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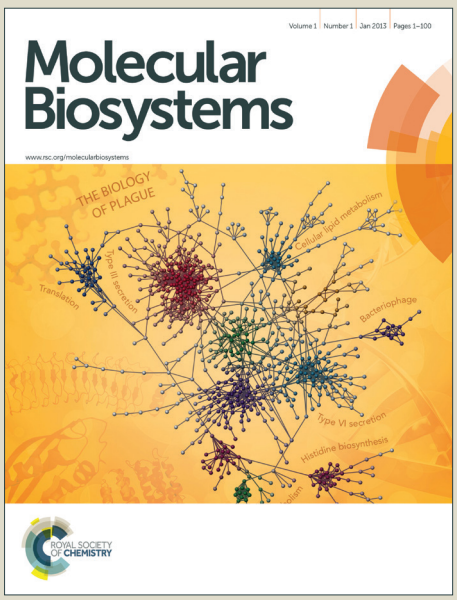

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Title: Role of Salivary and Candidal Proteins in Denture Stomatitis; an exploratory proteomic analysis
Running Title: Denture Stomatitis Proteomics

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Abstract

Denture stomatitis, inflammation and redness beneath a denture, affects nearly half of all denture wearers. Candida organism, the presence of a denture, saliva, and host immunity are the key etiological factors for the condition. The role of salivary proteins in denture stomatitis is not clear. In this study 30 edentulous subjects wearing a maxillary complete denture were recruited. Unstimulated whole saliva from each subject was collected and pooled into two groups (n=15 each); healthy and stomatitis (Newton classification II and III). Label-free multidimensional liquid chromatography/tandem mass spectrometry (2D-LC-MS/MS) proteomics on two mass spectrometry platforms were used to determine peptide mass differences between control and stomatitis groups. Cluster analysis and principal component analysis were used to determine differential expression among the groups. The two proteomic platforms identified 97 and 176 proteins (ANOVA; $p<0.01$) differentially expressed among the healthy, type 2 and 3 stomatitis groups. Three proteins including carbonic anhydrase 6, cystatin C, and cystatin SN were found to be the same as previous study. Salivary proteomic profiles of patients with denture stomatitis were found to be uniquely different from controls. Analysis of protein components suggests that certain salivary proteins may predispose some patients to denture stomatitis while others are believed to be involved in the reaction to fungal infection. Analysis of candidal proteins suggest that multiple species of candidal organisms play a role in denture stomatitis.

Keywords: dentures, mass spectrometry, proteomics, saliva, stomatitis

Introduction

Denture stomatitis (DS) refers to an inflammatory condition of the mucosal tissue underneath the denture. This condition occurs in about 1/2 to 1/3 of patients wearing a maxillary denture [1]. DS is classified clinically into three types, type 1 (DS I), type 2 (DS II) and type 3 (DS III), referring to clinical localized mild, localized moderate, and generalized tissue inflammation [2]. The main etiological factors are dentures, candidal organisms, and host responses [3, 4]. In healthy, non-immunocompromised, non-xerostomic, edentulous denture wearing population, we have previously shown that the severity and presence of DS is related to the quantity of *Candida albicans* present in dentures as well as the quantity of the organism in saliva. We also found that there is no correlation between the presence of the organism in the biopsy tissue and the presence or severity of DS [5]. This suggests that the denture acts as a "hiding place" for the organism, while saliva acts as a media to transfer the organism in contact with the mucosal tissue. Surprisingly, the DS tissue most often has no sign of *C. albicans*. While the immune system may have eliminated the organism in the tissue, it creates perhaps an inflammatory reaction toward the organism leaking out from the denture and into the saliva. It has been a common belief that *C. albicans* is the main player in DS development. We, and others, found that there are non-albicans organisms present in DS [6-8]. However, it is not entirely clear if these non-albicans candidal species play any role in the saliva of DS patients [8].

