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Short Communication

Immunoreactive pattern of *Staphylococcus epidermidis* biofilm against human whole saliva

Saliva is essential to interact with microorganisms in the oral cavity. Therefore, the interest in saliva antimicrobial properties is on the rise. Here, we used an immunoproteomic approach, based on protein separation of *Staphylococcus epidermidis* biofilms by 2DE, followed by Western-blotting, to compare human serum and saliva reactivity profile. A total of 17 proteins were identified by MALDI-TOF/TOF. Serum and saliva presented a distinct pattern of immunoreactive proteins. Our results suggest that saliva seems to have higher propensity to react against *S. epidermidis* proteins with oxidoreductase activity and proteins involved with L-serine metabolic processes. We show that saliva was a powerful tool for the identification of potential *S. epidermidis* biofilms proteins.

Keywords:
Human serum / Immunoproteomics / Saliva / *S. epidermidis* biofilm
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Despite enormous efforts have been made in the search of new diagnostic techniques and new therapeutic strategies, infections caused by bacteria still remain high. Biofilms are often defined as "a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface [1]." The extracellular matrix, where biofilm cells are embedded, contributes to bacteria survival in a hostile environment [2]. Thus, biofilms have a higher capacity than planktonic cells to tolerate immune response [3]. Moreover, the physiological heterogeneity and evasion to the host immune system also contribute to a hardly effective elimination of the microorganism [4]. Biofilm-associated infections may represent 80% of the chronic bacterial infections diagnosed [5]. *Staphylococcus epidermidis* is a commensal colonizer of skin and mucosae [6]. Nevertheless, *S. epidermidis* biofilms are among the major responsible for chronic infections since frequently adhere to indwelling medical device [7]. Nowadays, in order to find a more effective treatment to biofilms, there is an increased interest in identification and development of antimicrobial peptides. For example, a promising peptide was recently developed and presented antimicrobial efficacy over biofilms, including *Staphylococcus aureus* biofilms, since it targets (p)ppGpp, a nucleotide which is a signal of a stress condition, such as nutritional stress [8].

Due to its involvement in protection to microbial colonization [9], the interest in the saliva antimicrobial properties has been increasing [10]. Saliva is a plasma ultrafiltrate fluid, which includes specific proteins produced by salivary glands [11]. It is estimated that approximately 20% of total salivary proteins are also seen in plasma [12]. Immune markers of systemic infections, such as antigens, antibodies, and nucleic acids of infecting pathogens, are suspected to enter saliva from the blood through absorption and subsequent secretion by the salivary glands [13]. Additionally, salivary proteins are crucial to interact with oral cavity microorganisms [14]. The purpose of this work was to assess the saliva potential against *S. epidermidis* biofilm proteins, comparing the immunoproteomic profile of human whole saliva and human serum.

Whole proteome was obtained from *S. epidermidis* biofilms grown in a glucose-enriched medium, as described in [15]. To obtain whole proteome, biofilms were directly scrapped and resuspended with detergent extraction buffer,

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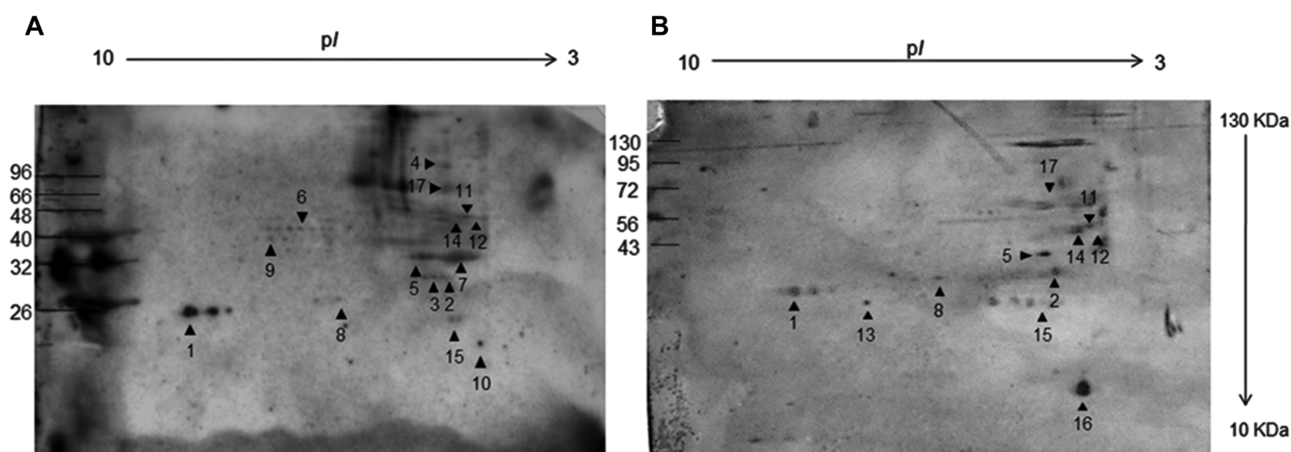

