Salivary Protein Profiles in Humans:
An Investigation of Salivary Proteins in
Response to Olfactory Stimuli

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Abstract

Previous studies have shown that there is indeed a sub-mandibular salivary reflex in response to olfactory stimuli as well as evidence for control of protein release in saliva by the human body. This study set out to determine if different olfactory stimuli will cause significant differences in the presence of salivary alpha amylase in human saliva samples. In a within-subject repeated measures design, 35 participants gave four saliva samples after smelling coffee, honey, jalapenos, and pickles. Total protein in each sample was found using a Bradford assay as well as quantification of salivary alpha amylase using immunoblotting and the software imageJ. There was not a statistically significant difference shown between the area/µg protein of each of the four different olfactory stimuli as determined by a repeated measures ANOVA with a Greenhouse-Geisser correction (F (2.589, 62.145) = 2.434, p = 0.082). Although, salivary alpha amylase presence was shown to be highest in the sour stimuli (pickles) and lowest in the bitter stimuli (coffee). Alternate experimental methods are discussed for future studies to more accurately determine if the relationship between olfactory stimuli and the presence of salivary alpha amylase exists.

Introduction

Saliva is commonly overlooked as playing a huge role in the digestive system of the human body. It is responsible for protecting the teeth, protecting against microorganisms, and interactions with food and stimulating their breakdown in the first step of the digestive cycle (Neyraud, 2014). Around 90% of the saliva produced in the human mouth comes from just three paired major salivary glands: parotid, submandibular, and sublingual (Pedersen, et al., 2002). It has been highly studied that taste is a main stimulant for formation of saliva (Pedersen, et al., 2002). The taste impulses are carried to the brain by parasympathetic nerves which then trigger the salivation response (Pedersen, et al., 2002). Taste has been traditionally divided into five main categories: sweet, salty, sour, bitter, and umami (Neyraud, 2014). Studies have revealed that the highest salivation response is seen when stimulated with a sour taste, followed by salt, then sweet, and bitter; umami was not tested in this study (Pedersen, et al., 2002).

Although there has been much research done on the effects of taste on salivation responses, the scope of olfactory, or smell, salivation responses and their effects on the content of the saliva has not been studied to the same degree. The University of Winnipeg concluded, by exposing subjects to food odors and non-food odors, that there is a human salivary reflex in response to food odors only (Legoff and Spigelman, 1998). Therefore, there is an olfactory-salivary reflex. Another study by Lee and Linden in 1992 took this information a step further to find out where exactly this reflex comes from in the oral cavity. Saliva was collected from the submandibular gland after exposure to six different odors of different types of food as well as

exposure to distilled water. A significant increase in saliva was seen in response to all stimuli except the distilled water (Lee and Linden, 1992). From this information, Lee and Linden concluded that there is an olfactory-submandibular salivary reflex in humans. In addition, studies on the effect of stress of salivary protein alpha-amylase concentration have shown that protein levels in saliva are regulated by the human body (Nater et al., 2005). From this research, one can conclude that there is indeed a salivary response to olfactory stimuli and the human body does indeed regulate protein levels in saliva. The goals of this study are to identify and quantify salivary alpha amylase in human saliva samples, as well as understand the relationship between olfactory stimuli and the presence of salivary alpha amylase in human saliva. Alpha amylase was chosen as the protein of interest because alpha-amylase breaks down carbohydrates and thus should be very abundant enzyme in saliva (Behringer, et al., 2013).

I hypothesize that upon exposure to different olfactory stimuli such as spicy, sweet, sour, and bitter there will be different amounts of salivary alpha amylase present in each salivary sample. In addition, I hypothesize that the sour stimulus will yield the highest amount of alpha amylase and the bitter stimulus will yield the lowest. Although much research has been done on the presence of a salivary response to olfactory stimuli in humans, there has not been studies performed to determine if these salivary responses differ in their protein concentrations upon the stimulus of varying scents.

