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THE INFLUENCE OF DIFFERENT CULTURE MEDIA ON *Pseudomonas aeruginosa*, *Escherichia coli*, AND *Staphylococcus aureus* BIOFILM FORMATION

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Abstract

Microorganisms such as *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* are frequently isolated in samples of urinary, blood, intestinal, and respiratory infections, among others. These bacteria are also associated with microbial biofilm formation. *E. coli*, *P. aeruginosa*, and *S. aureus* biofilm infections are particularly hard to manage and often associated with nosocomial problems. This study investigated the influence of different culture media on *E. coli*, *P. aeruginosa,* and *S. aureus* biofilm formation. Bacterial performance was evaluated in brain heart infusion broth, Mueller-Hinton broth, or tryptic soy broth, with or without supplementing with different glucose levels (1-5%). The study quantified biofilm biomass and the count of viable biofilm colonies. This is the first study that compares the biofilm formation of *E. coli*, *P. aeruginosa* and *S. aureus* in polystyrene using different culture media and with different glucose concentrations. The most robust growth of *E. coli*, *P. aeruginosa*, and *S. aureus* occurred in brain heart infusion broth supplemented with 5% glucose, Mueller-Hinton broth without glucose, and tryptic soy broth with 2% glucose, respectively. Our data demonstrate that behavioral and morphological characteristics of each bacterium require a specific broth to enhance the growth of these microorganisms. These findings will contribute to future tests for therapeutic alternatives with anti-biofilm potential.

Keywords: Adhesion. Gram-negative bacteria. Gram-positive bacteria. *In vitro* techniques.

1. Introduction

Since the discovery of penicillin, indiscriminate antibiotic use and hospitalizations have caused the emergence of various multidrug-resistant microorganisms (Yewale 2014; Mulani et al. 2019). Persistent bacterial cells are known for recurrent infections and present treatment challenges. These cells are metabolically inactive, and antibiotics cannot kill them (Keren et al. 2004; Lewis 2010).

The emergence of multidrug-resistant strains, such as *E. coli* and *P. aeruginosa*, and the ability of many pathogens to form biofilms during infection increase the risk of bacterial diseases not treatable with current antibiotics (Brown et al. 2012). The ability to form biofilms on various surfaces also complicates the eradication of *S. aureus*, an important nosocomial and foodborne pathogen (Lade et al. 2019). The capacity of these pathogens to organize as biofilm increases bacterial resistance, and they represent approximately

80% of persistent infections worldwide, cross-infections, bacteremia, endocarditis, gastritis, and urinary tract infections when present in catheters or tracheal tubes (Mirzaei et al. 2020).

Biofilm-related infections cause high population morbidity and mortality. Therefore, understanding the conditions of such biofilm formation is essential when searching for effective and innovative therapeutic alternatives (Azam and Khan 2019; Lesho and Laguio-Vila 2019; Mirzaei et al. 2020). Given the above, this study evaluated different conditions for *S. aureus*, *E. coli*, and *P. aeruginosa* biofilm formation.

2. Material and Methods

Microorganisms and growth media

Three American Type Culture Collection (ATCC) strains were used: Gram-negative *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* PA01 (ATCC 47085), and Gram-positive *Staphylococcus aureus* (ATCC 25923). The microorganisms were maintained in a culture medium with glycerol and cooled to -80°C. The sample was thawed, inoculated on brain heart infusion (BHI) broth, and incubated for 24 hours. It was then seeded on nutrient agar and incubated at 37°C for 24 hours.

Biofilm formation

Biofilm was formed according to the previously optimized and described conditions (Sandberg et al. 2008) with modifications. Microorganisms were inoculated on a 0.5 McFarland scale in saline, and 15 µL were added into flat-bottomed 96-well plates (Nunclon™ D-surface, Nunc, Roskilde, Denmark) containing 100 µL of BHI broth, Mueller-Hinton broth (MHB), or tryptic soy broth (TSB), with or without various glucose concentrations (1-5%). The negative control represented a culture medium only. The plate was incubated at 37°C for 24 hours.

Quantification of biofilm biomass

After incubation, the supernatant was removed and washed three times with distilled water. The treated biofilm was fixed with 95% methanol and stained with 150 µl of 1% crystal violet for 15 minutes at room temperature (RT). After incubation, the plate wells were washed with distilled water. Ethanol 95% was added to dissolve the stain, and after 10 minutes, 100 µL was transferred to another plate for spectrophotometrical measurement at 570 nm in a spectrophotometer (TP-Reader; ThermoPlate, Goiás, Brazil) (Sandasi et al. 2010).

