Original Research Article

Effect of chronic commercial sweeteners consumption in lymphocytes of Peyer's patches.

ABSTRACT

Aims: To know the effect of chronic commercial sweeteners consumption in lymphocytes of Peyer's patches.

Study Design: a prospective, longitudinal, comparative and experimental study.

Place and Duration of Study: The study was conducted in the Nutrition Research Laboratory of the Faculty of Medicine of Universidad Autónoma del Estado de México (UAEMéx) between August 2018 and May 2019 and was approved by the Bioethics committee.

Material and Methods: **We were used** Ttwo groups of mice (sex?) **with** of different strains were used: 1) Balb/c and 2) CD1, both at**from** 8 weeks old age. The groups were divided into 4 subgroups: 1) Control (without sweetener), 2) Sucrose …dosage….. (table sugar ….amount?), and two groups of commercial **sweetners**sweeteners 3) Splenda® (Purity and dosage), and 4) Svetia® (Purity and dosage). The mice consumed the supplementation for 8 weeks. In Addition, were quantified glucose (in plasma?), percentage of lymphocytes from Peyer's patches , water and food consumption (on a daily basis?).

Results: Mice increased their body weight after 6 weeks of treatment. The animals of

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Control and Sucrose subgroups showed a significant gain of 5g of weight, compared with the Splenda® and Svetia® subgroups, which increased 4g. In tThe subgroup treated with **of** Splenda the **significantly reduced** blood glucose was reduced significantly. Svetia and Control groups consumed more water without sweetener. Food consumption was variety. By the end, the percentage of lymphocytes from Peyer´s patches increased in the Sucrose subgroup, but decreased in other subgroups.

Conclusion: it is a fact The consumption of **that** sweeteners may modify the lymphocyte population of Peyer's patches in the small intestine and this variation depends to the frequency of consumption? , the strain of the rodents and the type of sweetener.

Comment [JFR Rev2]: Body weight gain?

Comment [JFR Rev3]: Trademarks, use the symbols everywhere in the manuscript.

Comment [JFR Rev4]: Meaning?

Comment [JFR Rev5]: Significantly or not?

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Keywords: sweeteners, Peyer´s patches, lymphocytes, body weight, blood glucose, water consumption.

1. INTRODUCTION

Sweeteners are chemical compounds that have the ability to produce a sensation of sweetness [1] and they have various effects on health [2, 3]. Sucrose (table sugar), is the oldest used sweetener and provides energy to the body [4]. The increase in chronic non communicable diseases and sedentary lifestyle are causing consumers to look for products that are reduced in energy and therefore in sugar, using more and more non-caloric commercial substitutes [5]. These offer a sweet taste to food, but with a lower energy content [6, 7]. The preference for sweet taste varies according to genetics and age [8], it is fundamental in the nutritional status [9], therefore, there is a need to look for sugar substitutes, with a similar effect on taste, but with less energy [10]. Sweeteners are classified as natural and artificial [11]. Artificial as sucralose, are produced by chemical synthesis, have little or no energy supply, with power than sucrose sweetener [12]. This sSweetener

was synthesized in 1976, and is approximately 600 times sweeter than sucrose [13]. It is manufactured by selective halogenation of sucrose, is thermostable, resists a wide variety of pH, is not metabolized or stored in the body, and is excreted unchanged in urine and feces [14]. 85% of sucralose is not absorbed, the remaining 15% is absorbed by passive diffusion [15]. Baird, IM et.al, in 2000, published a study related to the tolerance of sucralose in humans, they confirm that it does not generate adverse effects on health [16]. Among the natural we found stevia, it's come from vegetable products, give energy power and they have a sweetening power inferior or similar to sucrose (300 times sweeter than sucrose) [17, 18]. Steviol glycosides isolated from the leaves of the plant, *Stevia Rebaudiana Bertoni*, contains a *Stevioside* and *Rebaudioside A* [19]. Their metabolism begins in the intestine, they are broken down to steviol with help of the intestinal microbiota, mainly by *Bacteroides sp*., and they are absorbed by facilitated diffusion to the blood. Finally, steviol is secreted in the urine as steviol glucuronide and in feces like free esteviol [20, 21]. Stevia is safe when used as a sweetener, suitable for diabetic patients, with phenylketonuria, obese and for those who wish to avoid the consumption of sugar in the diet [22]. It is known that its use does not alter blood glucose concentrations [23], for which they are well accepted in diabetic patients [24], do not contribute to dental caries [25] and can be used in pregnant women [26].

