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Sour cherry extract inhibits human salivary α -amylase and growth of *Streptococcus mutans* (a pilot clinical study)[†]

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The purpose of this study was to determine whether cherry extract has any effect on salivary α -amylase activity (sAA) or on the level of *Streptococcus mutans* in human saliva. 70 patients (45 females and 25 males) in three age groups (22 children, 25 young adults, and 23 adults) were examined. All participants completed a questionnaire to obtain information on their oral health behaviour and life style. Clinical examination was performed to record the number of decayed, missing and filled teeth (DMF-T). Saliva samples were collected for the measurement of sAA and the salivary *S. mutans* level before and after chewing a gum with or without cherry extract. Statistical evaluation of data was performed. *S. mutans* and the sAA level of unstimulated saliva samples did not depend on either age or gender. The basal sAA value of adult patients was in linear correlation with the dental caries status. Habitual chewing-gum use decreased the resting sAA and the mean of DMF-T. The number of *S. mutans* cells was significantly lower in the resting saliva of allergic patients. The applied mechanical and gustatory stimuli by chewing gum resulted in higher sAA and *S. mutans* levels and a slow decrease of values was observed in the control group for the next 30 min. Thereafter, sAA and *S. mutans* levels decreased earlier in the presence of sour cherry extract than those of control cases. Chewing gum with sour cherry extract may be useful for the prevention of dental caries.

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1. Introduction

Caries is a multifactorial dental disease characterized by demineralization of the inorganic matrix and disintegration of the organic matrix of dental hard tissues. Caries is still the most common oral disease and can affect people of all ages,

ethnicities and socioeconomic backgrounds. Earlier caries was described as a transmissible infectious disease,¹ and although within ten years a fundamental paradigm shift had occurred, the role of oral bacteria in caries progression is obvious. β -Haemolytic *Streptococcus mutans* is one of the most important participants of the oral microflora and dental biofilm. In the oral cavity, biofilm formation is a permanent process with balanced metabolic activity, so caries is a natural phenomenon. Carbohydrates have a great impact on cariogenesis, from which fermentable mono- and disaccharides are produced due to the α -amylase activity of saliva. The adhesion of the acid resisting *S. mutans* to the tooth in the presence of sugar is significant. The glycosyl transferase enzymes promote the polymerization of the extracellular matrix and the adhesion of the *S. mutans*. One of the possible antimicrobial treatments is using targeted antibiotic therapy since broad spectrum killing disrupts normal microbial flora. Since oral pathogens are becoming increasingly resistant to current antibiotics, there is an accelerating search for finding new antibacterial compounds, and therefore some old sources are being reconsidered. Medicinal herbs are a potential source because of their proven antimicrobial activity. A systematic screening of herbs that may

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have therapeutic applications in dentistry has been reported by Chen *et al.*² Strong antimicrobial activity of *Glycyrrhiza uralensis* against *S. mutans* was found by He *et al.*³ Although cariogenic bacteria are transmissible and are necessary for dental caries to occur, caries is truly a multifactorial disease involving bacteria and many other factors. Caries is the result of a deviation from the healthy balance of the microflora, but caries is also a nutritional problem. The quality of the hydroxyapatite of the individual, the amount of carbohydrates, salivary flow and constituents of the saliva are all important parameters in the development of caries.

Saliva is an important body fluid which plays an unquestionable role in oral and dental health. Physiological and genetic variations result in a wide range of normal saliva composition.⁴ One of the most important salivary enzymes is α -amylase, which is an indicator of properly functioning salivary glands.⁵ Amylase is an extremely important enzyme found in human saliva, but its role in caries development is controversial. Some epidemiological studies found a positive correlation between dental caries and salivary amylase concentration,^{6,7} although several recent studies found that low α -amylase activity indicates early childhood caries.^{8,9} Similar to almost every salivary protein, which is multifunctional or even amphifunctional,¹⁰ human salivary amylase (HSA) plays more than one role. In addition to the hydrolysis of starch, HSA adheres to tooth enamel^{11,12} and selectively binds to pioneer bacterial colonizers of the oral cavity (*e.g.* amylase binding streptococci).¹³ Therefore, this HSA-*Streptococcus* interaction is essential for the development of dental biofilm. On the other hand, amylase together with MG2 mucin promotes the aggregation and clearance of *Streptococcus mutans*¹⁴ and performs a direct inhibitory effect on the growth of certain bacteria^{15,16} or viruses.¹⁷

Although human salivary amylase plays an indispensable role in the development of caries, the caries decreasing effect of its inhibition has been studied only for tea.¹⁸

In contrast, several studies aimed at examining the effect of amylase inhibition on blood glucose levels. Some of these research studies suggested and applied *in silico* screening for inhibitory compounds of natural origin¹⁹ or *de novo* design of new inhibitors based on the natural ones.^{20,21} These results may also be useful in the development of anti-caries agents.