This has lead to our idea that 1) host factors may play a role in the development of DS both in the tissue level and in the saliva level, and 2) there may be some interaction among different candidal species and the host. Proteins found in saliva have been

shown to play a role as biomarkers and antifungal proteins in the presence of oral candidiasis [5, 9-11]. In our previous studies, we focused on examining host proteins and compared the proteomic profiling of edentulous patients with and without DS using surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF/ MS) and further identified DS-associated salivary proteins using Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF/ MS) and Liquid Chromatography - Mass Spectrometry/Liquid Chromatography - Tandem Mass Spectrometry (LC-MS/MS) based on SELDI- TOF/MS profiling [6]. SELDI-TOF/MS identified 61 peptide masses differentially expressed between non-DS, DS II and DS III. Only 13 peptide masses are downregulated in DS compared to non-DS controls [6]. In this study, we used label-free quantitative mass spectrometry-based proteomic analyses to further examine the salivary proteomic profiles of DSII and III to controls. Two independent proteomic platforms, Thermo LTQ Orbitrap and Waters Synapt mass spectrometry systems, were used to determine peptide mass differences between the controls and stomatitis samples. The objective of this study is to further examine human salivary proteins that may have been missed from the previous study as well as to characterize proteins associated with DS from candidal organisms.

Materials and Methods

Study population and sample collections:

The study protocol was approved by the Office of Human Research Ethics, the University of North Carolina Institutional Review Board (IRB), No. 07-2014. Thirty edentulous patients wearing a maxillary denture were recruited, 15 healthy controls, and 15 denture stomatitis (DS); 8 DS II and 7 DS III. A written informed consent for saliva

sample collection, storage, and analysis was obtained from all participants. The clinical diagnosis of DS was complemented by histological assessments (biopsy and swabs of the lesion), and by culture (tissue, denture, and saliva). Subjects with chronic disease with oral manifestations other than denture/ mucosal stomatitis or that have overt denture abrasion associated with symptoms, were excluded. The subject demographics are shown in Supplementary Table 3. Approximately 4 ml of unstimulated whole saliva sample was collected in a 15 ml Falcon tube. The tube was then centrifuged to remove food and tissue debris. The supernatant was placed into aliquots of 250 ml and immediately frozen in liquid nitrogen to avoid enzymatic and bacterial degradation of the protein content.

Sample Preparation:

Pooled aliquots for the control, DS II and DS III materials were analyzed using a Bradford assay to determine the concentration of protein present using a Thermo Scientific Micro BCA Protein Assay kit. The samples were diluted to fall within the linear working range of the kit (5-200µg/mL) and the concentrations calculated based on absorbance values compared to a BSA standard curve. A volume of each sample corresponding to 35µg of protein (based on the protein quantitation results) was used. The sample volumes were normalized by adding 50mM ammonium bicarbonate (AmBic) to a volume of 29.8µL. A 1% solution of Rapigest (Waters Corporation, Milford, MA) was added to each sample to denature the proteins. The solution was placed in a shaking heated mixer at 40°C for 10 minutes. A total of 200mM dithiothreitol (DTT) was added to each sample and the tubes were heated to 80°C for 15 minutes to reduce the disulfide bonds. The free sulfur atoms were then alkylated by adding 400 mM

Iodoacetamide (IA) and placing the tubes in the dark for 30 minutes at room temperature. A tryptic digest was performed by adding 0.7µg of Trypsin Gold-Mass Spectrometry grade (Promega, Madison, WI) and incubating the samples at 37°C overnight. Alcohol dehydrogenase (ADH, Waters) digest from yeast was added to a final concentration of 50fmol/µg protein. The trypsin reaction was stopped and Rapigest degraded with the addition of 10% TFA/20% acetonitrile/70% water followed by heating the sample at 60°C for 2 hours. The samples were centrifuged and the supernatant pipetted into autosampler vials.

Differential Protein Expression LC/MS/MS Analysis:

The samples were pooled and analyzed using a simultaneous label-free differential protein expression approach, and analyzed on two independent instruments, a Thermo LTQ Orbitrap and Waters Synapt mass spectrometry systems. The Waters Synapt mass spectrometry system was coupled to a Waters nanoACQUITY UPLC system. The saliva samples were analyzed in triplicate, except for the DS II samples analyzed on the Waters Synapt which were analyzed in duplicate (a single replicate run failed). MS data was processed using Rosetta Elucidator (Rosetta Biosoftware). Protein database searching and identification was completed using Mascot, SwissProt human and Candida databases.

Data Analysis and Differential Protein Determination:

Collected proteomics data was evaluated using ANOVA (a cutoff value, $p \leq 0.01$) to determine differentially expressed protein biomarkers among three sample groups;

healthy (control), DS II and DS III. Principal component analysis (PCA) and cluster analysis were performed only for those differentially expressed biomarkers.