The gut-associated with lymphoid tissue (GALT) is located in the mucosa of the gastrointestinal tract [27], contains the largest surface area of exposure to microorganisms, as it contains a diverse and dense microbiota that are not pathogenic to the host [28, 29]. The mucosa of the gastrointestinal tract is able to identify pathogenic and nonpathogenic substances, and therefore discern between producing or not, an immune response [30]. The immunological defense in the intestine is carried out by the GALT lymphocytes, organized in compartments, the Peyer's patches (inductor site), the lamina propria (effector site) and the isolated lymphoid follicles (ILF) [31]. The most important of these structures is that they contain a large number of cells, derived from a cellular precursor generated in the bone

marrow [32]. In the small intestine, there are about 200 Peyer's patches (PP), each one consists in aggregates of B cells (lymphoid follicles), surrounded by rich areas in T cells and antigen-presenting cells (APCs) [33]. On its surface there are flattened epithelial cells with few villi and mucus-producing cells [34]. The PP can be considered as the immunological sensors of the intestine and are an initial contact site with the antigens [35]. When antigenic stimulation occurs in the PP, the lymphocytes migrate to the blood, proliferate and differentiate in the spleen before returning to the lamina propria and other areas of the mucosa [31].

The effect of sweeteners on the immune system is controversial and is not yet clear. It has been observed that the use of glucose, fructose and sucrose, cause reduction of phagocytic activity of peripheral blood neutrophils [36]. The effect of sucralose has been studied in lymphoid organs such as spleen and thymus [37], doses greater than 3000 mg/kg showed changes in the thymus [38] and reductions in peripheral white blood cells and lymphocyte count have been observed [39]. On the other hand, stevia administered at different doses increased phagocytic activity and proliferation of T cells [40]. In another study, they found that steviol has no effect on the release of TNF-α, and IL-1β in THP-1 human monocytic cells when stimulated by LPS [41]. In human colon carcinoma cell lines, the effect of stevioside on the release of IL-8 was studied, using TNF-α as a stimulator, they found that steviol reduces the expression of NF-kB [42].

With the intention of improving the quality of food, sugars are partially or totally replaced by sweeteners, this is seen in the increase of commercial products that contain them [43]. Splenda® contains sucralose (%? Purity?) and Svetia® has Stevia (%? Purity?), both are the most used commercial forms in Mexico, are distributed in restaurants and are sold in all markets and malls.

These sweeteners are used as additives in more than 50% of low caloriee commercial products (source? reference?) and taking into account that Peyer's patches are the first

immunological contact zone of sweeteners in the GI system, it is necessary to know the effect of chronic commercial sweeteners consumption in lymphocytes of Peyer's patches.

2. MATERIAL AND METHODS

2.1 Study design

A prospective, longitudinal, comparative and experimental study was carried out. Two different strains of mice were used: Balb/c and CD1, from 8 weeks old, weighing between 19.5 g and 22.3 g. Both groups were fed normal standard food Rodent Laboratory Chow 5001 from Purina and water ad libitum. They were kept in plastic cages in groups of 4 each, under pathogen-free conditions and with light/dark cycles of 12 hours. The study was conducted in the Nutrition Research Laboratory of the Faculty of Medicine of the Universidad Autónoma del Estado de México (UAEM) and was approved by the Bioethics Committee of the same faculty. The mice were managed based on NOM-062-ZOO-1999, Specifications for the production, care and use of laboratory animals [44].

2.2. Distribution of groups and administration of sweeteners

The mice were distributed into two groups: Group 1) Balb/c strain mice and Group 2) CD1 strain mice. Each group were divided in 4 subgroups (n=8): A) Control Group (CL), without sweetener, B) Sucrose Group (Suc), C) Splenda Group (Spl), D) Svetia Group (Svt).

