INTERNATIONAL JOURNAL OF ADVANCED BIOLOGICAL RESEARCH
© 2004 - 2012 Society for Science and Nature (SFSN). All rights reserved
www.scienceandnature.org

THE FREE AMINO ACIDS (PROLINE AND ARGININE) CONCENTRATION FROM HUMAN WHOLE UNSTIMULATED SALIVA PATIENTS

Essam F. A. Al-Jumaily & Muntaha A. Al-Safar
Biotechnology Dept., Genetic Engineering and Biotechnology, Institute for postgraduate studies ,Baghdad University, Baghdad, Iraq,
Institute of Technical Medicine , Baghdad, Iraq

ABSTRACT
The study was conducted to determine the concentration of two free amino acids (Proline and Arginine) in human whole unstimulated saliva of caries susceptible and caries resistant individuals using reversed phase HPLC, derivatizing the free amino acids using phenylisothiocyanate, and then finding out if there is an association between the concentration of theses amino acids in saliva and the prevalence of dental caries using the DMFT(Decayed, Missing, Filled, Teeth) caries index. The results were showed that there were no significant differences between the concentration of the two amino acids in the saliva of the patient group and the control group. Although insignificant , the median values of the two amino acids were higher in the control group than in the patient group. In the patient group, there was a significant positive correlation between age and proline and arginine ($p<0.05$), also there appears to be a high, through insignificant, correlation between arginine and DMFT, Also in the patient group, an increase in proline and arginine may cause an increased DMFT score.

KEY WORDS: Free amino acids, Human whole saliva, HPLC,Proline , Arginine.

INTRODUCTION

Dental caries, along with periodontal disease, are probably the most common chronic diseases in the world (Levine ,2011 and Soben , 2004). Although caries has affected humans since prehistoric times, the prevalence of this disease has greatly increased in modern times on a worldwide basis, an increase strongly associated with dietary change (Soben,2004). Tooth loss resulting from dental caries will lead to diminished chewing ability which can cause nutritional disorders. In addition, caries results in other significant, although intangible, costs in the form of pain, suffering, and cosmetic defects. The status of caries in third world countries represents the greatest challenge to dental science, in developing economies, income for basic health care needs is minimal, including dental care, the increase in caries may reach threefold to fivefold increase in children (Roberson *et al*., 2002).

Saliva has a dramatic effect on caries attack (Cornejo *et al*., 2008). The importance of saliva in controlling plaque pH is well demonstrated by the observation of Henskens *et al*.,1996. The role of saliva in caries protection is demonstrated in patients with diminished salivary flow due to several reasons, who usually suffer from increased caries rate (Carbrta *et al*., 2009).

There are several molecules in saliva that has the ability of reducing caries through killing of bacteria or raising plaque pH, of these molecules, are several peptides and free amino acids (Ferguson, 1999; Bowen, 2002).

The acidic proline rich proteins are found only in saliva, they are needed to maintain calcium homeostasis in the mouth by binding calcium and inhibiting hydroxyapatite crystal growth (Bennick *et al*., 1981), suggesting that, when adsorbed on the tooth surface, they block specific mineral growth sites (Gibbons . and Hay , 1989).

Glycosylated proline rich proteins act as lubricants (Hatton *et al*., 1985), while basic proline rich proteins precipitate tannins and thereby prevent absorption of this potential toxin from the alimentary canal (Lu and Bennick, 1998), also some of the basic proline rich proteins appear to play a role in dental caries (Ayad *et al*., 2000 and Boze *et al*., 2010).

The aims of this study are considered that tried to measure the concentrations of two free amino acids (proline, and arginine) in humans from whole unstimulated saliva, and see if there is any association between these amino acids and dental caries, and also the presence of certain peptides in the same saliva samples and their association with caries.

MATERIALS AND METHODS

In this study 25 individuals were enrolled, with ages ranging from 17-53 years, all samples For the measurement of the dental caries, a caries index was used, that is the DMFT index which is the most common epidemiologic measure of caries, and calculated by counting the number of teeth that are: diseased (D), missing (M), due to caries, and filled (F) also due to caries, while the (T) component of the index stands for teeth (T). Once the tooth is missing or filled, it becomes a permanent measure for the life of the patient, so they represent historical marker for the presence of disease. It is important to recognize the importance of this index in making decision concerning changes in caries in populations (Roberson *et al*., 2002).

