BioMed Research International

Research on Saliva Secretion and Composition

Lead Guest Editor: Elsa Lamy
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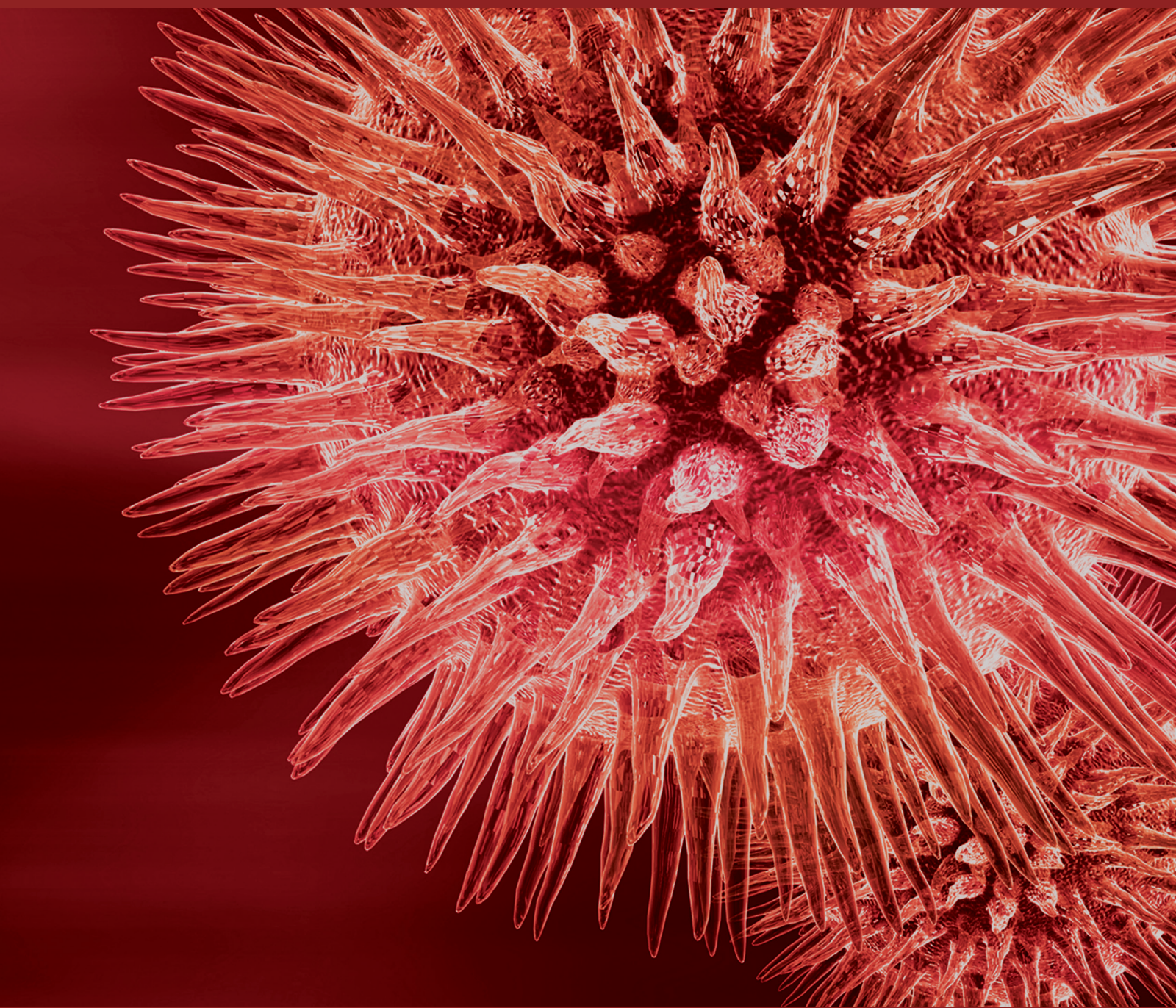

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Editorial

Research on Saliva Secretion and Composition

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In the last two decades, saliva gained enormous attention and has become focus of a high number of studies, which resulted in a considerable progress in the knowledge about this biofluid. The technological and analytical advances, including the application of omics approaches, allowed the identification of numerous molecules in saliva, many of which are present in this fluid in proportion to blood. This permitted saliva to be seen as a fluid with potential for diagnosis of different pathologies and physiological conditions. Moreover, the noninvasive nature of its collection is an advantage over serum and reinforced the interest in saliva research.

This special issue put together high-quality research, highlighting the diversity of areas in which the study of saliva is of interest.

Ten articles, three reviews and seven original research articles, were selected for publication. The review presented by Z. Zian et al., about the use of saliva in systemic sclerosis studies, focuses on the proteomic technologies, highlighting the potentials and limitations of using saliva as a tool of diagnostic of this disease. F. Asa'ad et al. reviewed the current knowledge in saliva research applied to psoriasis, emphasising that salivary biomarkers of this disease can be valuable in the future. Finally, the review of E. Kubala et al. presented information about the diagnostic value of saliva as a research material, in the field of dental treatment. The authors concluded that more and more internists, paediatricians, pharmacologists, clinical and forensic pathologists, endocrinologists, immunologists, psychologists, and dentists are discovering the benefits offered by saliva as diagnostic tool.

Two of the original articles presented results about salivary antioxidants variations during disease treatment. One of these works, reported by A. Tvarijonaviciute et al., studied the effect of *Chamaemelum nobile* on the oxidative stress parameters and their relationship to clinical symptoms in patients with oral lichen planus. The obtained results suggest that use of *Chamaemelum nobile* could stabilize this disease, at least in medium-low severity cases. Meanwhile, K. Fejfer et al. studied oxidative stress in saliva in morbid obese individuals, before and after bariatric surgery. They observed the presence of oxidative stress-related modifications of salivary biomolecules, which although improved after bariatric surgery was not completely effective in restoring redox balance in the oral cavity.

Besides its potential in the diagnosis of oral and systemic diseases, saliva also has a major importance in food perception. Evidences show that saliva modulates oral sensations, such as astringency and/or basic tastes, or even aroma perception, suggesting that more knowledge about this biofluid composition and the factors influencing its secretion should be evaluated. Under this topic, two articles present results about the relationship between saliva and taste: (1) S. Satoh-Kuriwada et al. evaluated the effects and mechanisms of tastes on labial minor salivary gland secretion and concluded that although all basic tastes cause a gustatory-reflex secretion in minor glands, sour and umami tastes have a major effect; (2) Y. Feng et al. characterized the composition, in microorganisms and their metabolites, of the film lining the tongue, and highlighted the importance in considering the salivary microbiome in studies evaluating relationship between oral medium characteristics and taste perception.

On the other hand, a number of studies exist dealing with the effects of salivary flow influence on the analytes of

interest. However, the possible effects that could result in salivary flow changes are less studied. With the aim of filling this gap of knowledge, R. H. Affoo et al. evaluated the effect of tooth brushing on salivary flow rate, comparing manual and electrical tooth brushing. The results indicate that the flow rate increases up to 5 minutes following this process. This observation was even more pronounced in older individuals.

Finally, salivary proteomics was performed in two different studies. K. T. B. Crosara et al. using proteomic approaches identified a number of interactions between amylase and other proteins present in saliva, pioneering the exploration of the vast salivary interactome. Interestingly, the authors suggest that salivary amylase may have other major functions besides carbohydrate digestion. The second article was reported by S. Lucena et al. being the only one treating nonhuman saliva. In this study, it was observed that dog saliva proteome is influenced by the breed of the animals. Moreover, acid stimulation, which is frequently used as a way of stimulating higher saliva secretion, induced changes in saliva protein composition that should be taken into account in dogs as well as other animal species and humans when designing the experiments.

Overall, studies that form the presented special issue, although coming from different fields, report the potential of saliva as a noninvasive biofluid and highlight the importance of going deep in this area.

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Research Article

The Associations between Biochemical and Microbiological Variables and Taste Differ in Whole Saliva and in the Film Lining the Tongue

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The objective of this work was to investigate whether the biological film lining the tongue may play a role in taste perception. For that purpose, the tongue film and saliva of 21 healthy subjects were characterized, focusing on microorganisms and their main metabolic substrates and products. In parallel, taste sensitivity was evaluated using a test recently developed by our group, and the links between biological and sensory data were explored by a correlative approach. Saliva and tongue film differed significantly in biochemical composition (proportions of glucose, fructose, sucrose, and lactic, butyric, and acetic acids) and in microbiological profiles: compared to saliva, tongue film was characterized by significantly lower proportions of Bacteroidetes ($p<0.001$) and its main genus *Prevotella* ($p<0.01$) and significantly higher proportions of Firmicutes ($p<0.01$), Actinobacteria ($p<0.001$), and the genus *Streptococcus* ($p<0.05$). Generic taste sensitivity was linked to biological variables in the two compartments, but variables that appeared influent in saliva (flow, organic acids, proportion of Actinobacteria and Firmicutes) and in tongue film (sugars and proportions of Bacteroidetes) were not the same. This study points to two interesting areas in taste research: the oral microbiome and the specific characterization of the film lining the tongue.

1. Introduction

Eating behavior is a key factor of health in humans: some of the major pathologies affecting the modern societies such as obesity, cardiovascular diseases, or type 2 diabetes have been reported to be strongly linked to it. Determinants of eating behavior are various and comprise, for example biological, psychological, and socioeconomic factors. Among biological factors, the sense of taste participates in the sensory perception of the food and, thus, influences food choices. Impairments in taste perception can, for example, lead to eating disorders and malnutrition [1]. Probably the most objective way of characterizing and classifying subjects according to their taste function is to measure their taste sensitivity and more precisely their individual detection threshold. This approach is thus widely used in patients with oral complaints [2, 3] or with diet-related conditions such as obesity [4, 5].

Taste perception occurs after activation of specialized receptors in the taste buds on the tongue. Saliva is the principal fluid that interacts with food and it is the medium that bathes the taste buds; thus it plays an important role in taste perception, through several mechanisms such as protection of the taste receptors or transport of taste compounds [6]. Several studies have indeed described the relationship between salivary composition and taste sensitivity, for example, to bitterness [7–9] or to the taste of oleic acid [10]. Besides free-flowing saliva, the biological film lining the tongue

Table 1: Primers used for qPCR assays.

Primer	Oligonucleotide sequence (5′-3′)	Target	Reference
SPU Fwd	AAACTCAAAKGAATTGACGG	All bacteria	a
SPU Rev	CTCACRRCACGAGCTGAC	All bacteria	a
ACT Fwd	TACGGCCGCAAGGCTA	Actinobacteria	a
ACT Rev	TCRTCCCCACCTTCCTCCG	Actinobacteria	a
BACT Fwd	CRAACAGGATTAGATACCCT	Bacteroidetes	a
BACT Rev	GGTAAGGTTCCTCGCGTAT	Bacteroidetes	a
FIRM Fwd	TGAAACTYAAAGGAATTGACG	Firmicutes	a
FIRM Rev	ACCATGCACCACCTGTC	Firmicutes	a
γ-P Fwd	TCGTCAGCTCGTGTYGTGA	γ-Proteobacteria	a
γ-P Rev	CGTAAGGGCCATGATG	γ-Proteobacteria	a
β-P Fwd	ACTCCTACGGGAGGCAGCAG	β-Proteobacteria	b
β-P Rev	TCACTGCTACACGYG	β-Proteobacteria	b
Fuso Fwd	CGCAGAAGGTGAAAGTCCTGTAT	*Fusobacterium* spp	c
Fuso Rev	TGGTCCTCACTGATTCACACAGA	*Fusobacterium* spp	c
Veil Fwd	A(C/T)CAACCTGCCCTTCAGA	*Veillonella* spp	d
Veil Rev	CGTCCCGATTAACAGAGCTT	*Veillonella* spp	d
Strep Fwd	GTACAGTTGCTTCAGGACGTATC	*Streptococcus* spp	e
Strep Rev	ACGTTCGATTTCATCACGTTG	*Streptococcus* spp	e
Prev Fwd	CACCAAGGCGACGATCA	*Prevotella* spp	f
Prev Rev	GGATAACGCCYGGACCT	*Prevotella* spp	f

[a] De Gregoris et al., 2011; [b] Pécastaings et al., 2016; [c] Suzuki et al., 2004; [d] Rinttilä et al., 2004; [e] Picard et al., 2004; [f] Marathe et al., 2012.

surface is even more intimately in contact with the taste buds. The numerous depressions of the tongue dorsum form a unique ecological site, which provides a large surface area for the accumulation of saliva, oral debris, and microorganisms [11]. Thus, the tongue is coated with a film comprising bacteria, desquamating cells, and residual saliva. The term "tongue coating" is also used in the literature although it very often refers to an undesirable excess of biological material. Some studies have linked tongue coating to taste perception. Lower overall taste performance (sensitivity to four tastants) was marginally observed in elderly patients with a coated tongue [3], and reduced recognition thresholds of saltiness and acid were observed in nursed elderly after light scraping the anterior half of the tongue [12]. Both cases support the assumption that the film lining the tongue may influence taste perception, and we formulate the hypothesis that more particularly the bacterial component of this film deserves attention. This is based on several direct or indirect observations suggesting that oral bacteria could modulate the sense of taste. Thus, Solemdal et al. [13] have made the connection between higher salivary counts of the bacteria *Streptococci* and *Lactobacilli* and reduced perception of sour taste. We also observed that a low sensitivity to the taste of fatty acids was associated with a high concentration of organic acids, probably of bacterial origin, in saliva [10]. Two possible mechanisms were mentioned: first, higher bacterial loads in the tongue film would set a barrier limiting the access of taste molecules to the taste receptors; second, bacterial metabolism may modulate the concentration of tastants (e.g., substrates such as sugars or amino-acids, end-products such as organic acids) near the taste receptors and thus modify taste sensitivity according to the sensorial adaptation mechanism.

In this context, this study had two main objectives: first, it aimed to characterize the composition of saliva and tongue film in healthy subjects, focusing on microorganisms and their main metabolic substrates and products (sugars and organics acids). Second, it aimed to investigate whether variability in these indices, especially microorganism profile, was related to variability in sensitivity to the five basic tastes (sweet, sour, salty, bitter, and umami).

2. Materials and Methods

2.1. Chemicals and Reagents. Fructose, lactose, sucrose, acetic acid, propionic acid, butyric acid, and lactic acid were obtained commercially from Sigma-Aldrich (Steinheim, Germany). Glucose was purchased from Merck (Darmstadt, Germany). The primers (Eurogentec, Belgium) used for preamplification reaction (PCR) are shown in Table 1.

2.2. Subjects and Sampling of Saliva and Tongue Film. The study and protocols were approved by a relevant ethical committee (Comité de Protection des Personnes Ouest V, n° 2016-A01954-47). Written informed consent was obtained from the participants. Twenty-one healthy subjects (11 females, 10 males, 22 to 60 years old) who all declared themselves to be in good oral health participated in this study. More precisely the exclusion criteria were as follows: smokers, pregnant women, food allergy sufferers, long term (over one month) medicated subjects, subjects who took an antibiotic course, had dental treatment, or used an antiseptic mouthwash in the preceding month, subjects who ever received head and neck

radiotherapy, and sufferers from pathologies affecting the oral cavity (e.g., Sjögren syndrome, lichen planus, gingivitis). In addition none of the subjects brushed their tongue as part of their oral hygiene routine. Donors were instructed not to eat or drink at least 2h before sample collection, which occurred between 10 and 11 a.m. Unstimulated whole saliva was collected by direct draining into a 5 ml weighed tube during 3 minutes. After a short rest, seated participants swallowed residual saliva, immediately stuck their tongue out as far as possible, and maintained this pose while the film was taken. All samples were collected by the same experimenter. Tongue film was collected by scraping the tongue with a plastic sterile stick from the root to the apex, applying one single scraping motion. The whole sampling procedure was applied on two separate days per subject. On the first day, weights were recorded and samples were prepared for biochemical and microbiological analyses. On the second day, the pH of saliva and film samples was measured immediately after collection with a microelectrode (IQ240 pH meter, IQ Scientific Instruments, Carlsbad, CA, USA).

2.3. Determination of Sugars and Acids Concentration by HPLC. Saliva was diluted 1/2 and tongue film 1/4 with Milli Q water. Samples were centrifuged at 15000 g for 15 min at 4°C, and the supernatant was further diluted 1:10 and filtered through a 0.22 μm nylon filter.

Sugars were analyzed using Dionex ICS-3000 ion chromatographic system (Dionex, Sunnyvale, USA) consisting of a gradient pump chromatography enclosure with a 5-μL injection loop and an electrochemical detector. The sugar composition of the sample was determined by pulsed amperometric detector (PAD), using a CarboPac 1 column (2 × 250 mm i.d., 5 mm, Dionex) and 100mM NaOH as mobile phase at a flow rate of 0.25 mL/min.

Organic acids were also analyzed by Dionex ICS-3000 ion chromatography system (Dionex, Sunnyvale, USA) equipped with an electroconductivity detector. Ion chromatography was carried out using IonPac AS11-HC column (Dionex, 4×250 mm). The mobile phase, 0.8 mM NaOH, was at a flow rate of 0.25 mL/min and at room temperature. The organic acids were detected by chemical suppressed conductivity using an anion-ICE micromembrane suppressor.

Sugars and organic acids were identified and quantified according to the retention time and signal intensity of reference compounds. The standard curves were obtained using glucose, fructose, sucrose, and lactose at concentrations ranging from 0.005 to 2.5 mg/L and lactate, acetate, propionate, and butyrate at concentrations ranging from 0.15 to 37.5 mg/L.

2.4. Enumeration of Colony-Forming Units (CFU). Serial dilutions (10^{-3} to 10^{-6}) of diluted saliva and film samples were prepared and 100 μl samples were plated on Columbia medium (Biokar) supplemented with glucose (10 g/l) and 5% (v/v) defibrinated sheep blood. Counting of colonies was performed after incubating the plates in two different conditions, aerobic and anaerobic (5% CO_2), at 37°C for 48 h. The total concentration of cultivable microorganisms was approximated by adding the concentrations of aerobic and anaerobic microorganisms.

2.5. DNA Extraction and qPCR. DNA extraction was performed using Nucleo-Spin DNA tissue kit (Macherey-Nagel, Germany) following the manufacturer's instructions with a minor modification; namely, in the lysis step, 0.1 μm glass beads were added and the mixture was vortexed at maximum speed for 10 min. Quality of the extracted DNA was determined using a NanoDrop ND-1000 spectrophotometer and gel electrophoresis. PCR amplifications were performed in triplicate in 20 μl reaction mixtures, containing 4 μl of DNA extract, 300 nM of each primer, and 10 μl of SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad) in a CFX96 Real-Time PCR Detection System (Bio-Rad, USA). Thermal cycling conditions were an initial denaturation at 95°C for 3 min followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. Efficiency of amplification was determined by running a standard curve for each primers couple. Primers were selected to target total bacteria and the most representative phyla, classes, or genera of oral bacteria (Table 1). All primers targeted 16S rRNA, except for *Streptococcus* spp whose primers targeted *tuf* gene. The percentage (P) of each specific group (SPE) was determined relatively to the total bacteria (SPU) with the equation: $P=(\text{Eff. SPU})^{Ctspu}/(\text{Eff.SPE})^{Ctspe}x100$, with Eff being the PCR amplification efficiency. Preliminary experiments revealed that α-Proteobacteria was not detected in the oral microbiota. Results for the phylum Proteobacteria were therefore calculated by adding results of the two classes β-Proteobacteria and γ-Proteobacteria. Similarly, results of the genus *Fusobacteria* were considered as an approximation for the phylum Fusobacteria because it is almost exclusively the only genus of this phylum found in the oral cavity [14].

2.6. Determination of Taste Sensitivity Scores. Taste sensitivity was evaluated for the five basic tastes: sweet (fructose), salty (sodium chloride), sour (citric acid), bitter (quinine hydrochloride), and umami (monosodium glutamate). All tastants were of food grade quality and deionized water was used as solvent. For each taste, the lowest perceived concentrations were determined thanks to a test (T@sty test™) recently developed by our group (Patent WO2015/165880). This test uses test-sheets made from edible wafer paper. A test-sheet consists of six series of three precut discs (18 mm diameter). For each series, one disc contains the tastant (the "tasty disc") and the two others are neutral. On one test-sheet, the tastant concentration increases gradually from the first series to the sixth series (Figure 1). The concentrations were chosen to obtain a Gaussian distribution of the individual scores across the general population.

For each series, subjects were instructed to taste the three discs by placing them on the tip of the tongue for a few seconds and to find the tasty disc by answering the following question: Which disc is different from the other two? Subjects were instructed to randomly answer if no difference was perceived. Answers recorded for a full test-sheet were converted to a score ranging from 0 (low sensitivity, highest concentration not perceived) to 6 (high sensitivity, all concentrations perceived). Calculation of the score was

Table 2: Salivary flow rates and weight of tongue film sampled (n=21).

	Mean	Min	Max	Median	Standard deviation (SD)	SD %
Salivary flow rates (g/min)	0.45	0.05	1.13	0.36	0.31	68.6
Weight of tongue film (g)	0.025	0.007	0.051	0.020	0.012	48.5

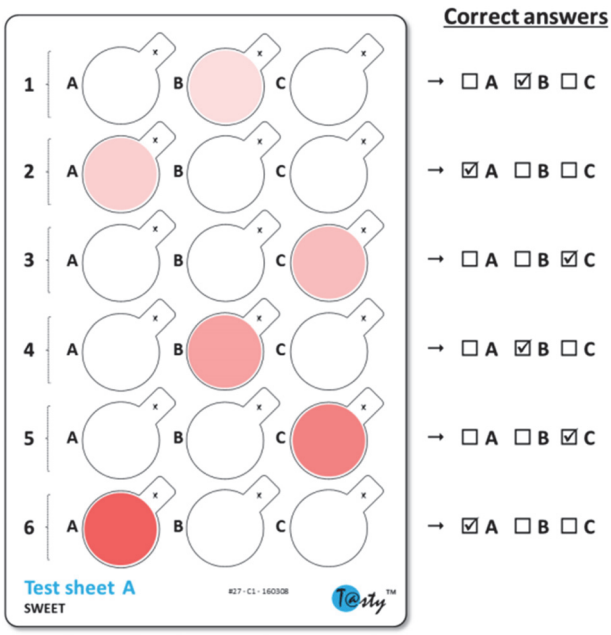

Figure 1: Example of a test-sheet. Tasty discs are indicated in color, with increasing color intensity corresponding to increasing concentrations of the tastant. Within each series of three discs, subjects are asked to identify which one is the tasty disc.

inspired by the Best-Estimate Threshold (BET) method: each test-sheet resulted in a series of 6 answers (correct/wrong) ordered by increasing concentration of tastant. The score corresponds to the number of consecutive correct answers after the highest concentration for which a wrong answer was given. For example, a score of 2 corresponds to the case where answers given for the sixth and fifth series are correct but answer for fourth series is wrong, whatever the answers given for the other series. For each taste, the sensitivity score was the average calculated on four replicates.

2.7. Statistical Analysis. The statistical analysis was conducted using Statistica (StatSoft). Paired t-tests were performed to evaluate the difference in pH and composition between saliva and tongue film. Pearson correlation was calculated between pH in saliva and pH in film. The correlation between taste sensitivity scores and biological data was evaluated using Spearman correlation tests. The choice of a nonparametric test in that case is justified by the presence of censored data (scores = 0 or 6) in the sensory dataset. A score of 0 indicates that the subject would perceive the tastant only above the highest concentration presented, while a score of 6 indicates that the subject could perceive the tastant even below the lowest concentration presented.

3. Results

3.1. pH of Saliva and Tongue Film. The pH of saliva varied from 5.89 to 7.00 (mean 6.47 ± 0.29) and it was significantly lower ($p < 0.001$) than the pH of tongue film which varied from 6.53 to 7.86 (mean 7.15 ± 0.36). Moreover, a significant correlation ($r = 0.839$; $p < 0.001$) was observed between the pH of saliva and pH of film.

3.2. Salivary Flow Rate and Weight of Tongue Film. Results are depicted in Table 2. Saliva flow rates showed large variations, ranging from 0.05 to 1.13 g/min (mean 0.45 ± 0.31 g/min). The wet weights of tongue film varied between 7 and 51 mg (mean 25 ± 12 mg).

3.3. Sugars and Organic Acids Profiles. The concentrations and percentages of four sugars (glucose, fructose, sucrose, and lactose) and four organic acids (lactic acid, acetic acid, propionic acid, and butyric acid) in saliva and tongue film are shown in Table 3. Glucose (16.9 ± 10.5 μg/g in saliva; 6.2 ± 7.0 μg/g in film) and acetic acid (145.8 ± 121.5 μg/g in saliva; 389.9 ± 242.3 μg/g in film) were the predominant sugar and acid, respectively.

As shown in Table 3, the proportion of glucose was significantly lower ($p < 0.05$) in film than in saliva while the proportions of fructose and sucrose were significantly higher ($p < 0.05$) in film.

The proportions of lactic and butyric acid were significantly lower ($p < 0.0001$ for lactic acid, $p < 0.01$ for butyric acid) in film than in saliva while the proportion of acetic acid was significantly higher ($p < 0.001$) in film (Table 3).

3.4. Concentrations of Cultivable Microorganisms. The concentrations in $\log_{10}$(CFU/g) of cultivable aerobic and anaerobic microorganisms in saliva and film are shown in Figure 2. In saliva the mean concentration of aerobic microorganisms (2.10×10^7 CFU/g) was slightly higher than that of anaerobic microorganisms (1.56×10^7 CFU/g), whereas in film, the mean concentrations of aerobic and anaerobic microorganisms were almost similar (1.94×10^8 CFU/g and 1.95×10^8 CFU/g, respectively). In other words, the ratio of anaerobic/aerobic microorganisms was higher in film than that in saliva.

Table 3: Concentrations and relative proportions (percentages) of sugars and organic acids in saliva and tongue film. The p value represents the level of significance when comparing the relative proportions of each metabolite between saliva and film (paired t-test).

Compound	Concentration in saliva (μg/g±SD)	Concentration in film (μg/g±SD)	Proportion in saliva (%± SD)	Proportion in film (%± SD)	p
glucose	16.9 ± 10.5	6.2 ± 7.0	95.4 ± 10.5	89.7 ± 7.0	<0.05
fructose	0.3 ± 0.3	0.3 ± 0.2	1.6 ± 0.3	3.6 ± 0.2	<0.05
sucrose	0.1 ± 0.1	0.2 ± 0.3	0.4 ± 0.1	2.4 ± 0.3	<0.05
lactose	0.5 ± 0.3	0.3 ± 0.5	2.7 ± 0.3	4.3 ± 0.5	ns
lactate	15.0 ± 19.1	5.1 ± 3.1	10.9 ± 11.5	1.3 ± 0.9	<0.001
acetate	145.8 ± 121.5	389.9 ± 242.3	67.6 ± 9.4	80.1 ± 7.1	<0.0001
propionate	44.9 ± 55.8	96.5 ± 96.9	16.2 ± 6.3	16.2 ± 6.3	ns
butyrate	12.6 ± 14.1	12.0 ± 15.1	5.4 ± 3.9	2.3 ± 2.9	<0.01

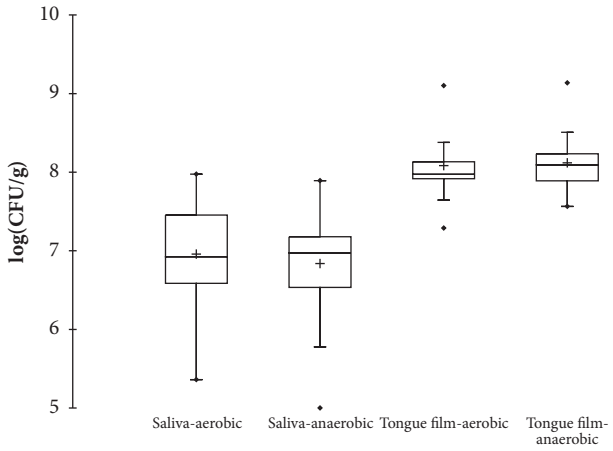

Figure 2: Box plot representation of cultivable aerobic and anaerobic microorganisms counts in saliva and tongue film.

3.5. Bacterial Communities in Saliva and Tongue Film. Results for the 5 phyla and 3 genera (*Veillonella* and *Streptococcus* belonging to Firmicutes and *Prevotella* belonging to Bacteroidetes) quantified are shown in Figure 3. Overall the most abundant phyla were Bacteroidetes (37.7 ± 15.7% in saliva, 19.6 ± 9.9% in film) and Firmicutes (9.2 ± 2.8% in saliva, 11.0 ± 3.1% in film). Saliva samples were characterized by significantly higher proportions of Bacteroidetes ($p<0.001$) and its main genus *Prevotella* ($p<0.01$) whereas tongue films samples exhibited significantly higher proportions of Firmicutes ($p<0.05$), Actinobacteria ($p<0.001$), and the genus *Streptococcus* ($p < 0.05$).

3.6. Sensory Evaluation. The mean sensitivity scores were 4.1 (sweet), 3.5 (salty), 3.4 (sour), 3.1 (bitter), and 2.4 (umami), respectively, with a wide distribution across subjects (Figure 4(a)). In addition, Figure 4(b) shows the biplot representation of the principal component analysis (PCA) performed on the correlation matrix. The first two factors represented 89% of the initial variability of the data. The F1 axis, explaining 76% of the variability, clearly differentiated subjects according to their overall sensitivity, indifferently from the taste considered. On axis 2, the main opposite contributors were scores of sensitivity to bitterness and umami. Accordingly, significant correlations ($p < 0.01$) were observed between sensitivity scores for all 5 tastes, at the exception of scores for bitterness and umami ($r = 0.419$).

3.7. Relationships between Taste Sensitivity Scores and Biological Variables. Table 4 shows the correlations between sensitivity scores, on the one hand, and biological data organized in 6 blocks (pH, saliva flow or film weight, concentrations of acids, concentrations of sugars, total microbial count, and proportions of the different bacterial phyla) on the other hand. To visualize the results, correlations are classified as close to null (0), weak (- or + for negative and positive correlations, respectively), or moderate (-- or ++).

Given the size of the population studied, it is not reasonable to focus on some specific correlations. However, this table provides an interesting view of some trends linking quite robustly some blocks and taste in general. In particular, saliva flow was negatively correlated to sensitivity for most tastes, and although not standing for all tastes, the negative trend was also observed for weight of the tongue film: overall, subjects with lower salivary flow and lower weights of film perceived the tastes better. There was a sharp contrast in the results linking organic acids and sensitivity scores. While the correlations were almost always positive (weak or moderate) in saliva, they were close to null or weakly negative in tongue film. Therefore, concentrations of organic acids appeared more influent in saliva than at proximity of the taste buds to explain taste sensitivity.

Looking at the sugars, there was also a clear contrast between the results in film and in saliva. This time, a lot of correlations were close to null in saliva (15 "0" out of 20 correlations), while only two "0" correlations were observed in tongue film. In tongue film, higher concentrations of glucose and fructose were systematically associated with lower sensitivity, while higher concentrations of sucrose and lactose were rather associated with higher sensitivity.

Finally, the last block where interesting and consistent correlations were found is the block of phyla proportions. In saliva, the proportions of Actinobacteria especially and Firmicutes to a slightly lesser extent were negatively associated with sensitivity scores. In tongue film, the proportion of Bacteroidetes was positively associated with three tastes.

Looking at the results from a different angle, some tastes appeared associated with more of the biological variables than

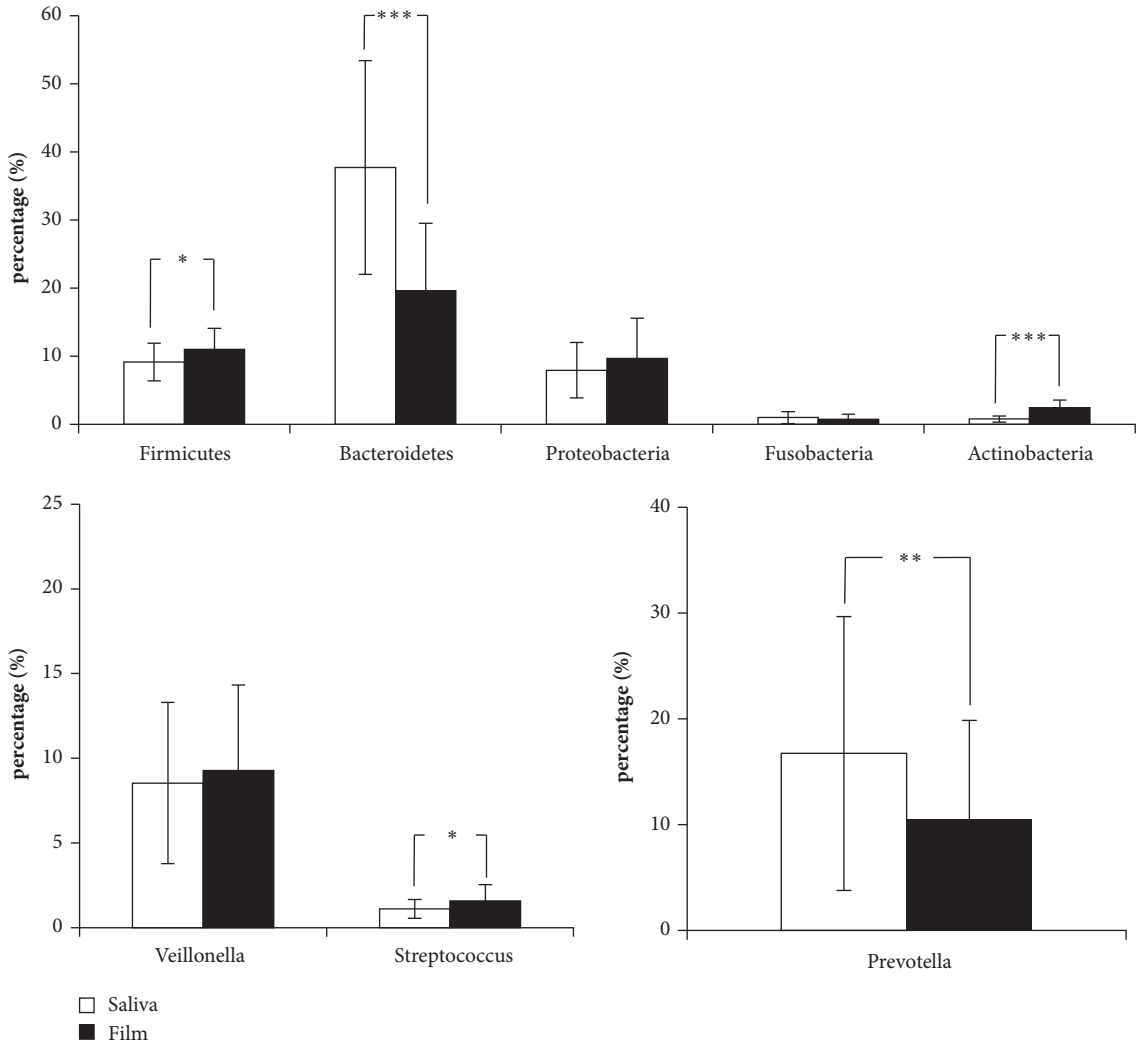

Figure 3: Composition of bacterial communities in saliva and tongue film (n=21): proportions of 5 phyla and 3 genera. Significant difference between saliva and tongue film is indicated as follows: $*$ $p<0.05$, $**$ $p<0.01$, $***$ $p< 0.001$.

others. In particular, it is interesting to note that the salty taste showed weak or moderate correlations for 14 out of the 16 variables studied in saliva, as opposed to only 7/16 for bitter and umami tastes, for example. In tongue film, the taste that showed the most and the highest correlations was bitterness.

4. Discussion

The objective of this work was to investigate whether the biological film lining the tongue, or tongue film, may play a role in taste perception. For that purpose, we first characterized jointly tongue film and saliva of healthy subjects and found that these two biological fluids differed in composition and microbiological profiles. Second, it was evidenced that taste sensitivity in general was linked to some biological variables in the two compartments, but variables that appeared influent in saliva (flow, organic acids, proportion of Actinobacteria and Firmicutes) and in tongue film (sugars and proportions of Bacteroidetes) were not the same.

The natural material found at the surface of the tongue is poorly described except when it is present in excess (it is then termed tongue coating), for example, in patients suffering from halitosis. The tongue film characteristics found in this study are slightly different from those of tongue coating. For example, pH of tongue coating was found to be more alkaline [15] than in the present study. The weight of film sampled here was lower than in another study [16], in which the mean weight was 173 mg (N=96). This first may be due to different sampling procedures: the tongue was scraped until no more coating could be dislodged in van Tornout et al. [16], while the tongue was scraped only once in this study. Second, subjects had different oral health status in the two studies, namely, halitosis patients versus healthy subjects. Other authors have reported an impact of oral diseases on tongue film quantity with higher wet weight of tongue film in a periodontal disease group (90.1 mg, n=17) compared to the control group (14.6 mg, n=6) [17]. The ratio of anaerobic/aerobic bacteria in tongue film here (close to 1) was also well below the value (2.29) found in tongue coating [18]. However, the authors also reported that the ratio decreased to 1.46 after treatment of halitosis by local antibiotics. Again this highlights that the

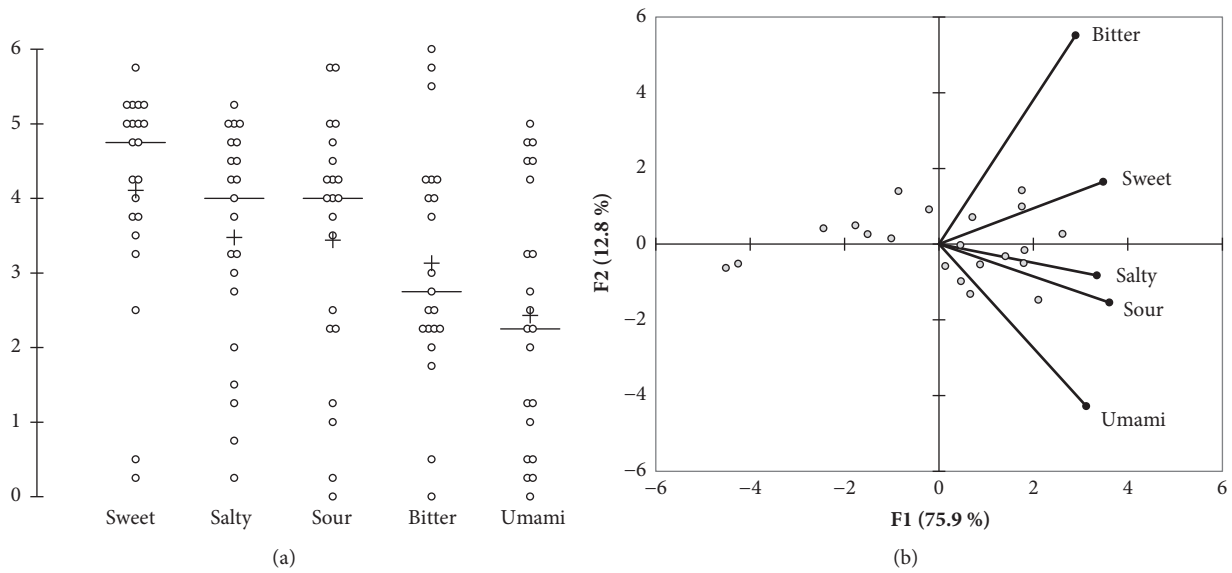

Figure 4: Taste sensitivity scores. Scattergram illustrating the distribution of scores across the panel (a) and biplot representation of the principal component analysis performed on the correlation matrix (b).

Table 4: Correlations between taste sensitivity scores and biological variables in saliva and in tongue film. The Spearman correlations coefficients are coded as follows: -- for -0.5< r < -0.3 (moderate negative correlation); - for -0.3< r < -0.1 (weak negative correlation); 0 for -0.1<r<0.1; + for 0.1< r<0.3 (weak positive correlation); ++ for 0.3< r < 0.5 (moderate positive correlation). A star indicates a significant correlation ($p<0.05$).

Salivary variables	Sweet	Salty	Sour	Bitter	Umami	Film variables	Sweet	Salty	Sour	Bitter	Umami
pH	0	+	-	+	0	pH	0	0	0	0	0
flow	-	--	--	0	--*	weight	-	0	-	0	--
lactate	++	+	+	+	+	lactate	-	-	0	-	0
acetate	+	++	+	+	0	acetate	-	0	0	-	0
propionate	+	++*	+	+	+	propionate	-	0	-	0	0
butyrate	++	++	+	+	0	butyrate	0	0	0	0	-
glucose	0	0	0	0	0	glucose	-	-	-	--	-
fructose	0	++	+	0	+	fructose	-	-	-	--	--
sucrose	-	-	-	0	-	sucrose	++	0	+	+	+
lactose	0	0	0	0	0	lactose	+	0	+	-	+
total bacterial count	0	+	0	0	0	total bacterial count	-	+	-	0	0
Actinobacteria	--	--*	-	-	--	Actinobacteria	0	0	-	+	-
Bacteroidetes	0	+	-	0	-	Bacteroidetes	+	+	0	++*	0
Firmicutes	--	-	-	-	0	Firmicutes	0	0	0	-	-
Proteobacteria	0	-	0	0	+	Proteobacteria	0	-	0	0	+
Fusobacteria	0	-	0	0	0	Fusobacteria	0	-	-	0	0

available data for tongue coating do not correspond to the tongue film in a healthy situation.

In terms of biochemical composition, the concentrations found in saliva are consistent with previous reports, particularly for glucose [19] and acetate [20], while, to our knowledge, no data is available for the organic acids' composition of tongue film or tongue coating. The origin of the organic acids in the oral cavity is repeatedly attributed to microbial metabolism. This has been described particularly for the production of lactic acid from glucose or sucrose in saliva and in dental plaque [21, 22] where the large increase (5 to 8-fold change) in lactic acid concentration is both rapid (within 5 minutes) and transient (returning to basal level in approximately 30 minutes). However, when the extracellular sugar supply is limited, for example, some time away after a meal like in our conditions, bacterial metabolism is not dominated by lactate production but shifts to production of mixed acids [23]. Thus both in saliva [22] and in dental plaque [21] the predominant organic acids in resting conditions are ordered as in the present study, i.e., acetic acid > propionic acid > lactic acid > butyric acid. Our results indicate that the tongue film of healthy

individuals is comparatively richer in acetic acid than saliva, which may be linked to different bacterial communities. For example, *Veillonella* utilizes lactate specifically and produces acetate and propionate as end-products. Other Firmicutes such as *Lactobacilli* and *Streptococci* can also convert lactate to acetate, as also *Actinomyces* belonging to the Actinobacteria phylum [23]. In our case, we indeed found that the two phyla Firmicutes and Actinobacteria were more represented in tongue film.

The 5 phyla quantified account for 80-95% of the entire saliva microbiome [24] and are also abundant in other oral samples [25, 26]. Our results differ from other reports in which Firmicutes was often the most abundant phylum in saliva [27], in tongue coating [28] or on the tongue dorsum [26]. This divergence is likely linked to different DNA extraction methods, but it does not prevent comparison between subjects or correlations with taste sensitivity scores.

Focusing now on the sensory results, the correlative approach we chose in this study has been seldom applied. Most articles linking saliva properties and composition and taste sensitivity have opted for an approach where groups were constituted based on sensory results and differences between groups were tested [7–10]. However, one recent study correlated salivary biochemical data and taste sensitivity [29]. Their main finding was that sweetness sensitivity correlated with salivary pH but only for 38% of subjects with the highest and lowest pH values. Keeping all subjects in our study, the link between pH and sensitivity is not clearly established neither in saliva nor in tongue film.

We found consistent negative correlations between taste sensitivity and saliva flow/tongue film weight. Concerning saliva, comparable correlations between flow and taste sensitivity have been previously described. For example, when an acid enters the oral cavity, a high salivary flow (usually associated with higher buffering capacity of saliva) diminishes the protons concentration at the receptors vicinity [6] and therefore sensitivity would be lowered. Similar associations were reported between salivary flow and sensitivity to oleic acid [30] or NaCl perception [6]. It is worth mentioning that methods used to determine taste sensitivity are most often performed with tastants in solution, which implies that the taste solution is diluted in saliva present in the oral cavity. In our case, the tastant is included in a solid matrix placed on the tongue, but the correlation also indicates that a low salivary flow improves sensitivity. It is therefore highly plausible that the amount of saliva on the tongue is related to saliva flow and that at low flow (yet within the healthy range), the local tastant concentration is higher. In addition, the tongue film weight was also negatively correlated to sensitivity, translating that the film also acts as a barrier limiting the diffusion of the taste molecules to the receptors on the tongue. This is, for example, consistent with Quirynen et al. [31] who found a lowering of taste identification threshold after mechanical cleansing of tongue treatment, resulting in a lower index of tongue coating. The removal of tongue coating by mild brushing has also been previously shown to improve sour taste recognition in older adults [12].

