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Effects of Glucose and Temperature on Exopolysaccharides, Extracellular Matrix Proteins Production and Biofilm Formation of Carbapenem

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ABSTRACT

Background and Aim: *Acinetobacter baumannii* is one of the most nosocomial pathogens as it can form biofilm on hospital surfaces. The objective of this work was to analyze the production of exopolysaccharides, extracellular proteins, and biofilm formation of carbapenem-resistant *A. baumannii* on silicone and ceramic surfaces.

Materials and Methods: Qualitative and quantitative tests were conducted to evaluate the production of exopolysaccharides in different culture conditions. The Biuret method was applied for protein determination. Furthermore, the count of viable cultivable cells was used to study biofilm formation. For physicochemical characterization, the surfaces were subjected to contact angle measurements.

Results: Incubation at 37°C with glucose (1.5%) was the optimal condition for producing exopolysaccharides. Glucose supplementation has also impacted the protein production by *A. baumannii*. Moreover, proteins were abundant in the extracellular matrix compared to exopolysaccharides (0.46 mg/mL for exopolysaccharides and 2.48 mg/mL for proteins). The strains formed biofilms on both surfaces but with different capacities, possibly due to the hydrophilic nature of ceramic and the hydrophobic nature of silicone. The addition of 1.5% glucose enhanced biofilm formation on ceramic for all strains. A positive correlation was established between the EPS concentration and the number of cells forming biofilm on silicone with 0.2% of glucose and between protein production and biofilm formed on ceramic with 0.2% and 1.5% of glucose. On the contrary, a negative correlation was detected between protein production and biofilm formation on the silicone surface with 0.2% glucose concentration.

Conclusion: The environmental conditions significantly affect *A. baumannii* biofilm and its extracellular matrix compounds.

Keywords: *Acinetobacter baumannii*, Biofilm, Ceramic, Exopolysaccharide, Proteins, Silicone

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1 Introduction

Acinetobacter is a genus of gram-negative bacteria belonging to the phylum of *Proteobacteria*. Its species are coccobacillus non-motile, non-fermentative, catalase and citrate positive, oxidase negative, growing under strictly aerobic conditions. Among these species, *Acinetobacter baumannii* (*A. baumannii*) is the most common nosocomial pathogen of hospital-acquired infections such as ventilator-associated pneumonia, bacteremia, urinary tract infections, burn wound infections, endocarditis, meningitis, and septicemia.

The Infectious Diseases Society has recently named *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *A. baumannii*, *Pseudomonas aerug-*

inosa, and *Enterobacter spp.* as'the ESKAPE pathogens. This is because of their resistance to antibiotics and their ability to escape from the antibiotic action.

In the last fifteen years, *A. baumannii* has received significant attention due to its several resistance mechanisms and high rates of morbidity and mortality. *A. baumannii* is naturally resistant to many antibiotics **(1)** and, due to the widespread use of antibiotics, the multidrug-resistant (MDR) and the extensively drug-resistant *A. baumannii* (XDR) have emerged worldwide **(2, 3)**. Consequently, the treatment of diseases caused by this bacterium is impeded by this fact, limiting the therapeutic options **(1)**.

Acinetobacter baumannii can adhere to several abiotic surfaces, including polystyrene, polypropylene, and borosili-cate. It was shown that *A. baumannii* colonizes various objects, including medical equipment and tools, hospital furniture, and even gowns and gloves of healthcare providers **(4, 5)**. This pathogen can also adhere to biotic surfaces such as erythrocytes **(6)** and human bronchial epithelial cells **(7)**.

In addition, the biofilm formation ability of *A. baumannii* is an essential factor contributing to the persistence in the hospital environment and the occurrence of nosocomial infections **(8, 9)**. This capacity favors survival in hostile environments and acts as a barrier by reducing the activity of drugs and biocides **(10)**.