Materials and Methods

Saliva Collection

Prior to the collection day, participants (N = 35) were asked to confirm they had no allergies to the food stimuli in which they would be subjected to. The food stimuli were coffee

grounds, honey, jalapenos, and pickles. Each one of the stimuli were selected because of their typical taste that differentiates them into taste categories: coffee grounds – bitter, honey – sweet, jalapenos – spicy/hot, pickles – sour. Participants were also asked to not consume food one hour prior to the scheduled collection time. Four collection tubes were labeled with participant number as well as each different stimulus. An oral cavity wash was performed with distilled water before exposure. Each participant was exposed to their own cup of coffee grounds for a 10-minute time interval. At the end of the time interval, participants offered a saliva sample of at least 1.5ml into the corresponding collection tube. Another oral cavity wash with distilled water was repeated before exposure to honey. This same process was repeated f

or the jalapeno and pickle stimuli. Samples were then stored at -20°C until analyzed. Each person offered all samples in one sitting and all samples were given in one of two rooms in Voigt Science Hall on the campus of McKendree University.

Bradford Assay

A Bradford Assay was used to quantify the amount of total protein in each of the given samples. This technique is simpler, quicker, and more sensitive than the Lowry method of protein quantification (Kruger, 2002). The Bradford assay, in comparison to the Lowry method, is also subject to less interference by common reagents and nonprotein components of biological samples (Kruger, 2002). The assay is reliant on the binding of the dye Coomassie Blue G250 to protein in the samples.

In this analysis, a standard curve was created using 1mg/ml BSA concentration. A dilution scheme of 11 standards were made ranging from 0.0mg/ml to 1.0mg/ml by adding the appropriate amount of BSA and distilled water to equal 100 µl total volume. This was added to

3ml of Bradford Reagent. 100 µl of each saliva sample was also combined with 3ml of Bradford Reagent. The absorbencies of each of the 11 standards and all samples were then measured and recorded using a spectrophotometer set at 595nm. The slope from the generated standard curve was then used to determine total protein in each sample.

1-D SDS Polyacrylamide Gel Electrophoresis

10 % polyacrylamide gels were used during the 1-D gel electrophoresis. The Smart Dual Color Pre-Stained Protein Standard from GenScript was used as a standard in each gel. This standard was selected because the pre-stained proteins ranged in size from 14kDa to 100 kDa. The protein of interest, alpha amylase, falls in the middle of this range at approximately 55 kDa (Iontcheva et. al, 1997). Each of the 7 proteins present in this standard are at concentrations of 0.4 µg/µl. Using the total protein determined from the Bradford assay, the maximum amount of protein per individual was loaded into each well. This means within any one individual, each well containing samples from the four stimuli would have the same amount of total protein, but there would be differences in total protein loaded between individuals. 10 µl of the standard was loaded into the corresponding well. Two participants samples (8 total) samples were loaded onto each gel. The gels were run at 200V for 30 minutes per the *Current Protocols in Cellular Biology* handbook (2007).

Immunoblotting and Immunodetection

After the electrophoresis of the gels were complete, a Western Blot was performed on each of the gels to determine the presence and quantity of the protein of interest, salivary alpha amylase. The proteins on the gels were electrophoretically transferred to nitrocellulose membranes by creating an immune-blot sandwich and running at 20V for 2.5 hours (Gallagher

et. al, 2011). After the transfer of the proteins to the nitrocellulose membrane, each membrane was submerged in 25ml of blocking buffer (1X Tris Buffered Saline with Tween and 5% w/v non-fat dry milk) for 30 minutes on a rocking platform. . The blocking solution was then discarded, and the membranes were subjected to 10ml of a 1/1000 dilution of Anti-Alpha Amylase (polyclonal Rabbit) primary antibody for 15 minutes on a rocking platform. The membranes were then washed with 50ml of 1X TBST. An incubation with 10ml of a 1/200 dilution of Anti-Rabbit secondary antibody was subjected to the membranes for 10 minutes on a rocking platform. The blots were then washed again with 50ml of 1X TBST. A final incubation with HRP (Horseradish peroxidase) substrate produced color development that was recorded by picture.

