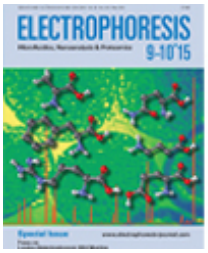

Immunoreactive pattern of *Staphylococcus epidermidis* biofilm against human whole saliva. Electrophoresis (2015) 36:1228–33

Virginia Carvalhais[a,b], Francisco Amado[a], Frederico Cerveira[c], Rita Ferreira[a], Manuel Vilanova[d,e], Nuno Cerca[b], Rui Vitorino[a*]

[a] QOPNA, Mass Spectrometry Center, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal

[b] CEB - Centre of Biological Engineering, LIBRO - Laboratory of Research in Biofilms Rosário Oliveira, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal

[c] Anatomia Patológica, Centro Hospitalar Baixo-Vouga, Avenida Artur Ravara, 3814-501 Aveiro, Portugal

[d] IBMC - Instituto de Biologia Molecular e Celular, Rua do Campo Alegre 83, Porto, Portugal

[e] ICBAS – Instituto de Ciências Biomédicas Abel Salazar, University of Porto, Rua de Rua de Jorge Viterbo Ferreira 228, 4050-313 Porto, Portugal

*Corresponding author: email: rvitorino@ua.pt

Abstract

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 *S. 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.

Introduction

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” (Costerton et al. 1999). The extracellular matrix, where biofilm cells are embedded, contributes to bacteria survival in a hostile environment (Vuong et al. 2004).Thus, biofilms have a higher capacity than planktonic cells to tolerate immune response (Cerca et al. 2005). Moreover, the physiological heterogeneity and evasion to the host immune system also contribute to a hardly effective elimination of the microorganism (Stewart et al. 2008). Biofilm-associated infections may represent 80% of the chronic bacterial infections diagnosed (Davies 2003). *Staphylococcus epidermidis* is a commensal colonizer of skin and mucosae (Otto 2012). Nevertheless, *S. epidermidis* biofilms are among the major responsible for chronic infections since frequently adhere to indwelling medical device (Otto 2009). 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 (Fuente-Nunez et al. 2014).
Due to its involvement in protection to microbial colonization (Devine et al. 2008), the interest in the saliva antimicrobial properties has been increasing (van 't et al. 2014). Saliva is a plasma ultra-filtrate fluid which includes specific proteins produced by salivary glands (Baum 1993). It is estimated that approximately 20% of total salivary proteins are also seen in plasma (Zhang et al. 2013). 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 (Schafer et al. 2014). Additionally, salivary proteins are crucial to interact with oral cavity microorganisms (Heo et al. 2013b). 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.

Material and Methods

Whole proteome was obtained from *S. epidermidis* biofilms grown in a glucose enriched medium, as described in (Carvalhais et al. 2015b). To obtain whole proteome, biofilms were directly scrapped and resuspended with detergent extraction buffer, 25mM Tris-HCl (pH=7.2), 10mM CHAPS, 0.5M NaCl, 5% glycerol and 1mM PMSF. Cells were disrupted by mechanical lysis using a FastPrep® cell disruptor (3 cycles of 30 sec and 6.5 m/s). After lysis, cell debris were removed by centrifugation (15,000*g* for 15 minutes at 4ºC) and proteins were precipitated with 20% of TCA-cold acetone and quantified using the RC-DC assay. Proteins were separated by 2-dimensional electrophoresis, as described in (Carvalhais et al. 2015c). 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 chemiluminescence ECL. Finally, immunoreactive proteins were excised from 2DE stained with colloidal Coomassie and *in-gel* protein digestion was performed as described in (Carvalhais et al. 2015a; Carvalhais et al. 2015b). Proteins were identified by MALDI-TOF/TOF, as described in (Carvalhais et al. 2015a). Gene Ontology (Ashburner et al. 2000) and KEGG pathways (Kanehisa et al. 2004) were determined by using STRING tool (Franceschini et al. 2013). 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)).

Results and Discussion

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 to 64% of healthy subject's plaque (Didilescu et al. 2005). Also *S. epidermidis* was found as a colonizer of subgingival plaque in periodontally healthy people (Kroes et al. 1999). Latter, Negrini et al. showed that *S. epidermidis* biofilms were able to stimulate inflammatory response of salivary epithelial cells (Negrini et al. 2014). Not surprisingly, the immunoreactive profile obtained by serum and saliva was distinct (Figure 1).