Biofilms are bacterial clusters irreversibly attached to a surface or between cells, wrapped by a matrix composed of exopolysaccharides (EPS), proteins, extracellular DNA, and water channel **(11).** The production of EPS variously termed capsule, glycolcalyx, or a slime substance is a significant virulence factor **(12)**. Bacterial adhesion, aggregation, and bacterial proliferation followed by slime production result in a biofilm capable of establishing a virulent and persistent infection on the implant **(13)**. EPS leads to an irreversible adhesion that occurs with the formation of micro-colonies, the maturation of the biofilm **(11)**, and the formation of three-dimensional biofilm structures **(14)**. On the other hand, the extracellular matrix is composed of a variety of proteins with different functions and structures, including flagella, pili type IV, and amyloid-like proteins (e.g., curli protein in *Escherichia coli* **(15)** and FapC in *Pseudomonas aeruginosa* **(16)**. Specifically, amyloid-like proteins have been recognized as one of the most important components involved in the assembly and integrity of the biofilm matrix **(17)**. Besides, the physicochemical properties of the surface also have a rather important role in this phenomenon, namely the hydrophobicity and surface tension components **(18)**.

The first aim of this work was to study the influence of the carbon source and temperature on exopolysaccharides and extracellular proteins production of Carbapenem-resistant *A. baumannii* strains. These strains were isolated from two hospital facility intensive care units (adult and neonatal intensive care units) **(18)**. The second aim was to determine the ability of these isolates to form biofilm on silicone and ceramic surfaces and the effect of glucose on this latter. Ceramic and silicone surfaces are frequently found in hospital environments for that they were chosen for this work. In addition, the physicochemical properties of silicone and ceramic surfaces were determined. Finally, the correlation between EPS, extracellular proteins and, biofilm formation was examined.

2.Materials and Methods

Bacterial Strains

A total of 6 Carbapenem (imipenem and meropenem) resistant *A. baumannii* (Ab) strains was studied. The strains were isolated from medical devices of two hospital intensive care units (adult and neonatal intensive care units) and identified by our laboratory **(18)**. Also, the carbapenemase encoding genes were detected and identified (LAKTIB *et al.* under publication).

Screening for EPS Production

To determine EPS production, pure cultures were streaked on Trypticase Soy Agar (TSA) plates containing 0.2, 0.8, 1, 1.5, or 2% of glucose or sucrose as carbon sources. They were then incubated at 25 or 37°C for 3 days. Strains that produced slimy colonies were recorded as capable of producing EPS **(19)**.

Quantification of EPS and Proteins Production

A. Extracellular Matrix (ECM) Extraction

The strains of *A. baumannii* were incubated on TSA plates with or without glucose supplementation (0.2% and 1.5%) for 72 h at 37°C or 25°C. Subsequently, they were scraped off, suspended in 1 mL of 1.5 M NaCl solution, and incubated for 3 min at 25°C. The suspensions were then centrifuged at 5000 g for 10 min at 25°C, and the supernatants were collected as ECM fractions **(20)**.

B. EPS Quantification

EPS was quantified by the phenol-sulphuric acid method with some modifications **(21)**. In the test tube, 0.5mL of ECM sample was added to 0.5 mL of 5% phenol, followed by adding 2.5 mL of concentrated sulphuric acid. The test tube containing the solutions was then incubated for 10 min at 25°C, and optical

density (OD) was measured at 492 nm. Glucose was used as a standard.

C. Protein Quantification

To quantify proteins in the ECM fractions, we used the Biuret assay **(22)**. After adding 0.5 mL ECM, 0.2 mL Biuret reagent was added, and the mixture was then incubated for 30 min at 27°C. Subsequently, the absorbance was measured at 570 nm. Bovine serum albumin (BSA) was used as a standard.

Growth Conditions and Preparation of Microbial Suspension

After incubation at 37°C on TSA plates, the cells were harvested and washed twice by centrifugation for 15 min at 8400xg, then resuspended in physiologic water (NaCl: 9 g/L) to obtain a bacterial suspension of 10^8 CFU/mL.