Results

A label-free differential expression LC/MS/MS method was used to quantitatively compare the protein expression between a processed, pooled aliquot for each of the control, DS II and DS III. We chose to do a pooled sample analysis because of the following two reasons similar to what was proposed by Heffernan et al[12]. First, the pooled sample analysis provides us an average global view and the major trend of the differences between control and DS subjects. Second, with our limited budget, pooled sample analysis is the most cost-effective way to measure the effects of denture stomatitis on salivary proteomes. The samples were analyzed on two different LC/MS systems. We chose to present our proteomic data based on two separate LC/MS systems because of the following three reasons. First, we recognize that different MS proteomic systems can give out different biomarkers. We therefore used two different systems to see if the results can be confirmed. While expression of strong biomakers will have the same trend in both systems, low abundant proteins may be seen in one system better than another. Second, we want to demonstrate the results as LC/MS system specific so that other investigators in the future can replicate our methods and perhaps validate our results. Third, since there are clear differences especially in the PCA results, we believe that we would gain little information from combined analysis. Analytical results from both systems were separately processed using the Rosetta Elucidator software, and, when applicable, mass signals were annotated with the corresponding peptide and protein information based on the database search results

using a 1% false discovery rate cutoff. Data processing of the Thermo Orbi data resulted in identification of 51, 474 features (mass signals). Of these, 3,174 were annotated with peptide information that corresponded to the identification of 814 peptides corresponding to 371 proteins. Of the detected isotope groups (identified and unidentified peptides), 42% had a coefficient of variance < 25. Data processing of the Waters Synapt data resulted in identification of 102,196 features (mass signals). Of these, 6,252 were annotated with peptide information that corresponded to the identification of 1435 peptides corresponding to 202 proteins. Of the detected isotope groups (identified and unidentified peptides), 25% had a coefficient of variance < 25%. ANOVA was used to determine mass patterns that correlated with the control, DS II and DS III samples. Signals with a p value < 0.01 were selected as tentative markers and summarized by protein. Principle component analysis (PCA) plots demonstrating replicate reproducibility and sample differences based on detected differentially expressed protein for the two MS systems are presented in Figures 1. Cluster heat maps based on these protein expression patterns. While we used ANOVA to define differentially express proteins, cluster analysis allows us to address the degree of differentiation in each protein and the PCA demonstrates the overall differentiation of each group in relation to other groups when combined all differentially expressed proteins together. A summary table of the corresponding tentative differentially expressed signals are presented in Figure 1, and Supplementary Tables 4 and 5 (176 proteins for the Thermo Orbi data and 97 proteins for the Waters Synapt data).

Data assessment determined mass signals with differential expression between the healthy and DS samples using ANOVA ($p<0.01$). Roughly 6% of the detected mass

signals in these differential expression studies were identified in a database search. These biomarkers include proteins from salivary glands, serum, and mucosal tissues. Cluster analysis and PCA demonstrate that each mass spectrometry system combination of salivary protein biomarkers can be used to distinguish control, DS II and DS III individuals. Proteins were detected as differentially expressed between the two LC/MS systems. Human salivary proteins and candidal proteins found in each system were described (Supplementary Tables 1 & 2). Note that of previously identified SELDI biomarkers, three of these (CAH6, CYTC and CYTN) were also found in the expression analysis as differentially expressed candidate biomarkers. In addition, several immunoglobulin (IG) regions were also detected, supporting the assessment that some of the SELDI biomarkers may be IG variable regions.

Discussion

In our previous study using SELDI-TOF analysis to investigate proteomic profiles of denture stomatitis, we identified several proteins that were upregulated in DS patients [6]. The proteins observed in the study were different than those that had previously been identified in other studies associated with other forms of oral candidasis [13-15]. Using the Thermo LTQ Orbitrap and Waters Synapt analyses, we have identified three of the same proteins that exhibited upregulation in the previous SELDI-TOF study--CAH6, CYTC, and CYTN. Several Ig fragments similar to previous study are also found in this study. Note that the different results, from the two MS platforms, are not unexpected as the compared systems use different methods for molecule ionization and detection, a situation that has been well documented in many comparative proteomic studies [16, 17]. The redundant proteins have higher expression among DS II and III. However, CAH6

