



# Quantitative and physiological behavior techniques to investigate the evolution of monospecies biofilm of pathogenic bacteria on material surfaces

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## Abstract

In most natural habitats, microbes are not discovered in the planktonic phase but in multispecies biofilm communities. Bacteria in diverse microbial biofilm may interact or conflict relying on the varieties and features of solid surfaces. Hence, monospecies biofilm formed some potentially Gram-negative pathogenic species, including *Escherichia coli*, *Salmonella enterica*, and *Pseudomonas aeruginosa*, on two different materials: stainless steel (SS) and polypropylene (PP) were investigated. The developed biofilm was comprehensively studied using different approaches. Results displayed that the biofilm developed upon SS was more intensive than on PP. Statistically, a compelling correlation with significance was recorded between the biofilm age and increasing bacterial biofilm populations formed upon PP and SS materials. Likewise, the excellent levels of produced adenosine triphosphate (ATP) from the biofilm formed upon both PP and SS were reached after 80 days. The scanning electron microscope (SEM) micrographs exhibited the surface structure of biofilm for *E. coli*, *S. enterica*, and *P. aeruginosa* developed upon two materials (PP and SS). The results show that, the formed biofilm cells for all tested bacterial strains grown upon PP material were more minor than SS. In conclusion, the existing investigation delivers better knowledge about the approaches that could be applied to investigate biofilm formation on various surface materials. Likewise, biopolymers such as extracellular polymeric substances (EPS) play a critical role in establishing clusters and microcolonies.

**Keywords** Biofilm formation · SS and PP materials · SEM · Metabolic activities · Pathogenic bacteria

## Abbreviations

ATCC American Type Culture Collection  
ATP Adenosine triphosphate  
CFU Colony forming unit  
cm<sup>2</sup> Square centimeter  
DNA Deoxyribonucleic acid  
EPS Extracellular polymeric substances  
h Hour  
HISA HiCrome Improved Salmonella agar  
HFA Hifluoro Pseudomonas agar  
HPC Heterotrophic plate counts  
mL Milliliter  
μL Microliter

PCA Plate count agar  
PE Polyethelene  
PP Polypropylene  
PVC Polyvinyl chloride  
RHA Rapid Hicoliform Agar  
RLU Relative light unit  
SEM Scanning electron microscope  
SS Stainless steel  
TBC Total bacterial counts  
TSB Trypticase soy broth

## Introduction

The establishment of biofilm is a paramount consideration throughout many disciplines and poses significant dangers to manufacturing, healthcare, and the general population. Bacterial biofilm development is a substantial ongoing concern since this raises death and disability (Srinivasan et al. 2021). Furthermore, of all the places in home surfaces, where

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bacteria are able to develop and proliferate biofilm, the kitchen and bathroom sinks are the most likely to subject us to a miscellaneous diversity of pathogens (Liu et al. 2018). Natural biofilm is formed by microbial growth ubiquitously in different environment and considered multi-dimensional microbial community that reside within EPS matrix or slime that adheres to the material surfaces (Du et al. 2017). Microorganisms frequently attach to substrates and create an extracellular polymeric matrix, producing a biofilm (Khattoon et al. 2018). In addition to this, biofilm development occurs from planktonic or floating bacteria that can connect, proliferate, and create bacterial community architecture on solid surfaces (Riau et al. 2019).

Gram-negative bacteria have generally been the emphasis of biofilm investigation, in clinical materials and environments (Røder et al. 2016; El-Liethy et al. 2020). In the UK, Portugal, France, and Latvia, biofilm samples taken from aging pipes of declining water distribution systems included *E. coli* (Juhna et al. 2007). Furthermore, it was found that the natural biofilm of the sink drainage pipes included *E. coli* and other disease-causing bacteria (El-Liethy et al. 2020). Nevertheless, biofilm samples collected from medical devices were found to contain *E. coli*, *Pseudomonas aeruginosa*, and *Staphylococci* spp. (Subramanian et al. 2012; D'Ugo et al. 2018). The coexistence of numerous microbial species on the inside of a multilayer of biofilm can profit its residents in a broad range of ways, such as improved adaptive immunity avoidance or the accessibility of metabolic substances created by the coexisting microbes (Hemdan et al. 2019). Interestingly, pathogens have distinct characteristics between the planktonic and biofilm states because adhesion to a surface causes a significant change in the expression of many genes involved in EPS formation and biofilm maturity. It immediately results in constructing a barrier that shields bacteria from harsh conditions, such as antibiotics (Gupta et al. 2016) and antimicrobial agents (El Nahrawy et al. 2021). A possible explanation is that antimicrobials have become less effective against microbial biofilm, leading to infections in patient populations with implanted medical devices. Experts may be able to improve clinical management if they identify the importance of biofilm in disorders (Dall et al. 2017). *Enterococcus faecalis*, *Staphylococcus aureus*, *E. coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* are the most prevalent bacteria that develop biofilm on medical equipment and water infrastructure (Chen et al. 2013). Likewise, *S. aureus* and *P. aeruginosa* are highly infectious and co-exist in biofilm communities linked to various illnesses (Limoli et al. 2016). The management, manufacture, and consuming of food are examples of immediate interaction points for pathogenic organisms. Secondary interaction routes comprise extensively contaminated areas via sources like raw food, food-stuff, and liquids (Hemdan et al. 2017).

Recently, advanced approaches have been used for determination of natural microbial biofilm in medical devices and water plumbing systems. The advanced detection methods such as using specific primers for the target microbes and also using phenotypic identification of biofilm bacterial isolate using Biolog GEN III (El-Liethy et al. 2020). Moreover, bacterial community structures have been determined in natural biofilm using next generation sequencing (Yergeau et al. 2012; Staley et al. 2013; Ziemińska-Buczyńska et al. 2019). Lately, biofilm-based sensors have been developed for bacterial biofilm determination (Funari and Shen 2022). Sensors are used to study the biofilm growth and measure the dynamics of some indicators such as temperature, pH, oxygen level, and some biomolecules in natural biofilm (Subramanian et al. 2020; Saccomano et al. 2021). Because it is difficult to study naturally formed biofilms, particularly in water distribution systems and medical instruments, simulation systems were developed to investigate the behavior of bacterial biofilm (Lührig et al. 2015; Hellweger et al. 2016). As a result, the primary goal of this study is to evaluate the stages of biofilm formation for some Gram-negative bacterial strains, such as *E. coli*, *S. enterica*, and *P. aeruginosa*, on two different materials (stainless steel, SS and polypropylene, PP) using multiple investigation approaches. The bacterial biofilm was determined using cultural based methods on enrichment agar media and selective agar media. Furthermore, the bacterial biofilm was evaluated using culture-independent methods using SEM, ATP, and protein analysis.

