Low-level laser and transcutaneous electrical nerve stimulation on salivary glands impact type 2 Diabetes Mellitus oral microbiome: An interim analysis of a Randomized Trial

Abstract

Purpose: Diabetes Mellitus (DM) can lead to significant reduction of saliva production, which increases the risk of oral diseases. Managing reduced salivary flow becomes indispensable for restoring microbial balance in this condition. This study aimed to investigate the effects of physical stimulation methods on salivary secretion rate and microbiome composition in individuals with type 2 DM (T2DM) having reduced salivary flow using an exploratory interim analysis of a randomized clinical trial.

Methods: Eight individuals provided a total of 15 stimulated or rest saliva samples and were randomly assigned to the following groups: low-level laser therapy (LLLT) (G1), transcutaneous electrical nerve stimulation (TENS)(G2), and a combination of both methods(G3), administered twice a week over a span of 10 sessions. Salivary flow was evaluated at three time points: recruitment, before the 1st session (baseline) and after the 10th session. Subjective dry mouth was evaluated using the TESS scale at baseline and after the final session. DNA was extracted from whole saliva immediately before treatment (baseline) and after the final stimulation session, 16S rRNA gene amplified and sequenced (Illumina MiSeq).

Results: N=7 individuals showed improvement in stimulated salivary flow (SSF), achieving or maintaining normal range (SSF>0.7ml/min). Bacterial diversity was significantly higher in saliva after stimuli with laser(G1) when compared to TENS(G2). The relative abundance of *Firmicutes* and *Fusobacteriota* increased after all treatments, while *Actinobacteriota* and *Proteobacteria* decreased. The abundance of minority taxa was reduced after stimulation.

Conclusion: This exploratory study provides initial indications that physical stimulation may help manage T2DM-related reduced salivary flow and could be associated with subtle changes in low-abundance taxa suggesting a potential “clearance” effect. These preliminary observations require confirmation through larger, long-term studies.

Trial Registration Number: RBR-3tqv8r3. *Date of registration:* 13th April 2023.

Keywords

Diabetes Mellitus; Transcutaneous Electric Nerve Stimulation; Low-Level Light Therapy; Xerostomia; Microbiota.

Introduction

Saliva acts as a ‘‘natural mouthwash’’ that removes food particles, debris, and microorganisms. A healthy salivary flow helps maintaining the pH balance in the mouth, promoting clearance and preventing microbial imbalances associated with conditions such as dental caries[1], periodontitis[2], and halitosis[3, 4]. Additionally, saliva possesses antimicrobial properties, modulates the oral microbiota[5], and serves as a valuable sample for assessing the composition and dynamics of the oral microbiome [6, 7]. Consequently, systemic conditions that alter the quantity and quality of saliva can affect quality of life and impair oral and systemic diseases.

In this context, people living with chronic diseases such as diabetes mellitus (DM) often experience oral disorders in addition to various systemic manifestations. These included reduced salivary flow and altered biochemical salivary parameters, such as high urea and glucose levels[8, 9]. Moreover, hyperglycemia can acidify the oral environment and disrupt microbial diversity[10]. Glycemic control also affects specific types of salivary flow: uncontrolled glycemic levels have greater impact on unstimulated salivary flow than controlled levels, likely due to hormonal imbalances, microvascular changes, and neuronal alterations[9, 11].

Accordingly, the rate of salivary flow can influence the effectiveness of oral clearance mechanisms and impact susceptibility to oral diseases in individuals with DM[12, 13]. Research shows that individuals with normal salivary flow rates have faster sugar clearance[14] and experience less dental caries[15] compared to those with reduced salivary flow. Decreased salivary clearance leads to higher microbial counts, disrupting microbiome balance[16]. Thus, salivary clearance removes microorganisms and help maintaining microbial balance in the oral cavity[17]. Managing salivary dysfunction by stimulating the residual function of the salivary glands is essential for restoring microbial balance in the oral cavity for individuals with DM [10, 18].

However, most dental professionals are unfamiliar with the clinical management of reduced salivary flow, and the topic is often overlooked in undergraduate curricula[5, 19]. Furthermore, standardized protocols for treating this condition are still scarce in the daily clinical practice[20, 21]. Different methods have been used to establish a healthy saliva and/or to improve the quality and quantity of saliva, such as gustatory, mechanical, chemical/drug and physical stimulation[22], which include low-level laser therapy(LLLT) and transcutaneous electrical nerve stimulation(TENS). LLLT and TENS have emerged as promising alternatives to chemical methods, offering a less invasive, more affordable approach with minimal or no side effects[20]. LLLT, in particular, influences various cellular signaling pathways, including several markers also influenced by the microbiome, such as TGF-β and cytokines. This interaction suggests that laser could also affect the microbiome, positioning photobiomodulation therapy as a potential safe and conservative treatment for conditions related to microbiome imbalance, such as metabolic and neurological diseases[23].

This study aimed to investigate the effects of physical stimulation methods on salivary secretion rate and microbiome composition in individuals with type 2 DM (T2DM) having reduced salivary flow: LLLT, TENS

and their combination. This is an exploratory interim analysis of a randomized clinical trial (RCT), where the microbial composition analysis using 16S rRNA Illumina sequencing was conducted before and after stimulation.

Materials and methods

Trial design

A RCT was designed to test the effects of TENS and LLLT on salivary gland stimulation and microbial composition. The parallel-group trial (1:1 allocation) was registered in the Brazilian Registry of Clinical Trials(ReBEC). Trial registration number: RBR-3tqv8r3 (registered in 13th April 2023||http://www.ensaiosclinicos.gov.br/). The report of this study follows CONSORT checklist (http://www.consort-statement.org).

Participants

This study received approval from the Ethics Committee of the [redacted], under the number 4.748.761 CAAE 45184721.7.0000.0030, in accordance with the Declaration of Helsinki. All participants were recruited from the dental clinics at the [redacted] signed an informed consent and received basic periodontal treatment — including subgingival and supragingival scaling (according to individual needs) and plaque control through professional prophylaxis — 45 to 60 days prior to the initiation of the salivary stimulation protocol. Participants who presented with active caries received appropriate oral health guidance and treatment.

