

Effect of antihistamine-containing syrup on salivary metabolites: an *in vitro* and *in vivo* study

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Declaration of Interests: The authors certify that they have no commercial or associative interest that represents a conflict of interest in connection with the manuscript.

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<https://doi.org/10.1590/1807-3107bor-2021.vol35.0032>

Submitted: June 2, 2020
Accepted for publication: October 22, 2020
Last revision: November 19, 2020

Abstract: This study tested the null hypothesis that antihistamine-containing syrup does not change salivary metabolites *in vitro* and *in vivo*. For the *in vitro* experiments, saliva from 10 volunteers was mixed with a syrup or pill suspension of loratadine (1 mg/ml Claritin®, Schering-Plough, Rio de Janeiro, Brazil). For the *in vivo* experiment, 10 volunteers performed a mouth rinse with 10 mL of antihistamine syrup (Claritin®; Schering-Plough, Rio de Janeiro, Brazil) for 20 seconds and then discarded the rinse water. After 20 seconds, 5 mL of unstimulated whole saliva was spit into a plastic tube kept on ice. The protein profile of *in vitro* and *in vivo* experiments was analyzed using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The samples were also analyzed by nuclear magnetic resonance (NMR) spectroscopy, followed by Principal Component Analysis and Wilcoxon test ($p < 0.05$). There were differences in salivary metabolites after syrup interaction. The salivary concentrations of acetate, n-caproate, arginine, glutamate, and lysine among other metabolites were reduced with the syrup in both *in vivo* and *in vitro* experiments ($p < 0.05$), but no differences were observed when the pill suspension was used ($p > 0.05$). Similar changes in metabolite profiles were observed in both *in vitro* and *in vivo* experiments. Electrophoresis revealed no difference in the salivary protein pattern. The null hypothesis was rejected because the intake of syrup medicine changes the salivary composition and influences oral homeostasis and susceptibility to oral diseases.

Keywords: Saliva; Anti-Allergic Agents; Proteins; Electrophoresis; Magnetic Resonance Spectroscopy

Introduction

Allergy is one of the most common causes of respiratory disease, and according to the World Health Organization, asthma kills around 1,000 people daily and affects 339 million. Low- and middle-income countries disproportionately suffer the most severe cases.^{1,2} Antihistamine, an allergy medication, is commonly used alone or in association with pseudoephedrine. Patients with allergies frequently need medication throughout their life,^{3,4} and it is important to assess the effect of these medicines on saliva.



Human saliva, a complex mixture of proteins and small molecules, performs important and well-recognized functions that are needed to maintain oral health and the homeostasis of the oral environment.^{5,6,7} Adsorption of salivary metabolites on the oral surface is influenced by tooth anatomy, chemical composition of saliva, and microbial flora and may lead to the development of oral disease, such as caries.^{8,9}

Liquid medicines or mouth rinses can interact with saliva components^{10,11} and influence oral homeostasis^{12,13} by promoting biochemical alterations that affect the tooth surface. In addition, the high sucrose concentrations of liquid medicines can increase bacterial growth.^{14,15} Pediatric medicines, in particular, are generally prescribed in a liquid form and include sucrose as a flavor adjunct and preservative. The association between medicinal syrups and the incidence of dental caries is well described in the literature.^{12,13,14,15,16}

Among several analytical techniques used for metabolomic studies, nuclear magnetic resonance (NMR) spectroscopy is suitable to identify low-molecular-weight salivary metabolites.^{7,9,17,18} NMR is widely used because it is a non-destructive technique that provides detailed information on molecular structure, both for pure compounds and complex mixtures such as salivary fluid.^{7,9,17,19,20}

Using high-resolution ¹H-NMR spectroscopy, this study tested the null hypothesis that antihistamine-containing syrup does not change salivary metabolites.

Methodology

Subjects of the study

This study was approved by the Local Ethical Committee (64/65-2009 – CEP NESC UFRJ), and written informed consent was obtained from each subject.