The correlation between taste sensitivity and organic acids concentrations were more numerous and stronger in saliva than in tongue film and clearly indicate that higher salivary levels of organic acids are associated with a higher sensitivity. This relationship is intriguing and opposite to a previous finding where higher organic acids concentrations in saliva were associated with lower sensitivity to the taste of fat [10]. To date, the mechanism linking organic acids and taste sensitivity remains unknown. In contrast to organic acids, the correlation between taste sensitivity and sugars were more numerous and stronger in tongue film than in saliva. First it should be noted that, contrarily to all the organic acids which may be produced within the oral cavity, three of the four sugars measured are brought to the oral cavity through food intake (fructose found in fruit, lactose found in milk, and sucrose produced from plant sources). It therefore seemed initially plausible that the variations in levels of these sugars on the tongue film corresponded to the capacity of naturally cleansing the tongue after food intake. However in the present study, the signs of correlations were different between taste and fructose, on the one hand, and taste and lactose and sucrose, on the other hand. This suggests that the correlations do not merely translate a generic capacity of tongue cleansing. Another explanation may therefore reside in sugar metabolism found on the tongue dorsum. The two sugars which tended to be positively associated with taste sensitivity were the disaccharides sucrose and lactose, while the monosaccharides fructose and glucose were negatively associated with taste sensitivity. In mammalians, the disaccharides can be hydrolyzed by intestinal enzymes, and most oral bacteria can also use disaccharides for glycolysis. Perhaps more interestingly, sucrose specifically can be converted by oral bacteria into glucan and fructan, which serve as "building material" for biofilms [23]. This phenomenon has been mainly described for *Streptococci* in the context of bacterial adhesion to the dental surfaces [32], but glycoproteins have also been observed surrounding bacteria adherent to mucosal cells of tongue rats [33]. If such glycoproteins are at least partly of bacterial origin, this would mean that the sucrose conversion to glucan/fructan participates in the strength of biofilms on the tongue surface. Therefore, a higher level of sucrose in the tongue film may indicate a lower proportion of bacteria capable of converting them into glucan and fructan (or a lower conversion rate) and therefore a less firmly structured biofilm. The physical barrier between tastants and taste receptors would, as a consequence, be less efficient and sensitivity increased. A finer characterization of the bacterial genera and species in the tongue film and targeted study of their sugar metabolisms would be necessary to test this hypothesis. This conclusion also stands when looking at the bacteriological results: at present, it is suggested that higher proportions of Actinobacteria and Firmicutes in saliva are linked to lower taste sensitivity, while a higher proportion of Bacteroidetes in the tongue film increases sensitivity (particularly to bitterness). Given the diversity of genera and species within a phylum, described elsewhere in detail for the oral microbiome [34], it is overall difficult to propose mechanistic explanations on those links. This is especially true for the oral cavity, where particularly high within-subject diversity was reported [26]. Again, a more detailed characterization of microbial communities and of their metabolism would be of

interest, but this study demonstrates that the oral microbiome deserves to be considered as an explanatory variable when investigating perireceptor events involved in taste perception. To conclude, one should keep in mind that only a few correlations between taste sensitivity scores and biological variables were significant, and such correlations were modest. In addition, this preliminary study was conducted on a limited number of subjects and should be extended to a larger panel. Nevertheless, in spite of such limitations, this work already points to two interesting areas in taste research: the oral microbiome in general and the specific characterization of the film lining the tongue.

Data Availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

The Effect of Breed, Gender, and Acid Stimulation in Dog Saliva Proteome

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Saliva gained interest as a potential noninvasive source of biomarkers in humans and that interest starts to be extended also to other animal species. For this purpose, the knowledge of the salivary proteome in healthy conditions and the factors that affect it and how they affect it are necessary. The aim of the present study was to assess the effect that gender and breed have in saliva proteome and the changes in it induced by stimulation with acid. Saliva from 4 different purebred dogs (Portuguese Podengo, Greyhound, Rafeiro Alentejano, and Beagle) of both genders was collected without and after stimulation with lemon juice. SDS-PAGE and two-dimensional gel electrophoresis (2-DE) profiles were compared and the proteins of interest in-gel digested and identified by mass spectrometry. Acid stimulation decreased total protein concentration and the relative amounts of some protein bands/spots. Gender appeared to have minimal effect in saliva proteome, whereas the influence of breed varies. Beagles and Portuguese Podengos were the two breeds with higher differences. In conclusion, stimulation procedures and dog breed should be considered in data analysis when using salivary proteins for diagnostic purposes.

1. Introduction

Physiological variables are of added value to assess the welfare and lifespan both in humans and in animals, as they provide important information for interpreting and validating emotional and biological responses, respectively [1]. Saliva has gained interest for biomarker identification, mainly due to the noninvasive nature of its collection; at the same time that it contains glandular and blood-born molecules that can change under different conditions [2]. In dogs, most of the studies have been focused on the evaluation of stress by measuring salivary cortisol levels [3]. Infectious agents, such as *Helicobacter* spp., *Bartonella* spp., or rabies virus, have also been evaluated [4–6]. In addition, canine saliva has been used for quantification of acute phase proteins [7] and allergen measurements [8] and in forensic studies for canine mRNA determination [9]. Furthermore, recently, healthy dog saliva proteome has been characterized by shotgun proteomics, with the identification of 2,491 proteins and peptides [10]. Despite this characterization, two-dimensional electrophoresis (2-DE) salivary protein profiles of dog saliva have been less explored. Although several researchers consider that gel-based approaches provide limited information, 2-DE continues providing reliable quantitative results on differential protein expressions as they display a high number of protein species, their isoforms, and posttranslational modifications at the same time [11]. It also has the advantage of allowing modifications of the protein mixtures caused by inadequate treatment or endogenous protease activities with physiological relevance to be easily recognized via pattern disturbances by 2D gels [11].

Table 1: Dog population for each breed by gender and age.

Breed	Average body weight (Kg)	Age (years)	Gender	
			Female	Male
Portuguese Podengo	4-5	0.5–10	7	6
Greyhound	26–40	1–7	9	6
Rafeiro Alentejano	35–50	0.5–8	8	7
Beagle	9–11	2–11	0	10

In humans, physiological and environmental factors, such as gender, age, interindividual variability, taste stimulation, and circadian rhythms, were identified to cause differences in the human salivary protein profiles [12]. However, to the best of the author's knowledge, in dogs such influences in salivary proteome are not deeply studied. The knowledge of the possible salivary proteome changes due to different factors would later permit correcting data interpretation for disease diagnostics.

Different methods of saliva sampling in dogs have been reported in literature: (1) without stimulation [10, 13]; (2) using different stimulating methods, such as citric acid in swabs [14] or in crystals spreader in the tongue [15], beef-flavoured cotton ropes [16], dogs' snack held in front of the dog's snout [17], or visualization and smell of food [18] what could result in different salivary proteomes. Acid stimulation, which is one of the mostly used methods for stimulating saliva production in humans, has been already reported to influence human salivary proteome [12]. However, its influence in dog saliva composition has not been reported.

The aims of this study were to evaluate the possible influence of biological factors, namely, breed and gender, and different saliva sampling conditions (with and without saliva stimulation with citric acid) on dog's saliva proteome.

2. Materials and Methods

2.1. Ethical Note. Dogs used in this study belong to three kennels and to a university (University of Murcia), whose gave their informed consent and participated in the collection procedures by handling the animals. The saliva collection and all animal procedures were carried out by researcher accredited by the Federation of European Laboratory Animal Science Association (FELASA) and conformed to legislation.

2.2. Dog Population. Dog population for each breed by gender and age is shown in Table 1. All were healthy and normal weight animals. Only male's pure breed Beagle were neutered animals.

2.3. Saliva Collection. Saliva samples were collected in the afternoon between 3:30 and 6:30 pm. Dogs did not eat for 16–18 hours prior to saliva sampling. Water was provided ad libitum. Saliva was collected by rolling a cotton cylinder (Salivette®, Sarstedt) inside each dog's mouth as described previously [19, 20]. The cotton cylinders were inserted under the dog's tongue for chew, until completely soaked with saliva, for a maximum of two minutes [21]. Two to three sample were collected in all animals, in different days. In one of these sample collections two to three drops of lemon juice were put under the tongue for stimulating saliva flow (acid stimulation). Only for Rafeiro Alentejano breed acid stimulated saliva collection was not possible. After collection, the cotton cylinders were immediately placed on ice, until laboratory arrival, which lasted no more than 30 minutes. In the laboratory saliva was extracted from the cotton roll by centrifugation at 4°C, at 5000 rpm, for 5 min, and immediately stored at −20°C for further analysis.

2.4. Total Protein Concentration. Bradford method protein assay [22] with BSA as the standard protein (Pierce Biotechnology, Rockford, IL, USA) was performed to determine the total protein concentration of each sample. Standards and samples were run in triplicate, in 96-well microplates. Absorbance was read at 600 nm in a microplate reader (Glomax, Promega).

2.5. SDS-PAGE. Proteins from individual saliva samples of all animals (both without and with acid stimulation) were separated by SDS-PAGE electrophoresis in 14% acrylamide gels in a mini-protean apparatus (BioRad) as described before [23]. Briefly, a total of 15 μg protein from each saliva sample was run in each lane. The samples were resuspended in sample buffer [Tris–HCl 0.125 M pH 6.8, 2% (w/v) SDS, 5% (v/v) 2-mercaptoetanol, 20% (v/v) glycerol traces of bromophenol blue], heated at 95°C for 5 minutes, and run at a constant voltage of 140 V until the dye front reaches the end of the gel. Gels were fixed in 40% methanol, 20% acetic acid, for one hour, stained with Coomassie Brilliant Blue (CBB) G-250 (0.125% CBB G-250, 20% ethanol) for two hours and destained in several washes with distilled water. A scanning Molecular Dynamics densitometer with internal calibration and LabScan software (GE, Healthcare) were used to acquire gel images and to determine the percentage of volume of each protein band; GelAnalyzer software (http://www.gelanalyzer.com/) was used to analyze the gel images. Molecular masses were determined in accordance with molecular mass standards (Bio-Rad Precision Plus Protein Dual Color 161–0394) run with protein samples.

2.6. Two-Dimensional Gel Electrophoresis (2-DE)

2.6.1. Protein Precipitation. Due to the limited amount of individual saliva samples, the unstimulated and acid stimulated saliva samples from each breed and gender were mixed in pools, constituting a total of 12 pools: (1) unstimulated

female Portuguese Podengo; (2) unstimulated male Portuguese Podengo; (3) stimulated female Portuguese Podengo; (4) stimulated male Portuguese Podengo; (5) unstimulated female Greyhound; (6) unstimulated male Greyhound; (7) stimulated female Greyhound; (8) stimulated male Greyhound; (9) unstimulated female Rafeiro Alentejano; (10) unstimulated male Rafeiro Alentejano; (11) unstimulated male Beagle; (12) stimulated male Beagle. Volumes of saliva from each pool containing 250 μg of total protein were used. The volume of each pool was mixed with equal volume of TCA 20% (m/v), incubated overnight, at −20°C, followed by centrifugation at 15,000g, 30 min, and two cold-acetone washes. This protocol as previously observed by us allows satisfactory results for preparation of dog saliva samples for 2-DE [24].

2.6.2. 2-DE Protein Separation. For 2-DE, the precipitates were mixed with 250 μL rehydration buffer [7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 2% (v/v), 60 mM DTT and traces of bromophenol blue] +5 μL IPG buffer +5 μL NaOH. Then the precipitates were sonicated until total resuspension and incubated during 1 h at room temperature, being subsequently centrifuged for 5 min at 10000 rpm. IPG strips (13 cm, pH 3–10 NL; GE, Healthcare) were passively rehydrated overnight with this solution. Focusing was performed in a Multiphor II (GE, Healthcare) at 20°C, with the programme (gradient): (1) 0–300 V for 2 h; (2) 300 V for 2 h; 300 V to 3500 V for 6 h; 3500 V for 6 h. Focused strips were equilibrated in two steps of 15 min each with equilibration buffer [50 mM Tris–HCl, pH 8.8; 6 M urea; 30% (v/v) glycerol and 2% (w/v) SDS], with the addition of 1% (w/v) DTT and 65 mM iodoacetamide in the first and second steps, respectively. After equilibration the strips were applied in the top of a SDS-PAGE gel 14% acrylamide and run at 150 V constant voltage in a mini-protean system (BioRad). Staining with CBB-G250 and destaining were done through the same protocol described for SDS-PAGE gels. Gel images were acquired using the same scan method and apparatus described for SDS-PAGE gels. ImageMaster 2D Platinum v7 software was used to analyze these gel images. Spot editing and the match were performed automatically and corrected manually. Spot volume was normalized to the total spot volume. Three laboratorial replicates of each pool were run.

2.7. Protein Identification. Bands and spots that differed among the factors tested were manually excised from gels and digested with trypsin following the protocol already described [25]. MALDI-TOF/-TOF mass spectrometry was used for protein identifications. Tryptic peptide mixtures were acidified with 5% (V/V) formic acid, desalted, and concentrated using home-made reversal phase (R2 pores-Applied Biosystems) microcolumns (R2 pores-Applied Biosystems). Peptides were eluted with the matrix solution (α-cyano-4-hydroxycinnamic acid Fluka) 5 mg/mL in 50% (v/v) acetonitrile and 5% (v/v) formic acid. MS and MS/MS data were acquired in positive reflector mode in a 4800 Plus AB SCIEX using the software 4000 Series Explorer, version 3.5.3.3 (Applied Biosystems).

Peptide mass spectra were acquired using a MALDI-TOF/TOF 4800 plus MS/MS (Applied Biosystems® Life Technologies, Carlsbad, United States of America). Data were acquired in positive MS reflector using a PepMix1 (LaserBio Labs, Sophia-Antipolis, France) to calibrate the instrument. Each reflector MS spectrum was collected in a result independent acquisition mode, using 750 shots per spectra in 800–4000 m/z range and fixed laser intensity to 3100 V. Fifteen of the strongest precursors were selected for MS/MS. MS/MS analyses were performed using CID (Collision Induced Dissociation) assisted with a collision energy of 1 kV and a gas pressure of $1 \times 10{-}6$ Torr. For each MS/MS spectrum, 1400 laser shots were collected, using fixed laser intensity of 4400 V. Processing and interpretation of MS and MS/MS spectra were performed with the 4000 Series Explored™ Software (Applied Biosystems® Life Technologies, Carlsbad, United States of America).

Protein identification was performed using MS and MS/MS spectral data and ProteinPilot (Applied Biosystems, version 3.0, rev. 114732) on Canis canis database (85118 sequences; 46,697,962 residues) retrieved from NCBI (downloaded in October 2017). Searches included trypsin as digesting enzyme; peptide mass tolerance of 50 ppm; fragment mass tolerance of 0.5 Da and possible oxidation, carbomidomethylation, or deaminidation as variable amino acid modifications with one missed cleavage. Peptides were only considered if the ion score indicated extensive homology ($p < 0.05$). Proteins were considered if the protein score indicated significant statistical confidence ($p < 0.05$). Protein identifications with only one matched peptide were considered if they were identified with >95% confidence.

2.8. Statistical Analysis. Multivariate analyse of protein bands, on one hand, and protein spots, on the other, were performed with MetaboAnalyst 3.6 to evaluate clustering of individuals or groups [26]. Data normalization was used when normal distribution was not observed, using transformation (log10) or scaling methods, alone or combined. The method chosen was the one that allowed data to be normally distributed. For univariate analysis, t-test, one-way ANOVA, and two-way ANOVA were used for comparison of protein profiles (band percentage volume or spots percentage volume) between unstimulated and acid stimulated saliva and among breeds and genders. For Multivariate Analysis, partial least squares discriminant analysis (PLS-DA) was used. Discriminant variables selection was done using variable importance in the projection (VIP) with a threshold of 1.0. Finally, paired-samples t-test was used for comparison of total protein concentration between saliva samples with and without stimulation. Statistical significance was considered for $p < 0.05$.

3. Results

3.1. Effect of Acid Stimulation on Salivary Proteome

3.1.1. Total Protein Concentration. Total protein concentration decreased significantly in stimulated saliva in males of both pure breeds Portuguese Podengo and Beagle. In females,

Table 2: Comparison of total protein concentration (mean ± standard error) between saliva with acid stimulation and saliva without stimulation, for each dogs breed and gender.

	Total protein concentration (μg/mL)		
	with acid stimulation	without stimulation	p
Breed			
Portuguese Pondego ($n = 6$)	843.0 ± 163.6	2385.7 ± 482.9	0.036*
Greyhound ($n = 7$)	961.7 ± 72.3	1146.7 ± 504.7	0.354
Beagle ($n = 7$)	1273.3 ± 161.8	1811.8 ± 246.3	0.033*
Gender			
Female (Podengo, $n = 4$, Greyhound, $n = 4$)	950.1 ± 115.3	1743.3 ± 404.3	0.112
Male (Beagle, $n = 7$, Podengo, $n = 2$, Greyhound, $n = 3$)	1049.2 ± 110.5	1737.3 ± 170.7	0.001*

*Statistically significant differences for $p < 0.05$.

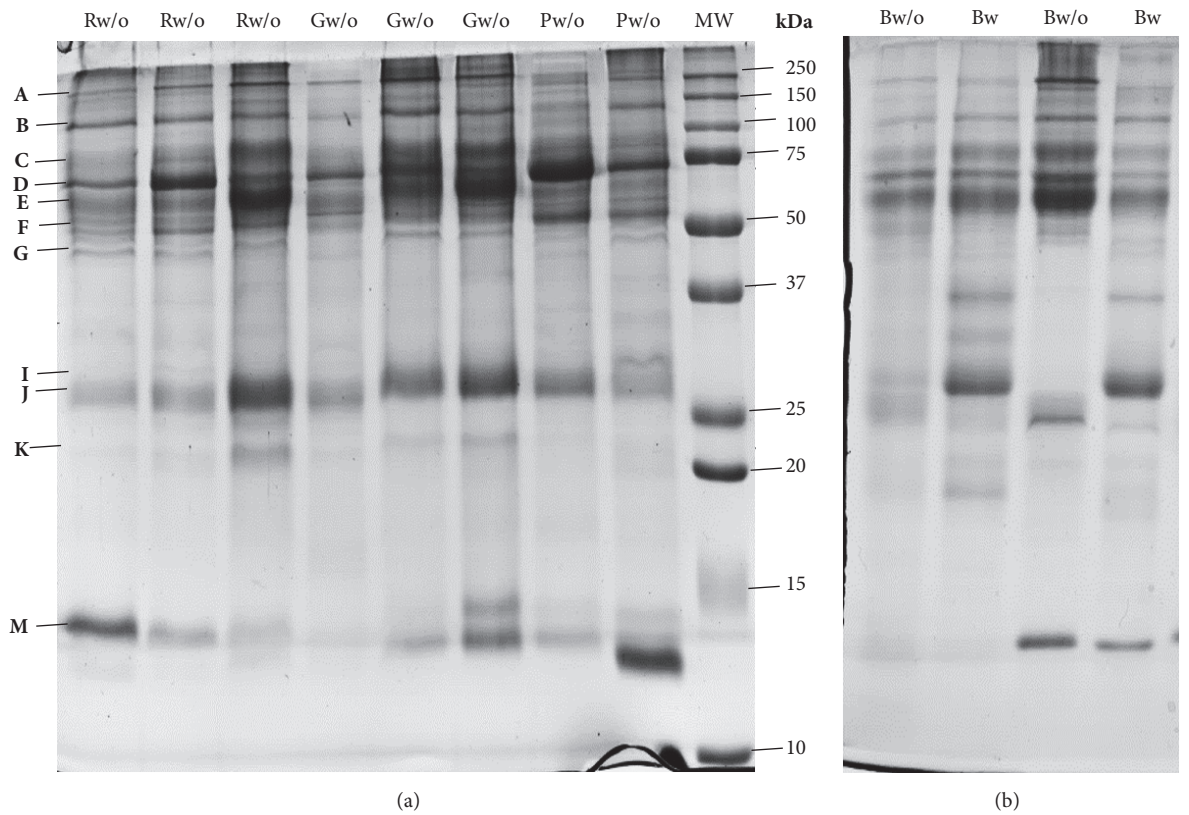

Figure 1: Representative SDS-PAGE profile of dog saliva: (a) from different breeds without stimulation (R: Rafeiro; G: Greyhound; P: Portuguese Podengo); (b) from Beagles without (w/o) and with (w) acid stimulation; MW: molecular mass marker; upper letters indicate the different protein bands.

no statistically significant differences were observed for saliva collected under the two conditions (Table 2). Concerning salivary flow rate, although this was not measured, it was possible to observe a tendency for higher salivary flow rates in big, comparatively to small breeds and higher salivary flow rate after lemon juice induction, in all breeds.

3.1.2. SDS-PAGE Profile. Among the 16 protein bands, with molecular masses between 20 and 245 kDa, observed in SDS-PAGE protein profiles (Figure 1), some presented changes in their intensities/volumes, which were induced by acid stimulation. Some of these changes were observed to be dependent on the dogs' breed and/or gender. Considering the total of the animals, 2 of the protein bands decreased (F and J) and one increased (I1) with acid stimulation (Table 3). Concerning bands F and J, the decreased levels were observed only in males and not in females.

By considering the dog breeds separately, changes induced by stimulation were observed only for Beagles: decreased expression levels of 4 protein bands (B, D, F, and J)

Table 3: Protein bands differently expressed (mean ± standard error) between saliva collected with and without acid stimulation.

Bands	% vol		p
	Without acid stimulation	With acid stimulation	
	Total of animals ($n = 20$)		
F	8.26 ± 0.46	5.61 ± 0.58	0.002*
I1	3.69 ± 0.51	7.62 ± 1.01	0.002*
J	12.81 ± 0.54	9.24 ± 0.63	0.0008*
	Beagles (only males) ($n = 7$)		
B	9.10 ± 0.79	6.13 ± 0.39	0.004*
D	10.28 ± 1.04	6.9 ± 0.39	0.004*
F	8.34 ± 0.91	4.15 ± 0.64	0.002*
I1	3.79 ± 1.00	10.10 ± 1.6	0.010*
J	11.88 ± 0.98	8.43 ± 0.70	0.002*
	Males (three breeds) ($n = 12$)		
F	8.34 ± 0.54	5.29 ± 0.69	0.003*
J	12.88 ± 0.68	8.56 ± 0.79	0.0003*

*Statistically significant differences for $p < 0.05$.

Table 4: Mass spectrometry identification of proteins present in bands from saliva SDS-PAGE profiles.

Band	Protein	NCBI Accession Code Accession n	Estim/theoret MW (kDa)#	ID Score*	Seq. Cov. (%)	Matched Peptides MS (MS/MS)
A	Mucin-19	XP_022267206.1	240.6/340.8	201	11	21 (5)
C	IgGFc-binding protein	XP_022261796.1	75/318.0	187	14	14 (9)
D	Chain A, Crystal Structure Analysis Of Canine Serum Albumin	pdb|5GHK|A	67.8/65.7	815	52	15 (11)
E	Serum albumin isoform X1	XP_005628024.1	61.3/68.6	661	44	12 (10)
F	IgGFc-binding protein	XP_022261796.1	52.6/318.0	313	8	13 (6)
M	Full-double-headed protease inhibitor, submandibular gland	sp|P01002.1|IPSG_CANLF	12.2/12.8	166	46	6 (3)

#MW values observed in gel versus theoretical ones. *Protein score is $-10*\log(P)$, where P is the probability that the observed match is a random event. Protein scores greater than 62 are significant ($p < 0.05$).

and increased expression level of 1 protein band (I1) (Table 3). Information about mass spectrometry details of identified proteins is present in Table 4.

Although, in the pure breeds Portuguese Podengo and Greyhound, none of the individual bands from SDS-PAGE protein profiles showed statistical significant intensity differences, between the saliva collected with and without acid stimulation, the multivariate PLS-DA model clustered separately unstimulated saliva from acid stimulated saliva, in these two breeds (Figure 2). The protein bands J, K, and M were the major contributors for the differences in Greyhounds. Band M was identified as containing full-double-headed protease inhibitor, whereas the other two bands resulted in no confident identification. The protein bands C, E, and G, identified as containing IgGFc-binding protein and serum albumin, were the major contributors for differences in Portuguese Podengos (Supplementary Figure 1).

3.1.3. Two-Dimensional Protein Profile (2-DE). By analyzing 2-DE salivary protein profiles (Figure 3), 3 protein spots were observed to be present in lower volume in the saliva collected after stimulation: spot 0 (34.4 ± 5.14 and 13.9 ± 2.97% vol., saliva without and with stimulation, respectively), spot 5 (0.73 ± 0.02 and 0.47 ± 0.05% vol., saliva without and with stimulation, respectively) and spot 81 (0.5 ± 0.24 and 0.21 ± 0.20% vol., saliva without and with stimulation). These spots were identified as serum albumin subunit A, cytoskeletal keratin, and one unknown protein (Table 5).

3.2. Effect of Dog's Breeds and Genders on Salivary Proteome

3.2.1. Total Protein Concentration. The four different breeds did not differ among them for the total protein concentration of saliva, as shown by univariate statistical analysis. Also, no differences were observed between genders, neither for saliva collected without nor saliva collected with acid stimulation.

3.2.2. SDS-PAGE Profile. Salivary protein profiles of the 4 dog breeds studied were compared for the saliva collected without acid stimulation. Six protein bands showed a different volume among dog breeds (Table 6): bands containing serum

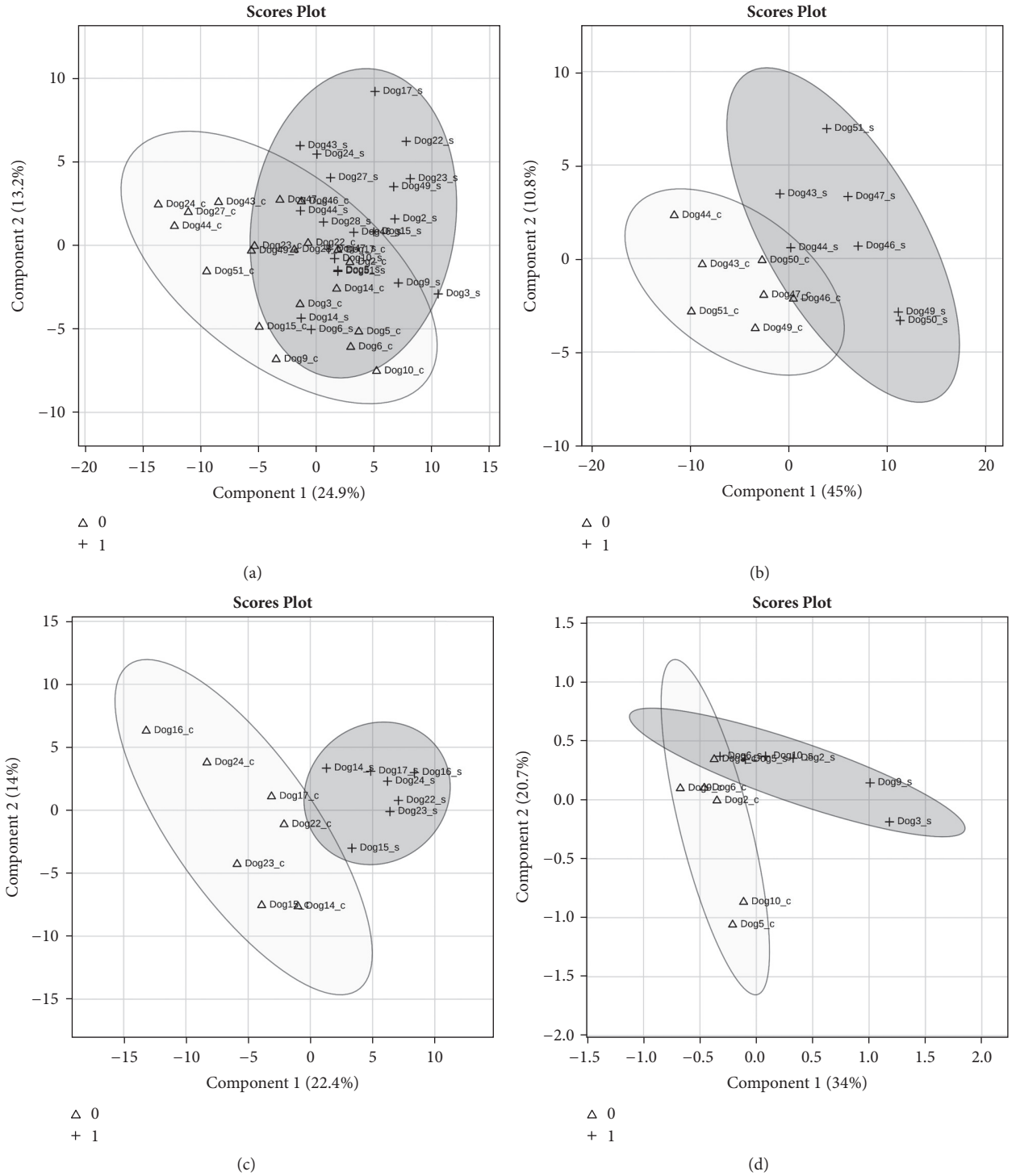

Figure 2: PLS-DA of saliva samples SDS-PAGE bands for all dogs (a), Beagles ($n = 7$) (b), Greyhounds ($n = 7$) (c), and Portuguese Podengos ($n = 6$) (d). Scaling was applied to rows when needed; X and Y axes show principal component 1 (PC1) and principal component 2 (PC2), respectively, and the total variance explained by each of them. Δ: with acid stimulation; +: without stimulation.

albumin were observed to be increased in Beagles, whereas a band containing a full-double-headed protease inhibitor was decreased, comparatively to the other breeds; bands containing albumin and IgGFc-binding protein were increased and one not identified was decreased in Portuguese Podengo. No trends for gender were found and no relationship between breed and gender was found, as well.

Through the multivariate PLS-DA model, that has into account the interrelationship among variables, it was possible to cluster Portuguese Podengos and Beagles more distant,

Table 5: Mass spectrometry identification of proteins present in spots from saliva 2-DE profiles differing between stimulation conditions and/or among breeds.

Spots	Protein	Entry reference	Estim/ theoret MW (kDa)	Estim/ theor pI	Score ID*	% Seq. Cov.	Matched Peptides MS (MS/MS)
0	Chain A, Crystal Structure Analysis Of Canine Serum Albumin	pdb|5GHK|A (NCBI)	78.1/65.7	4.9/5.3	263	42	18 (2)
5	Keratin, type I cytoskeletal 10	Q6EIZ0 (Uniprot)	18.5/57.7	7.8/5.1	207	30	10 (5)
8	double-headed protease inhibitor, submandibular gland	XP_022264993.1 (NCBI)	17.9/15.7	6.0/8.6	428	58	4 (8)
12	Immunoglobulin J chain	XP_532398.2 (NCBI)	30.1/18.3	4.4/4.7	125	40	3 (2)
16	Immunoglobulin lambda-1 light chain isoform X34	XP_005636600.1 (NCBI)	30.0/24.8	6.0/6.4	326	33	6 (4)
18	Immunoglobulin lambda-1 light chain isoform X25	XP_022266294.1 (NCBI)	31.0/24.9	5.5/5.1	198	35	8 (4)
37	IgGFc-binding protein	XP_022261796.1 (NCBI)	59.9/318.0	4.9/5.2	267	4	7 (4)
45	Uncharacterized protein	J9P732 (Uniprot)	25.0/21.4	5.8/6.0	192	28	4 (5)
81	Uncharacterized protein	F1PW98 (Uniprot)	19.1/55.0	8.0/5.7	111	29	15 (2)

*Protein score is $-10*\log(P)$, where P is the probability that the observed match is a random event. Protein scores greater than 62 are significant ($p < .05$).

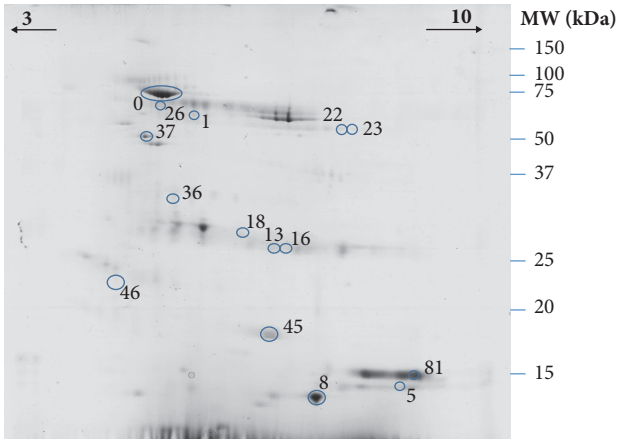

Figure 3: Representative dog's saliva 2-DE gel. Spots excised for digestion and identification by MS are numbered.

comparatively to the other breeds (Figure 4(a) and Supplementary Figure 2). The differences between Portuguese Podengo and Beagles for nonstimulated saliva were confirmed in the saliva collected after acid stimulation, by univariate analysis. In this case, it was also possible to observe that these breeds differ in saliva protein profile, with five proteins bands (E, F, I1, J, and M) observed to be differently expressed (Table 7 and Figure 4(b)).

3.2.3. 2-DE Saliva Profile. 2-DE salivary protein profiles of the several dog breeds evaluated presented differences in the percentage volumes of 7 protein spots. Through ANOVA (univariate analysis) it was observed that Portuguese Podengo presented higher levels of 5 salivary protein spots [1 ($p = 0.034$), 18 ($p = 0.041$), 22 ($p = 0.046$), 36 ($p = 0.036$), and 46 ($p = 0.014$)], comparatively to the other breeds. Among them, only spot 18 was identified (as a light-chain of immunoglobulin lambda-1). Spots 8 ($p = 0.043$) and 26 ($p = 0.015$) were present at different levels in Beagles, the spot 8 (identified as double-headed protease inhibitor) being in lower levels than in Greyhounds and the spot 26 (not identified) in higher levels than in the other breeds.

Besides these spots, multivariate PLS-DA model clustered Portuguese Podengo distinctly from Beagles and Rafeiro Alentejano breeds in 2-DE protein profiles (Figure 5). Spots 1, 18, 23, 45, and 82 were the ones that most contributed to these differences (Supplementary Figure 3). Detailed information about MS/MS identification of the referred spots is presented in Table 5.

In the case of spots 45 and 81, the identification resulted in unknown proteins. However, through BLAST analysis, it was possible to observe 83% homology between the protein present in spot 45 and a S100 calcium binding protein A9 and 83% homology between the protein present in spot 81 and keratin 8.

4. Discussion

In this study, the influence of gender and acid stimulation on the normal dog salivary proteome of different breeds was studied through in-gel based proteomics approach. For all the breeds, animals with a wide range of ages were included in the study. The number of proteins observed and identified in dog saliva through this methodology is much lower than the one reported in other studies, using LC-MS/MS [10, 27]. Nevertheless, in this study, dog gel protein profiles presented what can be of utility for studies where protein isoforms and/or posttranslational modifications (PTMs) are of interest [11]. SDS-PAGE and 2-DE protein separations were simultaneously performed in this study due to the limited amount of individual saliva. As such, SDS-PAGE was used for assessing variability and to make comparisons using individual information. Since this approach only allows

Table 6: Protein bands differently expressed (mean ± standard error) between dog breeds, in saliva collected without acid stimulation.

Bands	Breed		% vol		p
		Port. Pod.		6.07 ± .31[b]	
B	Beagle	Greyhound	9.02 ± 0.63[a]	6.71 ± .44[b]	0.005
		Raf. Alent.		6.86 ± .38[b]	
		Greyhound		8.16 ± 0.80[b]	
D	Port. Pod.	Raf. Alent.	12.59 ± 1.32[a]	8.2 ± 0.46[b]	0.005
		Beagle		10.71 ± 0.48	
		Port. Pod.		8.14 ± 0.53[b]	
E	Beagle	Greyhound	13.7 ± 1.29[a]	11.5 ± 0.98[a,b]	0.005
		Raf. Alent.		9.97 ± 0.45[b]	
		Greyhound		7.05 ± 0.62[a,b]	
F	Port. Pod.	Raf. Alent.	9.66 ± 0.75[a]	6.39 ± 0.56[b]	0.01
		Beagle		8.53 ± 0.90[a,b]	
		Greyhound		6.32 ± 0.51[a,b]	
G	Port. Pod.	Raf. Alent.	3.73 ± 0.72[a]	6.35 ± 0.35[b]	0.005
		Beagle		8.43 ± .99[b]	
		Port. Pod.		8.64 ± 1.04[b]	
M	Beagle	Greyhound	1.71 ± 0.005[a]	7.44 ± 1.25[b]	0.005
		Raf. Alent.		5.98 ± .83[b]	

Different letters mean statistically significant differences between pairs, for $p < 0.05$. Beagle ($n = 10$); Portuguese Podengo ($N = 7$); Greyhound ($n = 11$); Rafeiro Alentejano ($n = 13$).

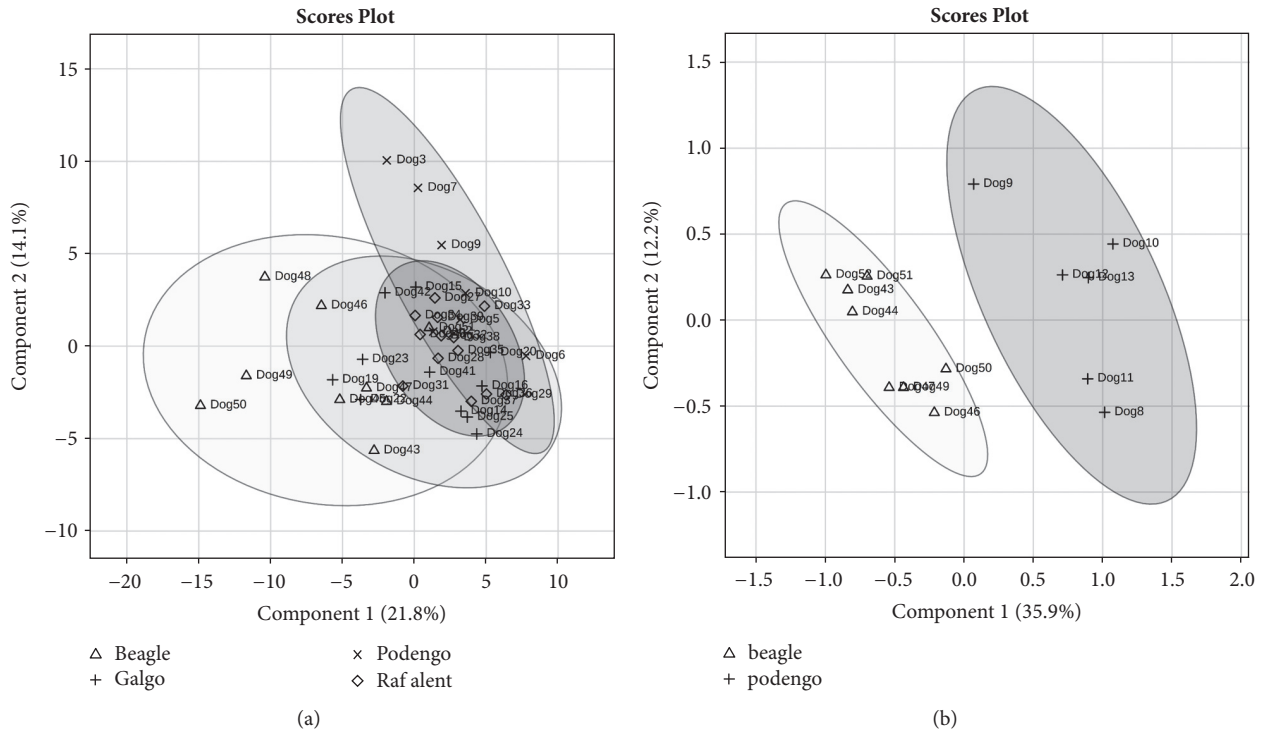

Figure 4: Partial Least Square Determinant Analysis (PLS-DA) model for all dog unstimulated saliva samples SDS-PAGE bands [Δ: Portuguese Podengo ($n = 7$); +: Greyhound ($n = 11$); ◊: Rafeiro Alentejano ($n = 13$); and x: Beagles ($n = 10$)] (a) and for stimulated saliva samples SDS-PAGE bands [+: Portuguese Podengo ($n = 6$) and Δ: Beagles ($n = 8$)] (b). Scaling was applied to rows when needed; X and Y axes show principal component 1 (PC1) and principal component 2 (PC2), respectively, and the contribution of each of them for explaining the total variance.

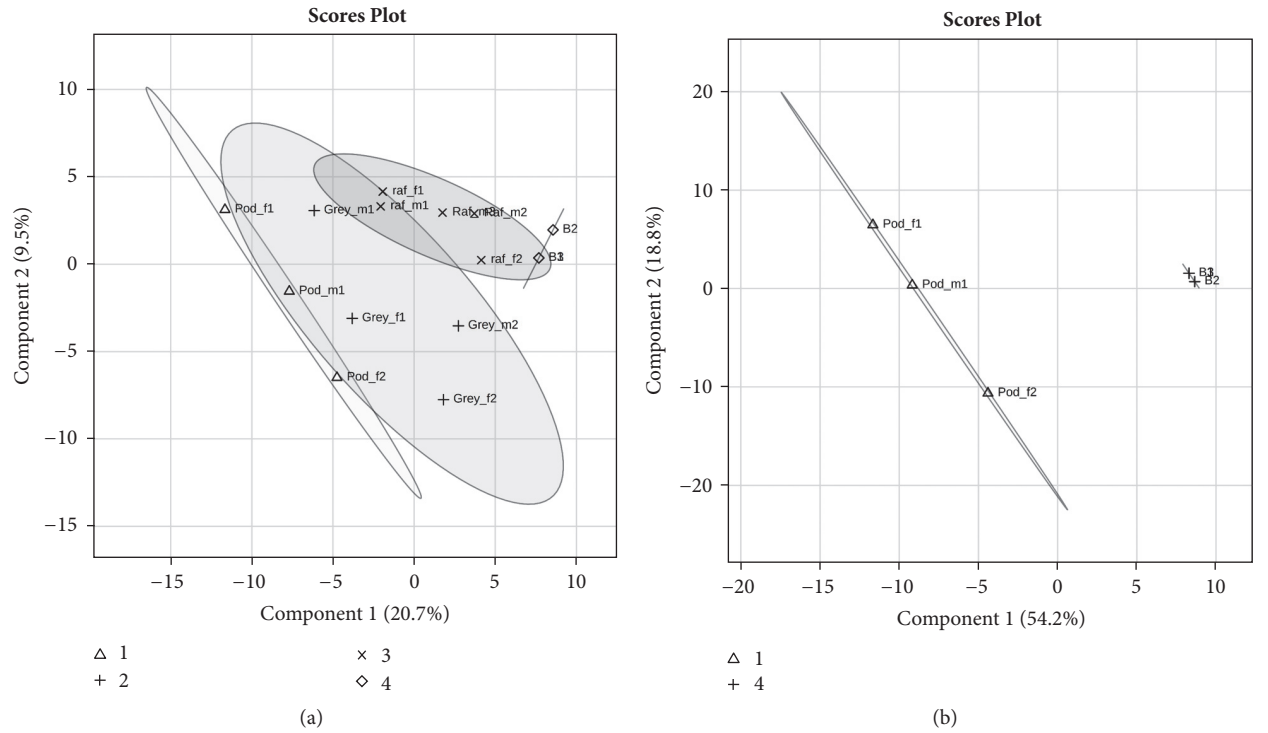

Figure 5: PLS-DA of dog saliva pool samples 2-DE spots of each breed (a) or considering only Portuguese Podengo and Beagles (b). Log transformation was applied to rows; *X* and *Y* axis show principal component 1 (PC1) and principal component 2 (PC2), and the respective % of explanation for the total variance. 1 – Portuguese Podengo; 2- Greyhound; 3- Rafeiro Alentejano; 4- Beagle.

Table 7: Protein bands differently expressed (mean ± standard error) in saliva samples with acid stimulation between Portuguese Podengo ($n = 6$) and Beagles ($n = 8$)[#].

