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Protocols

Modification of physico-chemical surface properties and growth of *Staphylococcus aureus* under hyperglycemia and ketoacidosis conditions

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ABSTRACT

Diabetes is a widely spread disease affecting the quality of life of millions of people around the world and is associated to a higher risk of developing infections in different parts of the body. The reasons why diabetes enhances infection episodes are not entirely clear; in this study our aim was to explore the changes that one of the most frequently pathogenic bacteria undergoes when exposed to hyperglycemia and ketoacidosis conditions. Physical surface properties such as hydrophobicity and surface electrical charge are related to bacterial growth behavior and the ability of *Staphylococcus aureus* to form biofilms. The addition of glucose made bacteria more negatively charged and with moderate-intermediate hydrophobicity. Ketone bodies increased hydrophobicity to approximately 75% and pathological concentrations hindered some of the bacterial surface charge by decreasing the negative zeta potential of cells. When both components were present, the bacterial physical surface changes were more similar to those observed in ketone bodies, suggesting a preferential adsorption of ketone bodies over glucose because of the more favorable solubility of glucose in water.

Glucose diabetic concentrations gave the highest number of bacteria in the stationary phase of growth and provoked an increase in the biofilm slime index of around 400% in relation to the control state. Also, this situation is related with an increase of bacterial coverage. The combination of a high concentration of glucose and ketone bodies, which corresponds to a poorly controlled diabetic situation, appears associated with an early infection phase; increased hydrophobic attractive force and reduced electrostatic repulsion between cells results in better packing of cells within the biofilm and more efficient retention to the host surface.

Knowledge of bacterial response in high amount of glucose and ketoacidosis environments can serve as a basis for designing strategies to prevent bacterial adhesion, biofilm formation and, consequently, the development of infections.

1. Introduction

How bacteria can begin the colonization of any material -biotic or abiotic- or even how they interact with each other is initially directed by interfacial properties such as hydrophobicity and the electrical charge of the surface. The balance between repulsive electrostatic forces and the hydrophobic attractive force has a major role in dictating the way bacteria interact with their surroundings. On the other hand, the stronger the attraction between bacteria, the more compact the layers they form on a surface, giving the bacteria the advantage of being able to resist external attacks or to make a more effective colonization of other nearby surfaces.

Teichoic and lipoteichoic acids, in addition to a thick layer of cross-linked peptidoglycans and other polysaccharides, form the surface of Gram positive bacteria [1]. These acids are composed of repeating units of ribitol phosphate, which are substituted with D-alanine and N-acetylglucosamine. They have a zwitterionic character because of the negatively charged phosphodiesters and the D-alanine ester modifications that are positively charged. However, the specific composition of the surface of bacteria is dependent on the strain, and different degrees

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of substitutions in the wall of the teichoic acids cause differences between surface properties of different bacterial strains. Nevertheless, in addition to these characteristics of the surface of the microorganisms, the composition of the media where cells are suspended has a relevant role in the interfacial behavior of bacteria. Retention of solutes on the very active surface of the bacteria can modify its hydrophobicity as well as its surface charge, or zeta potential, and in turn its interfacial behavior.

Diabetes mellitus is a chronic metabolic disorder characterized by the increase of blood glucose above the levels of healthy individuals, which are in the range of 0.70–0.99 g/L [2]. It is a wide-spread disease and a global problem affecting the quality of life of many patients. According to WHO, diabetes caused in 2016 1.6 million deaths directly related with this disease and a rise in prevalence is expected to affect 629 million people by 2045 [3].

Glucose provides the energy that the organism needs, and insulin facilitates its delivery to cells [4]. The reasons for high concentration of glucose or hyperglycemia, therefore, may be either due to an insufficient production of insulin, or because the insulin produced not functioning properly [5]. Poorly controlled diabetes is also associated to ketoacidosis. Insulin is responsible for the mobilization of glucose to tissues or organs, but in the case of insufficient insulin level, cells replace with fatty acids the lack of glucose as a source of energy. The results of this optional metabolic route are ketone bodies, which in turn can be metabolized.

Several authors [6–13] have shown that diabetes is linked to a higher risk of developing infections [14] in different parts of the body. There are several reasons behind the promotion or enhancement of infections by diabetes. Hyperglycemia adversely affect neutrophilic chemotaxis and phagocytosis, decreasing defenses of the immune system and making wound healing more difficult [15]. Experimental models have also evidenced deficient angiogenesis, reduced growth factor levels, poor vascularization and maintenance of a chronic inflammatory state limiting healing capacity [2]. However, very little attention has been paid to the possible impact that diabetic alterations, such as hyperglycemia and ketoacidosis, exert on the pathogenic bacteria, directly responsible for the infections.

Staphylococci is one of the most frequently isolated genera in infections in the context of diabetes [16–20], and in particular, the species *Staphylococcus aureus*. Casqueiro et al. review the pathogenesis of diabetic's infections. Within this extensive study, the authors found that *S. aureus* was the most common microorganism responsible for skin and soft tissue infections, especially in diabetic feet [21]. Additionally, due to its relevance in this context, *S. aureus* is the standard species selected for different *in vivo* animal studies to investigate the connection between infections and diabetes [22–25]. The success of microorganisms in colonizing a niche depends on their ability to change and adapt the expression of their virulence factors to their surroundings [26]. Temperature, pH and availability of nutrients from the media impose conditions on the metabolic activity of the cell [26], and in turn the waste products of the metabolism modify the surrounding pH and composition.