Preparation of Substratum Surface

We used Silicone and Ceramic. These latter were cut into 1 cm^2 square (10 _ 10 mm coupon-tests). Then 70% (vol/vol) ethanol solution was employed fo cleaning for 15 min and being then rinsed using distilled water. Finally, thesterilization of the substrates was performed by autoclaving for 20 min at 120°C.

Bacterial Adhesion

The incubation of 10 mL of bacterial suspension wa conducted in a Petri dish that contained substratum surface coupons (3 h at 37°C). After that, to remove the non-adhering bacteria, the coupons were rinsed 3 times using distilled water.

Biofilm Formation

The coupons with adhering bacteria were placed in a new Petri dish containing 20 mL of TSB with or without glucose (0.2% and 1.5%) and incubated for 72 h at 37°C. After that, they were washed three times with sterile distilled water and immersed in a test tube that contained physiological water (NaCl: 9 g/L). We detached the bacterial cells from the support utilizing a sonication bath (Power sonic 405) for 5 min. After sonication, CFUs were counted employing the serial dilution technique of the obtained bacterial susp-ension. The determination of the counts was done on nutrient agar plates after incubation for 24 h at 37°C by the plate count method. Each step was conducted 3 times.

Contact Angle Measurements and Hydrophobicity

Contact angle measurements were performed using a goniometer by the sessile drop method. One drop of a liquid was deposited onto a dry substratum surface, as it was done in he article by AZELMAD *et al, 2018*. Three to six contact angle measurements were made on each substratum surface for all probe liquids, including formamide (99%), diiodomethane (99%), and distilled water. The Lifshitz-Van der Waals (γ^{LW}), electron donor (γ^-), and electron acceptor (γ^+) components of the surface tension of the surfaces were estimated from the approach proposed by Oss *et al.* (1988). In this approach, the contact angles (θ) can be expressed as:

$$Cos\theta = -1+2(\gamma_S^{LW}\ \gamma_L^{LW})^{1/2}/\ \gamma_L + 2(\gamma_S^{+}\ \gamma_L^{-})^{1/2}/\ \gamma_L + 2(\gamma_S^{-}\ \gamma_L^{+})^{1/2}/\ \gamma_L$$

θ is measured by the contact angle. (S) and (L) denote surface and liquid respectively.

The cell surface hydrophobicity was evaluated through contact angle measurements and using the approach of Van Oss *et al.* **(23)**, as it was done in he article by AZELMAD *et al, 2018* . In this approach, the degree of hydrophobicity of a given material (i) is expressed as the free energy of interaction between two entities of that material when immersed in water (w) ΔG_{iwi}. If the interaction between the two entities is stronger than the interaction of each entity with water $\Delta G_{iwi} < 0$, the material is considered hydrophobic. Conversely, if $\Delta G_{iwi} > 0$, the material is hydrophilic. ΔG_{iwi} can be calculated through the surface tension components of the interacting entities, according to:

$$\Delta G_{iwi} = 2\ \gamma_{iw} = -2[(\gamma_i^{LW})^{1/2} - (\gamma_w^{LW})^{1/2})^2 + 2(\gamma_i^{+}\gamma_i^{-})^{1/2} + (\gamma_w^{+}\gamma_w^{-})^{1/2} - (\gamma_i + \gamma_w^{-})^{1/2} - (\gamma_w^{+}\gamma_i^{-})^{1/2}]$$

Statistical Analysis

Correlation analysis, one- and multi-ways ANOVA analysis of variance were applied using Statistica StatSoft version 6. The experiments were performed in triplicate. P-values of ≤ 0.05 were considered significant.