Bands	% vol		P^*
	Portuguese Podengo	Beagle	
E	7.39 ± 1.64	13.21 ± 0.54	0.003
F	8.89 ± 0.57	3.98 ± 0.60	9.075e − 05
I1	2.72 ± 1.07	10.64 ± 1.50	0.002
J	12.87 ± 1.21	8.40 ± 0.60	0.004
M	9.12 ± 0.68	2.85 ± 0.60	1.6369e − 05

[*] Statistically significant differences for $p < 0.05$. [#] *N* of Beagles used for comparison was different that the one reported in Table 3, since for 1 animal only saliva from the collection after stimulation contained enough amount for analysis, impeding that animal for being included in paired analysis reported in Table 3.

separation according to molecular masses, several proteins must be present in each band, making it difficult to know the one (or several) responsible for changes. 2-DE profiles of saliva pools were used to add such detail.

No significant differences among breeds or between genders were observed on total protein concentration of normal dog's saliva. However, a decrease in the total protein concentration after acid stimulation was observed, especially in males of both pure breeds Portuguese Podengo and Beagle. In terms of profiles, proteins such as cytoskeletal keratin, serum albumin, and IgGFc-binding proteins were identified in bands and/or spots whose levels decreased with acid stimulation. IgG Fc-binding protein has been recently identified as one of the more abundant proteins in dog saliva [10] being a protein involved in binding IgG on mucosal surfaces [28]. To our knowledge, there are no other reports, in the literature, concerning the effect of acid stimulation on salivary proteome of dogs or other animals. But, our results are in accordance with studies performed in humans [12], where it was observed that acid stimulation produced considerable major changes, namely, in proteins related to immune function, inflammation, and cell movement [12]. Also Lorenz et al. (2011) [29] observed significant decreases on the relative abundance of several protein spots, in human saliva, after citric acid stimulation. It is curious that keratin is a protein from the cytoskeleton and IgG Fc-binding protein is a gel-like component of the mucosa. Stimulation with lemon juice raised the total volume of saliva produced and, as such, the cotton roll needed less time in the mouth for getting enough saliva amounts. Such decreased time of saliva collection, associated with fewer movements, may have resulted in a lower incorporation of components from the epithelium in the samples. In fact, the possibility of variations in the levels of these proteins being done to this effect was recently suggested [13].

In dogs, saliva collection without stimulation has the constraint of allowing obtaining only limited volumes of saliva for performing some laboratorial techniques [30]. However, if stimulation is needed it is important to have in

mind the referred differences in protein composition that such stimulation is producing.

In the present study, we could observe that salivary protein composition varies among different dog breeds, but no major differences were observed between genders. The reduced impact of gender in dog salivary proteome observed is in agreement with others recently published [13]. Our results go in accordance with these observations.

Two of the breeds that most differed between them were Portuguese Podengo and Beagle. According to Federation Cynologique Internationale (FCI) (http://www.fci.be, accessed on January 31, 2018) purebred Portuguese Podengo is a primitive type of breed, hunting dog probably originating from the ancient dogs, traditionally used for helping in rabbit or birds hunting, but without working trial [31]. This breed is also used as a watch and companion dog. Despite being a pure breed, it is expected that individuals present higher genetic variability than Beagles, since this last has been bred in a controlled way, also for use in laboratory studies. Also according to FCI, purebred Beagles belong to a small-sized hound group with working trial. By using clustering analysis, to define phylogenetic tree, this breed belongs to a cluster comprised mostly by modern breeds used in hunting [32].

Bands containing chains of canine serum albumin and IgGFc-binding protein were proteins differently expressed among dog's breeds. One of the proteins observed to be present in lower amounts in Beagles, both in SDS-PAGE and in 2-DE protein profiles, was the full-double-headed protease inhibitor from the submandibular glands. This protein is a serine type endopeptidase, which has been assumed to protect mucosal cells in mouth and oesophagus against the action of proteinases from microbial origin and/or ingested with food [33].

In the present study only a limited number of proteins were observed to differ with stimulation and/or among breeds. Even some protein spots failed a positive identification, which can be related to a lower number of proteins present in curated protein databases, comparatively to other species, such as humans. On the other hand, in this study, dogs were available from pure breed kennels and some of the differences observed for the different breeds can be done to different types of dog food consumed. Further studies, with a higher number of animals per breed, higher number of breeds and controls for type of food, and other treatments are necessary to have a better characterization of each breed saliva proteome.

5. Conclusions

This work, in line with what was hypothesized, allowed us to conclude that dog salivary protein composition is influenced by different factors. Despite the need of procedures that allow the collection of higher amounts of saliva, it is necessary to be aware that techniques such as acid stimulation not only induce higher salivary flow rates, but also change the levels/proportion of various salivary proteins. It is also of interest to retain that dog salivary proteome should be considered according to dog breed, since this was observed to be a factor responsible for variations in the proportion of different salivary proteins. In fact, breed appears to have even more influence than gender. Nevertheless, that does not mean that gender should be ignored, in dog saliva analysis. Despite males and females presenting minimal differences in salivary profiles, in this study differences in the way each gender responded to stimulation were observed.

From our knowledge this is one of the first studies evaluating factors affecting dog saliva electrophoretic protein profiles. More studies are needed to increase the knowledge about dog saliva proteome, in order to use it in research and diagnosis.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

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Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Supplementary Materials

Supplementary 1. Supplementary Figure 1: PLS-DA loading plots (left) of the first two components for analysis of SDS-PAGE bands of profiles from saliva collected with and without acid stimulation in Beagles (a), Greyhound (b), and Portuguese Podengo (c); for each case, variable importance in the projection (VIP) is presented, with 1.5 score considered as thresholder (right).

Supplementary 2. Supplementary Figure 2: PLS-DA loading plots (left) of the first two components for analysis of protein bands of salivary profiles from the different dog breeds. Variable importance in the projection (VIP) is presented (right).

Supplementary 3. Supplementary Figure 3: PLS-DA loading plots (left) of the first two components for analysis of protein

spots of salivary profiles from the different dog breeds. Variable importance in the projection (VIP) is presented (right).

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Research Article

Salivary Antioxidant Status in Patients with Oral Lichen Planus: Correlation with Clinical Signs and Evolution during Treatment with *Chamaemelum nobile*

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Oral lichen planus (OLP) is a chronic inflammatory mucocutaneous disease, which manifests as a succession of outbreaks. OLP was associated with salivary oxidative stress. Randomized, double blind, parallel-group study was performed. The sample consisted of 55 clinically and histopathologically diagnosed OLP patients. Twenty-six patients were treated with 2% *Chamaemelum nobile* gel and 29 with a placebo. Nonstimulated (basal) saliva was collected on the first day of the study and 4 weeks later. Salivary total antioxidant status (TAS) was evaluated by four different methods: two TAC (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) equivalent antioxidant capacity methods (TAC1 and TAC2), cupric reducing antioxidant capacity (CUPRAC), and ferric reducing ability of plasma (FRAP). At baseline (T1), no statistically significant differences were detected in any of the TAS analytes between the two groups of patients. After four weeks of treatment, a statistically significant increase was detected in FRAP in the placebo group (0.323 [0.090–0.467] versus 0.406 [0.197–0.848] mmol/g $* 10^{-3}$) ($P < 0.05$). Significant correlations were observed between pain and drainage and TAC1, CUPRAC, and FRAP and between xerostomia and the TAC1, TAC2, CUPRAC, and FRAP. The results of the present study showed that in patients with OLP increases of TAS in saliva are associated with increase in pain and xerostomia and decrease in drainage, suggesting a worsening condition of the patient. The use of *Chamaemelum nobile* gel would be recommended for disease stabilization.

1. Introduction

Oral lichen planus (OLP) is a chronic inflammatory mucocutaneous disease. OLP manifests as a succession of outbreaks that can adopt a variety of clinical forms: reticular OLP with Wickham's Striae, erosive-ulcerous OLP, and atrophic OLP. Its high prevalence (0.5–2%), recurrent nature, and potential risk of malignancy have led to extensive research into the disease [1, 2].

The etiology of OLP is unknown, although its pathogenesis includes an immune disorder in which CD8 cytotoxic lymphocytes attack epithelial cells [2, 3]. There is a hypothesis that increased oxidative stress and an imbalance in the antioxidant defense system may be involved in the pathogenesis of OLP [4, 5]. For this reason, it is thought that determining the oxidative/antioxidant status of an inflammatory disease may be of value for assessing its severity and for monitoring the disease's evolution and response to treatment [6, 7].

In order to clarify the possible association between the oxidative stress and OLP pathogenesis, measurement of antioxidants and oxidants in saliva of patients suffering from this disease is increasing in the last years [8]. Saliva offers several advantages over serum as a diagnostic fluid: its collection is noninvasive and very easy, and sample collection can be repeated indefinitely. Recently, alterations in total antioxidant status (TAS) and reactive oxygen species (ROS) were described in saliva of patients with OLP when compared with healthy controls [8]. However, knowledge about the

behavior of analytes related to oxidative stress in saliva is lacking [7].

The most common therapeutic options for treating OLP include corticosteroids, retinoids, cyclosporine, tacrolimus, phototherapy, and surgery, although treatment often produces adverse effects [2, 3]. Chamomile has been used to deal with diverse inflammatory disorders and possesses a variety of active flavonoids such as alpha bisabolol, azulene, matricin, and chamazulene, all of which have antioxidant, anti-inflammatory, antispasmodic, antibacterial, and immunoregulatory capacities [9]. For this, chamomile was shown to be beneficial in the treatment of oral diseases, such as mucositis [10]. Furthermore, the topic chamomile application was shown to improve clinical presentation of OLP, including decreased pain, burning sensation, and itching [11].

This study investigated the correlation of TAS of saliva in patients with OLP measured by four different assays and its possible relation to clinical variables such as pain, draining, and xerostomy. Furthermore, the changes of TAS during OLP treatment were assessed.

2. Material and Methods

2.1. Study Design. This randomized, double blind, parallel-group study, of 4-week duration (Trial Registration Number Identifier: NCT02421770) was conducted in full accordance with ethical principles and was approved by the Bioethics Commission of the University of Murcia. Informed consent to take part was obtained from each subject.

Saliva samples were collected from patients diagnosed with OLP following established clinical and histologic criteria [6]. Included patients had not received any treatment for OLP in the previous two weeks in the case of topical treatments, or in the last four weeks in the case of systemic therapies. Exclusion criteria consisted of allergy to some ingredient of the products tested, the use of antioxidant drugs or medication capable of inducing lichenoid reactions, the presence of dysplasia in the histopathological study of OLP, periodontal disease, medication with an immunosuppressant, and a history of trauma and/or surgery.

2.2. Study Products. The product assayed was 2% *Chamaemelum nobile*, with a gel consistency supplied in 500 ml containers, as was the placebo [11]. Both products consisted of the same excipients and composition, water, hydroxyethyl, sorbitol < 0.1%, E-202 (potassium sorbate) < 0.1%, E-223 (sodium metabisulfite) < 0.1%, food coloring < 0.1%, and chamomile aroma < 0.1% (Ababbo, Murcia, Spain), except that the experimental gel included 2% chamomile and the placebo did not. Both preparations had the same colour. An operator external to the study coded the products in identical opaque containers. A randomization code was kept in an opaque envelope in a safe environment and opened only at the end of the study. Both patients and researchers were blind to group assignment (treatment/placebo). The gels (0.5 ml) were applied uniformly to the oral cavity with the finger three times a day in the areas that presented symptoms for a period of 4 weeks. After each application, patients were asked not to eat or drink for 20 min.

A clinical history was made for each patient and patients were examined clinically. Oral clinical examinations and data registration were performed by a single examiner, a specialist in oral medicine (CA). Patients were asked to indicate their estimated mean pain intensity at the beginning and the end of the trial. The pain was measured on a 10-point Visual Analogue Scale (VAS) (0 = no symptoms, 10 = severe pain) (López-Jornet et al., 2009). The patients were asked to draw a vertical line at the point on the horizontal line which best represented their symptoms. Draining was evaluated as previously described and salivary flow rates were measured in ml/min [12]. The xerostomy inventory was evaluated using a questionnaire consisting of 11 items. Patients respond by scoring from zero to five according to the absence (0) or severity (5) of the symptom, a higher score indicating greater severity [13].

2.3. Saliva Collection. Before collecting saliva, all participants rinsed their mouth with distilled water. Nonstimulated saliva was obtained using the draining method [12], without chewing movements, in dry plastic vials with the participant sitting in a relaxed position during 5 min. In all cases, saliva samples were taken in the morning between 10.00 and 12.00 hours. Samples with blood contamination (determined by visual inspection scale) were excluded, since unvisible blood contamination of saliva does not interfere with oxidative stress markers and antioxidant status [14]. Saliva was centrifuged immediately after collection at 3000g for 10 min. The supernatant was transferred into Eppendorf tubes and stored at −80°C until analyses.

2.4. Antioxidant Analysis. Salivary TAS was evaluated by measuring trolox equivalent antioxidant capacity (TAC1 and TAC2), ferric reducing ability of plasma (FRAP), and cupric reducing antioxidant capacity (CUPRAC) as previously described [8].

2.5. Statistical Analysis. All data were registered as medians and percentiles (unless otherwise stated). These were calculated using routine descriptive statistical procedures and software (GraphPad Software, San Diego, CA, USA). Results were evaluated for approximate normality of distribution using the D'Agostino and Pearson omnibus normality test, giving a nonparametric distribution; for T1 and T2 comparison within the groups, and to make comparisons between groups, data were log transformed and Student's t-test was used. Correlations between variables were estimated using Spearman correlation analysis. A value of $P < 0.05$ was considered statistically significant.

3. Results

Finally, saliva samples before and four weeks after the treatment were obtained from a total of 55 patients (Figure 1). In the treatment group, 10 subjects were male (38.5%) and 16 were female (61.5%); in the placebo group, 7 (24.1%) were male and 22 (77.9%) female ($P = 0.25$). Mean age in the treatment group was 63.1 ± 14.36 years and 62.8 ± 10.3 in the placebo group ($P = 0.91$).

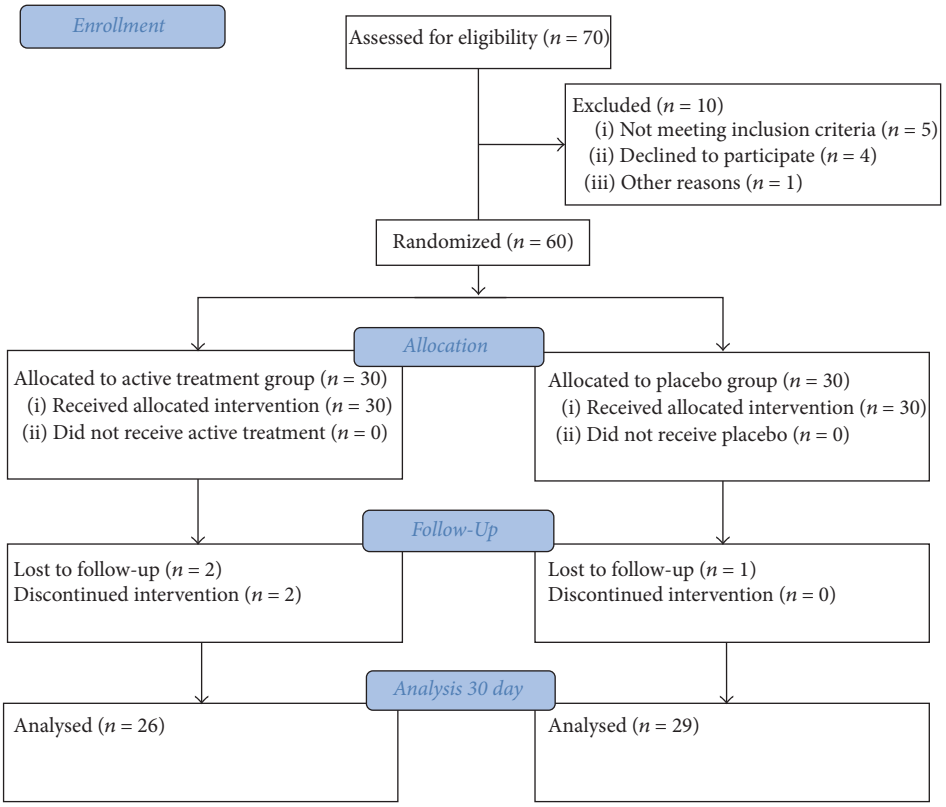

Figure 1: Flow diagram.

Table 1: Median (interquartile range) data for TAC1, TAC2, CUPRAC, and FRAP at baseline (T1) and four weeks after (T2) in placebo and treatment groups.

Analyte	Placebo			Treatment		
	T1	T2	*P*	T1	T2	*P*
Flow rate, ml/min	4.7 (1.4–7.8)	4.1 (2.0–7.5)	0,427	3.6 (1.5–9.0)	5.1 (1.2–7.5)	0.4672
Xerostomy	19 (11–30)	22 (13–33)	0.457	24 (12–34)	22 (12–31)	**0.048**
TAC1, mmol/L	0,201 (0,136–0,299)	0,233 (0,150–0,393)	0,390	0,288 (0,166–0,404)	0,278 (0,209–0,445)	0,477
TAC2, mmol/L	0,400 (0,258–0,589)	0,421 (0,223–0,522)	0,972	0,457 (0,295–0,513)	0,397 (0,276–0,526)	0,946
CUPRAC, mmol/L	0,130 (0,088–0,255)	0,191 (0,116–0,359)	0,401	0,199 (0,113–0,348)	0,225 (0,149–0,408)	0,391
FRAP, mmol/L	0,323 (0,090–0,467)	0,406 (0,197–0,848)	**0,039**	0,392 (0,195–0,685)	0,464 (0,298–0,568)	0,781

No statistically significant differences were found in either group with respect to OLP evolution time, which ranged from 6 months to 8 years. Median (range) OLP severity was 2 (2–4) in the treatment group and 2 (2–4) in the placebo group ($P = 0.58$)

Table 1 shows median (interquartile range) data of TAC1, TAC2, CUPRAC, and FRAP at baseline (T1) and at four weeks for both groups. At baseline (T1), no statistically significant differences were detected in any of the analytes between the two groups of patients. After four weeks of treatment, a statistically significant increase was detected in FRAP in the placebo group ($P < 0.05$). No other statistically significant changes were detected for any of the other analytes.

When data of the two groups and the two samplings were pooled, significant correlations were observed between pain and drainage and TAC1, CUPRAC, and FRAP, and between xerostomia and the TAC1, TAC2, CUPRAC, and FRAP (Table 2).

4. Discussion

There are evidences that OLP is associated with oxidative stress in saliva since various oxidative biomarkers such as TAS, malondialdehyde (MDA), uric acid, or gamma-glutamyl transferase [4, 8, 15–17] were shown to be altered in this disease. Salivary TAS was previously measured in healthy controls and patients with OLP [4, 8, 18]. However, no studies were reported about the dynamics of TAS in saliva of patients with OLP after treatment as well as the possible correlations between TAS values and severity of OLP clinical signs.

Table 2: Correlation between total antioxidant capacity and clinical parameters.

Variable	Pain	Flow rate	Xerostomy
	r; P	r; P	r; P
TAC1, mmol/L	NS	NS	0.233; 0.013
TAC2, mmol/L	0.239; 0.010	−0.323; 0.001	0.326; 0.001
CUPRAC, mmol/L	0.182; 0.040	−0.359; <0.001	0.271; 0.005
FRAP, mmol/L	0.331; <0.001	−0.345; <0.001	0.322; 0.001

Salivary antioxidant capacity can be measured with a variety of methods. The present study employed TAC1, TAC2, CUPRAC, and FRAP, which are the most widely used methods for measuring total antioxidant status. Furthermore, it was suggested that the best approach was to combine different assays when evaluating TAS [8, 19]. Determination of antioxidant status in a global way by TAS assays offers the benefit of evaluation of all individual antioxidant components of a sample, in contrast to determination of each antioxidant component separately, what is labor-intensive, time-consuming, and costly-expensive [20, 21]. However, different assays employed for TAS determination could produce diverse results and conclusions, since they measure different compounds. For instance, measurement of trolox equivalent antioxidant capacity (TAC) reflects plasma concentrations of albumin, urate, ascorbic acid, a-tocopherol, and bilirubin, while FRAP values mainly reflect levels of uric acid (up to 60%) and less ascorbic acid and a-tocopherol [21, 22]. For this reason, in the present study, salivary TAS was evaluated by four different methods. Although it should be stated that none of these methods are able to measure enzymes [23]. Therefore, the activity of some enzymatic oxidative stress markers, such as superoxide dismutase, catalase, or glutathione peroxidase, is not reflected in TAS measurements [23].

Patients included in the present study presented a mild to moderate form of the disease (median severity index was 2 out of 5 in both groups), what allowed the application of alternative treatment or including them in placebo group. Otherwise, it would be ethically not appropriate to include severely ill patients and maintain them during one month without any treatment (in case of the placebo group). This fact should be taken in account when extrapolating the results to other studies, since different severity of the disease could result in different behavior of antioxidants [24].

The increase in FRAP that occurred in the placebo group would indicate that an increase in TAS occurs when patients with OPL are not treated. Although unfortunately no markers of oxidative stress were measured in our study, it could be postulated that the increase in TAS in nontreated patients with median-low severity OLP would be related to an increase in the need of antioxidant protection secondary to an increase in oxidant compounds in saliva. Elevated concentrations of oxidant compounds in saliva were associated with lichen planus and correlated with the severity of the lesions [5]. In a previous study, we have demonstrated that the TAS concentrations in saliva are higher in patients with OLP compared to controls. And in line with our study, Agha-Hosseini et al. [18] found increased salivary TAS, using FRAP method, in patients with OLP in comparison with controls, although this was not statistically significant. In contrast, Ergun et al. [4] found no significant differences in salivary FRAP between healthy and OLP patients. This disagreement at least in part could be explained by the inclusion of patients with different severity OLP, although further studies would be indicated in order to clarify this topic.

The samples used in this study were banked samples of a previous report in which the use of chamomile topical treatment for one month was associated with the clinical improvement in patients with OLP [11]. Despite the clinical improvement, we did not detect changes in salivary TAS. However, the lack of increase of TAS in treated group could be considered as a beneficial effect of the chamomile resulting in disease stabilization and, thus, its use could be recommended for patients with OLP severity score of 2–4 out of 5. Further long-lasting studies using higher % preparations should be desirable in order to evaluate the possible effects of chamomile on salivary markers related to inflammation and oxidative stress in patients with OLP.

When clinical variables were evaluated in relation to salivary TAS levels, positive correlation was observed between TAS and pain, being the strongest when FRAP assay was used. This finding at least in part could be explained by the fact that pain results in increased cortisol (an endogenous glucocorticoid), which in turn was associated with increased antioxidant capacity of the serum [25, 26]. Furthermore, negative and positive correlations between salivary TAS and drainage and xerostomy, respectively, were observed. These two correlations complement and confirm one another and indicate that in cases where less saliva is produced and thus the feeling of dry mouth is increased, salivary TAS levels are higher.

In conclusion, the results of the present study showed that, in patients with OLP, the topical application of chamomile for one month had no effect on salivary TAS, while the lack of treatment (placebo group) was accompanied by the significant increase in antioxidants measured by FRAP assay. Furthermore, an increase in salivary antioxidants in patients with OLP, especially when measured by FRAP method, was related to an increased pain and xerostomy and a decreased drainage. Taken together, it could be suggested to use 2% chamaemelum nobile gel to stabilize patients with median-low severity OLP.

Abbreviations

CUPRAC: Cupric reducing antioxidant capacity
FRAP: Ferric reducing ability of plasma
OLP: Oral lichen planus
ROS: Reactive oxygen species
TAS: Total antioxidant status
TAC: Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) equivalent antioxidant capacity method.

Conflicts of Interest

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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Review Article

Saliva as a Future Field in Psoriasis Research

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Psoriasis is a skin inflammatory disease characterized by an increased body of comorbidities, including parodontopathy. Despite the visibility of skin lesions, prognostic biomarkers, related to disease monitoring and therapeutic effectiveness, are still missing. Although several markers have been studied, none of them has been identified as an independent prognostic factor. This concise review aims to summarize the current knowledge and results in saliva research applied to psoriasis. Combination of different markers could improve the prognostic prediction in patients with psoriasis. Future studies are needed to implement research on salivary biomarkers and their prognostic/therapeutic effects in the management of patients with psoriasis.

1. Psoriasis and the Need of New Biomarkers

Psoriasis is an immune-mediated systemic inflammatory skin disease, interpreted as complex, because of the genetic, immunological, and environmental aspects [1–5]. The clinically visible manifestations are objectivized on the skin where well demarcated, infiltrated, and peripherally erythematous skin plaques are present, due to altered differentiation and hyperproliferation to the keratinocytes together with multifarious inflammatory cells and neoangiogenesis [6, 7]. An increased body of evidence has linked psoriasis to several comorbidities such as cardiovascular and periodontal ones and suggested the need for an affordable set of biomarkers [8, 9]. Currently, many biomarkers were proposed for psoriasis; however, none of them was considered as a valid and accepted disease marker. The ideal biomarker is a biological hallmark that is sensitive, specific, reproducible, and capable of identifying a physiological or pathological status and/or a therapeutic response [10]. Furthermore, the biomarker assay should be validated, standardized, and easy to perform [10]. In the past years, the biomarkers research in psoriasis focused on assessing blood and skin samples, genetics, and transcriptomics with contrasting results [11]. Therefore, saliva with its two secretory pathways has gained growing interest as an alternative and available biological sample to analyze, looking for biomarkers (Figure 1) [12–14]. Available evidence, first maturated in the field of rheumatic diseases, suggests the relation between saliva, oral inflammation, and systemic health. In addition, oral microbiome is also related to both skin and gastrointestinal system, consequently modulating the systemic inflammation [15]. This concise review summarizes the current evidence regarding the applications of saliva in psoriasis and describes future approaches in the field.

2. Salivary Changes in Rheumatic Diseases

Several rheumatic diseases may compromise the physiological salivary function (Figure 2), causing acute and chronic disorders. They are analyzed in detail in Table 1. The most

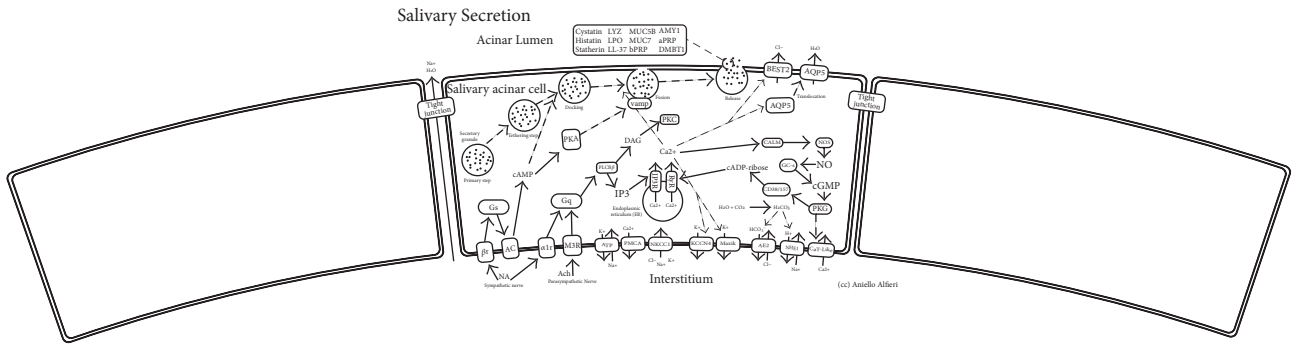

Figure 1: The two salivary secretory pathways: protein exocytosis and fluid secretion. Salivary secretion is modulated by the autonomous system: the sympathetic system activates the adenylate cyclase increasing the level of cAMP leading to the secretion of proteins from the secretory granules. The parasympathetic, instead, by the upmodulation of phospholipase C causes the raising of intracellular Ca2+ and fluid secretion. BEST2: bestrophin 2. AQP5: aquaporin 5. VAMP: vesicle associated membrane protein. CALM: calmodulin like 6. CD38/157: bone marrow stromal cell antigen 1. PKG: protein kinase, cGMP-dependent, type I. GC-s: guanylate cyclase 1 soluble subunit alpha 2. NOS: nitric oxide synthase 1. RYR: ryanodine receptor 3. IP3r: inositol 1,4,5-trisphosphate receptor type 1. Cat-like: transient receptor potential cation channel subfamily V member 6. NHE1: solute carrier family 9 member A1. MaxiK: potassium large conductance calcium-activated channel subfamily M alpha member 1. KCNN4: potassium intermediate/small conductance calcium-activated channel subfamily N member 4. NKCC1: solute carrier family 12 member 2. PMCA: ATPase plasma membrane Ca2+ transporting 1. ATP: ATPase Na+/K+ transporting family member beta 4. M3R: cholinergic receptor muscarinic 3. α1R: adrenoceptor α 1D. AC: adenylate cyclase 1. βR: adrenoceptor beta 1. Gs: guanine nucleotide-binding protein G(s) subunit α. Gq: guanine nucleotide-binding protein G(q) subunit α. PLCβ: phospholipase C β 1. MUC5B: mucin 5B, oligomeric mucus/gel-forming. MUC7: mucin 7, secreted. AMY1: amylase, alpha 1A. aPRP: proline rich protein HaeIII subfamily 1. bPRP: proline rich protein BstNI subfamily 1. DMBT1: deleted in malignant brain tumors 1 protein. Cystatin: cystatin-SN. Histatin: histatin 1. Statherin: statherin. LYZ: lysozyme C. LPO: lactoperoxidase. LL-37: cathelicidin antimicrobial peptide. See [12–14].

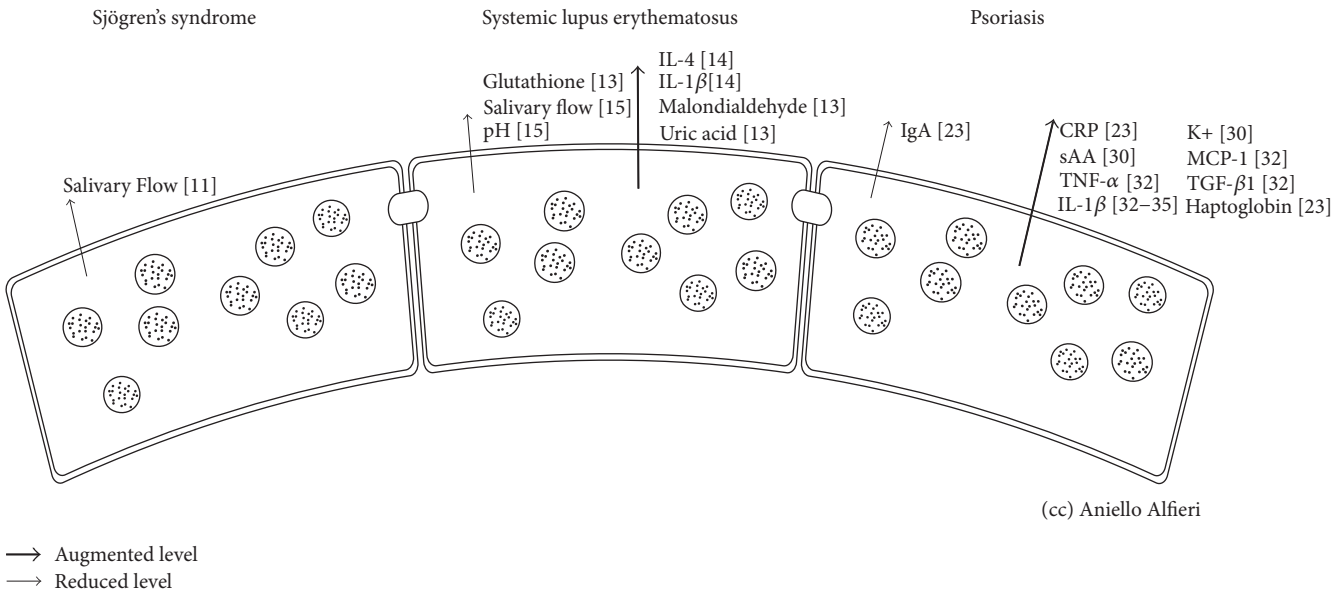

Figure 2: Component changes of salivary secretion in rheumatic diseases and psoriasis. sAA: alpha amylase; K+: potassium; CRP: C-reactive protein; TNF: tumor necrosis factor; TGF: transforming growth factor; MCP: monocyte chemoattractant protein; IL: interleukin.

common oral changes in rheumatic diseases are hyposalivation (low salivary flow) and xerostomia. In Sjögren's syndrome, the lymphocytic infiltration of salivary glands can lead to secretory hypofunction leading to xerostomia [16]. The progression of lymphocytic infiltration gives rise to focal sialadenitis, salivary gland dysfunction, and, in about quarter of patients, an expansion of the parotid and submandibular glands, which are often associated with a reduced salivary flow [16]. Furthermore, the reduced antimicrobial effect of the salivary secretion and the poor lubrication of the mouth facilitate the onset of oral infections, mucosal fragility, and burning mouth syndrome (BMS). Other complications include oral candidiasis, dental caries, dysphagia, and dyspepsia [17]. In systemic lupus erythematosus (SLE), saliva was demonstrated to be an incredible source of biomarkers (Table 1), revealing an impaired antioxidant

Table 1: Changes of salivary function and components in patients with rheumatic diseases. IL: interleukin.

Disease	Salivary change	Effect
Sjögren's syndrome	Salivary flow	↓[16]
Systemic lupus erythematosus	Glutathione	↓[18]
	Malondialdehyde	↑[18]
	Uric acid	↑[18]
	IL-1β	↑[19]
	IL-4	↑[19]
	Salivary flow	↓[20]
	pH	↓[20]

Table 2: Changes of salivary function and components in patients with psoriasis.

Disease	Salivary change	Effect
Psoriasis	IgA*	↓[25]
	CRP	↑[25]
	Haptoglobin	↑[25]
	K+	↑[26]
	sAA	↑[26]
	TNF-α	↑[27]
	TGF-β1	↑[27]
	MCP-1	↑[27]
	IL-1β	↑[27, 28]

*PASI > 10; sAA: alpha amylase; K+: potassium; CRP: C-reactive protein; TNF: tumor necrosis factor; TGF: transforming growth factor; MCP: monocyte chemoattractant protein; IL: interleukin.

power with reduced levels of glutathione and conversely high levels of malondialdehyde and uric acid [18]. This data was also confirmed by the high levels of proinflammatory salivary cytokines IL-1β and IL-4, which are also positive markers to periodontal disease [19]. Thus, the high levels of caries found in SLE patients might be justified by the inflammatory microenvironment of the oral cavity and by the decrease in salivary flow, pH, and buffer capacity [20]. The Behçet disease, instead, is correlated with dysbiosis of the salivary microbiota, probably implicated in recurrent aphthous stomatitis [21]. Xerostomia is also common in bullous pemphigoid and in mucous membrane pemphigoid. Despite a correlation between BP-180 salivary levels being discouraged in bullous pemphigoid [22], the correlation between skin disease severity, the Autoimmune Bullous Skin Disorder Intensity Score (ABSIS), and salivary levels of both desmogleins 1 and 3 remains a matter of debate in pemphigus vulgaris [22, 23]. Furthermore, saliva suggested a possible connection between herpes simplex and pemphigus vulgaris; Ruocco et al. detected herpes simplex DNA in very early stages of pemphigus vulgaris, leading to the hypothesis of a potential viral role in triggering the autoimmune response [24].

3. Saliva and Biochemical Characteristics in Psoriasis

In many studies, different salivary components have been evaluated in psoriasis patients (Table 2), such as salivary total protein, salivary immunoglobulin A (IgA), IgG, lysozyme, C-reactive protein (CRP), and Haptoglobin. However, findings have been inconclusive for some markers due to contradictory results between studies, which might be attributed to the difference in the type of investigated psoriasis [29–31]. Although Krasteva and colleagues reported no statistically significant difference in the salivary level of IgA, assessed by radial immunodiffusion, between psoriasis patients and healthy controls, they observed that patients with a PASI > 10 had a tendency to show lower levels of IgA, compared to patients with a PASI < 10, suggesting that patients with a PASI > 10 might be at high risk of developing microbial infections that could trigger psoriasis [25]. In the same study, the authors reported a statistically significant increase of salivary C-reactive protein (CRP), associated with the inflammatory nature of the psoriasis, and, as known, CRP has a prognostic significance for the worsening of psoriasis [32]. Similarly, increased salivary levels of Haptoglobin were reported, indicating a local defense mechanism against psoriasis [25]. The levels of salivary CRP and Haptoglobin were determined by an immunoturbidimetric method.

In another study, Soudan and coworkers assessed the alterations of other salivary components and their correlation with the severity of psoriasis [33]. Sodium (Na+), potassium (K+), chloride (Cl−), and alpha amylase (sAA) were analyzed using ISE (Ion Selective Electrode) technology for electrolyte measurements and LISA 500 plus systems for sAA. Findings showed a significantly higher K+ and sAA concentrations in psoriasis patients, with respect to controls, while there was no significant rise in the other investigated salivary ions. Nonetheless, these changes were not related to either the severity or the duration of psoriasis. Recently, Bottoni et al. analyzed the saliva proteomic components in psoriatic patients against diabetic patients and healthy controls, by attenuated total reflection (ATR) in conjunction with infrared spectroscopy. There were differences in the secondary structure composition of proteins between psoriatic and diabetic patients as compared to the control group [26]. Moreover, the authors concluded that the saliva spectra of the control group and that of the palmoplantar psoriatic patients differ from plaque psoriasis and diabetic patient spectra because of the absence of the amide II band and the presence of different secondary protein-structure conformations. Although all the previous findings suggest the difference in biochemical components between psoriasis patients and healthy individuals, and also between different psoriatic disease entities, more studies are required to support the preliminary results in literature.

4. Saliva and Correlated Biomarkers in Psoriasis

Although salivary biomarkers have been identified in various systemic diseases, evidence is still scarce on the biomarkers that appear in the saliva and are correlated to psoriasis [34, 35]. In this context, Ganzetti et al. evaluated the

expression levels of interleukin- (IL-) 1β, IL-6, transforming growth factor- (TGF-) β1, IL-8, tumor necrosis factor- (TNF-) α, interferon- (IFN-) c, IL-17A, IL-4, IL-10, monocyte chemoattractant protein- (MCP-) 1, macrophage inflammatory protein- (MIP-) 1α, and MIP-1β in salivary secretions from patients with psoriasis, using multianalyte ELISA Arrays [27]. The authors reported a statistically significant greater expression of TNF-α, TGF-β1, MCP-1, and IL-1β in saliva of patients with psoriasis than in healthy subjects, with a positive correlation between IL-1β, TGF-β1, and MCP-1 expression and oral disease severity [27]. In fact, it was suggested that the increased expression of IL-1β in patients with psoriasis might explain why such individuals show more missing teeth and more alveolar bone resorption and periodontitis than healthy individuals, as previously reported [36]. Periodontitis is a destructive disease of the tooth-supporting tissues induced by bacterial biofilm [37] and, in highlight of the reported findings, it seems that psoriasis and periodontal disease share the underlying inflammatory process, especially that IL-1β could be responsible for tissue destruction in periodontal disease by increasing the levels of matrix metalloproteinases [38]. Higher IL-β salivary levels in psoriasis with respect to healthy individuals were supported in another study; Mastrolonardo et al. observed higher basal IL-1β levels among psoriatic patients suggesting an increase in its production [28]. Such changes in cytokine activity may play an important role in propagating inflammation in psoriatic skin. Furthermore, the association between psoriasis and oral mucosa could be indicated by the increased salivary [27] and serum levels of TGF-β1 and MCP-1 in psoriasis patients [39, 40]. In a different investigation, Ganzetti and coworkers confirmed the validity of saliva as a noninvasive tool to monitor inflammation in psoriasis [41]. At baseline, psoriasis patients had higher salivary levels of IL-1β, evaluated via an enzyme-linked immunosorbent assay (ELISA), in comparison to healthy individuals. After 12 weeks of treatment with TNF-α inhibitors, IL-1β levels significantly reduced in comparison to that of baseline, but remained significantly higher than in healthy controls even after treatment. Nonetheless, larger cohort studies are still needed to confirm these findings.

5. Saliva as a Monitor for Antipsoriatic Drugs

For more than four decades, it was possible to predict plasma concentrations of drugs by measuring the salivary drug concentration, avoiding the need of venipuncture [42]. This is especially desirable in patients within the pediatric and geriatric population, in which venipuncture could be difficult to perform. The prediction of plasma concentration is mostly reliable if there is a documented correlation between plasma drug levels and clinical outcomes. In fact, this concentration is mostly reliable for nonionized drugs at normal plasma pH (phenytoin, phenobarbital, and antipyrine) but is unreliable for ionized drugs (chlorpropramide, tolbutamide, propranolol, and meperidine). In clinical practice, there is scarce evidence indicating whether this strategy is useful for antipsoriatic drugs. Pediatric patients with rheumatological diseases receiving methotrexate chronically have shown poor correlation between serum methotrexate concentrations and salivary levels [43]. Also, in a study that enrolled adults with various malignant diseases, the measurements of methotrexate salivary concentrations have shown no correlation with serum methotrexate levels, in monitoring patients after 24 hours of methotrexate infusions [44]. More recently, a sensitive and a simple quantifying method by liquid chromatography-tandem mass spectrometry (LC-MS/MS) was shown to be a valid method for determination of saliva excretion on samples obtained after an intravenous administration of 1 mg/kg/dose of methotrexate to six patients with acute lymphoblastic leukemia [45]. This method could be a promising tool for the quantification of methotrexate in the saliva of patients with psoriatic disease as well. The LC-MS/MS method has been also investigated for the measurement of cyclosporine in saliva and a correlation between the cyclosporine concentrations in fifteen paired blood-saliva samples from kidney transplant recipients was shown to be significant [46]. The method of saliva collection coupled with the LC-MS/MS quantification technique for cyclosporine analysis could be also beneficial if applied to psoriatic patients undergoing cyclosporine therapy. Less encouraging results were reported with the monoclonal fluorescent polarization immunoassay (FPIA) kit, adapted to salivary testing by using a novel extraction method developed and patented under the name of Middle East Research Institute (MERI) [47]. This method showed no significant correlation between blood and salivary cyclosporine levels [47]. Psoraderm 5, Meladinine, and Oxsoralen, three psoralens utilized during PUVA therapy, were well monitored with salivary sampling. This method is a noninvasive alternative to serum levels measurements and is suitable for routine applications; five or six salivary samples are sufficient to determine tmax in a patient starting photochemotherapy, while three samples are mandatory for Cmax [48]. Steroids, often prescribed for psoriasis treatment, may cause adrenocortical suppression; as a screening technique for iatrogenic adrenal suppression, morning salivary cortisol (MSC) has 100% sensitivity and 97% specificity [49]. MSC assay is an accurate tool for monitoring adrenal function and should be recommended for psoriatic patients who are on steroid therapy.

The techniques reviewed are well established methodologies for therapeutic drug monitoring (TDM). High-performance liquid chromatography (HPLC), capillary electrophoresis (EC), liquid chromatography (LC), gas chromatography (LC or GC) coupled with UV spectroscopy, or mass spectrometry (MS) has been extensively applied to TDM, optimizing protocols for the analysis of drugs administered and the analysis of drug metabolites if necessary. Compared to sophisticated and large-scale techniques, like high-performance liquid chromatography-mass spectrometry (HPLC-MS), immunoassays are easily and readily miniaturized by employing nanostructured surface sensors or micro- and nanoparticles (NP), and microfluidic versions of these analytical devices are already available for research purposes. Recent advancements in point-of-care (PoC) technologies show great transformative promises for personalized preventive and predictive medicine. However,

fields like TDM, which first allowed for personalized management of patients' disease, are still lacking in the widespread application of PoC devices [50].

Future studies may be warranted to design PoC technologies, more reliable and less invasive procedures, for therapeutic antipsoriatic drug monitoring.