Several studies show the importance of microbial hydrophobicity and electrical surface charge to define the virulence and success of a bacterial infection, and also how they are changed because of the characteristics of the surrounding media [27–34]. Within this context, this work will address the changes that *S. aureus* undergoes after being subjected to high concentrations of glucose and ketone bodies (hyperglycemia and ketoacidosis). We focus on how these conditions modify its growth behavior, its virulence factors such as hydrophobicity and surface electrical charge, and its capacity to form biofilms. This information may provide some clues to prevent infections in persons suffering from diabetic disorders.

2. Materials and methods

2.1. Bacterial strain and media

Gram-positive *Staphylococcus aureus* ATCC 29213, obtained from The American Type Culture Collection (ATCC, Manassas, VA, USA), was used in this study. *S. aureus* was stored at –80 °C in porous beds (Microbank Pro-Lab Diagnostics, Austin, TX, USA). From the frozen stock, blood agar plates (OXOID Ltd, Basingstoke, Hampshire, UK) were inoculated and incubated at 37 °C. Bacteria taken from these plates were incubated at 37 °C for 9 h with Trypticase Soy Broth (TSB) (OXOID Ltd, Basingstoke, Hampshire, UK). To inoculate 50 mL of TSB glucose-free (Sigma-Aldrich, St. Louis, MO, USA) with or without supplements, 25 μL of this pre-culture was used as described below. These cultures were incubated for about 14 h at 37 ºC to perform surface characterization experiments. Incubation time coincides with almost the end of the exponential phase of each supplemented growth medium.

To analyze the effect on *S. aureus* of high concentration of glucose and ketone bodies that could occur in diabetic environments, six supplements for culture mediums and/or suspending solutions were prepared based on glucose-free TSB. The choice of these concentrations was based on previous studies performed (data not shown) and on the literature where glucose and ketone body levels are indicated [35–37]. Fasting blood glucose values for diagnosis of diabetes indicate that a concentration < 1 g/L is considered non-diabetic, and if it is > 1.26 g/L it is diagnosed as diabetes, and it is called hyperglycemia. The glucose levels in growth media or suspensions were reached with glucose (Sigma-Aldrich, St. Louis, MO, USA) added at a concentration of 0.9 g/L (normal level, G1) or 1.9 g/L (hyperglycemic level, G2). Regarding ketone body concentrations, the procedures were the same as for glucose. The levels were taken from the literature [38,39] and then bacterial growth tests (not shown) were performed to choose the concentrations of this study. Normal ketone body levels for diagnosis are ≤ 1 mmol/L and for ketoacidosis concentration is found to be ≥ 3 mmol/L, as literature shows. The presence of ketone bodies in media was modeled by mixing acetone (Panreac Chemistry SLU, Barcelona, Spain), methyl acetoacetate (ACE) (Sigma-Aldrich, St. Louis, MO, USA) and hydroxybutyric acid (HA) (Sigma-Aldrich, St. Louis, MO, USA) at a ratio of 1:6 for HA:ACE and a 2% amount of acetone [39,40]. Level of ketone bodies was studied in growth media or solutions by adding this mixture at 1 mmol/L (normal level, K1) or 9 mmol/L (ketoacidosis level, K2). The combined action of glucose and ketone bodies was studied in media or solutions with added glucose at 0.9 g/L and ketone bodies at 1 mmol/L (GK1) or with added glucose at 1.9 g/L and ketone bodies at 9 mmol/L (GK2). The mixture of these concentrations was done because the clinical relevance sought, where low concentrations of the components could appear in non-pathological conditions. The high concentration combination was chosen based on bibliography [35,36], setting a diagnosis blood glucose level (1.9 g/L) and bearing in mind patients of uncontrolled levels of hyperglycemia with high ketoacidosis risk (9 mmol/L). Media or solutions to which neither glucose nor ketone bodies were added were taken as control (C). All the media were sterilized before use.

pH measurements of culture media and bacterial suspension with and without supplements were determined at 21 °C with a pH-meter GLP 21 (Crison Instruments™, MO, Italy). The pH data are reported as mean values ± standard error (SD) of the mean. The statistical analysis was done with the GraphPad InStat 3.0 (GraphPad Software, Inc., San Diego California USA), using multiple comparisons procedure, Dunnett's test.

2.2. Bacterial growth curve

Growth curves of *S. aureus* cultivated with and without the abovementioned supplements were studied. For this purpose, microorganisms were resuspended in tubes with 3 mL of TSB with and without

supplements at 63% transmittance at 492 nm measured in a horizontal-light spectrophotometer (Helios Epsilon, Thermo Fisher Scientific Inc., UK). Tubes were incubated with shaking at 37 °C in a Hotcold (J. P. Selecta, Barcelona, Spain). Bacterial growth was evaluated by the optical density of the culture in the tubes. Measurements were made at regular time intervals up to 10 h of incubation.

2.3. Biofilms assay

For biofilms analysis, microorganisms were resuspended in 9.5 mL glucose-free TSB at 63% transmittance at 492 nm with a 1×10^6 CFU/mL. The bacterial growth procedure was similar as that explained in "*Bacterial strain and media*" section. Then, 100 μL of this bacterial suspension and 100 μL of TSB supplemented with different conditions were added to polystyrene flat-bottomed 96–wellmicrotiter plates (Greiner bio-one, Frickenhausen, Germany). After 24 h of bacterial growth with shaking at 37 °C O.D. of microtiter plate were measured. The measurements were carried out with a microplate spectrophotometer reader (ELx800; Bio-Tek Instruments, Inc. Winooski, VT, USA). This O.D. data was called OD-growth, because it represents the measure of culture growth by estimating the increase in the medium turbidity. Next, wells were washed three times with phosphate buffered saline (PBS) with and without supplements to remove the non-adherent bacteria. The adhered biofilms were heat-dried and stained with violet crystal (Panreac Chemistry Barcelona, Spain). Excess of dye was eliminated by rinsing the plate with water. Once the dye was dried, 200 μL of 33% (v/v) glacial acetic acid (Panreac Chemistry. Barcelona, Spain) were added. OD-biofilm is the measurement made to this violet crystal at 492 nm. This value of OD-biofilm was associated to the amount of biofilm formed in each well. The experiments were carried out in duplicate and repeated at least four times with independent cultures to confirm reproducibility.