## Materials and methods

### Microorganisms used and growth conditions

Three tested bacterial species employed in the present study were *E. coli* ATCC 25922, *Salmonella enterica* ATCC 14028, and *Pseudomonas aeruginosa* ATCC 10145, which served as Gram-negative bacteria. According to El Nahrawy et al. (2019) every bacterial culture stored at  $-20\text{ }^{\circ}\text{C}$  is reactivated by inoculating 1 mL of suspension into a tube with 20 mL of trypticase soy broth (TSB). The inoculated tubes were incubated at  $37\text{ }^{\circ}\text{C}$  for 18–24 h.

### Biofilm formation

#### Biofilm formation on material coupons

To exam the biofilm formation stages, the three bacterial strains were prepared as described above and inoculated to develop biofilms on the inner surface of tested substances (stainless steel (SS) and polypropylene (PP)). Ten sterile coupons by 70% ethanol (3 cm wide and 10 cm length) from each studied material were prepared. The coupons

were immersed into flasks with 15 mL of sterile tap water. After that, 100  $\mu$ L of 24-h fresh culture of each tested bacterial strain which previously washed three times by sterile distilled water to remove any cell debris and nutrients residue was inoculated to each flask. The initial bacterial count was  $\sim 10^6$  CFU/mL. The experimentation interval reached 90 days; a coupon was taken every 10 days for examination. All laboratory trials were repeated three times. The results were expressed as CFU/cm<sup>2</sup> (Hemdan et al. 2017; El-Liethy et al. 2020).

### Biofilm formation on 96-well microtiter plates

A single bacterial species of biofilm was established on 96-well uncoated and transparent microplate (Alpha Laboratories, UK). Briefly, 190  $\mu$ L of TSB medium was loaded with a 10- $\mu$ L fresh prepared cell suspension ( $10^8$  CFU/mL), and 200  $\mu$ L of deionized water were injected into well to use as a negative control. The ready microplate was then maintained at 37 °C for 16 h. After the planktonic cells were removed, the plates were air-dried after washed twice with phosphate-buffered saline (PBS). Two hundred microliters of crystal violet solution (1%) was added to the wells, and the microplates were incubated for 10 min. The wells were washed three times with PBS; the bacteria biofilm was incubated with a mixture of ethanol and acetone (4:1) for 10 min (Coffey and Anderson 2014; Ebert et al. 2021).

### Determination of the microbial populations in biofilm using the culture-dependent technique

Pour and spread plate techniques conducted microbiological investigations of collected biofilm samples. Samples or appropriate dilutions were inoculated in non-selective agar media (plate count agar) and selective agar media (enzymatic-based culture media) (Hemdan et al. 2020). Results were registered as CFU/cm<sup>2</sup>. The detached biomass (biofilm cell suspension) was diluted with sterile saline solution.

To enumerate the total number of biofilm cells of all tested Gram-negative bacterial species, the suspension of detached biofilm cells was diluted with a sterile saline solution by tenfold serial dilutions to determine the suitable biofilm counts. Two different agar media were utilized to determine the connections between the ages of biofilm communities and the numbers of surviving cells. Total bacterial counts (TBC) at 37 °C for 24 h were enumerated using the pour plate technique (Walter 1961). The spread plate assay was applied to count the cell numbers of *E. coli* involved in biofilm. One hundred microliters of proper dilution was aseptically transferred onto the Rapid Hicoliform Agar (RHA), Hifluoro Pseudomonas agar (HFA), and Hicrome Improved Salmonella agar (HISA) (HiMedia-India) for enumerating *E. coli*, *P. aeruginosa*, and *Salmonella* spp.,

respectively. All the inoculated plates were incubated at 37 °C for 24–48 h (El-Liethy et al. 2020; El Nahrawy et al. 2022).

### Direct observation of biofilm using the culture-independent technique

#### The quantification of ATP level

The luciferase testing procedure was employed to measure the ATP concentration. The formed biofilm was swabbed from 1.0 cm  $\times$  1.0 cm of the tested material's surface area. Then, the swab was immersed in 5 mL of sterile distilled water. A photodetector cell was incubated with an aliquot of 270  $\mu$ L of luciferin-luciferase and 30  $\mu$ L of the biofilm suspension (Hemdan et al. 2017; Slavin et al. 2017). Luminescence intensity was represented as a relative light unit (RLU).

#### Scanning electron microscope (SEM) observation

SEM investigation (SEM model JEOL JXA-840A, electron probe micro-analyzer, Japan) was employed to investigate biofilm structure. SEM was utilized to explore a 1.0  $\times$  1.0 cm<sup>2</sup> of material coupons. The fixation and dehydration of biofilm on coupons were conducted according to Ma et al. (2019).

#### Composition of whole biofilm and EPS

Produced EPS, which is secreted from microbial biofilm, was extracted from the whole biofilm using the cation exchange resin technique (Jachlewski et al. 2015; Hemdan et al. 2017). The carbohydrate content of crude extracted EPS and whole biofilm was measured using the phenol–sulfuric acid technique (Dubois et al. 1956). The quantity of protein in EPS and complete biofilm was estimated using the Folin assay (Lowry et al. 1951).

#### Statistical analysis

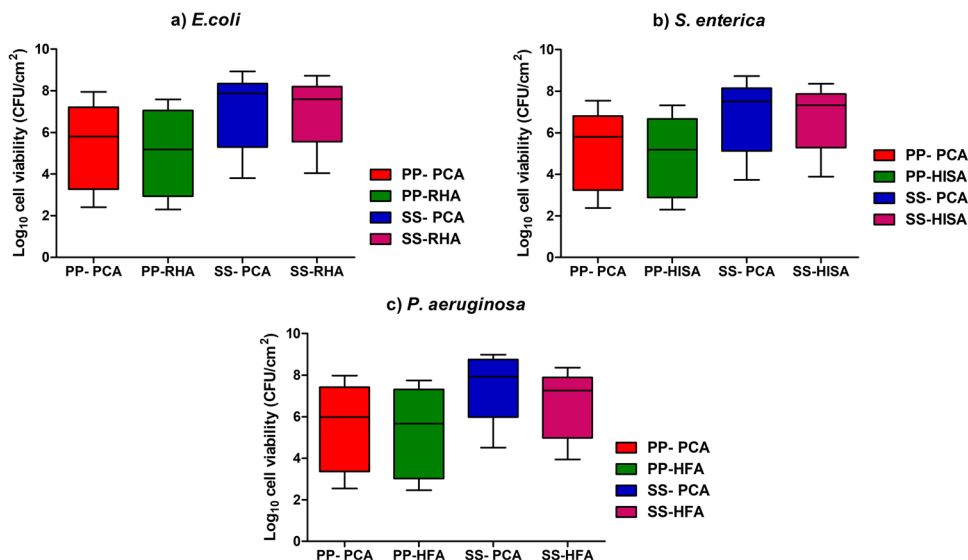
GraphPad Prism version 5.0 (USA) has been employed for the statistical studies.  $R^2$  and a Pearson correlation were used to determine significance.  $P$  value was set at  $< 0.0001$ .