Nevertheless, several proteins were reactive with both biological fluids. Sera have been used in immunoproteomics to identify *S. epidermidis* immunogenic proteins (Sellman et al. 2005). However, reactive pattern may diverse among sera samples, since it is strongly dependent on immune response of donors or previous exposure to bacteria (Newcombe et al. 2014; Pourmand et al. 2006; Sadovskaya et al. 2007; van Kessel et al. 2014; Vytvytska et al. 2002) . Nevertheless, here, we identified a total of 17 *S. epidermidis* proteins (Table 1), wherein 6 proteins were found reactive only to saliva and 9 proteins were found reactive to saliva and serum.

Gene Ontology analysis (Figure 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).

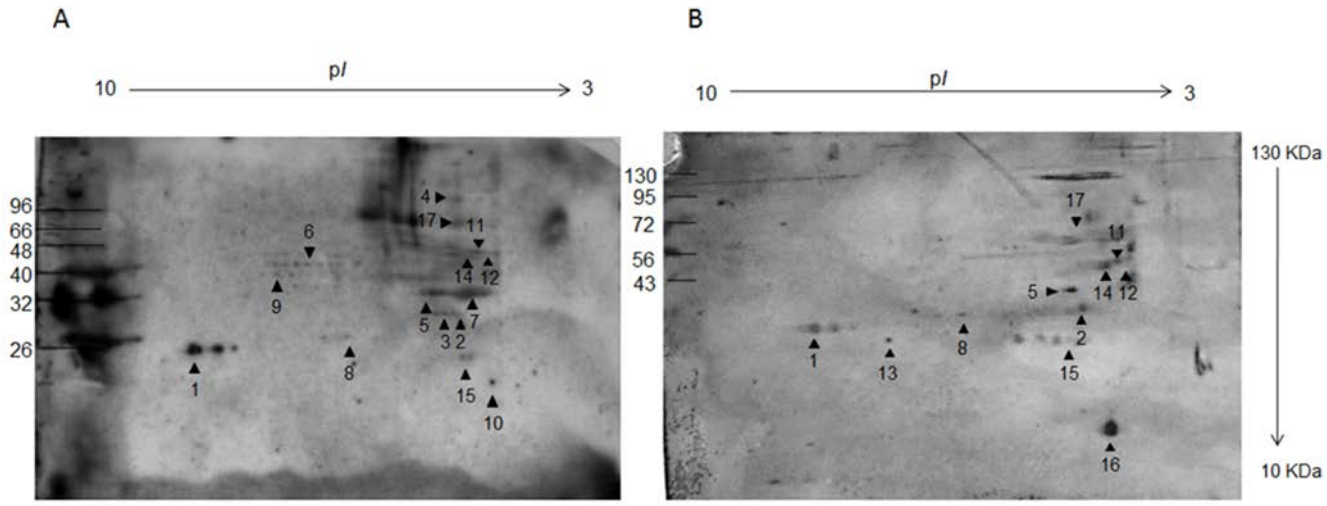

Figure 1 | Immunoblotting profile of whole proteins of *S. epidermidis* biofilms using whole human saliva (A) and human serum (B) as probes. Protein spot identification is mentioned in Table 1.

