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Effect of sweetened beverages intake on salivary aspartame, insulin and alpha-amylase levels: A single-blind study

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ABSTRACT

The objective was to assess aspartame excretion in saliva and the salivary insulin, total protein (TP), and alpha-amylase (AMI) levels in response to the ingestion of sweetened beverages (sodium cyclamate, aspartame, acesulfame, and sucrose). Fifteen healthy participants were included in a single-blinded trial with the intake of Diet soft drink, Regular soft drink, Water + sweeteners, Low sucrose content (3.5 g), and Water (blank) in 5 different days. In each day, saliva was collected at T0 (fasting), T1 (15 min after test-drink intake), T2 (30 min), T3 (60 min), and T4 (120 min) for the measurement of salivary aspartame (HPLC), TP, AMI (ELISA assays) and insulin levels (chemiluminescence). Chi-square, Friedman, ANOVA and Spearman correlation tests were applied. The late-perceived sweet/sour residual flavor was reported at a frequency of 80%, 60% and 20% after ingestion of artificially sweetened drinks, beverages with sucrose, and plain water, respectively ($p < 0.05$). Aspartame was detected in saliva after artificially sweetened drinks intake, with highest area under the peak for the Diet soft drink ($p = 0.014$). No change was observed for TP and AMI levels during the 120 min. Insulin levels increased 1 h after soft-drinks ingestion (regular and diet), while the levels did not change for Low sucrose content and Water + sweeteners test-drinks. Salivary aspartame correlated with insulin levels only after Diet soft drink intake (rho ≥ 0.7; $p < 0.05$). As aspartame can be detected in saliva and swallowed again until completely excreted, these results contribute to the knowledge of the biological fate of artificial sweeteners and the study of health outcomes.

1. Introduction

The frequency of consumption of foods and beverages containing sweeteners is high worldwide (Sylvetsky et al., 2017; González-Rodríguez et al., 2021; Oliveira and Canella, 2022), and people are widely exposed, sometimes unknowingly, to the consumption of low-calorie sweeteners (Logue et al., 2020). Although the safety of its use is established before regulatory approval, there is a debate about the consumption of sweeteners and their potential association with glucose intolerance in individuals with obesity (Toews et al., 2019). Recent recommendations from the World Health Organization state that non-sugar sweeteners should not be used as a mean of weight control because of the lack of evidence of its benefits in long-term prospective observational studies (WHO, 2023), highlighting the need to assess its potential health benefits and, more importantly, its metabolism and potential adverse effects (Magnuson et al., 2016; Lohner et al., 2017).

The sweet taste is preferred by many people and may be an innate preference in humans (Maone et al., 1990), resulting in the daily consumption of large amounts of sugar by the population. However, the harm that this food can bring to health is well known. Therefore, part of the population chooses to replace sugar with sweeteners with an intense sweet taste with less product (and calories) when compared to sucrose. Among the sweeteners most used in foods and beverages, such as soft drinks, are: sodium cyclamate, aspartame and acesulfame potassium, all synthetic.

Aspartame is the most commercialized synthetic sweetener in the world; its main components are phenylalanine and aspartate, and it is metabolized in the intestinal lumen. Sodium cyclamate is usually

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associated with other sweeteners because of its residual taste; its metabolism is still quite uncertain, since only a small portion of the population can metabolize it (Fatibello-Filho et al., 1996); although banned in some countries, it continued to be used as a food additive with an acceptable daily intake amount(Renwick et al., 2004). Acesulfame potassium is a stable synthetic potassium salt obtained from an acidic compound of the acetic acid family with high sweetening power, and is not metabolized.

In the human digestive tract, there are a number of substances responsible for the degradation and absorption of nutrients from ingested food and beverages (Hoyle, 1997). The digestion of sugars begins in the oral cavity with the action of enzymes responsible for the food bolus formation, mainly salivary alpha-amylase (Freitas and Le Feunteun, 2019). In addition, the sweet taste is also perceived (which may come from artificial sweeteners) through the stimulation of taste buds present in various regions of the oral cavity, initiating physiological processes that result in increased salivary secretion (Kusakabe et al., 2020; Yamada et al., 2023) and whole-body metabolic preparation for the absorption of nutrients - including the insulin secretion. The cephalic phase of insulin secretion is an early rise in the blood levels associated with oral exposure, which depends on the type and presentation of stimulus (Lasschuijt et al., 2020; Wiedemann et al., 2020). Fabre et al. (2012) found a high positive correlation between salivary and serum insulin levels, with salivary levels representing 10% of the serum ones. It has been hypothesized that, as low/no-calorie sweeteners also bind to and stimulate sweet-tasting receptors, the release of insulin might occur, which in turn could lower blood glucose levels and result in weight gain and increased appetite; however, the current knowledge is still inconclusive (Mehat et al., 2022).

The protein content of saliva participates in some aspects of perception and digestion of food (Carreira et al., 2020). Salivary alpha-amylase is directly associated with the oral perception of starchy foods, carbohydrate absorption and the glycemic index (Moriel et al., 2010; Lamy et al., 2020). Studies have shown that it plays an important role as a marker of nutritional status (Lamy et al., 2015) and its levels in saliva changes as a result of health interventions, such as gastroplasty (Marquezin et al., 2022). Recently, a previous study showed that the expression levels of cystatins and alpha-amylase in saliva increased due to bread olfactory stimulation, suggesting that odor can also promote anticipatory changes in saliva secretion related to food intake (Carreira et al., 2020), thus contributing to the understanding of their participation in taste and aroma perception and/or in the initial steps of digestion.

