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ORIGINAL ARTICLE

Comparative Proteomics in Caries Active and Caries Free Associated S. Mutans Strains for Metabolic Remodeling Favouring Colonization

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ABSTRACT

Introduction: A multifactorial condition, dental caries is primarily brought on by cariogenic bacteria that are frequently present in the mouth. Caries infections are commonly associated with the acid-producing bacteria Streptococcus mutans. If left untreated, dental caries can result in tooth decalcification, cavities, hypersensitivity, and even tooth loss. Comparative proteomics by Mass spectrometry was carried out to reveal caries-associated metabolic remodeling in S mutans isolates favouring biofilm formation and colonization.

Methods: We conducted a differential proteomic analysis to determine the differences in protein expression between S.mutans strains linked to dental caries and those present in a healthy oral microbiome. High-performance mass spectrometry (MS) using Orbitrap, has led to the development of proteomics in larger-scale protein analysis. Five clinical specimens from individuals who had caries disease (Caries active) were compared with two isolates from the healthy oral dental microbiota (Caries free). Protein samples were digested, and a peptide mixture investigation was done.

Results and Discussion: 3276 proteins that were expressed at comparable amounts in both groups of bacteria and were found by proteomic analysis. Only 39 of these proteins were unique and distinct to group 1 (those without caries), whereas 444 proteins were specific to group 2. (S.mutans from caries patients). Significant differences in the grouping of the control (Caries free) and caries (Caries active) samples were seen through PCA analysis. 23 Significantly regulated proteins in S. mutans bacteria were discovered ($p < 0.05$, t-test). Among these, 23 differentially expressed proteins, 10 were upregulated, and 13 downregulated. By Identifying differential peptides and proteins, their quantification, and appropriate bioinformatic analysis, there are insights into formation of biofilm by S. mutans in dental caries and potential targets for intervention using various antagonists/ nanoparticle approaches as alternatives to conventional antibiotics.

INTRODUCTION

Streptococcus mutans, an acid-producing bacterium, is frequently linked to tooth infections. It is a biofilm made of bacteria, saliva, and food particles coating tooth enamel (Nyvad et al., 2013; Vu et al., 2009). When the film becomes multi-layered, it forms dental plaque, a debilitating condition wherein many of the bacterial species can co-reside and further exacerbate the infection (Song et al., 2006; Yim et al., 2013). In a healthy person, oral microbiota is not the source of any oral pathology, but excessive biofilm production might result in tooth caries or periodontal disorders (Stoodley et al., 2002).

It has been shown that in dental caries, the biofilm formed by S. mutans undergoes active remodeling, allowing it to utilize sugars present in the food and promote colonization of the dentin (Marsh, 2006). This colonization process is exacerbated with metabolic remodeling, wherein the Streptococci (mutans) produce strong acids during metabolism, damaging the dental enamel and causing cavitation. Further, these bacteria also secrete extracellular polysaccharides (EPS) in the presence of high carbohydrates, which further enhances their cariogenicity (Flemming et al., 2007; Svensäter et al., 2003).

Aimed to understand the differentially expressed proteins in S. mutans strains associated with dental caries compared to those found in normal oral microbiome, we performed the differential MS-based proteomic analysis (Manoil et al., 2020). Two cultures from normal oral dental microflora were compared with five different clinical isolates from patients with extensive dental caries. To identify if the strains have any inherent metabolic differences, for proteomic comparison, we first grew all the strains in a static condition where biofilm formation is promoted for 48 hours. We then extracted proteins to compare expression differences using quantitative MS/MS analysis (Klein et al., 2012). We did not directly compare the differences in the proteome in the bacteria isolated from human subjects as it was not possible to get pure bacteria directly from patients. Our approach allowed us to obtain the necessary amount of pure material needed for comparative analysis and identified the inherent differences in normal vs. cariogenic strains (Hendrickson et al., 2012).