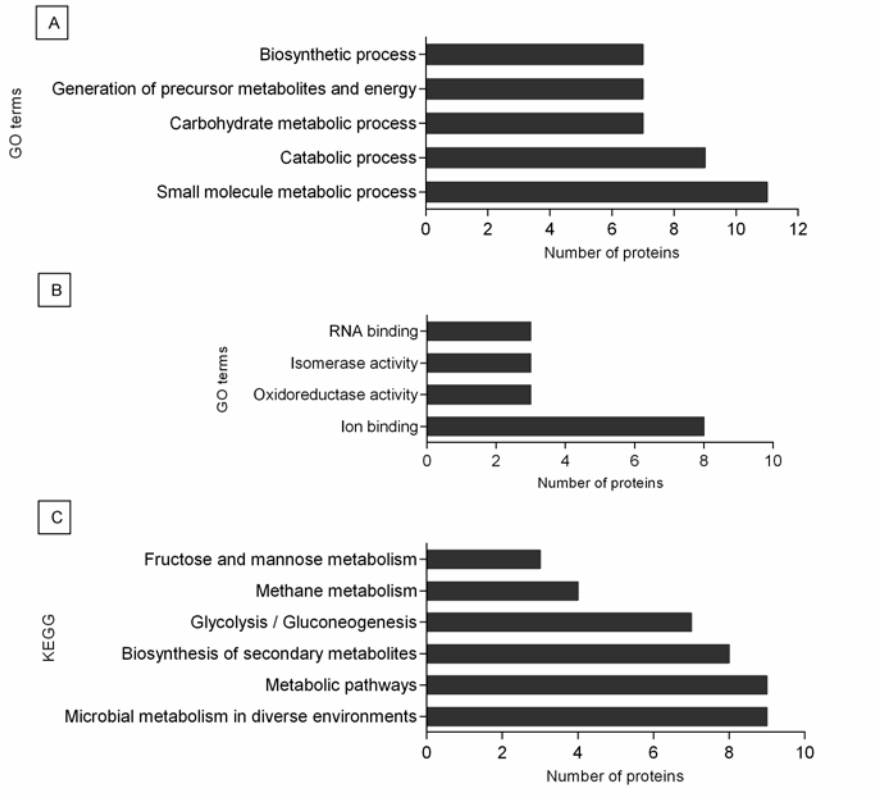

Figure 2 | Data analysis using STRING tool. The most representative GO terms of biological processes (A), molecular functions (B) and KEGG pathways (C) of immunoreactive proteins.

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 (Lang et al. 2000). CysK, FusA, GlyA, Gap, SERP0527 and AhpC proteins seems to be more reactive to human saliva than human serum (Figure 1). Half of them presents 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 aminoacid L-serine plays a role in cellular proliferation (de Koning et al. 2003).

This result may suggest that saliva contains factors with activity against growing bacteria. Indeed, saliva encompass a large panel of antimicrobial peptides to balance the bacteria growth in oral cavity, which may be determinant to establish homeostasis (van 't et al. 2014). Despite the saliva proteome is constituted of more than 2400 salivary proteins, there is inter-individual variability in the composition of saliva proteins (Amado et al. 2013). Interestingly, around 21% of proteins found in human saliva are associated with immunity (Castagnola et al. 2011). Heo and colleagues exposed *S. aureus* biofilm to human saliva in order to identify salivary protein binding (Heo et al. 2013a). 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 (Heo et al. 2013a). 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 (Trindade et al. 2014).

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 inter-individual variability, it would be of major importance to identify which salivary peptides are binding to those proteins and assess their influence over biofilms.

Acknowledgments

VC had an individual FCT fellowship (SFRH/BD/78235/2011). NC is an Investigator FCT. This work was funded by Fundação para a Ciência e a Tecnologia (FCT) and COMPETE grants PTDC/BIA-MIC/113450/2009, FCOMP-01-0124-FEDER-014309, QOPNA research unit (project PEst-C/QUI/UI0062/2013), RNEM (National Mass Spetrometry Network) and CENTRO-07-ST24-FEDER-002034. The authors also thank the FCT Strategic Project PEst-OE/EQB/LA0023/2013 and the Project “BioHealth - Biotechnology and Bioengineering approaches to improve health quality", Ref. NORTE-07-0124-FEDER-000027, co-funded by the Programa Operacional Regional do Norte (ON.2 – O Novo Norte), QREN, FEDER. The authors also acknowledge the project “Consolidating Research Expertise and Resources on Cellular and Molecular Biotechnology at CEB/IBB”, Ref. FCOMP-01-0124-FEDER-027462.

Conflict of interests

The author(s) declare that they have no conflict of interests.

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

Spot	Protein	Acession Number	Protein name	MW (KDa)	p*I*	Function	PSORTb localization	Cello 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 catalyses 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 which 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 hydroxymethyltransferase	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
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	Catalyses 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

References

Amado FM, Ferreira RP, Vitorino R (2013) One decade of salivary proteomics: current approaches and outstanding challenges. Clin Biochem 46:506-517

Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, Harris MA, Hill DP, Issel-Tarver L, Kasarskis A, Lewis S, Matese JC, Richardson JE, Ringwald M, Rubin GM, Sherlock G (2000) Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat Genet 25:25-29

Baum BJ (1993) Principles of saliva secretion. Ann N Y Acad Sci 694:17-23

Bustin SA, Benes V, Nolan T, Pfaffl MW (2005) Quantitative real-time RT-PCR--a perspective. J Mol Endocrinol 34:597-601