So far, it is not known how much of sugar substitutes are excreted in their unaltered form in saliva, re-stimulating the taste buds and being swallowed again, thus remaining longer in the digestive tract. It is also necessary to understand the secretion of salivary enzymes and hormones related to taste perception, digestion, and carbohydrate metabolism, such as alpha-amylase and insulin, in response to artificial sweeteners ingestion. Thus, this study aimed to evaluate the excretion of aspartame in saliva after the ingestion of sweetened test-drinks, in addition to assess alpha-amylase, total protein and insulin salivary levels in response to the ingestion of sweetened beverages (sodium cyclamate, aspartame, acesulfame, and sucrose) in a single-blinded, randomized trial.

2. Material and methods

This is a randomized, single-blinded trial approved by the Research Ethics Committee of the Federal University of São Paulo (UNIFESP), under protocol CAAE 22769319.6.0000.5505 and registered at the Brazilian Clinical Trials Registry (ReBEC; https://www.ensaiosclinicos.gov.br/).

2.1. Trials and saliva collection

Sample size was calculated considering the results of Sun et al. (2014); the results showed that at least 10 participants were needed to detect a 30% difference in the area under the insulin secretion curve between different treatments (intake of different foods), considering a test power of 80% and 5% alpha level.

Fifteen healthy young adult participants (18–45 years; 11 women and 4 men) were included, all students or staff of the University (not trained), normoglycemic individuals. The inclusion criteria were adults of both sexes with healthy oral conditions (no gingivitis, dental caries or any other oral condition that could interfere in salivary composition or taste perception, examined by a Dental Surgeon); the exclusion criteria were: presence of any systemic disease that could also affect salivary composition/flow and xerostomia symptoms; smoking or alcohol habit; presence of chronic diseases such as diabetes, hypertension, and cancer; chronic use of medications; use of oral or injectable contraceptives; dietary restrictions; daily use of sweeteners; acute diseases that could interfere on smell or taste perceptions, such as flu or COVID-19. None of the participants included had COVID-19 until the end of the trial.

Participants were asked to restrict the use of sweeteners in their diets in the previous week and during the trials, to avoid any influence on the results. They attended five sessions on different days for the ingestion of 200 mL of each test drink offered in plastic cups using a randomized procedure, at room temperature, blindfolded, as described below:

1. Diet soft drink (sweetener composition according to the manufacturer: 54 mg of sodium cyclamate, 30 mg of acesulfame potassium, and 24 mg of aspartame in 200 mL;
2. Regular soft drink (sucrose content according to the manufacturer: 21 g in 200 mL;
3. Water + sweeteners: 200 mL of solution containing water (natural mineral water), 54 mg of sodium cyclamate, 30 mg of acesulfame potassium, and 24 mg of aspartame (Merck KGaA, Darmstadt, Germany), similar to the concentration found in the Diet soft drink;
4. Low sucrose content/sucrose 0.02 g/mL: 3.5 g of sucrose in 200 mL of plain water (Merck KGaA, Darmstadt, Germany);
5. Water (blank): 200 mL of plain water. Test-drinks of Sucrose 0.02 g/mL and plain water (blank) were included to improve comparability and observe the sensitivity of the analysis.

At each scheduled session, the participant attended the laboratory in the morning (fasting) and remained without ingesting any type of food or drink until the end of the all saliva collections. On each day, five saliva collections were performed using a polyester roller: at baseline (T0; before ingestion of the test drink) and, after ingestion of the test drink: T1 (15 min later), T2 (30 min later), T3 (60 min later) and T4 (120 min later).

Prior to saliva collection, the oral cavity was cleaned with distilled water. A polyester oral swab/roll (*salivette*, Salimetrics, USA) was placed under the tongue (sublingual/submandibular gland) to collect saliva for the measurement of sweeteners; another oral swab was placed over the Stensen duct, bilaterally, for the collection of saliva with higher protein content directly from the parotid saliva (avoiding the contact with other oral fluids) and the dosage of insulin, alpha-amylase and total protein. Immediately after collection, the swabs were centrifuged at 5000 rpm for 5 min (4 °C) and the supernatant pipetted and aliquoted into eppendorfs to be frozen at −80 °C for further analysis.

Additionally, within the 120 min follow-up time of all trials, the participants were asked about any perceived residual taste in the mouth (sweet, sour, or other) using forced answer options (yes/no; qualitative approach). The participant was asked at every saliva collection. Any positive answer between 30 and 120 min was considered positive to sweet/sour/other late residual taste.

2.2. Salivary total protein and amylase measurement

The measurement of salivary total protein was carried out using a standard curve of albumin (Sigma-Aldrich) with six concentrations (2, 1.5, 1.0, 0.5, 0.25, 1.125 μg/μL) and squared regression coefficient of $R^2 = 0.989$. The colored complex was quantified spectrophotometrically at 595 nm (Synergy HT, BioTek). All the measurements were performed in triplicates using diluted saliva (1:5).

The determination of salivary alpha-amylase was performed using Elisa assay and specific kit for salivary samples (Salimetrics, CA, USA), according to the manufacturer's specifications. As these are samples collected from the parotid gland (where higher concentrations of proteins are expected), the amylase concentrations observed exceeded the detection limit of the equipment; therefore, in addition to the dilutions specified by the manufacturer, the samples were diluted another 3 times in order to enable reading. On each test day, the samples were thawed in a refrigerator between 4 °C and 5 °C, followed by centrifugation at 14000 rpm for 10 min at 4 °C before the analyses, which were performed in duplicates. The readings were performed spectrophotometrically at 405 nm (Synergy HT, BioTek) and 37 °C.