Figure 1. Immunoblotting profile of whole proteins of *S. epidermidis* biofilms using whole human saliva (A) and human serum (B) as probes. Protein spot identification is mentioned in Table 1.

Table 1. Immunoreactive proteins identified by 2DE-MALDI-TOF/TOF

Spot	Protein	Acession number	Protein name	MW (kDa)	p*I*	Function	PSORTb localization	Cell localization
1	SsaA	Q5HLV2	Staphylococcal secretory antigen SsaA	27.91	8.4	Not known; immunogenic protein expressed during sepsis and particularly during episodes of infective endocarditis	Extracellular	Extracellular
2	Fda	Q5HL21	Fructose-bisphosphate aldolase class 1	32.99	4.89	Glycolytic enzyme that catalyzes D-fructose 1,6-bisphosphate into glycerone phosphate and D-glyceraldehyde 3-phosphate	Unknown	Cytoplasmic
3	CysK	Q5HRP1	Cysteine synthase	33.15	5.18	Catalyzes the reaction that led to acetate formation	Cytoplasmic	Cytoplasmic
4	FusA	Q5HRK5	Elongation factor G	76.88	4.8	This protein promotes the GTP-dependent translocation of the nascent protein chain from the A-site to the P-site of the ribosome	Cytoplasmic	Cytoplasmic
5	PfkA	Q5HNK6	6-Phosphofructokinase	34.88	5.34	Catalyzes the reaction of D-fructose 6-phosphate into D-fructose 1,6-bisphosphate	Cytoplasmic	Cytoplasmic
6	GlyA	Q5HMB0	Serine hydroxymethyl-transferase	45.24	5.73	Catalyzes the reversible interconversion of serine and glycine with tetrahydrofolate serving as the one-carbon carrier	Cytoplasmic	Cytoplasmic
7	Gap	Q5HQV4	Glyceraldehyde-3-phosphate dehydrogenase 1	36.19	4.83	Catalyzes the conversion of glyceraldehyde 3-phosphate to D-glycerate 1,3-bisphosphate	Cytoplasmic	Cytoplasmic

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Table 1. Continued

Spot	Protein	Acession number	Protein name	MW (KDa)	p*I*	Function	PSORTb localization	Cello localization
8	CodY	Q5HPT7	GTP-sensing transcriptional pleiotropic repressor CodY	28.75	5.61	It is a GTP-binding protein that senses the intracellular GTP concentration as an indicator of nutritional limitations. At low GTP concentration it no longer binds GTP and stop to act as a transcriptional repressor	Cytoplasmic	Cytoplasmic
9	SERP0527	Q5HQM1	NADH dehydrogenase-like protein SERP0527	44.18	5.80	It catalyzes the transfer of a pair of electrons from NADH	Cytoplasmic membrane	Cytoplasmic
10	AhpC	Q5HRY1	Alkyl hydroperoxide reductase subunit C	21.0	4.58	Directly reduces organic hydroperoxides in its reduced dithiol form	Cytoplasmic	Cytoplasmic
11	Pgk	Q5HQV3	Phosphoglycerate kinase	42.74	4.76	Catalyzes the transference of a phosphate group from 3-phospho-D-glycerate to ADP	Cytoplasmic	Cytoplasmic
12	RpsA	Q5HP69	30S ribosomal protein S1	43.37	4.46	Binds mRNA, thus facilitating recognition of the initiation point. It is needed to translate mRNA with a short Shine-Dalgarno (SD) purine-rich sequence	Cytoplasmic	Cytoplasmic
13	GpmA	Q5HLI0	2,3-Bisphosphoglycerate-dependent phosphoglycerate mutase	26.7	6.46	Catalyzes the interconversion of 2-phosphoglycerate and 3-phosphoglycerate	Cytoplasmic	Cytoplasmic
14	Tuf	Q5HRK4	Elongation factor Tu	43.16	4.7	This protein promotes the GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes during protein biosynthesis	Cytoplasmic	Cytoplasmic
15	TpiA	Q5HQV2	Triosephosphate isomerase	27.37	4.9	Catalyzes the interconversion of D-glyceraldehyde 3-phosphate and glycerone phosphate	Cytoplasmic	Cytoplasmic
16	Asp23	Q5HM47	Alkaline shock protein 23	19	4.92	May play a key role in alkaline pH tolerance	Unknown	Cytoplasmic
17	GpmI	Q5HQV1	2,3-Bisphosphoglycerate-independent phosphoglycerate mutase	56.36	4.8	Catalyzes the interconversion of 2-phosphoglycerate and 3-phosphoglycerate	Cytoplasmic	Cytoplasmic