3.Results

Screening for Eps Production

All tested strains of *A. baumannii* showed a different ability to produce slimy colonies by changing the added sugar, its concentration, and the incubation temperature (Table 1). This production was also affected by the type of strain. Ab6 appeared to be the most productive strain of mucoid colonies. A significant difference was observed between sucrose and glucose in both temperatures. Indeed, incubation at 37°C with glucose (1.5%) was optimal for maximum viscosity for all strains (Table 1) and, in particular, Ab6. On the other hand, at 37°C, viscosity raises by increasing the glucose concentration from 0.2 to 1.5% and decreases again by maintaining 2% (Table 1). On the contrary, there was no significant change by increasing the sucrose concentration, and the viscosity was very low compared to glucose (Table 1).

By incubating the strains at 25°C, the colonies were much less slimy than 37°C for glucose, and there was no significant difference between the concentrations (Table 1). Even when increasing the concentration, a complete absence of viscosity was observed for sucrose (Table 1).

Table 1. Formation of slimy colonies by *A. baumannii* strains in the presence of different concentrations (0.2, 0.8, 1, 1.5, or 2%) of sugars

	Glucose										Sucrose									
	37°C					25°C					37°C					25°C				
Strains	0.2	0.8	1	1.5	2	0.2	0.8	1	1.5	2	0.2	0.8	1	1.5	2	0.2	0.8	1	1.5	2
Ab1	+	+	+	++	+	+	+	+	+	+	-	+	+	+	+	-	-	-	-	-
Ab2	+	+	+	++	+	+	+	+	+	+	-	+	+	+	+	-	-	-	-	-
Ab3	+	+	+	++	+	+	+	+	+	+	-	+	+	+	+	-	-	-	-	-
Ab4	+	+	+	++	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-
Ab5	+	+	+	++	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-
Ab6	+	++	++	+++	++	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-

Legend: -: no slimy, +: slimy, ++: high viscosity, +++: very high viscosity

EPS Quantification

EPS was quantified using the phenol-sulphuric acid method. At 37°C and in the absence of glucose, the EPS concentration ranged from 0.34 (Ab3) to 0.46 mg/mL (Ab6) (Figure 1a). At a concentration of 0.2%, the EPS produced by all strains decreased; for example, for Ab 6, the EPS production goes from 0.46 (without glucose) to 0.37 mg/mL (0.2% glucose). On the other hand, except for Ab3, the EPS production increased at 1.5% of glucose (Figure 1a). A significant difference between the concentrations was observed ($P \leq 0.01$).

At 25°C, the concentrations detected are very low compared to those found at 37°C ($P \leq 0.0002$). For both glucose concentrations, the EPS production decreased for all strains with significant differences ($P \leq 0,04$) (Figure 1b). In this case, it can be deduced that in addition to the stress caused by the temperature, glucose may have created another one, causing a decrease in EPS production.

To conclude, a glucose concentration of 1.5% and incubation at 37°C were the best conditions for optimal EPS production.

Protein Quantification

At 37°C, except Ab1, we observed that the proteins concentrations have increased for both glucose concentrations compared to its absence (Figure 1c). for the three strains (Ab2, Ab3, and Ab4), proteins concentration raised with the increase of glucose concentration. On the contrary, for Ab5 and Ab6, proteins production decreased when the glucose supplementation increased (Figure 1c). It appears that the maximum extracellular proteins concentration has been detected in the presence of glucose.

At 25°C, for all glucose concentrations, Figure 1d shows that adding glucose has caused an increase in proteins production for the three strains (Ab2, Ab3, and Ab6) and decreased this concentration for the others (Ab1, Ab4, and Ab5).

Biofilm Formation

All carbapenem-resistant *A. baumannii* strains have expressed their ability to form biofilm on ceramic and silicone surfaces but with different levels (Table 2).

Table 2. The number of *A. baumannii* adhered cells (10^4 CFU/cm^2) forming a biofilm.

Strains	Ceramic surface	Silicone surface
Ab 1	199.33	59
Ab 2	63	483.33
Ab 3	720	90
Ab 4	37	45.67

Strains	Ceramic surface	Silicone surface
Ab 5	43	41
Ab 6	64.33	65

This difference in biofilm-forming ability may be due to the physicochemical properties of the surfaces (Table 3).