## Results

### Determination of biofilm formation using culture-dependent methods

Biofilm colonization and development by *E. coli*, *S. enterica*, and *P. aeruginosa* on the surface of two different materials

**Fig. 1** Boxplots of the average bacterial biofilm viability over the 90-day period as estimated by  $\log_{10}$  CFU/ml of the three tested Gram-negative strains on PP and SS materials. **a** *E. coli* recovered on PCA and RHA media, **b** *S. enterica* recovered on PCA and HISA media, and **c** *P. aeruginosa* on PCA and HFA media



were examined, as illustrated graphically in Fig. 1. After 80 days of biofilm establishment, the higher growth rate of *E. coli* biofilm established on SS upon the material assessing with both plate count agar (PCA) and Rapid Hicoliform Agar (RHA) medium was  $8.50 \times 10^8$  and  $5.20 \times 10^8$  CFU/cm<sup>2</sup>, respectively, as shown in Fig. 1a. The growth kinetics rate was slower on PP material; however, the highest count of *E. coli* biofilm on PP material was  $8.9 \times 10^7$  CFU/cm<sup>2</sup>, compared to  $3.80 \times 10^7$  CFU/cm<sup>2</sup> on both PCA and RHA media during the same time frame. The rate of biofilm establishment of *S. enterica* on two pipe materials (PP and SS) was determined using two media PCA and Hicrome Improved Salmonella agar (HISA). The growth of biofilm on PP material was weaker than any other SS material studied, as indicated in Fig. 1b. Furthermore, after 80 days of biofilm formation, the more significant growth rate of *S. enterica* biofilm generated on PP material utilizing both PCA and RHA media was  $3.50 \times 10^7$  and  $2.10 \times 10^7$  CFU/cm<sup>2</sup>, respectively. While the growth rate of bacterial cells on SS material was the fastest, the most outstanding biofilm count on PCA and RHA media was  $5.30 \times 10^8$  and  $2.30 \times 10^8$  CFU/cm<sup>2</sup>, respectively. This indicated that the tested SS material has a rough surface, which might enable the biofilm to form more quickly and at higher concentrations. The numbers of sessile cells (Fig. 1c) revealed that adhered cells increased exponentially when the potential of *P. aeruginosa* to create biofilm was examined. Using PCA and HFA media, the maximum cell density of dispersed biofilm cells from PP material was obtained after 80 days of biofilm production:  $9.40 \times 10^7$  and  $5.60 \times 10^7$  CFU/cm<sup>2</sup>, respectively. Using the same media and biofilm production on SS material, the greatest cell density was achieved at  $9.50 \times 10^8$  and  $2.30 \times 10^8$  CFU/cm<sup>2</sup>.

From statistical analysis, results disclosed a positive correlation between the biofilm age and increasing bacterial

**Table 1** Correlation between *E. coli* biofilm ages and  $\log_{10}$  of survived biofilm cells growth rates

Correlation items	PP-PCA	PP-RHA	SS-PCA	SS-RHA
Number of XY pairs	9	9	9	9
Pearson <i>r</i>	0.9393	0.9354	0.9006	0.8991
<i>P</i> value (two-tailed)	0.0002	0.0002	0.0009	0.0010
<i>P</i> value summary	***	***	***	***
<i>R</i> squared	0.8823	0.8751	0.8111	0.8084
Is the correlation significant? (alpha = 0.05)	Yes	Yes	Yes	Yes

**Table 2** Correlation between *P. aeruginosa* biofilm ages and  $\log_{10}$  of survived biofilm cell growth rates

Correlation items	PP-PCA	PP-HFA	SS-PCA	SS-HFA
Number of XY pairs	9	9	9	9
Pearson <i>r</i>	0.9465	0.9460	0.8779	0.8859
<i>P</i> value (two-tailed)	0.0001	0.0001	0.0019	0.0015
<i>P</i> value summary	***	***	**	**
<i>R</i> squared	0.8958	0.8949	0.7707	0.7849
Is the correlation significant? (alpha = 0.05)	Yes	Yes	Yes	Yes

biofilm populations, implying that the highest biofilm formation could be obtained in the maturation state (after 80 days) (Tables 1, 2, and 3).

Based on the information provided, it appears that the biofilm growth ability and rate were compared between three different bacterial species in the same experiments, and the results showed that *P. aeruginosa* had the highest growth rate of the three species. However, it is also mentioned that

**Table 3** Correlation between *S. enterica* biofilm ages and log<sub>10</sub> of survived biofilm cells growth rates

Correlation items	PP-PCA	PP-HISA	SS-PCA	SS-HISA
Number of XY pairs	9	9	9	9
Pearson <i>r</i>	0.9362	0.9284	0.8849	0.9007
<i>P</i> value (two-tailed)	0.0002	0.0003	0.0015	0.0009
<i>P</i> value summary	***	***	**	***
<i>R</i> squared	0.8764	0.8619	0.7830	0.8113
Is the correlation significant? (alpha=0.05)	Yes	Yes	Yes	Yes

**Table 4** Variability in average biofilm growth using crystal violet staining assay along the columns in terms of absorbance at 570 nm (mean value ± standard deviation given in parenthesis)

Biofilm formation rate		Absorbance at 570 nm
Bacterial species	Incubation time	
<i>E. coli</i>	1 day	0.46 ± 0.25
	3 days	0.68 ± 1.13
<i>S. enterica</i>	1 day	0.41 ± 1.05
	3 days	0.73 ± 0.95
<i>P. aeruginosa</i>	1 day	0.59 ± 1.19
	3 days	0.84 ± 1.35

*P. aeruginosa* developed a significant amount of biofilm in the wells, which may indicate that it produced more biofilm than the other two species. It is important to note that the amount of biofilm produced is not necessarily the same as the growth rate of the biofilm. Growth rate refers to how quickly the biofilm is forming, while the amount of biofilm

produced is the total amount of biomass present in the biofilm. It is possible for a biofilm to have a high growth rate but a relatively low amount of biomass if the bacteria are not able to accumulate and produce significant amounts of EPS (Table 4).

