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Microbial alcohol-conferred haemolysis (MACH) occurring in Staphylococcus aureus biofilms

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ABSTRACT

Recently, the phenomenon called microbial alcohol-conferred haemolysis (MACH) was described for *Staphylococcus aureus*. It is characterized by increments in haemolysis when colonies are exposed to ethanol vapors. However, the confirmation of MACH by biofilms of *S. aureus* grown in presence of ethanol has been no reported. Methods: To respond such question, MACH phenomenon was evaluated in aerobic and anaerobic biofilms of *S. aureus* ATCC®25923™ grown in the presence of 0.5 mg mL-1 and 1 mg mL-1 ethanol and followed up for 24 and 48 h. Biomasses and haemolytic activities were measured. Results: Increments in the haemolytic activity for aerobic biofilms occurred in a time-dependent manner. Anaerobic biofilms did not show any increment in such parameters when ethanol was present at any concentration. Conclusions: These observations may be important for clinicians that treat chronic drinkers.

Keywords: *Staphylococcus aureus*, biofilm, ethanol, haemolysis, MACH.

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INTRODUCTION

Among the attributes of microbial virulence, the growth as biofilm certainly is the most important[1,2]. This phenotype provides numerous advantages as increased resistance to antimicrobials[3], increased expression of extracellular enzyme activity4 and greater ability to escape from the immune system[5,6].

Although constitutively expressed, haemolysins can also be over-expressed in the presence of ethanol (EtOH) or other alcohols, in a phenomenon called microbial alcohol-conferred haemolysis (MACH)[7]. Experimental evidence shows that during the metabolism of EtOH by yeasts an oxidative burst occurs[8] and supports that MACH phenomenon is worthy of further consideration because it raises the potential for microbial virulence[9], including *Staphylococcus aureus*[10].

This deserves special attention when taking in account chronic alcoholic individuals, in which the blood alcohol levels are found high for long periods. However, despite the evident importance, no data are available concerning to the influence of EtOH on *S. aureus* biofilm formation or its influence on the secretion of hemolysins by these biofilms.

MATERIAL AND METHODS

Ethics statement

This study only was carried out after the appreciation and approbation by the local Research Ethics Committee (protocol 6126/11). The blood donor has provided a written informed consent.

Biofilm formation under the influence of EtOH

We employed *S. aureus* ATCC®25923™ because of its known haemolytic11 and biofilm-forming behavior[12], as well as its ability to grow in absence of molecular oxygen[13].

Static biofilms were grown in polystyrene wells in normoxic (80% N2, 20% O2) and anoxic (90% N2, 10% CO2) atmospheres. Briefly, the strain was planktonically (200 rpm, 37 °C) grown in BHI under normoxic or anoxic conditions until an OD660nm∼1.00; then, the cells were washed and resuspended in sterile 145 mM NaCl until an OD660nm∼1.00. The cell adhesion was achieved transferring 1 mL aliquots of suspensions to 24-well polystyrene plates and incubating them at 37°C in normoxic or anoxic atmosphere, under 60 rpm. After 2h, supernatants were removed and the walls were washed twice with sterile 145mM NaCl. One-milliliter aliquots of Mueller-Hinton broth containing increasing concentrations of EtOH (0.0 mg mL-1, 0.5mg mL-1, and 1.0 mg mL-1) were finally introduced in the wells. Biofilms were grown in normoxic or anoxic atmospheres at 37°C for 24 h or 48 h.

After incubations, the supernatants were aspirated, clarified by centrifugation (10,000×g, 10 min) and transferred to sterile screw cap vials.

Estimation of biofilm biomasses

The cellular content remaining in the wells were gently washed with 145 mM NaCl to remove non-adherent cells and fixed with 100% methanol for 10 min. Methanol was aspirated and the bio-

films were dried drying with hot air. The adherent cells were stained with 50 μL of 0.4% (w/v) crystal violet (in 12% EtOH) for 5 min. The dye was removed with successive washes with 145 mM NaCl. Biofilm biomasses were estimated by colorimetry at 595 nm, after elution with 1% (w/v) SDS.