Aizawa and co-workers stated that acid production from cooked starch, due to the presence of oral streptococci, was decreased by α -amylase inhibitors.²² We have recently shown the α -amylase inhibitory effect of the sour cherry extract, and the tested anthocyanin compounds were competitive inhibitors of human salivary α -amylase.²³

In addition, the antimicrobial activity of sour cherry juice was verified against different oral or pathogen bacteria by several research groups,^{24–27} but its effect against β -haemolytic *S. mutans* has not been examined so far.

However, one of the cherry's anthocyanin components, malvidin-3,5-diglucoside, showed bactericidal activity and inhibited the acid producing activity of *S. mutans*.²⁸

In another study, the dissolution of anthocyanins incorporated into the dental copolymer and their inhibitory activity on *S. mutans* were confirmed.²⁹

Based on earlier results, a dual target therapy was planned with sour cherry extract against HSA and *S. mutans*. We searched for an appropriate formula to introduce the sour cherry extract into the mouth. Chewing gum was chosen as an appropriate carrier of the sour cherry extract since the slow-release of active ingredients is expected to be favourable for caries prevention.

In addition, gum chewing stimulates salivation both mechanically and chemically, due to the flavouring components of gum. These stimuli increase the oral levels of salivary defence factors, such as the buffering system (bicarbonate ions), salivary enzymes (peroxidase, lysozyme and amylase), pH-rise-promoting factors, proline-rich proteins and antimicrobial histidine-rich proteins. Chewing gums can be used to dispense pharmacological agents,³⁰ and such products may be regarded as functional foods, since chewing gum was defined as a food by the Federal Food, Drug, and Cosmetics Act. Foods with components which inhibit human oral microflora or the fermentation process may contribute to the reduction of dental caries. Functional foods should be tested in clinical experiments with appropriate controls to verify their preventive effect, but only a few *in vivo* human studies have been published to date.³¹ Only green tea was verified as a functional food for oral health, because clinical studies are still lacking for the other foods.³² We designed a clinical pilot study to gain experimental data *in vivo* about the effect of cherry extract on the amylase activity and the level of *S. mutans* of the oral cavity. The primary step of our present work was to gain baseline information about the salivary amylase and the β -haemolytic *S. mutans* levels of different age groups in a Hungarian population. Afterwards, we examined the effect of chewing as a stimulus on the amylase and *S. mutans* level. We wanted to know whether there was any impact of age or sex on the basal and stimulated amylase activity or the *S. mutans* level. In this study, we aim to demonstrate the possible beneficial effects of a sour cherry extract containing chewing gum on the sAA and β -haemolytic *S. mutans* level. Bactericidal and enzymatic activity lowering effects were assessed from samples, which were collected after chewing a gum enriched with sour cherry extract.

2. Materials and methods

2.1 Participants

The study was conducted in full accordance with the ethical principles of the Declaration of Helsinki and approved by the Institutional Ethics Committee (4260-2014 and 4383-2015, DE KK RKEB/IKEB, Regional and Institutional Ethics Committee, Clinical Center, University of Debrecen). All patients were verbally informed about the nature of the study and an informed written consent was signed prior to the study.

All children were accompanied by at least one of their parents, who also signed the informed consent. Seventy-five subjects took part in our study. Children, university students and adults were recruited from an experimental seminar held at the University of Debrecen, Hungary. Participants were excluded from the study if they suffered from salivary gland diseases, apparent oral infections, severe systemic diseases, or mental problems. Exclusion criteria also included: pregnant or breast feeding individuals, and subjects with intake of antibiotics, drugs or other psychotropic substances. Finally, 70 participants (45 women and 25 men) fulfilled the inclusion criteria. Clinical examination was performed to record the number of decayed, missing and filled teeth (DMF-T) and patients were categorised into two groups based on their decay surfaces (DSs). This index is used to measure the number of carious tooth surfaces in the oral cavity. DS = 0 means that the patient was free of caries, if DS > 0, the patient had minimally one carious tooth surface. Since the activity of salivary α -amylase can also be modified by food, stress and emotions,¹⁰ participants completed a standardised questionnaire to obtain information on their oral health behaviour and life styles (*i.e.* diet, oral hygiene, frequency of dental visits, stress level, and smoking habits). Patients were instructed to refrain from eating or drinking anything but water, from exercising, smoking and doing any oral hygiene practices 1 hour before saliva collection. Subjects were divided into three groups based on their age. The patients of the first age group (11 females and 12 males) were 12 years old, the second (20 females and 5 males) ranged from 18 to 20 years and the third (14 females and 9 males) were over 30 years of age.

