

1 **Original Research Article**

2

3 **Effect of chronic sweeteners consumption in**

4 **lymphocytes of Peyer's patches of two mice**

5 **strain**

6

7

8 **ABSTRACT**

9

Aims: To know the Effect of chronic sweeteners consumption in lymphocytes of Peyer's patches of two mice strain.

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 two groups of mice with different strains: 1) Balb/c and 2) CD1, both from 8 weeks old. The groups divided into 4 subgroups of non-nutritive sweeteners consumption: Control, Sucrose, Splenda and Svetia. The mice took the supplementation for 8 weeks. Were quantified glucose, percentage of lymphocytes, water and food consumption.

Results: Mice increased their body weight after 6 weeks of treatment. The animals of Control and Sucrose subgroups showed a significant gain of 5g of weight, compared with the Splenda and Svetia subgroups, which increased 4g. The same way the subgroup of Splenda

significantly reduced blood glucose, Svetia and Control groups that consumed more water without sweetener. Food consumption was variety. By the end, the percentage of lymphocytes increased in the Sucrose subgroup, but decreased in other subgroups.

Conclusion: It is a fact that sweeteners modify the lymphocyte population of Peyer's patches and this variation depends on the frequency, the strain of the rodents and the type of sweetener.

10

11 *Keywords: sweeteners, Peyer's patches, lymphocytes, body weight, blood glucose, water*
12 *consumption.*

13

14

15 **1. INTRODUCTION**

16 Sweeteners are chemical compounds that have the ability to produce a sensation of
17 sweetness [1] and they have various effects on health [2, 3]. Sucrose (table sugar), is the
18 oldest used sweetener and provides energy to the body [4]. The increase in chronic non
19 communicable diseases and sedentary lifestyle are causing consumers to look for products
20 that are reduced in energy and therefore in sugar, using more and more non-caloric
21 substitutes [5]. These offer a sweet taste to food, but with a lower energy content [6, 7]. The
22 preference for sweet taste varies according to genetics and age [8], it is fundamental in the
23 nutritional status [9], therefore, there is a need to look for sugar substitutes, with a similar
24 effect on taste, but with less energy [10]. Sweeteners are classified as natural and artificial
25 [11]. Artificial as sucralose (**Splenda**), are produced by chemical synthesis, have little or no
26 energy supply, with power than sucrose sweetener [12]. Among the natural we found stevia,
27 it's come from vegetable products, give energy power and they have a sweetening power
28 inferior or similar to sucrose [13]. With the intention of improving the quality of food, sugars
29 are partially or totally replaced by sweeteners, this is seen in the increase of commercial

Comment [11]: Is not indicated in any part of manuscript if sucralose is presented as Splenda (commercial name)

30 products that contain them [14]. It is known that its use does not alter blood glucose
31 concentrations [15], for which they are well accepted in diabetic patients [16], do not
32 contribute to dental caries [17] and can be used in pregnant women [18].

34 ~~Stevia~~

35 Steviol glycosides, natural sweeteners isolated from the leaves of the plant, *Stevia*
36 *Rebaudiana Bertoni*, contains a *Stevioside* and *Rebaudioside A* [19]. It is 300 times sweeter
37 than sucrose [20]. Their metabolism begins in the intestine, they are broken down to steviol
38 with help of the intestinal microbiota, mainly by *Bacteroides sp.*, they are absorbed by
39 facilitated diffusion to the blood, finally, steviol is secreted in the urine as steviol glucuronide
40 and in feces like free steviol [21, 22]. Stevia is safe when used as a sweetener, suitable for
41 diabetic patients, with phenylketonuria, obese and for those who wish to avoid the
42 consumption of sugar in the diet [23].