Slime index (SI) was determined to reveal differences in biofilm due to the action of supplements. It was obtained from the ratio between the OD-biofilm and OD-growth and the result was expressed as a percentage relative to the control without supplementation, according to equation [2.1]

$$SI = 100 \frac{\left(\frac{OD-biofilm\ \ with\ \ supplement}{OD-growth\ \ with\ \ supplement}\right)}{\left(\frac{OD-biofilm\ \ without\ \ supplement}{OD-growth\ \ without\ \ supplement}\right)} \quad [2.1]$$

Biofilm formation was estimated and was reported as mean values ± standard deviation of the mean (SD). The statistical analysis was done with the GraphPad Instat 3.0 (GraphPad Software, Inc., San Diego California USA), using unpaired samples T-student. Differences were considered statistically significant at p values ≤ 0.05.

2.4. Bacteria surface characterization

The effect of supplements on the adhesive capacity of bacteria was evaluated by the Microbial Adhesion to Hydrocarbons (MATH) test. To prepare bacterial suspensions for MATH assays, bacteria were grown in culture medium with or without supplementation. After approximately 14 h, all cultures were near to the end of their exponential phase. Then the bacterial cultures were centrifuged for 5 min at 1000 *g* (Sorvall TC6, Dulont, Newtown, Pennsylvania, USA) and washed three times with potassium chloride 0.1 mM conditioned at 37 °C. Next, bacteria were resuspended with 3 mL of potassium chloride (KCl) without supplement at an initial optical density (A_0) at 492 nm comprised between 0.6 and 0.9. Afterwards, 150 μL n-hexadecane was added to the tube and was vortexed for 1 min, allowed to settle for 10 min and the optical absorbance (A_f) measured. The relative hydrophobicity index, R, was calculated according to equation [2.2]

$$R = \left(\frac{A_f - A_o}{A_o} * 100\right) \quad [2.2]$$

The statistical analysis was done with the GraphPad InStat 3.0 (GraphPad Software, Inc., San Diego California USA), using Tukey-Kramer Multiple Comparisons Test.

Zeta potential of *S. aureus* growth in media with or without supplements was measured by electrophoresis with a Zetasizer Nano ZS, (Malvern Instruments, United Kingdom). To prepare bacterial suspensions for zeta potential assays, the bacterial growth was the same as in the MATH procedure. After growth, the bacterial suspension was centrifuged for 5 min at 1000 *g* and washed three times with potassium chloride (0.1 mM) conditioned at 37 ºC. Next, bacteria were resuspended in KCl without supplements. 1 mL of this KCl suspension at an O. D. at 492 nm of 63% was used to fill the measurement cell of the Zetasizer.

The experiments were carried out in duplicate and repeated at least three times with independent cultures to confirm reproducibility. The statistical analysis was done with the GraphPad InStat 3.0 (GraphPad Software, Inc., San Diego California USA), using compare selected pairs of columns procedure, Bonferroni's test.

2.5. Profilometry and Scanning Electron Microscopy (SEM)

Profilometry and Scanning Electron Microscopy experiments were carried out on the biofilms. After the biofilm formation on 22 mm diameter polystyrene samples, samples were washed twice with tempered TSB. Afterwards, these samples were fixed at room temperature with 3 wt% glutaraldehyde around 12–15 h, gently washed with PSB for 5 min and passed down with an ethanol gradient from 30 to 100 vol% for 1 h each.

Profilometry study was made with a confocal profilometer Leica DCM8 and images were analyzed with LeicaMap®. The topography of each biofilm condition was study from a 60×60 μm^2 area image taken at the highest magnification of the equipment (150x objective). In addition, to determine the bacterial packing-stacking caused by aggregates, the parameter biovolume ($\mu m^3/\ \mu m^2$) was used. This parameter is used in different researches as a measure of biofilm [41–43], in addition, we will use this parameter to give information on the average thickness (or average height) of single bacteria or aggregates appearing in the biofilm coated surface.

SEM experiments were performed with a scanning electron microscope Quanta 3D FEG (FEI, Hillsboro, US). Before imaging samples were subjected to the same fixation process described in Profilometry, then samples were subsequently vacuum-dried, sputter-coated with Au prior to be inserted in the high-vacuum electron chamber. Images with magnifications ranging from 150x to 50000x were taken randomly in different sections of the samples.

3. Results and discussion

3.1. Bacterial growth

Fig. 1 shows the growth curves of *S. aureus* in the conditions studied. In general, for all the media, the lag phase was almost finished after 90 min from the beginning of the culture, giving way to the appearance of the exponential growth phase which lasted for about 300 min. After this time, a quasi-plateau of the curve indicates the period of the stationary phase of growth.

The stationary phase in supplemented growth media attained higher bacterial concentration values (in optical density) than in the control (Fig. 1), and appeared to be highest for the medium with the highest concentration of glucose, G2. It should be noted that results in the growth curve corresponding to this period were affected by a high experimental uncertainty. This was especially important in the case of media where ketone bodies were present because of the formation of lumps of the planktonic bacteria which could not be dispersed even by vortexing. Tentatively, the order in the concentrations of *S. aureus* in the stationary phase can be given as: G2 > GK2 > G1 > GK1 > K1 > K2 > C.