shows higher expression in the healthy group in both Waters and Thermo systems, although Thermo LTQ analysis shows elevated expression in DS III as well. Note that lower expression of CAH6 (Carbonic anhydrase 6) has been associated with higher risks of caries and periodontitis--outcomes that favor bacterial growth. CAH6 is also one of the more common markers found in saliva--therefore, it may be nonspecific to DS [18]. The gene ontology of cystatin C (CYTC) is extensive and its involvement in disease has been well documented [19-23]. The role of CYTC in defense response has been inferred from direct assay. Inflammation and the presence of candida that are associated with DS are possible etiological factors of defense response. Among the Immunoglobulin regions detected, there was more agreement between the two LC/MS systems with DS III. Previously, we found that Ig regions were more elevated in DS II [6]. It is probable that the large presence of Ig's among DS subjects, reflects a B-cell mediated immune response to inflammation in the palatal mucosa. This suggests that anti-inflammatory medications should play a role in the overall treatment of DS if traditional treatment methods such as candida elimination and correcting denture fit prove unresponsive. Other differentially expressed proteins that we found as upregulated among DS groups expectedly show some interaction relating to inflammation and immune response (Supplementary Table 1). For instance, complement C3 was identified as unregulated in both DS II and DS III sample groups. It interacts with Integrin alpha-M/beta 2, which is implicated in adhesion of macrophages, monocytes, and is a receptor for fibrinogen, the precursor for blot clot formation [24]. One of the recognition systems of candida-host cell recognition has been described as involving the CR2/CR3 complement receptor of *C. albicans* [25-27]. The mechanism for opsonization of *C. albicans* involves the covalent

binding of complement C3. Through the alternative pathway, C5 activates a phagocytic and pro-inflammatory cytokine response to the yeast species [28]. However, *C. albicans* may also exhibit receptor mimicry whereby the yeast non-covalently binds to the complement C3, which inhibits the ability of C3 to opsonize the candida [29]. Non-specific glucose binding to lysine residues (glycation) at the active site of complement C3 would inhibit its function as an opsonizing agent and reduce immune response to yeast species [30, 31]. Therefore, groups exhibiting uncontrolled diabetes with higher levels of glycosylated protein, may be at greater risk for candidal infection and denture stomatitis.

Several salivary proteins (Supplementary Table 1) identified also suggest that the dysregulation of iron or iron metabolism, e.g. lactotransferrin, serotransferrin, hemopexin, and Neutrophil gelatinase-associated lipocalin, plays a role in DS. Lactotransferrin, a mucosal iron-binding protein, was found to be elevated in both stomatitis groups across the two analysis systems. Lactotransferrin for example is known to have some antifungal activity against *C. albicans* [32]. It also exhibits bactericidal properties and can inhibit microbial growth [33]. The up-regulation of lactotransferrin among DS II and DS III groups supports the bacterial and fungal component of denture stomatitis etiology. Inhibition of bacterial growth is achieved though sequester of free iron, however it also may directly bind to the cell surface of bacteria—which results in cell breakdown [34]. The mechanism for its anti fungal activity against *C. albicans* and other species is less clear. Iron is thought to play a role in cell-mediated immunity [35]. In the case of transferrin, iron-saturated transferrin enhances DNA replication in PHA-stimulated lymphocytes [36, 37]. Transferrin, in the absence of iron, however, does not enhance replication. It is possible, that iron deficiency,

therefore, may predispose patients to DS, through reduced cell mediated immune response, lowered cytotoxicity of anti fungal and antibacterial proteins such as lactotransferrin, and greater host susceptibility to infection.

While the proteomic profiles of DS II and DS III are similar in terms of human proteins, it appears the DS III group has a higher frequency and level of serum-originating proteins, e.g. ceruloplasmin, hemoglobins, serotransferrin, and albumin (Table 1 and Supplementary Table 1). This suggests higher vascular leakage and more extensive inflammation in DS III compared to DS II and control. This finding is similar to our previous study [6]. While both DS II and DS III have high levels of immunoglobulin fragments, our results again confirmed our previous finding that DS II seems to have higher immunoglobulin fragments. It is interesting to note that the control group appears to have more expression of proteins involved in innate immunity. Here we found higher level of lysozyme C and short palate, lung and nasal epithelium carcinoma-associated protein 2 (SPLUNC2). We report a similar trend of innate salivary proteins, e.g. short palate, lung and nasal epithelium carcinoma-associated protein 1 (SPLUNC1), in control edentulous subjects compared to edentulous subjects with diabetes [38]. Our findings here perhaps suggest that innate immunity proteins may protect patients from DS. Immunogloblins presents a more acute response in DS II, while serum proteins present a more chronic response in DS III. These proteins may in the future be examined for potential biomarkers or diagnostic tool development. Monitoring these proteins may also help in prevention and treatment of DS.