MATERIALS AND METHODS

Bacterial Isolation and culture: The study's ethical approval was granted by the institutional ethics committee of the PMNM Dental College and Hospital in Bagalkot, Karnataka, India (Ref. No. PMNMDCH/2402/2018-19). Both Caries active and Caries free dental plaque samples were processed as previously described by Wu et. Al. (2003) with minor modification. Colonies plated on MSA were picked for strain purification and identification.

The culture of each bacterial strain was obtained following an optimized method using brain heart infusion medium (BHI) grown in anaerobic conditions at 37 °C. An equal number of biofilm cells were collected. Once the cell pellet had been washed with water for two minutes to eliminate medium components, cells were added to the tubes. To verify cell weight, the Eppendorf tube's volume was weighed once again. Cell weight for each tube was recorded.

Proteomic Analysis: Equal quantities of biofilm pellets were collected for lysate preparation, and they were cleaned with 0.1 M Tris (pH 8.8) to get rid of any remaining medium components and debris. After adding three volumes of lysis buffer (10% SDS and 0.1M Tris, pH 8.8), lysates were boiled in a water bath for five minutes for full lysis. After being sonicated for five minutes, the lysate underwent immediate trypsin digestion. Protein samples were reduced with 5mM TCEP, alkylated with 50mM iodoacetamide, then trypsin was used to digest them for 16 hours at 37 °C (1:50 trypsin/lysate ratio). Digests were dried with a speed vacuum after being cleansed with a C18 silica cartridge to get rid of salts. In buffer A, dry pellet was reconstituted with 5% acetonitrile, 0.1% formic acid. Using C18 columns, sample cleaning was completed, and peptide mixture analysis was conducted. An Ultimate 3000 RSL Cnano system with an Orbitrap Eclipse was used for the experiments. 500ng of each sample was put onto a C18 column of 50 cm and a 3.0µm Easy-Spray Column from Thermo Fisher Scientific. At a flow rate of 300 nl/min, peptides were eluted using a 0–40% gradient of buffer B (80% acetonitrile, 0.1–2% formic acid), and was then injected for MS analysis. 100 minutes were spent running LC gradients.

MS1 spectra were collected in the Orbitrap. (R= 240k; AGQ target = 400000; Max IT = 50ms; RF Lens = 30%; mass range = 400 – 2000; centroid rate). For 10s, dynamic exclusion used to

eliminate all charge states for a certain precursor. In a linear ion trap, MS2 spectra were acquired (rate = turbo; AGQ target = 20000; Max IT = 50ms; NCE HD = 35%). On the QE Plus (Thermo Scientific, USA), a 100-minute MS Run with an MS1 resolution of 70k and an MS2 resolution of 17500 was conducted. Proteome Discoverer (v 2.4) was used to analyze raw data files in Thermo format against the Uniprot S. mutans reference proteome database. The precursor and fragment mass limitations for the Sequest search were set at 10ppm and 0.5 Da, respectively. Enzyme specificity was set for trypsin/P (cleavage at the C terminus of "K/R: unless followed by "P") and a maximum missed cleavage value of two for the protease that was utilized to create the peptides. For database searches, fixed modification carbamidomethyl on cysteine and the variable modifications methionine oxidation and N-terminal acetylation were both taken into consideration. Protein false discovery rate and peptide spectrum match were both set at 0.01 FDR. Analysis was done with a Database search using SEQUEST AND AMANDA. For Organism: S.mutans Uniprot Database was used. Differentials and Statistics are presented as Venn Diagrams and excel files (Hendrickson et al., 2012; Stoodley et al., 2002).

RESULTS

Two S. mutans strains were isolated from patients without caries and five from patients with caries. The strains were cultures in traditional BHI media in glass tubes in standing condition for biofilm formation. After growth the cells were harvested, washed, and then processed for proteome analysis. This design of experiment was based on limitation of low bacterial load in clinical material and with the expectation that expression level changes can be recapitulated for some generations even after samples are removed from native environment.