The same subjects participated in the *in vitro* and *in vivo* studies. Ten volunteers without any systemic disease and allergies were recruited from the Dental School (Universidade Federal do Rio de Janeiro) for *in vitro* and *in vivo* experiments. The systemically healthy subjects were 24–30-years old (mean age 26.6 years). Subjects who were

diagnosed with systemic comorbidities (*i.e.*, obesity, diabetes, renal failure, and Sjogren Syndrome), who had bleeding oral lesions, who were undergoing medicine administration such as antihistamine, or who had taken systemic antibiotics within 6 months prior to saliva sample collection were excluded. The subjects were free of periodontal disease or dental caries, as determined by visual-tactile examination by a single examiner. All volunteers provided informed consent, and the local research ethics committee approved the study.

In vitro experiments

Whole saliva (5 mL) was collected in the morning at the same time of the day (8:00 am–10:00 am) to avoid fluctuations in the results due to the circadian saliva cycle.^{21,22} Patients were instructed to expectorate saliva samples into a plastic tube kept on ice. Each patient was instructed to refrain from oral activities, such as eating or drinking (excluding drinking water), as well as to not brush their teeth for 2 hours prior to sample collection. The samples were centrifuged at 10,000 ×g for 60 minutes at 4°C to remove cells and debris.^{7,9,17,18} The supernatants were subsequently stored at –80°C until NMR analysis. For *in vitro* experiments, 1 mg/ml Claritin® syrup (Schering-Plough, Rio de Janeiro, Brazil) diluted in distilled water at pH 7.0 and mixed with 0.45 mL saliva was used. This concentration was selected on the basis of a pilot study that demonstrated a similar resonance intensity between *in vitro* and *in vivo* experiments (*data not shown*). Control samples were prepared as described using 5% distilled water instead of the drug solution. After 20 seconds of incubation, the mixture was centrifuged at 3,000 ×g for 3 minutes and the supernatant was used for NMR analysis. This protocol was according to that in a pilot study, in which no pellet formation was observed.

The second approach used a Claritin® pill (Schering-Plough, Rio de Janeiro, Brazil) that was dissolved in 1 mg/mL of citric acid (same concentration of the syrup), and after the extraction of loratadine, it was neutralized to pH 7.0 using sodium hydroxide. The suspension was centrifuged at 3,000 ×g for 5 minutes, and the supernatant contained

loratadine at ~2.61 mM. The resultant solution also contained lactose. The pill extract was then mixed with the saliva of a volunteer. The same pill extract was used for all samples. After 20 seconds of incubation, the saliva mixture was centrifuged at 3,000 \times g for 3 minutes and the supernatant was used for NMR analysis. This protocol was according to that in a pilot study, in which no pellet formation was observed.

In vivo experiments

Ten volunteers performed a mouth rinse with 10 mL of antihistamine syrup (Claritin[®]; Schering-Plough, Rio de Janeiro, Brazil) for 20 seconds and then discarded the rinse water. This rinse period was according to that in a pilot study (*data not shown*). After this period, 5 mL of whole saliva was expectorated from the mouth into a plastic universal tube placed on ice. The volunteers were instructed to rinse their mouth with water and spit it out to remove the residual syrup, and none of them reported any adverse effect. The samples were centrifuged at 10,000 \times g and 4°C for 60 minutes, and the supernatants were used for NMR analysis.^{7,9,17,18}

Nuclear magnetic resonance analysis

All samples were prepared by mixing 0.45 mL of the salivary material with 0.05 mL of deuterium oxide (Cambridge Isotope Laboratories Inc., Cambridge, USA), which provides a field-frequency lock, and 0.01 mL of 5 mM sodium 2,2-dimethyl-2-silspentane-5-sulfonate solution (DSS; Sigma-Aldrich, Milwaukee, USA). Deuterium oxide was used as a lock to the magnetic field with the sample (lock), and DSS was used as the chemical shift reference ($\delta = 0.00$ part per million [ppm]).