A clinical examination was performed on all individuals to estimate the DMFT index for each individual.

Collection of saliva samples:

10 ml of unstimulated saliva was collected from each individual by expectoration and they were collected

between 9 a.m. and 1p.m. Saliva was put in tubes and then frozen at -20 ºC .Studies showed that no differences were observed in the free amino acid concentration of freshly collected samples and those which had been frozen at -20 ºC (Van Wuyckhuyes *et al*., 1995) .

Determination of free amino acid concentration:

Preparation of the samples:

The samples were thawed and vortexed, one ml was taken from each sample and centrifuged at 14000 xg for 30 minutes at room temperature, 100 µl was removed from the supernatent and made 80% with respect to ethanol (100 µl of sample +400µl of absolute ethanol), according to the law (400/(100+400)) * 100, and left at -20ºC for 24 hours, ethanol insoluble material was removed by centrifugation at 14000 xg for 30 minutes, ethanol precipitation appear to result in no loss of free amino acid and removed substances which interfered with the chromatographic separation of amino acids (Van Wuyckhuyes *et al*., 1995) .

Preparation of phenylthiocarlbamyl derivations:

The method described by Heinrikson and Meredith (1984) was used in the preparation of the derivatives and as follows: The ethanol was evaporated from the sample using rotary evaporator under vacuum pressure and in a temperature of 50ºC until complete dryness, 100µl of the coupling buffer was added and then evaporated by rotary evaporator under vacuum pressure in a temperature of 50ºC until complete dryness of the sample, another 100µl of the coupling buffer was added along with 5µl of phenylisothiocyanate, the components were mixed and left for 5 minutes at room temperature, the sample was dried again using rotary evaporator under vacuum pressure in a temperature of 50ºC until complete dryness, finally 250µl of the injection buffer was added to the sample to be ready for injection in the HPLC.

High performance liquid chromatography (HPLC)

After the addition of the injection buffer to the sample, 10 µl was injected into the HPLC system, the amino acid derivatives were detected using a waters model 481 U.V. detector, a wave length of 254nM was used, the retention time and band areas were calculated using a type LKB 2221 integrator.

The separation conditions mentioned by McClung and Frankenberger (1988) were followed, using a reversed phase OSD2(C-18) column, with dimension: 4.6*250mm and a 5µm particle size. The amino acids were recognized by the retention time for each amino acid using standard amino acids prepared in the same way as samples, and injected into the HPLC system under the same separation conditions used with the samples.

A DMFT cut-off value was calculated according to Al-Murrani *et al*. (2000) in order to determine the caries susceptible (patient) group, and the caries resistant (control) group.

All the statistical analyses were carried according to Bland, (2003) using MINITAB version 14 (2006). The mean, standard error (SE), coefficient of variation (CV %), and median values for the amino acid concentrations and the DMFT score index were calculated.

Kolmogorov-Smirnov test was used to determine normality of the samples. Mean values for the amino acid concentrations and the DMFT score index were compared for differences using the two-sample two-sided t-test, while median values were compared using the Mann-Whitney U-test of significance between the patient and control groups.

To determine the quantitative relations between the age and the DMFT score, and between the amino acid concentrations and the DMFT score; the regression of the DMFT score on age and amino acid concentrations was performed in the patient group.

RESULTS AND DISCUSSION

In this research we tried to study the topics: the first one is the estimation of the concentration of two free amino acids, (proline, and arginine), in human whole unstimulated saliva in Iraqi samples using HPLC, and the relation between each amino acid and dental caries using the DMFT caries index.

Whole saliva was collected from 25 individuals, 4 females and 21 males, with ages ranging from 17 - 53 years old, and with a DMFT score ranging from 0 to 18, the number of samples that were analyzed for the amino acid concentration was 23 samples (duo to technical difficulties), and 25 samples were analyzed for the protein profile.For determining the caries sensitive and caries resistant individuals using the DMFT index, a DMFT cut-off value must be determined.