2.3. Salivary aspartame assessment

As no previous method existed in the literature for the dosage of sweeteners in saliva, the method proposed by Trandafir et al. (2009) was tested for its suitability in the measurement of aspartame and acesulfame potassium in saliva samples.

Initially, for the dosage of aspartame and acesulfame potassium in sublingual/submandibular gland saliva, an ultraviolet light (UV) scan was carried out in a spectrophotometer (UVmini-1240, Shimadzu, Japan) between 0 nm and 800 nm in duplicates to observe the wavelength, with standard solutions inserted in a quartz cuvette (Fig. 1). Both aspartame and acesulfame-K absorbed UV at similar wavelengths, enabling the improvement of the existing chromatographic method (Trandafir et al., 2009) to a single wavelength.

Further, the analytes in the saliva samples were separated by reversed-phase chromatography (Shimadzu Analytical System, Japan) with C18 column (150 mm length, 4.6 mm inner diameter, and 5 mm particle) and pre-column. Mobile phase with KH_2PO_4 0,02 M pH 4.3 – Acetonitrile [88:12] (injection volume: 10ul; flow rate: 1 mL/min) and certified reference materials (Merck KGaA, Darmstadt, Germany) were used.

Prior to injection, the samples were centrifuged for 5 min in 12000 rpm. This step is important to turn clean the solution, and only the supernatant was injected.

For the aspartame, peaks appeared on the chromatogram at 15 min, running at 217 nm and results were expressed as area under the peak. The evaluation of the reliability aspects of the bioanalytical method (selectivity, calibration curve, and limit of quantification) was based on the Bioanalytical Method Validation Guidance (Food and Drug Administration, 2018), obtaining the following results: i) Selectivity: blank was free of interference at the retention time of aspartame; ii) Calibration curve: the method has presented linear response in the range of 0.5 to 20 μg/mL presenting a correlation coefficient of 0.9838; iii) Limit of quantification: the lowest amount of an analyte that can be quantitatively determined with acceptable precision and accuracy; we have established as the low concentration of calibration curve (0.5 μg/mL).

Although the pilot results of measuring acesulfame potassium in saliva have shown good results, the performance and analytical aspects found during the analysis of the participants' saliva did not reached reasonable quality standards. For this reason, these results are not shown.

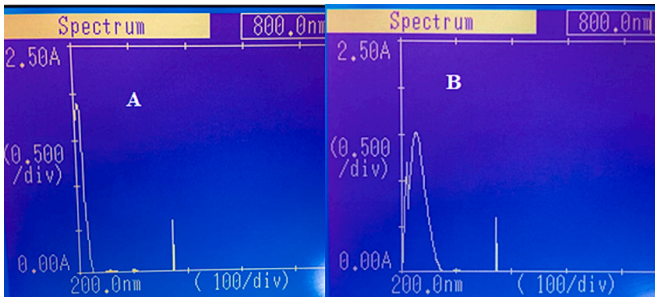

Fig. 1. Ultraviolet light (UV) scan of acesulfame potassium (left) and aspartame (right).

2.4. Salivary insulin measurement

The determination of insulin in parotid saliva was performed using the electrochemiluminescence technique at the Clinical Analysis Laboratory. A Roche Cobas 8000 Analyzer (Roche Diagnostics International AG Ltd, Switzerland) along with the Cobas Elecsys insulin immunoassay (ref. 07027559190) were used. According to the manufacturer, the detection limit of the method is 0.4-1000 μU/mL – 2.78-6945 pmol/L, with a blank limit of 0.2 μU/mL (1.39 pmol/L), and quantification limit equal to 1 μU/mL (6.95 pmol/L), defined in accordance with the protocol requirements of the Institute of Clinical and Laboratory Standards. The saliva samples were thawed and centrifuged at 2500 rpm for 10 min; 250 μL of the supernatant (pure saliva) were transferred to the micro sample cup and subsequently analyzed automatically based on the calibration curves defined by the equipment.

2.5. Residual interference test and possible artificial sweetener degradation

As a way of controlling a possible residual interference effect, a pretest was carried out to determine how much sweetener residue remains in the oral cavity after contact of these compounds with oral mucosa. For this, five participants performed a one-minute mouthwash with a solution of Water + sweeteners (15 mL of mineral water + 4.05 mg of cyclamate, 2.25 mg of acesulfame potassium and 1.8 mg of aspartame). After 15 min of clearance, the saliva samples of each participant were collected using swabs placed under the tongue as described above for further analysis. By analyzing the injection chromatograms of the collected samples, no signal was observed at predetermined retention times, concluding that there were no aspartame residues in saliva after the contact of these substances with the oral cavity.

Additionally, another test was performed to verify a possible degradation of sweeteners by enzymes present in saliva; for that, samples of fresh saliva were collected from three participants (fasting); after centrifugation (14000 rpm for 10 min at 4 °C), each sample was directly and individually added with solutions of acesulfame potassium and aspartame in four different concentrations (75, 25, 10, and 1 ug/mL); a total of 150uL of the solution was added to the vials. The analysis of the chromatograms showed that the peak areas are proportional to the injected concentrations; therefore, it was concluded that aspartame is not metabolized or degraded in saliva (Supplementary material).