Quantification of Protein

Digital images from the immunodetection were further analyzed to quantify the amount of salivary alpha amylase in each sample. The software "imageJ" was used to quantify this by using densitometry to measure the intensity of the bands of interest. This software used the area underneath a curve representing the density of the bands as a means for quantification. After each band from all samples was analyzed, the data was normalized by calculating the area per µg of protein loaded. It should be noted that only 32 of the participants samples were able to be analyzed to due to budgetary constraints.

Statistical Analysis

After the collection of the data, outliers were removed. The data sets from the Bradford Assay and the Immunoblot detection of salivary alpha amylase were then analyzed using a one-way repeated measures ANOVA, respectively, by using SPSS. The repeated measures analysis of variance (ANOVA) was used to determine whether there are any statistically significant

differences between the means of two or more related groups using a critical level of 0.05 (i.e. amount of salivary alpha amylase between the four different stimuli). The Mauchly's test of sphericity was performed to test whether the variances between each group were equal. A test of normality was also performed to test if the data followed a normal distribution of a bell-shaped curve.

Results

Bradford Assay

The data collected from the Bradford Assay (total protein of each sample) was analyzed using a One-Way repeated measures ANOVA to test for differences in total protein concentration between the four different stimuli (Table 1). There was not a statistically significant difference between the total protein of the four different stimuli with a Greenhouse-Geisser correction (F (2.258, 76.789) = 0.508, p = 0.626).

Table 1

Results from the one-way repeated measures ANOVA for the Bradford Assay. Noted values are highlighted.

Tests of Within-Subjects Effects

Measure: Protein_ugul

Source		Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Stimulus	Sphericity Assumed	.073	3	.024	.508	.678	.015
	Greenhouse-Geisser	.073	2.258	.032	.508	.626	.015
	Huynh-Feldt	.073	2.427	.030	.508	.639	.015
	Lower-bound	.073	1.000	.073	.508	.481	.015
Error(Stimulus)	Sphericity Assumed	4.902	102	.048			
	Greenhouse-Geisser	4.902	76.789	.064			
	Huynh-Feldt	4.902	82.529	.059			
	Lower-bound	4.902	34.000	.144			

Immunoblot and Detection

The blots resulting from the 1-D electrophoresis and sequential Western Blot were analyzed using the imageJ software. Figure 1 shows one of the blots that were analyzed to determine the density of the bands representing salivary alpha amylase.