DMFT cut-off value:

The highest value for the DMFT index is 28 (all the teeth are affected except the third molars), and the lowest value is 0 which is caries free. Different researchers take different high limits as caries sensitive or susceptible (Margolis and Moreno, 1992; VanWuyckhuyse *et al*., 1995).

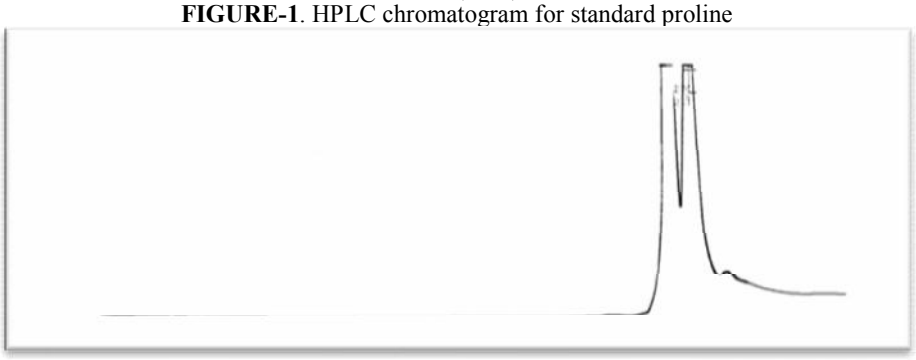
In this study, a DMFT score of less than 6 was considered as caries resistant and designated as the control group, and from 6 upwards are considered caries sensitive and designated as the patients group.

To justify this consideration, using Al-Murrani *et al*. (2000), for a suggested method for the calculation of the cut-off value for the Iraqi patients and control as a unified sample. The lowest 99% confidence limit was calculated using mean ± 2.57 SE for both limits. The lower limit was found to be 5.54 and the upper limit was 11.5, the lower limit (99%) was considered and our control was taken accordingly, any person with a DMFT score higher than 5 is considered caries sensitive and from 5 downwards is considered caries resistant. With the DMFT cut-off value determined, the samples were divided into two groups, a control group (caries resistant) which consisted of 6 samples, and a patient group which consisted of 17 samples for the HPLC analysis.

Figures (1-2) shows the amino acid concentrations' HPLC chromatogram for the two standard amino acids proline (RT 19.56 min), and arginine (RT 21.52 min), respectively), while figures 3 and 4 shows the amino acid concentrations' HPLC chromatogram for a control and a patient sample respectively.

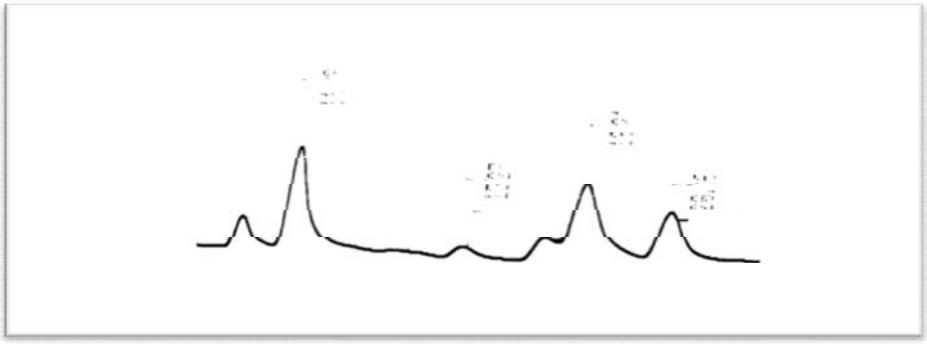
Time (min.)