NMR spectra were acquired using a Bruker 400 MHz Avance spectrometer (Bruker Biospin, Rheinstetten, Germany) equipped with a Bruker 5-mm high-resolution probe operating at a frequency of ¹H at 400.13 MHz and a probe temperature of 25°C. Carr-Purcell-Meiboom-Gill pulse sequence for ¹H spectrum and ¹H-¹H total correlation spectroscopy experiments with acquisition parameters of 70 ms mixing time and 2048 \times 256 points were performed according to Fidalgo et al.^{7,9} NMR signals were

determined over a range of frequencies defined by the resonance difference between a specific peak and a standard (DSS), expressed in ppm. After spectra acquisition, edge effects were evaluated by overlaying all spectra using Topspin (Bruker Biospin). In brief, each chemically distinct hydrogen nucleus of low-molecular-weight metabolites from salivary samples produce an NMR signal that is measured as a chemical shift, represented by ppm, which characterizes a metabolite.²³ The assignment strategy of the metabolites in the chemical shifts included the use of the Human Metabolome database (<http://www.hmdb.ca/>), assignment strategies used in previous studies,^{7,9,17,18} and ¹H-¹H total correlation spectroscopy experiments.

Electrophoresis analysis

To evaluate salivary protein interaction with pill or syrup compositions, mixtures of salivary samples from three subjects were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, performed according to the method by Laemmli,²⁴ using 12% running gel and 4% stacking gel, and the samples were stained using Coomassie blue.

Statistical analyses

Metabolite intensities were determined using the statistical program AMIX (Bruker Biospin). Saliva spectra were compared before and after antihistamine syrup treatment. Each spectral dataset was normalized to the total sum of the integrals to compensate partially for differences in concentrations, and the matrix was normalized by scaling the dataset using Pareto scaling.²⁵ Each NMR spectrum was reduced to a smaller number of variables, calculated by integrating regions of equal bucket size of 0.02 ppm. The water region was excluded to eliminate variation. Regions of syrup and pill suspension components, *i.e.*, 0.80–1.88, 2.33–4.03, 4.50–5.50, 6.95–7.95, and 8.27–8.74 ppm, were also excluded. The datasets were stored in a matrix E with 35 rows (35 data sets from the samples: 10 before drug application, 10 after *in vitro* syrup drug application, 10 after *in vivo* syrup drug application, and 5 after *in vitro* pill suspension) and 335 columns (335 chemical shifts). The bucket tables

were normalized using the sum of intensities, and the data were submitted to the Pareto scaling method²⁵, before applying the partial least squares-discriminant analysis (PLS-DA) method. PLS-DA used the initial input variables of each group that were stored in the y-table (0 and 1) and was performed using Metaboanalyst 3.0 software (www.metaboanalyst.ca/MetaboAnalysts).²⁶

Metaboanalyst 3.0 software was also used to determine the predictive performance of the models; in addition, each model was evaluated using Q^2 , R^2 , and accuracy (ACC), for the purpose of cross-validation.²⁷

Further, the intensity of each peak corresponding to the chemical shifts was tabulated using Statistical Package for the Social Sciences 20.0 (SPSS Inc., Chicago, USA) and subjected to normality analysis by applying Shapiro-Wilk test. Wilcoxon test was used to evaluate the differences in metabolite intensities before and after the antihistamine syrup or pill. The significance level was set at 5%. The power analysis was calculated on the basis of the acetate peak because this metabolite appeared frequently in the obtained spectra and is unambiguously assigned singlet. For this calculation, the means and standard deviations of the intensities (arbitrary unities) and the sample size of each group (syrup in *in vivo* mixture, syrup in *in vitro* mixture, and pill interaction) were obtained. The power analysis is capable of measuring the effect size that can be detected using a given sample size. For this purpose, a confidence interval of 95% was adopted and a two-tailed test using OpenEpi 3.04.04 software was performed.

Results

We used two different experimental strategies: *in vitro* and *in vivo*. *In vitro* experiments were performed by incubating whole saliva and antihistamine-containing syrup in a plastic tube. *In vivo* experiments required each patient to rinse with a medicinal solution prior to saliva collection. After 20 seconds, the subject expectorated the mixture of medicine and saliva. After centrifugation, each mixture was analyzed by NMR. Figure 1 illustrates the whole

saliva ¹H NMR spectra as well as resonance intensity differences before and after drug application for *in vitro* and *in vivo* experiments. For statistical analyses, the water region was excluded to eliminate variation. To avoid resonances from the syrup and pill components, the following regions were also excluded: 0.80–1.88, 2.33–4.03, 4.50–5.50, 6.95–7.95, and 8.27–8.74.