Eligibility criteria

Eligible participants were adults aged 30-65, of either sex, with a prior diagnosis of T2DM (controlled or uncontrolled), and controlled periodontal disease. The same experienced periodontist, evaluated periodontal status, and carried out the prior periodontal treatment, ensuring visual plaque index = 0 at baseline followed by low percentages of bleeding on probing as previously described[24, 25]. During recruitment (before periodontal treatment), participants also needed to exhibit a resting salivary flow (RSF)≤0.4ml/min and/or stimulated salivary flow (SSF)≤0.7ml/min[26], and report oral dryness based on Treatment Emergent Symptom Scale(TESS) questionnaire (after periodontal treatment, baseline, instrument detailed below) [27], i.e., participants were recruited based on any one or a combination of the following: low RSF OR low SSF, AND additional subjective complaints of dry mouth after periodontal treatment.

Exclusion criteria included severe systemic complications, such as heart disease, chronic liver or kidney disease, immunosuppression, chronic lung disease, lupus, Sjogren's syndrome, hypothyroidism, recent radiotherapy/chemotherapy, absence of a recent blood glycated hemoglobin (A1c) exam to assess glycemic control, smoking, organ transplantation, epilepsy, electronic device use (e.g., pacemaker), and motor coordination issues.

General health status

The identification of T2DM relied on self-reported information provided by the participants regarding a previous medical diagnosis. Glycemic status was evaluated using blood A1c (%) levels as previously described[1]. Additionally, the daily number of regularly used medications was recorded.

Oral health status

Periodontal evaluation was conducted as part of the eligibility screening, not the baseline data collection. a comprehensive periodontal examination was performed by an experienced periodontist, including assessment of bleeding on probing at six sites per tooth using a Williams-type periodontal probe, and evaluation of the visual plaque index [24]. By the time of the baseline assessment, all participants had undergone appropriate periodontal treatment and oral care control, being all clinically well-controlled for periodontal conditions (visual plaque index = 0 and bleeding on probing <10% of the sites)[24, 25]. Therefore, periodontal indices were not reassessed at baseline.

The number of teeth, caries experience and caries activity were evaluated by previously trained examiners, who performed a visual-tactile assessment of caries activity based on the Nyvad criteria, as previously described[28]. This method for differentiation between active and inactive lesions and the reproducibility are described elsewhere [28].

Interventions

After recruiting, participants were randomly distributed in three groups to receive the following treatments for reduced salivary flow:

• Group 1(G1) – Salivary stimulation through LLLT

• Group 2(G2) - Salivary stimulation through TENS

• Group 3(G3) - Salivary stimulation through both therapies: LLLT+TENS

Participants were then seated comfortably in a dental chair, and their facial skin was cleaned with 70% alcohol to prevent interference with LLLT or TENS electrode placement. All groups underwent 10 sessions twice weekly, following group-specific protocols. A single dentist performed all treatments.

Laser irradiation was applied as follows: participants lay back in the dental chair, and the RED LASER (100 mW, 660 nm – 0.5 J/point) was applied intraorally at 10 points on the lower labial mucosa, 6 points on the upper labial mucosa, 3 points on the buccal mucosa, 2 points on the floor of the tongue, and 4 points at the junction of the soft and hard palate. The INFRARED LASER(100 mW, 808 nm – 1 J/point) was applied extraorally at 6 points over the parotid region and 2 points in the submandibular and sublingual regions. Both the dentist and participants wore protective eyewear throughout the laser treatment. Device used: Laser DMC–Therapy EC.

For the TENS application, standardized electrode placement involved attaching the positive electrodes over the parotid gland regions and the negative electrodes over the submandibular and sublingual gland regions. All electrodes were coated with conductive gel and secured with medical tape. The TENS device (Neurodyn III CLASS A Medical Electrical Device–IBRAMED) was set to a frequency of 100 Hz and a pulse width of 300 nm in TENS mode, during 20 minutes. Intensity was adjusted according to each participant's tolerance level.

When the treatments were combined, the laser application was performed first, followed by the TENS application.

Sample Size

The sample size for the RCT was calculated based on the primary outcome, salivary flow. Estimates for the calculation were derived from unpublished pilot data. In this pilot, the mean difference in salivary flow before and after stimulation was 0.11 ml/min, with standard deviations of 0.09 ml/min and 0.002 ml/min. Based on these values, a significance level(α) of 0.05, a power(1–β) of 0.80, and an anticipated dropout rate of 40% to ensure adequate power despite potential loss to follow-up, the required sample size was estimated to be n=8 per group. These results represent an interim analysis focusing on a subgroup of participants(n=8) from one specific randomization block of the trial. This interim analysis is not representative of the full sample size determined for the main clinical outcomes.

Randomization and allocation

A sequence of random numbers generated with a free online tool at OpenEpi.com. These numbers were placed in sequentially numbered, opaque envelopes by a researcher not involved in the treatment procedures to ensure concealment. Each participant's treatment allocation was revealed to the operator only immediately before saliva stimulation and following periodontal treatment.

Blinding

A blinding protocol was deemed impractical due to the distinct nature of each treatment. As a result, both the dentist administering the treatments and the participant were aware of their assigned treatment groups.

Outcomes

Sialometry

RSF and SSF sialometry were performed in the morning (8-10am) to minimize the influence of circadian rhythms. Participants were instructed to refrain from eating, drinking, using mouthwash, or performing oral hygiene for 30-min prior to the procedure, as longer fasting was avoided due to hypoglycemia risk. RSF volume was measured after five minutes of passive drooling, as previously described [28]. For SSF, participants chewed a rubber device for five minutes while collecting the flow. The total volume collected in each condition was then divided by five to express the salivary flow rate in mL/min. Both RSF and SSF

measurements were taken for diagnosis of reduced salivary flow during the recruitment, immediately before the first stimuli session (baseline, 45 to 60 days after tailored periodontal treatment, table 1), after the sixth session (data not shown) and at the end of the tenth session (final, table 1), following the same protocol. Upon collection, 500µL of the RSF and SSF saliva samples were aliquoted, centrifuged, and the resulting pellets were stored at -20°C until further microbial DNA extraction and sequencing. Any increase in salivary secretion rate was considered a “clinical improvement.”