FIGURE-1. HPLC chromatogram for standard proline



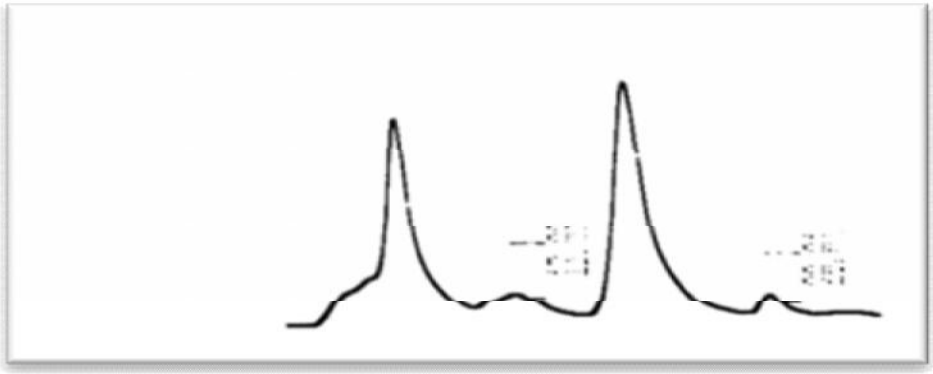
Time (min.)

FIGURE 2. HPLC chromatogram for standard arginine



Time (min.)

FIGURE 3. HPLC chromatogram for a control sample



Time (min.)

FIGURE-4. HPLC chromatogram for a patient sample

TABLE 1. Age, sex, amino acid concentrations (mg/ml), and DMFT score in patient group (n =17)

Sample	Age	Sex	Proline	Arginine	DMFT
1	35	Male	0.97	1.1913	15
2	41	Male	0.5584	0.7651	13
3	53	Female	0.0939	0	8
4	31	Male	0.0738	0.014	6
5	38	Male	0.3828	0	9
6	35	Male	0.0494	0	18
7	32	Male	0.0208	0	6
8	26	Male	0	0	11
9	44	Male	0.0272	0.0292	10
10	28	Male	0.1453	0	11
11	42	Male	0	0.0879	14
12	31	Male	0	0	10
13	20	Male	0.0467	0	6
14	31	Male	0.0291	0	17
15	28	Female	0.0308	0	13
16	17	Male	0.022	0	6
17	22	Male	1.701	0.0006	9

TABLE 2. Age, sex, amino acid concentrations (mg/ml), and DMFT score in control group (n =6)

Sample	Age	Sex	Proline	Arginine	DMFT
18	19	Female	0	0	0
19	19	Male	1.6796	0	4
20	26	Male	0.7363	0.2084	0
21	19	Female	0.5584	0	0
22	31	Male	0	0	0
23	26	male	0.0305	0	0

The amino acid concentration along with the age, sex, and the DMFT index of each sample is shown in Tables 1 for patients (n =17), and Table 2 for control (n =6).All amino acid concentrations are in mg/ml. The lowest value for each of the two amino acids studied in patients was 0, while for controls; the lowest value was 0 for proline and arginine and the highest values in patients were for proline (1.701), and for arginine (1.1913). While for the control group, the highest values of amino acid concentrations were for proline (1.6796), and arginine (0.2084).

To decide on the appropriate statistical analysis and tests, all samples were tested for normality using the Kolmogorov-Smirnov test. Results of this test showed that all samples of patients were not normally distributed (except age and DMFT); most of the control group were close to normality.

Table (2) shows the parameters calculated for the patients (n = 17) and the control (n = 6), both mean and median were included, the standard error (SE) was attached to each corresponding mean value, also the coefficient of variation (CV %) was also included to describe the size of sample variability and to allow comparison of the variances.

Comparisons were done using both the two-sample two-sided-t-test and the Mann-Whitney U-test of significance. The t-test assumes normality of the data, while the Mann-Whitney U-test does not need normality; the t-test compares the mean values, while the Mann-Whitney test compares the medians, results of both tests are shown in table (3).