2.2 Saliva collection

Unstimulated (resting) whole saliva was collected in the early afternoons, between 2 pm and 4 pm to avoid diurnal variation. Prior to the collection of saliva, participants were asked to rinse their oral cavity with water, then they were seated comfortably and allowed to rest for a few minutes. After 5 minutes, the subjects were instructed to swallow any saliva in their mouth, and then to allow saliva to accumulate in the floor of the mouth and to spit into a sterile test tube. After another 5-minute pause, the stimulated whole saliva was collected. The stimulus was both gustatory and masticatory provided by chewing a flavoured standard sized sugar-free gum. The volunteers were asked to chew the gum for 1 minute and to swallow the saliva produced. Thereafter, they removed the chewing gum from the oral cavity and spit the saliva volume secreted into a test tube immediately. The saliva collection was repeated with a smaller subgroup of patients ($n = 34$), who agreed to participate in a longer examination, after 10, 20, and 30 minutes. The samples were immediately transferred to the laboratory and stored at $-20\text{ }^{\circ}\text{C}$ until analysed.

2.3 Measurement of the α -amylase activity of the saliva samples

Kinetic experiments were carried out at $37\text{ }^{\circ}\text{C}$ in 50 mM MES buffer pH 6.0 containing 5 mM $\text{Ca}(\text{OAc})_2$, 51.5 mM NaCl and

152 mM NaN_3 . 200 μL aliquots of 5 mM 2-chloro-4-nitrophenyl-4- α -D-galactopyranosyl-maltoside (GalG_2CNP , SORACHIM S.A., Lausanne, Switzerland) substrate and MES buffer of 285 μL were mixed in a half-micro cuvette and incubated at $37\text{ }^{\circ}\text{C}$ for 5 min. Saliva samples (10 μL) were diluted 1 : 200 in an assay diluent and mixed well. The reaction was initiated by adding 15 μL diluted saliva into the mixture and the continuous measurement of absorbance started immediately. Kinetic curves of CNP release were measured continuously at 400 nm using the Parallel Kinetics Analysis program of a JASCO V550 (JASCO Tokyo, Japan) spectrophotometer. All experiments were repeated three or four times. Salivary α -amylase activity (sAA) values (1 unit = $\mu\text{mol min}^{-1}$) were calculated using $\Delta A \text{ min}^{-1}$ values of the initial rate.

2.4 Preparation of sour cherry extract

One type of Hungarian sour cherry variety was selected for examinations, namely "VN1", which was a selection of "Csengődi csokros". The ripe fruit samples were harvested from the orchard of the Research and Consulting Institute for Fruitgrowing, Újfehértó in July 2015. After picking, fruit samples were immediately transferred to the laboratory, then washed, deseeded and homogenized with a Braun Multiquick mixer. The fully homogenised samples were extracted with a double volume of acidified ethanol (0.1% HCl) by stirring with a magnetic stirrer (Heidolph, Germany) for 1 h. Thereafter, the samples were filtered, and the solvent was evaporated at $40\text{ }^{\circ}\text{C}$ under vacuum using a rotary evaporator. The sour cherry extract was characterized earlier in detail. The main components were cyanidin-3-*O*-rutinoside, cyanidin-3-*O*-glucoside and cyanidin-3-*O*-glucosylrutinoside/malvidin-3,5-diglucoside.²³

2.5 Preparation of chewing gum with and without sour cherry extract

The chewing gums were made by a melting procedure. For the formulation, Geminis T BHA gum base (CAFOSA), xylitol, citric acid, glycerol, saccharine (Sigma), peppermint volatile oil, and Kirsch Aroma (Akras) were used. The xylitol was dissolved in purified water at $80\text{ }^{\circ}\text{C}$, and then the gum base was added and melted in a water bath and mixed. The taste masking agents, flavouring agents (citric acid, glycerol, and saccharine) and 0.1 g anthocyanin rich sour cherry extract were dissolved in purified water. In the case of control gum products, the sour cherry extract was not added into the water phase. The water phase was emulsified in the melted gum base phase at $60\text{ }^{\circ}\text{C}$. Finally, the peppermint volatile oil was added at $40\text{ }^{\circ}\text{C}$. The uniform mixture was divided for 2.5 g pieces and formed ball-shape products. After 12 hours conditioning time at room temperature, the chewing gum balls were stored at $8\text{--}15\text{ }^{\circ}\text{C}$ in a plastic jar until application.