Assessment of Patient-Reported Dry Mouth (TESS scale)

The Treatment Emergent Symptom Scale (TESS) was used to evaluate patient-reported discomfort related to dry mouth. The questionnaire was administered by the same experienced clinician, ensuring consistency, and patients selected the score that best represented their symptoms for each item. Assessments were conducted at two time points: at baseline (defined as immediately before the first stimulation session, approximately 45 to 60 days after individualized periodontal treatment) and after the tenth stimulation session. The scale includes the following scores: 0= No complaints of dry mouth; 1= Suspicion or slight feeling of dryness at night or upon waking; 2= Mild complaint of dryness all the time that impedes normal oral functions; 3= Moderate complaint of dryness with some degree of functional impairment, but no perceived health risk, as well as difficulty in swallowing dry foods or speaking; and 4= Severe complaint, definite perception of diminished well-being, significant impairment or disability, as well as difficulty in swallowing any food, needs to drink water all the time and with complaint of pain in the mouth [27] (positive for discomfort any score>0).

Visual analysis of tongue coating

Tongue coating analysis was performed visually in virtual sextants on the lingual dorsum, which received the following scores: 0 = no substrate; 1= light substrate(visible papillae); 2= severe substrate(no visible papillae). The sum of the scores was the final score described for each participating individual[29]. At the end of the treatment, tongue coating score results were compared, with participants categorized as "better" if their tongue substrate decreased, "same" if it remained unchanged, and "worse" if it increased.

Salivary microbiome analysis using 16S rRNA

DNA was extracted from the pellets of the whole saliva (ideally 1 mL) using the QIAamp DNA Mini Kit(Qiagen). Both types of saliva (RSF and SSF) were assessed, and their microbial compositions were compared. The V4 region of the 16S rRNA gene amplified using the Q5 High Fidelity DNA polymerase kit(New England BioLabs Inc., Life Technologies Inc.MA) and the 564F(TCG-TCG-GCA-GCG-TCA-GAT-GTG-TAT-AAG-AGA-CAG-AYT-GGG-YDT-AAA-GNG) and 806R(GTC-TCG-TGG-GCT-CGG-AGA-TGT-GTA-TAA-GAG-ACA-GTA-CNV-GGG-TAT-CTA-ATC-C) primers(Eurogentec, Belgium), and sequenced paired end sequenced on the Illumina MiSeq platform(Illumina, San Diego, USA). Of the total samples collected, only the final samples were available for microbiome analysis. Among the 15 saliva samples analyzed, 8 were stimulated saliva and 7 were resting saliva (sample codes in Table 1).

Bioinformatics and statistical methods

The amplicon sequence variants(ASVs) were generated by DADA2[30]. Reads were trimmed, and filtered by quality and size, as previously described[10]. The taxonomy was assigned using the Silva v.138 database. ASVs assigned to Eukaryote, Chloroplast, and Mitochondria were removed before downstream analysis.

The alpha diversity was estimated for ASVs of samples rarefied by the rare curve function from vegan R package. The Shannon's index, Chao1's index, and the Pielou index of samples were determined using the Microbiome R package[31]. A square-root transformed relative abundances of taxa combined at the highest taxonomic level annotated were used to build a Bray-Curtis similarity matrices. Dissimilarities distances were plot in a metric multidimensional scaling(MDS) using the Phyloseq package in R[32].

Results

All participants completed their assigned protocols. Due to insufficient pre-treatment salivary flow in most cases(n=6) resulting in insufficient DNA yield, only final samples were available for microbiome analysis. Of the 15 total saliva samples analyzed, 8 were SSF and 7 were RSF, as indicated by the sample codes in Table 1. Sociodemographic, clinical, and A1c characteristics of this subset of patients are also shown in this table. The average ages of individuals that underwent LLLT, TENS, and LLLT+TENS were 57.0±6.3 years, 54.8±5.4 years, and 53.5±4.9 years, respectively. Although randomization was performed, only male participants were allocated to the laser and LLLT+TENS groups. In the TENS group, however, a higher number of participants led to a balanced male-to-female ratio. All participants reported oral dryness: one with suspected or mild dryness at night or upon waking, and most with mild dryness that impaired normal oral functions. Participants had 13 to 28 teeth (mean 21.8 ± 5.1), 0 to 5 active carious lesions, and 3 to 39 restorations, reflecting varied caries status and dental treatment histories. Among the eight participants included in the study, seven were using at least one antihypertensive medication, including ACE inhibitors, angiotensin receptor blockers, calcium channel blockers, beta-blockers, or diuretics. Six participants were using antidiabetic agents, and three participants were on statin therapy for dyslipidemia, and two reported the use of aspirin. Due to their systemic conditions, it was neither ethical nor feasible to discontinue medications. Self-reported oral hygiene habits were also documented, and nearly all participants reported brushing their teeth at least twice a day.

Table 1 shows the baseline and final salivary flow averages. Of the individuals with RSF ≥ 0.2 ml/min at baseline, most showed an increase in RSF after treatment or maintained similar values. An increase in SSF (or maintenance as normal parameters) was observed in most participants, with 7 out of 8 presenting post-treatment SSF values above 0.7 ml/min. Clinical improvement in saliva production was observed in one participant in the LLLT group, three in the TENS group, and one in the LLLT+TENS group.

The analysis of tongue coating before and after salivary stimulation treatments is also summarized in table 1. The LLLT group showed mixed results, with improvement and no change, the TENS group had both improvements and worsening, and the combined laser+TENS group demonstrated significant improvements in two participants.

Diversity and composition of the salivary microbiome

Shannon diversity analysis of rarefied data(30,000 sequences) revealed significant differences between the laser and TENS groups, though no differences were observed between these and the combined laser+TENS. When analyzing by saliva type(resting or stimulated), these differences were no longer significant. No differences were detected in Shannon diversity concerning saliva type or clinical improvement in salivary flow when comparing baseline and post-treatment salivary flow diagnoses(Fig. 1).