Both tests gave similar results; the differences that reached statistical significance were noted for DMFT, as expected from the method of scoring. Regarding the amino acid concentrations, there was no statistical significance between the patient and the control groups, which is the same finding, in stimulated saliva, by Varnic *et al*. (1991). Though none of the differences between patients and control were significant for the amino acid concentrations, but it can be noticed from table (1-2) that the mean of the amino acid concentrations in the patient group is generally higher than in the control group except for proline, figure (5). The ratio of the patient/control amino acid mean concentration values reflected that concentration of lysine is only slightly higher in the patient group (ratio 1.06), while for the histidine the ratio was more than twice (2.36 times) and arginine was more than 3 folds higher (3.51 times), proline was only 0.48 which means that proline is slightly more than 2 folds in the control group. The

coefficient of variation (CV%) for the amino acid concentrations is much larger in the patient group except for the arginine, which means that there is a great difference in the amino acid concentration of each amino acid in all the patient group, while in the control group the concentration values are more confined and closer to each other.

The ratio of the median values of the amino acid concentrations in the control group/the patients group was for the lysine more than 4 times higher (ratio 4.2), for the histidine it was more than 17 times (ratio 17.46), while for the proline it was more than 35 times (ratio 35.7), and finally for the arginine it was more than 2000 times (ratio 2080). In a previous study on dental plaque fluid, arginine and histidine are present in only trace amounts, higher is proline and lysine (Levine, 2011), which is the same finding in this study concerning saliva.

TABLE-3. Amino acid concentrations (mg/ml) mean ± standard error (SE), along with the coefficient of variation (CV %), and median values of patients and control for the parameters studied:

PARAMETER	PATIENT (N1)		CONTROL(N2)		MEDIAN	
	Mean ± SE	CV%	Mean ± SE	CV%	Patient	Control
Proline	0.244 ± 0.111	186.96	0.501 ± 0.269	0.434	0.047	1.680
Arginine	0.123 ± 0.080	0.001	0.035 ± 0.035	0.007	0.0001	0.208
DMFT	10.706 ± .931***	35.84	1.500 ± 0.500	1.500	10.000***	3.000

Two-sample-t-test (Means):
*** P < 0.0001 (significant at 0.1% level).
Mann-Whitney U-test (medians):
*** P < 0.001 (P < 0.0004). n1 = 17, n2 = 6

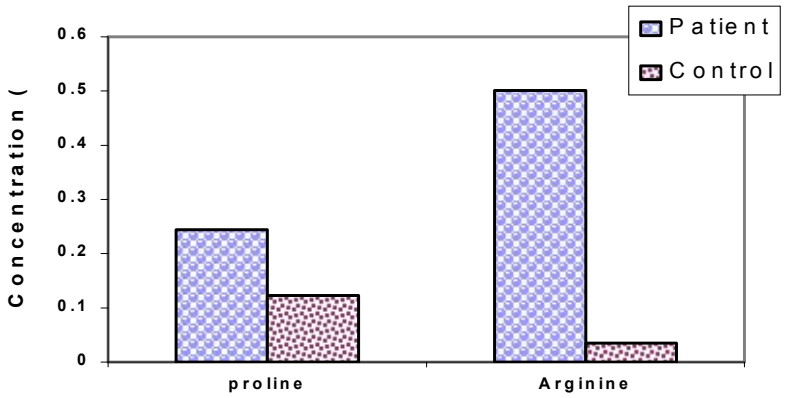


FIGURE 5. Whole saliva amino acid concentration mean values in patients and controls

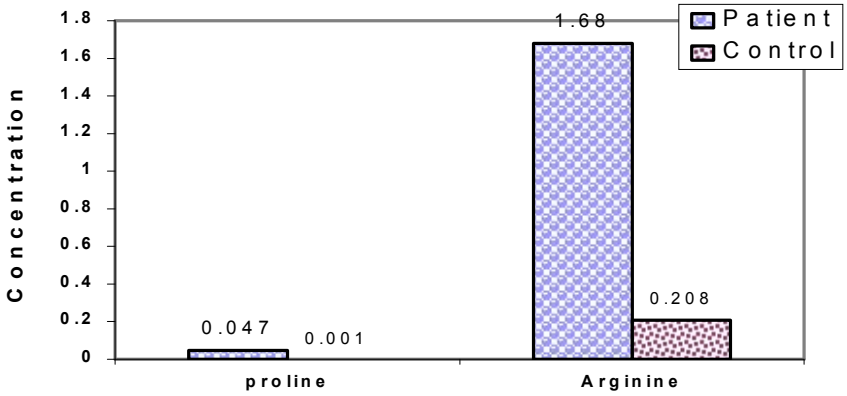


FIGURE 6. Whole saliva amino acid concentration median values in patients and controls