Comment [i2]: I suggest take out this. Is not necessary include subtitles in this part of the manuscript

44 ~~Sucralose~~

45 Sweetener synthesized in 1976, is approximately 600 times sweeter than sucrose [24]. It is
46 manufactured by selective halogenation of sucrose, is thermostable, resists a wide variety of
47 pH, is not metabolized or stored in the body, and is excreted unchanged in urine and feces
48 [25]. 85% of sucralose is not absorbed, the remaining 15% is absorbed by passive diffusion
49 [26]. Baird, IM et.al, in 2000, published a study related to the tolerance of sucralose in
50 humans, they confirm that it does not generate adverse effects on health [27].

Comment [i3]: May be this paragraph could be joined with text stating in line 26 ... *Among the natural we found stevia.....*

Comment [i4]: I suggest take out this. Is not necessary include subtitles in this part of the manuscript

52 ~~Gut-associated with lymphoid tissue (GALT)~~

53 The gut-associated with lymphoid tissue (GALT) is located in the mucosa of the
54 gastrointestinal tract [28], contains the largest surface area of exposure to microorganisms,
55 as it contains a diverse and dense microbiota that are not pathogenic to the host [29, 30].
56 The mucosa of the gastrointestinal tract is able to identify pathogenic and nonpathogenic

Comment [i5]: I suggest take out this. Is not necessary include subtitles in this part of the manuscript

57 substances, and therefore discern between producing or not, an immune response [31]. The
58 immunological defense in the intestine is carried out by the GALT lymphocytes, organized in
59 compartments, the Peyer's patches (inductor site), the lamina propria (effector site) and the
60 isolated lymphoid follicles [ILF] (32). The most important of these structures is that they
61 contain a large number of cells, derived from a cellular precursor generated in the bone
62 marrow [33]. In the small intestine, there are about 200 Peyer's patches (PP), each one
63 consists in aggregates of B cells (lymphoid follicles), surrounded by rich areas in T cells and
64 antigen-presenting cells (APCs) [34]. On its surface there are flattened epithelial cells with
65 few villi and mucus-producing cells [35]. The PP can be considered as the immunological
66 sensors of the intestine and are an initial contact site with the antigens [36]. When antigenic
67 stimulation occurs in the PP, the lymphocytes migrate to the blood, proliferate and
68 differentiate in the spleen before returning to the lamina propria and other areas of the
69 mucosa [32].

70

71 ~~Effect of sweeteners on the immune system~~

72 The effect of sweeteners on the immune system is controversial and is not yet clear. It has
73 been observed that the use of glucose, fructose and sucrose, cause reduction of phagocytic
74 activity of peripheral blood neutrophils [37]. The effect of sucralose has been studied in
75 lymphoid organs such as spleen and thymus [38], doses greater than 3000 mg/kg showed
76 changes in the thymus [39] and reductions in peripheral white blood cells and lymphocyte
77 count have been observed [40]. On the other hand, stevia administered at different doses
78 increased phagocytic activity and proliferation of T cells [41]. In another study, they found
79 that steviol has no effect on the release of TNF- α , and IL-1 β in THP-1 human monocytic
80 cells when stimulated by LPS [42]. In human colon carcinoma cell lines, the effect of
81 stevioside on the release of IL-8 was studied, using TNF- α as a stimulator, they found that
82 steviol reduces the expression of NF-kB [43]. With this previous context, and taking into
83 account that Peyer's patches are the first immunological contact zone of sweeteners, the

Comment [16]: I suggest take out this. Is not necessary include subtitles in this part of the manuscript

84 objective of this study was to compare the effect of chronic sweetener consumption on
85 Peyer's patches lymphocytes from two strains of mice.

86

87 **2. MATERIAL AND METHODS**

88 **2.1 Study design**

89 A prospective, longitudinal, comparative and experimental study was carried out. Two
90 different strains of mice were used: Balb/c and CD1, from 8 weeks old, weighing between
91 19.5 g and 22.3 g. Both groups were fed normal standard food Rodent Laboratory Chow
92 5001 from Purina and water ad libitum. They were kept in plastic cages in groups of 4 each,
93 under pathogen-free conditions and with light/dark cycles of 12 hours. The study was
94 conducted in the Nutrition Research Laboratory of the Faculty of Medicine of the Universidad
95 Autónoma del Estado de México (UAEM) and was approved by the Bioethics Committee of
96 the same faculty. The mice were managed based on NOM-062-ZOO-1999, Specifications
97 for the production, care and use of laboratory animals [44].