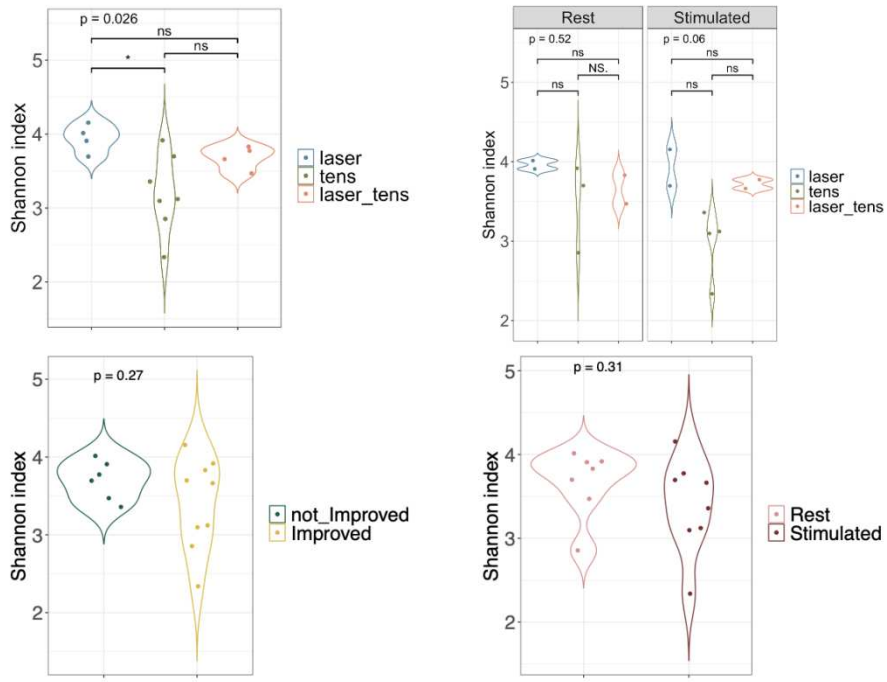

Fig.1 Alpha-diversity indexes displayed by sample group (Laser-LLLT N=4, TENS N=7, or laser+TENS N=4), clinical improvement of the salivary flow, and type of saliva (resting or stimulated) (Shannon index). Statistics using parametric test ANOVA (for groups), and Shapiro-Wilk normality test (W=0.91343, p-value=0.1528)

Beta diversity was measured using Bray-Curtis distances on an MDS plot and displayed by treatment type for reduced salivary flow and saliva type. The resting and stimulated saliva samples in the TENS group were more distantly clustered compared to those in the laser or LLLT+TENS groups. In the laser group, resting and stimulated saliva samples were clustered together, whereas in the combined treatment (LLLT+TENS), resting saliva samples were more distantly clustered than stimulated samples(Fig. 2). After performing statistical analysis using PERMANOVA(ADONIS) with 10,000 permutations(n=15) in a multivariate model that included salivary flow type, intervention group, and tongue substrate, a significant

effect on beta-diversity was observed due to the salivary flow type. When stratified by salivary flow, the intervention also showed a significant effect in the model.

Fig. 3 shows the relative abundance at the phylum and genus levels after treatments. *Firmicutes* and *Actinobacteriota* exhibited the highest relative abundance across all samples, regardless of treatment group. As for the genus level, *Streptococcus* as the most abundant across all samples as expected. Within the TENS group, the relative abundance of the predominant taxa varied more between samples than the other groups. When combining the abundance of all minority taxa (those with less than 1% abundance individually), they collectively emerged as the second most abundant group, showing the high diversity of the salivary microbiome.

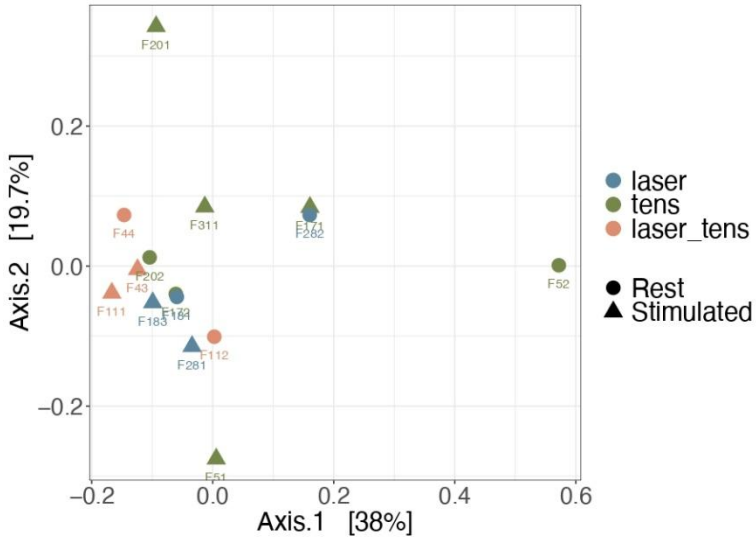

Fig.2 Beta diversity measured by Bray-Curtis distance on MDS plot, displayed by treatment (Laser, TENS or combining Laser and TENS), and in relation to the type of saliva (resting or stimulated). Statistics using PERMANOVA (ADONIS) with 10,000 permutations (n=15). Laser: samples F282, F281, F181 and F183; TENS: samples F51, F52, F201, F202, F171, F172 and F311; Laser + TENS:F111, F112, F44 and F43

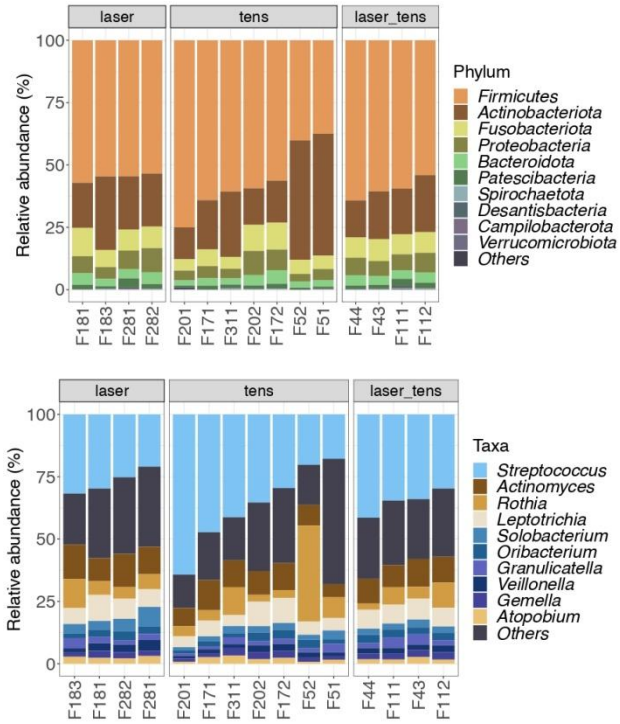

Fig.3 Most abundant phyla in the salivary microbiome after treatment with Laser (N=4), TENS (N=7) or combining Laser and TENS (n=4).Laser: samples F282, F281, F181 and F183; TENS: samples F51, F52, F201, F202; F171, F172 and F311;Laser + TENS: samples F111, F112, F44 and F43

Fig. 4 shows changes in the most abundant salivary microbiome phyla following treatment for reduced salivary flow, emphasizing saliva type and clinical outcomes. In two participants who had sufficient saliva both before and after treatment, we compared the microbiota regardless of treatment group or saliva type (Fig. 4, left most graphs). *Firmicutes* and *Fusobacteriota* showed a tendency to increase, while *Proteobacteria* tended to decrease. As for the genus level, *Streptococcus* remained stable after treatments regardless treatment modality. Notably, the proportion of “others” (low-abundance taxa) decreased after salivary stimulation (Fig. 4, bottom left most graph).