6. Future Perspectives

Salivary biomarkers levels tend to change in psoriasis (Figure 2), which reflect alterations in the their production/expression. Although salivary biomarkers in psoriasis patients have been evaluated in different studies, these investigations were only of a small sample size and focused only on specific biomarkers. Therefore, studies with wider salivary profiling and larger sample size are needed to confirm the preliminary findings in literature. Moreover, studies monitoring the salivary level changes of these biomarkers after psoriasis treatment are recommended, as more research on potential salivary prognostic biomarkers in psoriasis patients is still needed. In fact, salivary biomarkers might be helpful detectors of psoriasis severity and disease progression and could serve as valuable diagnostics in the future.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Review Article

A Review of Selected Studies That Determine the Physical and Chemical Properties of Saliva in the Field of Dental Treatment

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Physiological whole saliva is a unique body fluid constantly washing the mucous membranes of the mouth, throat, and larynx. Saliva is a clear, slightly acidic mucinous-serous secretion, composed of various electrolytes, small organic substances, proteins, peptides, and polynucleotides. There are many ways to use saliva as a biological fluid (biofluid). The significant advantages of saliva as a unique diagnostic material are its availability and the noninvasive method of collection. The aim of this review is to emphasize the diagnostic value of saliva as a research material in the configuration of its structure and secretion disorders. The data were obtained using the MEDLINE (PubMed) search engine, as well as an additional manual search. The analysis covered 77 articles selected from a group of 1986 publications and initially qualified for devising. The results were evaluated and checked for the correctness of qualifying in accordance with inclusion and exclusion criteria. The diagnostic use of saliva has attracted the attention of many researchers due to its noninvasive nature and relative simplicity of collection. In addition, it should be noted that the determination of chemical and physical saliva parameters can be effectively performed in the patient's presence in the dental office.

1. Introduction

Physiological whole saliva (WS) is a unique body fluid constantly washing the mucous membranes of the mouth, throat, and larynx. Apart from the gums and the anterior part of the hard palate, the entire oral mucosa contains fine salivary glands (from 200 to 400) which are responsible for the production of only 10% of the secretion. The large glands include 3 pairs of salivary glands, parotid, submandibular, and sublingual, which produce 90% of saliva [1]. These glands can also be classified in terms of the type of secretion produced as serous, mucus, and mixed. Saliva is a clear, slightly acidic mucinous-serous secretion, composed of various electrolytes, small organic substances, proteins, peptides, and polynucleotides [2]. About 65% of unstimulated (resting) saliva comes from the submandibular gland, 25% from the parotid gland, 4% from the sublingual gland, and 8% from other salivary glands [3].

Given the integrity of the hard and soft tissues of the oral cavity, this secretion is extremely important and constitutes a factor which is the most relevant to the maintenance of homeostasis in the mouth (due to the content of organic and inorganic components). It moisturizes oral tissues, allowing for articulation, digestion, and swallowing [4]. The fluid is also responsible for the protection of the surface of the teeth and mucous membranes against biological, mechanical, and chemical factors [5]. It participates in the perception of taste stimuli, temperature, and touch. The protective function of saliva is manifested in the removal of harmful products of bacterial metabolism, bacteria, and food debris from the oral

cavity and the surface of the teeth. The purification rate may vary from 0.8 to 8 ml/min. It is lower for the surfaces that are difficult for saliva to access. Saliva moistens the mucous membranes and teeth. Its proteins cover them with a thin coat, called the pellicle, which consists of amino acids and proteins. These substances are selectively absorbed on the surface of the teeth as a result of interaction with the enamel hydroxyapatite [6]. Moistening facilitates the formation and swallowing of bites and chewing of food, as well as reducing the harmful effects of mechanical, chemical, thermal, and biological injuries on the mucous membranes [4].

Saliva contains 99.5% water, 0.3% protein, and 0.2% inorganic and organic substances [7]. The most common inorganic constituents are sodium, potassium, calcium, magnesium, chlorides, and carbonates, while organic components include amylases, peroxidases, lipases, mucins, lysozyme, lactoferrin, kallikreins, cystatins, hormones, and growth factors [8]. In a healthy individual, daily salivation is estimated at 0.5 to 2 litres [9]. The regulation of salivary secretion takes place through the nervous pathway via the cholinergic system and a and b fibres of the sympathetic nervous system. While asleep, the rate of salivary secretion decreases and during chewing or speech it increases substantially. The secretion rate varies widely from person to person, even under normal conditions. After strong excitatory stimulation, for example, while eating, the salivation rate can increase markedly and after the administration of pharmacological agents the value can double [10].

Mucins (MUC) present in saliva protect the surface of oral mucous membranes from toxins and various types of irritants contained in stimulants or food. Recent studies also prove that these substances protect the mouth by various mechanisms influenced by unique polymer structures. First, mucins can interact with salivary proteins to change their location and retention and thus increase the protection of the mouth. In addition, MUC7 and MUC5B may interact with microorganisms in the oral cavity to facilitate their removal and/or reduce their pathogenicity [11]. However, such factors as neutral pH and the presence of Ca2+ and Mg2+ ions facilitate the healing of abrasions and wounds of the mucous membrane.

Saliva also plays a very important role in the inhibition and development of carious lesions of the teeth, improving remineralization of the tooth enamel and preventing demineralization [5]. When the pH of this secretion is within 6.8–7.2, it becomes a saturated solution of calcium phosphates, which results in quick and effective remineralization of the initial changes. However, if we slightly acidify the environment, saliva becomes an unsaturated solution and easily soluble calcium hydrogen phosphates are formed; thus the susceptibility of the teeth to caries increases [6]. Specific defensive factors include immunoglobulins: IgA (affecting phagocytosis of streptococci by leukocytes), IgG (together with IgA slow down the formation of tartar), and IgM (partially produced by the parotid, their presence indicates the existence of acute inflammation). In the group of nonspecific defensive factors, we can distinguish enzymes and bactericidal substances, such as lysozyme, lactoferrin, histatins, mucins, and salivary peroxidase [8]. Saliva also contains buffer systems responsible for maintaining proper acid-base balance. The most important role is played by the bicarbonate buffer. The buffers maintain the pH of resting saliva between 5.7 and 6.2, while the pH of stimulated saliva can reach 8 [6].

Inorganic ingredients of saliva come mainly from blood. Their content in the secretion is not constant and they always appear in the ionized form. Cations such as Na+ and K+ are involved in the active transport of compounds through cell membranes, while Ca2+ and Mg2+ activate some enzymes. The Cl^- anion activates α-amylase and F^- has anticarious activity, whereas I^- plays a role in defence mechanisms, mainly due to the presence of peroxidase in the system [12].

There are many ways to use saliva as a biological fluid (biofluid). The significant advantages of saliva as a unique diagnostic material are its availability and the noninvasive method of collection [13, 14]. Collection is fast, inexpensive, and safe. In addition, saliva as a "body mirror" can reflect the physiological and pathological state of the oral cavity. Therefore, it serves as a diagnostic and monitoring tool in many fields of science, such as medicine, dentistry, and pharmacotherapy [15]. By analyzing the image of saliva, we not only can assess the degree of caries risk but also obtain additional possibilities to diagnose specific diseases that give early symptoms in the mouth. In 2002, the National Institute of Dental and Craniofacial Research (NIDCR) eliminated all the obstacles by approving body fluids as a diagnostic tool to assess the state of health and disease [16].

2. Aim of the Study

The aim of this review is to emphasize the diagnostic value of saliva as a research material in the configuration of its structure and secretion disorders. This review summarizes the comprehensive literature search, which was conducted using databases, and presents the selected studies of the physical and chemical properties of saliva in the field of dental treatment in various physiological and pathological areas of human health.

3. Materials and Methods

The selected literature covers the years 2000–2018. The data were obtained using the MEDLINE (PubMed) search engine, as well as an additional manual search. The results were evaluated and checked for the correctness of qualifying in accordance with inclusion and exclusion criteria (Figure 1). The analysis covered 77 articles selected from a group of 1986 publications and initially qualified for devising.

4. The Essence of the Matter

The analysis of blood, which is the most common preparation in clinical chemistry, aims to identify diseases and monitor the progress of treatment. However, medical personnel increasingly appreciate and use saliva as a diagnostic material. Saliva is a clinically informative biological fluid that can be used in innovative laboratory and clinical diagnostics, as well

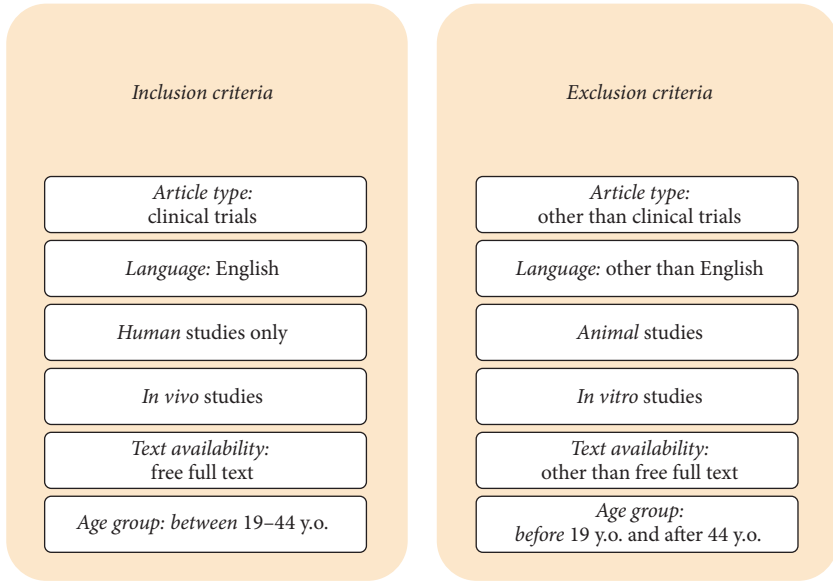

Figure 1: Criteria for including and excluding the correctness of qualifying the analyzed articles.

as in monitoring and treating patients with both oral and systemic diseases. The diagnostic use of saliva has attracted the attention of many researchers due to its noninvasive nature and relative simplicity of collection [17, 18].

Saliva as a diagnostic material is easy to collect, transport, and store. However, it should be remembered that samples of this material ought always to be collected from patients at the same time, preferably between 9:00 and 11:00 am (due to the highest physicochemical stability). A patient should not eat at least 90 minutes before the collection and stop taking medicines that affect salivary secretion 1 day before the procedure. Before sampling, the mouth should be rinsed with deionised water, and saliva should be collected for 10 minutes [19]. There are various methods of depositing saliva, depending on the type of material we want to obtain: the free flow of nonstimulated saliva from the mouth, spitting out using the Navazesh method [20] with little muscle stimulation, or sucking the saliva from the bottom of the oral cavity and its absorption [21]. In order to obtain stimulated saliva, we recommend chewing paraffin blocks or unflavoured and sugar-free chewing gum, which affects the secretion of saliva. The bacterial material and cellular impurities must be removed immediately after collecting the sample. The use of cotton filters for this purpose is not advisable, since hormones and proteins have an affinity for this type of material, and they may give false results or lower the diagnostic value of the test [22]. In order to determine the ionic composition and rheological properties of saliva, tests are carried out immediately after collecting the sample. In order to store saliva samples as a diagnostic material, they should be frozen, preferably in liquid nitrogen. Samples also ought to be collected into cooled containers. If samples are to be stored for a long time, the preferred temperature is −80°C. Potentially detrimental effects of freezing and defrosting can be compensated for by freezing samples at a temperature of −80°C, diluted in a ratio of 1 : 1 in glycerol [23].

Depending on the purpose of the material, glass or plastic containers are used, into which saliva can be drained, spat, or sucked. There are also absorption methods using commercially available kits, for example, Salivette. They include an absorbent cotton or synthetic cartridge, which the patient chews for a given period of time [24]. Because saliva sampling is noninvasive and gives an opportunity to carry out the procedure under various circumstances (large-scale studies, field tests, and the possibility of taking samples by people without medical training), it exceeds blood and urine tests in terms of usefulness. The simple, noninvasive method of obtaining the material for research and the repeatability of results make it useful to be applied in point-of-care tests [25].

Saliva diagnosis can be performed in the laboratory or directly in the dental office. Not only does the examination of the properties of this systemic secretion include the contained substances and chemical parameters, but also physical properties are increasingly explored (Figure 2).

4.1. Salivation Secretion Disorders. An adult person produces from 0.5 to 2 litres of saliva per day, but only 2–10% of the fluid is produced at night. During sleep, the rate of salivary secretion decreases to less than 0.25 ml/minute, while during chewing or speech these values oscillate around 10 ml/minute [26]. The basic salivary secretion is 0.33–0.55 ml/min on average and varies significantly between individuals, even under standard conditions. After strong excitatory stimulation, for example, under the influence of a food stimulus, the salivation can increase up to 1.5–2.3 ml/min, and after the administration of pharmacological agents, such as pilocarpine or methacholine, it reaches the value of 5.0 ml/min. The daily volume of saliva depends on the amount of sleep, the frequency and type of meals, and the action of emotional stimuli [27].

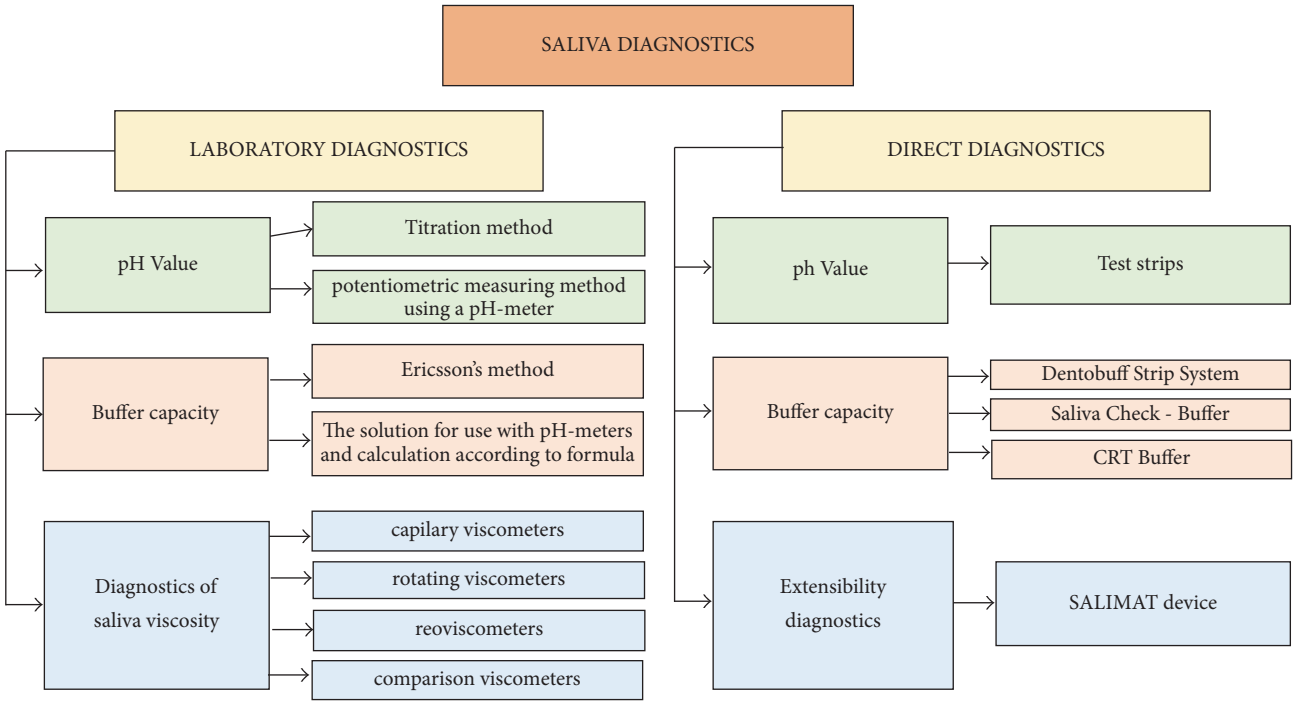

Figure 2: The original diagram of saliva diagnosis, which is also possible to be performed in the dental office.

In their studies, Fallahi et al. proved the insufficiency of cohesion forces in situations where the lowest value of adhesion forces was caused by the reduction of salivation, showing that not only the quality but also the amount of saliva secreted in adults using mobile prosthetic restorations is important [28]. The discussed issues were also raised by Turner et al. [29].

In their studies conducted in patients with xerostomia, Davis et al. demonstrated an increase in salivary secretion after the use of a toothpick by 440% compared to the baseline, while the growth was by 628% in subjects using toothpicks containing additional spilanthol, which is a fatty acid amide of plant origin obtained from the electric daisy (*Acmella oleracea*) [30].

The analysis of saliva performed by Khanum et al. immediately before and after extracorporeal dialysis in patients with renal insufficiency and significant changes in the oral environment showed an immediate effect of the stimulation of salivary secretion after the procedure [31], which was also investigated by other scientists [32, 33].

Various studies using physical stimuli were conducted in order to help people suffering from xerostomia. Aparna et al. observed that the production of saliva by the parotid glands increased in 76% of patients suffering from xerostomia after the use of percutaneous electrical stimulation (TENS currents) [34, 35].

Xerostomia is also associated with the consumption of some drugs, for example, anticoagulants, antidepressants, antiretroviral, and nonsteroidal anti-inflammatory drugs, as evidenced by numerous reports [36]. López-Pintor et al. have shown that the condition is also very common in patients suffering from diabetes in whom a decreased flow of saliva may additionally occur [37–39]. The studies on the causes and risk factors of xerostomia conducted by Niklander et al. demonstrated that the disease was more frequent in women above 60 years of age and in people who took medications for a long time, while menopause was considered as one of the risk factors [40].

Hypersalivation is extremely troublesome for patients struggling with Parkinson's disease. Nowadays, three mechanisms leading to increased salivation have been identified in this disease: elevated salivary gland production, disturbances of salivary absorption in the bottom of the oral cavity, and disorders of oral saliva removal. In refractory schizophrenia, hypersalivation is most often caused by clozapine [41].

Botulinum toxin is one of the most effective treatments for increased saliva secretion. In their studies, Mazlan et al. attempted to determine the most effective dose of this neurotoxin in reducing salivation in adult Asians suffering from neurological diseases. A prospective, randomized, double-blind controlled trial was conducted, which lasted for 24 weeks. The reduction in secretion was greater in the groups that received higher doses. The group taking 200 units showed the greatest decrease in salivation before week 24 and reported the most significant improvement [42–45]. In children, however, botulinum and Kinesio Taping [46, 47] can be applied to reduce salivation.

4.2. Saliva pH. Laboratory tests focus not only on the determination of substances in saliva but also on the analysis of its parameters. The pH value is one of them.

Saliva is a slightly acidic (pH 6-7) secretory fluid whose main ingredient is water (99%). Saliva collected without

stimulants, such as food, is hypotonic, and after stimulation, it becomes isotonic compared to plasma [48]. Saliva density is in the range of 1.002–1.012 g/ml, and its pH to a large extent depends on the rate of production. At night, when the salivation process is slower than that during the day, the pH reaches about 6.2–6.5. Saliva pH can increase up to around 8.0 due to the increased content of bicarbonate ions [49]. The concentration of hydrogen ions plays a significant role in the biophysicochemical processes taking place in the oral cavity. Saliva pH is not a constant value but undergoes significant changes under the influence of various factors (salivary secretion rate, daily cycle, diet, systemic diseases, and vegetative nervous system). The pH of mixed saliva is 6.38 (from 5.8 to 7.5) on average. The pH of whole saliva was shown to be higher in the morning than at midday and significantly higher after meals [48].

There was no relationship between the pH value and the age of patients; however, pH values differed in various areas of the oral cavity [50]. The highest value is found on the mucous membrane of the cheeks at the mouth of Stenson's duct, while the lowest value is found on the gums. There is the commonly known concept of critical pH, explaining the dissolution of enamel apatites at low concentration of hydrogen ions [51]. Maintaining the mineral balance between hydroxyapatite and saliva is important for the condition of the enamel. At pH = 5.5, the saliva is a saturated solution of calcium and phosphate ions, and under these conditions, these ions migrate to hydroxyapatites. When the pH drops, saliva becomes an unsaturated solution of these ions, which causes them to move in the opposite direction [5]. This leads to demineralization of the enamel, which stays unchanged for as long as these two processes remain in equilibrium. A drop in pH below the critical value (pH < 5.5) leads to superficial demineralization of the dental tissues [52]. Chemical dissolution of enamel hydroxyapatites without the use of bacteria is called enamel erosion. The erosion can be caused by acids supplied with food or internal acids formed in the body of patients suffering from bulimia, anorexia, and gastroesophageal reflux. The presence and intensity of erosive changes depend on the pH of saliva, its secretory capacity, buffering, composition, and the quality and duration of the action of a damaging factor [53].

The use of various oral care products also has an influence on pH. A phase IV clinical trial with three age groups was designed in order to analyze the effect of two rinses on saliva pH and to correlate the outcomes with age, buffer capacity, and salivary flow rate in healthy volunteers. A sudden significant increase in saliva pH was observed immediately after rinsing, while after 15 minutes the value dropped to an almost stable level. A huge increase in saliva pH value after using commercial rinses proves that saliva is a dynamic system and that the body is able to react to the stimulus by changing the content of this fluid. The results of this study increase the importance of in vivo measurements and strengthen the concept of the protective action of saliva [54].

The analysis of the latest literature reports led to the conclusion that the potentiometric method using pH meters is the most common laboratory technique for measuring saliva pH and that this parameter should be measured immediately after collecting the material [55]. There are also possibilities of direct diagnostics, for example, in the dental chair. This is a very easy and fast method of saliva pH diagnostics. Special test strips are used for this purpose; pH ought to be measured immediately after the material's collection; the result is obtained 10 seconds after the application of the material to the test strip, and data are read from the colour scale to an accuracy of 0.2.

4.3. Saliva Buffer Capacity. Given the modern methods of testing secretions, such as saliva, we should mention the possibilities of measuring buffering capacity. Hydrogencarbonate ions (HCO3−) are the most important in maintaining the homeostasis, chemical stability, and buffer capacity of saliva. Their concentration increases with the volume of saliva, proportionally reaching a peak of 40–60 mmol/l, which clearly exceeds the concentration of this anion in the blood plasma [56]. An increase in the HCO3− concentration is combined with a growth of salivary pH. This value is about 5.6 at the basal secretion and up to 7.8 during maximal secretion. In unstimulated saliva, the concentration of H+ ions is relatively constant. The stable pH level is maintained also thanks to buffering agents like bicarbonates, phosphates, proteins, free amino acids, ammonia, and urea [57].

The main buffering systems are bicarbonates, phosphates, and proteins. Bicarbonates are secreted only by large salivary glands, and their concentration depends on the rate of secretion. The second buffering agent is phosphate, whose concentration decreases with the increasing secretion rate. Because, contrary to the level of phosphates, the concentration of bicarbonates grows with the rate of saliva secretion, they are responsible for about 50% of the resting saliva buffer capacity and about 80% of stimulated capacity. Proteins and free amino acids have little effect on the buffering capacity of saliva [26].

In their studies, Nishihara et al. evaluated the effect of lactic acid bacteria *Lactobacillus salivarius* on the risk factors of caries. The participants were randomly divided into four groups and took *L. salivarius* WB21, *L. salivarius* TI 2711, Ovalgen® DC, or xylitol-containing tablets. They placed the pill on the tongue for a few minutes and allowed it to dissolve. The levels of streptococci and lactobacilli as well as the salivary flow, pH, and buffering capacity were evaluated before and after taking appropriate tablets. The level of streptococci was evaluated using Dentocult® SM Strip mutans, and the level of lactobacilli waas evaluated with Dentocult LB; the pH values were investigated using the CheckBuf test kit and the amount of salivary flow was assessed by a gum test. No significant differences were found between the groups in terms of the salivary flow and pH. The saliva buffering capacity increased significantly in the group of *L. salivarius* and Ovalgen® DC compared to the xylitol group [58].

In his study, Mummolo et al. assessed the bacterial levels of *Streptococcus mutans* and *Lactobacillus* spp., dental plaque, salivary flow, and buffer capacity of saliva before and during orthodontic treatment. The plaque index (PI) increased over time in each group, just like the saliva flow, and mainly

in subjects treated with self-ligating brackets, suggesting a difference between these devices and conventional systems. The authors of the study call for periodic microbiological monitoring and the control of salivary parameters during orthodontic treatment [59].

Diagnosis of saliva buffer capacity can be carried out in the laboratory or, as it more often happens, in a dental office through direct diagnosis using special test kits. The saliva buffer capacity can be tested in three ways:

(1) The Ericsson method: it is certainly the most accurate of the three methods mentioned; however, it is also the most time-consuming. A saliva sample is collected from the patient and then transferred and processed in a medical laboratory. The results are then sent back to the dental practitioner for consideration. Precisely, 1 ml of freshly collected saliva should be transferred to 3 ml of HCl (0.005 M for stimulated saliva and 0.0033 M for resting saliva). To prevent the foaming of the solution, add one drop of 2-octanol and mix it for 20 minutes to remove CO_2. The final pH of the solution is then measured using a pH meter

(2) The Dentobuff Strip System method: this method is much faster; a result can be obtained within a 5-minute period; however, this is neither as accurate nor as comprehensive as the aforementioned Ericsson method. Buffer capacity is determined based on the colour of the test strip 5 minutes after the application of a saliva drop onto the strip

(3) The Saliva Check-Buffer method: it is decidedly the fastest of the three methods with results being available after only two minutes. Interestingly, it is also more precise than the Dentobuff Strip System due to it having more test areas. Buffering capacity is determined based on the colour of the test strip windows two minutes after the application of saliva is applied to the strip.

5. Elasticity of Saliva

Mucins are mainly responsible for the elasticity of saliva. They are compounds rich in proline, glycoproteins, and electrolytes, as well as water, and are considered protective agents of the oral mucosa. These substances are one of the most important proteins in the mouth. In nonstimulated saliva, they constitute 20–30% of the total amount of proteins. With their increasing amount, the density and viscosity of the secretion grow. The viscosity of saliva depends primarily on the MG1 glycoprotein [60, 61].

No method has been introduced into general use which would allow for the unambiguous qualitative and quantitative diagnosis of saliva in terms of the biophysical properties mentioned above. From a technical point of view, a determination of the viscosity of saliva in a laboratory test does not cause major problems because viscosimeters are used for this evaluation. Different models of the tools are distinguished, capillary, rotational, with a falling ball, and comparative, allowing for the measurement of relative viscosity. Another approach to assess the biomechanical properties of saliva is to measure its elasticity [62].

It is known that carious lesions in the teeth can in various ways affect the properties of saliva. Aminabadi et al. examined how the elimination of active tooth decays in people with more than five tooth caries surfaces influenced saliva parameters. The selected parameters were evaluated in the samples of unstimulated saliva. One month after the treatment of caries, during which all the affected surfaces were removed, saliva samples were collected and reanalyzed. The salivary viscosity significantly decreased, while the buffering capacity and saliva pH significantly increased. It should be concluded, therefore, that tooth caries can be prevented by improving the quality of saliva, including its physical parameters [63–65].

SALIMAT (Poland), which is a prototype device used to test the direct viscosity of saliva, consists of a digital calliper as a supporting structure and reader to examine saliva (Figure 3). This test is a simple and effective measurement method and generally comes down to the determination of the length of the stretched saliva sample, of a specified volume, until it is broken. The device allows for the manual and automatic measurement of the length at which the sample breaks. It can be synchronized with a computer or work independently. The value at which the length of the saliva sample is broken can be read on the device display with an accuracy of ±0.001 mm. The moving part of the device is equipped with a drive wheel that allows it to move smoothly, which is important for automatic and precise measurement. Special overlays are mounted coaxially on the arms of the calliper. The upper one is formed by a flat-ended cylinder with a diameter of 9 mm, and in the lower one having a diameter of 10 mm a recess is milled (diameter 8 mm, depth 1 mm, and volume 50.26 mm^3), and here the sample of saliva is placed.

6. Determination of Hormones

6.1. Cortisol. The determinations performed in saliva can be used to diagnose infectious diseases, autoaggressive diseases, and cancer, as well as endocrine and cardiac disorders. Saliva can also be applied as a material in the examination of drug levels or tests for the presence of narcotics. Cortisol is a hormone most commonly determined in saliva. The diagnosis and monitoring of the therapy are based on changes in the levels of concentrations of this hormone [66]. Cortisol, which is the most important glucocorticoid, is synthesized by the adrenal cortex. Its production is regulated by the ACTH hormone, secreted from the pituitary gland, which in turn is controlled by CRH released from the hypothalamus, ACTH-releasing hormone. Over 90% of serum cortisol is connected with corticosteroid binding globulin (CBG, transcortin). During pregnancy or oestrogen therapy, the CBG concentration decreases, and consequently the total cortisol concentration increases. Free cortisol is biologically active; it affects the metabolism of proteins, fats, and carbohydrates and has anti-inflammatory and antiallergic properties. Cortisol has an impact on carbohydrate, fat, protein, calcium, and phosphate metabolism [67].

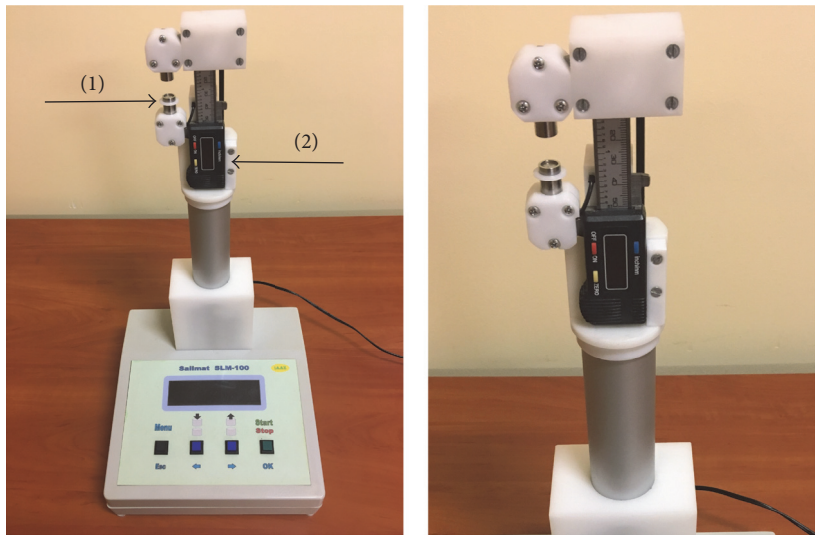

Figure 3: SALIMAT-SLM-100 (Poland), a prototype device for measuring the direct viscosity of saliva. (1) A saliva container. (2) Electronic measurement display.

Both increased and decreased levels of cortisol have a negative impact on the body and produce various symptoms and consequences. Cortisol tests are closely related to oral health. Lopez-Jornet et al. demonstrated that patients with lichen planus located in the oral cavity suffered from sleep disturbances and had higher levels of cortisol and proteins compared to the control group of healthy patients [68].

Cortisol determination is recommended if we suspect the excessive production or the lack of cortisol production by the adrenal cortex (in Cushing's and Addison's disease, hypothyroidism, hypoplasia of the adrenal glands, or neoplasms). The secretion of cortisol shows large daily changes; the highest values are observed early in the morning and the lowest ones around midnight. Thus, a single measurement of cortisol is of little importance—we should take samples in the morning and in the evening. Cortisol determination also plays an important role in certain stimulatory and suppressive tests that are used to examine the activity of the hypothalamic-pituitary-adrenal cortex axis [69].

In order to test saliva, we should collect the fluid in special tubes and centrifuge it. The examination is performed in 7 saliva samples collected throughout the day at specific times. Seven determinations throughout the day allow for the accurate assessment of changes in cortisol levels during the day. The measurement of cortisol in saliva is a simple, convenient, and accurate technique with a potential value in monitoring patients with hypercortisolemia. This analysis shows the late-night changes in salivary cortisol. Cortisol, determined in salivary samples, can be a simple, convenient biomarker for diagnosing and assessing the response to treatment in patients with Cushing's disease [70, 71]. Elias et al. also proved that salivary cortisol was more sensitive and reliable than urine cortisol, and it should be the basic biochemical test in diagnosing this disease [72].

Lokhmatkina et al. and Trickett et al. focused on the measurement of cortisol in salivary samples obtained from women who were victims of domestic violence and sexual abuse. The authors claim that this measurement may allow for a better understanding of the pathophysiological mechanisms of mental disorders related to violence in women and inform researchers and practitioners about the possibility of using salivary cortisol as a biological marker of prognosis, diagnosis, and treatment in those who are victims of this abuse [73, 74].

In their research, Peterson et al. confirmed the fact that the analysis of cortisol level can be used in psychiatric and psychological examinations. In the laboratory protocol, men were exposed to neutral stimuli causing negative and positive effects. The concentration of cortisol in saliva was measured during the whole laboratory procedure. The results of this study were consistent with the view that male sexual aggression is connected with physiological hyporeactivity and physiological profile associated with psychopathic behaviours and features [75]. Feinberg et al. confirmed these reports by analyzing the level of cortisol in the saliva of couples expecting their first child and who were experiencing emotional violence in the relationship. In these studies, which focused on the assessment of the hypothalamic-pituitary-adrenal axis activity, the authors showed that a persistent increase in the activity of this structure in men, with the simultaneous reduction of this activity in women, led to the onset of male violence in a later period [69].

Vreeburg et al. analyzed the level of cortisol in patients with various anxiety disorders. Three groups of patients were compared: control, without psychiatric disorders, and remitted, with no current anxiety disorder and with current anxiety disorder. Cortisol levels were measured in seven saliva samples. A slight but extremely significant difference was demonstrated in patients suffering from panic attacks with coexistent agoraphobia and depression [76].

The level of cortisol also changes under the influence of various stressful situations and the preparation for such events. This was confirmed by Meunier et al. in a study that concerned, among others, the analysis of salivary cortisol levels before, during, and after a simulated task related to intense emotional arousal. Communication skills training has an effect on physiological arousal. After simulated training, the cortisol level significantly increased compared to the control group [77]. Bedini et al. subjected a group of emergency response coordination centre operators to similar examinations. They noted higher levels of cortisol during shift work and in the subgroup of men. Higher cortisol levels are also observed during a serious life-threatening condition [78].

Tecles et al. and Preuß et al. used biomarkers contained in saliva to determine the level of students' stress related to public speaking, as well as a written exam. They showed that the highest concentration of cortisol was observed 20 minutes after the end of the presentation, and it had no connection with the level of stress indicated by students in the surveys, gender, or the quality of the presentation. In the case of written exams, the level of cortisol was high already on the day before the exam and dropped immediately after its completion [79, 80].

By means of serial cortisol measurements, we can also determine the adrenal cortex response to CRF [81]. Huhn et al. examined the level of this hormone in women during labour. They measured the level of cortisol in saliva before and after the occurrence of an acute painful stimulus. This is the measurement of the so-called biological response to pain. The response to pain was generally lower than expected and completely resolved within 72 hours [82].

The examination of salivary cortisol is also important in research into numerous disorders involving hygiene and quality of life. On the other hand, Lipschitz et al. proved that educational training on sleep hygiene caused a decrease in the cortisol levels in salivary samples in patients after cancer [83].

6.2. Leptin. Leptin is a substance that has been frequently tested and measured over the past few years. It is a protein secreted mainly by fat cells, and as a hormone it plays a role in the regulation of food intake and energy management. Gröschl et al. showed that leptin is also produced, stored, and secreted by the salivary glands, and its level increases with the flow of saliva [84].

Similar conclusions were presented by Jayachandran et al. who proved that obese people had higher levels of leptin in saliva. Their research also demonstrated a close relationship between the level of leptin and the range of movement of the teeth supplied with orthodontic devices. Smaller dislocations were reported in obese subjects than in those with normal Body Mass Index (BMI) [85].

Schapher et al. also demonstrated a higher level of leptin in all types of salivary gland neoplasms and thus indicated that it was a marker in the diagnosis of salivary gland tumours [86]. However, lower levels of the hormone were observed in patients with advanced periodontal disease [87].

The studies conducted by Rodrigues et al. on the level of leptin in saliva and taste sensation demonstrated that this level was higher in a group of girls but only those with normal body weight and a reduced sense of sweet taste. In a group of boys who were more sensitive to the bitter taste, elevated levels of this hormone were reported. The authors also found a tendency to higher concentrations of this hormone in obese people [88].

7. Determination of Enzymes

7.1. Amylase. Amylase is another substance very often determined in saliva in the laboratory. It is a protein that in human saliva is responsible for the formation of the glycoprotein complex within the pellicle which is formed on the surface of the teeth immediately after cleaning. It has a high affinity for bacteria living in the mouth and is associated with them by adhering to the surfaces in the mouth [89]. It also takes part in the initial digestion of food and helps remove carbohydrate fibres from the interdental spaces. Contreras-Aguilar et al. revealed that both activity and concentration of amylase vary depending on the stress situation and the method of determination and interpretation. These differences may result from many factors, such as changes in the salivary flow, the concentration of all salivary proteins, and various stressful situations [90]. Stress and pain are often interrelated events. Investigators have attempted to distinguish them using a variety of model systems that induce either stress or pain and subjects are monitored for changes in salivary biomarkers. A typical marker that has been identified is salivary amylase.

In their studies focused on the diagnosis of saliva in physically active people, Rutherfurd-Markwick et al. showed that the resting activity of amylase was higher in a group of women than in men. However, in men, a relationship was observed between the secretion of amylase and cortisol [91].

Among members of a Paralympic swimming team, Edmonds et al. reported an increase in the level of amylase during 14 weeks of preparation for international competitions, probably due to the impact of the parasympathetic system [92].

Higher levels of mothers' amylase secretion after caesarean section and with direct intraoperative contact with the child were demonstrated, compared to a group of mothers who had contact with their babies only after the procedure [93].

It was also shown that the consumption of coffee, albeit not being a stress factor, resulted in an amylase activity increase in saliva, without changes in the level of cortisol, which may suggest the activation of the sympathetic nervous system. As the level of amylase increases after drinking coffee without a simultaneous rise in cortisol, the authors of the abovementioned publication suspect that it may have antistress properties [94].

Not only caffeine but also methamphetamine (METH) aroused the interest of researchers in terms of the influence on salivary amylase. Haile et al. assumed that METH must affect the concentration of amylase because it activates the sympathetic system by increasing the central and peripheral

concentration of norepinephrine. It should be noted that it is a biomarker of the sympathetic system activation, which correlates with the level of norepinephrine in plasma. The researchers managed to show that METH increased the level of amylase and was dependent on age, BMI, and the amount of METH taken per kilogram of body weight. Thus, they demonstrated that the activity of amylase, which is a peripheral norepinephrine biomarker, is closely related to the subjective effects of METH [95].

The results of amylase determination also revealed changes in the profile of salivary proteins in morbidly obese women who were qualified for bariatric surgery. Lower levels of amylase were noted in a group of obese women who underwent surgery; the activity of this protein was also lower than that in a group of obese patients [96].

7.2. Lysozyme. Lysozyme is an enzyme originating from the large salivary glands, gingival fluid, and digested leukocytes. Lysozyme is also known as muramidase or N-acetylmuramide glycanohydrolase, which is an antimicrobial enzyme produced by animals which forms part of the innate immune system. Lysozyme is a glycoside hydrolase that catalyses the hydrolysis of 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in peptidoglycan, which is the major component of Gram-positive bacterial cell walls. This hydrolysis in turn compromises the integrity of bacterial cell walls causing lysis of the bacteria. In humans, the lysozyme enzyme is encoded by the LYZ gene. Elevated salivary lysozyme levels, a biomarker for oral infection and hyperglycemia, have also shown a significant association with hypertension, an early stage of cardiovascular disease.

Gillum et al. also demonstrated that overproduction of leukocytes in saliva is not necessary to increase the amount of lysozyme [97]. Kim et al. determined the effect of xylitol and sorbitol on lysozyme activity. Both analyzed carbohydrates inhibited the enzymatic activity of lysozyme in a solution of whole saliva, while they did not affect the properties of this protein on the surface of enamel hydroxyapatites [98].

In research on new drugs aimed at inhibiting the growth of cariogenic colonies of bacteria living in the oral cavity, Tonguc-Altin et al. proved that, thanks to the appropriate carriers and in combination with lactoferrin, lysozyme can be used to reduce the amount of *S. mutans* and *L. acidophilus* [99].

Studies showed that, in diabetes, which is caused by increased glucose (Glc) concentration, glycation of proteins, including lysozyme, leads to the formation of advanced glycation end products (AGEs), which in turn significantly increases the risk and pace of complications development. Mirmiranpour et al. demonstrated in their work the possibility of inhibiting glycation of lysozyme by L-lysine, which gives an opportunity to alleviate pathological changes in this disease [100].

In a study on salivary defence proteins in women with or without human papilloma virus (HPV) infection, Haukioja et al. proved that the level of this protein was higher in patients with the chronic infection and that the HPV infection of the oral cavity might be associated with the elevated concentration of IgG and lysozyme [101].

In their research, Ligtenberg et al. have demonstrated that physical effort also affects the flow of saliva and the secretion of proteins (including lysozyme), amylase, and 5B mucin. Exercises of moderate intensity increase the levels of proteins and lysozyme, as well as amylase and 5B mucin. The flow of saliva also improves. On the other hand, very strenuous effort leads to a reduction in the amount of proteins in the secretion [102].

8. Determination of Proteins

8.1. Chromogranin A. Chromogranin A is an acidic secretory protein that can be found in many types of neuroendocrine tissues. It is secreted by sympathetic nerves together with catecholamines and can be used as a marker for sympathetic nervous system activity [103, 104]. Chromogranin A (CgA) is produced in submandibular salivary glands; therefore, taking the sample of material to determine its level is simple, fast, and painless for the patient [105]. A circadian rhythm can be observed in the secretion of this protein. The highest values were recorded around 11:00 pm and the lowest ones at about 8:00 am [106].

In their research, Yoto et al. investigated the antistress effectiveness of two kinds of green tea against a mental stress task load. A group of patients participating in the study drank warm water, green tea (Sagara), and shaded white tea. Saliva was collected before the fluid intake and after mental stress load tasks. Their studies showed that concentration of CgA in saliva increased after mental stress, but drinking green tea can inhibit the growth [107].

In their studies, Matsumoto et al. examined the soothing effects of fragrance from yuzu. The salivary CgA was used as a stress marker showing the activity of a sympathetic nervous system. The study group was exclusively women. Participants were examined twice, firstly using the yuzu scent and secondly using unscented water as a control in the follicular phase. They proved that inhalations of the yuzu scent significantly decreased salivary CgA levels [108].

8.2. Immunoglobulin A. Immunoglobulins A are an immunoglobulin class that is characterized by the presence of a heavy α chain in its structure. The main physiological role of IgA is to participate in immune reactions within the mucous membranes. The amount of IgA synthesized in the human body throughout the day is greater than all other immunoglobulins combined. Although the concentration of this immunoglobulin in the blood plasma is small, the vast majority of it is secreted on the surface of mucous and serous membranes. There are two IgA subclasses, IgA1 and IgA2, which can be produced as monomeric or dimeric form. The secretory IgA (sIgA) is dimeric form of this immunoglobulin. sIgA can be found in mucous secretions, like saliva, tears, sweat, and so forth [109]. It is produced by B-lymphocytes adjected to the mucosal cells, transported through cells, and released into the secretions. The highest levels of sIgA in humans can be found in the minor salivary glands [110].

The secretory circadian rhythm reaches the highest level in the morning and the lowest level in the evening [111]. Stress affects the secretion of sIgA in saliva. The level of this immunoglobulin also depends on the saliva flow rates [112].

Research carried out by van Anders has shown that chewing gum significantly decreased saliva production time by 3–6 min, but it also leads to lowering the IgA level, in both men and women groups [113].

Laurent et al. in their studies focused on understanding the effect of stress factors on secretion of sIgA in children and adolescents. In saliva samples obtained after exposure to a series of performance or interpersonal stressors in laboratory sessions, the levels of sIgA, cortisol, and alpha amylase were determined. Participants displayed SIgA reactivity to and recovery from acute stress. The relations of sIgA with cortisol and alpha amylase reflected different forms of cross-system linkage. Youth SIgA trajectories followed a normative pattern of reactivity and recovery around the stressors; however, these responses were blunted in young people with higher externalizing scores. In contrast to SIgA, neither cortisol nor sAA related significantly to behavioral problems [114].

9. Conclusions

Even though saliva is not a popular body fluid, more and more dentists, internists, paediatricians, pharmacologists, clinical and forensic pathologists, endocrinologists, immunologists, psychologists, and dentists are discovering that the liquid is an easily available, noninvasive diagnostic tool. Newly emerging and fast-growing technologies, such as the latest point systems, RNA sequencing, and fluid biopsy, can provide new diagnostic solutions in the field of saliva diagnostics. These recent advances have broadly widened the possibilities of saliva testing in the oral cavity, making clinical salivary diagnosis a reality that can be very precise and useful in the assessment of oral health. In addition, it should be noted that the determination of chemical and physical saliva parameters can be effectively performed in the patient's presence in the dental office.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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Review Article

Salivary Biomarkers in Systemic Sclerosis Disease

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Scleroderma or systemic sclerosis (SSc) is frequently detected at an advanced stage due to diagnosis difficulties. Salivary biomarkers, if existing, could be used for predictive diagnosis of this disease. Human saliva contains a large number of proteins that can be used for diagnosis and are of great potential in clinical research. The use of proteomic analysis to characterize whole saliva (WS) in SSc has gained an increasing attention in the last years and the identification of salivary proteins specific for SSc could lead to early diagnosis or new therapeutic targets. This review will present an overview about the use of WS in SSc studies. The proteomic technologies currently used for global identification of salivary proteins in SSc, as well as the advantages and limitations for the use of WS as a diagnostic tool, will be presented.