2.6. Statistical analyses

Statistical analysis was performed with SPSS 28.0 (IBM Microsoft, USA) and PAST4 (Natural History Museum, Norway) software considering an alpha level of 5% (PMC). Exploratory analysis consisted of means, standard deviation, median, quartiles, and plots. Non-monotone missing pattern occurred in the aspartame data due to insufficient sample volume (which occurred in<10% of the samples); imputation was performed by predicting the missing data by linear regression.

The Chi-square test was used to verify the association between the intake of test-drinks or water and the late perception of sweet/sour taste in the mouth.

Considering the total protein, amylase, and insulin levels, the comparison between times (T0, T1, T2, T3, and T4) was performed using the Friedman test. Spearman's correlation test was used to verify the correlation between the aspartame and insulin salivary concentrations at times T2, T3 and T4.

Finally, the aspartame area under the curve after the ingestion of Water + sweeteners and Diet soft drink was compared using the Two-way Mixed model ANOVA, considering 'time' as within-subject factor.

3. Results

Fifteen healthy participants were included (11 women and 4 men, 18–45 years, mean age 30.3 ± 8.9 years; median = 29 years), with mean body mass index of BMI = 23.1 ± 4.6 Kg/m^2.

The report of perceived sweet or sour late residual taste between 30 and 120 min of each trial was more frequent for artificially sweetened drinks – both Water + sweetener and Diet soft drink (80%), when compared to beverages with sucrose – Low sucrose content and Regular soft drink (60%), and only Water (20%) (X2 = 75; DF = 2; $p < 0.0001$) (Fig. 2).

Table 1 shows the salivary levels of total protein, alpha-amylase, and insulin before (T0) and after (T1-T4) the ingestion of test-drinks. Both total protein and alpha-amylase levels did not differ in response to any ingested test-drink ($p > 0.05$). Insulin levels increased 60 min after the ingestion of the soft-drinks, both Regular and Diet soft-drinks ($p < 0.001$ and $p = 0.017$, respectively), while the levels did not significantly change for Low sucrose content and Water + sweeteners test-drinks. Conversely, the salivary insulin levels decreased within the two hours after Water intake (blank) ($p < 0.001$). It is possible to see in Fig. 3 that the insulin secretion curves of Regular and Diet soft drinks are very close, while a steady drop was observed for Water intake.

Salivary aspartame curves (mean area under the peak) from samples collected within 120 min after the ingestion of Diet soft drink and Water + sweetener drink are shown in Fig. 4 (T0 to T4); an upward Diet soft drink curve can be observed (F = 3.26; $p = 0.015$; power = 82%), with significant differences between test-drinks in T3 ($p = 0.014$) and T4 ($p = 0.044$). For the other test-drinks (Regular soft drink, Low sucrose content and Water), no peak was detected in the respective retention time.

The analysis of the correlation between aspartame area under the peak from samples of Water + sweetener and Diet soft-drink trials and the salivary insulin levels showed moderate positive correlations between aspartame levels at T2 and insulin concentration at T3 and T4 ($rho > 0.7$; $p < 0.01$) and aspartame at T3 with insulin concentration at T4 ($rho = 0.66$; $p = 0.026$) only for the Diet soft-drink intake (Fig. 5), suggesting a secretion response of insulin to aspartame ingestion. No significant correlation between aspartame area under the peak and salivary insulin levels was observed for the Water + sweetener intake ($p > 0.05$).

4. Discussion

This is the first study that explored the aspartame excretion in saliva and the effect of sweetened test-drinks intake on salivary total protein, alpha-amylase, and insulin levels. Aspartame was detected in salivary samples collected after the ingestion of Diet soft drink and Water + sweetener drink, with a steady rise of the Diet soft drink curve within the 120 min of follow-up. Although previous studies have reported that its metabolism and absorption occur rapidly in the intestinal lumen (Magnuson et al., 2016), even after 120 min it was possible to detect significant amounts of aspartame in saliva, evidencing that it remains for a longer period in the digestive tract, is secreted again in saliva and swallowed until completely excreted.

The study also explored the residual taste perceived by many people after the ingestion of no– or low-calorie sweetened drinks, demonstrating that, indeed, the perceived sweet/sour residual taste in the