Table 3. Contact angles (in degrees) of water (θ_w), formamide (θ_F), diiodomethane (θ_D), the surface tension of Lifshitz-Van der Waals (γ^{LW}), electron donor (γ^-), electron acceptor (γ^+) of silicone and ceramic surfaces and their free energy of interaction with water (ΔG_{iwi}).

Surface	Contact angles (°)			Surface tension: components and parameters ($mJ.m^{-2}$)			ΔG_{iwi} ($mJ.m^{-2}$)	Hydrophobicity
	θ_D	θ_F	θ_w	γ^{LW}	γ^+	γ^-		
Ceramic	69,73	61,17	63,03	23,00	0,67	27,33	7,30	Hydrophilic
Silicone	88,30	96,80	104,27	13,47	0,00	4,53	-52,30	Hydrophobic

The effect of glucose on *A. baumannii* biofilm formation was also studied (Figure 1e, f). In the absence of glucose, the *A. baumannii* strains expressed different preferences towards the two types of surfaces, taking the examples of Ab1, which adhered better to ceramic than silicone (P=0,001), and the opposite case of Ab2 (P=0,0002). By adding glucose, all strains formed a biofilm on silicone but to a lesser extent compared to ceramic except for Ab6, which formed more biofilm on the silicone with 0.2% of glucose than on ceramic. The greatest ability to form biofilm on the ceramic surface was expressed at a glucose concentration of 1.5% in all tested strains (P≤0.02). However, there was no significant difference in the majority of strains between the absence and 0,2% of glucose concentration (P≥0.06) except for Ab1(P=0.0004) and Ab3 (P=0.0009). For silicone surfaces, the addition of glucose did not significantly influence the biofilm format, except Ab2 and Ab6. We also observed that Ab6 reached its maximum at a glucose concentration of 0.2% (P=0.0002).

Biofilm Formation, Eps and Proteins Production

The correlation of the biofilm formation with the quantities of EPS and proteins produced at 37°C in different glucose concentrations was also studied. There is a positive correlation between EPS production and the numbers of bacterial cells forming a biofilm on the silicone with 0.2% of glucose concentration (R= 0.79).

For proteins, a positive correlation was established between the biofilm formed on the ceramic and 1.5% of glucose (R=0.85) (Figure 2a), knowing that the highest capacity of biofilm formation for the ceramic was found at this concentration (Figure 1). With a concentration of 0.2% glucose, a negative correlation was detected between protein production and biofilm formation on the silicone surface (R=-0.63) (Figure 2b) and a positive one on the ceramic surface (R=0.54) (Figure 2c). For the rest, no correlation was detected.