Quantification of cultivable cells

The number of cultivable cells in the biofilm was determined by counting colony-forming units (CFU) (Lopes et al. 2019). After incubation, the wells were washed three times with phosphate buffer saline to remove non-adherent cells. Then, 100 µL of phosphate buffer saline was added, and the biofilm was scraped off with a tip. The cell suspension was shaken, diluted in phosphate buffer saline, plated on brain heart infusion agar, and incubated at 37°C for 24 hours. After incubation, the total CFUs were counted, determining the log CFU per milliliter ($\log_{10}$ CFU/mL).

Statistical analysis

A two-way ANOVA followed by Dunnet and Tukey's tests compared the effect of glucose supplementation on each medium and biofilm formation of different media with the same glucose concentration (95% confidence interval). The experiments were performed in three replicates and three independent experiments.

3. Results

Quantification of biofilm biomass

Absorbance was read after staining, showing significant differences in the amount of biofilm. The experiment with *E. coli* showed that adding 5% glucose increased biofilm formation in BHI by 67%. Also, among the unsupplemented media, TSB formed 57% more biofilm than the others (Fig. 1A). Regarding *P. aeruginosa*, glucose concentration did not affect biofilm formation, but MHB formed biofilm with higher biomass than the one in BHI and TSB (33% and 16%, respectively) (Fig. 1B). Adding 2% glucose resulted in 86% more biofilm in *S. aureus* than TSB without glucose. In all tested media, glucose supplementation increased biofilm formation significantly (Fig. 1C).