2.6 Microbiology measurements

In order to detect and quantify living β -haemolytic *Streptococcus mutans* strains, saliva samples were cultured on trypticase soya base agar (Sigma Aldrich) enriched with 5%

defibrinated sheep blood (Biocenter). On this medium, β -haemolytic *S. mutans* strains have a characteristic colony morphology. The complete lysis of the red blood cells was manifested by transparent, yellow hollow circles around the unique *S. mutans* colonies facilitating the morphological identification of these strains. To obtain unique bacterial colonies, we performed a 10^4 fold dilution in a class II laminar-flow cabinet to avoid environmental contamination. Agar plates were incubated at 37 °C and under aerobic conditions containing 5–10% CO₂ for 4 days. Afterwards, morphological identification was performed and, in the end, the number of β -haemolysing *S. mutans* strains was counted and multiplied by 10^4 . In the case of clean agar plates, we repeated the culturing at one magnification of dilution.

2.7 Statistical analysis

Descriptive statistics were used to summarize all measurements of sAA and *S. mutans* levels. The mean values and SD from the unstimulated saliva at each time point were calculated. The distribution of data was tested using the Kolmogorov–Smirnov test. *t*-Tests of two independent samples were used for the comparison of the differences of sAA levels between categories in each parameter. Pearson's correlation coefficient (*r*) and linear regression methods were used to analyse the relationship between the age and oral status (DMF-T), resting the sAA level and DMF-T in groups of different ages.

In the three age groups, the oral status/DMF-T was examined using Welch's ANOVA instead of the usual ANOVA. When the ANOVA was significant, it was followed by a *post-hoc* test for multiple comparisons; if equal variances were assumed, the Bonferroni test was used, whereas if equal variances were not assumed, the Games–Howell test was used.

The assessment of the time effects between categories (control and treated) by one-way ANOVA with repeated

measures was performed to compare the mean differences of sAA levels. Two-way ANOVA with repeated measures was performed to assess the time effects between categories (control and treated), among the three age groups and the interaction effect to compare the mean differences of *S. mutans* levels.

Any differences found were corrected for multiple comparisons according to the Bonferroni method. Two-tailed tests with a significance level of 5% were used throughout. All statistical calculations were performed using the Statistical Package for Social Sciences version 23.0 (SPSS, Chicago, IL, USA).

3. Results and discussion

The first statistical evaluation of resting sAA values was made according to age and gender. The median and range of resting sAA in patients of three different age groups are displayed in Fig. 1A. Atypical values were plotted separately. Mean values obtained from groups of different ages did not differ significantly from each other (first age group 40.6 ± 40.9 , second age group 31.2 ± 22.6 , third age group 58.5 ± 49.4 , $P > 0.05$). The mean values of resting sAA levels were not significantly higher in males than females in all age groups (Fig. 1B) (first age group male/female $56.2 \pm 49.9/35.1 \pm 24.5$, second age group male/female $27.5 \pm 18.9/32.1 \pm 23.8$, third age group male/female $63.9 \pm 66.6/55.6 \pm 39.9$, $P > 0.05$). The composition of saliva exhibits an explicit inter-individual variability. The variability of sAA was found to be considerably high, as a result of the multitude of factors acting on the acute amylase level. The amount and activity of basal or resting HSA were found to be influenced by several short- or long-term factors, such as sex, age, diet, habits (tobacco, coffee, alcohol or medication use), oral health status, physical exercise, somatic and psychiatric diseases/status and acute stress.³³ Some of these factors might

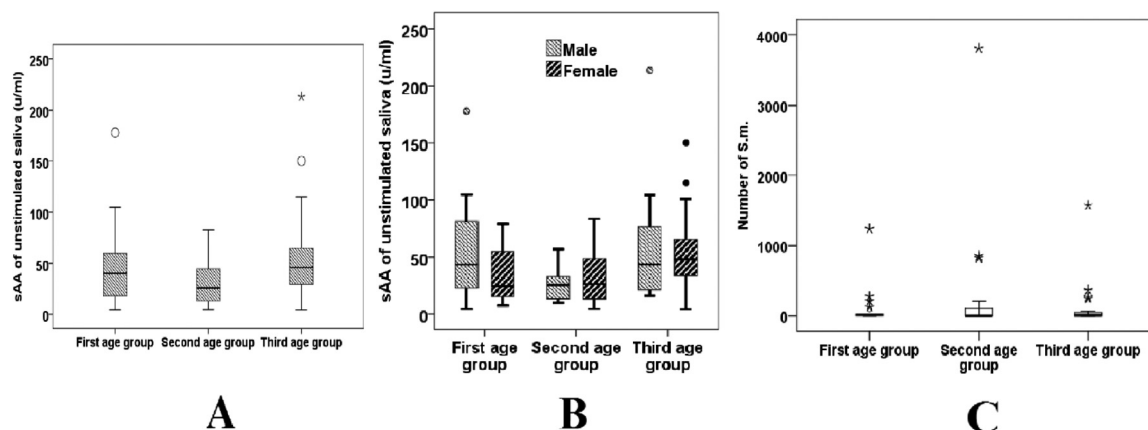


Fig. 1 Quantitative comparison of the median resting sAA levels of different age groups (A), and age groups divided by gender (B) and mean of the *S. mutans* number of different age groups (C) first age group: children of 12 years, second age group: young adults: 18–20 years, third age group: adults >30 years. The box represents the first and third quartiles (rectangular boxes); the line within the box is the median; and vertical lines show the smallest and largest values inside the limits (without the outlier values). Empty circles (○) above these levels represent atypical values, asterisks (*) mark extreme values.