Haemolysis assay

Samples of peripheral blood were obtained from three male volunteers, healthy and abstemious by aseptic venipuncture (10 mL, each). Sodium citrate (final concentration 0.38%) was used to prevent blood clotting and the purification of erythrocytes was performed using Hanks' balanced salt solution as wash buffer (HBSS: 4.2 mM $NaHCO_3$, 5 mM KCl, 0.4 mM KH_2PO_4, 138 mM NaCl, 0.34 mM Na2HPO_4, 5 mM glucose, pH 7.0). Erythrocyte preparations were carried out within the first hour after collection and experiments were performed up to 1 h post-purification[14]. Erythrocyte suspensions (haematocrit of 40%) were combined with supernatants from cultures of biofilms (1:1) and gently agitated at 25°C for 16h. The hemolytic activities of biofilm supernatants were determined by colorimetry at 545 nm. Haemolytic indexes (HI) were determined as ratio between released hemoglobin in the treatments and those of released hemoglobin from erythrocytes (haematocrit of 40%) and Mueller-Hinton broth-2M HCl (in a 1:1 ratio).

Specific haemolytic indexes (SHI) were determined by dividing the haemolytic indexes by the estimated respective biomasses (1U = HI biomass^{-1}).

Statistics

All tests described above were conducted in triplicate in at least three different situations. Numerical data were tabulated in MSExcel® spreadsheets (Microsoft Co.). All the data were tested for normality of distribution by the Levene's test and subjected to simultaneous multiple comparisons by the Tukey's test. A limit of 0.05 was considered to establish statistical differences between groups.

RESULTS AND DISCUSSION

The MACH phenomenon in *S. aureus* has been described exclusively in blood-agar plates[15]. To our knowledge, this study is the first contribution that prospected the possibility and occurrence in *S. aureus* biofilms. Although growths on semi-solid culture media are considered as biofilms[16], we believe that sensu stricto biofilms are much better considered when formed on inert abiotic surfaces (e.g. plastic surfaces). In this context, we proposed to investigate the occurrence of MACH phenomenon in sensu stricto biofilms.

Some evidence shows that microbial biofilms tend to increase the expression of soluble virulence factors, when compared to planktonic cultures[4,17]. In the case of *S. aureus*, despite its known medical importance, very little has been explored.

The results showed that aerobic biofilms respond in a time-dependent manner, secreting proportionally (HI biomass^{-1}) more extracellular haemolytic factors when grown in the presence of 0.5 mg mL^{-1} EtOH than the control ($p<0.05$) (Figure 1).

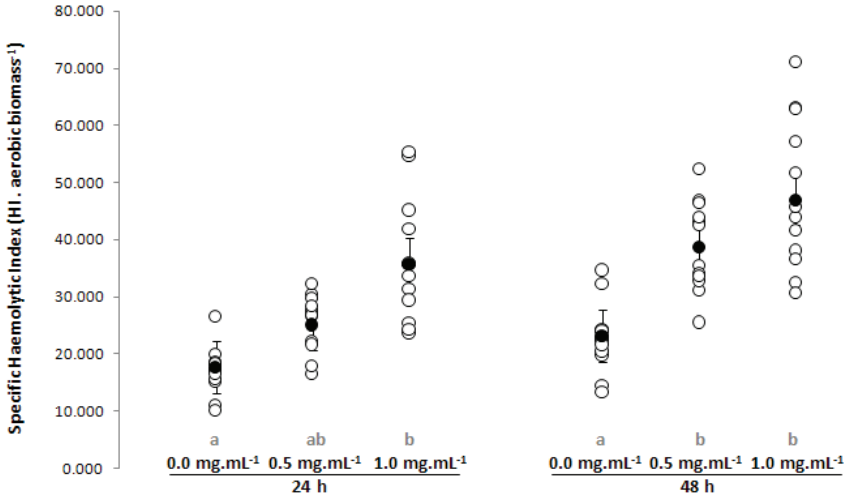

Figure 1. Specific Haemolytic Indexes for aerobic biofilms of *S. aureus* ATCC®25923™ grown in presence of EtOH. Error bars indicate 95% confidence intervals surrounding the averages

These same biofilms, when formed in 1mg mL^{-1} EtOH increased their haemolytic activities within the first 24h ($p<0.05$), which is stabilized until the 48th hour of incubation ($p>0.05$). In aerobic biofilms, the proportional increases in SHI result from EtOH-induced higher rates of haemolysis and concomitant reduction in biomasses (Figure 2).