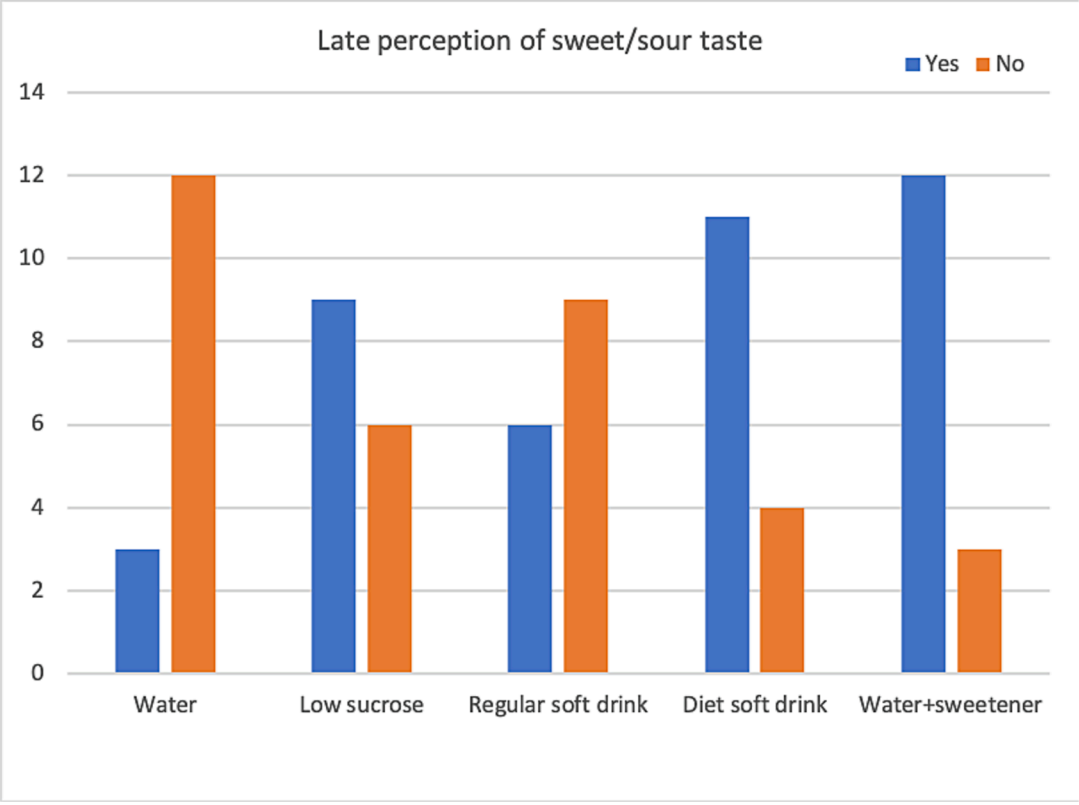

Fig. 2. Frequencies (n, absolute number) of positive responses of late sweet/sour/other taste perception within the 30–120 min of each trial (X2 = 75; DF = 2; $p < 0.0001$).

Table 1
Comparison of total protein, alpha-amylase, insulin, and aspartame over time: a Friedman test.

Analyte		Water	Low sucrose content	Water + sweeteners	Regular soft drink	Diet soft drink
	Time	**Median (25–75%)**				
Total protein (ug/uL)	T0	1.9 (1.4–2.8)	2.1 (1.6–4.0)	1.6 (0.7–2.1)	2.0 (1.2–3.0)	1.6 (1.2–2.1)
	T1	1.7 (1.2–2.5)	1.9 (1.4–3.1)	1.6 (1.2–3.1)	2.0 (1.5–4.2)	1.6 (1.1–2.6)
	T2	1.7 (1.2–2.5)	1.8 (1.6–3.5)	1.6 (1.1–2.7)	2.7 (1.5–4.4)	1.5 (1.1–2.3)
	T3	1.8 (1.4–2.9)	1.8 (1.4–4.3)	1.6 (0.7–3.4)	1.7 (1.4–4.0)	1.3 (0.9–5.3)
	T4	2.0 (1.3–2.4)	2.1 (1.5–2.9)	1.9 (0.7–2.5)	2.1 (1.4–3.1)	1.7 (1.1–3.3)
p-value		*0.994*	*0.784*	*0.644*	*0.398*	*0.741*
Alpha-amylase (U/ml)	T0	250.9 (207.6–303.1)	301.1 (278.5–477.2)	221.4 (141.7–305.0)	194.8 (148.6–479.2)	267.7 (141.7–366.1)
	T1	261.7 (206.6–531.4)	309.5 (226–6-457.3)	283.4 (99.4–618.4)	279.5 (182.0–485.1)	303.1 (186.0–390.7)
	T2	284.4 (195.8–379.8)	315.9 (260.8–444.8)	225.3 (136.8–369.0)	193.9 (165.3–439.9)	299.1 (137.8–391.6)
	T3	281.4 (188.9–431.0)	311.0 (188.9–536.3)	189.9 (119.1–450.7)	220.4 (158.4–482.2)	258.8 (157.4–469.4)
	T4	299.1 (178.1–345.4)	284.4 (223.4–389.7)	275.5 (143.7–300.1)	246.0 (179.1–429.0)	176.1 (104.6–497.4)
p-value		*0.459*	*0.926*	*0.781*	*0.654*	*0.778*
Insulin (μU/mL)	T0	13.4 (7.2–26.9)	8.5 (4.6–17.2)	11.5 (7.5–21.2)	13.1 (5.8–34.9)	11.8 (7.4–27.8)
	T1	11.9 (8.8–22.0)	10.8 (5.5–14.1)	8.8 (5.4–25.1)	13.2 (7.8–26.7)	10.9 (5.5–22.3)
	T2	11.2 (9.1–20.2)	8.3 (5.6–17.7)	5.7 (3.8–14.9)	14.9 (7.9–31.1)	12.3 (5.6–16.0)
	T3	8.1 (4.9–18.0)	8.6 (4.7–18.9)	8.0 (4.5–18.9)	**18.5** (10.0–31.1)	**12.8** (5.4–19.7)
	T4	**5.9** (4.5–15.9)	7.1 (3.0–16.5)	5.4 (3.4–11.5)	11.3 (6.5–17.1)	10.7 (4.5–19.5)
p-value		*<0.001*	*0.467*	*0.094*	*<0.001*	*0.017*
Aspartame (area under the peak)	T0	0	0	0 (0–490.6)	0	0
	T1	0	0	1605 (1174–3766)	0	3892 (984–9954)
	T2	0	0	2489.6 (2101 (2101–3459)	0	7159 (2282.5–25840.3)
	T3	0	0	2279.5 (1914–2678)	0	4326.5 (1462.8–37485.4)
	T4	0	0	**4219.9** (3824–4268.1)	0	**5704.6** (1025.3–46646.8)
p-value		–	–	*0.049*	–	*0.005*