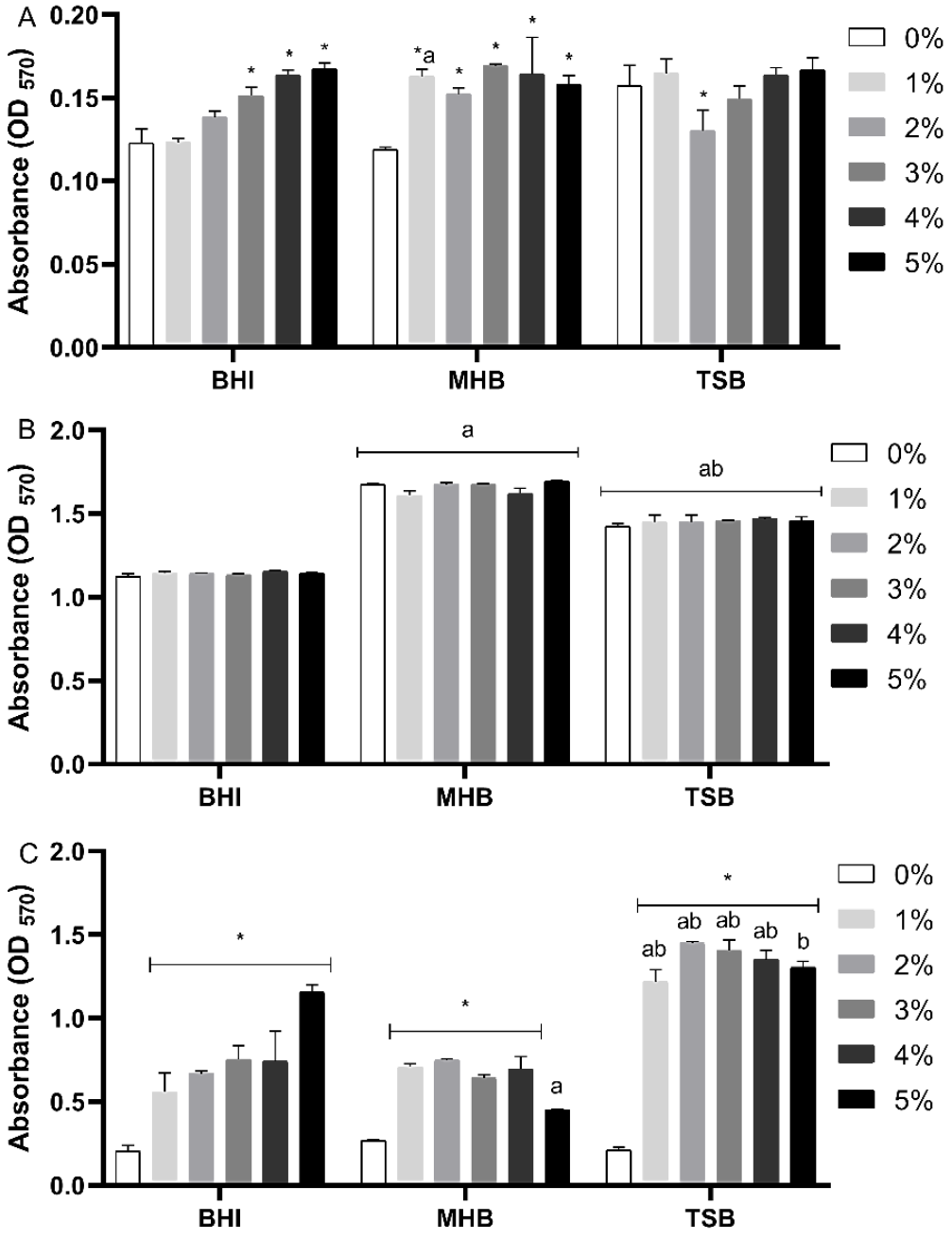

Figure 1. Quantification of the biomass of A - *E. coli*, B - *P. aeruginosa*, and C - *S. aureus* in different media with various glucose concentrations. The results are expressed as Mean ± Standard deviation. An analysis of variance (ANOVA) followed by Dunnet and Tukey's test was used considering $p < 0.05$ statistically significant compared to biofilm formation without glucose supplementation (0%) (*), BHI at the same glucose concentration (a), and MHB at the same glucose concentration (b).

Quantification of cultivable cells

The quantification of viable cells in biofilm showed that glucose supplementation benefited *E. coli* biofilm in MHB. As for *P. aeruginosa*, supplementation showed no significant differences at some concentrations, but MHB had more viable cells than the other culture media. TSB was an optimal medium for *S. aureus* biofilm formation, and glucose addition efficiently increased this biofilm production (Figure 2).