Figure 2 shows the PLS-DA plot of ¹H NMR results of saliva samples with and without antihistaminic mixture with the syrup or pill. The percentage of variance in Component 1 (CP1) was 29.9%, and Component 2 (CP2) was 28.6%. The accuracy was 85.71%, R^2 was 94.79%, and Q^2 was 69.15%. The syrup interacted with saliva in both the *in vitro* and *in vivo* experiments. The differences in metabolites were similar regardless of the protocol used.

To understand the differences, we determined which metabolite was modified in the presence of the syrup. The *in vitro* and *in vivo* experiments show similar changes in salivary metabolite levels. The intensity of resonances from components such as acetate, amino-butyrate, arginine, glutamate, lysine, n-caproate, proline, iso-caproate, and isoleucine was statistically significant ($p < 0.05$). Resonance assignments of these salivary metabolite components before and after antihistamine application are shown in Table. Some liquid medicines, such as the antihistamine syrup used in this study, are chemically stable at low pH, and citric acid is generally used for this purpose.¹³ Citric acid commonly acts as a salivary-stimulating agent by increasing salivary flow; thus, some metabolites could be diluted in saliva.^{11,25} However, our study demonstrated that both *in vitro* and *in vivo* drug application resulted in a reduction of specific metabolite concentrations. For this reason, we suggest that the antihistamine syrup also interacts with specific metabolites, reducing their concentrations in the saliva. The power analysis demonstrated an effect size of 100% for the syrup in the *in vivo* experiment, 99.99% for the mixture in the *in vivo* experiment, and 4.24% for pill interaction.

The electrophoresis analysis revealed no differences in the salivary protein pattern between control samples

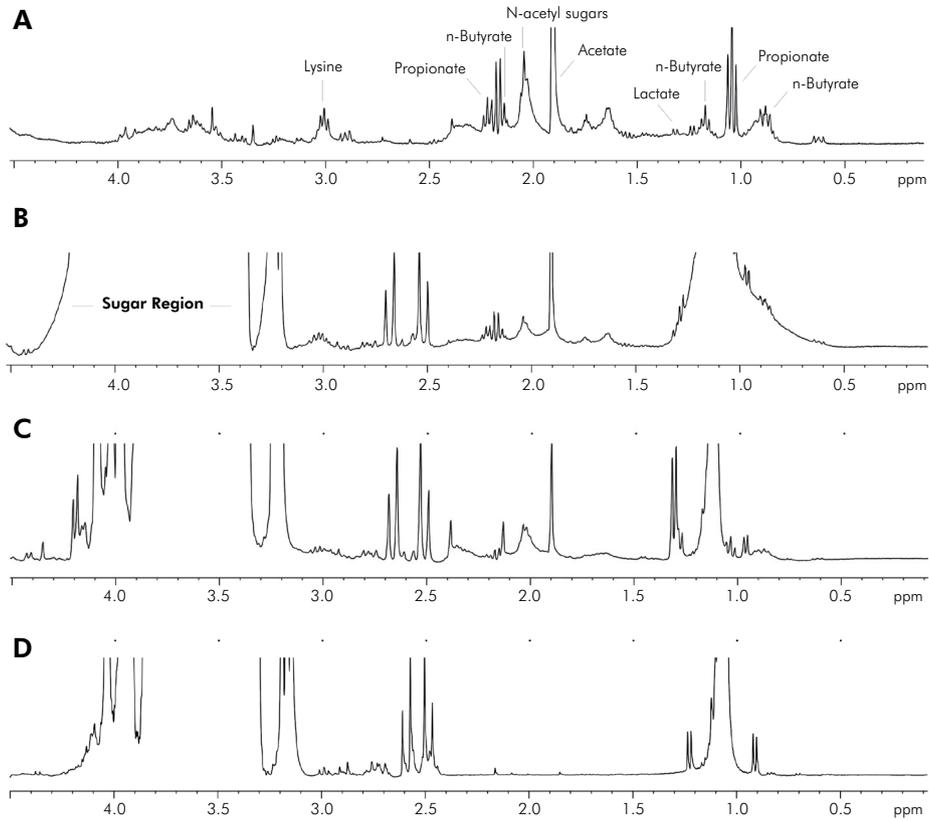


Figure 1. Representative ^1H NMR spectra of whole saliva before and after the administration of antihistamine syrup or pill mixture (0–4.5 ppm). A: Spectrum of whole saliva; B: Spectrum with *in vitro* syrup mixture; C: Spectrum after *in vivo* syrup experiment (rinse); D: Spectrum with *in vitro* pill suspension.