### Estimation of biofilm formation using direct culture-independent methods

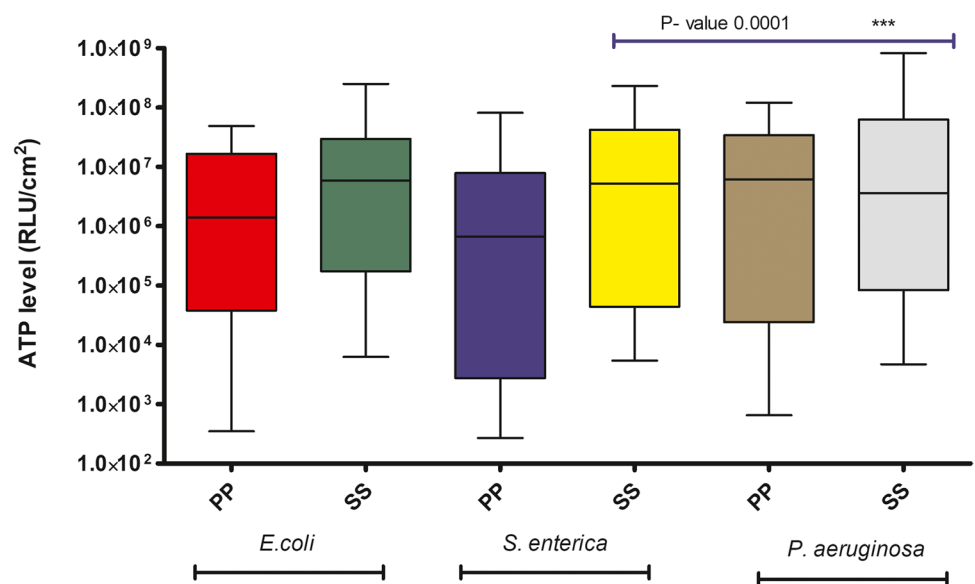
#### ATP quantification

Data presented in Fig. 2 demonstrated that the highest levels of produced ATP from the biofilm formed upon both PP and SS were reached after 80 days. Moreover, the most copious amounts of ATP were 8.20 × 10<sup>8</sup> RLU/cm<sup>2</sup> for *P. aeruginosa* biofilm developed on SS followed by 2.50 × 10<sup>8</sup> RLU/cm<sup>2</sup> for *E. coli* and 2.30 × 10<sup>8</sup> RLU/cm<sup>2</sup> for *S. enterica* on the same material. While the highest levels of ATP for biofilm formations of PP were 4.90 × 10<sup>7</sup>, 8.10 × 10<sup>7</sup>, and 1.20 × 10<sup>8</sup> RLU/cm<sup>2</sup> for *E. coli*, *S. enterica*, and *P. aeruginosa*, respectively. This data supports that the ATP assay may be a viable option for determining bacterial communities, which may be used as a rapid method for determining presence of bacteria. The strongest correlation between ATP and heterotrophic plate counts (HPC) was reported with extreme significance (*P* < 0.05).

#### Visualization of biofilm developed using SEM

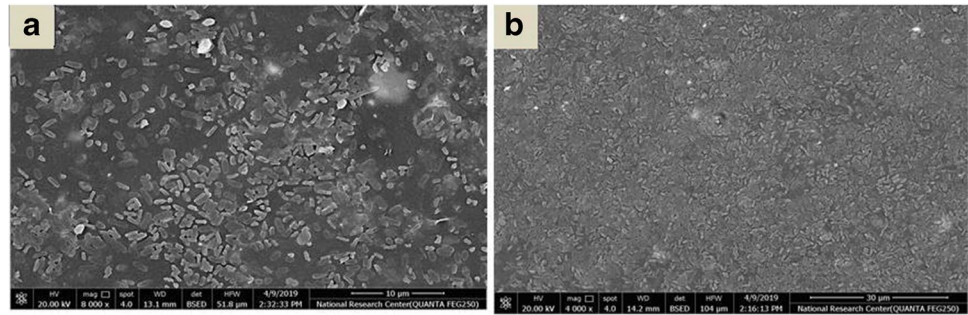
When analyzing the SEM images after 90 days, there are more cells attached on SS than PP (Figs. 3, 4, and 5), further supporting that SS surfaces may encourage biofilm formation more than PP. The obtained results evidenced

**Fig. 2** Minimum, maximum, and average of ATP level (RLU/cm<sup>2</sup>) of biofilm formation of *E. coli*, *S. enterica*, and *P. aeruginosa* grown on two various polypropylene (PP) and stainless steel (SS) materials

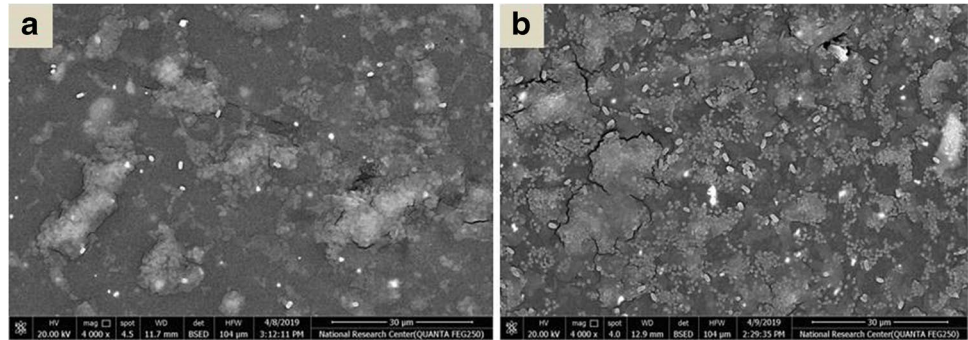




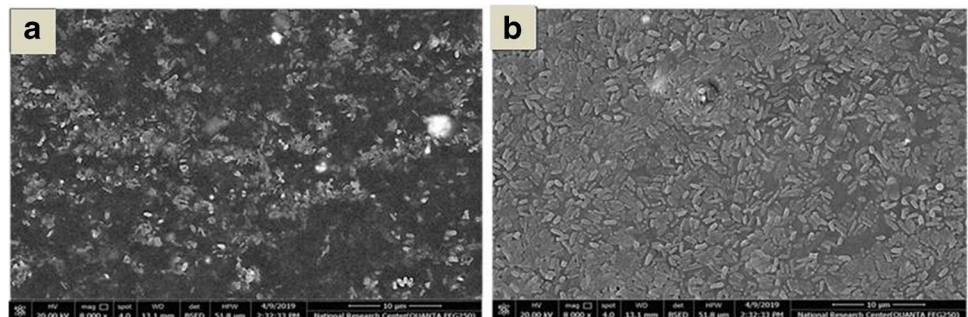
**Fig. 3** FESEM images of the formed biofilm of 90 days old for *E. coli* grown upon **a** polypropylene (PP) material and **b** stainless steel (SS) material