Splenda®)(Brand, supplier, purity in active ingredient) and Svetia® Brand, supplier, purity in active ingredient) are **the** commercially available in México. **names of the products that contain Sucralose and Svetia in Mexico**. The solutions were prepared with the treatments (sweeteners) in ultrapure water, they were placed in the drinkers daily, for oral consumption during the 24 h the 7 days of the week. The concentration used was 41.66 mg / mL of **Sucrose** Sucrose (Brand, supplier, purity) and 4.16 mg / mL of Splenda and Svetia in accordance with the recommendations of Official Mexican Standard NOM-218-SSA1-2011

Comment [JFR Rev7]: Define the strains, for the readers not familiar with the subject, include a brief definition/description of the strains of mice.

Comment [JFR Rev8]: Sex?
Males or females mice?
The growth, food and water consumption and metabolic response is different in males and females, and indication about the sex of the mice is not included in any part of the work.

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Comment [JFR Rev9]: Why these strains were selected?
What is the rationale behind the selection of these two strains?

CD1 is a multipurpose type of mice model, but Balb-c is more commonly used in experiments with cancer.
We can guess that CD1 can be a «regular» mice and t he Balb-c a «prompt-to-be-sick» model, but I would like the authors to include a brief description/explanation of the rationale behind selectiong these two types.

The work is about sweetners, why instead of using Balb-c, a model such as db/db or ob/ob was not used? More connected with the problems of diabetes or obesity or both?

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for non-alcoholic flavored drinks (45). The treatment was administered for 6 weeks, starting on the 60th day old of the animals.

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2.3 Determination of body weight and blood glucose

Quantification of body weight was performed weekly, starting at week 8. Weight measurements were made with anesthetized mice (0.1 mL of 1% sodium pentobarbital).

The concentration of peripheral blood glucose was quantified weekly with an Accu-Chek Perform glucometer. The sample was collected from the middle third of the tail.

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2.4 Water consumption quantification

The water consumption was done by placing 250 mL of water with or without sweetener in each drinker, at 24 h the volume of water consumed was measured and subtracted from the water that remained in the drinking fountain.

2.5 Obtaining samples

After 6 weeks of treatment, the animals were anesthetized with 0.1 mL of 1% sodium pentobarbital and sacrificed by cervical dislocation. One millilitre of blood was obtained by direct cardiac puncture (using a syringe with 50 µl of heparin); from the millilitre of blood, the lymphocytes were purified by density gradient with Lymphoprep ™ (Axis-Shield) (46). The small intestine was removed, and Peyer's patches were removed from it.

Once the Peyer's patches were removed, they were placed in Petri dishes with RPMI medium (3 mL), manually homogenized and filtered with nylon mesh (40-µm) to eliminate the remaining connective tissue. Centrifuged at 2500 rpm / 5 min, the cell button obtained from the Peyer's patches was placed in a hypotonic buffer solution (8.26 g/L of NH_4Cl, 1 g/L

of $KHCO_3$ and 0.037 g/L of EDTA-4Na, with a pH of 7.4) to lyse the erythrocytes. The cell suspension isolated from the Peyer's patches was washed with PBS. The cell viability of the isolated lymphocytes was immediately evaluated with a trypan blue assay. The lymphocytes were counted with Neubauer chamber to obtain the cellular percentage *per* mL of cell suspension.

2.6 Statistic Analysis

The statistical package SPSS version 19 for Windows was used to analyze the data. Tests were made of central tendency (mean), dispersion (standard deviation) and means were compared by means of one-way analysis of variance ANOVA, with Tukey's post hoc test to evaluate intra-group differences. Significance was considered with $p<0.05$.

3. RESULTS

3.1. Changes in body weight after consumption of sweeteners

All mice in group 1 significantly increased their body weight after 6 weeks of treatment. The animals of Control and Sucrose subgroups showed a significant gain of 5 g of weight ($p<0.001$), compared with the Splenda and Svetia subgroups, which increased 4 g (Table 1). In group 2 the increase in weight was similar, the mice of the Control and Sucrose subgroups increased on average 4 g of weight and the subgroups of Splenda and Svetia only 3 g ($p<0.014$). Svetia's group had the lowest weight gain (3 g), compared to Control ($p<0.009$), as shown in table 1. When comparing group 1 with group 2, significant differences were found ($p<0.001$), the weight of animals of group 1 was lower than those of group 2, although the behavior of weight gain was similar.