T0: fasting (before ingestion); T1: 15 min after test-drink ingestion; T2 (after 30 min); T3 (after 60 min); T4 (after 120 min).

mouth was associated with the ingestion of artificially sweetened drinks, probably due to the excretion of aspartame and other artificial sweeteners in saliva, as observed in this study for the case of aspartame. Previous studies have explored the aftertaste perception following artificial sweeteners ingestion(Såmündsen, 1985; Tan et al., 2019), characterized on a sensory level as bitter and/or metallic (Riera et al., 2007), but no other study related the late perception of sweet/sour residual taste with the sweetener excretion in saliva. These findings can be explained by the fact that acesulfame K is not metabolized by the body and is excreted by the kidneys unchanged with no known adverse effects (Pang et al., 2021), and only a small portion of the population can metabolize cyclamate (Fatibello-Filho et al., 1996).

It is of note the greater aspartame area under the peak for the Diet soft drink compared to the Water + sweetener test-drink, which was prepared with the same artificial sweetener concentrations. This finding probably explains why a correlation was found between aspartame area under the peak and insulin levels for the Diet soft drink intake, but not for the Water + sweetener drink intake. Moreover, insulin levels did not change significantly after the ingestion of Water + sweetener test-drink. Collectively, the results suggest a faster aspartame metabolization of Water + sweetener test-drink compared to added to Diet soft drink.

One can also hypothesize the potential stimulatory effect of other ingredients present in soft drinks. Although one previous study has shown that the carbonation of a simple glucose solution did not increase glycemic response nor alter gastric emptying or subjective feelings of satiety (Lau and Henry, 2017), greater oro-sensory exposure of foods increases insulin responsiveness (Lasschuijt et al., 2020). It should be considered that besides carbonation, the soft drinks have lower pH than the Water + sweetener test-drink and, thus, can offer other physiological stimuli than sweet taste. In addition, other ingredients present in the soft drinks such as cola, flavourings, and caffeine (bitter), may represent another source of stimuli for the secretion of enzymes and hormones. Oral sensory stimulation can trigger (or improve) insulin release, as demonstrated by Teff et al. (1993), who compared plasma insulin during fasting, food intake ('fed') and sensory stimulation without ingestion ('sham-fed') and found increased in plasma insulin and C-peptide levels under the conditions of food intake and sensory stimulation without ingestion, concluding that the insulin release occurs in the cephalic phase independently of plasma glucose changes.

The studies by Dhillon et al. (2017) and Ren et al. (2021) also evaluated the neuroendocrine response to exposure to food and beverages with sweeteners in humans and animal model, respectively. The exposure to sucralose and sucrose caused equally significant increases in serum insulin two minutes after exposure ('sham-fed') and significant decreases after two minutes in participants with overweight or obesity, both in oral exposure with beverages and sweetened solid foods, and the

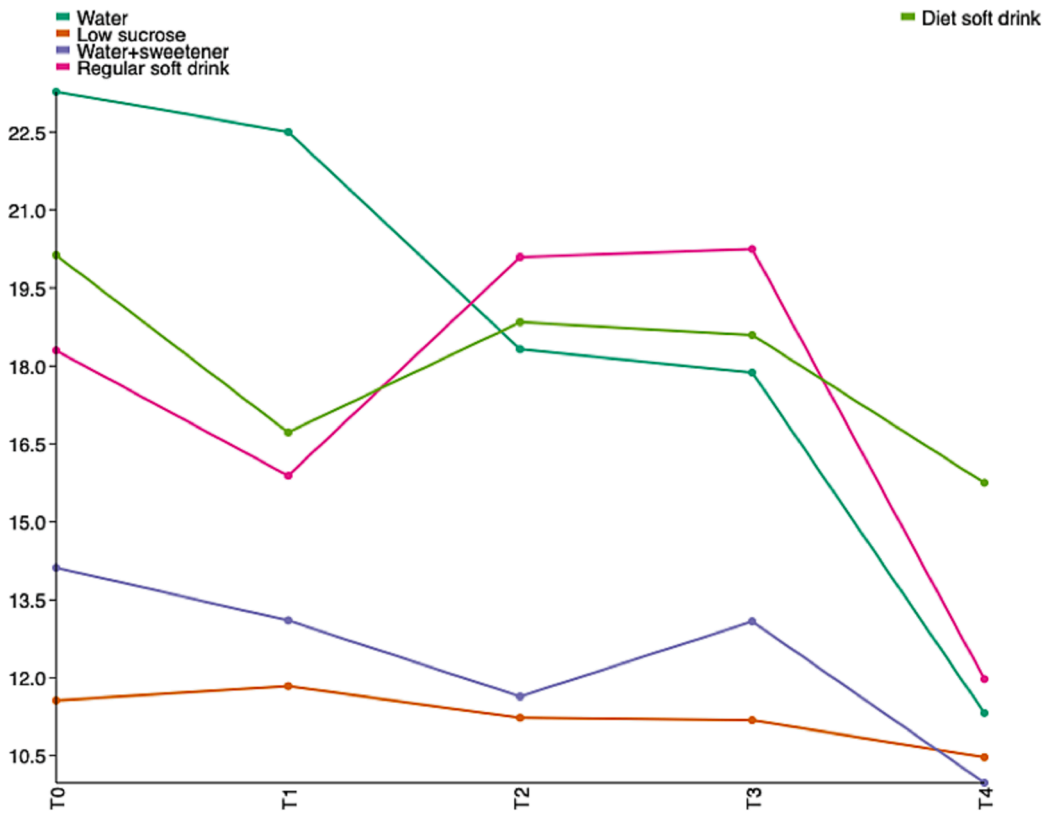

Fig. 3. Salivary insulin secretion curves (μU/mL) showing the levels before (T0) and after (T1-T4) the ingestion of test-drinks (means). T0: fasting (before ingestion); T1: 15 min after test-drink ingestion; T2 (after 30 min); T3 (after 60 min); T4 (after 120 min).