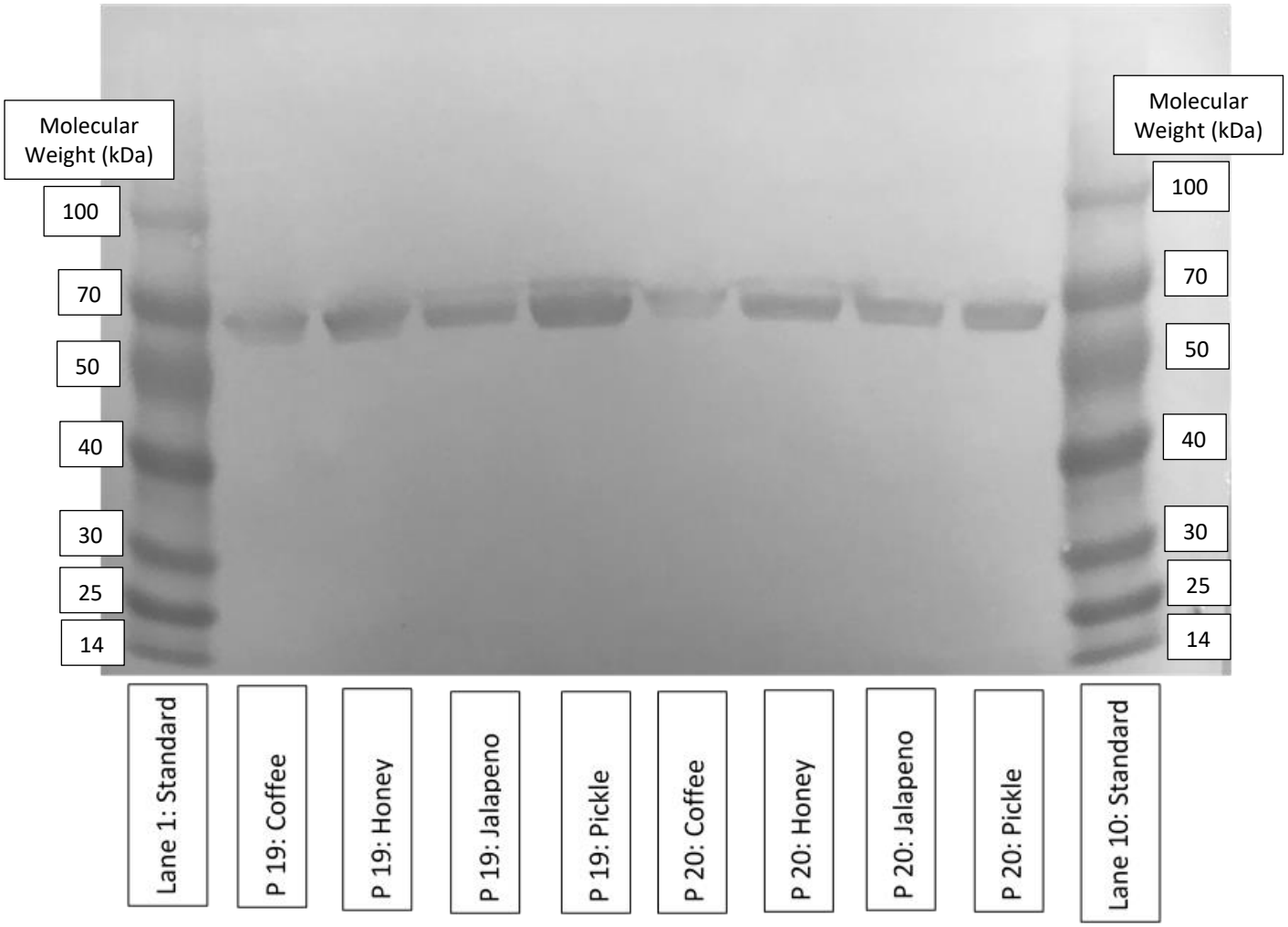

Figure 1: Immunoblot of two participants (19 & 20) along with two identical standards in the first and last lanes. The eight middle lanes represent the detected amount of salivary alpha amylase in each sample. The molecular weights of the standard lanes are labeled.

The means of the area/µg protein between the four groups of different olfactory stimuli were calculated then analyzed to determine if there were any differences. There was not a statistically significant difference between the area/µg protein of each of the four different olfactory stimuli as determined by a repeated measures ANOVA with a Greenhouse-Geisser correction (F (2.589, 62.145) = 2.434, p = 0.082). Samples from the coffee, honey, jalapeno, and pickle stimuli had means of 2690.1 (n = 25), 2938.3 (n = 26), 3592.3 (n = 29), and 4023.0 (n = 27) respectively (Tables 2 & 3).

Table 2

Descriptive statistics for each of the four groups of different olfactory stimuli. Noted values are highlighted.

Descriptives

Area_ugProtein

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Coffee	25	2690.0864	1482.73020	296.54604	2078.0455	3302.1274	465.12	5287.56
Honey	26	2928.3341	1765.63245	346.26901	2215.1797	3641.4884	575.30	6501.36
Jalapeno	29	3592.2554	2625.92702	487.62241	2593.4062	4591.1046	725.75	9105.54
Pickle	27	4022.9998	2422.50769	466.21182	3064.6877	4981.3119	1172.22	9247.53
Total	107	3328.8341	2184.31198	211.16541	2910.1781	3747.4901	465.12	9247.53

Table 3

Results from the one- way repeated measures ANOVA for the Immunoblot detection of salivary alpha amylase. Noted values are highlighted.