**Fig. 4** FESEM images of the formed biofilm of 90 days old for *S. enterica* grown upon **a** polypropylene (PP) material and **b** stainless steel (SS) material



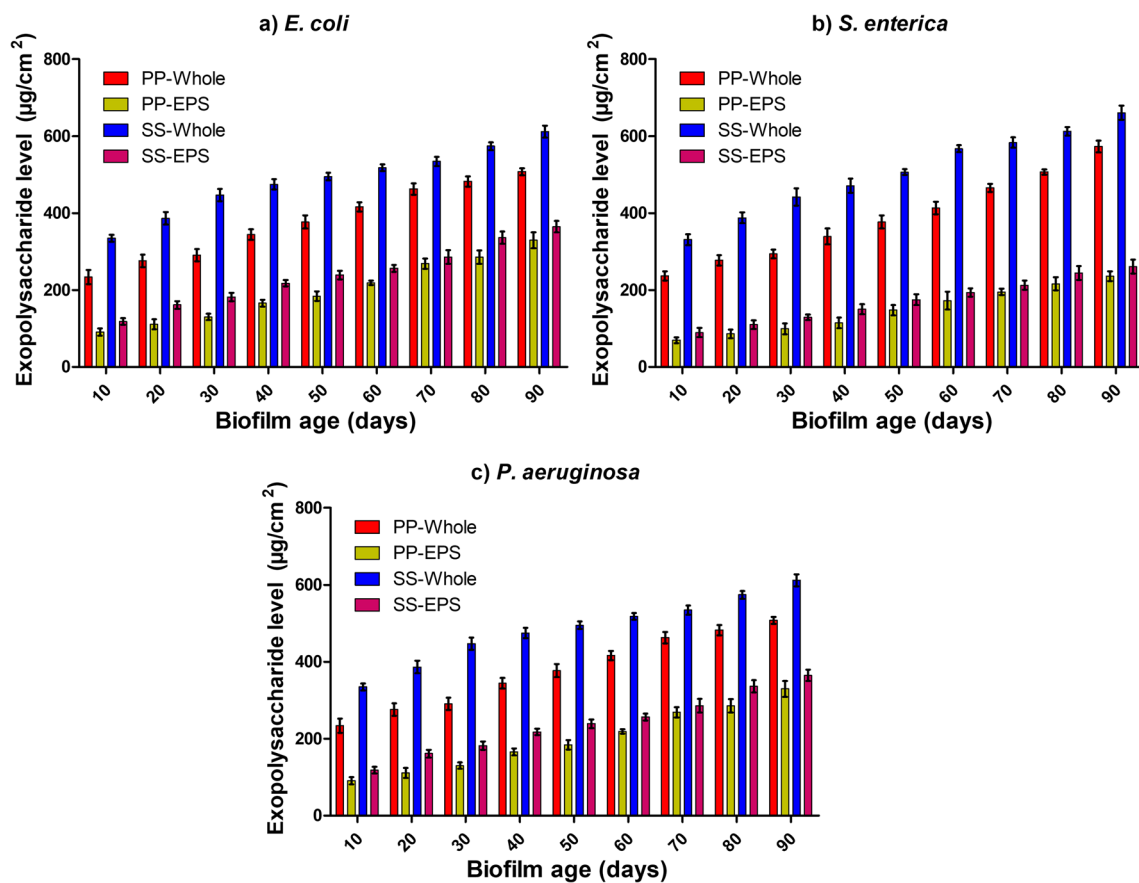
**Fig. 5** FESEM images of the formed biofilm of 90 days old for *P. aeruginosa* grown upon **a** polypropylene (PP) material and **b** stainless steel (SS) material



that the formed biofilm cells for all tested bacterial strains grown upon PP material were lower densities than that formed on SS material. In addition, *P. aeruginosa* biofilm appears denser than other species on SS, suggesting that SS material promoted biofilm formation. When SEM analyzed the same samples, it was observed that after 90 days, the SS surface could facilitate biofilm formation more than PP (Fig. 5). The amount and structure of polysaccharides released by bacteria into the EPS are influenced by environmental factors and cell concentrations. Additionally, the extrapolymeric substance thickness and agglomerates' diameters were measured.

### The quantification of microbial biofilm components

**Results of polysaccharides** The quantity of polysaccharides in whole *E. coli* biofilm developed on PP material ranged between 245 and 514  $\mu\text{g}/\text{cm}^2$ , while the amount of polysaccharide extracted from EPS was between 97.3 and 344.2  $\mu\text{g}/\text{cm}^2$ . Regarding the amounts of polysaccharides, the biofilm grown upon SS, result revealed that the quantity of polysaccharides was 341 and 623  $\mu\text{g}/\text{cm}^2$  in the whole biofilm and EPS, respectively, but the amount of polysaccharides extracted from EPS was 124.6 and 375.5  $\text{mg}/\text{cm}^2$  across the entire biofilm and EPS, respectively (Fig. 6a).



**Fig. 6** The average amount of exopolysaccharide ( $\mu\text{g}/\text{cm}^2$ ) of whole biofilm and extracted EPS of **a** *E. coli*, **b** *S. enterica*, and **c** *P. aeruginosa* as mature biofilm each 10 days until 90 days old biofilm grown upon polypropylene (PP) and stainless steel (SS) material

As to the development of biofilm by *S. enterica*, data illustrated in Fig. 6b the quantity of exopolysaccharide developed on PP material in both whole and EPS biofilm ranged from 230 to 584  $\mu\text{g}/\text{cm}^2$ , while the amount of exopolysaccharide extracted from EPS was between 75 and 245  $\mu\text{g}/\text{cm}^2$ . Throughout the development of biofilm formed on SS, the findings showed that the production of exopolysaccharide in whole and EPS biofilm was 325 and 639  $\mu\text{g}/\text{cm}^2$ , respectively. Still, the quantity of polysaccharide produced from EPS was 98 and 274  $\mu\text{g}/\text{cm}^2$  in the entire biofilm and EPS, respectively.