The proteomic analysis found 3276 proteins were not altered between the bacterial groups. Overall coverage revealed the presence of more than 3500 proteins in both groups of strains (Figure 1 – Venn diagram). Of these, only 39 proteins were specific and unique to Group 1 (without caries), and 444 proteins were determined to be present differentially or only in Group 2 (S. mutans from caries patients).

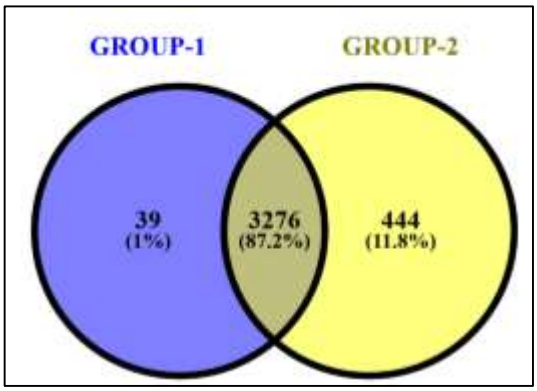

Figure 1: Venn diagram showing overlap as well as unique proteins in normal (group 1) vs. Caries (group 2).

Interestingly when the samples of group 2 were examined for internal homogeneity, it was revealed that they were quite heterogeneous and only 227 proteins were common to all. Each sample had many unique as well as shared proteins with one or more samples (Figure 2a, Venn diagram of group 2 samples). Similar differences were also observed in samples from group 1, and here 427 common proteins were recorded (Figure 2b, Venn diagram of group 1 samples). The relative paucity of the number of proteins in the samples revealed an overall remodeling of protein expression wherein a limited set of genes is expressed in the biofilm condition.

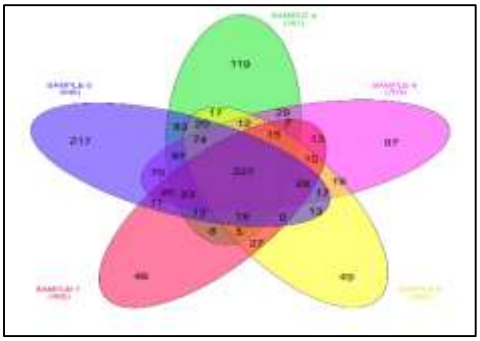

Figure 2a: Venn diagram of group 2 samples

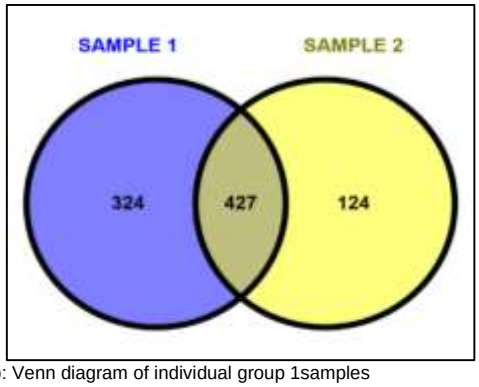

Figure 2b: Venn diagram of individual group 1samples

Overall, as revealed from PCA analysis and correlation plot, clusters of samples based on similarity, grouping analysis and replicate classification were good. The PCA analysis showed a distinct grouping of control and caries samples (Figure 3, PCA plot). Even though the inter-sample heterogeneity was high for caries samples, they were still distinctly different from the control and clustered together (blue for the caries samples and brown for the control samples). The correlation plot (Figure 4) revealed that while control samples (group 1) showed high correlation with each other (depicted in blue), the caries group showed low correlation scores with control samples (depicted in red), in agreement with PCA analysis. Despite this, the samples in group 2 still grouped distinctly from the control non-caries samples (indicated by blue in the group 2 caries samples). The correlational heat map distinctly showed that all non-caries samples had a negative correlation score with caries samples and all caries samples had a positive correlation score. These scores suggested that distinct protein expression signatures mark the caries samples as they are different from non-caries samples.