Tests of Within-Subjects Effects

Measure: Area_Protein

Source		Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Stimuli	Sphericity Assumed	28004456.93	3	9334818.977	2.434	.072	.092
	Greenhouse-Geisser	28004456.93	2.589	10815159.51	2.434	.082	.092
	Huynh-Feldt	28004456.93	2.930	9557664.028	2.434	.073	.092
	Lower-bound	28004456.93	1.000	28004456.93	2.434	.132	.092
Error(Stimuli)	Sphericity Assumed	276145888.4	72	3835359.561			
	Greenhouse-Geisser	276145888.4	62.145	4443581.129			
	Huynh-Feldt	276145888.4	70.321	3926919.012			
	Lower-bound	276145888.4	24.000	11506078.68			

Each stimulus was plotted vs. the mean area/µg of protein for each. Standard deviation bars were included to show variance within groups (Figure 2).

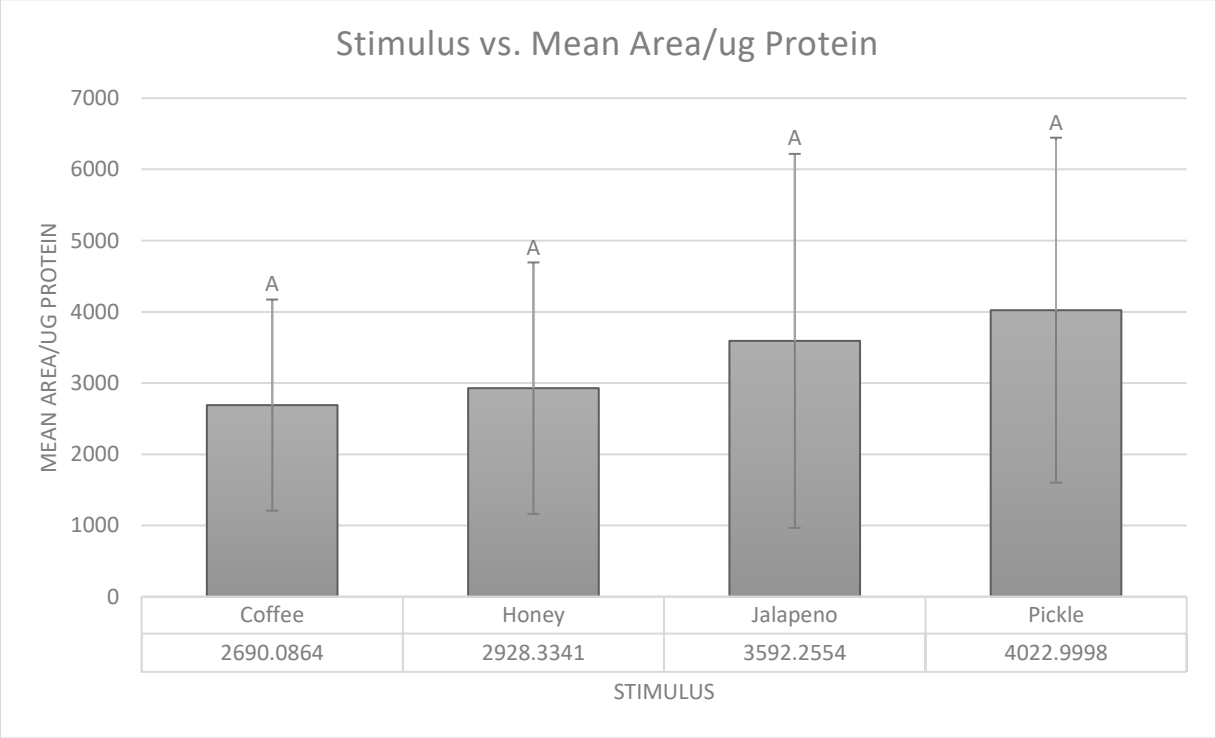

Figure 2: Graph showing the stimuli vs. the means of the area/µg protein. Standard error bars show high variation between groups. Stimuli labeled with the same letter are noted to not be significantly different from others with the same letter. Mean values are reported underneath respective stimulus.