Table 1 Statistical evaluation of the answers to the questionnaire via comparison of the mean resting sAA and *S. mutans* levels between categories for each parameter

Parameter	Category	N	sAA mean $\pm$ SD	<i>P</i> *	<i>S. mutans</i> mean $\pm$ SD	<i>P</i> *
Chewing gum use	0	47	51.32 $\pm$ 43.52	0.058	88.68 $\pm$ 261.39	0.265
	1	23	32.06 $\pm$ 27.99		290.09 $\pm$ 826.32	
Bleeding gum	0	44	39.35 $\pm$ 33.84	0.125	200.59 $\pm$ 641.30	0.344
	1	26	54.54 $\pm$ 47.78		77.46 $\pm$ 185.31	
Chronic illness	0	62	40.10 $\pm$ 32.16	0.128	144.94 $\pm$ 522.23	0.661
	1	8	82.88 $\pm$ 69.74		231.75 $\pm$ 547.87	
Using dental floss	0	39	38.94 $\pm$ 36.00	0.151	73.59 $\pm$ 153.61	0.195
	1	31	52.60 $\pm$ 43.82		257.10 $\pm$ 759.66	
Allergy	0	51	48.52 $\pm$ 44.38	0.228	202.78 $\pm$ 604.69	0.045
	1	19	35.51 $\pm$ 22.70		26.21 $\pm$ 65.44	
Dry mouth	0	58	47.09 $\pm$ 41.89	0.336	183.88 $\pm$ 569.65	0.310
	1	12	34.82 $\pm$ 27.90		14.58 $\pm$ 29.76	
Using mouthwash	0	38	41.14 $\pm$ 36.29	0.383	135.24 $\pm$ 339.45	0.734
	1	32	49.56 $\pm$ 44.04		178.16 $\pm$ 683.81	
Regular medication	0	56	42.09 $\pm$ 32.94	0.403	160.18 $\pm$ 547.75	0.866
	1	14	56.59 $\pm$ 60.80		133.57 $\pm$ 418.90	
DS-category	D-S = 0	43	47.82 $\pm$ 39.73	0.458	176.91 $\pm$ 613.20	0.659
	D-S > 0	27	40.48 $\pm$ 40.61		119.74 $\pm$ 336.50	
Current medication	0	53	42.94 $\pm$ 33.55	0.564	168.91 $\pm$ 562.04	0.694
	1	17	51.39 $\pm$ 56.27		111.06 $\pm$ 380.93	

Category 0: no 1: yes, *significance level $P < 0.05$.

actually change the amylase activity, but others might influence the basal amylase activity in general. The mean value and standard deviation of sAA were lower in the case of young adults (second age group), probably due to the greater social and age homogeneity of this group, where members were dental students, but the difference was not significant. Similar to the earlier published results,^{10,33,34} the resting sAA levels were influenced neither by age nor by sex.

The statistical analysis of *S. mutans* levels measured in resting saliva resulted in a similar tendency, the number of *S. mutans* cells did not differ significantly in the three age groups and was not influenced by gender (Fig. 1C). Although in all age groups, there were some patients with extremely high *S. mutans* cell numbers, the median of the *S. mutans* level in resting saliva was low, which reflects the fact that *S. mutans* is a classical adhering microorganism, rather present in the biofilm than in a planktonic form as published by Wessel and coworkers.³⁵ Neither the resting sAA nor the *S. mutans* level varies with age and gender, which allowed handling of data, obtained from different groups, together. The statistical evaluation of answers to the questionnaire gave some interesting results, as summarized in Table 1. All participants were non-smokers according to the questionnaire. The parameters were sorted according to the P value of sAA levels. P values showed the results of the two independent t -tests. Surprisingly, habitual chewing-gum use decreased the level of resting sAA. The effect was not significant, but this P value was the lowest, close to the significance level. The negative effect of habitual chewing on resting sAA was not published earlier. Chewing sugar-free gum between meals helps protect the teeth and gums since it can increase the flow of saliva and lower the amount of acid that attacks the teeth due to the increased sali-

vary bicarbonate buffer capacity. Lowering the basal level of sAA by habitual chewing may be an additional long-term protection for teeth. To confirm the caries reducing effect of chewing, an independent two-sample t -test was used. The comparison of the mean DMF-T values of chewing and non-chewing groups resulted in a significant difference between the two groups (mean $\pm$ SD in the chewing group 7.6 ± 7.8 , non-chewing group 14.2 ± 15.4 , $P < 0.05$). The teeth of patients in the chewing group were healthier due to the combination of short-term and long-term protecting effects. The sAA increasing effect of two other parameters, bleeding gum and regular use of dental floss, can be explained by their inflammatory impact.