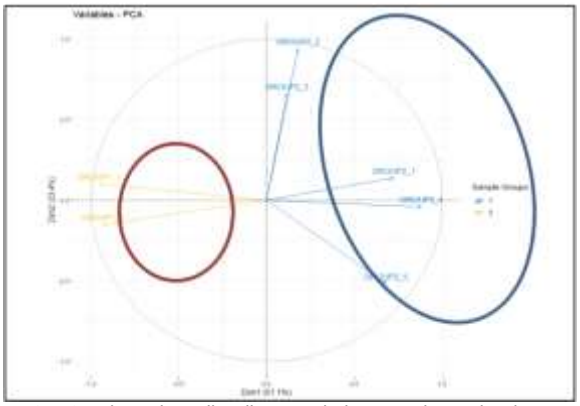

Figure 3: PCA plot. The yellow lines mark the control samples (grouped in brown) and blue the caries samples (grouped in blue).

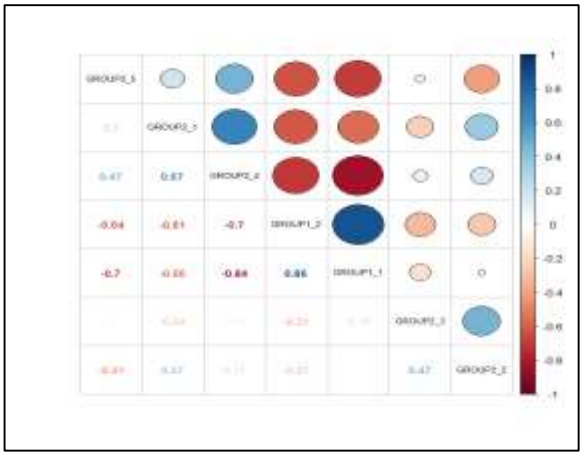

Figure 4: Correlation plot. The diagonal row lists the sample id and the bottom left section enlists the correlation score between each of the sample. This information is shown as a heat map on the top right portion. Group 1 samples, control samples and group 2, caries samples.

After significance and FDR analysis to correct for multiple comparisons, as presented in the Volcano plot (Figure 5), expression level changes of all the proteins were plotted as a function of expression changes in reference to the control samples. Post significance analysis, several proteins were marked as either distinctly higher or lower in levels, shown as green or red dots on the plot . Box and whisker plot (Figure 6) shows z score abundance values for all the samples depicting that the abundance is more for the samples from caries than control samples. Specific changes for these proteins were individually plotted as a heat map (Figure 7, heat map). It is evident from the heat map is that despite the inherent differences in the samples, comparative expression analysis revealed enrichment of some specific proteins in all the caries samples and the reduction in the expression of a few. Overall good distribution of samples was obtained (Figure 7: heat map and box plot for distribution profile), and apart for 2 samples, negative skew for samples were recorded and as presented before, all caries samples have distinct profile compared to normal samples (compare first five with last two in box plot). Approximately one-third of the differentially expressed proteins (7 proteins) are reportedly associated with biofilms. The enriched proteins mainly included enzymes involved in metabolic remodeling, including hydrolases such as alpha/ beta hydrolase; GTPase; peptidases like serine peptidases; dextran sucrase, and cppA family protein. Amongst the downregulated proteins were enolase; Ketol-acid reductoisomerase; Putative glutamyl-aminopeptidase endo-1,4 beta-glucanase and protein translocase subunit SecA.