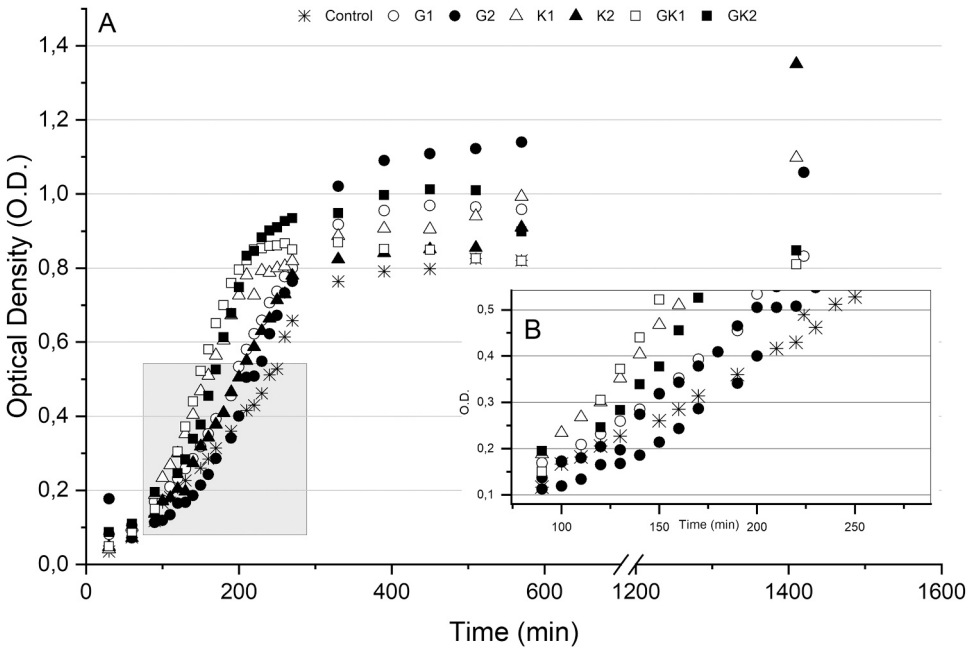

Fig. 1. A) Representative *S. aureus* growth curves in each culture media; B) Zoom of the exponential phase of bacterial growth.

Additionally, in order to consider any change in the OD due to the bacterial size, the diameters of the bacteria grown in the different culture media were measured by profilometry. Table 1 shows the mean diameters with the standard deviations, and it can be observed that the size of all bacteria is the same within experimental uncertainty, consequently, not affecting to the OD measured after growth.

Fig. 1 A shows the growth curves of *S. aureus* for each culture medium, and moreover in Fig. 1B we can see in more detail the exponential growth phase for each one. The exponential phase of bacterial growth has different slopes depending on the nutrients in the media (Fig. 1B), indicating that the rates of proliferation of bacteria in each media were different. Exponential growth phase can be characterized by the following equation [3.1]:

$$\log N = \log N_0 - \mu * (t_f - t_0) \quad [3.1]$$

Where N is the final absorbance of growth, N_0 the initial absorbance of bacterial growth, μ an index of growth rate which is called the specific growth rate constant and has units of the inverse of time (min^{-1}). In our case, it was more informative to obtain the generation time (g), which is an indicator of the observed behavior. The parameter g was obtained with the following equation [3.2]:

$$g = \frac{0.693}{\mu} \quad [3.2]$$

This variable (g) gives the time it takes for the population to double (Fig. 2).

Fig. 2 shows that, within the experimental uncertainty, generation times were between ca. 75 min for control and 47 min for GK1 medium. Addition of glucose to the growth media increased the rate of *S. aureus* growth respect to control, but g for G1 and G2 were not statistically different. Thus, the rate of growth in glucose does not appear to be dependent on the amount of glucose in the medium. This unbiased behavior indicates that even the low amount of glucose in the G1 medium, which corresponds to the concentration present in TSB in a non-pathological state, is sufficient to trigger the fast growth rate of *S. aureus* when glucose is its single nutrient. This behavior agrees with the observations of Xie et al., who found that the rate of growth in the exponential phase and also the level found in the stationary phase of *S. aureus* was mostly independent of the concentrations of glucose in media of between 2 and 8 g/L [44]. However, we found that bacterial growth, as shown by the level in the stationary phase, was favored in the media with a hyperglycemic concentration, G2, over G1 media.

Ketone bodies are known as alternative energy reserves for several microorganisms [45–47] and according to Fig. 2 they appear to provide a faster growth rate in K1 than glucose. Interestingly, media in which ketone bodies and glucose were added together, GK1 and GK2, decreased the generation time compared to media within only ketone bodies, K1 and K2, in the same proportion as did the addition of glucose on the g in the control medium, respectively. The highest rate of *S. aureus* growth corresponded to the GK1 medium, the generation time in this medium being about 28% less than for control media.

Effect of ketone bodies on microorganisms appears to be dependent on the bacteria and the concentration in the suspension. Potezny et al. found that ketone bodies act as bactericide for the gram-negative

Table 1
Zeta potential (mV) and hydrophobicity (%) values for *S. aureus* grown in the different supplemented culture media are in the table. In addition, pH values and the means diameters of the bacteria belonging to the different biofilms are shown. Significant differences ($p < 0.05$) are indicated by: + for initial values with respect to the control; ^ for values after bacterial growth; *within each supplemented medium to compare before and after bacterial growth effect. For significant differences ($p < 0.05$) with respect to the control in zeta potential and hydrophobicity, it marked with **.