1. Introduction

Scleroderma or systemic sclerosis (SSc) is a rare systemic autoimmune disease affecting the connective tissue and characterized by involvement of the skin, blood vessels, and visceral organs. It is associated with dysfunction of the immune cells, fibroblasts, and endothelial cells. The etiology of SSc remains unknown [1]. SSc affects preferentially women, more often during and after their childbearing age [2]. Sex ratio varies in published series from 3 to 9 women for a man [3].

There are two main clinical forms of SSc that differ primarily in their degree of skin involvement: limited cutaneous scleroderma (lcSSc) and diffuse cutaneous scleroderma (dcSSc), which are associated with different clinical complications [4]. Oral manifestations are frequent in SSc [5], and the majority of oral clinical features start with tongue rigidity and facial skin hardening [6]. On the other hand, it was shown that SSc affects salivary glands [7], and these latter can also be subject to fibrosis in SSc patients [8].

The medications and fibrotic changes in salivary glands of patients with SSc can contribute to reduced salivary flow in these patients [9]. This diminishing of saliva production in SSc patients is mostly related to concomitant Sjögren's syndrome [10]. Although a little knowledge about salivary gland involvement in SSc has been reported in the literature, it was demonstrated, in a previous study, that salivary gland changes (increased expression of E-selectin and TNF-α and infiltration by mast cells) are detectable in the early stages of the disease, before the onset of skin changes and when the criteria for a diagnosis of SSc are absent [11].

The identification of salivary protein profiles could lead to early diagnosis or new therapeutic targets of SSc [12]. Furthermore, the presence of the biomarker may correlate with different clinical symptoms of the disease due to its absence in healthy subjects [13]. In the last years and with technological and analytical development, saliva has attracted an increased interest for use in diseases diagnosis and treatment. To date, there have been few reports aimed to use the WS in SSc research [7, 12–14]. Giusti et al. [14] performed, for the first time, a study in an attempt to characterize the WS protein profiles of patients with SSc using a proteomic research approach.

In this review we will give an overview of the use of WS in SSc research. The proteomic technologies currently used for global identification of salivary proteins in SSc, as well as the

advantages and limitations for the use of WS in the disease, will be presented.

2. Whole Saliva

A number of proteomics researches contributed to the clarification and knowledge of the salivary proteome, and more than 2000 proteins and peptides have been found in WS and individual salivary glands [15]. Saliva is a mucoserous exocrine fluid produced by three major salivary glands (parotid, submandibular (SM), and sublingual (SL)) and other minor glands located under the oral mucosa [16]. Besides, WS comes also from local and systemic sources [17]. It is a combination of the secretions from the major and minor salivary glands, oral mucosa transudate, the gingival fold, desquamated epithelial and blood cells, nasal secretions, viruses, fungi, bacteria, and food debris [18]. WS contains hormones, immunoglobulins, proteins, enzymes, and mucosal glycoproteins [18]. It also contains a number of antimicrobial proteins which play an important role in reducing oral infections [19]. Its role in protecting oral structures has been well reported. Although saliva includes blood derived products, differently to serum, this oral fluid contains many locally secreted proteins which may be specific markers for some local diseases [16]. The presence in WS of many molecules that are circulating in the blood presents several advantages for disease diagnosis and prognosis as the collection of this fluid is noninvasive, safe, and easy; relatively low amounts of sample are needed and storage and transportation are not complicated [20, 21]. That is why, nowadays, WS is used as a diagnostic tool in clinical diagnosis, monitoring disease progression and management of patients [22, 23].

Passive drool saliva collection method is considered as the gold standard, but there are other saliva collection devices such as Salivettes [24, 25] which present a less viscosity and allow an easy handling as well as a better sample processing, particularly in some special cases such Xerostomia [26]. Unlike blood, due to its noncoagulating nature, saliva is easier to handle for diagnostic analysis procedures. The noninvasive collection procedures for saliva contribute to the procurement of repeated samples to follow the patients over time. Various collection and storage protocols of WS were described in published studies [7, 12–14, 27–36]. Due to the presence of circadian rhythms in WS flow rate and composition, WS collection should be made under standard conditions [37]. The variations in collection and/or storage procedures can change the salivary proteomic profiles after collection and therefore alter the biomarkers content and their detection [21], from where the need for adopting standardized procedures in saliva analysis. In fact, in order to avoid protein degradation, some authors have added 0.2% trifluoroacetic acid (TFA) to saliva sample [38]. Others have used a protease inhibitor cocktail and have stored the saliva at −20°C [39]. Furthermore, minimizing the time between collection and analysis of the sample has been proposed by some groups research [12–14, 36, 40]. Thus, it has been indicated that instead of using chemical inhibitors of proteolysis, collection of saliva into ice cold tubes could minimize proteolysis, as well as storage of the samples at −80°C rather than −20°C [41], in particular for storage at longer durations [42].

3. Applications of Whole Saliva for Systemic Sclerosis Study

In the recent years, proteomic approaches started to be used in the study of WS from patients with SSc. Knaś et al. [7] study was the first to describe the alteration on the salivary glands function in both subsets of SSc.

Among the different proteomic approaches, saliva has been studied using several techniques, either separately, or, more often, in combination such as one-dimensional polyacrylamide gel electrophoresis (1D-PAGE), two-dimensional polyacrylamide gel electrophoresis/mass spectrometry (2-DE/MS), capillary electrophoresis (CE), 2D-liquid chromatography/mass spectrometry (2D-LC/MS), nuclear magnetic resonance (NMR), matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF/MS), surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF/MS), Western blot, electrospray ionization (ESI), immunoassays (radio-immune assays, immunoradiometric assays, enzyme immuno-assays, and enzyme-linked immunosorbent assays) and lectin binding assays on PAGE gels [15, 22, 43–54]. The choice for the technique is depending on the objectives of the study and on the salivary proteins of interest. It has been reported that 2-DE combined with mass spectrometry has been widely used to study salivary proteins [14, 15, 49]. However, due to its limitations it is not the most important tool used in this field and does not allow the study of the complete proteome. In fact, other techniques have been shown to be more performant, including a variety of chromatographic combinations that has successfully characterized more than 3000 different components in saliva [38, 55–57]. Surface chromatography combined with MALDI-TOF/MS has allowed rapid and high-throughput detection of important proteins and peptides [58]. From our knowledge, only four studies focused on WS in SSc have been published, and only one study [14] has identified the salivary biomarkers in these patients. In these proteins separation was achieved by using 2-DE technique with subsequent protein identification being made by MALDI-TOF-MS (Table 1).

4. Salivary Proteins in Systemic Sclerosis

To date, there is a lack of early diagnostic markers, and the time between the diagnosis and symptom onset can be translated by years. Identification of the salivary proteins biomarkers involved in SSc may contribute to the early detection of the disease. The specific salivary markers identified so far were reported by Giusti et al. [14] in a study including 15 patients with dcSSc, in which they compared the differences between WS of SSc patients and control subjects. Indeed, it was reported that both previously identified and newly identified proteins occurred in WS of SSc patients but did not match with healthy subjects. Some of these proteins, such as keratin 6L, psoriasin, TPI, and Arp2/3 complex, might play a pathological role in SSc, suggesting that some of them may be

Table 1: Summary of published studies using the WS in SSc.

Study	Saliva sample	Patients/controls	Analytical methods	Findings
Giusti et al. [14]	UWS	15/15	2-DE, MALDI-TOF/MS	Presence of 9 proteins only in SSc (calgranulin A, calgranulin B, psoriasin, Arp2/3 complex, ß2-microglobulin, TPI, GAPDH, cyclophilin A, and cystatin B).
Baldini et al. [13]	UWS	44/80	SDS/PAGE, Western blot	Significant association of psoriasin with pulmonary involvement in dcSSc.
Knaś et al. [7]	UWS/SWS	97/55	ELISA, Spectrophotometrically	(i) In UWS of dcSSc and lcSSc: (1) Salivary flow, the output of total protein, and peroxidase activity were significantly lower. (2) sIgA and lactoferrin were significantly higher. (ii) In SWS: (1) In lcSSc, the total lysozyme and peroxidase activity were significantly higher. (2) In dcSSc, the salivary flow was significantly lower and the total sIgA and peroxidase activity were significantly higher.
Giusti et al. [12]	UWS	134/74	ELISA	Significant correlation between salivary psoriasin and DLCO in SSc.

dcSSc, diffuse cutaneous systemic sclerosis; DLCO, diffusion capacity of carbon monoxide; ELISA, enzyme-linked immunosorbent assay; lcSSc, limited cutaneous systemic sclerosis; MALDI-TOF, matrix-assisted laser desorption ionization; MS, mass spectroscopy; SDS/PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SSc, systemic sclerosis; SWS, stimulated whole saliva; UWS, unstimulated whole saliva; 2-DE, two-dimensional gel electrophoresis.

considered as new therapeutic targets or diagnostic markers for SSc. It was found also that, except Keratin 6L, the expression of most of the called typical salivary proteins like α-amylase, prolactin-inducible protein precursor, albumin, or cystatins remained unchanged between control subjects and patients. In contrast, the same research team showed that the expression of these normal proteins was altered in Sjögren's syndrome patients compared to the controls, with decreased levels of some salivary proteins [36] (Table 2).

Among those proteins, three that belong to the S100 calcium- and zinc-binding protein family related to inflammation have been identified: calgranulin A (S100A8), calgranulin B (S100A9), and psoriasin (S100A7) [12, 14, 45, 59]. S100A8 and S100A9 are mainly localized in the cytosol of neutrophils and are involved in the metabolism of arachidonic acid in human neutrophils [43, 60]. Some findings suggest that high concentrations of S100A8 and S100A9 might play a role in inhibiting the matrix metalloproteinases activity by the sequestration of zinc [59, 61]. This inhibition or reduced activity of MMP plays a crucial role in reducing extracellular matrix degradation in SSc individuals and leads to extensive fibrosis of this disease. Regarding psoriasin (S100A7), it was firstly identified by Madsen et al. [62], as a protein expressed in epithelial cells of the psoriatic skin. Increase of psoriasin expression has been also observed in WS of patients with dcSSc [14]. Although the biological effect of psoriasin in SSc remains unknown, a significant association of this protein and pulmonary involvement of dcSSc has been demonstrated [13]. Arp2/3 complex has been newly identified in WS [14]. This complex plays a role in the regulation of actin polymerization in cells, and it is necessary for neutrophil chemotaxis and phagocytosis [63]. The ß2-microglobulin is a component of MHC class I molecules which may play a role in the immune dysregulation of SSc [14]. Triose phosphate isomerase (TPI) and glyceraldehyde-3 P-dehydrogenase (GAPDH) 190 are glycolytic enzymes present in cytoplasm that may act as autoantigens in SSc and also in other autoimmune diseases such as systemic lupus erythematosus [64]. Regarding cyclophilin A, its contribution to the pathogenesis of immune-mediated endothelial activation and dysfunction was suggested by Kim et al. [65]. It is involved in the expression, folding, and degradation of proteins and catalyzes the cis-trans isomerization of proline imidic peptide bonds in oligopeptides [66]. Cystatin B is an intracellular thiol proteinase inhibiting [14, 44, 67]. However, its role in SSc has not been reported so far.

5. Advantages and Limitations

In the recent years and with the advances in proteomic technologies, salivary research emerged as an important area for the diagnosis of several local and systemic diseases. As mentioned above, saliva showed several advantages for systemic diseases research as well as for SSc including mainly safety and easy collection using simple, inexpensive, and noninvasive methods. The presence of several serum components in saliva has great benefits for research of new biomarkers. Moreover, due to its noncoagulating nature, saliva is easier to

Table 2: Salivary proteins identified in WS of SSc patients according to Giusti et al. [14].

Proteins	Swiss-Prot/NCBI	Function
Calgranulin A	P05109	Present in chronic inflammation and in epithelial cells constitutively or induced during dermatoses. Involved in the metabolism of arachidonic acid in human neutrophils. Seem to have a major role in inflammatory and immunological responses. May interact with components of the intermediate filaments in monocytes and epithelial cells. May play a role in inhibiting the matrix metalloproteinases activity.
Calgranulin B	P06702	Present in acute and in chronic inflammation. Stimulate neutrophil adhesion. Involved in the metabolism of arachidonic acid in human neutrophils. May play a role in inhibiting the protein kinases and the matrix metalloproteinases activity. May interact with components of the intermediate filaments in monocytes and epithelial cells.
Cystatin B	P04080	Proteinase inhibiting properties. Tightly binding reversible inhibitor of cathepsins L, H, and B.
Psoriasin	P31151	Present in fetal ear, skin, and tongue and human cell lines. Highly expressed in psoriasis and in other inflammatory skin diseases. Seem to participate in tumor progression. Also highly expressed in the urine of patients with bladder squamous cell carcinoma.
ß2-Microglobulin	Q6IAT8	Component of the MHC class I molecules.
Cyclophilin A	P62937	Involved in expression, folding, and degradation of proteins. Catalyze the cis-trans isomerization of proline imidic peptide bonds in oligopeptides.
Glyceraldeyde-3 P-dehydrogenase	P04406	Glycolytic enzymes present in the cytoplasm. Play a role in degradation of carbohydrate and glycolysis.
Triose phosphate isomerase	P60174	A highly conserved glycolytic enzyme. Mediated by glycolysis in red blood cells and in brain cells. Biosynthesis of carbohydrate and gluconeogenesis.
Actin-related protein 2/3 complex subunit 2	O15144	Strong candidate for the control of actin polymerization in chemotaxis.

Table 3: Advantages and limitations of WS as a diagnostic tool.

Advantages	Limitations
(i) Readily accessible and informative biofluid.	(i) Many informative molecules in lower amounts of saliva.
(ii) Easy, safe, inexpensive, and noninvasive diagnostic approach.	(ii) Centrifugation may also remove other proteins.
(iii) Noncoagulating nature.	(iii) Presence of several proteases degrading protein biomarkers.
(iv) More sensitive and more specific markers for oral diseases.	(iv) Difficult to have saliva completely free of stimulation which influences the results.
(v) Simple collection and minimal equipment required.	(v) Possibility of assaying proteins only after recent Exposure.
(vi) Storage and transportation at low cost.	(vi) Difficult detection with low concentrations of proteins of interest in saliva.
(vii) Less amounts of sample	
(viii) Contains serum constituents	

handle in diagnostic analysis procedures [15, 20, 22, 23]. Our knowledge about specific advantages and limitations of the use of this tool with diagnosis purposes in SSc is still limited. We mention in this review a raised concern for the use of WS in SSc patients with Sjogren's syndrome. These latter were shown to have generally a reduced salivary flow rate that could limit the collection and use of WS as research material for this group [7], but this needs to be more investigated. The most known advantages and limitations of WS that are likely to be extrapolated for SSc are presented in Table 3.

6. Conclusion

In conclusion, salivary biomarkers study is becoming an important part of the diagnosis of several diseases. Identification of salivary proteins in SSc is a promising finding that paved

the way to new diagnostic biomarkers for this pathology, but this needs to be more investigated as there are so far only few studies published in this regard.

Several approaches (SELDI-TOF/MS, HPLC, and other affinity chromatography techniques) hold promise for salivary proteomic analysis to discover and validate new biomarkers or therapeutic targets.

Deeper and more comprehensive studies are required to elucidate the functional significance of these proteins during the SSc and to improve diagnosis as well as treatment. In addition, larger populations are needed to validate and generalize these results in future studies of salivary proteomic. On the other hand, research to the salivary proteome in SSc patients from different regions of the world is required due to the variability in genetic and environmental factors of each subject.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

The Effects of Tooth Brushing on Whole Salivary Flow Rate in Older Adults

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Objectives. (1) To determine whether manual (MTB), or electric, tooth brushing (ETB) modulates whole salivary flow rate in older adults who are free of systemic disease. (2) To determine the duration of the brushing-related modulation of salivary flow rate. (3) To compare salivary flow rate modulation associated with MTB and ETB. *Method*. Twenty-one adults aged 60 years and older participated in two experimental sessions during which they used a manual, or electric, toothbrush to brush their teeth, tongue, and palate. Whole salivary flow rates were determined using the draining method before, during, and after brushing. Differences in salivary flow rates across time periods, and between conditions, were examined using paired samples t-tests applying a Holm-Bonferroni sequential procedure ($p_{corr} < 0.0045$). The relationship between tooth brushing and age with respect to maximum salivary flow rate increase was examined using Pearson's correlation coefficient ($p < 0.05$). *Results/Conclusion*. Whole salivary flow rates increased during, and for up to 5 minutes following, tooth brushing in adults aged 60 years and older who were free of systemic disease. The salivary effects of MTB and ETB were not significantly different. A moderate, positive correlation was observed between tooth-brushing-related maximum salivary flow rate increase and age.

1. Introduction

The oral tissues are among the most richly innervated of any in the human body. Sensory receptors in the tongue, periodontal ligament, gingiva, and palate convey an extensive range of sensory information including pressure, stretch, vibration, pain, and temperature [1–3]. These forms of sensory stimulation are thought to modulate salivary flow rate [4–6].

Tooth brushing has the capacity to produce pressure, stretch, and vibratory mechanical stimulation on the tongue, periodontal ligament (through pressure on the teeth), gingiva, and palate. Thus, tooth brushing may be hypothesized to modulate salivary flow rate. Current literature examining tooth brushing as a form of saliva stimulation is limited. Hoek and colleagues [7] identified a transient increase in salivary flow rate in 14 healthy adults during the initial five minutes following manual brushing. Ligtenberg and group [8] reported that, in 80 healthy students, salivary flow rates increased significantly after brushing with water and after brushing with dentifrice, and salivary flow rates remained increased for 60 minutes after brushing. In older adults with

clinically significant hyposalivation, Papas et al. [9] reported that an electric toothbrush (ETB) tended to stimulate greater salivary flow rates compared with a manual toothbrush (MTB) for up to 45 minutes after stimulation. Taken together, these studies provide conflicting findings with regard to the duration of increased salivary flow following tooth brushing. Furthermore, salivary responses to tooth brushing in older adults without clinically significant hyposalivation have not been examined. A clearer understanding of the effects of tooth brushing on salivary flow rate would inform consideration of the feasibility of this form of stimulation as an oral health intervention.

Therefore, the present study (i) examined whether manual, or electric, brushing of the teeth, tongue, and palate modulates whole salivary flow rate in older adults free of major systemic disease, (ii) determined the duration of brushing-related modulation of salivary flow rate, and (iii) compared the salivary flow rate modulation associated with manual and electric tooth brushing. We hypothesized that tooth brushing would increase salivary flow rate during, and for 5 to 30 minutes following, tooth brushing [7–9], with the ETB conferring a larger effect compared with the MTB. Given that older adults have a lower unstimulated whole salivary flow rate than younger adults [10], we further hypothesized that the maximum salivary flow rate increase in response to tooth brushing would decrease with increasing participant age. A version of this study has been published in thesis [11] and abstract form [12].

2. Methodology

Twenty-one nonsmoking adults who were free of major systemic disease volunteered as participants. Candidates were excluded if they had fewer than 20 natural teeth, complained of xerostomia, or had been seen by a dentist in the seven days immediately prior to the experimental session. The sample size was based on a power calculation indicating that a sample of $N = 20$ was sufficient to detect a difference of one standard deviation ($d = 1.0$) in a two-level, within-subjects independent variable 80.8% of the time, using a 0.01 alpha level and assuming a within-subjects correlation of 0.30.

Relevant participant data relating to age, medical history, and dental history were collected during a brief interview prior to the experimental session. Participants were instructed to eat breakfast and complete their morning oral hygiene routine by 0800 and to refrain from eating or drinking prior to the study session. Each session commenced at 0900 and lasted approximately 120 minutes. Each subject gave written informed consent before participating in the study, which was approved by the Western University Research Ethics Board for health sciences research involving human subjects.

Each person participated in two experimental sessions. Participants were randomly assigned to one of two groups. Group one used a MTB in the first experimental session and an ETB during the second experimental session. Group two followed the same procedure in reverse order. The two experimental sessions were separated by at least one day, and no more than 21 days, across subjects.

A visual inspection of the oral cavity was completed to rule out gross anatomic abnormalities and to ensure each participant had at least 20 natural teeth. Participants then rinsed their mouth with distilled water.

Participants were seated in a chair that was stationed in front of a table. Transducers were positioned on the participant: belt-mounted movement sensors positioned around the participant's neck (Model 1585, CT2 Pediatric Piezo Respiratory Effort Sensor, Pro-Tech Services, Inc., License Number 69444) and upper abdomen (Model 1582, CT2 Adult Piezo Respiratory Effort Sensor, Pro-Tech Services, Inc., License Number 69444) recorded neck and respiratory movements, respectively. An omnidirectional electret microphone (F-SM Snore Electret Microphone, Pro-Tech Services, Inc., License Number 69446), affixed to the participant's neck with medical tape, monitored the acoustic signal from the upper airway through the tissues of the neck. These physiologic signals were recorded continuously throughout the session using an AS40 Comet Series PSG/EEG Portable System (Astro-Med Inc., License Number 65827). In addition to these physiologic signals, a lateral-plane video recording of the participant that included the head, neck, shoulders, and chest enabled researchers to observe whether participants swallowed during the saliva collection periods.

Each of the two study sessions was comprised of a habituation period, control condition, experimental condition, washout period, and eleven salivary collection periods (Figure 1). During the five-minute habituation period, the participant sat at rest and was instructed to minimize their orofacial movements, and video recordings and neck movement, respiratory, and acoustic data were collected. Participants provided saliva samples, using the draining method, into a preweighed beaker [13]. A five-minute saliva-draining collection was performed at baseline following the habituation period and at 0 to 5 minutes, 10 to 15 minutes, 20 to 25 minutes, and 30 to 35 minutes following the control and experimental conditions. The participants were instructed not to swallow their saliva during the draining period. Following the study, the video and physiologic signals were reviewed by RHA for evidence of swallow-related respiratory and laryngeal movement patterns to verify that swallowing had not occurred.

Participants completed the control condition by placing either a Colgate Sensitive Pro-Relief MTB or a Colgate Sonic Power ETB, in the oral cavity (without dentifrice, bristles touching the superior surface of the tongue) and holding it stationary for two minutes. The experimental stimulation condition involved the participant actively brushing their teeth, tongue, and palate (without dentifrice) for two minutes. Participants were instructed not to swallow their saliva during the control and experimental tooth-brushing conditions. Immediately following both conditions, participants expectorated their saliva into a preweighed beaker. These salivary collections will be referred to as the salivary flow rates collected "during" the control and experiment. Participants sat for a five-minute washout period between the control condition and the experimental intervention.

Participants were trained to use a standardized tooth-brushing protocol when completing each two-minute oral

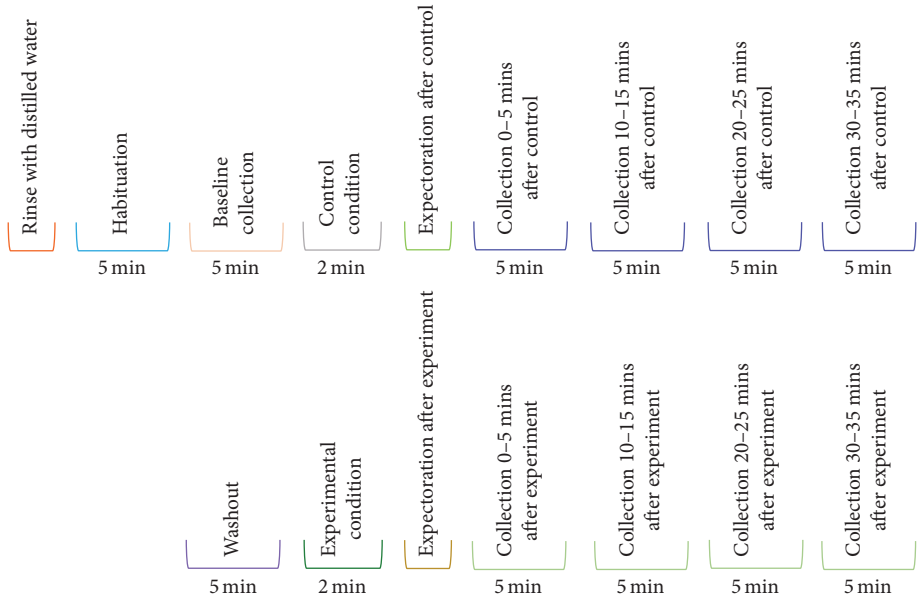

Figure 1: Experimental protocol.

cavity stimulation intervention. For the MTB intervention, participants were instructed to use a modified Bass technique [14] and systematically brush the outer, inner, and chewing surfaces of the teeth, as well as the tongue and palate. Similar instructions were used for the ETB intervention. The instructions for each of the standardized tooth-brushing protocols were presented in a video prior to the experimental intervention.

Subject data relating to age, medical history, and dental history were analyzed by RHA.

All beakers were weighed immediately prior to the experiment and immediately following each saliva-draining period.

Whole salivary flow rates were calculated for each collection period in g/min. Planned contrasts were completed using paired samples t-tests and a Holm-Bonferroni sequential procedure to correct for familywise error ($p_{corr} < 0.0045$) [15]. The comparisons of interest included (i) baseline rates compared with the flow rates during the control and experimental conditions and (ii) baseline rates compared with the flow rates from 0 to 5 minutes, 10 to 15 minutes, 20 to 25 minutes, and 30 to 35 minutes after the control and experimental conditions for both the MTB and ETB protocols. The effects of the control and treatment conditions on salivary flow rate were estimated using Cohen's d [16].

Differences between manual and electric tooth-brushing effects on salivary flow rate were examined through descriptive comparisons of the effect sizes (Cohen's d) calculated for the MTB and ETB conditions. Additionally, the maximum salivary flow rate increases (i.e., the difference between salivary flow rates during tooth brushing and baseline) associated with the MTB and ETB were compared using a paired samples t-test ($p < 0.05$). The relationship between the maximum salivary flow rate increases associated with the manual and electric tooth brushing was examined using a Pearson's correlation coefficient ($p < 0.05$).

The relationship between age and the maximum salivary flow rate change was examined using Pearson's correlation coefficient ($p < 0.05$). Medication use was explored using descriptive statistics and a chi-square test ($p < 0.05$).

Statistical analyses were completed using SPSS [17] and Microsoft Excel.

3. Results

3.1. Participants. Twenty-one adults participated in the study (62–83 years of age, M = 71.33 years, SD = 6.46 years; 11 female). All participants had at least 20 natural teeth (range = 22–28, M = 25.67, SD = 1.93). No participants were observed to swallow during the salivary collection periods. Seven of the 21 participants reported taking no medications with potential xerogenic effects and 14 of the 21 participants reported taking xerogenic medications (range = 0–4, M = 1.25, SD = 1.11) [18]. The results of a chi-square test ($\chi^2 = 3.19$, $p = 0.36$) indicated that the numbers of participants taking zero, one, two, or three or more medications were not significantly different (data not shown). Xerogenic medications used by the participants included antihyperlipidemic, antiulcer, antihypertensive, and anti-inflammatory agents.

3.2. Effects of Tooth Brushing on Salivary Flow Rate. The mean whole salivary flow rates for each collection period are presented in Figure 2 (MTB) and Figure 3 (ETB).

Planned contrasts were completed using paired samples t-tests. The Holm-Bonferroni sequential procedure was used to correct for familywise error ($p_{corr} < 0.0045$) (Table 1).

A significant, large increase ($d = 2.50$) in salivary flow rate was observed during manual tooth brushing compared with the baseline salivary flow rate (M = 0.63, SD = 0.34, $p < 0.0045$) and with the control condition salivary flow rate (M = 0.58, SD = 0.33, $p < 0.005$). The salivary flow rate

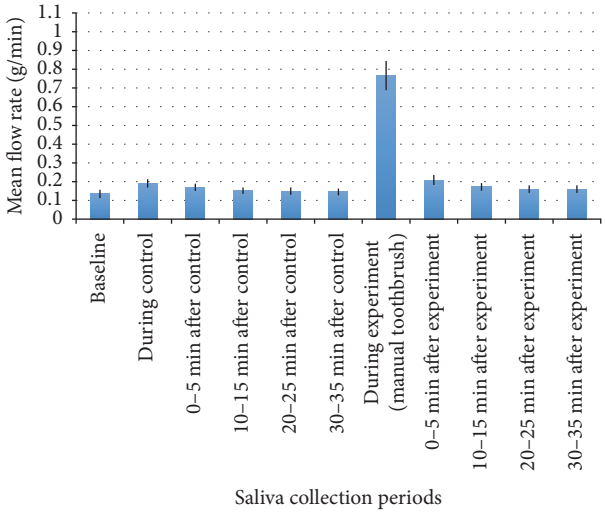

Figure 2: Mean whole salivary flow rate collected before, during, and after control and manual tooth brushing. Mean flow rate (g/min) is represented by the bars and the error bars indicate SE.

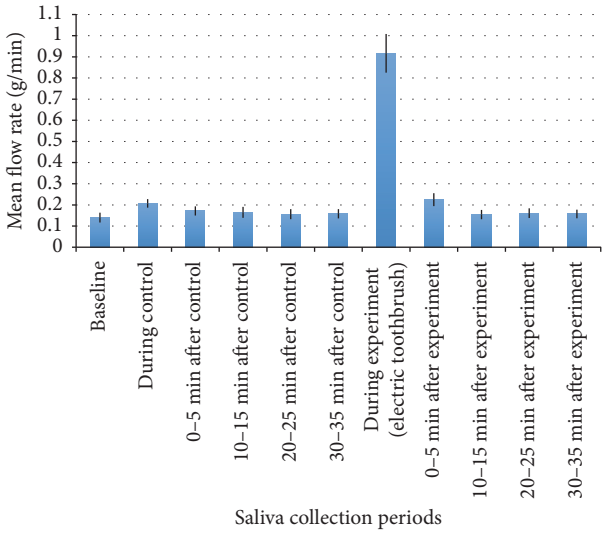

Figure 3: Mean whole salivary flow rate collected before, during, and after control and electric tooth brushing. Mean flow rate (g/min) is represented by the bars and the error bars indicate SE.

collected from 0 to 5 minutes after manual tooth brushing was significantly, moderately increased ($d = 0.661$) compared with the baseline salivary flow rate (M = 0.07, SD = 0.07, $p < 0.0055$).

A significant, moderate increase ($d = 0.672$) in salivary flow rate was observed during the control condition compared with the baseline salivary flow rate during the electric toothbrush protocol (M = 0.07, SD = 0.08, $p < 0.006$).

A significant, large increase ($d = 2.54$) in salivary flow rate was observed during the electric tooth brushing compared with the baseline salivary flow rate (M = 0.78, SD = 0.37, $p < 0.0045$) and with the control condition salivary flow rate (M = 0.71, SD = 0.35, $p < 0.005$). The salivary flow rate collected from 0 to 5 minutes after electric tooth brushing was significantly, moderately increased ($d = 0.681$) compared with the baseline salivary flow rate (M = 0.08, SD = 0.08, $p < 0.0055$).

Table 1: Salivary flow rate comparisons and the Holm-adjusted p values.

Comparison	Mean difference	Holm-adjusted p
Manual toothbrush		
Baseline, during brushing	0.63	0.0045
During control, during brushing	0.58	0.0050
Baseline, 0 to 5 minutes after brushing	0.07	0.0055
Electric toothbrush		
Baseline, during brushing	0.78	0.0045
During control, during brushing	0.71	0.0050
Baseline, 0 to 5 minutes after brushing	0.08	0.0055
Baseline, during control	0.07	0.0060

3.3. Manual Compared to Electric Tooth Brushing. The effect sizes associated with the salivary flow rate collected during manual and electric tooth brushing were similar (MTB: $d = 2.40$; ETB: $d = 2.54$). The maximum salivary flow rate increases associated with the MTB and ETB were not significantly different (M = 0.15, SD = 0.42, $p = 0.129$). A small correlation between the maximum salivary rate increases associated with the MTB and ETB protocols ($r(19) = 0.30$, $p = 0.184$) was identified.

3.4. Age and Salivary Flow Rate. Baseline salivary flow rate data for the MTB protocol were not normally distributed, as assessed by Shapiro-Wilk's test ($p = 0.001$). Baseline data for the ETB protocol, however, were normally distributed ($p > 0.05$). A Spearman rank-order correlation was completed using the MTB data and a Pearson's correlation coefficient was completed with the ETB data. Age was not significantly correlated with the baseline salivary flow rate for the MTB ($r_s(19) = 0.05$, $p = 0.84$) or ETB protocols ($r(19) = 0.1$, $p = 0.68$).

Age was moderately correlated with the maximum salivary flow rate increase ($r(19) = 0.55$, $p = 0.01$) for the MTB condition (Figure 4). A small, nonsignificant correlation was observed ($r(19) = 0.18$, $p = 0.44$) for the ETB condition.

4. Discussion

The results of the current study suggest that manual or electric tooth brushing is associated with short-duration increase in whole salivary flow rate in healthy older adults. Whole salivary flow rates increased significantly for up to five minutes following either manual or electric brushing of the teeth, tongue, and palate. The increase in salivary flow rate immediately following the two-minute brushing period was large whereas the increase at five minutes following the brushing period was moderate. The present study also found

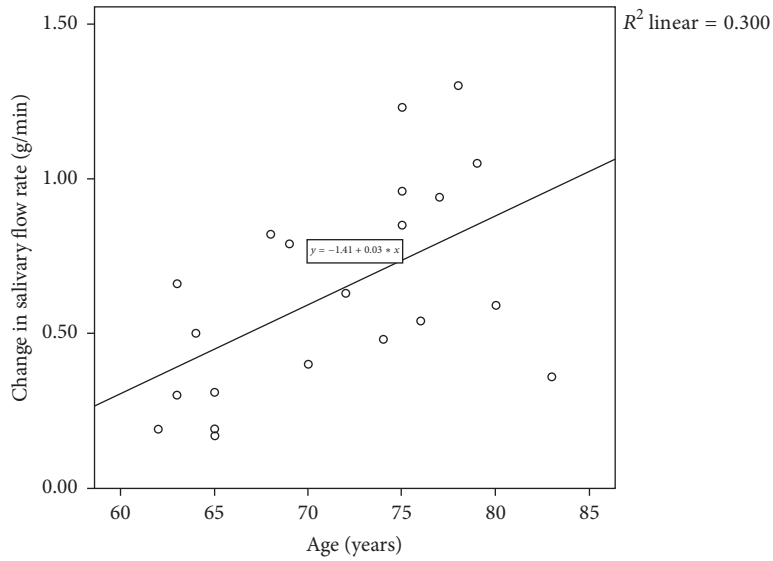

Figure 4: Scatterplot illustrating the correlation between age (in years) and the maximum salivary increase (g/min) associated with manual tooth brushing.

that holding a deactivated ETB in a stationary position in the oral cavity resulted in a transient increase in whole salivary flow rate.

These results are similar to those of Hoek et al. [7] who reported that tooth brushing induced a transient increase in saliva flow rate during the following five minutes. Our results are not consistent with those of Ligtenberg et al. [8], however, who reported that, after brushing with water or dentifrice, the salivary secretion rate increased significantly for 60 minutes. This inconsistency may be due to the additional oral stimulation associated with brushing with water or dentifrice which could have caused gustatory or thermal stimulation in addition to the mechanical stimulation associated with tooth brushing, potentially influencing the duration of increased salivary flow rates following stimulation.

We hypothesized that electric tooth brushing would have a larger effect on salivary flow rate compared with manual tooth brushing. Papas et al. [9] reported that Sonicare ETB users tended to have increased salivary flow rates after stimulation compared with MTB users. In contrast, Hiraba et al. [6] found that increasing the frequency of vibratory stimulation applied to the facial skin overlying the belly of the masseter muscles did not result in greater salivation, possibly because individual mechanoreceptors differ in their threshold sensitivity to vibration. We found no significant differences between the two tooth-brushing protocols with regard to increasing whole salivary flow rates which suggests that the increased vibration associated with ETBs may not result in a greater salivary response compared with MTBs.

In our post hoc analysis, we examined the relationship between age and the maximum salivary flow rate increase (i.e., the maximum difference between salivary flow rates during baseline and tooth-brushing conditions). We hypothesized that, as age increased, maximum salivary flow rate increase would decrease. This hypothesis, however, was not supported by the results. Instead, a moderate, positive correlation was observed between age and maximum salivary flow rate increase. That is, as age increased, so too did the maximum salivary flow rate increase observed during tooth brushing. In order to explain this phenomenon, we hypothesized that the older participants had reduced baseline salivary flow rates compared with the relatively younger participants. A lower baseline salivary flow rate might increase the potential for response to stimulation, resulting in a stimulated salivary flow rate similar to the relatively younger participants. When we then explored the relationship between age and baseline salivary flow rates, however, no significant correlations were identified. The oldest adults in our sample demonstrated a more robust salivary response to tooth brushing compared with the relatively younger subjects. Although parotid and minor salivary flow rates do not decline with increasing age [10] and aspects of somatosensation also do not decline with advancing age [19, 20], this evidence alone does not explain why we observed a positive relationship between age and maximum salivary flow rate increase. Older subjects were not observed to have more teeth compared with the younger subjects, younger subjects were not observed to be taking more xerogenic medications compared with the older subjects, and the males and females were equally distributed with regard to age (data not shown).

The positive relationship between age and maximum salivary flow rate increase was found to be moderate for manual tooth brushing but small for electric tooth brushing. This finding is provocative given that comparison of the salivary flow rate increases associated with the two types of brushing revealed no statistically significant differences. We examined the relationship between maximum salivary flow rate increases associated with the MTB and ETB and identified only a small correlation.

4.1. Limitations. Epithelial cells are continually being shed from the oral mucosa into saliva and it has been estimated

that the surface cells stay attached for only about three hours before being desquamated [21]. Participants in our study reported completing their early morning oral hygiene routine at least one hour prior to the experiment and all participants rinsed their oral cavities with distilled water immediately prior to participating. The elements of the present experimental protocol reduce the likelihood of epithelial cells making a significant contribution to the salivary collection and adding to the weight of the saliva samples. Nevertheless, it is possible that epithelial cells as well as plaque and residual food debris in the mouth, displaced by tooth brushing, may have contributed to the weight of the saliva samples.

One limitation of this study is that we did not complete a comprehensive dental exam on participants prior to study enrollment. We do not therefore have detailed information regarding the periodontal status of our participants.

Participants were mainly recruited from an exercise program, introducing a potential bias in that the participants may have been more health-conscious than the general population.

The ETB used in the experiment had a brush head shaped similarly to the MTB and was not circular in shape. A circular brush is a popular shape among name brand ETBs. Therefore, we may not have employed a representative ETB.

Glandular saliva was not collected in the present study. Thus, it is unclear which glands contributed more saliva to the increased flow rates in response to the tooth brushing. Based on previous work in this area, however, showing that the percentage contribution from the parotid gland increases to more than 50% of total salivary secretions during stimulation [22–24], we would predict that the parotid glands contributed the greatest percentage of saliva to the increased flow rates observed.

4.2. Clinical Implications. The present study showed a significant increase in whole salivary flow rate during, and for up to five minutes following, tooth brushing in older adults. Although the salivary flow rate modulation was transient, this increase in salivary flow would be expected to contribute to reducing oral bacterial load and increasing oral lubrication.

Increasing salivary volume may affect salivary clearance in that the volume of oral saliva contributes to triggering of the pharyngeal stage of swallowing [25] and increasing swallowing rate [26]. The act of swallowing secreted saliva reduces the concentration of exogenous substances in the oral cavity and is beneficial for oral health [27]. Thus, an increase in salivary volume would be expected to stimulate increased salivary clearance in an individual who has potentially harmful substances in the oral cavity, with beneficial effects on oral and overall health.

The correlation between age and maximum salivary flow rate increase indicates that the older adults in the present study experienced greater salivary responses to tooth brushing compared with the relatively younger participants. Given that aging is associated with reduced salivary flow [10], and that reduced salivary flow may lead to impaired oral health [28], older adults are at greater risk of developing poor oral health. Therefore, the capacity to increase salivary secretions among older individuals has important clinical implications with regard to improving the oral health of this cohort.

5. Conclusion

The present study suggests that tooth brushing stimulates saliva production for up to five minutes in adults aged 60 years and older who are free of systemic disease. Older participants had a more robust salivary response to tooth brushing compared with younger participants, suggesting that older adults may particularly benefit from tooth brushing to stimulate salivary secretion.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Research Article

Revealing the Amylase Interactome in Whole Saliva Using Proteomic Approaches

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Understanding proteins present in saliva and their function when isolated is not enough to describe their real role in the mouth. Due to protein-protein interactions, structural changes may occur in macromolecules leading to functional modulation or modification. Besides amylase's function in carbohydrate breakdown, amylase can delay proteolytic degradation of protein partners (e.g., histatin 1) when complexed. Due to its biochemical characteristics and high abundance in saliva, amylase probably interacts with several proteins acting as a biological carrier. This study focused on identifying interactions between amylase and other proteins found in whole saliva (WS) using proteomic approaches. Affinity chromatography was used, followed by gel electrophoresis methods, sodium dodecyl sulfate and native, tryptic in-solution and in-gel digestion, and mass spectrometry. We identified 66 proteins that interact with amylase in WS. Characterization of the identified proteins suggests that acidic (pI < 6.8) and low molecular weight (MW < 56 kDa) proteins have preference during amylase complex formation. Most of the identified proteins present biological functions related to host protection. A new protein-amylase network was constructed using the STRING database. Further studies are necessary to investigate individualities of the identified amylase interactors. These observations open avenues for more comprehensive studies on not yet fully characterized biological function of amylase.

1. Introduction

Whole saliva (WS) is a complex solution that results from secretions from major and minor salivary glands, oral mucosa cells, microorganisms, and elements from the plasma, which reach saliva via gingival crevicular fluid [1]. WS participates in different mechanisms related to the processing of food, the protection of hard and soft oral tissue, and the oral microorganisms' homeostasis [2]. In fact, most of the functions attributed to WS are executed by the salivary proteins [2]. An example of the functionality of salivary proteins is the formation of the Acquire Enamel Pellicle (AEP), a protein layer formed mainly by salivary proteins with higher affinity for hydroxyapatite [3]. Primarily, the AEP works as a physical and chemical barrier that protects the teeth. However, oral microorganisms also use the AEP as a platform to selectively adhere to the tooth surface leading to the formation of the oral biofilm (dental plaque) [3–8].

The presence of the oral biofilm is determinant for the development of the two most prevalent oral diseases: dental caries and periodontal disease. These diseases are the result of an unbalanced situation regarding the host's ability, in part provided by the salivary proteins, to control the growth of pathogenic oral bacteria when compared with the presence of indigenous microorganisms [9].

Several salivary proteins have been explored as key factor for the development of oral diseases based on biofilm formation [8, 10–16]. For example, carbonic anhydrase VI has

been investigated as a potential modulator for dental caries progression [14, 15]. This protein is involved in the maintenance of the salivary physiological pH, by the bicarbonate buffer system, and in the neutralization of acid produced by cariogenic microorganisms present in the biofilm [14, 17]. It has been suggested that reduced abundance or activity of carbonic anhydrase VI could be associated with higher risk to develop dental caries [15, 18, 19].

Salivary amylase is another protein with potential correlation with oral diseases. Amylase is the most abundant protein found in human saliva. Amylase is also present in the secretion of mammary and lacrimal glands [20]. Despite the vast literature on salivary amylase, the main function of salivary amylase as an efficient initiator of food digestion in the oral cavity is still debatable [21, 22]. Mechanisms that associate salivary amylase with the clearance of microorganisms from the oral cavity [22–24] and participation in the formation of the AEP [3, 22] and in the modulation of the oral biofilm via bacteria adhesion [8, 23–25] are well explained if considering the protein isolated. However, studies on the salivary proteome have indicated that understanding the individual proteins present in saliva, as well as how they function when isolated, is not enough to describe their real role when in the oral cavity. In fact, most proteins interact with other proteins originating protein complexes. Such interactions may cause structural changes in the macromolecule leading to the modulation or modification of the original individual function of the protein. For instance, when the *in vivo* identified amylase-histatin 1 complex was tested *in vitro*, amylase maintained its enzymatic activity on the hydrolysis of starch, while histatin 1 showed reduced killing activity against *Candida albicans [26]*. Also, it was shown that the lifetime of histatin 1, when complexed with amylase, was significantly increased when exposed to WS [26]. The observation that amylase can delay the proteolytic degradation of salivary protein partners when complexed suggests that this salivary protein may behave as an ideal carrier for important proteins throughout the oral cavity, while maintaining their integrity [26–28].