The metabolic remodeling was also reflected in the GO pathway analysis (Figure 8), where pathways involved in primary metabolic processes, including nitrogen compound metabolism; carboxylic acid metabolism, were determined to be maximally influenced (Figure 8 – GO biological process tree). The same was also captured in the biological processes network, and it was evident that most differential proteins are associated with metabolic remodeling (Figure 9- GO network map). The biological processes represented were mainly associated with metabolic processes, DNA replication, the immune response, and the defence system. In the molecular function category, the observed terms were mainly involved in prosthetic group binding, of acyl carrier proteins (ACP), and modified amino acid binding. In the cellular components analysis, extracellular region, cell wall, and external encapsulating structure showed up to be most significantly enriched. Molecular functions describe activities occuring at the molecular level, such as “catalysis” or “transport”. Activities are represented in GO molecular process tree (Figure 10) and molecular function network (Figure 11). Hydrolase, ligase, binding and catalytic activities are highlighted.

Apart from GO analysis, differentially expressed proteins were mapped into KEGG pathways. Amongst the differential proteins, D-alanine-poly (phosphoribitol) ligase (dltC) in the pathway for D-alanine metabolism, and SMU82_04583 and dextranase (dexA) were in the pathway for starch and sucrose metabolism. dexA was identified in the metabolic process, catalytic activity, extracellular region, cell part, extracellular region, external encapsulating structure, and extracellular region. Also, dltC was identified in the single-organism process, biological regulation, D-alanyl carrier process, binding, cell macromolecule metabolic activity, and macromolecular metabolism.

Our proteomic analysis has revealed extensive metabolic remodeling in the Streptococcus mutans in patients with caries compared to those isolated individuals without caries. Dextransucrase, Protein translocase subunit SecA, Ketol-acid reductoisomerase (NADP(+), Glutamyl-aminopeptidase endo-1,4-beta-glucanase, Hydrolase_4 domain-containing protein, Prolyl oligopeptidase family serine peptidase, Alpha/beta hydrolase, GTPase Obg, CppA family protein are some of the significantly different targets. The remodeling agrees with previous evidence, which suggests that in the case of caries, the bacteria generates strong acids and enhances its ability to utilize sugars to promote further growth and aggressive pathology (Marsh, 2006; Steinberg, 2004) and their differential expression can disturb the adhesion of bacteria, architecture of biofilm and development of plaque.

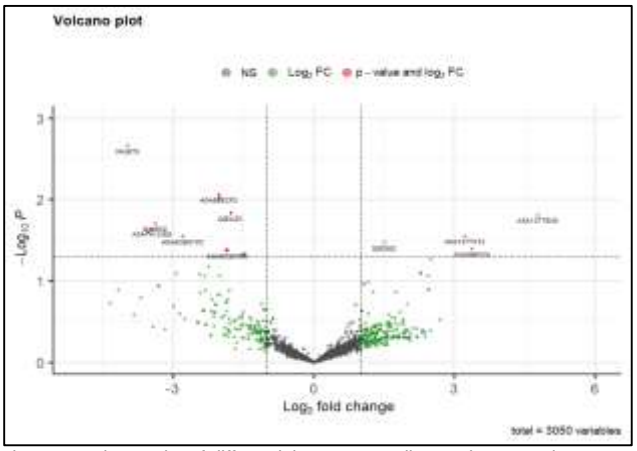

Figure 5: Volcano plot of differential proteome. All spots in green show more than 2-fold changes in the amount of the protein between the control and caries samples and spots marked in red show maximal changes or significant changes.

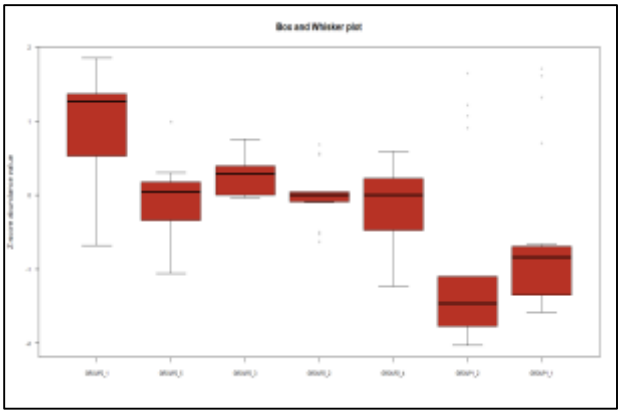

Figure 6: Box and whisker plot showing significance of the collected data (z score abundance values for all the samples)