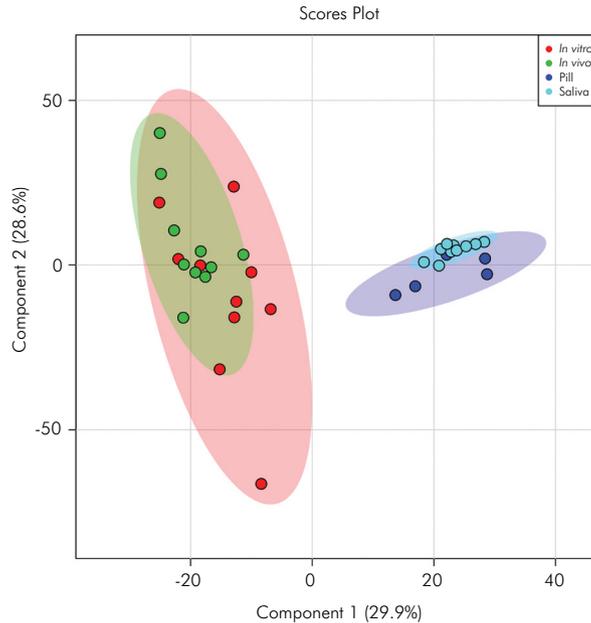


Figure 2. Two-component Principal Component Analysis model showing sample clustering of saliva without any medicine (black) and after treatment with antihistamine syrup *in vitro* (blue) and *in vivo* (green). *In vitro* treatment with the antihistamine pill extract is shown in red. The percentage of variance captures by 36.38% and Principal Component 2 (PC2) was 25.81%.

Table. Resonance assignments of salivary metabolite components before and after application of antihistamine mixture.

Metabolites	¹ H Chemical Shift (ppm)	Multiplicity*	Assignment	Syrup ^a	Syrup <i>in vivo</i> ^b	Pill interaction ^c	p-value**
Acetate	1.92	s	0	↓	↓	NC	< 0.01 ^a < 0.01 ^b 0.94 ^c
Aminobutyrate	1.88, 2.29 3.02	m, t t	β -CH ₂ , α-CH ₂ , γ -CH ₂	↓	↓	NC	< 0.01 ^a < 0.01 ^b 0.92 ^c
Arginine	1.71, 1.93, 3.25, 3.68	m, m, t, t	γ -CH, β-CH ₂ , δ-CH ₂ , α-CH	↓	↓	NC	< 0.01 ^a < 0.01 ^b < 0.64 ^c
Glutamate	2.10, 2.37, 3.79	m, m, t	β-CH ₂ , γ-CH ₂ , α-CH	↓	↓	NC	< 0.01 ^a < 0.01 ^b 0.25 ^c
Lysine	1.49, 1.73, 1.90, 3.01, 3.76	m, m, m, t, t	γ-CH ₂ , δ-CH ₂ , β-CH ₂ , -CH ₂ , α-CH	↓	↓	NC	< 0.01 ^a 0.03 ^b 0.47 ^c
n-caproate	0.83, 1.35, 1.58, 2.15	t, m, m, t	-CH ₃ , δ-CH ₂ 's, β -CH ₂ , α-CH ₂	↓	↓	NC	< 0.01 ^a < 0.01 ^b 0.32 ^c
Proline	2.07, 2.07 2.35, 3.40 4.16,	m, m m, m,	α-CH ₂ , β-CH ₂ , β-CH ₂ , δ-CH ₂ α -CH ₂	↓	↓	NC	< 0.01 ^a < 0.01 ^b 0.76 ^c
Iso-caproate	0.91, 1.55, 1.58, 2.15	d, m, m, t	δ-CH ₃ 's, β-CH ₂ , γ -CH ₂ , α-CH ₂	↓	↓	NC	0.27^a < 0.01 ^b 0.98 ^c
Isoleucine	0.94, 1.01, 1.47, 1.99, 3.68	t, d, m, m, d	CH ₃ , β-CH ₂ , -CH ₂ , β-CH, α-CH	↓	↓	NC	< 0.01^a < 0.01^b 0.78 ^c

*Multiplicity: s – singlet, d – duplet, t – triplet, q – quadruplet; **Wilcoxon test; p < 0.05. p-values indicate the comparisons with the control group; NC: no change.