The LC/MS systems identified several non-human originating proteins that exhibited higher expression levels. All of the proteins identified originated from candida

organisms (Supplementary Table 2). The most common candida source proteins found came from *Candida glabrata* (8 of 20). *Candida albicans* was also identified. *Candida albicans* has been associated with DS and has been detected at elevated expression in affected saliva [5]. In the study published in 2008 by Coco *et al*, the investigators sought to determine the connection between the diversity and quantity of yeast species related to oral pathology specifically, of denture stomatitis [8]. From their denture sonicate, swab, and oral rinse samples, 75% of the oral yeasts isolated were identified as *C. albicans* and 30% were *C. glabrata.* The *C. albicans* increased in proportion as the Newton classification level (inflammation prevalence) increased. Also, *C. glabrata*, while never isolated by itself, was found with *C. albicans* over 80% of the time [8]. The prevalence of *C. glabrata* associated with *C. albicans* and a corresponding increase in inflammation level suggests that the yeast species *C. glabrata* has some pathogenic relationship with *C. albicans* related to biofilm formation and inflammation. Our study supports this hypothesis as 40% of the candidal proteins that were identified (and found among the Stomatitis II and III groups) originated from *C. glabrata*. Most of the proteins found from *C. glabrata* are involved in transcriptional regulation. It has been suggested that *C. glabrata* and *C. albicans* have a "synergistic" relationship in the pathogenesis of oral infection [39]. It may be possible that proteins from *C. glabrata* have some mechanism of control over expression of inflammatory factors originating from *C. albicans* species. It is unclear, however, if the proteins identified here are directly related to host inflammatory response.

C. glabrata has shown a greater tendency to adhere to denture surfaces compared to *C. albicans* [40]. Luo and Samaranayake found that *C. glabrata* exhibited a

four-fold greater cell surface hydrophobicity (CSH) and two fold greater adherence to denture acrylic surfaces when compared to *C. albicans* [40]. *C. glabrata* on its own may generate a lower cytokine response in oral epithelial cells than *C. albicans* [41] however, its lower susceptibility to anti fungal drugs and host immune response potentiates *C. glabrata* as an initial colonizer of denture surfaces. Its presence on the denture may make it easier for *C. albicans,* which is more susceptible to lactotransferrin and anti-fungal drugs, to colonize. Our findings that proteins from *C. glabrata* were the most prevalent of candidal proteins, support this idea of *C. glabrata's* importance in denture adhesion and colonization. DS condition is known to be resistant to treatment and often reoccurs. Targeting both *C. albicans and C. glabrata* may reduce the refractory nature of DS. Further investigation is needed into the cooperative relationship between the two fungal species in the pathogenicity of DS.

Conclusions

While this exploratory study requires further validation with a larger population, it is a proof-of-principle that salivary proteomics analysis can be used to examine the role of proteins in saliva in DS development. The results suggest the presence of salivary biomarkers specific for candidiasis in denture wearers who are in good general health. This may provide insight into the role of dentures and salivary proteins in DS development in particular innate immunity and specific immunity proteins as well as proteins involved in iron metabolism. Understanding the complex role of salivary proteins may lead to novel diagnostic and therapeutic tools not only for DS patients, but also for other patients prone to oral candidiasis. Our results further suggest that while *C. albicans* is the main species in DS, there are other candidal species that may play a

symbiotic role in initial colonization, biofilm formation and DS development with *C. albicans*.

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Table and Figure Legends

Table 1 Selected differentially expressed proteins. List of selected host proteins that were differentially expressed that may be related to denture stomatitis.

Supplementary Table 1 Salivary Proteins. Functions of salivary proteins that were identified as differentially expressed among groups using both Waters and Thermo analysis.

Supplementary Table 2 Candidal Proteins. Functions of non-human originating proteins that were identified as differentially expressed in saliva samples using both systems.

Supplementary Table 3 Patient demographic data.

Supplementary Table 4 Protein differentially expressed in the ThermOrbitrap MS system.

Supplementary Table 5 Protein differentially expressed in the WaterSynapt system.