	C	*G1*	*G2*	*K1*	*K2*	*GK1*	*GK2*
Relative Hydrophobicity (%)	7 ± 6	49 ± 5**	47 ± 5**	75 ± 4**	77 ± 10**	72 ± 3**	82 ± 3**
Zeta potential (mV)	-39 ± 2	-47 ± 2**	-49 ± 5**	-38 ± 2	-19 ± 5**	-39 ± 4	-22 ± 8**
pH before growth	7.3 ± 0.09	7.2 ± 0.05$^{+,*}$	7.1 ± 0.05$^{+,*}$	7.2 ± 0.02^{+}	6.0 ± 0.04$^{+,*}$	7.0 ± 0.08$^{+,*}$	5.9 ± 0.03$^{+,*}$
pH after growth	7.3 ± 0.02	5.4 ± 0.02$^{\hat{},*}$	4.9 ± 0.03$^{\hat{},*}$	7.2 ± 0.01	6.6 ± 0.03$^{\hat{},*}$	5.5 ± 0.28$^{\hat{},*}$	4.9 ± 0.007$^{\hat{},*}$
Mean Diameters (μm)	0.98 ± 0.06	1.02 ± 0.07	0.98 ± 0.09	1.07 ± 0.14	1.02 ± 0.08	0.95 ± 0.12	0.92 ± 0.14

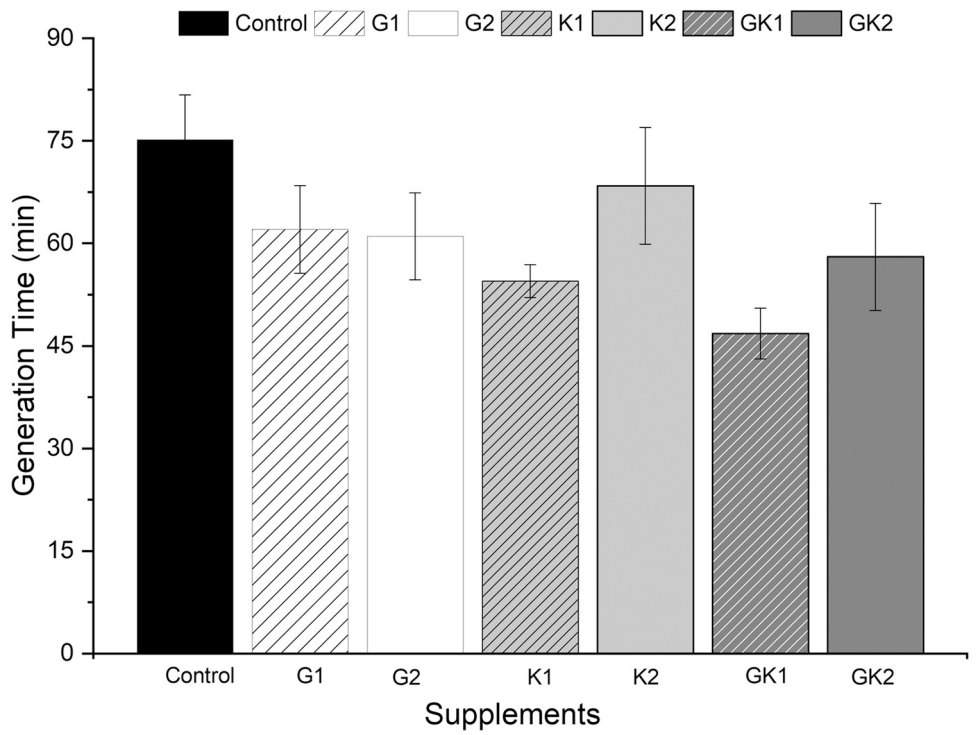

Fig. 2. Generation time of *S. aureus* in each culture media.

bacteria *E. coli* [48]. Nevertheless, growth of *S. aureus* with ketone bodies was enhanced with respect to the control medium, in agreement with the results of Dubos et al. who found that *S. aureus* can grow better in a medium with ketone bodies at pH as low as 5.5 than in a medium without extra nutrients [47]. But in spite of the high rate of growth of *S. aureus* because of the consumption of these metabolites, the total quantity yielded in the stationary phase in K1 and K2 have the lowest values, except for C, which means that ketone bodies are not as effective as glucose in increasing the population of bacteria despite the faster growth rate they provide to the cells.

3.2. Surface properties

Table 1 summarizes the relative hydrophobicity and zeta potential of *S. aureus* grown in media with and without supplements after 14 h of growth (the end of exponential growth phase with 50 mL of culture medium). This table also contains information about the pH of the culture medium, before and after bacterial growth. It should be noted that when the bacterial culture is in the exponential phase, the suspension media still have a large amount of nutrients to maintain bacterial proliferation, but they also contain waste compounds produced by the metabolic activity of the bacteria due to their growth.

Bacteria growth in the control medium does not modify the pH of the medium. Also, the cells grown in this condition appears very hydrophilic and with a negative surface charge. However, these properties are modified if the cells grow in supplemented media. Addition of glucose to the media in concentrations equivalent to healthy or hyperglycemic conditions results in bacteria with a moderate-intermediate hydrophobicity (ca. 50%), and with a more negatively charged surface than control bacteria. The structure of glucose provides the molecule with an electronegative and amphiphilic character, which makes glucose with a moderate hydrophobicity [49]. These properties appear to be transferred to the bacteria because of retention on their surface of glucose from the media. The pH of TSB does not change through the incorporation of glucose to the media, but the pH of the suspension after bacterial growth drops 1.8 and 2.2 points in G1 and G2 media, respectively, compared to the control media. A waste product of metabolism of glucose by bacteria is lactic acid [50], so it was to be expected that the observed change of pH was related to the presence of this acid in the media. Consequently, it cannot be discarded that some of these waste molecules were also present in the interfacial layer on the cell. The influence of these parameters has been studied by Djerebi et al. [51]. In their work they analysed how pH influences the physico-chemical properties of *Acinetobacter baumannii*. Their results show how pH influences the hydrophobicity of the microorganism and consequently bacterial adhesion.