The only significant difference in the starting *S. mutans* level was among allergic and non-allergic patients. Furthermore, the incidence of dry mouth and bleeding gums decreased, while using dental floss or habitual chewing gum use increased the number of *S. mutans* cells. As an endogen, a locally affecting factor, saliva plays a prominent role both in the promotion and inhibition of caries. An appropriate amount of saliva secretion cleans the oral cavity from microorganisms. In dry mouth with decreased saliva secretion, the extended mucin and mucoid content elevates the viscosity, but the washing efficiency and the amount of planktonic bacteria may decrease. In contrast, dental floss use or habitual chewing ensures strong mechanical clearance and does not allow the formation of mature biofilms, and bacteria are rather in a planktonic form in the oral cavity.

The oral status was examined in the three age groups handling males and females together (using Welch Robust Tests of Equality of Means and Games–Howell *post hoc* test). Not surprisingly, the mean of the DMF-T value was higher for older patients (mean $\pm$ SD in the first age group 2.8 ± 3.8 ; second

age group 7.6 ± 6.4 ; third age group 25.7 ± 15.2 , $P < 0.05$). The inter-group correlation between the mean sAA level in the resting saliva and the oral status (DMF-T) was analyzed using Pearson's correlation coefficient (r). The resting sAA values of adult patients with more caries affecting teeth were higher, while no effect was found for children (first age group $r = -0.124$, $P > 0.05$, second age group $r = 0.546$, $P < 0.05$, third age group $r = 0.415$, $P < 0.05$).

Correlations were significant for adults and a positive linear relationship was found in both adult age groups (second age group $y = 2.81 + 0.15x$, $P < 0.05$; third age group $y = 18.18 + 0.13x$, $P < 0.05$, Fig. 2). The slopes of the linear regression curves were approximately the same and showed that the increase of sAA per unit may enhance DMF-T by 0.15 or 0.13.

Our results, that higher sAA resulted in higher DMF-T of adults, were consistent with previously published studies,^{4,36} which showed a positive correlation between caries and the high sAA level for children and adolescents.

Although the recommendations of Rohleder drew attention to the importance of sampling interval and frequency, "Samples for assessing acute responses should be obtained at baseline and frequently (every 10 min) after that until 20–30 min after the end of the intervention,"³³ there are hardly any examples of such an experiment. In our study, the effect of the sour cherry extract containing chewing gum was examined for 30 min (three sampling) and was compared to the results of the control group (Fig. 3 and 4). During evaluation, the sAA values of different age groups and males and females were averaged in control and treated groups since basal sAA in the saliva of males and females was similar and was not influenced by age. The control and treated groups were similar in composition based on the age and gender. There was no difference between the resting enzyme levels of the two groups. The stimulation resulted in a significant increase of HSA activity in both groups (Fig. 3). An one-way repeated-measures analysis of variance showed a significant time effect, ($F(4,128) = 11.81$, $P < 0.05$, 1.: 50.06 ± 45.45 , 2.: 90.56 ± 62.99 , 3.: 84.11 ± 70.33 , 4.: 81.89 ± 66.66 , 5.: 81.13 ± 77.73). Only the mean of the unstimulated saliva differed from the other means in the case of the control group using the related t -test when a Bonferroni adjustment was made for the number of comparisons.

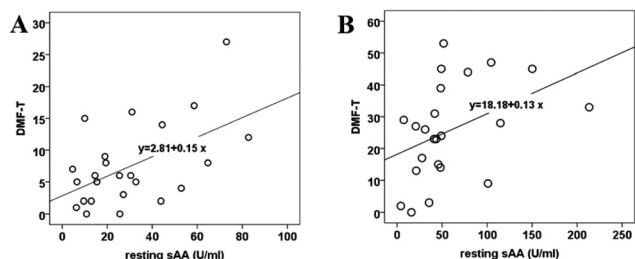


Fig. 2 Correlation between the resting salivary alpha-amylase activity (sAA) and DMF-T in the groups of (A) young adults (18 to 20 years), and (B) adults (over 30 years of age).