Regarding biofilm improvement on PP by *P. aeruginosa*, the quantity of exopolysaccharide developed on PP material in both whole and EPS biofilm ranged from 278 to 684  $\mu\text{g}/\text{cm}^2$ , while the amount of exopolysaccharide extracted from EPS was between 125 and 373  $\mu\text{g}/\text{cm}^2$ . Throughout growth of biofilms formed on SS, the findings showed that production of exopolysaccharide in whole and EPS extraction was 325 and 639  $\mu\text{g}/\text{cm}^2$ , respectively, but the quantity of exopolysaccharide produced from EPS was 142 and 412  $\mu\text{g}/\text{cm}^2$  in the entire biofilm and EPS, respectively (Fig. 6c).

The type of bacteria that form a biofilm can have a significant impact on the amount of EPS present in the biofilm. EPS are polymers produced by bacteria that help to hold the biofilm together and provide protection against environmental stresses. Different types of bacteria produce different types and amounts of EPS. For example, some bacteria produce EPS that are highly viscous and sticky, which can help to create a more robust and stable biofilm. Other bacteria may produce EPS that are less viscous and more easily dispersed, leading to a less stable biofilm. Additionally, some bacteria may produce EPS that are more resistant to degradation, which can lead to the accumulation of EPS over time. Overall, the type of bacteria that form a biofilm can have a significant impact on the amount and type of EPS present in the biofilm. This can, in turn, affect the properties and behavior of the biofilm, including its stability, ability to resist environmental stresses, and interactions with other organisms.

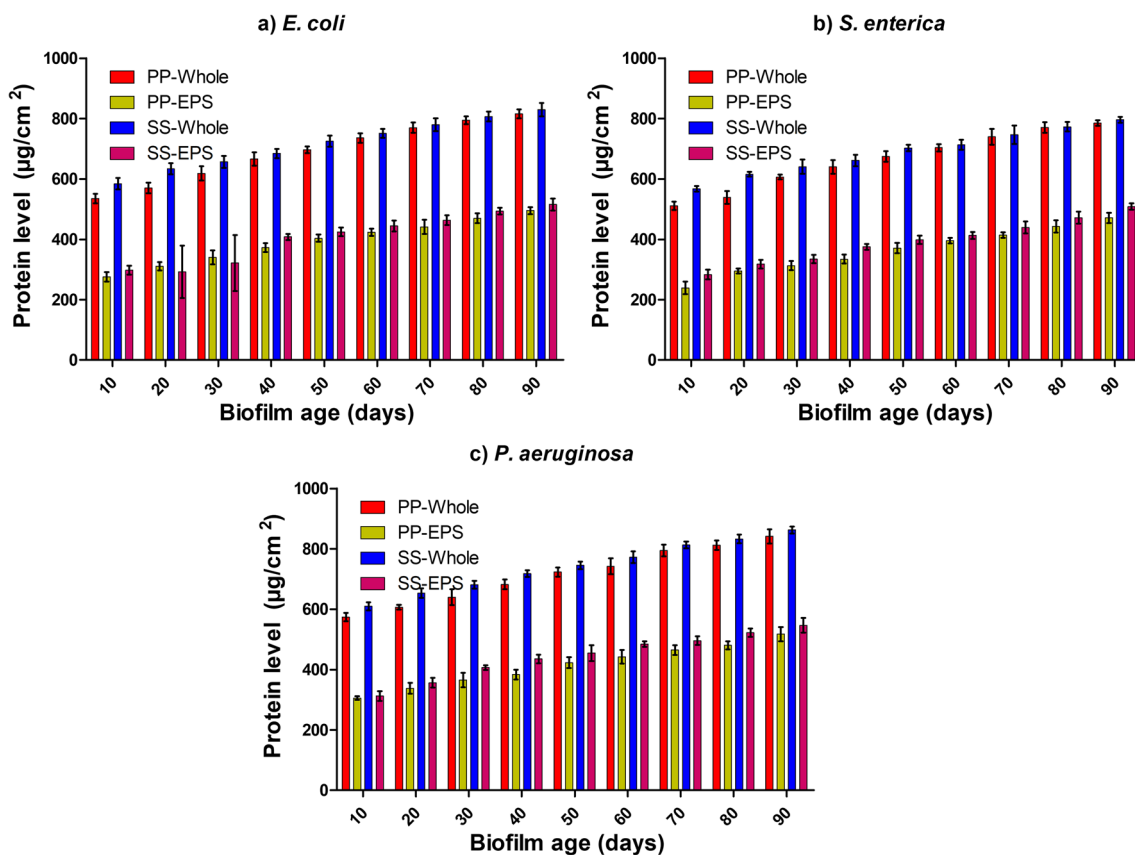
**Results of protein** The quantity of protein in whole *E. coli* biofilm developed on PP material ranged between 546 and 826  $\mu\text{g}/\text{cm}^2$ , while the quantity of extracted protein from

EPS was between 287 and 503  $\mu\text{g}/\text{cm}^2$ . Regarding the amounts of protein while biofilm grown on SS, it was found that the amount of protein was 598 and 845  $\mu\text{g}/\text{cm}^2$  in the whole biofilm and EPS, respectively, but the quantity of protein from EPS was 308 and 529  $\mu\text{g}/\text{cm}^2$  in the entire biofilm and EPS, respectively (Fig. 7a). The protein concentration formed on PP material by *S. enterica* during the biofilm construction spanned from 521 to 792  $\mu\text{g}/\text{cm}^2$ , whereas the protein concentration recovered from EPS was around 254 and 483  $\mu\text{g}/\text{cm}^2$  (Fig. 7a). The results indicated that the levels of protein in the whole biofilm and the EPS were 574 and 803  $\mu\text{g}/\text{cm}^2$  in the whole biofilm and the EPS, respectively, during the circumstance for biofilm produced on SS. The total biofilm and EPS made 295 and 516  $\mu\text{g}/\text{cm}^2$  of protein from EPS. When *P. aeruginosa* established a biofilm on PP, the amount of protein produced on the material ranged from 584 to 858  $\mu\text{g}/\text{cm}^2$ , while the amount of protein recovered from EPS was between 310 and 534  $\mu\text{g}/\text{cm}^2$ . However, the quantity of protein produced from EPS was 324 and 564  $\mu\text{g}/\text{cm}^2$ , respectively, in the condition for biofilm developed on SS. The results indicated that the concentration of protein in the entire and EPS biofilm was 619 and 871  $\mu\text{g}/\text{cm}^2$  in the whole biofilm and EPS, respectively (Fig. 7c).