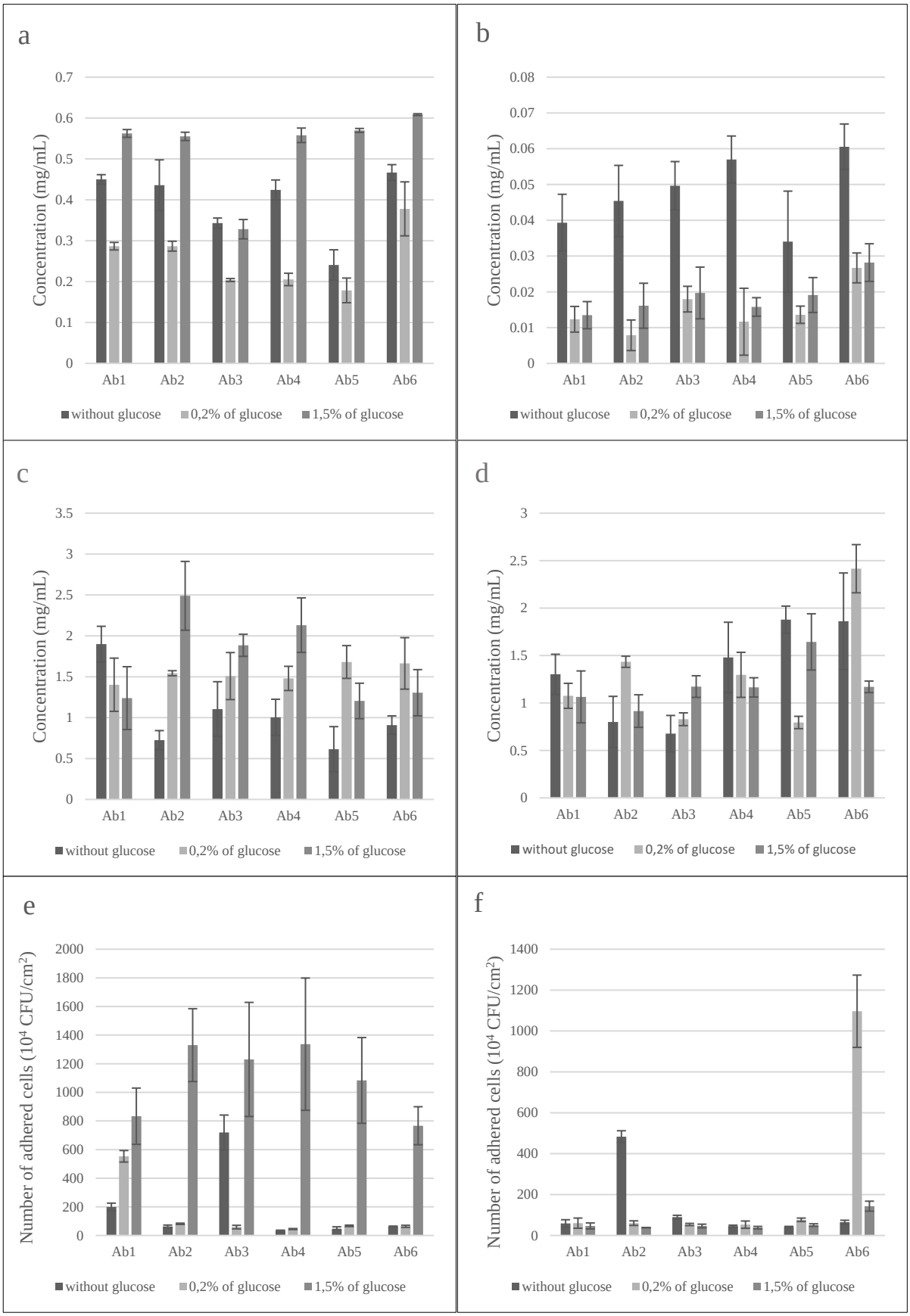

Figure 1. Concentrations of EPS and extracellular proteins produced by *A. baumannii* strains at 37°C (a, c) and at 25°C (b, d) respectively, and biofilm formation on ceramic (e) and silicone (f).