At low concentrations (≥0.1% vol/vol), close to those here studied, ethanol can delay the obtaining of viable cells in the post-stationary phase as well as compromising the transition of primary (sugars) to secondary catabolism (amino acids), with a reduction in the uptake of glutamate, ornithine, and proline[18]. These evidences explain, at least partly, why aerobic *S. aureus* bio-

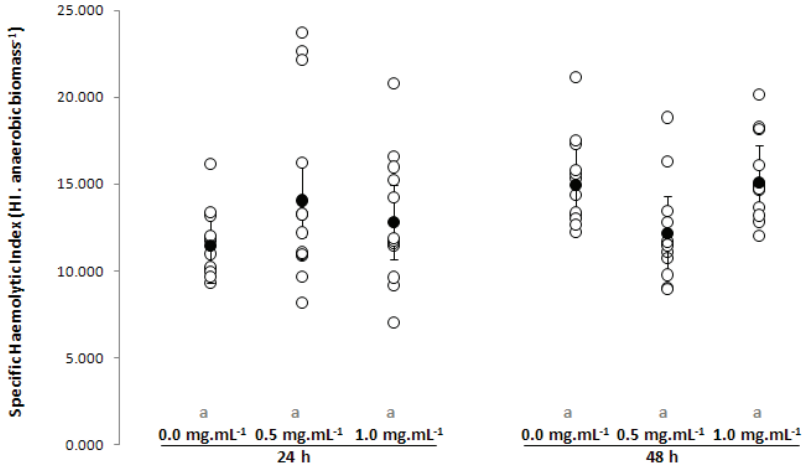

Figure 2. Specific Haemolytic Indexes for anaerobic biofilms of *S. aureus* ATCC®25923™ grown in presence of EtOH. Error bars indicate 95% confidence intervals surrounding the averages.

films become less cellularized in function of exposure time and ethanol concentration.

The increase in haemolytic indexes of aerobic biofilms under the influence of ethanol may result from the up-regulation of genes such as hld (which encodes delta-haemolysin), *hlb* (encoding beta-haemolysin), *hlgB* (encoding gamma-haemolysin), *sspA* (encoding V8 serine protease), *sspB* (encoding the precursor of cysteine protease), *plc* (encoding 1-phosphatidylinositol phosphodiesterase) and exotoxins[10]. The combination of these events, presented in the last two paragraphs, seems to be the most suitable to explain the rise in the values of SHI (Figure 1).

Unlike aerobic biofilms, biofilms grown in the absence of molecular oxygen have not experienced variations in SHI ($p>0.05$) (Figure 2). Such invariability results from the fact that treatments that led to higher biomasses also implicated in higher haemolytic indexes (Figure 3). The contrary also occurred and reductions in biomasses resulted in reductions in haemolytic activities.

The assumption that ethanol may act by increasing the biomass of staphylococcal biofilms[19-22] only showed concordant for anaerobic biofilms grown in 0.5 mg•mL^{-1} EtOH ($p<0.05$), in this study. In addition, unlike expectations[23-25], control anaerobic biofilms were not more cellularized than their aerobic equivalents ($p>0.05$). These inconsistencies are certainly due to the physiological polymorphism of strains employed in the different studies.

Summarizing, our results indicate the possibility of increasing the virulence of aerobic biofilms of S. aureus with increased haemolytic activity, even with the involvement of fewer bacterial cells. These findings may have some relevance, especially in chronic alcoholic individuals.

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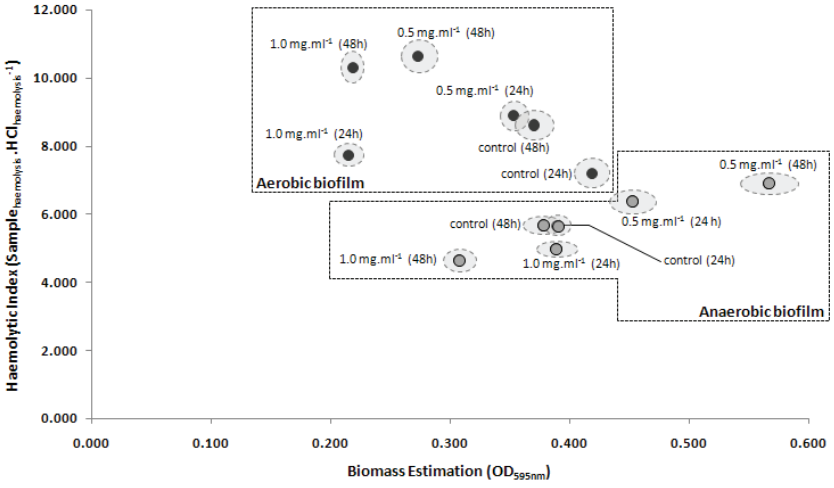

Figure 3. Cartesian dispersion of Haemolytic Indexes by estimative of biofilm biomasses of *S. aureus* ATCC®25923™ grown in presence of EtOH. Ellipses and circles surrounding coincident points represent 95% confidence intervals area.

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