Table 1. Average weight of mice after 6 weeks of supplementation with sweeteners.

	Control	Sucrose (Brandname®?)	Splenda®	Svetia®	
	Mean ±SD (g)	Mean ±SD (g)	Mean ±SD (g)	Mean ±SD (g)	*p* Value
Body Weight					
		Before Intervention			
Group 1	23.16±0.956	23.98±1.0	20.87±0.587	20.58±1.42	0.001*
Group 2	40.55±0.597	37.85±1.17	40.16±3.49	37.5±1.8	0.009*
		After Intervention (6 weeks)			
Group 1	28.33±1.05	28.81±1.23	24.5±0.609	24.92±1.29	0.001*
Group 2	44.48±0.448	41.45±1.54	43.68±4.22	40.67±2.03	0.014*

One-way ANOVA was performed to determine the differences between the subgroups, it was considered significant with p<0.05. A Bonferroni *post hoc* test* was performed to observe intra-group differences.

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3.2. Glycaemia

The glucose in group 1 showed no significant differences (p<0.122) between the subgroups. In group 2, the blood glucose concentration was higher, the subgroup of Splenda significantly reduced blood glucose (p<0.001), compared with the Control, Sucrose and Svetia subgroups. When comparing the groups, differences were found between them (p<0.001), group 1 had lower glucose concentrations, even in the control groups (Table 2).

Table 2. Blood glucose after 6 weeks of treatment with sweeteners.

	Control	Sucrose	Splenda®	Svetia®	
Glucose	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD	*p* value
Group 1	110.75±13.9	100±16.33	96.87±10.88	108.5±9.59	0.122**
Group 2	174.12±33	201.62±43.89	**133.25±40.73**	205.75±47.33	0.001*

One-way ANOVA was performed to determine the differences between the subgroups, it was considered significant with p <.001. A Bonferroni *post hoc* test* was performed to observe intra-group differences.

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3.3. Water with and without sweetener

Group 1 consumed more water with Sucrose and little water with Splenda ($p<0.001$), compared with the Svetia and Control groups that consumed more water without sweetener (Table 3). In contrast, group 2 consumed more water with Svetia after intervention, without differences between water consumption with Sucrose, Splenda and Control group, as shown in table 3. When comparing the groups, it can be seen that group 1 consumed more water with Sucrose than group 2, in both periods before and after interventions ($p<0.004$), as shown in table 3.

Table 3. Water consumption with and without of sweetener for 6 weeks of treatment.

	Control Mean ±SD mL	**Sucrose** Mean ±SD mg/mL	**Splenda** Mean ±SD mg/mL	**Svetia** Mean ±SD mg/mL	*p* value
Water consumption with and without of sweetener					
		Before Intervention			
Group 1	47.68±0.972	**101±1.32***	**31.83±0.987***	43.29±0.896	0.001**
Group 2	61.65±0.481	**65.95±0.481***	62.95±1.87	60.1±1.17	0.001**
		After Intervention (6 weeks)			
Group 1	43.29±1.0	**166.31±1.16***	48.37±1.36	47.15±1.88	0.001**
Group 2	69.1±0.320	69.1±0.962	69.1±0.320	**72.3±0.641***	0.001**

One-way ANOVA** was performed to determine the differences between the subgroups, it was considered significant with $p<0.001$. A Bonferroni *post hoc* test* was performed to observe intra-group differences.

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3.4 Food consumption

The subgroups of Sucrose and Splenda consumed less food ($p<0.001$), compared to the Control and Svetia subgroups. At the end of the 6 weeks of supplementation, the mice of group 1, subgroup of Sucrose, further reduced their feed intake ($p<0.001$). In group 2, at the beginning they consumed less amount of food in the Sucrose subgroup, although the Svetia subgroup increased their food consumption. At the end of the treatment, the Splenda subgroup consumed more food ($p<0.001$). When comparing group 1 with group 2, it can be

seen that there are no differences (p<0.60) between the groups regarding the amount of consumption, the differences observed are between the subgroups.