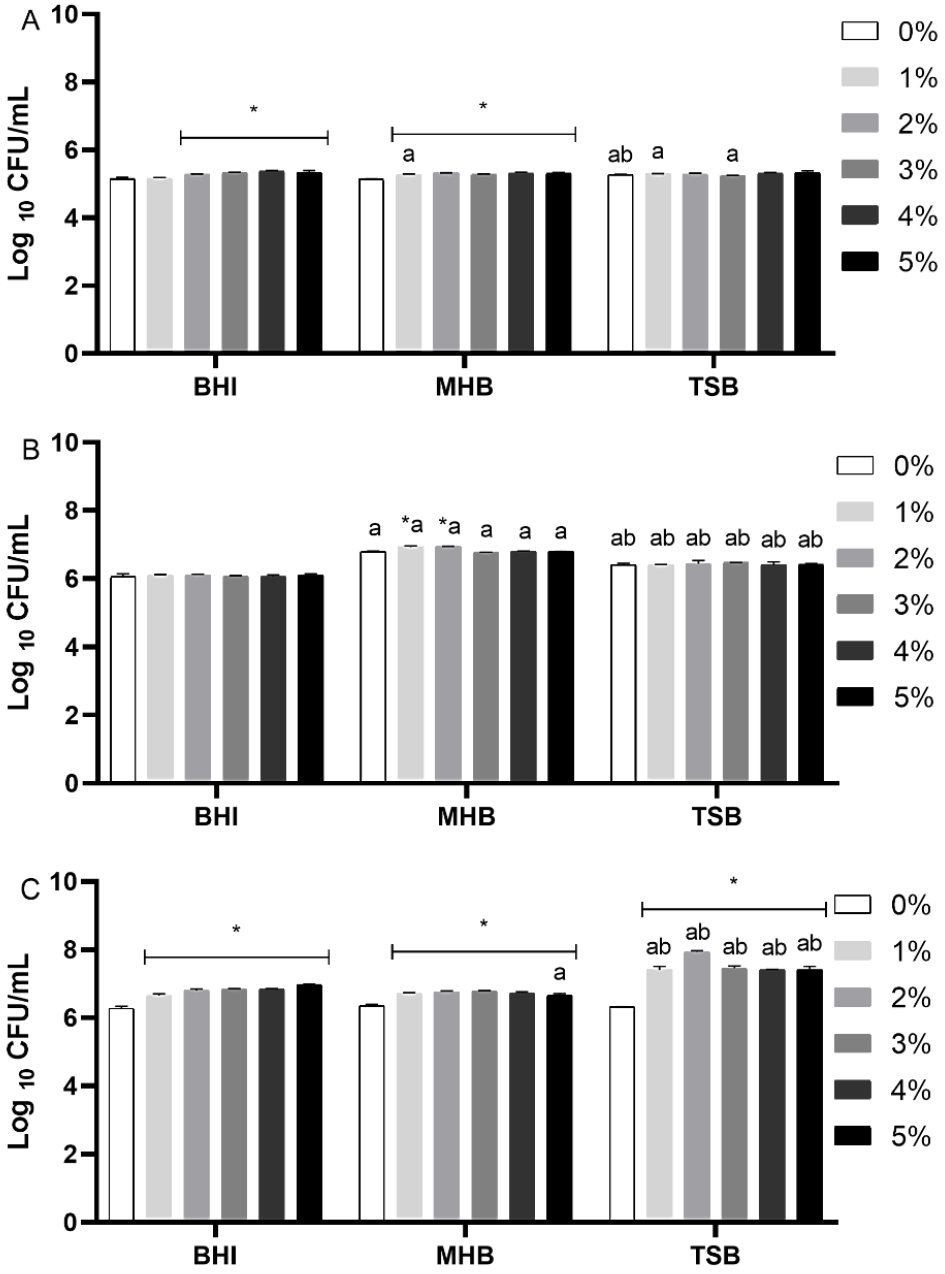

Figure 2. Quantification of cultivable cells A - *E. coli*, B - *P. aeruginosa*, and C - *S. aureus* in different media with various glucose concentrations. The results are expressed as Mean ± Standard deviation. An analysis of variance (ANOVA) followed by Dunnet and Tukey's test was used considering $p < 0.05$ statistically significant compared to biofilm formation without glucose supplementation (0%) (*), BHI at the same glucose concentration (a), and MHB at the same glucose concentration (b).

4. Discussion

S. aureus is a major clinically relevant pathogen that causes several infections, including mild soft skin infections, endocarditis, bacteremia, and severe pneumonia. *P. aeruginosa* colonizes very efficiently in

the human respiratory tract and is highly prevalent in patients with cystic fibrosis (CF), bronchiectasis, and chronic obstructive pulmonary disease (Lozano et al. 2020). *E. coli* is one of the main pathogenic bacteria that cause serious foodborne diseases in humans worldwide from contaminated and raw food consumption (Bae et al. 2021).

They are structured as biofilms, conceptually described as microorganism aggregates surrounded by an exopolysaccharides matrix that develops under biotic and abiotic surfaces, allowing their interaction. The biofilm formation of these species is significant because they produce virulence factors for protection and increase intrinsic resistance to antimicrobial products (Mirzaei et al. 2020; Lesho and Laguio-Vila 2019).

Therefore, biofilm studies must standardize culture techniques in clinical and research laboratories. Studies show that the nutrient composition of the culture medium highly impacts biofilm growth and development (Wijesinghe et al. 2019).

Regarding *S. aureus*, glucose contributes to bacterial growth, increasing glucose fermentation that helps *S. aureus* aggregation (Luo et al. 2020). Several studies have supplemented the culture media, and TSB (which already contains 2.5 g/L of glucose and 5.0 g/L of NaCl) often receives glucose (0.25%, 0.4%, 0.5%, and 1.0%) (Yu et al. 2017; Fernández-Barat et al. 2018; He et al. 2019).

Our study corroborates Lade et al. (2019), who reported high biofilm formation of 40 *S. aureus* clinical strains with different genetic origins *in vitro*. Such good glucose-induced biofilm formation for *S. aureus* was evidenced and later confirmed (Regassa et al. 1992; Tasse et al. 2018).

These results are probably due to the potential alteration in the *agr quorum-sensing* system. *Agr* regulates the expression of several toxins, including δ-toxin (a molecule with surfactant-like properties), which contributes to *S. aureus* adhesion and biofilm development (Lade et al. 2019).

To the best of our knowledge, no study evaluated the *E. coli* bacteria in the tested media and glucose concentrations to identify optimal biofilm formation. Most studies assess progressive changes in pH and sugar availability in the medium of a bacterial culture affecting cellular heterogeneity within the microbial community and the gene expression profile of the microbial population (Smith et al. 2018).

Glucose is one of the most abundant monosaccharides and the most accessible carbon source to be consumed by heterotrophic microorganisms such as bacteria. Furthermore, changes in glucose-lactose induce the downregulation of amino acid biosynthesis and aerobic metabolism genes (Sánchez-Clemente et al. 2020). Fernández-Gutierrez et al. (2020) evaluated glucose fermentation in four different media (LB, M9, M63, and MOPS) supplemented with glucose at different concentrations (4, 12.5, and 25 g/L). All showed maximum glucose conversion in the presence of a genetically modified *E. coli* strain.

Unlike *E. coli, P. aeruginosa* eschews glucose consumption (Manzo et al. 2021). Moreover, although the preferred metabolic strategy for *P. aeruginosa* may be aerobic respiration, which may contribute to growth in the presented conditions, a more substantial role for anaerobic processes is expected. Only one study evaluated glucose added to different culture media. Manzo et al. (2021) reported that adding glucose to MHB was associated with a modest increase in the osmoprotectant glycine betaine but without affecting cell wall components (Manzo et al. 2021).

5. Conclusions

Among the commonly used media, BHI broth supplemented with 5% glucose is the most conducive growth medium for studying *E. coli* biofilm *in vitro*, followed by MHB without glucose for *P. aeruginosa* and TSB with 2% glucose for *S. aureus.*

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