Differences at the phylum level among treatment groups in the final samples were negligible, as well as comparing saliva type (resting or stimulated) or categorized by clinical outcomes of salivary flow improvement post-treatment. An exception was observed for the phyla composition of the participants that presented worse tongue coating after treatment, that presented a tendency of higher levels of *Actinobacteriot*a(Fig. 4, top rightmost graph). The LLLT group showed a greater number of taxa in the predominant microbiota, confirmed by significantly higher alpha diversity. No significant changes were observed in the dominant taxa between rest and stimulated samples, or in relation to salivary flow improvement post-treatment (categorized as better, same, or worse clinical presentation of reduced salivary flow).

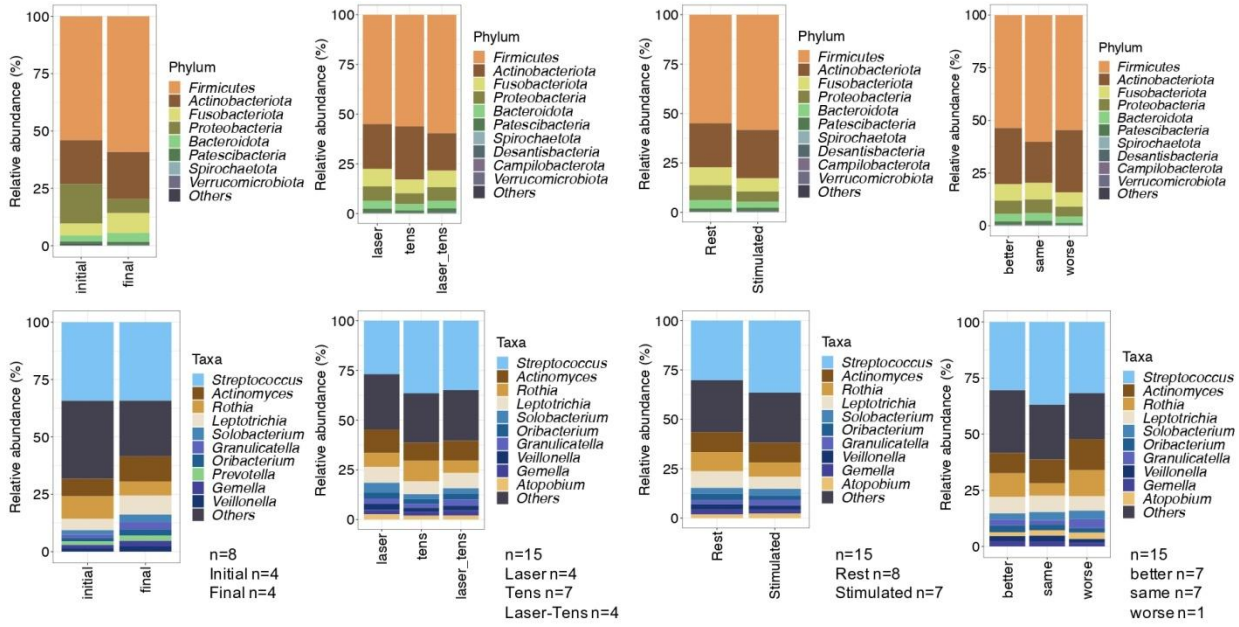

Fig.4 Composition of the most abundant phyla and genera in the salivary microbiome, analysed across the following conditions (from left to right): 1) before and after salivary stimulation, regardless treatment group, 2) following treatment with Laser - LLLT, TENS, or Laser + TENS, 3) based on saliva type (rest or stimulated), and 4) categorized by clinical outcomes post-treatment: less tongue substrate (labelled as “better”), unchanged tongue substrate (labelled as “same”), or increased tongue substrate (labelled as “worse”)

Discussion

This exploratory study investigated the oral microbiome following non-invasive, non-pharmacological therapies aimed at improving salivary function in individuals with T2DM. TENS, LLLT, and their combination were used to stimulate reduced salivary gland activity. Most participants showed stable or increased SSF, and LLLT may have contributed to a reduction in low-abundance microbial taxa, possibly reflecting an enhanced clearance effect. However, the small sample size due to insufficient DNA yield for high-quality NGS sequencing, mostly related to the low saliva volume, resulted in predominantly descriptive data, making before-and-after comparisons difficult. Only two participants provided sufficient saliva for quality in the DNA extraction and paired microbiome assessment (Fig.4 only). This underscores the clinical challenge we aim to address: the need for effective, clinically viable, and economically reasonable methods to manage salivary flow and composition in individuals with T2DM-related reduced salivary flow. The fact that several participants presented with near-zero baseline flow highlights the severity of the condition and reinforces the importance of studying and refining stimulation protocols. By focusing on strategies to restore salivary output, this exploratory work lays essential groundwork for future interventions targeting both functional improvement and downstream effects on the oral microbiome, which may ultimately influence disease risk and oral health outcomes.

Reduced salivary flow in T2DM is multifactorial, potentially influenced by autonomic dysfunction, glandular damage, microcirculatory changes, neuropathy, medication use, and glycemic variability[19, 33, 34]. Despite the majority of participants having uncontrolled T2DM and ongoing medication use, qualitative

findings indicated some improvement in SSF. Even participants who did not show increased flow maintained stable values, and all concluded the intervention with SSF above 0.7 ml/min, a threshold considered clinically acceptable. This improvement, however, did not appear to significantly alter the core microbial composition during the treatment. Importantly, participants did not alter their medication regimens, and drug use did not appear to hinder the effects of physical stimulation. The therapies were generally well tolerated, and all participants completed the full protocol despite the need for frequent clinic visits. Nonetheless, the long-term efficacy of these interventions remains uncertain, underscoring the need for larger, controlled studies to assess durability and to explore the potential benefits of repeated or combined treatments.