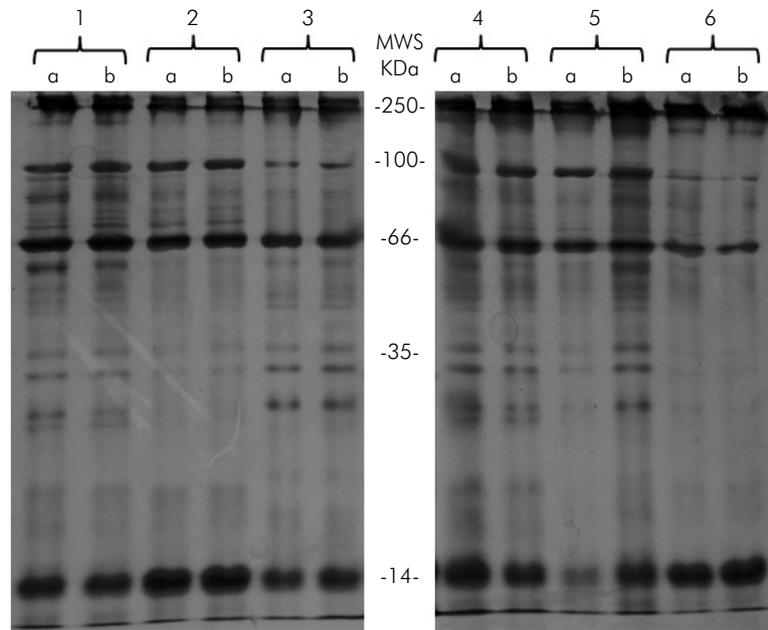
and after mixture of the samples with the syrup or pill suspension (Figure 3).

Discussion

In our study, high-resolution ¹H NMR spectra demonstrated changes in metabolite levels after application of antihistamine syrup using both *in vitro* and *in vivo* methods.

Principal Component Analysis is often used in metabolomic studies to classify subjects with different

conditions.^{28,29,30} In our study, principal component analysis illustrated differences in the metabolite profile of saliva before and after application of antihistamine syrup, using both *in vitro* and *in vivo* methods. Additionally, the analysis demonstrated that the pill suspension assay induced no statistically significant changes compared with the control group. Therefore, our experiments indicated that the active drug, loratadine, did not cause changes in salivary metabolite levels. We also evaluated the antihistamine pill in citric acid, the same acid found



The letter "a" indicates control group, the 1b, 2b, and 3b are the salivary protein pattern after mixture with pill suspension and 4b, 5b, and 5b after mixture with syrup.

Figure 3. Electrophoresis demonstrating no difference among groups.

in the syrup, and we observed no difference in the saliva component. The differences in metabolites were similar regardless of the protocol used. Furthermore, the changes in concentration of metabolites were related to the syrup composition because the same experiment performed with the antihistamine pill did not change the salivary composition. We conclude that the changes in saliva are related to the syrup formulation. Although 20 seconds is sufficient time to swallow a liquid medicine, the clearance of a liquid in the oral cavity takes approximately 5–10 min.³¹ Further, the duration of 20 seconds was used according to that in a pilot study.

The clearance of sucrose in the oral cavity is slow, and sucrose can be used by oral biofilms for energetic metabolism, producing organic acids.³² Our results indicate that children using antihistamine syrup should be encouraged to brush their teeth after use. To minimize tooth damage in children who frequently use antihistamine syrups, oral hygiene should be practiced after administration of liquid medicines.^{33,34} This procedure will remove sugar contained in the syrup and prevent the development of dental caries. Administration of syrup-based medicines at night

should be avoided because salivary flow is naturally lower and its protective capacity is reduced.^{33,35}

The interaction of salivary proteins with oral medicines has been previously studied.^{10,36,37} In contrast, limited information is available regarding changes in low-molecular-weight metabolites after interaction with oral medicines. A study using NMR demonstrated differences in the metabolic profile of human saliva before and after treatment with a mouth rinse.³⁰ The chemometric approach could identify alterations in metabolites such as glutamine, proline, leucine, valerate, acetoacetate, and arginine.