## Discussion

Restricting and controlling bacterial proliferation and biofilm formation in medical systems and water distribution networks is essential for maintaining public health (Puzon et al. 2009). Corrosion, deterioration in water quality, odor and taste, and deterioration of disinfectants in the distribution system are triggered by several different kinds of pathogens in the pipe biofilm (Wang et al. 2012, 2014; Hemdan et al. 2015). The category of pipeline materials used and their characteristics could be the principal cause of this, as they may influence the type, thickness, and rate of biofilm growth (Hemdan et al. 2016). As a result, a sufficient acquaintance of the biofilm features of various materials is crucial to guarantee potable water to consumers and the control of biofilm on medical supplies (Hemdan et al. 2017).

In the current study, the tested SS surface forms the biofilm more quickly than the tested PP material surfaces. The density of bacterial counts was higher on SS than PP. This may be due to the fact that the surface of SS is more rough than PP; moreover, the quantity of biofilm may



**Fig. 7** The average amount of protein ( $\mu\text{g}/\text{cm}^2$ ) of whole biofilm and extracted EPS of **a** *E. coli*, **b** *S. enterica*, and **c** *P. aeruginosa* as mature biofilm each 10 days until 90 days old biofilm grown upon polypropylene (PP) and stainless steel (SS) materials



reach up to 25 times higher on rough than smooth surfaces. A wide range of parameters can influence microbial colonization on solid surfaces. The degree of colonization of certain surfaces, for example, has been found to increase with surface roughness because the “valleys” present allow microbes to reside in a protected area with reduced shear forces and the surface roughness provides a surface with increased surface area for bacterial attachment (Donlan 2002; Vu et al. 2009). Nolan et al. (2018) discovered that biofilm grow much faster in PP than in SS materials, but such distinctions could not be discovered in aged pipe networks. Similarly, after decades of operations, there was no significant difference in the colonization of the investigated materials (SS, polyvinyl chloride (PVC), and PP) (Gloag et al. 2013). Moreover, with the plastic-based materials including PP, previous investigation reported that it can support less biofilm development than tarred steel and cement-based materials; others showed no significant differences between the biofilm of the SS, polyethylene (PE), and PVC (Gomes et al. 2014; Buse et al. 2017).

Although the bacterial biofilm counts on PCA were still in the same  $\log_{10}$  as the counts recovered by selective agar media in the current study. The addition of a secondary culture media was used as confirmatory results, nonetheless, the recovery of bacterial biofilm counts on PCA was found to be slightly higher than that determined on selective media, for example, the results of the present study showed that *E. coli* biofilm counts on SS material were  $8.50 \times 10^8$  CFU/cm<sup>2</sup> on PCA as enrichment agar medium. This could be because PCA is high enrichment medium with high nutrients, whereas selective media contain some selective agents that may inhibit the recovery of the target bacteria (Degirmenci et al. 2012). Rocelle et al. (1996) and Silk and Donnelly (1997) discovered no significant difference in bacterial population recovery using selective or enrichment agar media.

The earlier studies on biofilm in distribution networks have only used methods that rely on cultivating media to characterize the existing bacteria (Hemdan et al. 2021). The obtained results of cultural-dependent methods showed that the biofilm counts of *E. coli*, *S. enterica*, and *Pseudomonas aeruginosa* on SS material using enrichment agar media were  $8.50 \times 10^8$ ,  $5.30 \times 10^8$ , and  $9.50 \times 10^8$  CFU/cm<sup>2</sup>, respectively. On the other hand, the biofilm counts for *E. coli*, *S. enterica*, and *Pseudomonas aeruginosa* on PP material using enrichment agar media were  $8.90 \times 10^7$ ,  $3.50 \times 10^7$  and  $9.40 \times 10^7$  CFU/cm<sup>2</sup>, respectively. Researchers studied the density, species composition, and population dynamics of bacteria in biofilm produced in distinct pipe materials (e.g., iron, copper, SS, and PVC) using culture-based and culture-independent approaches (Kirmusaoğlu 2019). Many molecular techniques, which avoid using culture media, have been employed to investigate the occurrence of bacterial species

in biofilm samples, including high-throughput amplicon sequencing, microscopic investigations, and metabolic activity analyses (Mei et al. 2018). In the current investigation, it was discovered that measuring the quantities of ATP in SS and PP biofilm samples is highly recommended for determining the bacterial density. Moreover, ATP is regarded as a rapid method for figuring out bacterial densities in biofilm samples (Zhang et al. 2022). ATP bioluminescence approach is among the most often used substitute approaches for microbial population assessments. It may be performed as a quantitative assay to estimate the bacterial population densities in a broad range of used materials (Johani et al. 2018). Moreover, compared to the traditional approaches for quantifying the populations of bacterial cells in water and biofilm samples, such a technique is easy, affordable, and efficient. It has a strong association with heterotrophic plate count (HPC) statistics (Koopman et al. 2015). Biofilm is typically assumed from the standpoint of the enclosed microbial cells' physiological and safety needs. They can, however, be well-thought-out as biophysical materials, with the cells acting as colloids and the EPS functioning as a cross-linked hydrogel matrix. This paradigm has established weak substance physics parallels, enabling the current knowledge of biofilm communities as viscoelastic materials (Gloag et al. 2020). Further, mechanical analysis is challenging because biofilms are tiny and highly variable, both within and between biological replicates and species (Guerra et al. 2017). The composition of biofilm is also affected by the availability of nutrients in a specific environment and genetic factors of the microorganisms within the community (Hall-Stoodley et al. 2004; Melaugh et al. 2016).

In this study, it was found that *P. aeruginosa* strain was able to form biofilm on the inner surface of the wells. Moreover, *P. aeruginosa* was the most efficient at forming biofilm in terms of growth rate, but it also produced a significant amount of biomass, as determined by crystal violet of biofilms formed on microtiter plates (Table 4). On the other side, least biofilm biomass formed was for *S. enterica*. The processes of bacterial biofilm development follow the same general pattern (Dos Santos et al. 2018). Initially, the motile bacteria approach the surface that is to be colonized and explore it by moving. This is the phase of the reversible adhesion. From one moment on, some bacteria lose their flagella and attach irreversibly to the substratum (Eroshenko et al. 2017). This is followed by the attached growth of the bacteria, starting with the formation of microcolonies, accumulation of biofilm biomass, and, finally, mobilization of some cells and their detachment from the biofilm in search of novel niches. The latter process is little known; expectedly, it is related to processes of degradation or loosening of the biofilm matrix (Huang et al. 2018).