Carvalhais V, Cerca N, Vilanova M, Vitorino R (2015a) Proteomic profile of dormancy within *Staphylococcus epidermidis* biofilms using iTRAQ and label-free strategies. Appl Microbiol Biotechnol 99:2751-2762

Carvalhais V, Franca A, Pier GB, Vilanova M, Cerca N, Vitorino R (2015b) Comparative proteomic and transcriptomic profile of *Staphylococcus epidermidis* biofilms grown in glucose-enriched medium. Talanta 132:705-712

Carvalhais V, Cerveira F, Vilanova M, Cerca N, Vitorino R (2015c) An immunoproteomic approach for characterization of dormancy within *Staphylococcus epidermidis* biofilms. Mol Immunol

Castagnola M, Picciotti PM, Messana I, Fanali C, Fiorita A, Cabras T, Calo L, Pisano E, Passali GC, Iavarone F, Paludetti G, Scarano E (2011) Potential applications of human saliva as diagnostic fluid. Acta Otorhinolaryngol Ital 31:347-357

Cerca N, Martins S, Cerca F, Jefferson KK, Pier GB, Oliveira R, Azeredo J (2005) Comparative assessment of antibiotic susceptibility of coagulase-negative staphylococci in biofilm versus planktonic culture as assessed by bacterial enumeration or rapid XTT colorimetry. J Antimicrob Chemother 56:331-336

Costerton JW, Stewart PS, Greenberg EP (1999) Bacterial biofilms: a common cause of persistent infections. Science 284:1318-1322

Davies D (2003) Understanding biofilm resistance to antibacterial agents. Nat Rev Drug Discov 2:114-122

de Koning TJ, Snell K, Duran M, Berger R, Poll-The BT, Surtees R (2003) L-serine in disease and development. Biochem J 371:653-661

Devine DA, Cosseau C (2008) Host defense peptides in the oral cavity. Adv Appl Microbiol 63:281-322

Didilescu AC, Skaug N, Marica C, Didilescu C (2005) Respiratory pathogens in dental plaque of hospitalized patients with chronic lung diseases. Clin Oral Investig 9:141-147

Franca A, Melo L, Cerca N (2011) Comparison of RNA extraction methods from biofilm samples of *Staphylococcus epidermidis*. BMC Research Notes 4:572-

Franceschini A, Szklarczyk D, Frankild S, Kuhn M, Simonovic M, Roth A, Lin J, Minguez P, Bork P, von Mering C, Jensen LJ (2013) STRING v9.1: protein-protein interaction networks, with increased coverage and integration. Nucleic Acids Res 41:D808-D815

Fuente-Nunez C, Reffuveille F, Haney EF, Straus SK, Hancock RE (2014) Broad-spectrum anti-biofilm peptide that targets a cellular stress response. PLoS Pathog 10:e1004152-

Heo SM, Choi KS, Kazim LA, Reddy MS, Haase EM, Scannapieco FA, Ruhl S (2013a) Host defense proteins derived from human saliva bind to *Staphylococcus aureus*. Infect Immun 81:1364-1373

Heo SM, Ruhl S, Scannapieco FA (2013b) Implications of salivary protein binding to commensal and pathogenic bacteria. J Oral Biosci 55:169-174

Junttila S, Lim KJ, Rudd S (2009) Optimization and comparison of different methods for RNA isolation for cDNA library construction from the reindeer lichen *Cladonia rangiferina*. BMC Res Notes 2:204-

Kanehisa M, Goto S, Kawashima S, Okuno Y, Hattori M (2004) The KEGG resource for deciphering the genome. Nucleic Acids Res 32:D277-D280

Kroes I, Lepp PW, Relman DA (1999) Bacterial diversity within the human subgingival crevice. Proc Natl Acad Sci U S A 96:14547-14552

Lang S, Livesley MA, Lambert PA, Littler WA, Elliott TS (2000) Identification of a novel antigen from *Staphylococcus epidermidis*. FEMS Immunol Med Microbiol 29:213-220

Lloyd KG, Macgregor BJ, Teske A (2010) Quantitative PCR methods for RNA and DNA in marine sediments: maximizing yield while overcoming inhibition. FEMS Microbiol Ecol 72:143-151