The authors suggested that this type of study design could benefit further investigation in the metabolomic field. In our study, some organic acids, such as acetate, n-caproate, and iso-caproate, also had reduced concentrations after mixing with antihistamine syrup. Metabolites such as acetate, n-caproate, and iso-caproate are probably from microorganism metabolism.⁷⁹ These salivary metabolites decrease the salivary pH of dental plaque, and if this pH reaches the critical pH, it can lead to enamel demineralization.³⁸ Although these metabolites were immediately reduced after syrup interaction, it is important to point out that

syrup contains high levels of sugar, and therefore, it potentially increases organic acids with time.

Similarly, the concentrations of amino acids such as arginine, glutamate, lysine, isoleucine, aminobutyrate, and proline were significantly lower after antihistamine syrup application, as determined using *in vitro* and *in vivo* methods. The role of amino acids in oral diseases remains unclear. Massoudi et al.³⁹ suggested that the presence of various amino acids prevents bacterial growth. Amino acids can also exert a beneficial effect against dental caries owing to the enhancement of NH_4^+ . Furthermore, free amino acids exhibit different chemical properties and their combination can produce proteins with a large variety of functions in the oral cavity;⁴⁰ thus, their functions in the oral cavity must be thoroughly understood.

In the present study, the local effect of antihistamine-containing syrup on salivary metabolites was evaluated. The antihistamine syrup loratadine presents a pKa of ~4.33 (<https://hmdb.ca/metabolites/HMDB0005000>) and is a weakly ionizable base with pH-dependent solubility, and its solubility decreases exponentially with an increase in pH. Therefore, loratadine is more soluble in the stomach, which has a lower pH than the oral mucosa, which has a pH of ~7.0.⁴¹ It is important to highlight that not all drugs can be efficiently absorbed through the oral mucosa.⁴² For example, the systemic bioavailabilities of peptides and proteins are typically less than 5% of the administered dose with transmucosal delivery because of the physicochemical barrier of the oral mucosa, which contains enzymes that break down peptides. In addition, the barrier properties of the epithelium result in the oral mucosa being an efficient barrier to drug penetration, allowing only small quantities of a drug to penetrate. Furthermore, oral mucosal delivery is suitable only for drugs with a high potency. These layers provide a unique challenge for drug delivery via the oral mucosa.⁴²

A limitation of this study was the population studied. The antihistamine syrup is often used by children; however, in this study, for the first time, we aimed to analyze and compare the effects of antihistamine both *in vitro* and *in vivo*. Therefore, a young adult population was selected for this purpose because children have a limited ability

to spit. There is limited information available regarding the differences between the low-molecular-weight metabolites in saliva in children and those in the adult population. However, Fidalgo et al.⁷ demonstrated differences in metabolites based on phases of life, ages, and different dentitions. The authors demonstrated that individuals with permanent dentition (older children) presented higher levels of butyrate, ambiguous assignment, lysine, sugar region, and phenylalanine and propionate in comparison to primary dentition (younger children). In addition, Ellul et al.⁴³ identified differences in the concentration of specific metabolites in the serum between children and adults. These findings indicate that the effect of antihistamine-containing syrup in salivary metabolites from a child population can be different from the data reported here; therefore, further studies in a younger population are needed.

In vitro studies are often difficult to correlate with clinical data because of the complex environment of the human saliva.⁵ In our study, the *in vitro* and *in vivo* interactions between human salivary components and antihistamine syrup were similar. Therefore, our *in vitro* assays were validated and could be used to study the interaction of other medicines with salivary metabolites. In addition, we found that NMR is a reliable method to study small molecules.

Conclusions

The null hypothesis was rejected because the antihistamine syrup altered the salivary metabolite profile. The observed changes are likely related to syrup formulation rather than the active component loratadine. Similar changes in metabolite profiles were observed in both *in vitro* and *in vivo* experiments.

Acknowledgments

The authors acknowledge the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ), Centro Nacional de Ressonância Magnética Nuclear (CNRMN), and Instituto Nacional de Ciência and Tecnologia de Biologia Estrutural e Bioimagem (InBEB) for their financial support.

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