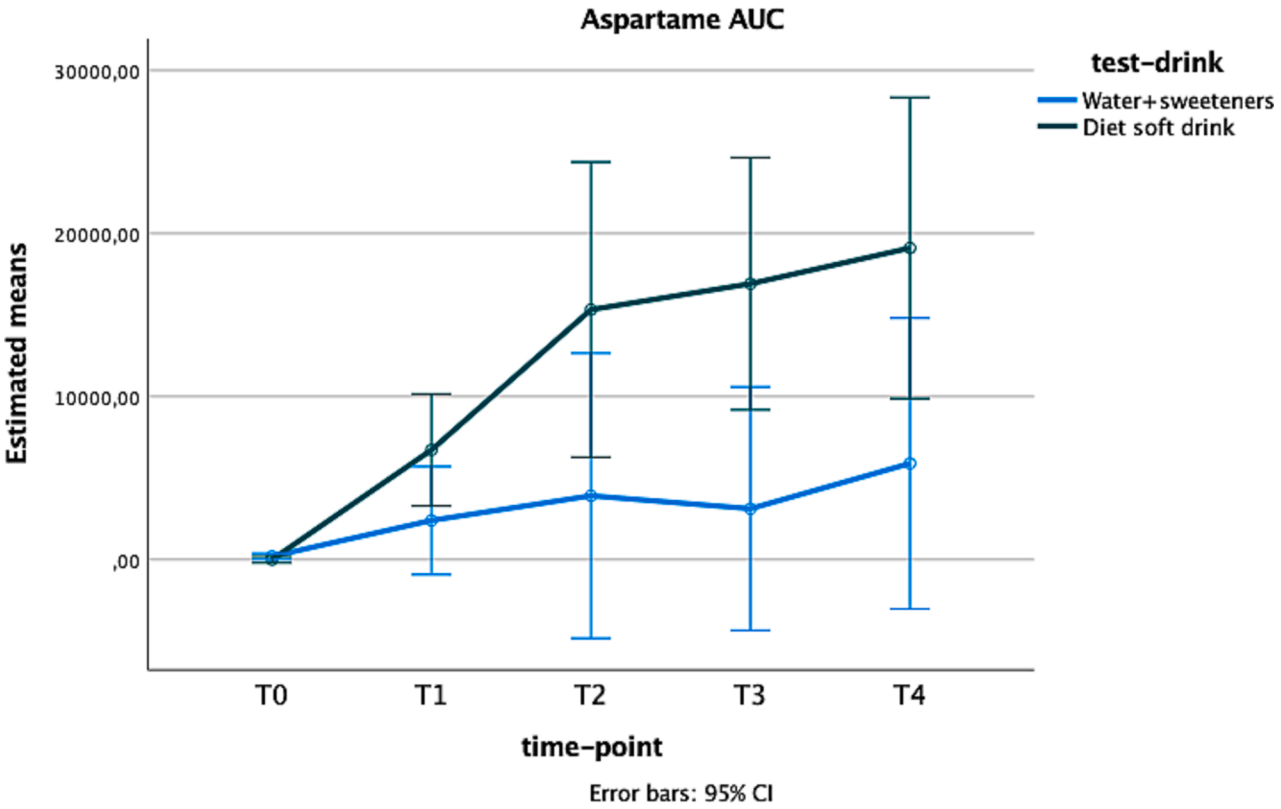

Fig. 4. Salivary aspartame secretion curves before (T0) and after (T1-T4) the ingestion of sweetened test-drinks (means and CI). T0: fasting (before ingestion); T1: 15 min after test-drink ingestion; T2 (after 30 min); T3 (after 60 min); T4 (after 120 min). Significant differences were found in T3 ($p = 0.014$) and T4 ($p = 0.044$; Two-way Mixed model ANOVA). Y axis: aspartame area under the curve.

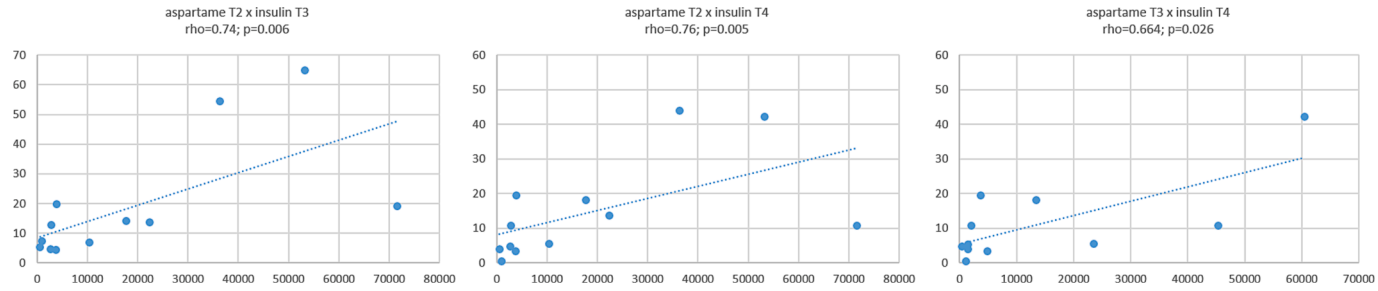

Fig. 5. Scatterplots of the correlation between salivary aspartame area under the peak (X axis) and insulin levels (μU/mL; Y axis) for the Diet soft-drink intake. T0: fasting (before ingestion); T1: 15 min after test-drink ingestion; T2 (after 30 min); T3 (after 60 min); T4 (after 120 min).