The data collected from the Immunoblot detection was also subject to a test for normality, the normal distribution of data points, as well as a test for homogeneity of variance, whether the variances between each group were equal (Tables 4 & 5).

Table 4

Outputs from testing for normal distribution of data points. Significance values form the Shapiro-Wilk test for normality are highlighted for each stimulus.

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Stimulus	Statistic	df	Sig.	Statistic	df	Sig.
Area_ugProtein	Coffee	.104	25	.200*	.952	25	.282
	Honey	.140	26	.200*	.926	26	.062
	Jalapeno	.154	29	.075	.888	29	.005
	Pickle	.181	27	.023	.895	27	.010

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Table 5

Mauchly's test for sphericity or normal variance. The significance value is highlighted.

Mauchly's Test of Sphericity[a]

Measure: Area_Protein

					Epsilon[b]		
Within Subjects Effect	Mauchly's W	Approx. Chi-Square	df	Sig.	Greenhouse-Geisser	Huynh-Feldt	Lower-bound
Stimuli	.769	5.973	5	.309	.863	.977	.333

Tests the null hypothesis that the error covariance matrix of the orthonormalized transformed dependent variables is proportional to an identity matrix.

Discussion

The aim of this study was to investigate the production of salivary alpha amylase in response to different olfactory stimuli. The results were unable to show a difference in the means of each group by statistical analysis using a one-way repeated measures ANOVA (p = 0.082). There was no relationship seen between stimulus and the amount of total protein given in a sample (p = 0.626) as given by results of analysis using a one-way repeated measures ANOVA on the data collected from the Bradford Assay protocol in the research study. This would lead one to conclude that different types of olfactory stimuli (bitter, sweet, spicy, sour) do not have an effect on the amount of salivary alpha amylase produced in humans.

These results contrast the main original hypothesis that was based upon evidence of an olfactory-submandibular salivary reflex in humans (Lee and Linden, 1992). The results from the following test should be noted. The test for sphericity of the data, or the normal variance of the differences across groups was not statistically significant, p = 0.309. This allows for the acceptance that the data had normal variance. In addition, the Shapiro-Wilk test for normality showed that only the data collected from the coffee stimulus and honey stimulus followed a normal distribution (p = 0.282, p = 0.062 respectively). The significance values for jalapenos and pickles (p = 0.005, p = 0.010 respectively) rejects the null hypothesis that the data is normally distributed. Although, lack of normal distribution does not immediately lead to invalid results. An ANOVA requires only *approximately* normal data because it is robust to violations of normality. This output does not take the same weight in interpretation as other statistical tests.

Post hoc tests using the Bonferroni correction revealed that there was the most difference between the means of data collected from coffee stimuli and pickle stimuli (2690.1 ± 1482.7 area/µg protein vs 4037.1 ± 2483.4 area/µg protein, respectively), but was not

statistically significant (p = 0.086). Although not significant, this data combined with the means of each stimulus group does correlate with an additional original hypothesis stating that samples from sour stimuli (pickle) will have the most salivary alpha amylase while samples from bitter stimuli (coffee) will have the least salivary alpha amylase. This hypothesis was based on previous studies about salivary responses to olfactory stimuli (Pedersen, et al., 2002).

There are a few design elements of the study that could be potentially affecting the results of this study and could be changed in further research on this topic. Each person gave samples in all four of the stimuli data sets. This violates independence of observations, which means that there is actually a relationship between each group because each participant was in all four groups. This requires the use of the non-parametric one-way repeated measures ANOVA. If this experiment would be repeated, having more participants and each participant only smelling one olfactory stimuli and only giving one saliva sample could be suggested. In addition, no saliva samples were collected before smelling the olfactory stimuli, thus there is no control to see if total protein content in the saliva changed in response to the olfactory stimuli. In future experiments, a control should be added to confirm that the salivary response due to olfactory stimuli is indeed present as indicated by past research. Further studies should investigate if these changes in experimental design have an effect on the significance of olfactory stimuli on salivary alpha amylase. This research could help in providing possible causes of many disorders and diseases relating to saliva. Saliva is a key component in taste sensitivity (Matsuo, 2000). Taste dysfunction disorder is a taste anomaly that deals with sensory functions that affect health not only through effects on intake of food, but also because of the