The sole presence in the growth media of the hydrophobic molecules of ketone bodies caused the surface of bacteria to increase its hydrophobicity, up to values of around 75%. When retained on the bacteria, the lower polarity of these molecules made the zeta potential of cells to decrease, probably because the adsorbed molecules hindered some of the charge of the molecules in the cell wall [32,52]. Nevertheless, the modification of the zeta potential respect to control bacteria was observed only in the media with the highest concentration of ketone bodies (K2), the zeta potential change being from -39 ± 2 mV for control bacteria to -19 ± 5 mV for bacteria grown in a medium rich in ketone bodies. It is likely that the concentration of ketone bodies in the range simulating healthy conditions, K1, was not able to sufficiently affect the bacterial surface charge. Acetoacetate and (R)− 3-hydroxybutyric acid, whose pKa were 3.58 and 4.7, respectively are relatively strong acids [53]. When they were added to the media in the concentration of K2, it caused the pH to drop by up to 1.3 points, despite the buffering ability of TSB, unlike in glucose concentrations where the drop in pH occurs due to bacterial growth caused by glucose metabolism. However, the lower concentration of ketone bodies in K1 was not sufficient to decrease the pH of TSB. The pH in bacterial suspensions in the exponential phase in the media K2 increased by 0.6 respect to the initial suspension, probably because of the withdrawal of some ketone bodies due to their metabolic consumption by bacteria. As expected, no change was detected for the pH of the K1 media after bacterial growth, because there was no change in pH in the initial suspension either. Therefore, the modification of pH because of the metabolism of ketone bodies confirms again the higher consumption of these metabolites in K2 than in K1. The waste products of metabolism of ketone bodies appear to have a more

basic pH than the ketone bodies themselves. However, their release by cells growth or removal of the ketone bodies from the medium, due to their consumption, do not seem to be sufficient in K1 to overcome the initial pH, but to slightly increase the pH in K2 medium.

For bacteria in mixed media, hydrophobicity and zeta potential values are similar to those for bacteria growth in ketone bodies as a single nutrient. This suggests that there is a preferential adsorption of ketone bodies over glucose on the bacterial surface because of the more favorable solubility of glucose in water. As in the case of suspensions in the exponential phase with glucose as a single additive, the pH of the suspensions with both glucose and ketone bodies in the exponential phase drops as a result of the lactic acid produced by the metabolism of glucose by bacteria. In the medium with low concentration of glucose and ketone bodies, GK1, there was a drop of pH of 1.5 points, close to the pH drop produced in G1 medium, which was 1.7. For the medium with the highest concentration of glucose and ketone bodies, GK2, the pH after bacterial growth dropped to 4.9, the same value as in medium G2, even though the initial pH in G2 and GK2 differed by 1.2 units. The highest hydrophobicity as well as the lowest absolute zeta potential are associated with the highest amount of ketone bodies and, within the experimental uncertainty, the presence of glucose in mixed media did not cause any significant difference.

3.3. Biofilm formation

Fig. 3 shows the results on the study of biofilm formation. These data provide further information on the influence of glucose and ketone supplements.

The ability of *S. aureus* to form biofilms in each media was analyzed through the Slime Index, which provides information about the relative capacity for biofilm creation independent of bacterial growth (Fig. 3), and it is useful to study the influence of supplements on biofilm creation with respect to the control. Additionally, the use of SEM images (Fig. 4) can provide a qualitative idea of the biofilm configuration on the surface, and the use of profilometry images (Fig. 5) can help in the quantification of the bacterial biofilm coverage and the biofilm biovolume (Table 2).

The addition of any supplement increased the Slime Index of biofilms. This effect was especially noticeable in the case of the pathological glucose concentration, G2. Despite differences in metabolism between planktonic and sessile bacteria, consumption of glucose favored a higher production of poly-N-acetyl-β-(1−6)-glucosamine (PNAG) [46], essential in the virulence of *S. aureus* biofilm, as denoted by the SI (Fig. 3), and a higher biofilm settlement (Fig. 4). The higher percentage on the SI under the G2 condition than under G1 indicates that availability of glucose is also decisive in the production of slime in the biofilm. Profilometry analysis (Table 2) also provided a higher bacterial coverage in G2, 65.3 ± 0.8%, than in G1, 32.3 ± 0.3%.

The impact of ketone bodies on biofilm formation has been analyzed for some Gram-negative bacteria and acetoacetate, and they have been described as an inhibitor of biofilm formation by some Gram-negative strains [48]. Horne et al. studied the action of acetoacetate and ethyl acetoacetate on biofilms of three different pathogenic bacterial strains [54]. They observed that a high concentration of acetoacetate, about 20 and 35 mg/mL depending on the strain, caused an inhibitory effect on biofilm growth, despite the fact that a moderate concentration would cause an increase in biofilm. In our research, biofilms grown with the sole presence of ketone bodies as nutrient had lower bacterial coverage than for the biofilm grown in the control media, as shown by SEM images (Fig. 4) but their slime index was even higher than for biofilm grown under healthy glucose conditions (G1) (Fig. 3). Quantitatively, in K1 the percentage of bacterial coverage is lower than in the control (Table 2) but in the case of K2 the percentage of bacterial coverage is similar to G1 and it is the 3rd highest (33% ± 8), similar to the "position" occupied in the SI results. This behavior suggests that concentration of ketone bodies plays a role in the fixation of biofilm to the substrata, although in the case of planktonic bacterial growth (Fig. 1) ketone bodies had the lowest values of optical densities in the stationary phase (excluding cultures in control media).