The amino acids studied participate in a variety of reactions that contribute to changing the pH of the plaque environment, as will be discussed below. The pH curves of plaque in response to sugar which was first described by Stephan (1944) and named after him (Stephan curve) clearly shows that the shape of the curve is similar in caries active and caries free individuals, but the resting pH in caries free individuals is more alkali (higher) than in caries active individuals. In all individuals there was a sharp drop in the pH of dental plaque following rinsing the

mouth with glucose solution, this drop in pH was greatest and lasted for the longest time in caries active individuals (Stephan, 1944). Experiments using sucrose rinses in caries susceptible and caries resistant individuals and measuring acidogenesis in plaque *in vivo* showed that when there is no salivary access, plaque pH levels are similar in caries resistant and caries susceptible groups, but as the access to saliva increased, the observed pH increased to a greater degree in caries resistant individuals than was noted in the caries susceptible individuals. This indicates that saliva (notably stimulated saliva) plays a major role in modifying plaque pH and quantitatively reflects caries status (Abelson and Mandel, 1981).

The median values of the amino acid concentrations for the patient and control group may be explained generally by the studies of Stephan (1944), and Abelson and Mandel, (1981), and also by the extensive studies which have demonstrated that low molecular weight constituents like amino acids are likely to exchange with the aqueous phase of dental plaque (plaque fluid) and enhance microbial glycolysis leading to a more rapid cessation of acid production and an earlier formation of base (Madapallimattam and Bennick, 1990), it has been observed that enhancement of glycolysis by microorganisms is brought about by a mixture of amino acids, peptides and proteins present in saliva (Messana *et al.,* 2004).The proteins in saliva are susceptible to proteolytic breakdown (Perinpanayagam *et al*., 1995), so saliva proteins may serve as an additional source of amino acids in saliva (Susana *et al,*. 2011), a possible explanation of the enhanced glycolysis by small molecules is that it may be easily hydrolyzed and processed by microorganisms (Kiran *et al.,* 2010).

Since mean values for pH and NH_4^+ concentrations of dental plaque fluid derived from caries free individuals are significantly higher than those observed for caries susceptible individuals (Kaufman and Keller, 2004), and the effect of amino acids in dental plaque fluid is raising the pH through the decarboxylation of these amino acids since decarboxylation is a proton consuming process (Eric *et al.*, 2006), then it would be reasonable to find that the level of the free amino acids in the saliva of the control (caries resistant) group is higher than the levels found in the saliva of the patient (caries susceptible) group.

From table (2), it can be seen that the median value of the arginine concentration in the control group is more than 2000 times higher than in the patient group, this can be explained in the light of the arginine deiminase system, The arginine deiminase system provides a source of ATP derived from catabolism of arginine to ornithine, CO_2, and NH_3 in a variety of organisms, including many streptococci (Abdelal, 1979). The arginine deiminase system appears to play a role in allowing predominantly aerobic microorganisms to grow anaerobically (Vander Wauven *et al*., 1984).

These findings show that the arginine deiminase system can play an important role in the acid-base physiology of organisms in that it could allow for recovery from acid stresses sufficiently severe to stop growth and glycolysis (Marquis *et al*., 1987). Even when streptococci are grown in media with repressive levels of glucose, there still is production of ammonia and degradation of arginine when the pH values are low (Phan and Marquis, 2006).

Dental plaque bacteria were tested for the arginine deiminase system, and found to be present in some organisms, however, acid tolerant organisms such as *Streptococcus mutans* probably do not need this system, because they can function at lower pH values in dental plaque using the proton translocating membrane ATPase, while organisms such as *Streptococcus sangius* usually can not function and are damaged by low pH values, so the arginine deiminase system may help these organisms to survive in the oral environment (Sofia *et al.,*2010 and Aas *et al.,* 2005). While *Streptococcus mutans* rarely required arginine (Kiran *et al.,*2010). All amino acid biosynthetic pathways were identified in the genome of *Streptococcus mutans* strain UA159, so it was not surprising to find that this microorganism can grow on minimal medium devoid of amino acids if thiosulfate is provided for cysteine biosynthesis (Ajdic *et al*., 2002).