solid food form elicited a greater response at 2, 6 and 10 min than the beverage form (Dhillon et al., 2017). In animal model, increased leptin and insulin levels was observed after short-term ingestion of sucralose, maltose, stevioside, and saccharin (Ren et al., 2021).

Insulin levels were increased 60 min after the ingestion of Regular and Diet soft-drinks; it was possible to see that both curves are very close, although the Regular one showed a greater rise between 30 and 60 min and a subsequent drop between 60 and 120 min, similarly to the results found in a glucose tolerance test. Conversely, the intake of 3.5 g sucrose solution (amount similar to that added in a cup of coffee) did not change the insulin levels over the 120 min, remaining relatively stable over time, while the insulin curve of Water showed a steady drop over the same period corresponding to and corroborating the fasting state of the participants. Although recent studies did not find significant effects on glycemic response, insulin sensitivity, Glucagon-like Peptide-1 release, and body weight after short-term (2–4 weeks) artificially sweetened water or soft drink intake (Kim et al., 2020; Orku et al., 2023), when the objective is weight control, one should consider that the observed increase in salivary insulin levels following soft drinks intake promote glucose internalization, that is, increased caloric absorption.

Alpha-amylase participates in the digestion of carbohydrates, being secreted mainly by the parotid glands. Its enzymatic action begins in the oral cavity and is inactivated in the stomach (Moriel et al., 2010). It was expected to observe a change in the alpha-amylase activity following the intake of sweet beverages, which was not found in any of the test-drinks of the trial, including the sucrose-based drinks (Regular soft drink and Low sucrose content test-drink). A previous study showed that total protein and alpha-amylase concentrations in saliva, and specifically alpha-amylase activity, were influenced by the type, but not by the concentration of the gustatory stimulus, with citric acid stimulation resulting in the lowest concentrations (Froehlich et al., 1987). As the Regular soft drink has a large amount of sucrose but potent acid taste and very low pH, these previous findings can help explain the results found in the present study. Furthermore, the secretion of alpha-amylase seems to be greatly elicited by starchy foods and the maltose generated after mastication (Aji et al., 2019; Lamy et al., 2020), thus varying according to the oral sensing of simple and complex carbohydrates.

The main limitation of this study was the impossibility of accurately determining acesulfame concentrations in saliva; although the method has provided reasonable results in the pilot study, the results found in the participants samples did not reach acceptable quality standards. The repetition of the test was not possible due to the insufficient amount of saliva samples at the end of the experiments. Thus, the improvement of acesulfame analytical method and the development of a method to measure the cyclamate levels in saliva are welcome.

It is important to emphasize that the comparison of 'Diet soft-drink', 'Regular soft-drink' and 'Water + sweeteners' has intrinsic limitations, because it does not consider all the ingredients present in the soft-drinks. Finally, it is worth mentioning that the imbalanced number of women and men in the study group may have represent another limitation, which was caused by the restricted number of volunteers that met the inclusion criteria, especially those without any sign/symptom/diagnosis of COVID-19 during pandemic.

With the dramatic increase in the consumption of sweeteners, it is necessary and timely to assess their potential health benefits and, more importantly, their metabolism fate (Lohner et al., 2017). Non-nutritive sweeteners are much more potent than table sugar, and a very small amount produces a sugar-like sweet taste without calories. The intensity of artificial sweetness could lessen the appeal of the natural sweetness found in healthier foods such as fruits and vegetables, which in turn can lead people to make less healthy choices. The novel results open new avenues and formulate hypotheses for the study of artificial sweeteners fate and their impact on health outcomes in future studies.

5. Conclusion

This is the first study that report aspartame secretion in saliva. The results found a greater salivary aspartame area under the peak after Diet soft drink compared with Water + sweeteners intake, and the former correlated with the concentration of salivary insulin levels. Furthermore, higher levels of salivary insulin were observed after the ingestion of Regular and Diet soft drinks compared to Low sucrose content and Water test-drinks.

Statements.

Data availability statement: The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to ethical restrictions.

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Permission to reproduce material from other sources: Not applicable.

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CRediT authorship contribution statement

Carolina Martins Finassi: Methodology, Investigation, Data curation, Writing – original draft. **Leandro A. Calixto:** Conceptualization, Methodology, Investigation, Data curation, Writing – review & editing. **Wilson Segura:** Investigation, Data curation, Visualization, Writing – review & editing. **Mariana Zuccherato Bocato:** Investigation, Data curation, Visualization, Writing – review & editing. **Fernando Barbosa Júnior:** Data curation, Resources, Visualization, Writing – review & editing. **Fernando L.A. Fonseca:** Methodology, Resources, Investigation, Writing – review & editing. **Elsa Lamy:** Conceptualization, Methodology, Writing – review & editing. **Paula Midori Castelo:** Resources, Visualization, Project administration, Formal analysis, Writing –

original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2023.113406.

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