loss of morale accompanying the loss of an important source of pleasure – taste (Bartoshuk, 1978). Salivary proteins also play a huge role in protecting the oral cavity, so with a better understanding of salivary proteins such as when they should be present and in what amount could be helpful in treating those who have disorders that have diminished their oral cavity defenses (van Nieuw Amerongen, et al., 2004).

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References

Bartoshuk, Linda M. (1978). The psychophysics of taste. *American Journal of Clinical Nutrition*, 31(6), 1068-77.

Behringer, V., Claudia Borchers, Tobias Deschner, Erich Mostl, Dieter Selzer, and Gottfried Hohmann. (2013). Measurements of salivary alpha amylase and salivary cortisol in hominoid primates reveal within-species consistency and between-species differences. *PLOS ONE*, 8(4), 1-8.

Contreras-Aguilar, M., Fernando Tecles, Silva-Martinex-Subiela, Damian Escribano, Luis Jesus Bernal, and Jose Ceron. (2017). Detection and measurement of alpha-amylase in canine saliva and changes after an experimentally induced sympathetic activation. *BMC Veterinary Research*, 13(226), 1-6.

Gallagher, S.R., (2007). One-dimensional SDS gel electrophoresis of proteins. *Current Protocols in Cell Biology*, 6.1, 1-37.

Gallagher, S.R., Winston, S.E., Fuller, S.A., and Hurrell, J. (2011). Immunoblotting and immunodetection. *Current Protocols in Cell Biology*, 6.2, 1-28.

Hu, S., Xie Yongming, Prasanna Ramachandran, Rachel Ogorzalek Loo, Yang Li, Joseph Loo, and David Wong. (2005). Large-scale identification of proteins in human salivary proteome by liquid chromatography/mass spectrometry and two-dimensional gel electrophoresis-mass spectrometry. *Proteomics*, 5, 1-15.

Iontcheva, I., Oppenheim, F.G., and Troxler, R.F. (1997). Human salivary mucin MG1 selectivity forms heterotypic complexes with amylase, proline-rich proteins, statherins, and histatins. *Journal of Dental Research*, 76 (3), 134-743.

Kruger, N. (2012). The Bradford Method for Protein Quantification. In Walker, J. (Ed.), *The Protein Protocols Handbook* (pp. 15-22). Totowa, NJ: Humana Press.

Lee, V. M., Linden, R. W. A. (1992). An Olfactory-Submandibular salivary reflex in humans. *Experimental Physiology*, 77, 221-24.

Legoff, Daniel B., Spigelman, M. N. (1998). Salivary response to olfactory food stimuli as a function of dietary restraint and body weight. *Appetite*, 8, 29-35.

Matsuo, R. 2000. Role of saliva in the maintenance of taste sensitivity. *Critical Reviews in Oral Biological Medicine*, 11(2), 216-29.

Nater, U.M., Rohleder, N., Gaab, J., Berger, S., Jud, A., Kirschbaum, C., and Ehlert, U. (2005). Human salivary alpha-amylase reactivity in a psychosocial stress paradigm. *International Journal of Psychophysiology*, 55, 333-342.

Neyruad, E. (2014). Role of saliva in oral food perception. *Monographs in Oral Science*, 24, 61-70.

Pedersen, A. M., A. Bardow, S.B. Jensen, and B. Nauntofte. (2002). Saliva and gastrointestinal functions of taste, mastication, swallowing, and digesting. *Oral Diseases*, 8, 117-29.

van Nieuw Amerongen A., J.G.M. Bolscher, and E.C.I. Veerman. (2004). Salivary Proteins: protective and diagnostic value in cariology? *Caries Research*, 38, 247-53.