Microbial decarboxylation of arginine and lysine are proton consuming events that result in the formation of polyamines, plaque levels of these amines were significantly higher than those observed for caries susceptible individuals (Phan and Marquis, 2006).

REFERENCES

Levine, M .(2011) " Dental caries " in Topic in Dental Biochemistry , pp. 267 – 283, Springer, Heidelberg , Germany.

Soben, P. (2004) Epidemiology etiology and prevention of dental caries . In : Sobin , p. (ed) Essentials of preventive and community dentisty . 2 ed edn . Arya , New Delhi , 241- 278 .

Cornejo, L. S. Brunotto M .and Hilas E. (2008) Salivary factors associated to the prevalence and increase of dental caries in rural schoolchildren . Rev Saude Puplica vol 42 ,pp. 19- 25

Henskens , Y . M. C. Van den keijbus , E. C. I. and Veemrman. (1996) Protein composition of whole and parotid saliva in healthy and periodontitis subjects : determination of cystatins , albumin , amylase and IgA . Journal of Periodontal Research , vol (31) .no (1) , pp. 57-65

Carbrta ,T., Pisano, E.,and Roberto. (2009) Age dependent modification of the human salivary seretory protein complex . Journal of proteome Research 8 , 8 ,pp. 4134-4134.

Levine ,M. (2011) Susceptibility to dental caries and the salivary proline rich protein. International .J. of Dentistry . 2011, Article ID 953412.

Messana ,I., Cabras,T., and Inzitari, R. (2004) Characterization of the human salivary basic proline rich proyein complex by a proteomic approach .J.of proteome Res 3,4, pp. 792-800.

Susana, S., Rui, V., and Hugo, O. (2011) Reactivity of human salivary protein families toward food polyphenols. J. Agr. And food chemistry Article ASAP.

Kiran , S. Ambatipudi, F. K.,Hagen, C.M. and Delahunty. (2010) Human common salivary protein 1 (csp-1) promotes binding of *Streptococcus mutans* to experimental salivary pellicle and glucans formed on hydroxyapatite surface . J.of proteome Research .9,12.pp, 6605-6614.

Kaufman , E. D.L.,and Keller , P.J.(2004) The diagostic application of saliva a review . Crit, Rev Oral Bio Med, 13, pp. 197-212.

Eric , N.,Sayd,T.,Marzel , M., and Dransfield.E. (2006) Proteomic analysis of human whole and parotid salivas following stimulation by different tasts. J.of proteome Res 5,9,pp.2474-2490.

Phan , T.N.,and Marquis , R. E. (2006) Triclosam inhibition of membrane enzyme and glycolysis of *Streptococcus mutans* in suspension and biofilms . Can .J. Microbiol . 52, pp. 977-983.

Sofia .D.F., Marik ,B., and Arthur ,C.O. (2010) *Streptococcus mutans* , caries and simulation models. Nutrients .2, pp. 290-298.

Aas,J. A., Paster , B . J., Stokes , L. N., Olsen, I.,and Dewhirst , F. E.(2005) Defining the normal bacterial flora of the oral cavity .J. Clin . Microbial .43, pp, 5721- 5732.

Schenck ,K (1993) Levels of salivary IgA antibodies reactiye with bacteria from dental plaque are associated with susceptibility to experimental gingivitis .J. Dent. Res .19 ,2 , pp, 95-98.

Ferguson, D. B. (1999) ORAL BIOSCIENCE. 1st edition. Churchill Livingstone. London. pp: 40, 118-165.

Gibbons, R. J.; and Hay, D. I. (1989) Adsorbed salivary acidic proline-rich proteins contribute to the adhesion of *Streptococcus mutans* JBP to apatitic surfaces. J. Dent. Res. 68: 1303-1307.