Figure 1 Proteomic analysis for Denture Stomatitis. (A) PCA analysis using WaterSynapt System; (B) Cluster analysis using WaterSynapt System; (C) PCA analysis using ThermOrbitrap System; and (D) Cluster Analysis usingThermOrbitrap System.

Figure 2 Proposed model of DS development demonstrating the interaction between host and candida organisms through proteins found in saliva.

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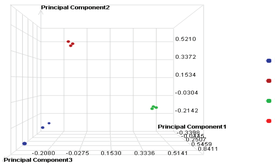
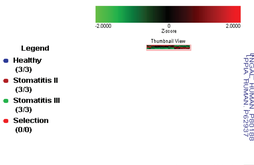
Selected upregulated proteins differentially expressed among groups	
Healthy	Carbonic anhydrase 6* Beta-2-microglobulin Homeobox protein DBX2 Lysozyme C Short palate, lung and nasal epithelium carcinoma-associated protein 2
Stomatitis II	Kallikrein-1 Neutrophil gelatinase-associated lipocalin Submaxillary gland androgen-regulated protein 3B Thioredoxin
Stomatitis III	Ceruloplasmin Cystatin-B Hemoglobin subunit delta Keratin, type II cuticular Hb3 Serotransferrin Serum albumin Uteroglobin (found in Healthy and Stomatitis III)
Stomatitis II and III	Alpha-2-macroglobulin Complement C3 Cystatin-SN* Haptoglobin Hemopexin Lactotransferrin Prolactin-inducible protein Cystatin-D Hemoglobin subunit beta Hemoglobin subunit alpha Cystatin-C*

* *Selected protein identified in previous work

A





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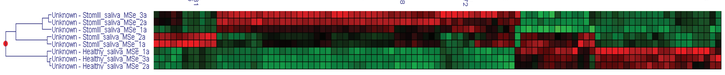

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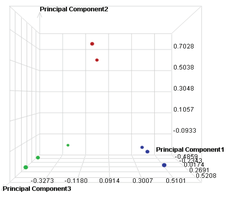
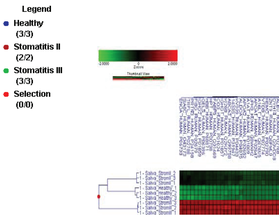
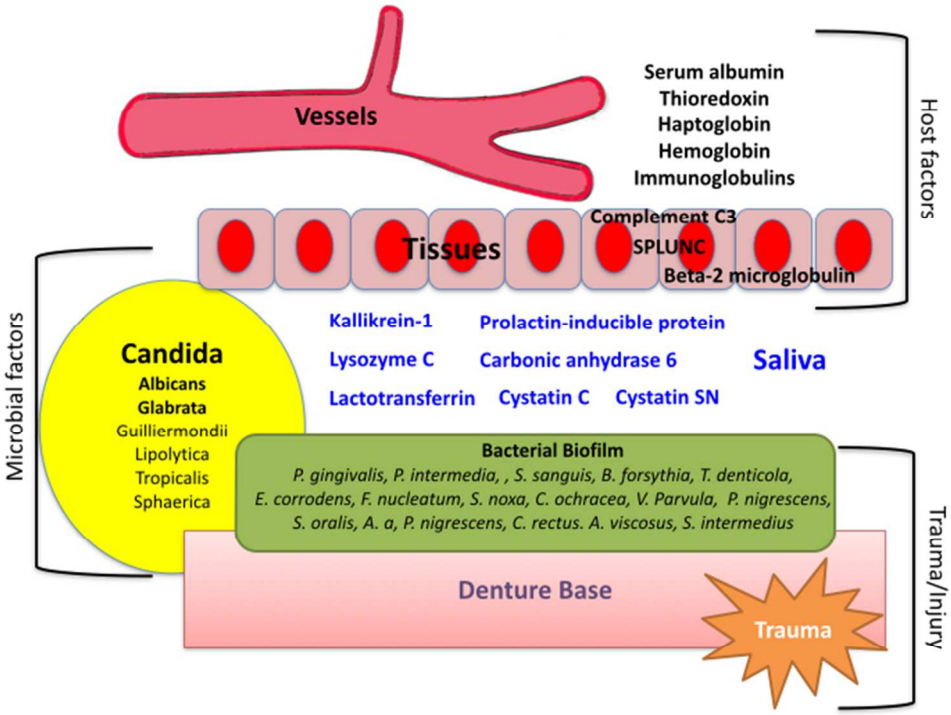




D



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