Heterotypic complexes in saliva between amylase and MUC 5B [28], MUC 7 [29], histatin 1 [26], and histatin 5 [27] have been previously described. Due to the biochemical characteristics and abundance of amylase in saliva, it is very likely that amylase interacts with several other proteins forming complexes. The objective of our study was to reveal the interactions among amylase and other salivary proteins in WS. A comprehensive identification of *in vivo* salivary amylase complexes opens new avenues for further studies related to potential protein degradation stability and how these physiological complexes can be translated to an emerging area related to protein/peptide protection and delivery in a target area.

2. Materials and Methods

2.1. Ethics Approval for Human Participants. This research was approved by the Research Human Ethics Board of the University of Western Ontario (review number 16181E).

2.2. Collection of Whole Saliva (WS) and Preparation of Sample Pools. Stimulated saliva samples were collected from three healthy, nonsmoking adult volunteers, ranging in age from 38 to 42 years (one male and two females). All volunteers exhibited good oral health and overall good systemic conditions. The collection of WS was done between 10:00 AM and 11:00 AM, to reduce the effects of the circadian cycle. Volunteers chewed on a 5×5 cm piece of parafilm until 7 mL of saliva was reached. Centrifugation at 14000 ×g for 20 min at 4°C was used to separate pellet and the WS supernatant (WSS). Only WSS were pooled together. Pellets were discarded. Each pool was made with 5 mL of WSS from each volunteer. Three pools were prepared, in different dates. Detailed scheme is shown in Figure 1. Saliva was used fresh for all experiments and was kept on ice from collection to the preparation of aliquots [30]. No protease inhibitors were added to the saliva samples.

2.3. Separation of Amylase Complex from WSS Using an In-House Affinity Chromatography. Affinity Chromatography (AC) was employed to enrich amylase when complexed with its protein partners. Potato starch (Acros Organics, New Jersey, USA) was used as ligand and amylase as a target. The used in-house AC method was designed and optimized, inspired by previous study [31]. A sample of 1 mL of pooled WSS was submitted to the column containing 700 μg of starch and hand-pressed slowly, the column was washed with distilled water, and amylase and its complex partners enriched solution was eluded with 1 mL of 0.1% trifluoroacetic acid (TFA). The eluate was subjected to bicinchoninic acid assay (BCA) (Pierce Chemical, Rockford, USA) for total protein concentration measurement. Bovine serum albumin was used as protein standard. Aliquots of 20 μg protein were prepared and subjected to further separation and characterization. Following the enrichment with AC, the amylase-enriched samples were subjected to three distinctive methods: (1) in-solution tryptic digestion, (2) further separation in SDS-PAGE and in-gel tryptic digestion, and (3) confirmation of the complex formation by molecular mobility in the native-PAGE and in-gel tryptic digestion of the amylase complex.

2.4. In-Solution Digestion. Aliquots of 20 μg of total protein each were denatured and reduced by addition of 50 μL of 4 M urea, 10 mM DTT, and 50 mM NH_4 HCO_3, pH 7.8, and incubated for 1 hour at room temperature (RT). The solution was diluted with the addition of 150 μL of 50 mM NH_4 HCO_3, pH 7.8. After tryptic digestion, carried out for at least 16 hours, at 37°C, with 2% w/w sequencing-grade trypsin (Promega, Madison, WI, USA), samples were desalted (Zip Tip C-18, EMD Millipore Inc., Germany) and submitted to mass spectrometric analysis (LC-ESI-MS/MS).

2.5. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) Separation Followed by In-Gel Digestion. SDS-PAGE was used to separate our protein mixture based on the individual molecular weight (MW) of our proteins. Before loading in the 12% SDS-PAGE, all samples were resuspended in 20 μL of sample buffer (0.4 M Tris-HCl pH 6.8, 4% SDS, 10% glycerol, 0.4% bromophenol blue, and 2%

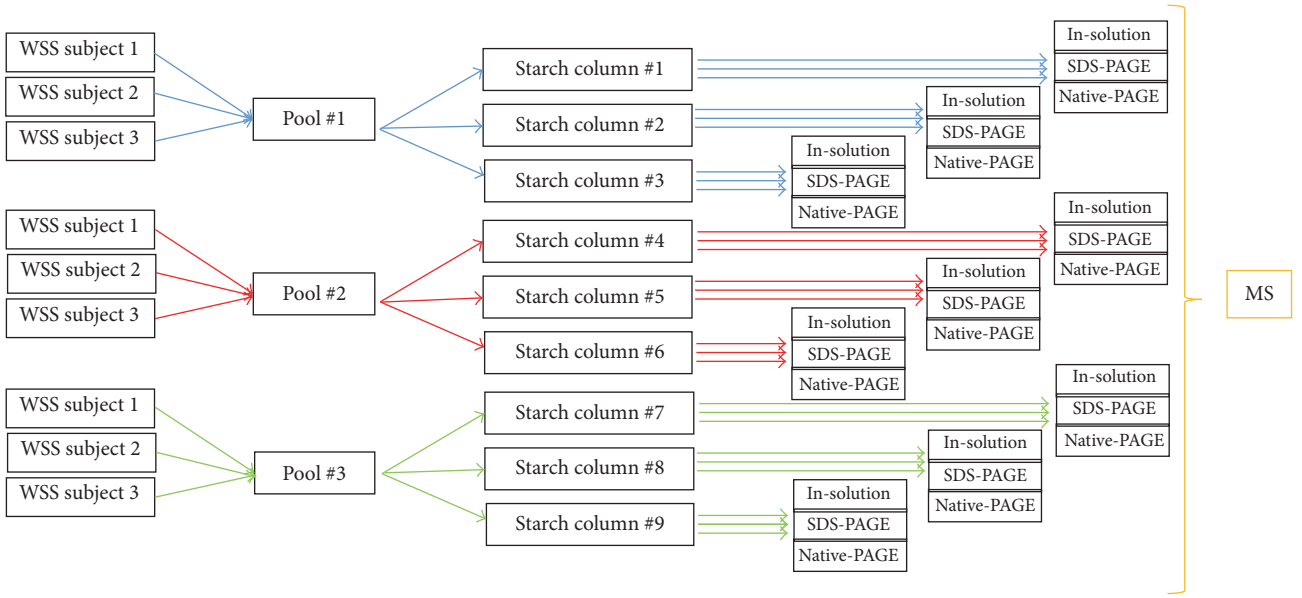

Figure 1: Schematic representation of the adopted methodology.

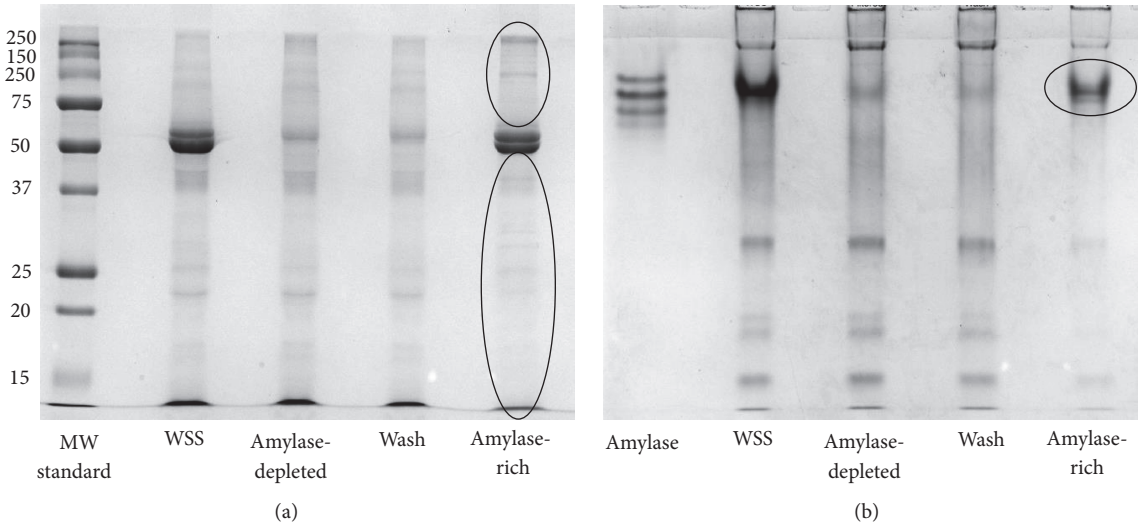

Figure 2: (a) SDS-PAGE 12% and (b) native-PAGE 8% showing areas of interest for identification of protein partners of amylase. The potential protein partners of amylase are expected to be found in the areas marked with an oval shape.

2-mercaptoethanol) and boiled for 5 min. Each sample was loaded in a separate well. From left to right, the first well was loaded with 5 μL of protein standard (Precision Plus Protein™ All Blue Prestained Protein Standards, Bio-Rad, California, USA), the second well was loaded with a sample from our original solution (WSS), the third well showed the amylase-depleted saliva, the fourth well had sample from the wash of the column, and the last well was loaded with an aliquot containing our amylase-enriched solution (amylase recovered from the starch column along with its partners) (Figure 2(a)). The voltage was kept constant at 100 V during electrophoresis. Immediately after the run, all gels were stained with Coomassie Blue (40% methanol, 10% acetic acid, and 2 g Coomassie Blue) overnight with shaking at RT. Destaining was done the following morning (40% methanol, 10% acetic acid), for 1 hour and 30 min with shaking at RT. After destaining protocol, the gels were kept in Milli-Q water until scanning.

Using a razor blade, regions containing potential partners of amylase were excised from the gels. In the SDS-PAGE, the partners are expected to be found dispersed in the entire lane representing the "amylase-enriched" solution (Figure 2(a)). Each lane was separated into six band regions, and a template was used to ensure that the spots from all gels were extracted at the same MW range. After placement in separate polycarbonate tubes, each band region was cut into approximately

1×1 mm pieces. Gel pieces were then destained using 25 mM NH_4 HCO_3 in 50% acetonitrile (ACN), shrunk with 100% ACN, and subjected to in-gel tryptic digestion. The digestion was carried out in 25 mM ammonium bicarbonate solution containing 0.01 μg/μL sequencing-grade trypsin (Promega, Madison, WI), for 16 hours at 37°C. Peptide extraction was achieved. Samples were desalted (Zip Tip C-18, EMD Millipore Inc., Germany) before mass spectrometry.

2.6. Native-PAGE and In-Gel Digestion. A native gel was used to ensure that amylase would run still complexed with its protein partners. For the 8% native-PAGE, after resuspending the samples of 20 μg of protein with 20 μL of sample buffer (0.4 M Tris-HCl pH 6.8, 10% glycerol, and 0.4% bromophenol blue), the same order used in the SDS-PAGE was observed when loading the samples into the wells from left to right (Figure 2(b)). Native-PAGE running buffer was added to the electrophorese unit, and the voltage was kept constant at 100 V. The same staining method was used with Coomassie Blue overnight as described above. Destaining was done the following morning (40% methanol, 10% acetic acid), for 1 hour with shaking.

For the native-PAGE, the protein partners of amylase are expected to be found in the dark band correspondent to the molecular mobility of the amylase complex (Figure 2(b)). Only the band about the molecular mobility of amylase-protein complex was studied. As described above, gel bands were cut into small 1×1 mm pieces, destained, and subjected to in-gel tryptic digestion. The digestion was carried out in the same manner that was described for the SDS-PAGE. Peptides were recovered and samples were desalted (Zip Tip C-18, EMD Millipore Inc., Germany) before mass spectrometry.

2.7. MS Analysis. Samples from all three described approaches were resuspended in 97.5% distilled water/2.4% ACN/0.1% formic acid and then subjected to RP nLC-ESI-MS/MS, using a LTQ-Velos (Thermo Scientific, San Jose, CA, USA) mass spectrometer. LC aligned with the C18 column of capillary-fused silica (column length 10 mm, column id 75 m, 3 m spherical beads, and 100 A° pores size) was used, linked to the MS through ESI. The survey scan was set in the range of m/z values 390–2000 MS/MS. Peptides were eluted from the nanoflow RP-HPLC over a 65 min period, with linear gradient ranging from 5 to 55% of solvent B (97.5% ACN, 0.1% formic acid), at a flow rate of 300 nL/min, with a maximum pressure of 280 bar. The electrospray voltage was 1.8 kV and the temperature of the ion-transfer capillary was 300°C. After a MS survey scan range within m/z 390–2000 was performed and after selection of the most intense ion (parent ion), MS/MS spectra were achieved via automated sequential selection of the seven peptides with the most intense ion for CID at 35% normalized collision energy, with the dynamic exclusion of the previously selected ions. The MS/MS spectra were matched with human protein databases (Swiss-Prot and TrEMBL, Swiss Institute of Bioinformatics, Geneva, Switzerland, https://ca.expasy.org/sprot/) using SEQUEST algorithm in Proteome Discoverer 1.3 software (Thermo Scientific, USA). The searches were performed by selecting the following SEQUEST parameters: (1) trypsin as protease enzyme, (2) 2 Da precursor ion mass tolerance, (3) 0.8 Da fragment ion mass tolerance, and (4) dynamic modifications of oxidized cysteine and methionine and phosphorylated serine and threonine. A maximum of four dynamic modifications per peptide were accepted. The SEQUEST score filter criteria applied to the MS/MS spectra for peptides were absolute XCorr threshold 0.4, fragment ion cutoff percentage 0.1, and peptide without protein XCorr threshold 1.5. Any nontryptic peptides passing the filter criteria were discarded. Only proteins for which two or more peptides were identified are reported in this study.

2.8. Identification of Protein Partners of Amylase. After MS analysis and interpretation, comparison of the common partners among the used methods allowed the construction of a list with proteins that participate with amylase in salivary complexes. Three in-house AC columns (technical triplicate) were used for each one of the 3 saliva pools (biological triplicate) prepared in different dates, making a total of 9 replicates for each one of the used approaches (in-solution digestion, SDS-PAGE followed by in-gel digestion, and native-PAGE followed by in-gel digestion). For the approach using in-solution digestion, the proteins identified by MS for the 9 replicates were compared, and proteins that were identified in at least 2 of the replicates were listed as common proteins for this first approach. Similarly, the proteins identified by MS from the dark bands of the 9 replicates submitted to native-PAGE, followed by in-gel digestion, were compared and the proteins that appeared in at least two replicates were considered common protein partners for this second method. Last, to identify the amylase partners from the lines representing the amylase-enriched sample in the 9 replicates submitted to the SDS-PAGE approach, followed by in-gel digestion and MS, a template was used to extract the bands from all the 9 gels at approximately the same molecular weight range. The lanes with enriched sample were divided into 6 areas. Each area was analyzed separately and the 6 protein lists for each line were combined into one single protein list for each replicate; duplicate proteins were excluded. Like the other two approaches, proteins identified in at least two of the 9 replicates were deemed common for this third approach. After this triage, a Venn diagram was used to verify similarities among the common proteins listed from each described approach. Inclusion criterion for positive identification of proteins as complex partners of amylase was that the same protein was found in at least two of the used approaches.

2.9. Bioinformatics Characterization of Amylase Complex Partners. The proteins identified in at least two of the described approaches were then characterized based on their calculated isoelectric point (pI) and molecular weight (MW). Using the physiologic salivary pH as reference (pH 6.8), the identified proteins were grouped based on their pI (pI below and above 6.8). In addition, the MW of amylase (56 kDa) was assumed for our MW cut-off and the same proteins were divided in three groups: proteins with 0–20 kDa, proteins with 20–56 kDa, and proteins with MW above 56 kDa. Whenever available, pI and MW were

calculated after removal of signal peptide given by UniProt database. Otherwise, pI and MW informed in the MS report were adopted. The identified amylase complex partners were also classified based on their biological functions using data from UniProt (http://uniprot.org) assessed on August 2017. Four major groups were formed including proteins that exhibit antimicrobial activity, protection against chemical aggression, participation in host immune response and/or regulation of inflammation, and physical protection of the oral mucosa and/or wound healing.

2.10. Simulation of Amylase-Protein Network Using STRING Database. STRING database was used to provide a schematic representation of the interactions among amylase and other proteins found in the human WS as described elsewhere [32]. First, a comprehensive search was performed in eight different databases (BioGRID, HPRD, APID, EMBL-EBI, FpClass, STRING, IntAct, and BioPlex) (Table 1) to provide a solid list with both known and predicted protein-amylase interactions. Second, a simulated amylase hub containing only the 66 proteins identified in this study was constructed using the STRING database. Last, a more inclusive network was created by merging the hub containing the proteins identified in this study with the possible partners of amylase listed in all eight searched databases. The filter was set to match with human databank, and the confidence score was set to 0.4 (medium) in all representations.

3. Results

The selectivity of our in-house AC starch columns towards amylase is demonstrated in Figure 2, where the band related to amylase's MW (56 kDa) in Figure 2(a) and the amylase complex in Figure 2(b) practically disappear in the lines representing saliva depleted from amylase and the wash with distilled water. On the other hand, dark bands are seen in the corresponding areas with the amylase-enriched samples. Although slight bands can be seen in areas besides that of the amylase complex in Figure 2(b) where the enriched sample is represented, such faded bands might be related to proteins that either show weak interaction with the complex which was disrupted during processing of samples, or may be related to "contaminants" that remained in the column after wash. To ensure a precise identification of proteins from the complex, only the proteins listed in the dark band in the native-PAGE (Figure 2(b)) were considered.

The data obtained after LC-ESI-MS/MS analysis of samples from the three described approaches identified 66 different proteins found in WS that form complex with salivary amylase. All identified proteins are listed in Table 2, along with the corresponding approach used for the identification, protein MW and pI. When results from all approaches were combined, 375 different proteins were recognized. In-solution digestion provided 164 proteins that probably interact with amylase: SDS-PAGE, followed by in-gel tryptic digestion, 237 potential partners; native-PAGE, followed by in-gel digestion, 67 possible complex partners. After selecting only proteins that were identified in two or more of the used approaches, results were narrowed down to 66 proteins,

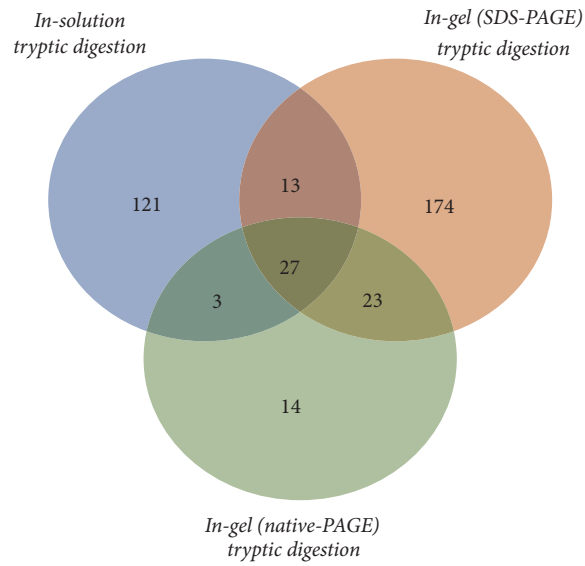

Figure 3: Venn diagram distribution of identified proteins in each of the three proteomic approaches used in this study.

where 27 proteins were detected in all three methods, besides amylase itself, and 39 other proteins were concomitantly identified in only two of the used approaches. A total of 13 unique proteins were identified using both in-solution tryptic digestion and SDS-PAGE, followed by in-gel tryptic digestion; 23 proteins were found in both PAGE approaches; and 3 proteins were uniquely found concomitantly in the samples from in-solution tryptic digestion and native gels, followed by in-gel tryptic digestion (Figure 3).

Moreover, the 66 common proteins displayed MW ranging from 4.31 kDa to 3340.16 kDa (Table 2), where most of the identified amylase partners (56%) presented a MW below 56 kDa, amylase's MW (Figure 4(a)). The identified proteins were also grouped based on their isoelectric points (pI). Clearly, most of the 66 proteins (67%) presented pI below 6.8. One-third (33%) of the identified amylase-protein partners exhibited basic characteristics ranging in pI above 6.8 (Figure 4(b)).

Interestingly, the characterization of the 66 identified proteins based on their biological functions indicated that most of the proteins participating in complex with amylase exhibit protective roles towards the maintenance of the host's health. In fact, from the 66 identified proteins, 37 display oral defensive functions: 13 proteins have antimicrobial activities, 9 elements are capable of neutralizing chemical aggressions to the host's tissues, 10 proteins participate in mechanisms that initiate or modulate the host's immune response and inflammatory process, and 10 proteins contribute to the physical protection of the host's tissue and/or wound healing (Table 3).

The amylase interactome simulation using STRING database demonstrated that not all 66 proteins were linked to the protein-amylase network (Figure 5(a)). Three distinct isolated groups of 3 to 4 proteins were formed apart from the network, along with other lonely individual nodes. MUC

Table 1: List of proteins with known and predicted interactions with amylase, identified by search in eight databases (BioGrid, HPRD, APID, EMBL-EBI, FpClass, STRING, IntAct, and BioPlex).

Protein name	Database
Sucrase-isomaltase (alpha-glucosidase)	STRING
Amylo-alpha-1,6-glucosidase,4-alpha-glucanotransferase	STRING
Lactase	STRING
Bactericidal/permeability-increasing protein	STRING
Collagen, type X, alpha 1	STRING
Glycogen phosphorylase, muscle form	STRING
Acetyl-CoA carboxylase beta (2)	STRING
Uridine monophosphate synthetase	STRING
Acetyl-CoA carboxylase alpha	STRING
S-phase kinase-associated protein 1	STRING
Glucan (1,4-alpha-), branching enzyme 1	STRING
Glycogen phosphorylase, liver form	STRING
Glycogen phosphorylase, brain form	STRING
A kinase (PRKA) anchor protein 8	HPRD, BioGRID, APID, EMBL-EBI, FpClass, STRING
Cyclin-dependent kinase 2 associated protein 1	BioGRID, String
A kinase (PRKA) anchor protein 1	HPRD, BioGRID, APID, EMBL-EBI, STRING
Mucin 5B, oligomeric mucus/gel-forming	BioGRID, APID, EMBL-EBI
Ras association (RalGDS/AF-6) domain family member 6	HPRD, BioGRID, APID, EMBL-EBI
Putative oral cancer suppressor, deleted in oral cancer 1	IntAct, HPRD, APID, EMBL-EBI
Superoxide dismutase (Mn), mitochondrial	IntAct, BioGRID, APID, EMBL-EBI
Uncoupling protein 2 (mitochondrial, proton carrier)	IntAct, APID, EMBL-EBI
ARP8 actin-related protein 8 homolog (yeast)	BioGRID, BioPlex, APID, EMBL-EBI
beta-1,3-N-Acetylgalactosaminyltransferase 1 (globoside blood group)	BioGRID, BioPlex, APID, EMBL-EBI
General transcription factor IIB	BioGRID, BioPlex, APID, EMBL-EBI
Killer cell immunoglobulin-like receptor, two domains, short cytoplasmatic tail, 2	BioGRID, BioPlex, APID, EMBL-EBI
Mab-21-like 1 (*C. elegans*)	BioGRID, BioPlex, APID, EMBL-EBI
Starch binding domain 1	BioGRID, BioPlex, APID, EMBL-EBI
Protein kinase, cAMP-dependent, regulatory subunit, type II, beta	BioGRID, BioPlex, APID, EMBL-EBI
Peptide (mitochondrial processing) beta	BioGRID, BioPlex, APID, EMBL-EBI
Trafficking protein particle complex 12	BioGRID, BioPlex, APID, EMBL-EBI
Ubiquitin-like 7	BioGRID, BioPlex, APID, EMBL-EBI
Calcium/calmodulin-dependent protein kinase ID	BioGRID, BioPlex, APID, EMBL-EBI
Receptor-interacting serine-theorine kinase 3	BioGRID, BioPlex, APID, EMBL-EBI
Chemokine (C-C motif) receptor-like 2	BioGRID, BioPlex, APID, EMBL-EBI
Pleckstrin homology domain containing, family G (with RhoGef domain) member 6	BioPlex, APID
Regulator of calcineurin 1	BioPlex, APID
Vasohibin 1	BioPlex, APID
Gastrokine 1	BioPlex, APID
Zinc-finger, B-box domain containing	BioPlex, APID
DEAD (Asp-Glu-Ala-Asp) box helicase 17	BioPlex, APID
Rho-related BTB domain containing 1	BioPlex, APID
Ts translation elongation factor, mitochondrial	BioPlex, APID
Tumor necrosis factor receptor superfamily, member 19	BioPlex, APID
Neuropeptide B	BioPlex, APID
Forkhead box N4	BioPlex, APID
FERM domain containing 1	BioPlex, APID
WD repeat domain 6	BioPlex, APID
DNA replication licensing factor MCM2	FpClass
Probable ATP-dependent RNA helicase DDX5	FpClass

Table 1: Continued.

Protein name	Database
G1/S-specific cyclin-D3	FpClass
cAMP-dependent protein kinase type II-alpha regulatory subunit	FpClass
Histatin 1	FpClass
Salivary acidic proline-rich phosphoprotein 1/2	FpClass
Statherin	FpClass

Websites: STRING: https://www.string-db.org; HPRD: http://www.hprd.org; BioGRID: https://www.thebiogrid.org; APID: http://apid.dep.usal.es; EMBL-EBI: https://www.ebi.ac.uk; FpClass: http://dcv.uhnres.utoronto.ca/FPCLASS/ppis/; IntAct: https://www.ebi.ac.uk/intact; BioPlex: http://bioplex.hms.harvard.edu.

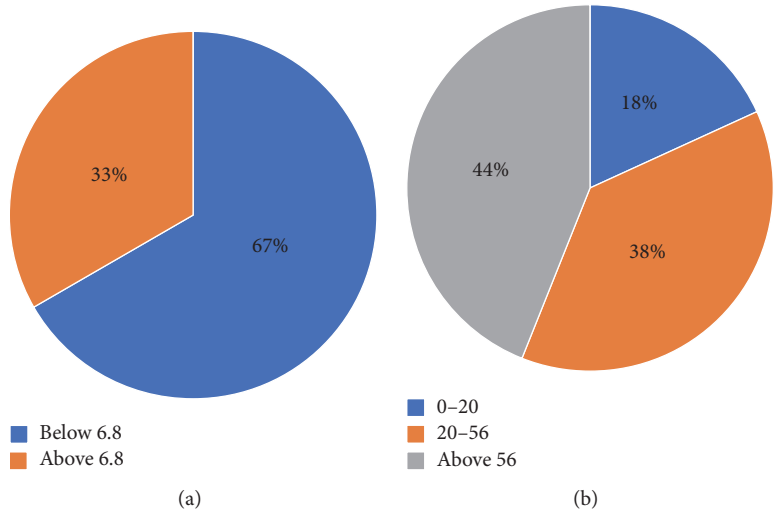

Figure 4: Percentage distribution of the identified proteins according to the biochemical characteristic of salivary amylase (pI 6.8 and 56 KDa). (a) pI distribution of the identified proteins using salivary amylase pI as comparison value. (b) Molecular weight distribution of the identified proteins using salivary amylase molecular weight as comparison value.

7 and MUC 5B were among the identified proteins. When the newly identified amylase network was merged with the previously documented interactors, only two groups of proteins were not linked to the network (Figure 5(b)). One group contained NUCB2 (nucleobindin-2) and CAMK1D (calcium/calmodulin-dependent protein kinase type 1D), and the other cluster formed by four keratins.

4. Discussion

A total of 66 proteins that participate in protein complex with amylase in WS were identified with the application of three different proteomic approaches. Initially, AC was used to enrich amylase along with its partners from the complex saliva solution. The reaction between amylase and starch is an enzymatic reversible mechanism [20], allowing for the recovery of intact amylase after its reaction with the starch. The use of a starch column was previously described as a mean for depletion of amylase from saliva [31]. However, in this study, we demonstrated that the mentioned method enriches amylase from saliva still complexed with other proteins. This observation was first suggested by the different bands present in the SDS-PAGE when the samples eluted from the starch column were separated by MW (Figure 2(a)). Later, this observation was confirmed by MS analysis of the same amylase-enriched samples where many proteins besides amylase were identified (Table 2). Thus, AC starch column alone should not be recommended for the depletion of amylase from saliva, unless a careful dismemberment of protein complexes can be performed earlier in ways that do not interfere with the activity of salivary amylase.

Moreover, the importance of using different methods for the identification of proteins was here demonstrated. Combining all used approaches, a total of 375 unique proteins were identified as potential members of the amylase complex. Interesting to note, SDS-PAGE was the method where the largest number of proteins was identified (237 proteins). From the 66 proteins that were identified in at least two of the used approaches and therefore are more likely to interact with amylase, only 3 proteins were not identified in the approach with SDS-PAGE. This demonstrates that additional sample separation based on the MW of each protein, together with the MS analysis of independent bands from different areas of the gel, prevents highly abundant proteins from masking or hiding low abundant ones, therefore improving the method specificity. On the other hand, while using directly in-solution tryptic digestion uniquely, 24 proteins from our final list of 66 interactors were not identified (Figure 3), once

Table 2: List of all identified potential amylase protein partners according to the used proteomic approach.

Accession number	Protein name	In-solution	In-gel (SDS-PAGE)	In-gel (native-PAGE)	MW (KDa)	Calc. pI
C0JYZ2	Titin	x	x	x	3340.16	6.09
B4E1M1	cDNA FLJ60391, highly similar to lactoperoxidase	x	x	x	73.88	8.15
Q9HC84	Mucin-5B	x	x	x	593.84	6.20
P04080	Cystatin-B	x	x	x	11.14	6.96
B4DVQ0	cDNA FLJ58286, highly similar to actin, cytoplasmic 2	x	x	x	37.30	5.71
P01037	Cystatin-SN	x	x	x	14.32	6.92
Q6PJF2	IGK@ protein	x	x	x	23.32	6.98
Q0QET7	Glyceraldehyde-3-phosphate dehydrogenase (fragment)	x	x	x	24.60	8.51
A0A075B6K9	Ig lambda-2 chain C regions (fragment)	x	x	x	11.30	7.24
P05109	Protein S100-A8	x	x	x	10.70	6.57
P12273	Prolactin-inducible protein	x	x	x	13.52	5.40
Q96DR5	BPI fold-containing family A member 2	x	x	x	25.05	5.19
A0A0C4DGN4	Zymogen granule protein 16 homolog B	x	x	x	17.21	5.39
Q9UGM3	Deleted in malignant brain tumors 1 protein	x	x	x	258.66	5.19
P01833	Polymeric immunoglobulin receptor	x	x	x	81.35	5.59
P01876	Ig alpha-1 chain C region	x	x	x	37.66	6.51
P23280	Carbonic anhydrase VI	x	x	x	33.57	6.41
C8C504	beta-Globin	x	x	x	15.87	7.98
A7Y9J9	Mucin 5AC, oligomeric mucus/gel-forming	x	x	x	645.90	6.27
P01834	Ig kappa chain C region	x	x	x	11.60	5.87
H6VRF8	Keratin 1	x	x	x	66.00	8.12
P13645	Keratin, type I cytoskeletal 10	x	x	x	58.83	5.13
P01036	Cystatin-S	x	x	x	14.19	4.83
B2R4M6	Protein S100	x	x	x	4.31	4.55
P35908	Keratin, type II cytoskeletal 2 epidermal	x	x	x	65.43	8.07
B1APF8	cAMP-dependent protein kinase catalytic subunit beta (fragment)	x	x	x	20.56	9.56
B5ME49	Mucin-16	x	x	x	1519.17	5.13
P25311	Zinc-alpha-2-glycoprotein	x	x		32.14	5.58
F6KPG5	Albumin (fragment)	x	x		66.49	6.04
B2R7Z6	cDNA, FLJ93674	x	x		50.34	7.05
E9PKG6	Nucleobindin-2	x	x		37.50	5.01
P02647	Apolipoprotein A-I	x	x		28.08	5.27
Q9Y6V0	Protein piccolo	x	x		553.28	6.09
P09228	Cystatin-SA	x	x		14.35	4.85
A0A024R9Y3	HECT, UBA, and WWE domain containing 1, isoform CRA_a	x	x		479.90	5.21

Table 2: Continued.

Accession number	Protein name	In-solution	In-gel (SDS-PAGE)	In-gel (native-PAGE)	MW (KDa)	Calc. pI
E7ETD6	Nucleosome-remodeling factor subunit BPTF	x	x		307.90	6.04
Q8TAX7	Mucin-7	x	x		36.81	9.30
P06733	alpha-Enolase	x	x		47.04	6.99
P10599	Thioredoxin	x	x		11.61	4.82
Q9UPN3	Microtubule-actin cross-linking factor 1, isoforms 1/2/3/5	x	x		838.31	5.28
O95661	GTP-binding protein Di-Ras3	x		x	25.50	9.46
A7E2D6	NAV2 protein	x		x	261.56	8.98
G3CIG0	MUC19 variant 12	x		x	802.68	4.96
Q8N4F0	BPI fold-containing family B member 2		x	x	47.13	8.48
P01024	Complement C3		x	x	184.95	6.00
H7BY35	Ryanodine receptor 2		x	x	562.25	6.19
Q07869	Peroxisome proliferator-activated receptor alpha		x	x	52.23	5.86
B4E1T1	cDNA FLJ54081, highly similar to Keratin, type II cytoskeletal 5		x	x	58.81	5.97
A8K2U0	alpha-2-Macroglobulin-like protein 1		x	x	159.33	5.50
Q6P5S2	Protein LEG1 homolog		x	x	35.86	5.79
B4E3A8	cDNA FLJ53963, highly similar to leukocyte elastase inhibitor		x	x	38.69	6.22
F8WA11	CLIP-associating protein 1		x	x	162.66	8.72
B7ZAL5	cDNA, FLJ79229, highly similar to lactotransferrin		x	x	73.10	7.78
P02533	Keratin, type I cytoskeletal 14		x	x	51.56	5.09
B2R825	alpha-1,4-Glucan phosphorylase		x	x	97.01	7.30
A0A087WWT3	Serum albumin		x	x	43.03	5.69
B7Z759	cDNA FLJ61672, highly similar to proteoglycan-4 (fragment)		x	x	92.09	9.44
J3QLC9	Haptoglobin (fragment)		x	x	39.03	5.54
P01877	Ig alpha-2 chain C region		x	x	36.50	6.10
A8K739	cDNA FLJ77339		x	x	24.84	5.06
B7Z747	cDNA FLJ51120, highly similar to matrix metalloproteinase-9		x	x	64.09	6.42
B7Z565	cDNA FLJ54739, highly similar to alpha-actinin-1		x	x	94.72	5.69
B4DI70	cDNA FLJ53509, highly similar to galectin-3-binding protein		x	x	44.37	5.03
P35527	Keratin, type I cytoskeletal 9		x	x	62.06	5.14
P80188	Neutrophil gelatinase-associated lipocalin		x	x	20.55	9.02
P04040	Catalase		x	x	59.62	6.95

again reinforcing the hypothesis of high abundance proteins preventing the identification of low abundance ones unless further separation is performed before MS analysis. Also, 13 of the 66 identified proteins from the amylase complex were not identified in the native-PAGE approach. Since native gels provide a sample separation based on the molecular mobility and charge of the complex, the absence of some of the identified proteins may be a consequence of weaker

Table 3: Distribution of proteins identified to interact with salivary amylase forming complex based on their biological functions.

Biological function	Accession number	Protein name
Defense response to bacterium, virus, and fungus (n = 13)	Q9HC84	Mucin-5B
	B4E1M1	cDNA FLJ60391, highly similar to lactoperoxidase
	P05109*	Protein S100-A8
	Q96DR5	BPI fold-containing family A member 2
	Q8N4F0	BPI fold-containing family B member 2
	Q9UGM3	Deleted in malignant brain tumors 1 protein
	P01876	Ig alpha-1 chain C region
	P01834*	Ig kappa chain C region
	P01877*	Ig alpha-2 chain C region
	B7Z759	cDNA FLJ61672, highly similar to proteoglycan-4 (fragment)
	Q8TAX7*	Mucin-7
	B7ZAL5	cDNA, FLJ79229, highly similar to lactotransferrin
	B4DI70	cDNA FLJ53509, highly similar to galectin-3-binding protein
Neutralization of chemical aggression (n = 9)	P04080	Cystatin-B
	P01037	Cystatin-SN
	P23280	Carbonic anhydrase VI
	P01036	Cystatin-S
	P09228	Cystatin-SA
	A8K2U0	alpha-2-Macroglobulin-like protein 1
	B4E3A8	cDNA FLJ53963, highly similar to leukocyte elastase inhibitor
	P04040	Catalase
	A7Y9J9*	Mucin 5AC, oligomeric mucus/gel-forming
Immune response and regulation of inflammation (n = 10)	P05109*	Protein S100-A8
	P12273	Prolactin-inducible protein
	P01834*	Ig kappa chain C region
	B2R4M6	Protein S100
	P01024	Complement C3
	P80188	Neutrophil gelatinase-associated lipocalin
	P01877*	Ig alpha-2 chain C region
	A0A075B6K9	Ig lambda-2 chain C regions (fragment)
	B7Z747	cDNA FLJ51120, highly similar to matrix metalloproteinase-9
	Q6PJF2	IGK@ protein
Mucosa protection and wound healing (n = 10)	B5ME49	Mucin-16
	P01833	Polymeric immunoglobulin receptor
	P35908	Keratin, type II cytoskeletal 2 epidermal
	P25311	Zinc-alpha-2-glycoprotein
	Q07869	Peroxisome proliferator-activated receptor alpha
	Q9UPN3	Microtubule-actin cross-linking factor 1, isoforms 1/2/3/5
	A7Y9J9*	Mucin 5AC, oligomeric mucus/gel-forming
	Q8TAX7*	Mucin-7
	P02647	Apolipoprotein A-I
	Q9UPN3	Microtubule-actin cross-linking factor 1, isoforms 1/2/3/5

Table 3: Continued.

Biological function	Accession number	Protein name
	G3CIG0	MUC19 variant 12
	A0A0C4DGN4	Zymogen granule protein 16 homolog B
	A0A024R9Y3	HECT, UBA, and WWE domain containing 1, isoform CRA_a
	E7ETD6	Nucleosome-remodeling factor subunit BPTF
	P06733	alpha-Enolase
	A7E2D6	NAV2 protein
	H7BY35	Ryanodine receptor 2
	Q6P5S2	Protein LEG1 homolog
	F8WA11	CLIP-associating protein 1
	P02533	Keratin, type I cytoskeletal 14
	B2R825	alpha-1,4-Glucan phosphorylase
	B4DVQ0	cDNA FLJ58286, highly similar to actin, cytoplasmic 2
	Q0QET7	Glyceraldehyde-3-phosphate dehydrogenase (fragment)
Biological functions not directly related to host protection or unknown ($n = 29$)	J3QLC9	Haptoglobin (fragment)
	B7Z565	cDNA FLJ54739, highly similar to alpha-actinin-1
	C0JYZ2	Titin
	C8C504	beta-Globin
	H6VRF8	Keratin 1
	P13645	Keratin, type I cytoskeletal 10
	B1APF8	cAMP-dependent protein kinase catalytic subunit beta (fragment)
	F6KPG5	Albumin (fragment)
	E9PKG6	Nucleobindin-2
	Q9Y6V0	Protein piccolo
	P10599	Thioredoxin
	O95661	GTP-binding protein Di-Ras3
	B4E1T1	cDNA FLJ54081, highly similar to keratin, type II cytoskeletal 5
	A0A087WWT3	Serum albumin
	A8K739	cDNA FLJ77339
	P35527	Keratin, type I cytoskeletal 9

*Proteins involved in more than one process related to host protection.

bindings, thus damaging the stability of some protein-protein interactions and preventing all proteins that were originally in the complex from being identified in this method.

Amylase-protein-protein interactions with histatins (histatin 1 and histatin 5) and with mucins (MUC 5B and MUC 7) were described previously [26, 28, 29, 33]. Mucins (MUC5B and MUC7), a protein family only present in mucous glands such as submandibular and sublingual glands, were here identified among the partners of amylase in WS, confirming previous studies [28, 29]. Contrarily, histatins were not identified in this study probably because of their short lifespan in the oral cavity due to protein degradation by endogenous oral proteases [26, 34, 35]. Protease inhibitors can be used in an attempt to prevent proteolytic degradation. However, in saliva, it has been shown that short-term storage of freshly collected saliva samples on ice is more effective in preventing proteolytic degradation, without interfering with the chemistry of the proteome, than the use of protease inhibitors [30]. Therefore, no protease inhibitors were added to the saliva samples as they could promote chemical alterations on our protein complexes leading to changes in the stability of the complex and to an incorrect identification of the proteins that participate in complexes with amylase.

To distinguish a protein profile among the identified partners of amylase, biochemical characterization was performed according to the calculated pI and MW of the proteins and to their biological functions. Using the prevailing physiological salivary pH as reference (pH 6.8), the identified proteins were divided into two groups: pI below and pI above 6.8. Most of all identified proteins (67%) presented isoelectric points below 6.8 and therefore exhibited negative charge in a solution with pH 6.8. On the other hand, one-third of the identified amylase-protein partners (33%) exhibited more basic characteristics with pI above 6.8, showing positive

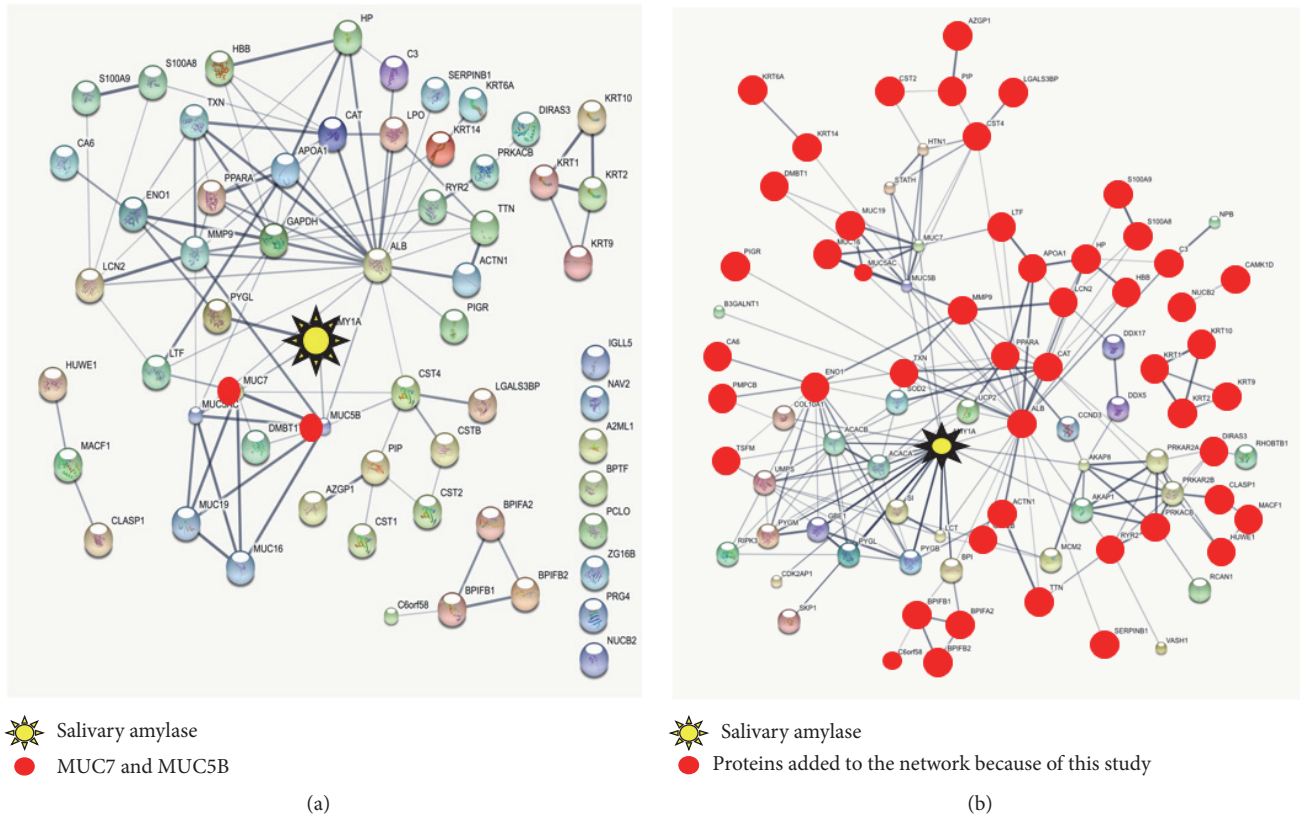

Figure 5: In silico view of amylase interactome using STRING database. (a) All proteins identified herein interact with amylase forming complex where represented. (b) An inclusive amylase interactome was constructed merging the proteins identified in this study with all known (*in vitro* studies) and predicted proteins (in silico databases) previously mentioned to form complex with amylase.

charges in pH 6.8. Therefore, there appears to be a preference for acidic proteins (pI < 6.8) to participate in the identified amylase complex. Knowing that ionic forces and hydrogen bonding, both electrostatic interactions, are involved in the formation of protein complexes, shifts in the net charge of salivary proteins possibly interfere with the nature and abundance of the proteins present in complexes. Differences among the pH of the secretions from the major salivary glands have been described [36, 37]. Also, changes in the pH of saliva have been suggested as biomarkers for systemic diseases [38]. In tumors, for example, there seems to be a shift in pH towards being acidic, acting as a favorable factor for tumor cells [38]. The proposition that variations in the salivary pH might interfere in the formation of salivary complexes suggests a new research and diagnostic avenue combining salivary proteome/interactome and salivary pH.