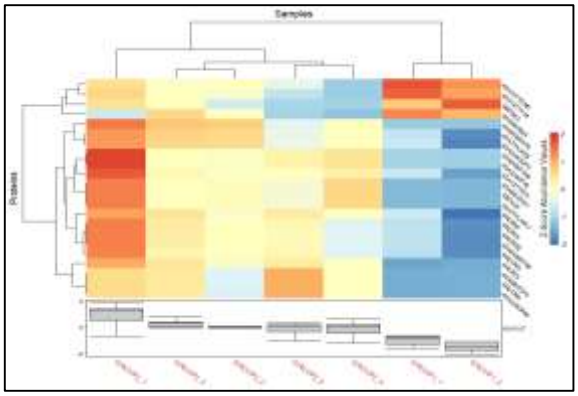

Figure 7: Heat Map of differential proteome. The genes are listed on the right and sample ids are provided at the bottom. Group 1 is the samples from control samples and Group 2 are from samples from caries.

Figure 8: GO biological process tree based on the differentially expressed proteins and their enrichment.

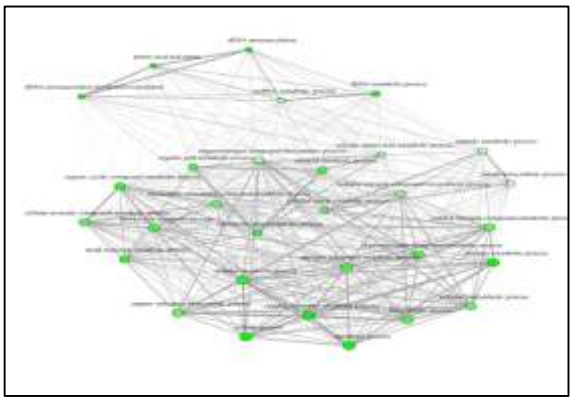

Figure 9: GO biological process network

Figure 10: Molecular process tree

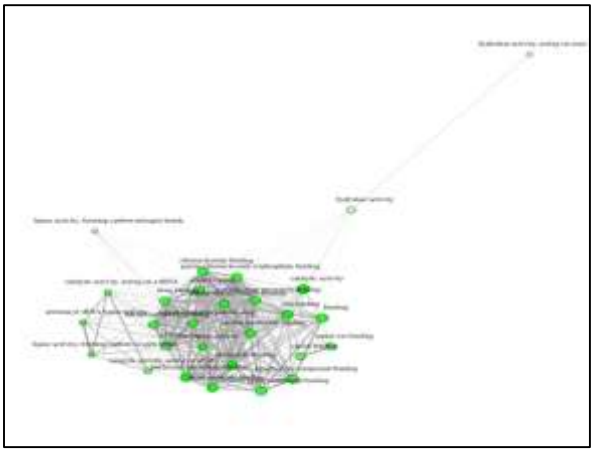

Figure 11: Molecular function network

DISCUSSION

Streptococcus mutans is suspected as the primary l agent for dental caries etiology. It achieves its pathogenicity through the ability to form biofilm on the tooth surface, commonly known as plaques (Francolini & Donelli, 2010). The ability of plaque formation of S. mutans has been proposed to be linked to the presence of many unique proteins in the bacteria, proteins that can bind to the host proteins such as collagen, glucan binding proteins, etc., which help them adhere and colonize (Xiao et al., 2012). S. mutans utilizes carbohydrates generating acidic metabolites leading to destruction and demineralization of tooth leading to dental caries. Factors of the bacteria contributing to development of oral biofilm is the capability to produce glucosyltransferase B, an enzyme responsible for the formation of glucan from sucrose. The glucan that is synthesized causes adhesion of S. mutans to each other and the enamel of the tooth, helping the bacteria to be tolerant to different physiological conditions. Such conditions include nutrient availability, aerobic or anaerobic conditions and variations in pH and providing protection against manual cleaning forces and the effect of antimicrobial treatment (Ito et al. 2020). S. mutans change their protein expression based on protein-protein interactions involving sucrose dependent and the sucrose independent mechanism. Sucrose dependent mechanism involves glycosyltransferases (GTFB, GTFC and GTFD) responsible for the synthesis of glucan from sucrose, providing sticking of bacteria to each other and bacteria adhering to tooth enamel. The sucrose independent mechanism is started by interaction of salivary agglutinins and the bacteria with surface associated protein P1 (SpaP or Pac1). Sucrose is the main metabolism for S. mutans and is the most cariogenic. Carbohydrates are shunted by two transporters: phosphoenol pyruvate sugar phosphotransferase and the ATP binding cassette transporters. S. mutans can mount and regulate response to low pH due to the ATPase translocating proton causing aciduricity. Lower the pH at which ATPase can function as the metabolic end products bulk, more aggressive is the bacteria in the biofilm formation. S. mutans is one of the top acidogenic microorganism found in biofilm since it produces acids from fermentation of carbohydrates different than any other streptococci. S. mutans also produce mutacins (bacteriocins) contributing to their colonization in biofilm formed in caries (Matsumoto-Nakano 2018). However, what kind of changes occur in the bacterial metabolism when they induce caries vs when they are just present on the dental enamel have not been examined till now. With this objective, our studies shed light on proteome modelling in the bacterial cells from plaques with caries and without caries. The anticipation was that the bacterial cells with caries would have access to higher nutrition and protein signatures indicative of aggressiveness if any.

Interestingly we did not record any differential enrichment for cell adhesion proteins, quorum proteins, or immunity-related

proteins. We did observe elevated levels of dextran sucrases, glycosyltransferase enzyme which is involved in sucrose metabolism and catalyzes the generation of fructose. In S. mutans, it has been shown to be responsible for their agglutination, which may facilitate their colonization *Gibbons 1969, PMC284819). Further, the enzyme also catalysis utilization of sucrose to generate organic acids linked with aggressive cavitation and formation of intracellular polysaccharides (IPS), which promotes bacterial survival in biofilm. It has been shown that the presence of IPS promotes cariogenesis by facilitating long-term acid production by serving as a carbon source even when the external dietary sugars are depleted. Based on this presence of higher levels of dextransucrases was not completely unanticipated. Another altered enzyme was enolase, which catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate, a process that feeds into glycolysis and increases carbon utilization, reducing IPS formation.

Although the number of proteins found to be differential was relatively few, their involvement in the caries formation can be ascertained and correlated with the sample source. The findings also suggested that there will be value in designing potential interventional entities that can target dextran sucrases critical for colonization, carbon flow management, and ultimate pathology. Proteomics has been progressively useful for studies of the antimicrobial activity as well as toxicity of antibiotics or Nanoparticles, a targeted approach based on proteomics can be beneficial in exploring the mechanism of biofilm formation for intervention against S. mutans (Jiang N et al., 2021). The chemical antibiofilm treatments currently used are quaternary ammonium salts, fluoride and iodine compounds. These tend to manifest a variety of side effects (Mehrishi et al. 2020). Higher dosages with long term usage of antimicrobial agents leads to antimicrobial resistance, known to be a drastic issue (Fajriani et al. 2020). Therefore, to overcome virulent biofilm forming bacteria, moving towards usage of herbal plants as antibiofilm agents. These products are organic and are less expensive with lesser side effects. This could lead to development of antibiofilm agents that are biofilm specific, and are nontoxic, such that the development of drug resistance is prevented.

Acknowledgments

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