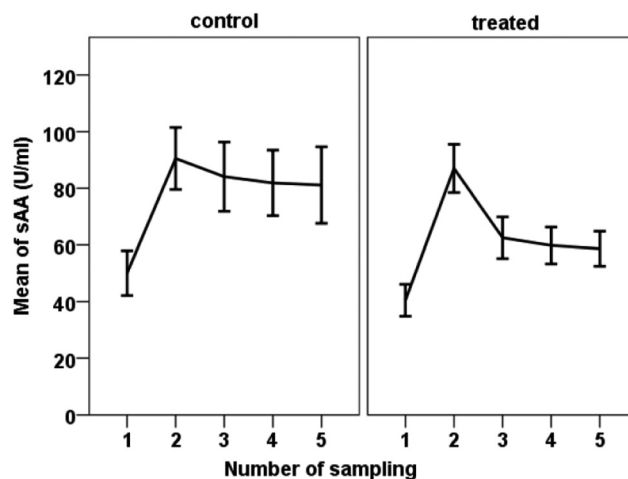


Fig. 3 The means of the sAA level in the control ($n = 34$) and treated ($n = 36$) groups of patients before and after stimulation. Sampling 1.: unstimulated saliva, 2.: stimulated saliva at 0 min, 3.: stimulated saliva at 10 min, 4.: stimulated saliva at 20 min, 5.: stimulated saliva at 30 min after stimulation. Data are presented as mean $\pm$ standard error (SE) of the mean.

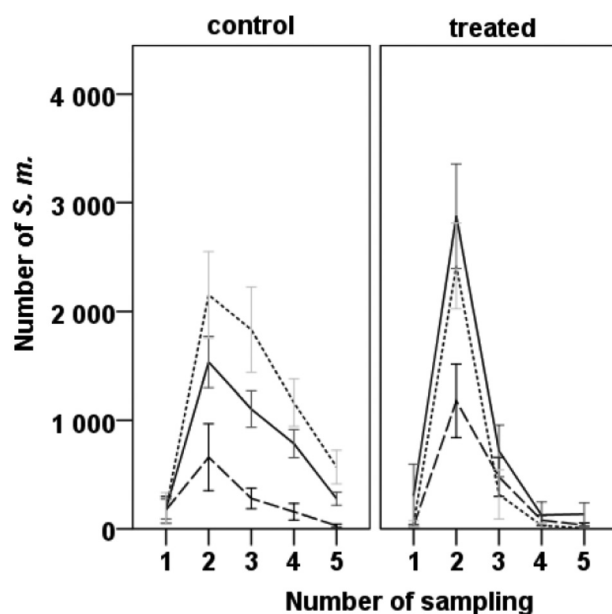


Fig. 4 The means of the *S. mutans* level in the three different age groups before and after stimulation. First age group: children (12 years old) (---) dashed line; second age group: young adults (18 to 20 years) (—) continuous line; third age group: adults (over 30 years of age) (.....) dotted line. Sampling: 1 unstimulated saliva, 2: stimulated saliva at 0 min, 3: stimulated saliva at 10 min, 4: stimulated saliva at 20 min, 5: stimulated saliva at 30 min after stimulation. Data are presented as mean $\pm$ standard error of the mean.

The elevated sAA level after stimulation can be interpreted by the detailed analysis of the saliva production process. The vast majority of the HSA enzyme (80%) is synthesized in the parotids and the remainder in the submandibular glands

(20%).⁶ The percentage of contribution to the saliva production of the glands changes during stimulation. Unstimulated saliva originates mainly (65–70%) from the submandibular glands, and only 20% is produced by the parotids.³⁷ After stimulation, there is an alteration in the ratio of contribution of each gland, the secretion of the parotids will exceed over 50% of the total salivary secretion.^{13,33} Similar results, a statistically significant increase in sAA due to stimulation, were obtained in the case of young (20–39 years old) adults,⁷ and this result is consistent with several other research studies dealing with the stress-induced HSA expression^{14–16} and the HSA level in eating disorders.³⁸ In contrast, *S. mutans* levels were handled separately according to age in the control and treated groups, since the number of cells after chewing were significantly different between age groups (Fig. 4). The *S. mutans* level was higher in stimulated saliva samples of both the control and treated groups. The masticatory effect of chewing liberated the bacterial cells from the biofilm and resulted in higher counts of *S. mutans* in the saliva samples. Significant differences were observed between children and adults in the control group and in the treated group, as well. In the case of *S. mutans* counts, the comparison of results with other studies was not simple, as a disparity between the results exists. This could be attributed to the differences in the ages of patients, distinctness in the technique of sampling saliva and the cultivation of bacteria. Most studies have dealt with children or adolescent and have used plaque or stimulated saliva samples. The results shown by Sullivan *et al.*³⁹ and Mundroff *et al.*⁴⁰ prove that the number of *Streptococcus mutans* in dental plaque samples explains the variation in caries similar to the number in stimulated whole saliva.