Table 4. Consumption of food for 6 weeks of supplementation with sweetener.

	Control Mean ±SD (g)	**Sucrose** Mean ±SD (g)	**Splenda** Mean ±SD (g)	**Svetia** Mean ±SD (g)	*p* value
Food consumption					
		Before Intervention			
Gruop 1	32.08±0.02	**24.08±0.011***	**25.68±0.03***	29.92±0.034	0.001**
Gruop 2	27.1±0.32	**25.6±0.641***	26.52±0.293	**29.7±0.641***	0.001**
		After Intervention			
Gruop 1	32.9±0.755	**16.07±0.939***	31.12±0.649	32.73±1.5	0.001**
Gruop 2	29.7±0.641	28±0.641	**30±2.77***	**27.7±0.320***	0.006**

One-way ANOVA** of one factor was performed to determine the differences between the subgroups, it was considered significant with p<0.05. A Bonferroni *post hoc* test* was performed to observe intra-group differences.

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3.5. Percentage of lymphocytes of Peyer's patches

In group 1, the percentage of lymphocytes increased in the Sucrose subgroup, but decreased in the Splenda and Svetia subgroups, although the differences are not significant (p<0.077). In group 2, a significant decrease can be seen in the subgroups that consumed sweeteners (p<0.028), particularly in the Sucrose subgroup (p<0.022), compared with the control subgroup. When comparing groups 1 and 2, differences in lymphocyte percentages can be appreciated, as well as the different behavior between strains.

Table 5. Percentage of Peyer patches lymphocytes in mice supplemented with sweeteners during 6 weeks.

Control Mean ±SD	**Sucrose** Mean ±SD	**Splenda** Mean ±SD	**Svetia** Mean ±SD	*p* Value

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	%	%	%	%	
Lymphocytes					
Group 1	28.66±3.9	30±4.8	26.1±4.1	26.48±4.3	0.238
Group 2	74.37±4.3	**30.62±1.5***	43.87±2.2	49.12± 2.0	0.028**

ANOVA** of one factor was performed to determine the differences between the subgroups, it was considered significant with $p<0.05$. A Bonferroni *post hoc* test * was performed to observe intra-group differences.

4. Discussion

4.1. *Changes in body weight, food and water consumption*

The results presented in this study showed that mice of group 1 and 2 gained weight with Sucrose consumption, compared with the subgroups of Splenda and Svetia. In group 2, the Svetia subgroup had lower weight gain compared to the Sucrose and Splenda subgroups. Group 2 had greater weight gain, this may be due to the characteristics of the strain. In addition, mice of group 1 had a greater predilection for the consumption of sweeteners, particularly of Sucrose, and lower for Splenda. Group 2 had a greater predilection for the consumption of water with Svetia. This behavior probably is derived from the absence or low energy content of Splenda and Svetia respectively [47, 48], therefore, there was no increase in weight in these groups, compared with the group of Sucrose. It is a fact that drinks with high Sucrose content promote weight gain [49], and is associated with other metabolic disorders that cause states of inflammation and some types of cancer, such as colon cancer [50]. This effect may be due to the fact that carbohydrates interact with receptors of the small intestine that cause secretion of satiety peptides such as the glucagon-like peptide 1 (GLP-1) [51], in addition to gastric distension caused by high water intake with sucrose.

The preference for water with sucrose in rodents is documented [52, 53], and it has been linked to the discovery of sweet taste receptors T1R3 or gusducin in the intestine [54]. In contrast, in the study conducted by Bello and Hajnal in 2005 with rats, they showed that rats do not like drinks with Sucralose, since the consumption of water without sucralose was

similar to the consumption of water with Sucralose [55]. The preference of rodents to sweeteners like Stevia was also studied and it was observed that it has better acceptance compared to other non-caloric sweeteners such as saccharin [56]. This shows that there is variation in the preference between different non-caloric sweeteners and even between species such as mice and rats. Preference also varies between genera; females have a better response to sweetness than males [57].

In groups 1 and 2, Sucrose subgroups consumed less food, but in group 2, Splenda and Svetia increased food consumption. This situation can be attributed to the energy contribution of each sweetener, sucrose provides greater energy content, which causes a satiety sensation in rodents and inhibits appetite. Groups of non-nutritive sweeteners, which contribute little or very few calories, could cause an increase in appetite [49].