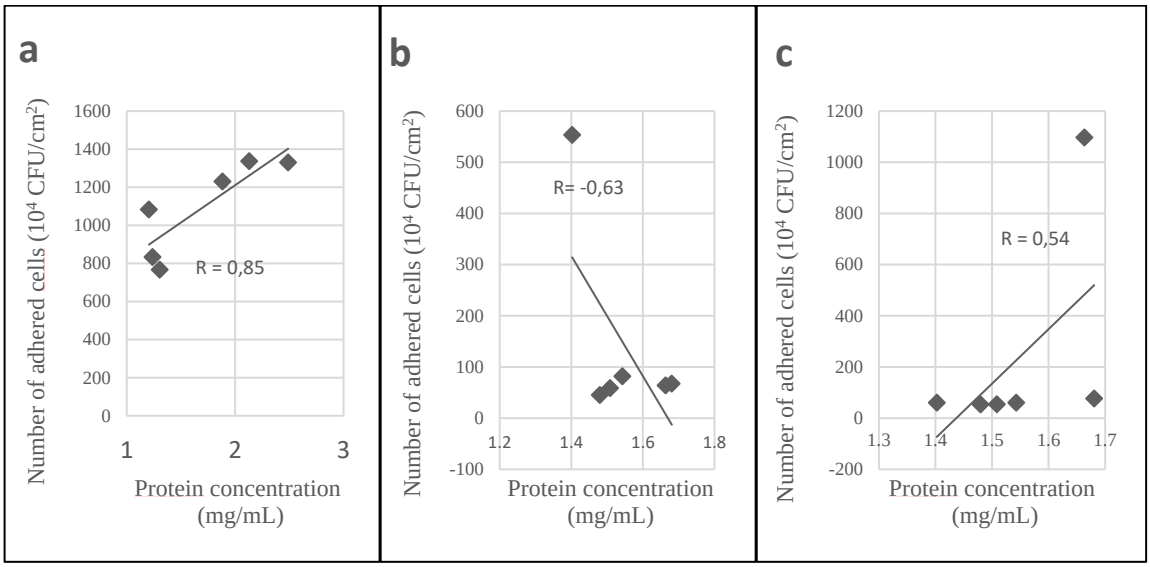

Figure 2. Correlation between proteins production and biofilm formation, on ceramic with 1.5% of glucose (a) and on silicone (b) and ceramic (c) with 0.2% of glucose concentration.

4.Discussion

Our results have proved the powerful action of glucose on *A. baumannii* EPS and protein production and its biofilm-forming ability. Glucose can be both a carbon source and a signal molecule by affecting the expression of several genes and those involved in carbon utilization. In *Pseudomonas aeruginosa,* glucose availability is linked to the production of virulence factors **(24)**. This may also be the case for *A. baumannii* following our results. In a study based on the connection between exogenous glucose and virulence in the opportunistic pathogen *A. baumannii*, 199 genes exhibited significantly differential expression levels when incubating this pathogen in glucose-supplemented Luria Bertani medium **(25)**. 85% had higher transcription levels, while only 15% were down-regulated in this medium. In addition, the few genes down-regulated in the medium do not define any specific metabolism or biological processes while the other up-regulated are involved in cell surface composition, peptidoglycan, lipopolysaccharide, EPS production, biosynthesis of EPS precursors, and secretion systems **(25)**.

In most of the works available on databases, the EPS screening test based on slimy colonies was used for detecting lactic bacteria-produced EPS. Contradictorily to our finding when glucose was the best for *A. baumannii*, a study showed that all strains of lactic bacteria were able to produce EPS only in the presence of sucrose, and no production was detected for other types of sugar **(19)**. Sucrose was also optimal for EPS production by *Leuconostoc lactis* **(26)**. Similarly, Jung *et al.* (2013) have also detected, by quantifying EPS production under various conditions, the highest levels of EPS in the presence of sucrose by *Cronobacter sakazakii* ATCC 12868 **(27)**.

Besides the presence of glucose, the temperature change also influenced EPS production by the tested strains since 37°C was the most suitable. Similar to our work, in the study by Junkins and Doyle, wild-type *Escherichia coli* O157:H7 strain 932 produced the greatest amounts of EPS when grown at 37°C **(28)**. On the other hand, EPS production by *Lactobacillus rhamnosus* strain C83 was 37% higher when it was incubated at 25°C instead of 37°C (100 mg/L compared with 63 mg/L) **(29)**.

Comparing the EPS and proteins produced quantities, we can observe that proteins were more important than EPS (0.46mg/mL for EPS and 2.48-mg/mL for proteins as a maximum) even with changing culture conditions (Figure 1). This result is in line with the one described by another work which showed that proteins are the major component of the extracellular matrix of multidrug-resistant *Pseudomonas aeruginosa* DC-17 isolated from dental caries, with 1.928 mg/mL to 162.3 mg/L only of EPS **(30)**. Despite the crucial role of EPS, proteins have dominated the matrix more than EPS, which probably reflects the main function of these proteins. However, the structural information for many of these proteins is poorly understood. The amyloid-forming proteins (amyloid-like proteins) are one of the most important components of the extracellular matrix involved in the formation and stability of the biofilm **(17)**.