Biofilms growth under the highest glucose concentrations, G2 and GK2, had the highest bacterial coverages, especially in the case of the biofilm growth in GK2 (81.1% ± 0.3). Cells grown in GK2 media showed the highest hydrophobicity and the lowest negative zeta potential among the different cultures. The hydrophobic attractive force and a

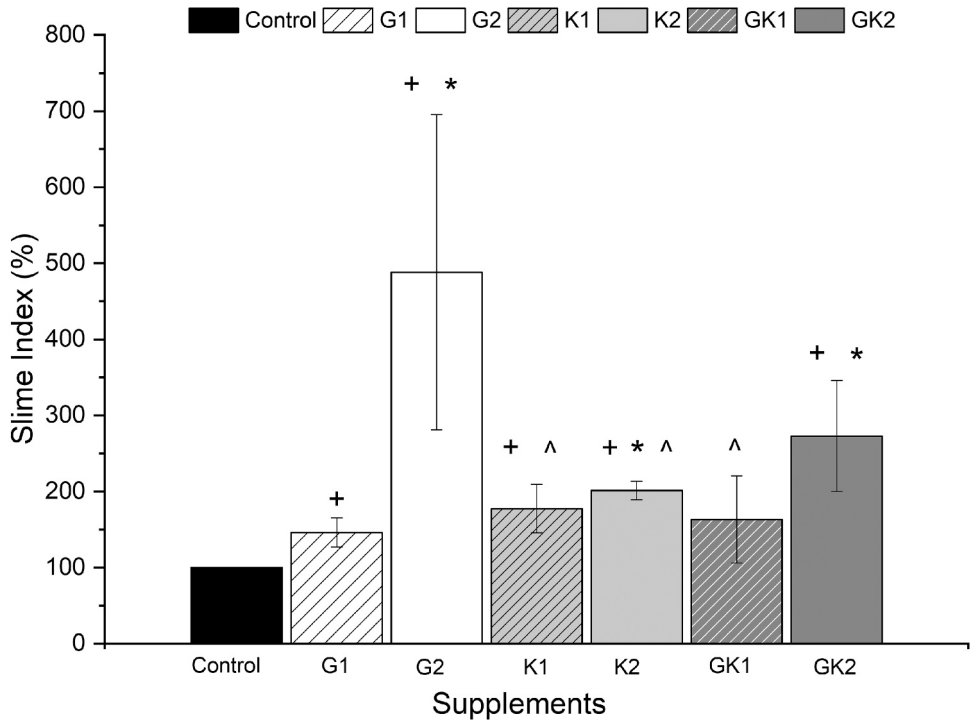

Fig. 3. Percentage of SI obtained for each condition. Significant differences $p < 0.05$ from control (+), from G1 (*), from G2(^).

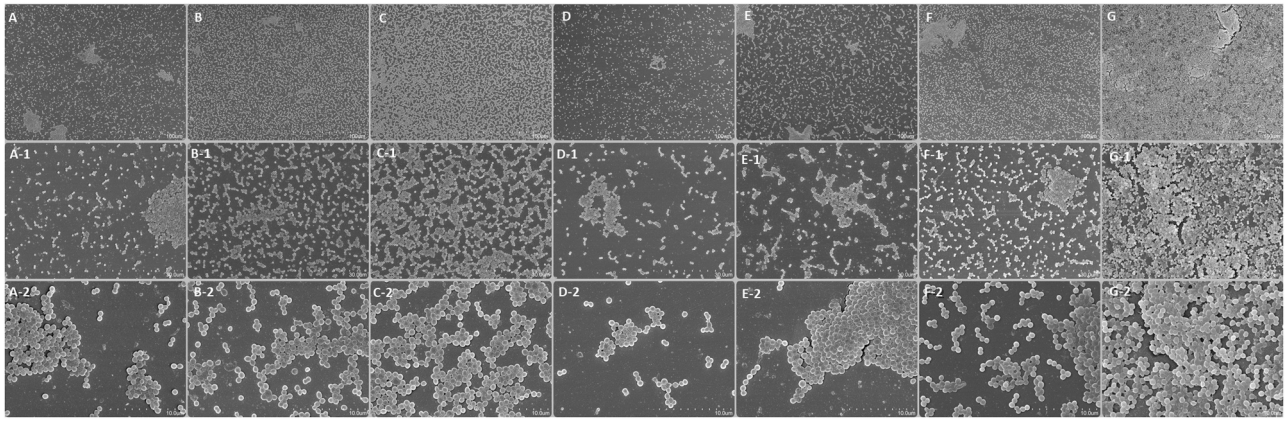

Fig. 4. SEM images taken at 100, 30 and 10 μm magnification for the biofilms formed by *S. aureus* in TSB supplemented with the different concentrations. A, A-1 and A-2, for biofilm formed with control medium; B, B-1 and B-2 for biofilm formed with G1 medium; C, C-1, C-2 for biofilm formed with G2 medium; D, D-1, D-2 for biofilm formed with K1; E, E-1, E-2, are from the biofilm made with K2 medium; F, F-1, F-2, are from GK1 medium; and finally images G, G-1, G-2 are from the biofilm created by the bacteria in GK2 medium.