4.2 Blood glucose changes

In group 2, sucralose showed a lower concentration compared to the other subgroups. In the Chang *et.al.* study, in 2010, they evaluated the proximal small bowel exposure to sucralose, applied an intraduodenal glucose infusion in ten healthy subjects, took blood samples at frequent intervals and determined that Sucralose does not modify the glycemic response rate [58]. In addition to Sucralose other artificial sweeteners report a glycemic index similar to Sucrose [59]. In another study conducted by Wang *et.al*. in 2011, they investigated the effect of steviol on insulin resistance and the pro-inflammatory status of adipose tissue in mice fed a high-fat diet; oral administration had no effect on body weight, basal insulin levels, glucose tolerance, and insulin sensitivity improved and decreased secretion of inflammatory cytokines in adipose tissue [60], concluded that the use of Stevia is beneficial and helps control blood glucose levels.

A study designed to evaluate the effects of stevia on blood glucose concentration and blood pressure (BP) with active treatment of steviol glucoside or placebo for 3 months. There were no changes in systolic/diastolic blood pressure, glucose concentration and glycosylated

hemoglobin (HbA1c), therefore, oral stevia is well tolerated and has no pharmacological effect [19].

4.3. Changes in the percentage of lymphocyte from Peyer's patches

Studies on the effect of sweeteners on the immune system of the small intestine and particularly Peyer's patches are still scarce. In the study by Sehar *et.al.*, in 2008, they report that Stevia can stimulate the proliferation of T and B cells, increasing humoral and cellular immunity [40], in lymphocytes from the spleen, in Balb/c mice of both sexes, evaluated viability by stimulating lymphocytes *in vitro* directly with stevioside and did not decrease viability. This study was carried out on lymphocytes purified from Peyer's patches, as a site of first contact with the ingested and absorption sweeteners. In addition, the response between strains was different, in Balb/c mice (group 1) sucrose increased the percentage of lymphocytes from Peyer's patches, and in group CD1 (group 2), sucrose reduced this percentage. Another possible explanation for the decrease is found in the type of study and sweetener used. In *in vitro* studies where the product used not for commercial use (Esvetia/Truvia) if not reactive grade, stevia was administered at different doses, some superior to those used in this work, without differences in the results [61]. These results could be extrapolated to the human being since the metabolism of Stevia is similar between rodents and humans. On the other hand, the consumption of sucrose has been related to a decrease in the phagocytic index in neutrophils [36], which means that the consumption of sucrose can alter the function of the cells and particularly in the Peyer's patches as the first contact site of the sweetener. The effect of Sucralose on the immune response of inflammatory bowel diseases has been observed [62], in chronic inflammatory processes as a consequence of an increase in intestinal permeability [63] which causes immunological reactions against diet antigens and components of the intestinal microbiota [64]. In the study carried out by Abou-Donia *et.al*., in rats indicated that Splenda has adverse effects such as reduced microbiota, increased fecal pH, and over-expression of proteins that limit the

bioavailability of drugs [65]. The cause of the inhibition of the bacteria of the intestine is related to the deterioration of the digestive proteases caused by the consumption of Sucralose [66] that increases the intestinal permeability that causes inflammation of the mucous membranes and that leads to the excessive activation of the lymphocytes, which contributes to the pathogenesis of the Intestinal Inflammatory Disease and the Crohn's disease [67, 68].

4. CONCLUSION

It is a fact that The consumption of sweeteners may modify the proportion of lymphocytes from Peyer's patches and this variation depends directly significantly on the on dose, frequency, and type of sweetener. Splenda® decreased significantly the proportion of lymphocytes in Peyer´s patches, particularly in the CD1 strain. As well, we found differences between strains in weight, preference of consumption of sweeteners and water with Splenda®, Svetia® and Sucrose when compared with the consumption of water free of without sweetener.

Comment [JFR Rev21]: Only from a preliminary study with mice model, without any additional safety/toxicology analysis, is not appropriate to make it a «fact».

COMPETING INTERESTS

Authors have declared that no competing interests exist.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NOM-062-ZOO-1999) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

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