25 mM Tris-HCl (Pharmacia Biotech, Uppsala, Sweden) (pH 7.2), 10 mM CHAPS (Sigma-Aldrich, St. Louis, MO), 0.5 M NaCl (VWR, Radnor, PA), 5% glycerol (Sigma-Aldrich), and 1 mM PMSF (Sigma-Aldrich). Cells were disrupted by mechanical lysis using a FastPrep® (Thermo Fisher Scientific, Waltham, MA) cell disruptor (three cycles of 30 sec and 6.5 m/s). After lysis, cell debris was removed by centrifugation (15 000 × *g* for 15 min at 4°C) and proteins were precipitated with 20% of TCA-cold acetone (Sigma-Aldrich) and quantified using the RC-DC assay (Bio-Rad, Hercules, CA). Proteins were separated by 2D electrophoresis, as described in [16]. Then, proteins were transferred to a nitrocellulose membrane in transfer buffer. Immunoblotting was performed with human saliva or human serum. Secondary antibody against Human IgG was used (A0170, Sigma-Aldrich). Immunoreactive spots were detected by enhanced ECL (Amersham Pharmacia Biotech, UK). Finally, immunoreactive proteins were excised from 2DE stained

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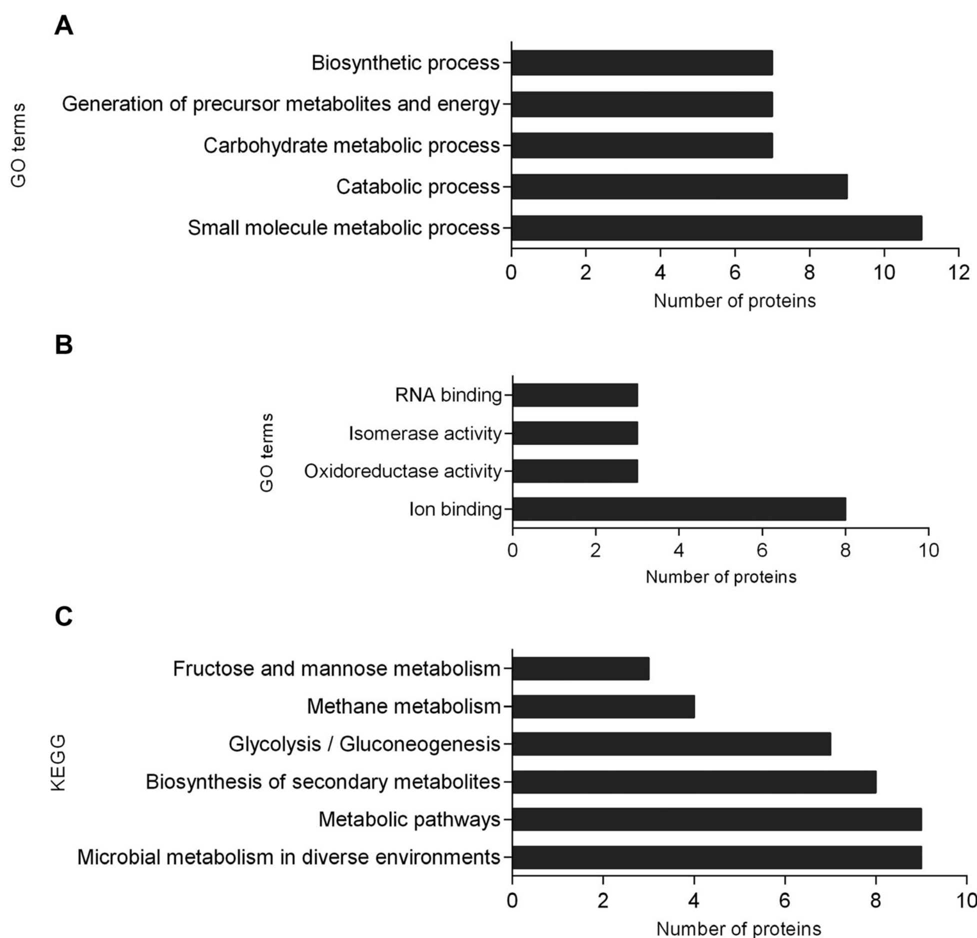