Since only subjects with overall good systemic and oral health were included in this study, it is implied that all our results were acquired around physiological salivary pH. In this condition, the characterization of the 66 identified proteins based on their biological functions reinforced the possible function of amylase as an important biological carrier. In total, 56% (37 proteins) of the identified partners of amylase exhibited important roles towards the maintenance of oral health. Four main mechanisms were recognized: antimicrobial activities, protection against chemical aggressions, immune response and regulation of inflammation, and physical protection of the mucosa and wound healing. About the debatable participation of amylase in the development of dental caries, this study did not aim to clarify the direct involvement of salivary amylase in the carious process. Contrarily, a new question is here proposed on the potential indirect participation of amylase in the protection against dental caries via functional modulation and/or protection of "anticariogenic" proteins from early proteolytic degradation in the oral cavity. A possible example of such proteins identified in this study is carbonic anhydrase VI. Besides carbonic anhydrase VI's involvement in taste sensation, this isoenzyme maintains the physiological salivary pH by catalyzing the hydration of carbon dioxide (bicarbonate buffer system), assisting in the recovery from acidic, more cariogenic, salivary challenges [17]. Carbonic anhydrase VI can also penetrate in the biofilm to facilitate the neutralization of acids secreted by the bacteria [14]. Carbonic anhydrase VI was identified among the proteins that participate in salivary complex with amylase. However, the direct binding of amylase and carbonic anhydrase VI and the possible consequences of such interaction are yet to be investigated. Other proteins identified in this study were cystatins B, SN, S, and SA. Cystatins are proteins that inhibit cysteine proteases secreted by the host, bacteria, and viruses [39]. Cystatins SA and SN are particularly involved in the

control of the proteolytic events *in vivo* such as periodontal tissue destruction [40]. The presence of cystatins B, SN, S, and SA in salivary complexes with amylase suggests that amylase may contribute indirectly against periodontal diseases.

Open proteomics/interactomics databases have been developed to assist in the study of protein-protein interactions and to accelerate discoveries in the field. Using the STRING database, a simulation of the amylase interactome with the identified partners of amylase was constructed. Out of the 66 members of the amylase complex listed in this study, only two proteins had been previously reported in the literature to present direct interactions with amylase; they were MUC5B [28] and MUC7 [29]. No direct binding between amylase and any of the other 64 proteins identified herein has been described up till now. Therefore, additional studies are needed to determine if any of these other proteins bind directly to amylase forming the first shell of the protein complex, as well as the exact position of each of the identified members of the amylase complex in the protein-protein network. Furthermore, the creation of a second amylase-protein-protein network merging the newly identified amylase-protein network with the previous known and predicted amylase interactors demonstrated that, using in silico approach based on molecular affinity prediction and prior *in vivo* and *in vitro* experiments, most the 66 proteins identified herein fill the gap in the amylase interactome present in WS.

It is important to highlight that the proteins identified herein in complexes with amylase, the most abundant salivary protein, were detected using three different proteomic approaches, with nine replicates, using saliva from three subjects, collected in three different dates (Supplemental Table 1). On the other hand, it is likely that each salivary protein has a different binding affinity with amylase. In fact, it is well known that changes in the salivary flow rate, person's overall health, and emotional state can promote qualitative and quantitative variations in the salivary proteome [41–45] and, consequently, in the amylase interactome. Future studies need to address the amylase interactome in different physiological/pathological conditions.

In summary, this study pioneered the exploration of the vast salivary interactome. It is important to remember that some of the proteins identified herein may interact with amylase indirectly, having one or more proteins as mediators of such interactions. Unfortunately, very little is known about the dynamics of these interactions. Transient protein complexes are less likely to be identified than permanent protein complexes. Additional studies are needed to confirm how the proteins listed in this manuscript interact with each other and with amylase. Amylase's ability to protect such partners from proteolytic degradation and/or modulate their biological functions while in the complex is yet to be studied comprehensively.

5. Conclusion

The large number of amylase complex partners identified herein reinforces the hypothesis that the real role of amylase in the oral cavity might not be related to carbohydrate digestion. Instead, amylase's most important role may be associated with protein transport and possible protection and functional modulation of its partners. In an era of more personalized and targeted medicine, this study opens the hypothesis for a novel therapeutic avenue where amylase can offer information for the development of an ideal carrier for functionally important peptides/proteins towards the prevention of oral diseases. Moreover, the salivary interactome may function as a foundation for the development of more efficient artificial saliva and/or mouth washes and provide more reliable models to design drugs directed to amylase or dependent on its function.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Supplementary Materials

Supplemental Table 1: detailed information regarding the identification of the 27 proteins that were common in all three used proteomic approaches. *(Supplementary Materials)*

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Research Article

Effects and Mechanisms of Tastants on the Gustatory-Salivary Reflex in Human Minor Salivary Glands

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The effects and mechanisms of tastes on labial minor salivary gland (LMSG) secretion were investigated in 59 healthy individuals. Stimulation with each of the five basic tastes (i.e., sweet, salty, sour, bitter, and umami) onto the tongue induced LMSG secretion in a dose-dependent manner. Umami and sour tastes evoked greater secretion than did the other tastes. A synergistic effect of umami on LMSG secretion was recognized: a much greater increase in secretion was observed by a mixed solution of monosodium glutamate and inosine 5′-monophosphate than by each separate stimulation. Blood flow (BF) in the nearby labial mucosa also increased following stimulation by each taste except bitter. The BF change and LMSG secretion in each participant showed a significant positive correlation with all tastes, including bitter. Administration of cevimeline hydrochloride hydrate to the labial mucosa evoked a significant increase in both LMSG secretion and BF, while adrenaline, atropine, and pirenzepine decreased LMSG secretion and BF. The change in LMSG secretion and BF induced by each autonomic agent was significantly correlated in each participant. These results indicate that basic tastes can induce the gustatory-salivary reflex in human LMSGs and that parasympathetic regulation is involved in this mechanism.

1. Introduction

The minor salivary glands are vital for the maintenance of oral health because they secrete abundant mucin, which acts as a lubricant [1], and are involved in immunoactivity through secretion of immunoglobulin A [2]. Although the minor salivary glands contain less volume than the major salivary glands [3], they are widely distributed throughout the oral mucosa [4].

Eating is a strong stimulus for the secretion of saliva by the major salivary glands [5]. Large volumes of saliva are secreted before, during, and after eating via the gustatory-salivary reflex, masticatory-salivary reflex, olfactory-salivary reflex, and esophageal-salivary reflex. Parasympathetic efferent activities induced by taste stimuli have been shown to involve salivation and vasodilation in the major salivary glands [6]. However, the details of secretion mechanisms in the minor salivary glands remain unclear because of difficulties in collecting and quantifying the minute secretion volume from the minor salivary glands. We previously developed a new technique for measuring the minor salivary gland flow using a simple iodine-starch filter paper method [7] and demonstrated that the subjective feeling of dry mouth was more strongly related to a reduction in minor salivary gland flow than in whole salivary flow [8]. This finding suggests an important role of the minor salivary glands in xerostomia.

In the present study, we examined the effects of five basic taste stimuli (sweet, salty, sour, bitter, and umami) on reflex salivation in the human labial minor salivary glands (LMSGs). Specifically, we studied the synergistic effect of the umami taste on reflexive LMSG secretion because the combined umami taste of monosodium glutamate (MSG) and inosine 5′-monophosphate (IMP) is widely known to have a strong effect on taste perception as a characteristic feature of the umami taste [9]. Additionally, we investigated the nervous control of LMSG secretion using autonomic agents while monitoring the nearby blood flow (BF) in the labial mucosa where LMSG secretion was observed.

2. Materials and Methods

2.1. Participants and Exclusion Criteria. In total, 64 healthy participants were initially recruited from the students at Tohoku University and the residents and staff members at Tohoku University Hospital. Individuals with systemic disease (e.g., endocrine, infectious, or immunological disease or a history of chemotherapy and/or radiation therapy for head and neck cancer), who had been prescribed medications that could directly affect dry mouth, or who had a feeling of oral dryness were excluded. Individuals with hyposalivation identified by measuring the LMSG flow and those with a history of psychological problems were also excluded after careful interviews and psychological testing (Self-Rating Depression Scale) to avoid psychogenic oral dryness. Consequently, 56 individuals (average age, 31.2 ± 8.3 years; age range, 19–42 years; 44 men, 12 women) were finally included. These participants were divided into four groups to evaluate the LMSG responses to taste ($n = 21$), the synergistic effect of umami ($n = 10$), the relationship between LMSG secretion and the BF change where the LMSG are located ($n = 14$), and the involvement of the autonomic nervous system in LMSG secretion and the BF change ($n = 11$). This study was designed and conducted in complete accordance with the World Medical Association Declaration of Helsinki (http://www.wma.net) and was approved by the Ethics Committee of the Tohoku University Graduate School of Dentistry. Written consent was obtained from each participant after they had received an explanation of the purpose of the study.

2.2. Quantification of LMSG Secretion. The LMSG secretion responses to distilled water (DW), tastants, or autonomic agents were quantified using the iodine-starch filter paper method as previously described [7]. Briefly, a strip of test paper (3 × 1 cm, Filter paper 1; Toyo Roshi Kaisha, Ltd., Tokyo, Japan) painted with a solution of iodine in absolute alcohol and a fine starch powder mixed with castor oil was applied onto the lower lip for 2 min [7]. The blackened areas of each test paper, imprinted by the iodine-starch reaction, were scanned and digitized at 8 bits using a GT-9500 ART image scanner (Seiko Epson Corp., Nagoya, Japan) with the scanning resolution set at 144 dpi. The total area was measured using image analysis software (Scion Image Beta 4.02; Scion Corporation, Frederick, MD, USA). Each total area value was converted to a flow rate (μL/cm^2/min) using the calibration line $Y = -0.084 + 24.992X$ (Y, area in mm^2; X, volume in μL) previously described [7].

2.3. BF Measurements in Labial Mucosa. BF changes in the lower labial mucosa, where quantification of the LMSG secretion was undertaken, were continuously monitored using reflection-mode laser Doppler flowmetry (SNF12007; Cyber Firm Med, Inc., Tokyo, Japan) before and after the administration of DW, tastants, or autonomic agents. During measurement of the BF, the participants were asked to keep their mouths open, and the lower lip was everted using an angle widener. The test areas were isolated with rolled gauze and then dried with a cotton gauze pad immediately before the recording was performed. A sensor probe was firmly anchored to the angle widener with surgical tape, and the tip of the probe was kept at a distance of 0.5 mm from the lip surface. All recordings were electrically calibrated to zero BF. Laser Doppler signals from the lower labial mucosa were continuously monitored, together with the systemic blood pressure (BP) (Finometer; Finapres Medical Systems, Amsterdam, Netherlands). The outputs from the flowmeter and BP monitor were recorded on a multichannel chart recorder (Recti-Horiz-8K; NEC San-ei, Tokyo, Japan).

2.4. Taste Stimulation

2.4.1. Five Basic Tastes. Five well-established taste substances were used. For the four basic tastes (sweet, salty, sour, and bitter), ready-made test solutions of Taste Disc™ (Sanwa Chemical Co., Ltd., Nagoya, Japan) were used. Each concentration of the four basic tastes was as follows:

(i) Sweet (sucrose): 8.7 mM (No. 1: S1), 73.0 mM (No. 2: S2), 292.1 mM (No. 3: S3), 584.2 mM (No. 4: S4), and 2337.1 mM (No. 5: S5)

(ii) Salty (NaCl): 51.3 mM (No. 1: N1), 213.8 mM (No. 2: N2), 855.5 mM (No. 3: N3), 1711.1 mM (No. 4: N4), and 3422.3 mM (No. 5: N5)

(iii) Sour (tartaric acid): 1.3 mM (No. 1: T1), 13.3 mM (No. 2: T2), 133.2 mM (No. 3: T3), 266.5 mM (No. 4: T4), and 533.0 mM (No. 5: T5)

(iv) Bitter (quinine): 0.03 mM (No. 1: Q1), 0.5 mM (No. 2: Q2), 2.5 mM (No. 3: Q3), 12.6 mM (No. 4: Q4), and 100.7 mM (No. 5: Q5)

For umami taste, MSG aqueous solution previously developed for an umami taste sensitivity test [10] was used. The concentrations of the umami taste were 1 mM (No. 1: G1), 5 mM (No. 2: G2), 10 mM (No. 3: G3), 50 mM (No. 4: G4), and 100 mM (No. 5: G5). Each taste number (Nos. 1–5) was set up so that the intensity of the participant's perception of the taste was equivalent in spite of the different taste qualities based on previously reported data of the Taste Disc [11] and our previously described findings regarding umami [10]. Thus, the intensity of perception of the same taste number (Nos. 1–5) was equal among the five tastes.

A 5 mm diameter cotton ball containing 50 μL of each taste solution or DW was applied onto the posterior tongue for 2 min, and the LMSG secretion was then measured. The participants were asked to rinse their mouth with water for at least 15 min between each taste stimulation. The next taste stimulation was applied after the measurement value had returned to the baseline level. All participants ($n = 21$) were asked to refrain from eating or drinking (except water), smoking, and brushing their teeth for at least 3 h before testing.

2.4.2. Combined Umami Tastes of MSG and IMP. To investigate the well-known synergistic effect of combined umami tastes on LMSG secretion, 5 mM of MSG aqueous solution, 5 mM of IMP aqueous solution, and a solution containing a mixture of the two (5 mM MSG and 5 mM IMP) were

prepared. Combined umami tastes that have been shown to evoke a synergistic effect [9] were made using an aqueous solution containing 10 mM MSG and 10 mM IMP. Changes in LMSG secretion were quantified following each administration of MSG, IMP, or MSG + IMP solution onto the posterior tongue of 10 participants. The procedure was similar to the above-described experiment involving the five basic tastes.

2.5. Relationship between LMSG Secretion and Nearby BF Change following Taste Stimulation. The LMSG secretion and nearby BF changes in the labial mucosa following application of the highest concentration (No. 5) of each of the five basic taste solutions onto the posterior tongue were observed for 14 participants. The LMSG secretion was first measured, and then the BF change to the tastant was measured until the BF had recovered to the prestimulus value.

2.6. Use of Autonomic Agents. The following four autonomic agents were prepared:

(i) 0.1% adrenaline: a sympathomimetic agent (Adrenaline Injection 0.1%; Terumo Corporation, Tokyo, Japan)

(ii) 3% cevimeline hydrochloride aqueous solution: a muscarinic (M3) receptor agonist (Saligren® capsule 30 mg; Nippon Kayaku, Tokyo, Japan)

(iii) 1% atropine sulfate hydrate: a cholinergic blocking agent (atropine ophthalmic solution 1%; Nitten Pharmaceutical Co., Ltd., Nagoya, Japan)

(iv) 2.5% pirenzepine hydrochloride aqueous solution: a muscarinic (M1) receptor antagonist (pirenzepine hydrochloride tablets 25 mg; Nichi-Iko Pharmaceutical Co., Ltd., Toyoma, Japan)

The concentration of each autonomic agent was based on the manufacturer's medical package insert for clinical use. The LMSG flow rate and BF were measured following application of a 3 × 1 cm filter paper soaked in 50 μL of each agent or DW on the labial mucosa for 5 min in 11 participants. Stimulation with the next agent was applied after the measurement values had returned to the baseline level. The participants were asked to rinse their mouth with water, and an interval of at least 30 min was set between each stimulation.

2.7. Data Analysis. The LMSG secretion after the administration of DW, tastants, or autonomic agents is presented as a percentage of the resting value (mean ± standard deviation). To compare each mean to the control (DW) mean, the data were analyzed by one-way analysis of variance followed by Dunnett's multiple-comparison test. Tukey's honestly significant difference test was used to analyze the differences in LMSG secretion and BF changes following stimulation with various tastants.

The BF changes in the labial mucosa after the administration of DW, tastants, or autonomic agents are presented as a percentage of the baseline value recorded with no administration (mean ± standard deviation). To compare each mean to the control (DW) mean, the data were analyzed by one-way analysis of variance followed by Dunnett's multiple-comparison test.

The normality of the data was assessed using the Shapiro-Wilk test, and the correlation between the changes in LMSG secretion and the nearby BF was then statistically analyzed using Spearman's rank correlation. All statistical analyses were performed using SPSS 18.0 (SPSS Inc., Chicago, IL, USA). The criterion for significance was defined as $p < 0.05$.

3. Results

3.1. Changes in LMSG Secretion following Stimulation with Five Basic Tastes. Low concentrations of the five basic tastes (sweet, salty, sour, bitter, and umami) caused no significant changes in LMSG secretion; however, high concentrations (Nos. 3–5) of all tastes evoked significant increases in LMSG secretion (Figure 1). Table 1 shows the detailed results.

As shown in Table 2, sour and umami tastes evoked significantly larger increases in LMSG secretion than did the other tastes (sweet, salty, and bitter) at high concentrations (Nos. 4 and 5), although low concentrations (Nos. 1–3) they did not.

3.2. Changes in LMSG Secretion following Stimulation with Mixed Umami Substances. Mixed umami substances of 5 mM MSG and 5 mM IMP caused a significant increase in LMSG secretion ($p < 0.0001$), while each solution alone elicited no significant change (MSG G2: 104.1 ± 6.2, $p = 0.985$; IMP: 106.6 ± 6.8, $p = 0.886$) as compared with DW stimulation (101.8 ± 4.5) (Figure 2).

3.3. Relationship between LMSG and Nearby Lip BF following Taste Stimulation. The highest concentration (No. 5) of each of the five basic tastes evoked a significant increase in LMSG secretion (sweet S5: 131.3 ± 19.3, $p = 0.045$; salty N5: 130.8 ± 11.1, $p = 0.049$; sour T5: 264.8 ± 76.4, $p < 0.0001$; bitter Q5: 131.0 ± 36.3, $p = 0.048$; umami: 266.8 ± 47.6, $p < 0.0001$) compared with DW stimulation (96.7 ± 0.8) (Figure 3(a)). All tastes except bitter evoked a significant increase in lip BF (sweet S5: 134.5 ± 9.5, $p = 0.013$; salty N5: 128.9 ± 8.6, $p = 0.047$; sour T5: 238.5 ± 43.8, $p < 0.0001$; umami G5: 224.4 ± 56.4, $p < 0.0001$) compared with DW stimulation (99.0±2.5), while bitter did not elicit a significant change in BF (118.9 ± 29.1, $p = 0.295$) (Figure 3(b)). As shown in Tables 3 and 4, sour and umami tastes evoked significantly larger increases in both LMSG secretion and BF changes than did the other tastes (sweet, salty, and bitter). Some participants showed increases in both LMSG secretion and BF change in response to bitter taste, but others showed decreases in both LMSG secretion and BF change. Comparison of the changes in the same participants revealed a significant correlation between the amount of changes in salivation and BF in response to each taste stimulus (sweet: $r = 0.802$; salty: $r = 0.751$; sour: $r = 0.806$; bitter: $r = 0.805$; umami taste: $r = 0.853$) (Figure 4).

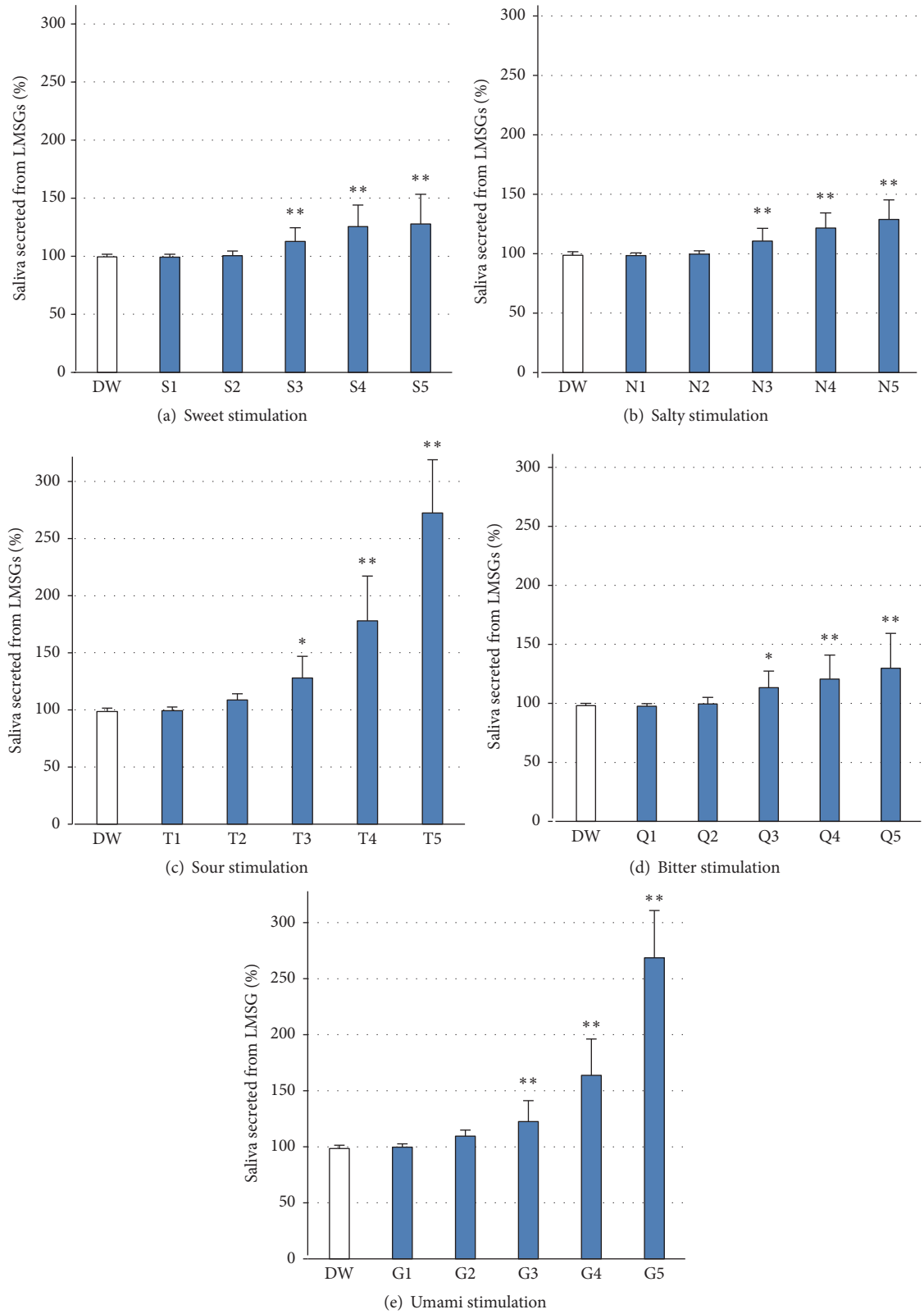

Figure 1: Changes in LMSG secretion following stimulation with five basic tastes. High concentrations (Nos. 3–5) of each of the five basic tastes (S, N, T, Q, and G) elicited a significant increase in LMSG secretion in human participants ($n = 21$), although lower concentrations (Nos. 1 and 2) of each solution caused no significant change. Ordinate: a percentage (%) of the resting saliva. $^{*}p < 0.05$, $^{**}p < 0.0001$.

Table 1: Changes in LMSG secretion induced by DW or tastants.

	DW	1	2	3	4	5
				S (sweet)		
% change	99.5 ± 2.3	99.1 ± 2.6	100.4 ± 4.1	112.8 ± 8.7	125.5 ± 16.6	126.4 ± 25.5
p values	-	0.999	0.998	**<0.0001****	**<0.0001****	**<0.0001****
				N (salty)		
% change	100.6 ± 3.0	98.5 ± 2.2	108.9 ± 8.7	114.1 ± 12.5	118.7 ± 14.1	119.8 ± 16.6
p values	-	0.999	0.966	**<0.0001****	**<0.0001****	**<0.0001****
				T (sour)		
% change	98.7 ± 2.8	99.3 ± 3.2	111.1 ± 7.2	134.2 ± 15.8	174.2 ± 35.7	272.4 ± 42.5
p values	-	0.999	0.696	**0.007***	**<0.0001****	**<0.0001****
				Q (bitter)		
% change	104.0 ± 1.9	97.7 ± 1.9	105.1 ± 9.7	115.1 ± 18.9	119.0 ± 26.8	121.1 ± 34.3
p values	-	0.999	0.994	**0.001***	**<0.0001****	**<0.0001****
				G (ummai)		
% change	98.5 ± 2.9	99.6 ± 3.1	109.4 ± 5.4	122.5 ± 18.6	163.7 ± 32.5	268.6 ± 42.1
p values	-	0.999	0.998	**<0.0001****	**<0.0001****	**<0.0001****

Statistical differences were analysed by one-way ANOVA and Dunnett's multiple-comparison test ($^{*}p < 0.05$, $^{**}p < 0.001$); % change indicates a percentage of the resting value (mean ± standard deviation); $n = 21$.

Table 2: Increases in LMSG secretion induced by high concentration of tastants (No. 4 and 5).

		S (sweet)	N (salty)	T (sour)	Q (bitter)	G (umami)
No. 4	S (sweet)	-	0.977	<0.001*	0.949	<0.001*
	N (salty)		-	<0.001*	1.000	<0.001*
	T (sour)			-	<0.001*	0.21
	Q (bitter)				-	<0.001*
	G (umami)					-
No. 5	S (sweet)	-	1.000	<0.001*	1.000	<0.001*
	N (salty)		-	<0.001*	1.000	<0.001*
	T (sour)			-	<0.001*	0.999
	Q (bitter)				-	<0.001*
	G (umami)					-

Statistical differences were analysed by one-way ANOVA and Tukey's honestly significant difference test; numerical value means p value between one tastant and another: ∗ (asterisk) means significant; $n = 21$.

3.4. Changes in LMSG Secretion and Nearby Lip BF Change following Stimulation with Autonomic Agents. Administration of cevimeline chloride (parasympathetic agonist) caused a significant increase in LMSG secretion (170.3 ± 22.1, $p < 0.0001$), while adrenaline (sympathetic agonist) (33.2 ± 3.8, $p < 0.0001$), atropine (parasympathetic inhibitor) (64.0 ± 6.1, $p < 0.0001$), and pirenzepine (parasympathetic antagonist) (42.3 ± 8.4, $p < 0.0001$) evoked a significant decrease in LMSG secretion compared with DW stimulation (103.4 ± 3.4) (Figure 5(a)). These changes induced by the different agents were consistent with those of nearby lip BF changes; that is, cevimeline chloride caused a significant increase in the BF (198.9 ± 37.1, $p < 0.0001$), while adrenaline (37.7 ± 9.2, $p < 0.0001$), atropine (61.9 ± 12.2, $p < 0.0001$), and pirenzepine (49.6 ± 18.8, $p < 0.0001$) elicited a significant decrease in the BF compared with DW stimulation (106.8 ± 7.2) (Figure 5(b)). Significant correlations were found between the amount of change in LMSG secretion and the BF for each autonomic agent in the same participant (adrenaline: $r = 0.893$; cevimeline: $r = 0.882$; atropine: $r = 0.797$; pirenzepine: $r = 0.788$) (Figure 6).

4. Discussion

4.1. Responses of Minor Salivary Gland Secretion to Stimulation with Five Different Tastes. The gustatory-salivary reflex (i.e., taste-initiated secretion of saliva) is important for tasting, masticating, and swallowing food. This vital reflex has been mainly studied in the saliva secreted from the major salivary glands or mixed saliva secreted from the major and minor salivary glands. Kerr [12] showed that the human major salivary flow response to citric acid, salt, and sucrose was 10, 7, and 4 times higher than the resting saliva response, respectively. Hodson and Linden [13] also demonstrated that the five basic taste qualities (sweet, salty, sour, bitter, and umami) induced the gustatory-salivary reflex in the parotid gland and that parotid salivary flow increased in a dose-dependent manner in response to umami taste (MSG).

Table 3: Increases in LMSG secretion induced by tastant of No. 5.

		S (sweet)	N (salty)	T (sour)	Q (bitter)	G (umami)
No. 5	S (sweet)	-	0.999	<0.001*	0.826	<0.001*
	N (salty)		-	<0.001*	0.697	<0.001*
	T (sour)			-	<0.001*	0.967
	Q (bitter)				-	<0.001*
	G (ummai)					-

Statistical differences were analysed by one-way ANOVA and Tukey's honestly significant difference test; numerical value means p value between one tastant and another: ∗ (asterisk) means significant; $n = 14$.

Table 4: Increases in BF change induced by tastant of No. 5.

		S (sweet)	N (salty)	T (sour)	Q (bitter)	G (umami)
No. 5	S (sweet)	-	0.991	<0.001*	0.717	<0.001*
	N (salty)		-	<0.001*	0.926	<0.001*
	T (sour)			-	<0.001*	0.791
	Q (bitter)				-	<0.001*
	G (ummai)					-

Statistical differences were analysed by one-way ANOVA and Tukey's honestly significant difference test; numerical value means p value between one tastant and another: ∗ (asterisk) means significant; $n = 14$.

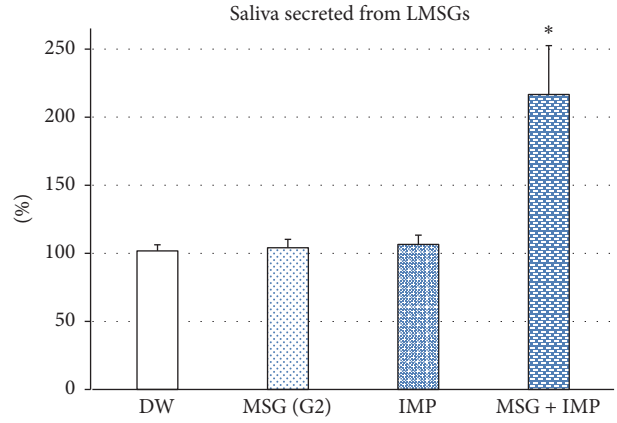

Figure 2: Changes in LMSG secretion following stimulation with umami substances. Neither MSG (G2) nor IMP elicited a significant change in LMSG secretion, while mixed umami substance (5 mM MSG + 5 mM IMP) caused a significant increase in LMSG secretion ($p < 0.0001$) ($n = 10$). $^*p < 0.0001$. Ordinate: a percentage (%) of the resting saliva.

Few reports have provided a detailed comparison of gustatory-salivary reflex salivation in response to the different taste stimuli in the minor salivary glands, except our preliminary report [14], because of the difficulty in measurement of the minute secretion volume from the minor salivary glands. In the present study, we used a newly developed method for measuring the LMSG flow rate [7] and demonstrated that (1) each of the five basic taste stimuli elicited a significant increase in saliva secreted from the LMSG in a dose-dependent manner, and (2) sour and umami tastes elicited significantly larger increases in LMSG secretion than did sweet, salty, or bitter. These results are consistent with previous reports demonstrating the major salivary flow response [13, 15, 16].

Allen [17] reported a correlation between gustatory-salivary reflex salivation in the parotid gland and the intensity of the taste stimulus. Therefore, the taste intensity of each different taste quality must be equivalent to compare the differences in the amount of saliva produced by the gustatory-salivary reflex. In the present study, each different taste quality solution, including umami, was administered at five different intensities (Nos. 1–5) based on a previous study that established the cumulative distribution of each tastant [10, 11]. For example, the specific taste quality of the No. 2 concentration of each taste solution can be recognized by 50% of participants. Thus, using the same number of taste test solutions, it becomes possible to supply an equal intensity of taste perception in spite of the differences in taste quality.

4.2. Responses of LMSG Secretion to Stimulation with Mixed Umami Substance. Mixed umami solution containing MSG and IMP caused a significant increase in LMSG secretion, whereas stimulation with MSG or IMP alone did not increase LMSG secretion at these concentrations. The synergism of umami tastes between MSG and guanylate was first reported by Kuninaka [18, 19], and Yamaguchi and Ninomiya [9] indicated that the detection threshold of umami taste perception of MSG was markedly lower in the presence of IMP. A recent electrophysiological study involving mice demonstrated the occurrence of marked enhancement of the glossopharyngeal nerve responses to MSG by the addition of guanylate [20, 21]. This is in line with our result on the synergism of umami tastes when the posterior tongue is stimulated by a mixture of MSG and another umami substance (e.g., IMP). It has been suggested that the human taste receptor, a T1R1 + T1R3 heterodimer, induces potentiation of the synergism between MSG and IMP. A recent study suggested the existence of separate binding sites for MSG and IMP within the same T1R1 Venus flytrap domain, which is important for umami taste synergism [22, 23]. In T1R1-knockout mice, the synergism

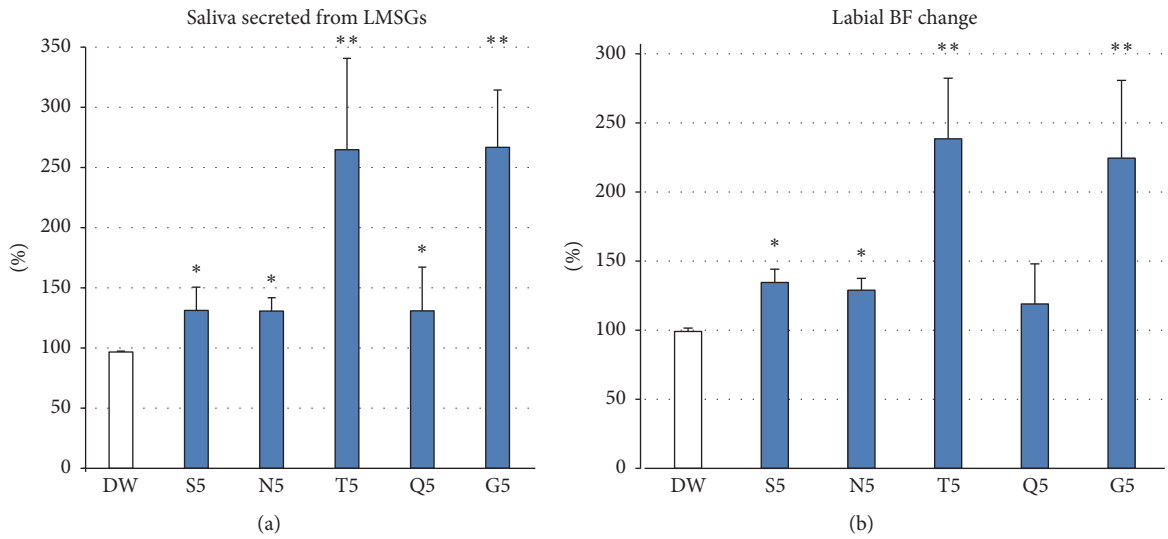

Figure 3: Changes in (a) LMSG secretion and (b) nearby BF change following stimulation with five basic tastes. (a) The highest concentration (No. 5) of each of the five basic tastes (S, N, T, Q, and G) evoked significant increases in MSG secretion ($n = 14$). (b) The same concentration of each of the basic tastes except bitter elicited a significant increase, but not a significant change, in labial mucosal BF ($n = 14$). Ordinate: a percentage (%) of (a) the resting saliva and (b) the baseline BF value. $^{*}p < 0.05$, $^{**}p < 0.0001$.

between MSG and IMP is considerably reduced in the anterior tongue [24]. Thus, the umami taste has a quite distinguished synergistic effect exhibited by no other taste quality. We demonstrated that the synergistic effect of umami not only showed sensory perception but also evoked the gustatory-salivary reflex in the LMSGs. This synergistic effect has also been shown to be elicited not only between MSG and IMP but also between MSG and other nucleotides of guanylate [18, 19]. Therefore, further studies of the effect of different mixtures of MSG and other nucleotides on reflex secretion in the LMSGs are needed.

Umami has another specific characteristic, that is, its residual aftertaste, which differs from other taste qualities [25]. In a preliminary study, we examined the time course of the salivary flow of LMSG secretion in response to the five basic tastes and found that the umami taste evoked a long-lasting increase in LMSG, whereas sour taste evoked a prominent increase in the LMSG flow that immediately diminished [14]. It seems likely that these long-lasting effects on LMSG secretion incidental to the umami taste are due to the residual aftertaste. The synergism and residual aftertaste of the umami taste may be beneficial for patients with dry mouth based on our previous finding that xerostomia is more strongly related to the LMSG flow than the major salivary gland flow [8].

4.3. Relationship between LMSG Secretion and Nearby Lip BF Change following Taste Stimulation. The salivary glands are supplied by a dense capillary network equivalent to that of the heart; thus, vasodilatation of these capillaries surrounding the salivary glands might be necessary to ensure that large volumes of saliva are produced by the secretory cells [26, 27]. We considered that the circulation surrounding the LMSGs is closely related to the LMSG secretory system. Consequently, we examined the nearby lip BF where the LMSG secretion measurement was performed using laser Doppler flowmetry. Our measurement of the BF included the labial glandular BF because laser Doppler flowmetry can measure the erythrocyte flux through an approximately $1\,mm^3$ volume of the capillary bed without touching the tissues [28]. This can be accomplished because the LMSGs densely exit via the superficial oral mucosa, which is very thin. We demonstrated that stimulation with all tastes except bitter caused an increase in the nearby lip BF consistent with the increase in the LMSG secretion. In addition, sour and umami tastes induced prominent increases in the BF in the same manner as the LMSG secretion. Taste stimulation evoked no BP changes, indicating that vasodilation in the stimulated area was induced.

Interestingly, we observed a correlation between the rate of changes in the BF and the LMSG response to each taste stimulus in different participants (Figure 4). As shown in Figure 4, sweet, salty, sour, and umami tastes evoked correlated increases in the BF and LMSG secretion (>100% in the figure) in all participants; however, bitter caused a correlated decrease in the BF and LMSG secretion (<100% in the figure) in some participants. Thus, bitter only evoked a BF decrease in some participants. A previous study showed that the BF in the orofacial area is uniquely controlled by a double autonomic system; that is, vasoconstriction mediated via sympathetic nerve fibers and vasodilation mediated via parasympathetic efferent nerve fibers [29]. Bitter taste can evoke both sympathetically induced reflexive vasoconstriction and parasympathetically mediated vasodilation, while the other tastes prominently induce reflex vasodilation. In this respect, the hedonic dimension to the taste reportedly plays various roles in the many taste-mediated whole-body responses. Interestingly, an unpleasant bitter taste can

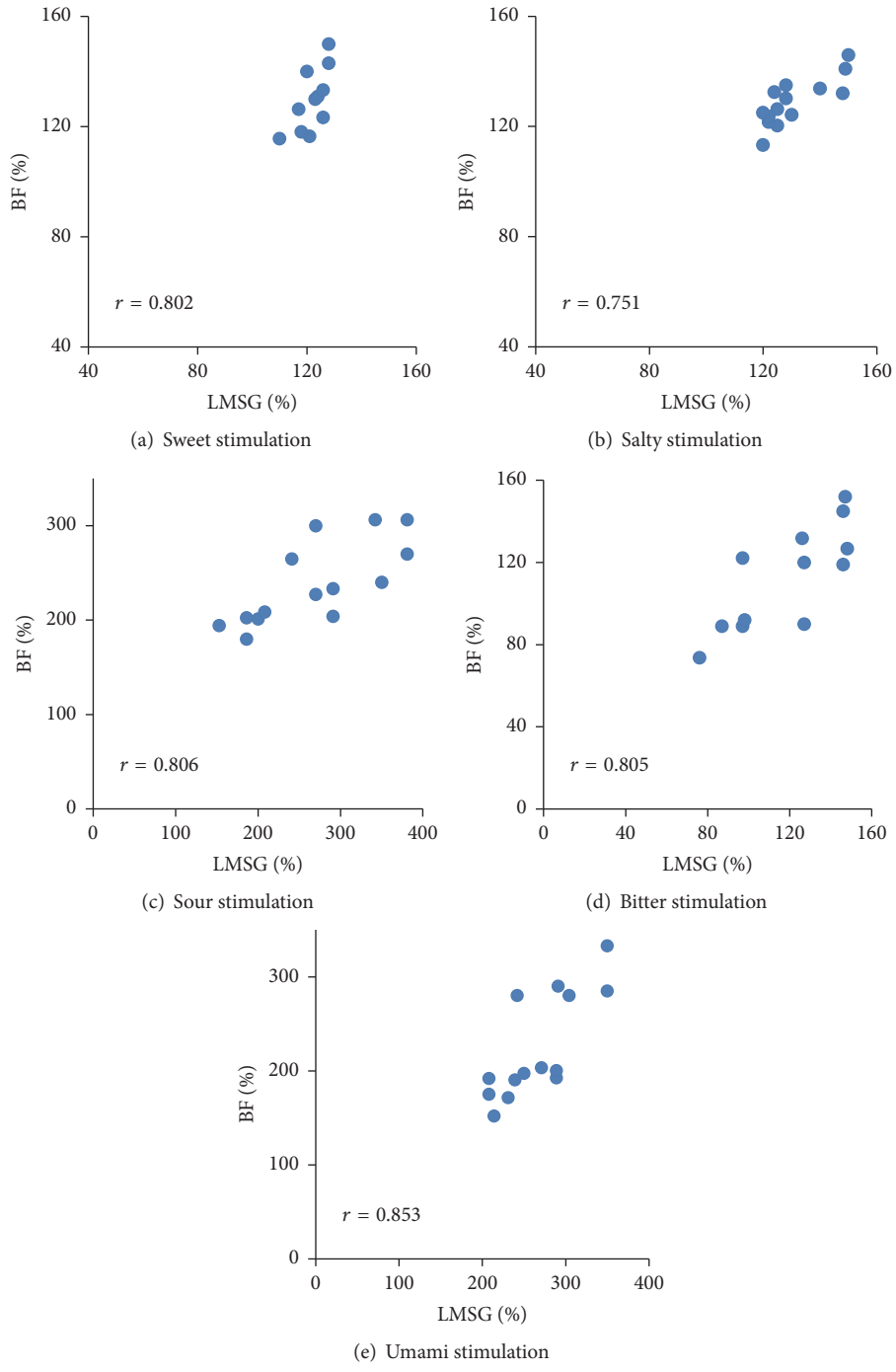

Figure 4: Relationship between LMSG secretion and BF response in lip following taste stimulation. Significant correlations were present between the amount of change in LMSG secretion and BF evoked by the highest concentration (No. 5) of each taste stimulus ($n = 14$). Ordinate: a percentage (%) of the baseline BF value; Abscissa: a percentage (%) of the resting LMSG saliva.

reportedly induce sympathetically mediated physiological changes in skin BF and skin temperature, instantaneous heart rate, and skin potential and skin resistance much more strongly than other taste qualities (sweet, salty, and sour) [30]. Additionally, pleasant stimuli were found to elicit approach and acceptance, whereas unpleasant stimuli induced avoidance and rejection, thus determining taste preferences and aversions [30]. Although we did not record the participants' liking of each tastant in this experiment, some participants indeed hated the bitter taste. Such individuals may show stronger decreases in LMSG secretion and the BF as a sympathetic effect. Further studies are needed to clarify the role of unpleasant taste sensations in the control of taste-mediated responses related to food rejection.