The rate of clearance is influenced by factors such as salivary flow rate, saliva volume, and swallowing frequency[14]. High salivary flow rates, both resting and stimulated, aid in the effective removal of fermentable carbohydrates, acids, and desquamated epithelial cells, each carrying around 100 microorganisms[17]. A healthy individual sheds approximately 8×10^{10} bacterial cells per day through normal saliva flow[17]. This process helps eliminate potentially harmful bacteria, contributing to the maintenance of a balanced oral microbiome in symbiosis with its host[17]. If LLLT can enhance these processes while increasing salivary flow, it may offer benefits beyond volume alone. Further research is needed to evaluate its long-term clinical relevance and potential role in preventing oral diseases or modulating the oral microbiome.

It is expected some impact on the oral microbiome composition following the increase in salivary flow. We observed significant lower diversity after laser when compared to TENS(Fig.1). The ability to enhance salivary flow and modulated the microbiome may have influence of the mechanisms of each physical method employed. While TENS was designed to target the major salivary glands through neural stimulation of the autonomic nervous system, laser therapy was applied to stimulate both the major and minor salivary glands. LLLT stimulates electron excitation and creates an electromechanical field in mitochondria, raising calcium levels and promoting cell proliferation while modulating inflammation - an important benefit given that inflammation often accompanies reduced salivary gland activity[35]. A previous study demonstrated that laser alleviates hyposalivation and enhances salivary flow and/or indicators of salivary function, including the levels of H+, Cl-, bicarbonate ions or proteins present in saliva[36].

In another study, different laser's wavelengths, such as 830nm and 685nm, were efficient in the management of hyposalivation, after ten consecutive days of treatment[37]. Then, we opted for the use of different wavelengths here: the shorter wavelength of the red laser offers superficial penetration, making it more effective for stimulating the minor salivary glands; while the longer wavelength of the infrared laser was used for extraoral applications, where its deeper penetration can effectively target the major salivary glands[38].

TENS delivers electrical current via electrodes on major glands, mimicking physiological signals to boost blood flow, oxygenation, and ATP production[39]. It seems more effective at accelerating rather than initiating salivary flow, thus benefiting cases of reduced, not absent, gland function[40, 41]. Electrode positioning affects parotid saliva output, as positive poles produce stronger pulses, potentially improving

RSF. Although studies suggest promising effects, clear evidence linking TENS mechanisms to efficacy is lacking due to low quality of the studies[20]. Our results also showed sample heterogeneity, indicating that individual or external factors may influence outcomes, highlighting the need to clarify these interactions and define when TENS can effectively modulate the microbiome.

Studies have linked microbiota changes to reduced salivary flow, regardless of the underlying exocrinopathy[42, 43]. For instance, aciduric and acidogenic microorganisms are enriched in the oral cavity of individuals living with T2DM compared to healthy ones[44]. Their salivary microbiome is influenced by systemic hyperglycemia, as well as changes in salivary pH, which may be linked to local hyperglycemia[10]. A study by our group demonstrated significant reduction in the salivary flow and salivary acidification of individuals living with T2DM when compared to healthy ones[9]. Consequently, salivary stimulation in T2DM may enhance salivary flow and comfort, aid in pH regulation, and also promote microbiome clearance, even with a modest increase in salivary flow. This speculated "washing effect" observed may involve low-abundance microorganisms, possibly part of a transient microbiota that, under favorable conditions, can become pathobionts and drive oral dysbiosis. Although the salivary flow increase was minimal, it may still help explain the enhanced washing effect and its microbiome impact. Even small flow increases—from zero to 0.8 ml/min (stimulated) or from 0.0006 ml/min (resting)—could relate to microbiological shifts. Notably, individuals treated solely with laser showed a significant rise in microbial diversity, likely due to its anti-inflammatory action, which is relevant given the reduced diversity typically seen in T2DM[45]. As laser acts at the cellular level, the resulting oxygenation may alter the oral environment and, in turn, microbial diversity. The beta-diversity results suggest that the type of salivary flow is a significant variable in explaining differences in the observed composition. When the analysis was stratified by salivary flow type, the effect of the intervention also proved significant in the model. This implies that the applied treatments impacted composition or diversity in a manner dependent on the salivary flow type, highlighting a potential interaction between the intervention and the functional state of the salivary glands.

The sample size, the absence of a control group without T2DM, and the lack of blinding protocols, limit the generalizability of the results. Despite that, variations in caries status and medication use offer a realistic representation of clinical conditions and may serve as an exploratory analysis of real-life scenarios. At the individual level, it is not possible to determine whether reduced salivary flow is due solely to medication use, poor glycemic control, or the interaction of both - especially given that these factors often coexist in this population. As this is an interim analysis of a RCT, we are currently unable to isolate the impact of each variable. Multivariable regression analyses in the future could identify which factors are independently associated with reduced salivary flow. For this reason, we have refrained from drawing direct associations between salivary flow and either medication use or A1c levels at this stage, as such conclusions would be premature and potentially speculative. However, we strongly believe that the microbiological findings remain promising; to our knowledge, this is the first study to present clinical follow-up after salivary stimulation treatments using 16S rRNA amplicon NGS sequencing, paving the way for future research with more robust designs.

Another limitation is the use of the TESS questionnaire, which is not validated for assessing xerostomia; however, it was applied to recruit the participants (confirming dry mouth complaining at baseline - immediately before the first stimulation session) and again at the end of the stimulation treatment, allowing us to understand the level of patient-reported discomfort related to dry mouth. While the smallest detectable change in the salivary flow rate is naturally limited by the resolution of the measurement tools (syringe gradation and time standardization), this method is widely used and accepted in both clinical and research contexts for detecting subtle changes in salivary flow [28]. Also, baseline measurements were taken after the completion of periodontal treatment - approximately 45 to 60 days post-recruitment. It is possible that the periodontal treatment per se resulted in clinical improvements of salivary flow contributed to the observed increase in salivary flow at baseline, and this should be better explored in the future. However, we believe the improved baseline values does not mean the person did not need salivary stimuli, as a recent systematic review shows that the average RSF in healthy individuals may be substantially higher than traditionally cited thresholds [26]. The review identified 0.4 mL/min as a meaningful clinical cutoff to distinguish between healthy individuals and those with hyposalivation-associated conditions such as diabetes.