We counted similarly low starting *S. mutans* numbers in the control and treated groups at first sampling (see values of 1 sampling in Fig. 3 and 4), which significantly increased after stimulation (chewing a gum) due to the liberation of biofilm embedded cells (see values of 2 sampling in Fig. 3 and 4). The adhesion of cells after the chewing resulted in a slow decrease of the cell number in all age groups of the control group. After 30 minutes, the *S. mutans* level reached the starting value only in the first age group (children), where the increasing was the lowest. In the case of the treated group, whose members chewed gum containing the sour cherry extract, the high *S. mutans* values caused by chewing decreased rapidly in all three age groups and reached the low resting value after 20 minutes. The values of the two adult groups were almost the same at sampling of 1., 2., 4. and 5. The elevated cell number after chewing was significantly lower in the children groups (first age group) of both experiments.

We obtained interesting results for bacterial counts, which were statistically evaluated based on the answers to the questionnaire (Fig. 5). Significant differences were found between the control and treated groups in three cases. The *S. mutans* level of regular dental floss users was higher in all sampling times (Fig. 5A), indicating a less stable state of the biofilm due

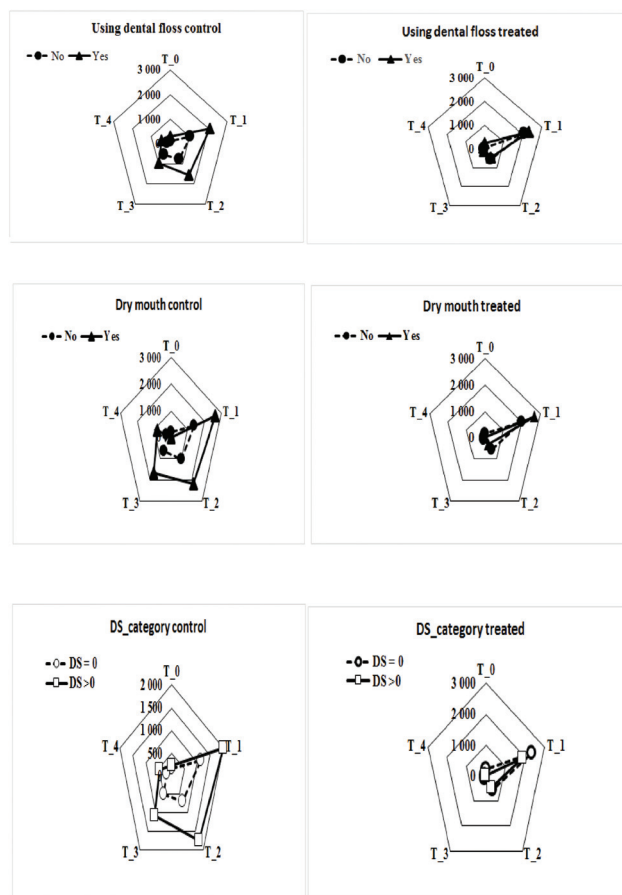


Fig. 5 Polar plots of the *S. mutans* cell number in the control and case groups. Comparison between the categories of questionnaire. Using dental floss control/treated (A), dry mouth control/treated (B), DS_category control/treated (C), two DS categories were: non-caries group DS = 0 and caries group DS > 0.

to the systematic removal with flossing. The situation was similar in the case of the different DS categories, with the bacterial level being higher in patients with caries (DS > 0). The difference was significant/higher in stimulated saliva. As was mentioned earlier, dry mouth decreased the number of *S. mutans* cells in resting saliva, but after chewing, the numbers were always higher in patients with dry mouth. These differences between the categories completely disappeared in the treated groups.

4. Conclusions

We proved in a human pilot study that anthocyanin containing sour cherry extract inhibited the human salivary α -amylase enzyme delaying the starch degradation in the oral cavity. In addition, sour cherry extract rich in anthocyanin prevented the growth of *S. mutans*. Our results revealed that the mechanical effect of chewing, the washing effect of saliva and the chemical inhibition effect of anthocyanins complemented each other and resulted in increased efficiency.

The chewing gum has proved to be a suitable carrier for inhibitor compounds and can be considered as a vehicle for other naturally occurring inhibitor compounds. α -Amylase can be the biological target for anti-caries interventions. The known structure of α -amylases and modern chemo-informatics tools allow testing the inhibition potential of numerous naturally occurring compounds.⁴¹ Virtual screening is a useful approach to search even very large libraries of small molecules which can bind to the enzyme active centre and to filter the inhibitor candidates.⁴² After an isolation or concentrating process and an activity validation, similar food products can be developed from the selected natural compounds.

Conflicts of interest

There are no conflicts to declare.

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