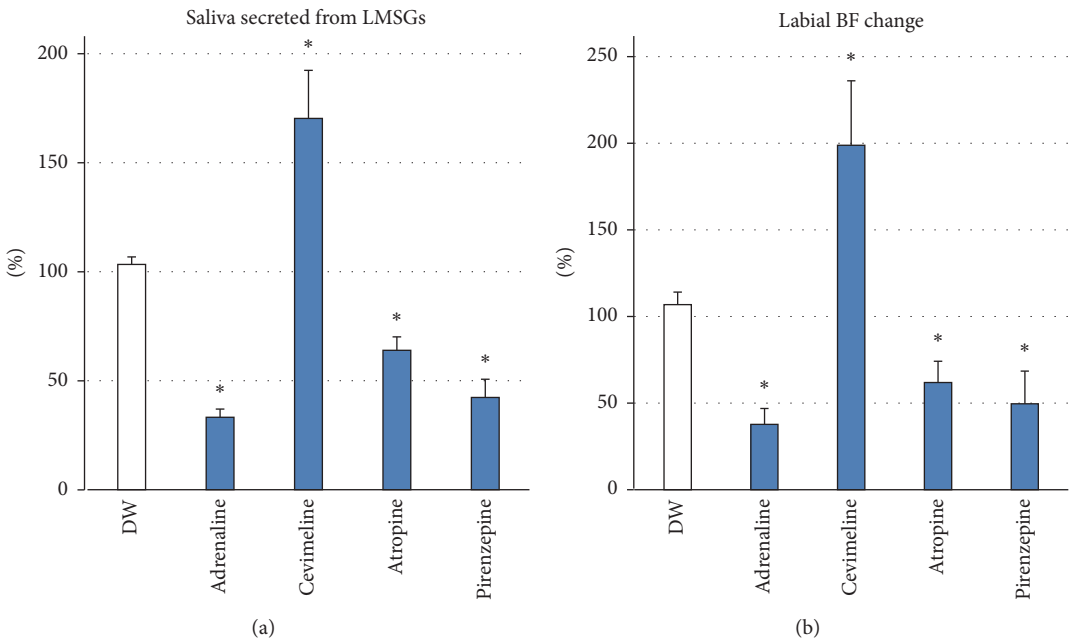

Figure 5: Changes in (a) LMSG secretion and (b) nearby BF change following stimulation with autonomic agents onto the lip. (a) Administration of cevimeline chloride caused a significant increase in LMSG secretion ($p < 0.001$), while adrenaline, atropine, and pirenzepine evoked significant decreases in LMSG secretion ($n = 11$). (b) Cevimeline chloride caused a significant increase in labial mucosal BF, while adrenaline, atropine, and pirenzepine elicited significant decreases ($n = 11$). Ordinate: a percentage (%) of the (a) resting saliva and (b) baseline BF. $^*p < 0.0001$.

Overall, our results indicate that the BF change surrounding the LMSGs is an important factor in the salivary secretory system in the LMSGs.

4.4. LMSG Secretion and Nearby Lip BF Changes Mediated by the Autonomic Nervous System. Salivary secretion is controlled by the parasympathetic and sympathetic autonomic nervous systems [31]. In the human parotid gland, the gustatory-salivary reflex involves the activity of both types of autonomic nerves, while the masticatory-salivary reflex preferentially activates the parasympathetic nerves [32]. Mobilization of the intracellular messenger calcium by stimulation of muscarinic receptors (M1, M3) is associated with fluid secretion, particularly large volumes in response to muscarinic agonists, via exocytosis in the rat parotid gland [33]. However, the neural regulation of the gustatory-salivary reflex in human LMSGs remains unknown. In the present study, application of cevimeline hydrochloride hydrate (an agonist of the muscarinic M3 receptor) onto the lip elicited an increase in LMSG secretion. Furthermore, pirenzepine (an antagonist of the muscarinic M1 receptor) and atropine (a competitive inhibitor of the muscarinic acetylcholine receptor) elicited a decrease in LMSG secretion. Thus, we conclude that muscarinic receptors (M1, M3) are engaged in human LMSG secretion. However, the application of adrenaline (an agonist of α and β adrenergic receptors) certainly decreased LMSG secretion. This phenomenon differs from that described in a report on sympathetic nerve-induced secretion by the parotid gland, suggesting that the human LMSGs may lack sympathetic secretion. This idea is supported by a histochemical study indicating that few adrenergic nerves have been identified in the human LMSGs [34].

Nervous control of the orofacial BF is regulated by both the parasympathetic and sympathetic autonomic nervous systems [29]. In the cat, labial BF is controlled by two groups of parasympathetic fibers (the facial and glossopharyngeal nerves) for vasodilatation [29] and by sympathetic α-adrenergic fibers for vasoconstriction [35]. We also examined the neural regulation of the BF in the human labial mucosa because salivary secretion appears to be related to the nearby BF, as mentioned above. In our pharmacological analysis, application of cevimeline hydrochloride hydrate (an agonist of the muscarinic M3 receptor) elicited a prominent increase in the BF without a change in the BP, and pirenzepine (an antagonist of the muscarinic M1 receptor) and atropine (a competitive inhibitor of the muscarinic acetylcholine receptor) elicited a significant decrease in the BF without a change in the BP, indicating that muscarinic receptors (M1, M3) are engaged in vasodilatation in the human labial mucosal tissues surrounding the LMSGs. Furthermore, adrenaline (an agonist of α and β adrenergic receptors) elicited a significant decrease in the nearby BF without a change in the BP, indicating that α-adrenergic receptors are involved in vasoconstriction in this region. Interestingly, correlations were found between the dynamics of the saliva secreted from the LMSGs and the nearby lip BF changes in response to each chemical agent in the same participants (Figure 6), although vascular responses monitored by laser Doppler flowmetry should include not only the labial glandular BF

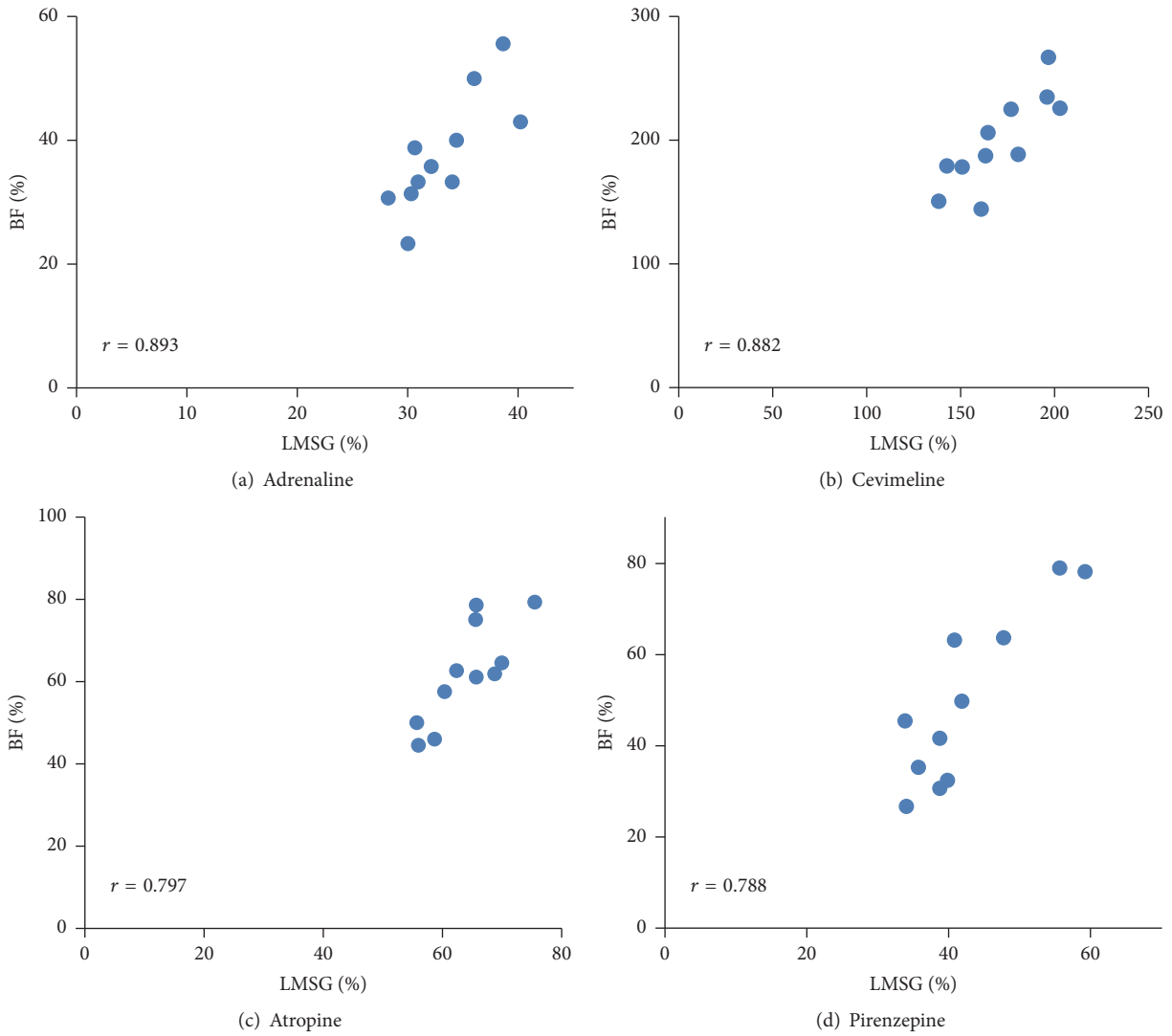

Figure 6: Relationship between LMSG secretion and BF response in lip following stimulation with autonomic agent. Significant correlations were present between the amounts of change in LMSG secretion and BF evoked by each autonomic agent in the same participant ($n = 11$). Ordinate: a percentage (%) of the baseline BF value; abscissa: a percentage (%) of the resting LMSG saliva.

but also the mucosal capillary BF. These results show that parasympathetic activation can simultaneously increase the salivary secretion from the LMSGs and induce vasodilatation in the mucosal tissues surrounding the LMSGs. Conversely, decreases in the saliva secreted by the LMSGs may be caused by a decrease in BF incidental to the vasoconstriction because the human LMSGs possibly lack sympathetic secretion, as discussed above [34]. Thus, we consider that LMSG secretion is strongly influenced by the nearby BF. Further detailed studies are necessary to clarify the effects of the relationship between LMSG secretion and nearby BF changes on the autonomic nervous system.

The present study has shown that each of the five basic taste sensations can induce human LMSG secretion. This LMSG secretion is an autonomic nervous system-induced reflex that spontaneously arises at meals and may be beneficial to various functions of eating, such as smooth chewing and formation of a food bolus. Moreover, LMSG secretion provides lubrication and protection of the oral mucosa because the LMSG secretions contain high concentrations of protective substances such as mucin and immunoglobulin A. This LMSG secretion induced by taste substances contained in food at meals would thus be beneficial for maintaining oral health.

5. Conclusions

Taste stimulation can cause a gustatory-reflex secretion in the human LMSGs. In particular, sour and umami tastes cause larger increases in LMSG secretion than do other tastes. Umami has a synergistic effect on the LMSG secretion reflex. Parasympathetic regulation is involved in the gustatory-salivary reflex in the LMSGs in association with the changes in BF near the LMSGs.

Conflicts of Interest

All authors declare no conflicts of interest regarding the publication of this paper.

Acknowledgments

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Research Article

Oxidative Modification of Biomolecules in the Nonstimulated and Stimulated Saliva of Patients with Morbid Obesity Treated with Bariatric Surgery

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Morbid obesity leads to progressive failure of many human organs and systems; however, the role of oxidative damage to salivary composition is still unknown in the obese patients. In this study, we assessed the effect of bariatric surgery on oxidative damage in nonstimulated (NS) and stimulated (S) whole saliva. The study included 47 subjects with morbid obesity as well as 47 age- and gender-matched healthy volunteers. Oxidative modifications to lipids (4-hydroxynonenal (4-HNE) and 8-isoprostanes (8-isoP)), proteins (advanced oxidation protein products (AOPP) and protein carbonyl groups (PC)), and DNA (8-hydroxy-D-guanosine (8-OHdG)) were analyzed in morbidly obese patients before and after bariatric surgery as well as in the healthy controls. The concentrations of 8-isoP, AOPP, PC, and 8-OHdG were significantly higher in both NS and S of patients with morbid obesity than in the control patients and compared to the results obtained 6 months after bariatric surgery. The levels of oxidative damage markers were also higher in S versus NS of morbidly obese patients. In summary, morbid obesity is associated with oxidative damage to salivary proteins, lipids, and DNA, while bariatric treatment generally lowers the levels of salivary oxidative damage.

1. Introduction

Overweight and obesity are chronic diseases characterized by excessive accumulation of adipose tissue. According to the WHO, at least 50% of adults and 20% of children are overweight (BMI > 25 kg/m^2), and more than 400 million are obese (BMI > 30 kg/m^2). Moreover, the number of people with morbid obesity (BMI > 40 kg/m^2) has increased by 4-5 times compared to the 1990s. Complications of morbid obesity are extremely dangerous to human health and include metabolic syndrome, cardiovascular disease, insulin resistance (IR), and type 2 diabetes [1]. Treatment of morbid obesity requires multidirectional actions, but the most effective method is surgical treatment, including also bariatric surgery [2]. There are a number of surgical methods; however, the presented study only included laparoscopic gastric sleeve resection. This technique is well tolerated by patients, which allows for their faster recovery. There is a decrease in the number of postoperative complications, satisfactory reduction of body weight, and associated diseases [3].

Not only the aforementioned excess of the adipose tissue, but also, above all, its dysfunction is central to obesity-related complications. It was shown that adipose tissue in

obese patients develops an inflammatory milieu, which drives oxidative stress (OS) due to a large increase in reactive oxygen species (ROS) formation by immune cells as part of the immune response [4, 5]. Oxidative stress is a situation in which chronically elevated ROS levels lead to disturbances in cellular metabolism and degradation of cellular components such as lipids, proteins, and DNA [4–6]. The cell membrane is the first element to be exposed to contact with free radicals; therefore, the earliest symptom of developing OS is lipid peroxidation. Most often the lipids with one or more double bonds are subject to oxidative modifications leading to the formation of peroxides (4-hydroxynonenal (4-HNE) and 8-isoprostanes (8-isoP)). Oxidative modifications of proteins and free amino acid residues are also observed at high ROS concentrations. Oxidation of proteins results in breaking of protein chains, formation of cross-links within single or multiple polypeptide chains, and modification of amino acid residues. Oxidative damage to proteins can be assessed by measuring concentrations of, for example, protein carbonyls (PC) or advanced oxidation protein products (AOPP). Oxidative DNA modifications are observed in a form of, for instance, increased concentrations of 8-hydroxy-D-guanosine (8-OHdG) [7, 8].

It is believed that OS causes damage to the salivary gland components and promotes chronic systemic and local inflammation. It leads, inter alia, to the initiation and progression of pathological changes within the oral cavity. Indeed, it was shown that more than half of people with morbid obesity are diagnosed with diseases of oral cavity, the pathogenesis of which may be associated with excessive ROS [9–11]. Moreover, there is evidence indicating that salivary gland dysfunction is already manifested at the obesity stage [10]. Taking into account the importance of saliva in maintaining oral homeostasis, it becomes clear that, starting with the obesity stage, IR and diabetes can adversely affect oral health and the quality of life. The pathogenesis of the salivary glands dysfunction in the course of morbid obesity is still unknown.

The influence of oxidative stress in the pathogenesis of salivary gland dysfunction in the course of insulin resistance or type 2 diabetes has been confirmed [7, 12, 13]. To the best of our knowledge, there are hardly any sources describing salivary OS in the course of morbid obesity.

The purpose of this experiment is to assess the occurrence and intensity of oxidative stress in unstimulated and stimulated saliva of patients with morbid obesity before and 6 months after bariatric surgery by evaluating the concentrations of oxidative damage markers of lipids, proteins, and DNA. The relationship between the oxidative stress markers and the secretory function of salivary glands in the course of morbid obesity and its treatment has not been studied so far. Our goal is to better understand the relationship between oxidative stress and salivary gland dysfunction in the course of morbid obesity.

2. Materials and Methods

The research was approved by the Bioethics Committee of the Medical University of Bialystok, Poland (permission number: R-I-002/175/2012 of 31 May 2012). Every patient was informed about the purpose of the study and consented in writing to participate in the project.

2.1. Patients. The study involved 47 patients with morbid obesity (14 men and 33 women, aged 34 to 55). Patients were treated in the 1st Clinical Department of General and Endocrine Surgery of the Medical University of Bialystok. From 2012 to 2014, the patients underwent bariatric surgeries (laparoscopic gastric sleeve resection) performed by the same qualified surgeon (H.R.H.). Immediately before and 6 months after bariatric surgery, each patient had a blood test and a dental examination, and their unstimulated and stimulated mixed saliva was collected. The control group (C) consisted of 47 healthy, age- and gender-matched adults under the supervision of the UMB Department of Restorative Dentistry. Dental examinations as well as the collection of unstimulated and stimulated salivary samples from the control group were performed on a one-off basis.

Criteria for inclusion in the study group were as follows:

(i) BMI > 40 kg/m^2

(ii) Waist circumference ≥ 94 cm in men and ≥80 cm in women

(iii) Total cholesterol concentration ≥ 200 mg/dL

(iv) LDL cholesterol concentration ≥ 160 mg/dL

(v) HDL cholesterol concentration < 40 mg/dL

Criteria for inclusion in the control group were as follows:

(i) BMI 18–25 kg/m^2

(ii) Total cholesterol concentration < 200 mg/dL

(iii) LDL cholesterol concentration < 160 mg/dL

(iv) HDL cholesterol concentration > 40 mg/dL

Criteria for inclusion in the study and control group were as follows:

(i) Age 34–55

(ii) HOMA-IR (Homeostatic Model Assessment of Insulin Resistance) < 1

(iii) Blood uric acid concentration 180–420 μmol/L

(iv) AspAT and ALAT activity < 510 nmol/L/s

(v) TSH concentration in serum 2–5 mU/L

(vi) Lack of inflammatory condition in the mouth, including gingivitis (no bleeding while probing gingival pockets; pale pink gingivae) and periodontitis (PPD: periodontal pocket depth measured from the gingival margin to the pocket bottom < 4 mm [14]; CAL: clinical attachment loss < 3 mm)

Criteria for exclusion from the study and control group were as follows:

(i) Smoking

(ii) Alcohol consumption (except occasional use, once every 2-3 months)

(iii) Taking medicines (including antibiotics and glucocorticosteroids) and dietary supplements affecting saliva secretion and its antioxidant status in the last 3 months

(iv) Comorbidities of inflammatory etiology and involvement of oxidative stress (including diabetes, hypertension, diseases of kidneys, liver, thyroid gland, gastrointestinal tract, and immune system)

(v) Pregnancy

2.2. Dental Examination. The dental examinations took place at the UMB Department of Restorative Dentistry. The examinations were performed by the same qualified dentist (A. Z.), in artificial light, by means of an explorer, a mirror, and a periodontal probe. The DMFT index (total number of decayed, missing, and filled teeth) was calculated in accordance with the criteria of the WHO [15] as well as the SBI (Sulcus Bleeding Index) [16], PPD, and CAL. The intrarater reliability for DMFT was $r = 0.97$, for SBI $r = 0.96$, for PPD $r = 0.92$, and for CAL $r = 0.96$.

2.3. Saliva Collection. The study material was mixed stimulated and unstimulated saliva. It was collected via the spitting method at least 2-3 hours after tooth brushing or food and fluid intake (except water), always between 8 a.m. and 10 a.m. Saliva collection was performed in one separate room for all the patients, without exposing them to the effects of additional aromatic, taste, and visual stimuli. The patients had their saliva collected in a comfortable seated position, with the head slightly inclined forward. After rinsing the mouth three times with distilled water, the patients spat the saliva gathered at the bottom of the oral cavity for 15 minutes in total. Saliva was collected into sterile centrifuge tubes placed in a container with ice. The saliva collected for the first minute was discarded [17]. Saliva secretion was stimulated with a 2% citric acid solution placed at the back of the tongue (100 μL every 30 seconds). Stimulated saliva was collected for a total of 5 minutes [18]. The volume of unstimulated and stimulated saliva was measured with a pipette set to 100 μL. The saliva minute flow was calculated by dividing the volume of the secreted saliva by the time needed for its collection. To prevent oxidation of the sample during processing and storage, the solution of butylated hydroxytoluene (BHT) (10 μL 0.5 M BHT in acetonitrile per 1 mL of saliva) was added to saliva samples [19]. Immediately upon collection, the saliva samples were centrifuged at 12000 ×g (4°C). The supernatant, frozen to −80°C immediately after centrifugation, was maintained for biochemical determination or further study.

2.4. Biochemical Analysis. In samples of unstimulated and stimulated saliva, the concentration of lipid peroxidation products (4-HNE protein adducts and 8-isoprostanes (8-isoP)), protein oxidation products (advanced oxidation protein products (AOPP) and protein carbonyl groups (PC)), and DNA oxidation products (8-hydroxy-D-guanosine (8-OHdG)) was marked. All assays were performed in duplicate samples and standardized to 100 mg total protein.

The concentrations of 4-HNE protein adduct, 8-isoP, and 8-OHdG were determined by the ELISA method (enzyme-linked immunosorbent assay) using ready-made reagent kits (Cell Biolabs, Inc., San Diego, CA, USA; Cayman Chemicals, Ann Arbor, MI, USA; USCN Life Science, Wuhan, China, resp.) in accordance with the manufacturer's instructions. The coloured end-product absorption was measured at 450 nm wavelength using the Mindray Microplate Reader, Shenzhen, China.

AOPP concentration was determined by colorimetric method according to Kalousová et al. [20], measuring the total oxidation capacity of iodide ion. Immediately before the assay, saliva samples were diluted 1 : 5 (v : v) with PBS (pH 7.4). The absorbance was measured at 340 nm wavelength using the Infinite M200 Pro microplate reader, Life Science, Tecan.

PC concentration was determined by colorimetric method according to the Reznick and Packer method [21]. In the presence of 2,4-dinitrophenylhydrazine (2,4-DNPH), PC forms a stable complex connections having a maximum absorption at 355–390 nm wavelength. The absorption of the resulting complexes was measured at 360 nm wavelength using the Infinite M200 Pro microplate reader, Life Science, Tecan. The milimolar absorption coefficient for 2,4-DNPH (22 $mM^{-1}\,cm^{-1}$) was used to evaluate the PC content.

The total protein content was determined colorimetrically by the bicinchoninic method (BCA), using a ready-made reagent kit (Thermo Scientific Pierce BCA Protein Assay Kit, Rockford, IL, USA) and a bovine serum albumin standard (BSA).

2.5. Statistical Methods. Statistical analysis was performed using the Statistica 10.0 (Cracow, Poland). Due to the fact that the obtained results were characterized by normal distribution, the following parametric tests were used: ANOVA post hoc NIR test for comparing multiple groups and the t-test for comparing two groups. The Cohen Kappa (online calculator) was used to establish an intrarater agreement between the two measurements of the assessed dental indicators performed by one researcher at an interval of two days. Pearson correlation coefficients were used to determine the association between the two variables. The results are presented as mean ± SD. The assumed statistical significance was $p < 0.05$.

3. Results

3.1. General Characteristics of the Patients. Bariatric surgery returned the BMI, total cholesterol, LDL, HDL, and triglycerides to the values observed in the control group (Table 1). The values of the stomatological parameters did not differ between the control group and obese patients before and after bariatric surgery (Table 1). Bariatric surgery did not affect dental findings (Table 1).

Mean unstimulated salivary flow in morbid obese individuals was significantly lower compared to the normal weight control and 6 months after bariatric surgery (p =

Table 1: Demographic data, general health, stomatological findings, salivary flow, and protein concentration of the control and morbid obese patients at the baseline and 6 months after bariatric surgery (mean ± standard deviation).

	C ($n = 47$)	O ($n = 47$)	ABS ($n = 47$)
Male/female	14/33	14/33	14/33
Age	42.80 ± 13.10	44.52 ± 10.51	45.12 ± 11.11
BMI (kg/m^2)	20.61 ± 2.31*	47.10 ± 0.81**	22.30 ± 3.4
TC (mg/dL)	147.51 ± 12.8*	210.70 ± 10.05**	160.3 ± 11.10
LDL (mg/dL)	105.70 ± 25.30*	180.40 ± 15.81**	119.30 ± 24.11
HDL (mg/dL)	43.23 ± 2.15*	29.31 ± 1.12**	38.23 ± 2.10
DMFT	20.50 ± 7	18.50 ± 9	18.50 ± 9
SBI	0.84 ± 0.10	0.9 ± 0.10	0.9 ± 0.10
CAL (mm)	2.7 ± 0.3	2.8 ± 0.5	2.8 ± 0.5
PPD (mm)	2.5 ± 1.1	2.7 ± 0.5	2.7 ± 0.5
NS (mL/min)	0.41 ± 0.10*	0.28 ± 0.04**	0.39 ± 0.17
S (mL/min)	1.21 ± 0.10*	0.74 ± 0.20	0.76 ± 0.21***
TP (NS) (mg/mL)	0.84 ± 0.01*	0.64 ± 0.02**	0.80 ± 0.05
TP (S) (mg/mL)	1.04 ± 0.25*	0.75 ± 0.04**	0.97 ± 0.22

C, control; O, morbid obese patients; ABS, patients after the bariatric surgery; BMI, body mass index; TC, total cholesterol; LDL, low density lipoprotein; HDL, high density lipoprotein; DMFT, decayed, missing, filled teeth; SBI, Sulcus Bleeding Index; CAL, clinical attachment loss; PPD, periodontal pocket depth; NS, unstimulated saliva secretion; S, stimulated saliva secretion; TP, total protein. $^*p < 0.05$ C:O, $^{**}p < 0.05$ O:ABS, $^{***}p < 0.05$ ABS:C.

Table 2: Comparison of oxidative damage products in unstimulated and stimulated saliva of patients with morbid obesity before and after bariatric surgery as well as healthy controls.

	C		O		ABS	
	NS	S	NS	S	NS	S
4-HNE (μg/100 mg of protein)	34.62 ± 8.80	49.72 ± 8.34	39.49 ± 6.92	66.17 ± 15.38	34.16 ± 7.94	52.22 ± 9.85
	p < 0.001		**p < 0.001**		**p < 0.001**	
8-isoP (pg/100 mg of protein)	4.65 ± 1.71	3.30 ± 0.90	14.76 ± 5.41	13.05 ± 3.14	12.80 ± 4.74	10.63 ± 2.12
	p < 0.001		**p < 0.01**		**p < 0.001**	
AOPP (μmol/100 mg of protein)	2.07 ± 1.59	1.16 ± 0.69	7.80 ± 11.37	6.45 ± 13.07	2.28 ± 1.06	1.56 ± 0.75
	p < 0.001		$p > 0.05$		**p < 0.001**	
PC (nmol/100 mg of protein)	253.31 ± 103.25	282.76 ± 185.58	1579.51 ± 403.26	1164.21 ± 454.74	314.07 ± 104.20	398.32 ± 100.54
	$p > 0.05$		**p < 0.001**		**p < 0.001**	
8-OHdG (ng/100 mg of protein)	0.43 ± 0.19	0.40 ± 0.16	1.14 ± 0.83	1.77 ± 1.34	0.47 ± 0.22	0.63 ± 0.18
	$p > 0.05$		**p < 0.001**		**p < 0.001**	

C, control; O, morbid obese patients; ABS, patients after the bariatric surgery; NS, unstimulated whole saliva; S, stimulated whole saliva; 4-HNE protein adducts; 4-hydroxynonenal protein adducts; 8-isoP, 8-isoprostanes; AOPP, advanced oxidation protein products; PC, protein carbonyl groups; 8-OHdG, 8-hydroxy-D-guanosine.

0.001 and $p = 0.01$, resp.). Mean value of stimulated salivary secretion in morbid obese at the baseline and 6 months after surgery was significantly lower compared to control patients ($p = 0.002$ and $p = 0.003$, resp.) (Table 1). However, the mean protein concentration in the unstimulated and stimulated saliva of patients with morbid obesity prior to surgery was significantly lower in comparison with control patients ($p = 0.03$ and $p = 0.01$, resp.) and 6 months after bariatric surgery ($p = 0.03$ and $p = 0.04$, resp.) (Table 1).

3.2. Lipid Oxidation Products. The mean 4-HNE-protein adduct concentration was significantly higher in unstimulated and stimulated saliva of patients with morbid obesity before the surgery compared to healthy controls ($p = 0.003$ and $p = 0.001$, resp.) and patients with morbid obesity 6 months after bariatric surgery ($p = 0.0001$ and $p = 0.00001$, resp.) (Figure 1(a)).

The mean 4-HNE-protein adduct concentration was significantly higher in stimulated compared to the unstimulated saliva in the control ($p = 0.00001$) as well as in morbid obese at the baseline ($p = 0.000001$) and 6 months after surgery ($p = 0.000001$) (Table 2).

The 8-isoP concentration in patients with morbid obesity prior to the surgery was significantly higher in both unstimulated and stimulated saliva compared to the controls ($p = 0.0000001$ and $p = 0.001$, resp.) and results obtained 6 months after bariatric surgery ($p = 0.03$ and $p = 0.000001$, resp.). 8-isoP concentrations in unstimulated and stimulated saliva in patients with obesity after bariatric surgery were

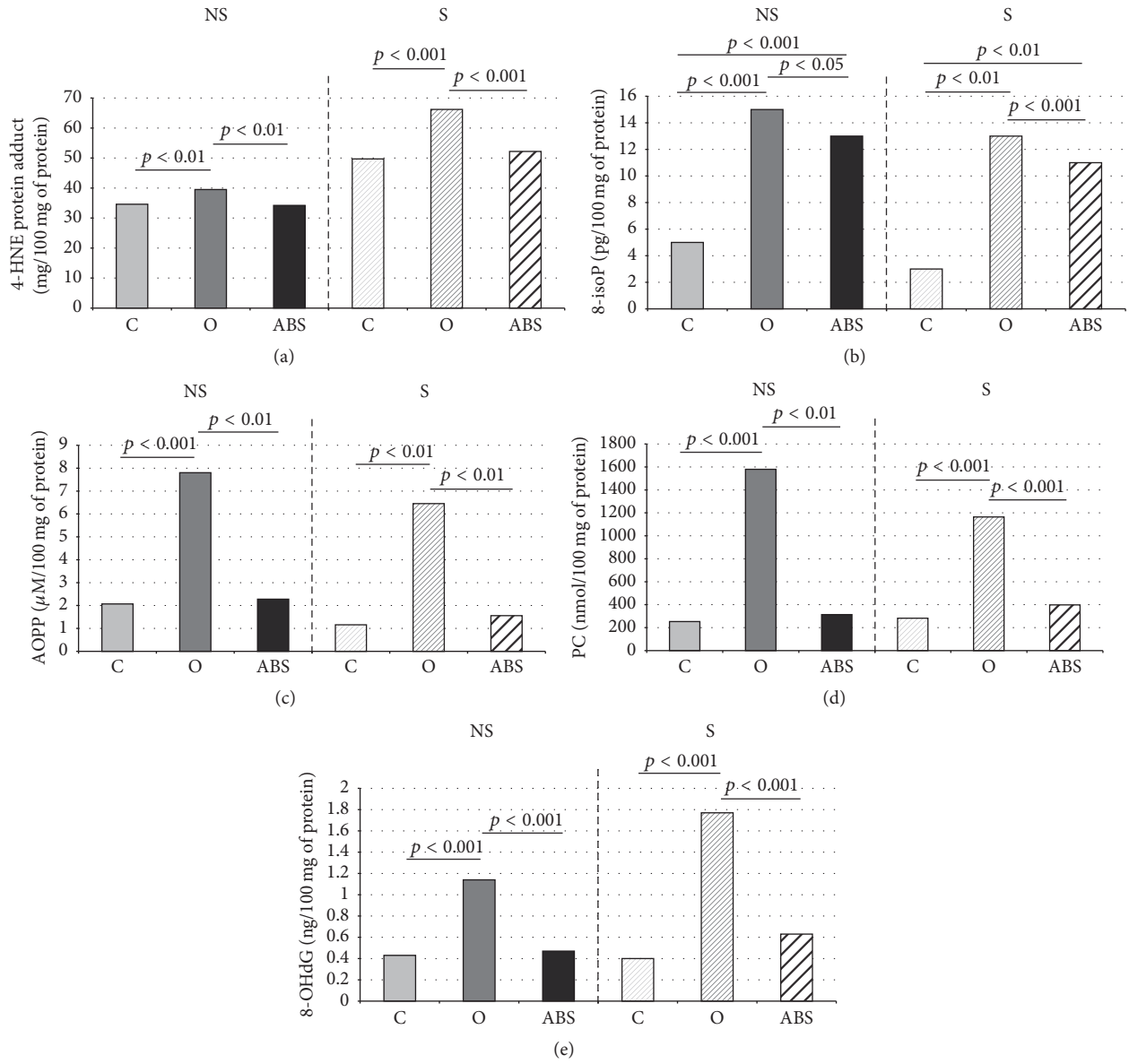

Figure 1: *Oxidative damage to lipids (a, b), proteins (c, d), and DNA (e) in unstimulated and stimulated saliva of patients with morbid obesity at the baseline and 6 months after bariatric surgery as well as the healthy controls.* C, control; O, morbid obese patients; ABS, patients after the bariatric surgery; NS, unstimulated whole saliva; S, stimulated whole saliva; 4-HNE protein adducts, 4-hydroxynonenal protein adducts; 8-isoP, 8-isoprostanes; AOPP, advanced oxidation protein products; PC, protein carbonyl groups, 8-OHdG, 8-hydroxyguanosine.

significantly higher than in the control group ($p = 0.0000001$, $p = 0.001$, resp.) (Figure 1(b)).

The 8-isoP concentration was significantly higher in unstimulated compared to the stimulated saliva in the control ($p = 0.000001$) as well as in morbid obese at the baseline ($p = 0.001$) and 6 months after surgery ($p = 0.0001$) (Table 2).

3.3. Protein Oxidation Products. In the group of patients with morbid obesity prior to surgical treatment, the mean AOPP concentrations in both unstimulated and stimulated saliva were considerably higher than in the control patients ($p = 0.00005$ and $p = 0.0009$, resp.) and compared to the results obtained 6 months after bariatric surgery ($p = 0.00009$ and $p = 0.002$, resp.) (Figure 1(c)).

The mean AOPP concentration was significantly higher in the unstimulated saliva compared to the stimulated saliva of the control ($p = 0.0006$) and morbid obese patients 6 months after surgery ($p = 0.0002$) (Table 2).

In both unstimulated and stimulated saliva of morbidly obese patients, the mean PC concentration was significantly higher compared to the control group ($p = 0.0000001$ and $p = 0.00000001$, resp.) and results obtained 6 months after bariatric surgery ($p = 0.001$ and $p = 0.0000001$, resp.) (Figure 1(d)).

The mean PC concentration was significantly higher in the unstimulated saliva compared to the stimulated saliva of the morbid obese patients at the baseline ($p = 0.00001$) and it was significantly higher in the stimulated saliva compared to the unstimulated saliva of the morbid obese patients 6 month after surgery ($p = 0.0001$) (Table 2).

3.4. DNA Oxidation Products. The mean value of 8-OHdG concentration in unstimulated and stimulated saliva of patients with morbid obesity at the beginning of the experiment was considerably higher than in the control patients ($p = 0.0000001$ and $p = 0.00000001$, resp.) and compared to the results obtained 6 months after bariatric surgery ($p = 0.0000001$ and $p = 0.0000001$, resp.) (Figure 1(e)).

The mean 8-OHdG concentration was significantly higher in stimulated saliva compared to unstimulated saliva of the morbid obese patients at a baseline ($p = 0.0002$) and six months after bariatric surgery ($p = 0.0001$) (Table 2).

3.5. Correlations. There was a negative correlation between 4-HNE protein adduct and unstimulated and stimulated salivary flow of morbid obese patients ($p = 0.02$, $r = -0.67$ and $p = 0.03$, $r = -0{,}54$, resp.). There was a negative correlation between 8-isoP and stimulated salivary flow of morbid obese patients six months after bariatric surgery ($p = 0.01$, $r = -0{,}69$).

There was no correlation between DMFT, SBI, PPD, CAL, blood parameters and 4-HNE protein adduct, 8-isoP, AOPP, PC. and 8-OHdG concentrations in the unstimulated and stimulated saliva of morbid obese patients before and after bariatric surgery.

4. Discussion

This is the first study displaying that morbid obesity increased while its treatment with bariatric surgery generally decreases oxidative damage to lipids, proteins, and DNA both in unstimulated and in stimulated human saliva.

Saliva is a secretion from salivary glands that forms the environment of the oral cavity and is responsible for preliminary digestion of food, cleansing both mucous membrane and teeth as well as maintaining proper pH in the oral cavity. Moreover, saliva participates in both specific and nonspecific immune defense as well as showing very effective antioxidative systems that protect oral cavity environment from harmful effect of reactive oxidative species and reactive nitrogen species (RNS). The coexistence of ROS overproduction and impairment of antioxidative systems is called oxidative stress. It is believed that OS leads to damage to the components of salivary gland cells and enhances chronic systemic and local inflammatory condition (through the increase in the production of proinflammatory cytokines). Therefore, oxidative stress results in the initiation and progression of numerous pathological changes within the oral cavity which most commonly include caries, gingivitis, periodontitis, candidiasis, and dysfunction of salivary glands. The latter can be observed in form of changes in both quality and quantity of secreted saliva [9, 11, 22].

It was shown that, in more than 50% of persons with morbid obesity, the presence of oxidative stress-related oral cavity diseases can be observed [23, 24]. Interestingly, no studies confirming OS presence in the oral cavity in these patients can be found. According to the research by Knaś et al. [10], impairment of antioxidative systems in the saliva of morbid obesity patients as well as a normalizing effect of bariatric surgery on these conditions can be observed. Although the authors evaluated also MDA concentration, the results of their research fail to allow evaluating the scope and predicting the effects of oxidative stress in the oral cavity. Not only did the applied method of evaluating MDA concentration show a minor diagnostic value, but it also should be emphasized that the test of single redox biomarker in isolation has limited value in the diagnosis, staging, and prognosis of the oxidative stress-related human diseases. Numerous approaches of the measurement of oxidatively changed cellular components have been described so far. In this experiment we used the most common assessment to evaluate oxidative damage: oxidized lipids (8-isoP and 4-HNE protein adduct), proteins (AOPP and PC), and DNA (8-OHdG).

To begin with, it should be emphasized that we found no relationship between the concentration of the examined parameters of OS and the local inflammatory process and general parameters. According to the results, the changes observed in the unstimulated and stimulated saliva may be caused by the dysfunction of the salivary glands, regardless of general and local inflammatory processes.

It is well accepted that human parotid gland produces saliva mainly after stimulation while submandibular gland provides unstimulated saliva [25, 26]. Therefore, it is believed that disorders of the content or secretion of stimulated saliva reflect abnormalities in the function of parotid salivary gland. Analogically, disorders related to secretion of unstimulated saliva are related to the dysfunction of submandibular gland.

Our study showed that both unstimulated and stimulated saliva of morbidly obese patients were characterized by an increased concentration of 4-HNE protein adduct, 8-isoP, AOPP, PC, and 8-OHdG compared to the obtained data pertaining to unstimulated and stimulated saliva of normal weight control. A greater percentage increase in the concentration of the majority of the analyzed oxidative products in the stimulated (4-HNE protein adduct ↑33%, 8-isoP ↑295%, AOPP ↑456%, and 8-OHdG ↑342%) versus unstimulated saliva (4-HNE protein adduct ↑14%, 8-isoP ↑217%, AOPP ↑276%, and 8-OHdG ↑165%) may prove increased oxidative damage to parotid gland compared to submandibular gland in morbidly obese patients. This increase in oxidative damage in stimulated saliva may be related to a considerable insufficiency of antioxidative systems of parotid versus submandibular glands in morbidly obese patients described by Knaś et al. [10]. On the other hand, more intense oxidative damage observed in stimulated saliva versus unstimulated saliva of morbidly obese patients may be related to the observed by other researchers enhanced storage of adipocytes in the parotid parenchyma which is almost absent in submandibular glands [27]. Adipocytes by monocyte chemoattractant protein-1 (MCP-1) activate the

influx of monocytes and their conversion into macrophages. Macrophages release cytokines TNF-α, IL-6, and IL-1β and thus inflammation develops. This leads to the activation of NADPH oxidase in the phagocytic cells and the enhanced formation of ROS, which in a seriously defected antioxidant barrier leads to the oxidative damage and dysfunction of the parotid gland.

Six months following the procedure we observed a significant decrease in BMI and the concentrations of total cholesterol, fractions of HDL, LDL, and triglycerides compared to the values observed in the control group. Complete therapeutic success was not accompanied by prevention from salivary oxidative damage and failed to restore redox balance in stimulated and unstimulated saliva to the values observed in control group. Six months following bariatric surgery we observed a maintaining increased 8-isoP concentration in both stimulated and unstimulated saliva compared to the control group. However, selectively increased 8-isoP concentrations prove that six months after bariatric surgery salivary glands are subject to lower intensity of the oxidative stress in comparison to preoperative status. It has been shown that the earliest symptom of the oxidative stress is lipid peroxidation since the lipids of cellular membrane are the first ones to be exposed to the harmful effects of free radicals. Only in case of an increase in the concentration of ROS, the concentration of lipid peroxidation products increases and proteins, and later DNA, undergo oxidation [28].

It should be stressed that our study showed a high degree positive effect of the loss of body mass in decreasing oxidative stress in unstimulated and stimulated saliva. The bariatric surgery related body loss protective action was noted in significantly lower concentrations of 4-HNE protein adduct, 8-isoP, AOPP, PC, and 8-OHdG in stimulated and nonstimulated saliva 6 months after the procedure compared to preoperative status. Furthermore, the concentrations of 4-HNE protein adduct, AOPP, PC, and 8-OHdG of both stimulated and unstimulated saliva showed the same values as the ones observed in the control group.

We observed a negative correlation between 4-HNE protein adduct and unstimulated and stimulated salivary flow of morbid obese patients. It is an interesting correlation considering the fact that 4-HNE protein adduct activates the expression of proinflammatory cytokines (TGF-β1 and IL-1) and metalloproteinases through ROS-mediated stimulation Akt-kappaB signaling pathway. TGF-β1 inhibits DNA synthesis and expression of Na^+/K^+ ATPase, both being essential for epithelial proliferation [29]. As a result, the loss of acinar cells and the exchange of parenchyma function with fibrous tissue occur. Inflammatory condition and reconstruction of extracellular matrix resulting from the effect of increased activity of metalloproteinases are a known factor that leads to a decreased response of residual acinar cells to Ach (acetylcholine), NA (noradrenaline), or receptor reconstruction. All these processes may lead to the reduced secretion of both stimulated and nonstimulated saliva and impaired mechanism involved in the synthesis/secretion of protein, the phenomena we observed in the saliva of morbidly obesity patients. Similarly, a negative correlation between 8-isoP concentration and stimulated secretion in patients 6 months after surgical procedure may be responsible for the fact that the secretion of stimulated saliva was significantly higher compared to preoperative values yet was also significantly lower compared to control group. Isoprostanes are a potential factor that impairs the integrity and liquidity of the mucous membrane as well as the function of membranous receptors [30].

There are certain limitations to our study. There are a number of different markers of ROS-related modification, yet our analysis included only those used most commonly. Using other markers of OS may partially or completely alter our observations and conclusions. Changes in oxidation marker concentrations may also result from increased postoperative intake of fruit and vegetables, as recommended by the surgeon, that are rich in polyphenols. The latter are known to stick to oral mucosa and increase salivary total antioxidative capability and thus prevent biomolecule oxidative modifications [13]. Undoubtedly, an advantage of this work is a relatively high number of patients carefully selected in terms of carbohydrate-lipid metabolism and accompanying diseases and the fact that this is the first study evaluating the scope of oxidative damage in the saliva of patients before and after bariatric surgery.

5. Conclusions

(1) Oxidative modification of cellular components was greater in the stimulated saliva versus unstimulated saliva of morbidly obese patients.

(2) Six months after bariatric surgery a decreased oxidative modification of biomolecules in unstimulated and stimulated saliva could be observed, yet bariatric surgery related weight loss was not effective in restoring redox balance in the oral cavity.

(3) The presence of oxidative stress within salivary glands in morbidly obese patients may be indicative of the fact that antioxidant supplementation could decrease/remove hypofunction of salivary glands in this group of patients.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

Katarzyna Fejfer and Piotr Buczko had equal contribution to the study.

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