Hatton, M. N.; Loomis, R. E.; Levine, M. J.; and Tabak, L. A. (1985) Masticatory lubrication. The role of carbohydrate in the lubricating property of a salivary glycoprotein–albumin complex. Biochem. J. 230: 817-820.

Roberson, T. M.; Heymann, H. O.; and Swift, JR. E. J. (2002) STURDEVANT'S ART AND SCIENCE of OPERATIVE DENTISTRY. 4th Edition. Mosby. St.Louis. Missouri. pp: 65, 66, 89, 90.

VanWuyckhuyse, B. C.; Perinpanayagam, H. E. R.; Bevacqua, D.; Raubertas, R. F.; Billings, R. J.; Bowen, W. H.; and Tabak L. A. (1995) Association of free arginine and lysine concentrations in human parotid saliva with caries experience. J. Dent. Res. 74: 686-690.

Heinrikson, R. L.; and Meredith, S. C. (1984) Amino acid analysis by reverse-phase high-performance liquid precolumn derivatization with phenylisothiocyanate. Anal. Biochem. 136: 65-74.

McClung, G.; and Frankenberger, W. T. JR. (1988) Comparison of reverse-phase high-performance liquid chromatographic methods for precolumn-derivatized amino acids. J. liq. chromatogr. 11: 613-646.

Al-Murrani, W. K.; Al-Shummari, A.; Al-Obaidi, A.; and Mustafa, A. M. (2000). New approach for the calculation of the cut-off point (value) in immunological and diagnostic tests. Iraqi Journal of Microbiology. 12: 1-8.

Margolis, H. C.; and Moreno, E. C. (1992) Composition of pooled plaque fluid from caries-free and caries-positive individuals following sucrose exposure. J. Dent. Res. 71: 1776-1784.

Varnic, L.; Granic, P.; and Rajic, Z. (1991) Basic amino acid in the pathogenesis of caries. Acta. Stomatol. Croatica. 25: 71-76.

Stephan, R. M. (1944) Intra-oral hydrogen-ion concentrations associated with dental caries activity. J. Dent. Res. 23: 257-266.

Abelson, D. C.; and Mandel, I. D. (1981) The effect of saliva on plaque pH in vivo. J. Dent. Res. 60: 1634-1638.

Madapallimattam, G.; and Bennick, A. (1990) Phosphopeptides derived from human salivary acidic proline-rich proteins. Biological activities and concentration in saliva. Biochem. J. 270: 297-304.

Perinpanayagam, H. E. R.; VanWuyckhuyse, B. C.; Ji, Z. S. and Tabak, L. A. (1995) Characteristics of low molecular weight peptides in human parotid saliva. J. Dent. Res. 74: 345-350.

Abdelal, A. T. (1979) Arginine catabolism in microorganisms. Annu. Rev. Microbiol. 33:138-168.

Vander Wauven, C.; Pierard, A.; Kley-Raymann, M. and Haas, D.(1984) *Pseudomonas aeruginosa* mutantsaffected in anaerobic growth on arginine: evidence for a four-gene cluster encoding the arginine deiminase pathway. J. Bacteriol. 160: 928-934.

Marquis, R. E.; Bender, G. R.; Murray, D. R. and Wong, A. (1987) Arginine deiminase system and bacterial adaptation to acid environments. Applied and Environmental Microbiology. 53: 198-200.

Ajdic, D.; McShan, W. M.; McLaughlin, R. E.; Savic, G.; Chang, J.; Carson, M. B.; Primeaux, C.; Tian, R.; Kenton, S.; Jia, H.; Lin, S.; Qian, Y.; Li, S.; Zhu, H.; Najar, F.; Lai, H.; White, J.; Roe, B. A.; and Ferretti, J. J. (2002) Genome sequence of *Streptococcus mutans* UA159, a cariogenic dental pathogen. P.N.A.S. 99: 14434–14439.

Bland M. (2006) Introduction to Medical Statistics. 3rd Edition. Oxford University Press. London. pp: 47-123, 162, 185-222.