SEM was already a beneficial model for examining the substratum structure and tracking bacterial adhesion

and biofilm development on various substrates. Certainly, SEM has been used to investigate and describe biofilms on medical equipment since its beginnings (Hemdan et al. 2017). In the present work, SEM showed that, the biofilm of the three tested bacteria species (*E. coli*, *Salmonella enterica*, and *P. aeruginosa*) have been formed on both PP and SS materials. In addition, the three tested bacterial strains adhered well on both PP and SS materials. This is back to the fact that bacterial cell adhesion is influenced by factors such as physiology, cell morphology, and the physicochemical properties of the contact surface. Gram-negative bacteria adhere to surfaces more easily than Gram-positive bacteria because they have pili, flagella, and fimbriae, as well as an outer membrane (Santâ et al. 2014). Iibuchi et al. (2010) found that *Salmonella* sp. has ability to form biofilm on PP surface; they also observed formation of EPS by SEM. Furthermore, the results of SEM showed that the densities of the formed biofilm of the three tested bacteria species were higher on SS than PP materials. This may be due to the surface of stainless steel material is hydrophobic that facilitates the adhesion of microorganisms more easily than other surface compared to polypropylene surface (Santâ et al. 2014). Further, SEM possesses the magnification and accuracy sufficient to examine the overall shape of the microbes involved in forming biofilm and their spatial configuration (Ferreira Ribeiro et al. 2016). Despite conventional techniques that would provide bulk measurement, SEM provides a spatial analysis that renders it an appealing technique for evaluating biofilm formation on heterogeneous surfaces (where there is a junction between two materials) (Gomes and Mergulhão 2017).

Extracellular polymers, widely known as extracellular polymeric substances (EPS), are the structure that binds vital microorganisms together, adheres to the surface, and protects them from stressful events (Park and Hu 2010). The dimensional strength of the bacterial communities is supported by EPS, composed of carbohydrates, proteins, deoxyribonucleic acid (DNA), and lipids in varying proportions (Flemming and Wingender 2010). The EPS acts as a physical barrier to protect the enclosed bacterial community from environmental stress by facilitating cell–cell adhesion and surface attachment (Poulin and Kuperman 2021). *E. coli*, *Salmonella*, and *Pseudomonas* spp. are able to produce EPS (Wang et al. 2013; Pang et al. 2017). The results revealed that the significant average amounts of polysaccharides in the whole and EPS biofilm were discovered in the biofilm formed on SS. This could be owing to a difference in microbial numbers that can play a role in biofilm development, with a positive relationship between microbial numbers and microbial metabolic activity (protein and polysaccharide). These results are consistent with those of Hemdan et al. (2017). They found that the levels and amounts

of protein and polysaccharides in the whole biofilms are higher than in harvested EPS of biofilm.

Moreover, the EPS promotes the establishment of three-dimensional biofilm construction by serving as nutrition storage reservoirs and supporting bacteria in adhering to several substrates. It effectively shields bacteria from a variability of hostel and environmental situations. Under specific ecological parameters, bacteria may produce an abundance of EPS components with various EPS with various structural and functional properties (Vu et al. 2009). *E. coli* and *P. aeruginosa*, for example, create substantial amounts of colonic acid or alginate in response to environmental stressors such as dehydration and osmotic stress (Rabin et al. 2015). The EPS, proteins, and extracellular DNA that comprised the biofilm matrix facilitated the formation of a community-based biofilm (Wilson et al. 2017).

As a consequence of changing microbial species attaching to substrates, biofilm structures develop into organized communities. Biofilm infestation with various dangerous microorganisms plagues industrial facilities, including water treatment systems and healthcare institutions, having a fatal effect on public health. As a result, research into microbial biofilm utilizing various monitoring techniques is crucial from a medical and financial perspective. These results shed light to better understand the physiological behavior and densities of biofilm. While *P. aeruginosa* has frequently been observed to outcompete other tested species, its rate of biofilm production was higher than that of the other species. *S. enterica* had the slowest rate of biofilm growth at the same time. Particularly for *P. aeruginosa*, biofilm production on SS materials was much higher than on PP materials. From the obtained results, it was observed that *P. aeruginosa* generated biofilm with higher protein levels and polysaccharides than *E. coli* and *S. enterica*. This may be due to the ability of *P. aeruginosa* to produce different kinds of polysaccharides for instance alginate, Psl and Pel. These polysaccharides are responsible for the production and strength of biofilm (Batoni et al. 2016; Passos da Silva et al. 2019). It is well known that Psl-like polysaccharide is considered the main composition of some *Pseudomonas* spp. EPS and also plays an important role in the virulence intensity of these *Pseudomonas* spp. (Heredia-Ponce et al. 2020). The bacterial biofilm cell counts using culture-dependent methods were matched with the results of microbial metabolic activities. The obtained result recommends the direct techniques for deeply investigation of biofilm as they are easy to use, low-cost, and reliable.

## Conclusion

We evaluated 90-day formed biofilms *E. coli*, *S. enterica*, and *P. aeruginosa* on SS and PP materials. Biofilm formation has been assessed using culture-dependent methods on both

selective and nonselective agar media, as well as culture-independent methods such as scanning electron microscope (SEM) and ATP and protein quantification. Using both selective and nonselective agar media, the biofilm of *E. coli*, *Salmonella enterica*, and *P. aeruginosa* counts on SS was greater than on PP materials. Furthermore, SEM revealed that SS materials encourage biofilm formation more than PP materials after 90 days. Moreover, SEM revealed that the density of *P. aeruginosa* biofilm was greater on both SS and PP materials than that of *E. coli* and *Salmonella enterica*. On the other hand, the ATP amounts of *E. coli* ( $2.50 \times 10^8$  RLU/cm<sup>2</sup>), *S. enterica* ( $2.30 \times 10^8$  RLU/cm<sup>2</sup>), and *P. aeruginosa* ( $8.20 \times 10^8$  RLU/cm<sup>2</sup>) on SS materials were higher than that formed on PP materials. Finally, it can be concluded that PP materials are advantageous for industrial applications, such as those used in drinking water distribution systems and medical devices. The biofilm evaluation in simulated system using cultural dependent approaches such as PCA and specific agar media and using cultural independent approaches such as SEM, ATP, and protein determinations is highly recommended.

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**Data availability** The data that support the findings of this study are available on request from the corresponding author.

## Declarations

**Conflict of interest** The authors declare no competing interests.

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