTIR) spectroscopy for functional groups.

CONCLUSION

In our investigation, *Klebsiella pneumoniae* showed higher biofilm biomass (> 28-72%) than three other bacterial species. Experimental values of dual-species biofilm biomasses decreased (by 6%) or increased (by 30%) compared to theoretical values. We also found that cell density of a particular species significantly affects the biomass of dual-species biofilm. We did not find any correlation between either SMP or EPS with the biofilm biomasses of the four studied bacterial species. Future research should be more in-depth and analytical on the interactions of mixed bacterial strains.

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DECLARATION

The authors declare that they do not have any type of conflict of interest.

AUTHOR CONTRIBUTION

MBSA performed the work, prepared the draft of first submission and revised draft as per the editor/reviewers' comments. NRM designed, supervised the work and edited the first and revised draft. All authors have read and approved the final manuscript.

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