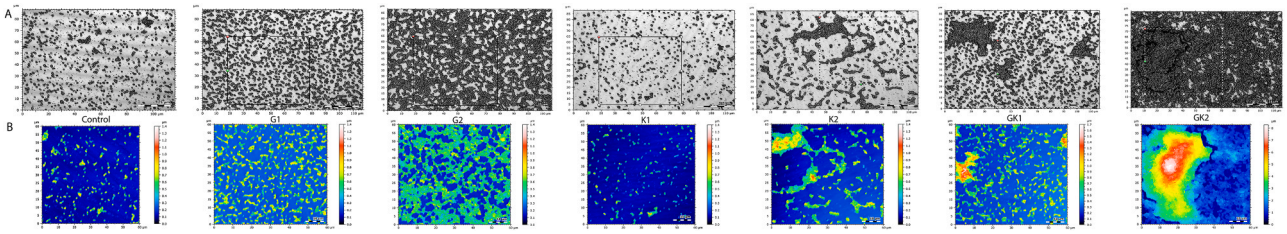

Fig. 5. A) Confocal Profilometer images of each biofilm without and with supplements, taken at maximum magnification (150x objective) B) 60 × 60 area extracted from the, previous image where the topography is analyzed. From left to right we find the biofilm images of the Control, G1, G2, K1, K2, GK1 and GK2 supplements.

Table 2
Bacterial coverage (%) and biovolume ($\mu m^3/\mu m^2$) measured on profilometry images of bacterial biofilms created with different culture media. Significant differences ($p < 0.05$) are indicated by: a with respect to the control; b with respect to the G1, c with respect to the G2; d with respect to the K1; e with respect to the K2; f with respect to the GK1.

	C	*G1*	*G2*	*K1*	*K2*	*GK1*	*GK2*
Bacterial coverage (%)	16.1 ± 0.4	32.4 ± 0.3^{a,b}	65.3 ± 0.8^{a,b}	5.5 ± 0.8^{a,b,c}	33 ± 8^{a,c,d}	23.5 ± 0.8^{a,b,c,d}	81.1 ± 0.3^{a,b,c,d,e,f}
Biovolumen ($\mu m^3/\mu m^2$)	1.1 ± 0.1	1.08 ± 0.01	1.3 ± 0.4	0.94 ± 0.07	0.96 ± 0.02	1.4 ± 0.1	3.8 ± 1.9

reduction of the electrostatic repulsion among cells allow better packing of cells in biofilm and a more efficient retention on the surface. It cannot be ruled out that the wide dispersion in SI results for biofilm growth in G2 and low biofilm retention observed by SEM for these cultures could be due to the very different surface characteristics of G2 respect to GK2. This lower retention results in less bacteria on the surface: bacteria grown in G2 medium had only an intermediate value of hydrophobicity and a very much higher negative surface charge than bacteria grown in GK2. The more efficient electrostatic repulsion and the lower hydrophobic attraction among bacteria from G2 culture compared to those from the GK2, make it easier for the cells to be unexpectedly removed from the biofilm on the surface of the polystyrene wells. A complementary information can be extracted from the profilometry images of Fig. 5, which support the idea that surface properties of bacteria under GK2 are optimal for virulence, promoting bacterial coverage and packing. Topographical images show that only in the case of GK2 the Z-scale changes from 0 to 1.4/1.7 nm to 0–8 nm, which means that bacteria in the zones of higher accumulations of GK2 have a multi-layer configuration, bearing in mind the mean bacterial diameter. In this sense, also the biofilm biovolume (Table 2) matches with the visual topographical information: it increases from a biovolume of about 1.1 $\mu m^3/\mu m^2$ to 3.8 $\mu m^3/\mu m^2$, which is about a 245% of increment.

The treatments with a mixture of both components have a combination of the characteristics observed in the treatments with the single supplements: The additive activity of glucose and ketone bodies forms stronger biofilm. In the case of GK2, hyperglycemia provides better surface coverage and ketoacidosis increases the packing of the bacterial cells in a multi-layered configuration.

From these results the importance of the role played by the medium in which bacteria grow can be confirmed, since it alters the growing capabilities and surface properties of bacteria. Glucose is an effective nutrient for *S. aureus* as it favors its growth as well as biofilm formation. Ketone bodies can also be used by the cells as nutrients and appear to provide energy to cells at a higher rate than glucose. However, despite the fact that it is not so favorable for the metabolism of bacteria it makes the surface of bacteria very hydrophobic and with a lower electrical charge, thus providing more virulence to the bacteria.

4. Conclusions

In the pathological situation in which hyperglycemia and ketoacidosis are acting together, a combined action from the availability of both nutrients on bacteria appears. On the one hand, the activity of bacteria, as reflected in its growth and biofilm formation capacity, appears to be determined by the availability of glucose in the media. The level of bacteria production reached in the stationary phase was similar for

cultures in GK2 and G2, but higher than in K2. pH after growth in medium GK2 was almost the same level as for G2 medium, even taking into account the basification that the metabolism of ketone bodies produced, as shown by the pH change which occurred in the medium K2. However, surface characteristics such as hydrophobicity and zeta potential appear to be determined by the metabolism of the ketone bodies. Under the pathological environment GK2 *S. aureus* takes advantage of a high capacity for growth of the glucose metabolite, but the intervention of the ketone bodies causes significant modifications in the surface properties of the bacteria, giving way to the most compact and thickest biofilm, which is likely to have the worst infection evolution.

CRediT authorship contribution statement

María Fernández-Grajera: Conceptualization, Validation, Formal analysis, Investigation, Writing - Original Draft and Review & Editing and Funding acquisition. Miguel A. Pacha-Olivenza: Methodology, Writing - Original Draft, Project administration and Funding acquisition. Amparo M. Gallardo-Moreno: Writing - Original Draft and Review & Editing, Project administration, Data Curation and Funding acquisition. M. Luisa González-Martín: Writing - Original Draft and Review & Editing, Supervision and Funding acquisition. Ciro P é rez-Giraldo: Resources, Writing - Review & Editing and Funding acquisition. M. Coronada Fernández-Calderón: Resources, Writing - Original Draft and Review & Editing, and Supervision.

Declaration of Competing Interest

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

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