Our results proved that these carbapenem-resistant *A. baumannii* strains could form biofilm on both ceramic and silicone surfaces. The ability of *A.*

baumannii to form a biofilm on ceramic was also investigated by Ivanković *et al.* (2017) who marked the essential role of disinfectant/surface material interactions c in the survival of *A. baumannii* biofilms on this type of support. Moreover, ceramic surfaces may be an important source of infection in hospital environments because biofilm populations on ceramic were more resistant compared with those on glass **(31)**. Silicone has excellent biocompatibility and biodurability properties, making it useful for implantable biomedical devices. M'hamedi *et al.* (2014) have found that *A. baumannii* isolates could form biofilm on silicone **(32)**. Another work compared biofilm formation on polyvinyl chloride, silicone, and rubber latex endotracheal probes, have shown that *A. baumannii* had more susceptibility to rubber latex followed by polyvinyl chloride and silicone last **(33)**.

Contact angle measurements suggested that ceramic is a hydrophilic surface while silicone is a hydrophobic one, which could affect biofilm formation on these surfaces. It has already been proved that the physicochemical parameters of both surface and bacteria can largely affect the adhesion of bacteria and, subsequently, the formation of biofilm **(34)**. An *A. baumannii* strain that is more hydrophobic has been shown to adhere better to silicone catheters and has also produced more biofilm **(35)**. According to Azelmad *et al.* (2018), the adhesion of *P. aeruginosa* differs from one strain to another depending on the properties of the surfaces and of the strain itself **(34)**.

Concerning the glucose effect on biofilm, the most remarkable ability to form biofilm on ceramic was detected at a glucose concentration of 1.5%. However, for silicone surfaces, the addition of glucose did not greatly influence the biofilm formation. Generally, biofilm-producing bacteria are often associated with the increased synthesis of different compounds of ECM. ECM of biofilm received important attention from scientists in these recent years because of their crucial functions. In *A. baumannii*, increased production of ECM creates a protective environment that prevents antibiotic penetration and thus leading to resistance development.

Despite the intervention of EPS and proteins in the biofilm formation and the effect observed by the previous glucose results on the latter, the correlation analysis did not prove much. This lack of correlation may reflect some of the variations with this pathogen, which may pose a challenge in the treatment of biofilm formation. From these results, it can also be deduced that biofilm formation by these *A. baumannii* strains depends on several factors such as the strain itself, its intrinsic factors, the surface nature, and the surrounding environment.

5. Conclusion

In conclusion, the results showed that all clinical isolates of carbapenem-resistant *A. baumannii* tested were slimmer in the presence of glucose at 37°C. Glucose has also improved these strains' exopolysaccharides and proteins production and biofilm formation. *A. baumannii* can form biofilms on both silicone and ceramic surfaces. The results obtained in this paper confirmed that biofilm is a multifactorial character. It involves different factors like bacteria, its determinants, and the capacity of these bacteria to produce EPS and proteins. In addition, the growing conditions (i.e., glucose amount and temperature) and surface properties of both cell surface and substratum also have a significant effect on this phenomenon. Depending on these factors, Carbapenem-resistant *A. baumannii* probably uses different approaches to form a biofilm, increase its hospital colonization, and increase its morbidity and mortality. Therefore, we must understand these approaches more to select an appropriate anti-*A. baumannii* biofilm therapy. Further work is necessary to elucidate the effects of growth conditions on biofilm formation, EPS, and proteins production and discover the different strategies used by this bacterium to persist and infect in the hospital environment. On the other hand, the environment and its components must be considered when studying the formation of biofilm, especially when it concerns pathogens because a simple change can alter the bacterium's behavior.

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Conflict of Interest

We declare that there are no conflicts of interest.

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