Conclusion

This interim exploratory analysis provides initial indications that physical stimulation may assist in managing T2DM-related reduced salivary flow. While no major shifts were observed in the dominant oral microbiota, subtle changes in low-abundance taxa suggest a potential “clearance” effect, possibly influenced by increased salivary flow or alterations in saliva composition. Although these microbiome findings are preliminary, they offer valuable insight and generate important hypotheses for future research. As the first study to investigate this specific saliva stimulation protocol using clinical and microbiological outcomes, these results may inform the design of larger, well-powered studies. Given the short duration of treatment and known inter-individual variability, further investigation is essential to confirm and expand upon these observations.

Declarations

Ethics approval

The study received approval from the Ethics Committee of the University of Brasília, under the number 4.748.761 CAAE 45184721.7.0000.0030, in accordance with the Declaration of Helsinki.

Consent to participate

Informed consent was obtained from all individual participants included in the study.

Funding Statement

This study was funded by the Foundation for Research Support of the Federal District (FAP-DF) [process no.16991.78.45532.26042017] and National Council for Scientific and Technological Development and Brazilian Hospital Services Company (CNPq/EBSERH) [project no. 408020/2021-0].

Clinical Trial Number

This study was registered in the Brazilian Registry of Clinical Trials (ReBEC). Clinical Trial Number: RBR-3tqv8r3 (registered in 13th April 2023‖http://www.ensaiosclinicos.gov.br/).

References

1. Vieira Lima, C., et al., *Can hyperglycemia be associated with caries activity and root caries in adults?* Primary care diabetes, 2023. **17**(1).

2. Carramolino-Cuéllar, E., et al., *Salivary flow and xerostomia in patients with type 2 diabetes.* Journal of Oral Pathology and Medicine, 2018. **47**(5): p. 526-530.

3. Memon, M.A., et al., *Aetiology and associations of halitosis: A systematic review.* Oral Diseases, 2022(January): p. 1-7.

4. Jo, J.K., et al., *Identification of salivary microorganisms and metabolites associated with halitosis.* Metabolites, 2021. **11**(6).

5. Wolff, A., et al., *A Guide to Medications Inducing Salivary Gland Dysfunction, Xerostomia, and Subjective Sialorrhea: A Systematic Review Sponsored by the World Workshop on Oral Medicine VI*, in *Drugs in R and D*. 2017.

6. Gomar-Vercher, S., et al., *Stimulated and unstimulated saliva samples have significantly different bacterial profiles.* PLoS One, 2018. **13**(6): p. e0198021.

7. Zaura, E., et al., *Defining the healthy "core microbiome" of oral microbial communities.* BMC Microbiol, 2009. **9**: p. 259.

8. Mozaffari, H.R., et al., *Salivary profile in adult type 2 diabetes mellitus patients: A case-control study.* Journal of the Pakistan Medical Association, 2019. **69**(2): p. 190-194.

9. Marques, R.C.R., et al., *Salivary parameters of adults with Diabetes Mellitus: a systematic review and meta-analysis.* Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology, 2022.

10. Vieira Lima, C.P., et al., *Enrichment of sulphate-reducers and depletion of butyrate-producers may be hyperglycaemia signatures in the diabetic oral microbiome.* J Oral Microbiol, 2022. **14**(1): p. 2082727.

11. Chávez, E.M., et al., *A longitudinal analysis of salivary flow in control subjects and older adults with type 2 diabetes.* Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics, 2001. **91**(2): p. 166-173.

12. Dawes, C., *Salivary flow patterns and the health of hard and soft oral tissues.* J Am Dent Assoc, 2008. **139 Suppl**: p. 18S-24S.

13. Britse, A. and F. Lagerlof, *The diluting effect of saliva on the sucrose concentration in different parts of the human mouth after a mouth-rinse with sucrose.* Arch Oral Biol, 1987. **32**(10): p. 755-6.

14. Dawes, C., *A mathematical model of salivary clearance of sugar from the oral cavity.* Caries Res, 1983. **17**(4): p. 321-34.

15. Lagerlof, F. and C. Dawes, *The volume of saliva in the mouth before and after swallowing.* J Dent Res, 1984. **63**(5): p. 618-21.

16. Singh, M., et al., *Characterizing Microbiota from Sjogren's Syndrome Patients.* JDR Clin Trans Res, 2021. **6**(3): p. 324-332.

17. Lynge Pedersen, A. and D. Belstrøm, *The role of natural salivary defences in maintaining a healthy oral microbiota.* Journal of dentistry, 2019. **80 Suppl 1**.

18. Grisi, D.C., et al., *The Complex Interrelationship between Diabetes Mellitus, Oral Diseases and General Health.* Current diabetes reviews, 2022. **18**(3).

19. Sreebny, L.M., et al., *Xerostomia in diabetes mellitus.* Diabetes Care, 1992. **15**(7): p. 900-911.

20. Melo, J.L.D.M.A., et al., *A scoping review on hyposalivation associated with systemic conditions: the role of physical stimulation in the treatment approaches.* BMC Oral Health, 2023. **23**(1).

21. Pinho e Souza Coelho, C., et al., *Clinical management of salivary gland hypofunction in head and neck cancer patients: a scoping review of physical salivary stimulation methods*. 2023: Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology.

22. Napeñas, J.J., M.T. Brennan, and P.C. Fox, *Diagnosis and treatment of xerostomia (dry mouth).* Odontology, 2009. **97**(2): p. 76-83.

23. Bicknell, B., et al., *Photobiomodulation of the microbiome: implications for metabolic and inflammatory diseases.* Lasers in Medical Science, 2019. **34**(2): p. 317-327.

24. Ainamo, J. and I. Bay, *Problems and proposals for recording gingivitis and plaque.* International dental journal, 1975. **25**(4).

25. Tonetti, M. and M. Sanz, *Implementation of the new classification of periodontal diseases: Decision-making algorithms for clinical practice and education.* Journal of clinical periodontology, 2019. **46**(4).

26. da Silva, J., et al., *What Is the Normal Salivary Flow Rate in Healthy Adults? A Systematic Review with Meta-Analyses.* JDR clinical and translational research, 2025.

27. Wang, S., et al., *Investigation of the clinical value of total saliva flow rates.* Archives of oral biology, 1998. **43**(1).