Negrini TC, Arthur RA, Waeiss RA, Carlosa IZ, Srinivasan M (2014) Salivary epithelial cells as model to study immune response against cutaneous pathogens. Clin Transl Sci 7:48-51

Newcombe J, Mendum TA, Ren CP, McFadden J (2014) Identification of the immunoproteome of the meningococcus by cell surface immunoprecipitation and MS. Microbiology 160:429-438

Nour AM, Barbour EK, Depint F, Dooms M, Niang K, Dulac A, Niamba CN, Chaaya G, Pouillart PR (2010) Comparison of five RNA extraction methods from rabbit's blood. Agriculture and Biology Journal of North America 1:448-450

Otto M (2009) *Staphylococcus epidermidis*--the 'accidental' pathogen. Nat Rev Microbiol 7:555-567
Otto M (2012) Molecular basis of *Staphylococcus epidermidis* infections. Semin Immunopathol 34:201-214
Pinto FL, Thapper A, Sontheim W, Lindblad P (2009) Analysis of current and alternative phenol based RNA extraction methodologies for cyanobacteria. BMC Mol Biol 10:79-
Pourmand MR, Clarke SR, Schuman RF, Mond JJ, Foster SJ (2006) Identification of antigenic components of *Staphylococcus epidermidis* expressed during human infection. Infect Immun 74:4644-4654
Rump LV, Asamoah B, Gonzalez-Escalona N (2010) Comparison of commercial RNA extraction kits for preparation of DNA-free total RNA from *Salmonella* cells. BMC Res Notes 3:211-
Sadovskaya I, Faure S, Watier D, Leterme D, Chokr A, Girard J, Migaud H, Jabbouri S (2007) Potential use of poly-N-acetyl-beta-(1,6)-glucosamine as an antigen for diagnosis of staphylococcal orthopedic-prosthesis-related infections. Clin Vaccine Immunol 14:1609-1615
Santiago-Vázquez LZ (2006) Comparison of two total RNA extraction protocols using the marine gorgonian coral *Pseudopterogorgia elisabethae* and its symbiont
Symbiodinium sp. Electronic Journal of Biotechnology 9:598-603
Schafer CA, Schafer JJ, Yakob M, Lima P, Camargo P, Wong DT (2014) Saliva diagnostics: utilizing oral fluids to determine health status. Monogr Oral Sci 24:88-98
Sellman BR, Howell AP, Kelly-Boyd C, Baker SM (2005) Identification of immunogenic and serum binding proteins of *Staphylococcus epidermidis*. Infect Immun 73:6591-6600
Sieber MW, Recknagel P, Glaser F, Witte OW, Bauer M, Claus RA, Frahm C (2010) Substantial performance discrepancies among commercially available kits for reverse transcription quantitative polymerase chain reaction: a systematic comparative investigator-driven approach. Anal Biochem 401:303-311
Stewart PS, Franklin MJ (2008) Physiological heterogeneity in biofilms. Nat Rev Microbiol 6:199-210
Tavares L, Alves PM, Ferreira RB, Santos CN (2011) Comparison of different methods for DNA-free RNA isolation from SK-N-MC neuroblastoma. BMC Res Notes 1:140-145
Trindade F, Amado F, Pinto da CJ, Ferreira R, Maia C, Henriques I, Colaco B, Vitorino R (2014) Salivary peptidomic as a tool to disclose new potential antimicrobial peptides. J Proteomics 115:49-57
van 't HW, Veerman EC, Nieuw Amerongen AV, Ligtenberg AJ (2014) Antimicrobial defense systems in saliva. Monogr Oral Sci 24:40-51
van Kessel KP, Bestebroer J, van Strijp JA (2014) Neutrophil-Mediated Phagocytosis of *Staphylococcus aureus*. Front Immunol 5:467-
Vuong C, Voyich JM, Fischer ER, Braughton KR, Whitney AR, DeLeo FR, Otto M (2004) Polysaccharide intercellular adhesin (PIA) protects *Staphylococcus epidermidis* against major components of the human innate immune system. Cell Microbiol 6:269-275
Vytvytska O, Nagy E, Bluggel M, Meyer HE, Kurzbauer R, Huber LA, Klade CS (2002) Identification of vaccine candidate antigens of *Staphylococcus aureus* by serological proteome analysis. Proteomics 2:580-590
Zhang A, Sun H, Wang P, Wang X (2013) Salivary proteomics in biomedical research. Clin Chim Acta 415:261-265