Figure 2. Data analysis using STRING tool. The most representative GO terms of biological processes (A), molecular functions (B), and KEGG pathways (C) of immunoreactive proteins.

with colloidal Coomassie and in-gel protein digestion was performed as described in [15,17]. Proteins were identified by MALDI-TOF/TOF, as described in [17]. Gene Ontology [18] and KEGG pathways [19] were determined by using STRING tool [20]. Biological samples were collected from healthy volunteers after written informed consent (approved by the Ethics Committee of Instituto Ciências Biomédicas Abel Salazar (document number 081/2014)). Complete Material and methods section may be found in Supporting Information.

It is known that human saliva and serum have different contact with *Staphylococcus* spp. Among the high microbiome diversity, oral mucosa is frequently colonized by *S. aureus,* which is found in 4–64% of healthy subject's plaque [21]. Also *S. epidermidis* was found as a colonizer of subgingival plaque in periodontally healthy people [22]. Later, Negrini et al. showed that *S. epidermidis* biofilms were able to stimulate inflammatory response of salivary epithelial cells [23]. Not surprisingly, the immunoreactive profile obtained by serum and saliva was distinct (Fig. 1). Nevertheless, several proteins were reactive with both biological fluids. Sera have been used in immunoproteomics to identify *S. epidermidis* immunogenic proteins [24]. However, reactive pattern may diverse among sera samples, since it is strongly dependent on immune response of donors or previous exposure to bacteria [25–29]. Nevertheless, here, we identified a total of 17 *S. epidermidis* proteins (Table 1), wherein six proteins were found reactive only to saliva and nine proteins were found reactive to saliva and serum. Gene Ontology analysis (Fig. 2 B) showed that these proteins were mainly involved in small molecule metabolic process (GO:0044281) and catabolic processes (GO:0009056). Their main molecular functions were catalytic activity (GO:0003824) and ion binding (GO:0043167). Similarly, the main representative KEGG pathways were microbial metabolism in diverse environments (ser01120), and metabolic pathways, such as biosynthesis of secondary metabolites (ser01110) and glycolysis/gluconeogenesis (ser00010). More than a half of the identified proteins were both reactive to saliva and serum. Amongst them, there is a well-known immunogenic protein, SsaA [30]. CysK, FusA, GlyA, Gap, SERP0527, and AhpC proteins seem to be more reactive to human saliva than human serum (Fig. 1). Half of them present oxidoreductase activity (Gap, AhpC, and SERP0527 proteins). Additionally, L-serine metabolic process (including GlyA and CysK proteins) was a biological process found only in saliva experiment. It is known that the amino acid L-serine plays a role in cellular proliferation [31]. This result may suggest that saliva contains factors with activity against growing bacteria. Indeed, saliva encompasses a large panel of antimicrobial peptides to

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balance the bacteria growth in oral cavity, which may be determinant to establish homeostasis [10]. Despite the saliva proteome is constituted of more than 2400 salivary proteins, there is interindividual variability in the composition of saliva proteins [32]. Interestingly, around 21% of proteins found in human saliva are associated with immunity [33]. Heo and colleagues exposed *S. aureus* biofilm to human saliva in order to identify salivary protein binding [34]. Their main aim was to decipher the mechanism by which the microorganism can colonize the oral cavity. They found that a limited number of salivary proteins, mainly involved in specific or innate immune defense, interact and influence *S. aureus* metabolism, contributing to host–pathogen interplay [34]. Conversely, Staphylococcal protein A (SpA) was the main responsible for binding salivary immunoglobulins. Furthermore, Trindade et al. showed the potential antimicrobial activity of different saliva peptides isolated from mammals on *S. aureus* exponential planktonic cells [35].

To the best of our knowledge, the present study represents, in general, the first attempt to use saliva as a probe for immunoblotting against bacterial proteins. Our results suggest that human saliva seems to be more reactive to proteins from *S. epidermidis* biofilm with oxidoreductase activity. Despite high interindividual variability, it would be of major importance to identify which salivary peptides are binding to those proteins and assess their influence over biofilms.

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The authors have declared no conflict of interest.

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