28. Ribeiro, B.A., et al., *Impact of detection criteria on coronal and root caries estimates in adults with and without type 2 diabetes mellitus.* Clinical oral investigations, 2022. **26**(4).

29. Shimizu, T., T. Ueda, and K. Sakurai, *New method for evaluation of tongue-coating status.* Journal of oral rehabilitation, 2007. **34**(6).

30. Callahan, B.J., et al., *DADA2: High-resolution sample inference from Illumina amplicon data.* Nat Methods, 2016. **13**(7): p. 581-3.

31. Lahti, L. and S. Shetty, *Tools for microbiome analysis in R*. 2017.

32. McMurdie, P.J. and S. Holmes, *phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data.* PLoS One, 2013. **8**(4): p. e61217.

33. Meurman, J.H., et al., *Saliva in non-insulin-dependent diabetic patients and control subjects.* Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology, 1998. **86**(1): p. 69-76.

34. de Lima, A.K.A., et al., *Diabetes mellitus and poor glycemic control increase the occurrence of coronal and root caries: a systematic review and meta-analysis.* Clinical Oral Investigations, 2020. **24**(11): p. 3801-3812.

35. Fidelix, T., et al., *Low-level laser therapy for xerostomia in primary Sjögren's syndrome: a randomized trial.* Clinical Rheumatology, 2018. **37**(3): p. 729-736.

36. Golež, A., et al., *Effects of low-level light therapy on xerostomia related to hyposalivation: a systematic review and meta-analysis of clinical trials.* Lasers in Medical Science, 2021.

37. Brzak, B.L., et al., *Different Protocols of Photobiomodulation Therapy of Hyposalivation.* Photomed Laser Surg, 2018. **36**(2): p. 78-82.

38. De Carvalho E Silva, R.M., et al., *Photobiomodulation for the management of xerostomia and oral mucositis in patients with cancer: a randomized clinical trial.* Lasers in Medical Science, 2023. **38**(1).

39. Johnson, M.I., C.H. Ashton, and J.W. Thompson, *An in-depth study of long-term users of transcutaneous electrical nerve stimulation (TENS). Implications for clinical use of TENS.* Pain, 1991. **44**(3): p. 221-229.

40. Hargitai, I.A., R.G. Sherman, and J.M. Strother, *The effects of electrostimulation on parotid saliva flow: a pilot study.* Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 2005. **99**(3): p. 316-20.

41. Dyasnoor, S., S. Kamath, and N.F.A. Khader, *Effectiveness of Electrostimulation on Whole Salivary Flow Among Patients with Type 2 Diabetes Mellitus.* Perm J, 2017. **21**: p. 15-164.

42. AlmståhI, A., et al., *Oral microbiota associated with hyposalivation of different origins.* Oral microbiology and immunology, 2003. **18**(1).

43. Saúco, C., et al., *Hyposalivation but not Sjögren's syndrome associated with microbial dysbiosis in women.* Frontiers in microbiology, 2023. **14**.

44. Cena, J., et al., *Enrichment of Acid-Associated Microbiota in the Saliva of Type 2 Diabetes Mellitus Adults: A Systematic Review.* Pathogens (Basel, Switzerland), 2023. **12**(3).

45. Sabharwal, A., et al., *The salivary microbiome of diabetic and non-diabetic adults with periodontal disease.* Journal of Periodontology, 2019. **90**(1): p. 26-34.

Table 1 Characteristics of the participants with Diabetes Mellitus and their salivary characteristics before (baseline) and after management of salivary gland hypofunction using of physical stimulation methods

Treatment/Group	**Laser (G1)**		**TENS (G2)**				**Laser + TENS (G3)**	
Participants	1	2†	3	4	5	6	7	8†
Sample label	F282* F281**	F181* F183**	F51** F52*	F201** F202*	F171** F172*	F311**	F111** F112*	F43** F44*
Participants characteristics								
Gender	Male	Male	Male	Female	Male	Female	Male	Male
Age	53	62	55	51	56	63	50	57
Age (Mean age ± SD)	57.5 ± 6.36		54.8 ± 5.40				53.5 ± 4.94	
A1c (%)	5.8	6	7.9	8.4	7.8	8.3	8	7.1
Number of teeth	26	17	21	26	24	28	13	20
Number of active caries lesions (surfaces)	1	1	3	4	0	0	2	0
Number of inactive caries lesions (surfaces)	2	10	2	0	5	4	2	1
Number of restorations (surfaces)	39	5	3	30	11	18	4	12
Baseline (immediately before the treatment)***								
Baseline SSF (ml/min)	1.00	1.20	0.50	1.0	0.62	0.28	2.54	0.80
Baseline RSF (ml/min)	0.02	0.4	0.0	0.18	0.3	0.0	0.2	0.2
Initial toague coating score	0	6	8	3	1	0	12	10

Initial dry mouth complaint-TESS	4	2	2	2	2	4	4	1
After treatment								
Final SSF (ml/min)	1.50	1.20	0.80	1.5	1.10	0.34	1.60	1.60
Final RSF (ml/min)	0.02	0.4	0.0006	0.2	0.6	0.01	0.3	0.15
Final tongue coating score	3	6	1	7	3	0	6	2
Final dry mouth complaint-TESS	2	1	1	1	1	2	1	1

SSF= stimulated salivary flow; RSF = resting salivary flow. * = final RSF; ** = final SSF; *** = baseline data collected post-recruitment, specifically 45 to 60 days following tailored periodontal treatment. Therefore, participants may have shown improved salivary flow at baseline compared to their values at the time of recruitment. †=Patients who exhibited both initial and final SSF and RSF, as analyzed in the first graph of Fig. 4. Analysis of the lingual substrate was performed visually in virtual sextants on the lingual dorsum, which received the following score: 0 = no substrate; 1= light substrate (visible papillae); 2= severe substrate (no visible papillae). The sum of the scores was the final score described for each participating individual. Dry mouth complaint according to TESS questionary involved the following options: 1 = No complaints of dry mouth; 2= Suspected or mild feeling of dryness at night or upon waking; 3= Mild complaint of dryness all the time that prevents normal oral functions; 4= Moderate complaint of dryness with some degree of functional impairment but no perceived health risk, as well as difficulty in swallowing dry foods or speaking; 5= Severe complaint, definite perception of diminished well-being, significant impairment or disability, as well as difficulty swallowing any food, patient needs to drink water all the time and complaint of mouth pain