98

99

100 **2.2. Distribution of groups and administration of sweeteners**

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

104 The solutions were prepared with sweeteners in ultrapure water, they were placed in the
105 drinkers daily, for oral consumption during the 24 h 7 days of the week. The concentration
106 used was 41.66 mg / mL of sucrose and 4.16 mg / mL of Splenda and Svetia. The treatment
107 was administered for 6 weeks, starting on the 60th day old of the animals.

108

109 **2.3 Determination of body weight and blood glucose**

110 Quantification of body weight was performed weekly, starting at week 8. Weight
111 measurements were made with anesthetized mice (0.1 mL of 1% sodium pentobarbital).
112 The concentration of peripheral blood glucose was quantified weekly with an Accu-Chek
113 Perform glucometer. The sample was collected from the middle third of the tail.

114

115 **2.4 Water consumption quantification**

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

119

120 **2.5 Obtaining samples**

121 After 6 weeks of treatment, the animals were anesthetized with 0.1 mL of 1% sodium
122 pentobarbital and sacrificed by cervical dislocation. One millilitre of blood was obtained by
123 direct cardiac puncture (using a syringe with 50 µl of heparin); from the millilitre of blood, the
124 lymphocytes were purified by density gradient with Lymphoprep™ (Axis-Shield) (45). The
125 small intestine was removed, and Peyer's patches were removed from it.

126 Once the Peyer's patches were removed, they were placed in Petri dishes with RPMI
127 medium (3 mL), manually homogenized and filtered with nylon mesh (40-µm) to eliminate
128 the remaining connective tissue. Centrifuged at 2500 rpm / 5 min, the cell button obtained
129 from the Peyer's patches was placed in a hypotonic buffer solution (8.26 g/L of NH₄Cl, 1 g/L
130 of KHCO₃ and 0.037 g/L of EDTA-4Na, with a pH of 7.4) to lyse the erythrocytes. The cell
131 suspension isolated from the Peyer's patches was washed with PBS. The cell viability of the
132 isolated lymphocytes was immediately evaluated with a trypan blue assay. The lymphocytes
133 were counted with Neubauer chamber to obtain the cellular percentage *per* mL of cell
134 suspension.

135

159 **3.2. Glycaemia**

160 The glucose in group 1 showed no significant differences ($p < 0.122$) between the subgroups.
 161 In group 2, the blood glucose concentration was higher, the subgroup of Splenda
 162 significantly reduced blood glucose ($p < 0.006$), compared with the Control, Sucrose and
 163 Splenda subgroups. When comparing the groups, differences were found between them
 164 ($p < 0.001$), group 1 had lower glucose concentrations, even in the control groups (Table 2).

Comment [i11]: May be is Stevia? Because the values of Splenda in group 2 are very low compared with others subgroups (See table 2)

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

	Control	Sucrose	Splenda	Svetia	
Glucose	Mean \pm SD	Mean \pm SD	Mean \pm SD	Mean \pm SD	<i>p</i> value
Group 1	110.75 \pm 13.9	100 \pm 16.33	96.87 \pm 10.88	108.5 \pm 9.59	0.122**
Group 2	174.12 \pm 33	201.62 \pm 43.89	133.25\pm40.73	205.75 \pm 47.33	0.010*

Comment [i12]: This *p* value is correct? What about 0.006 indicated in the text (line 159)?

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

171 **3.3. Water with and without sweetener**

172 Group 1 consumed more water with Sucrose and little water with Sucralose ($p < 0.001$),
 173 compared with the Svetia and Control groups that consumed more water without sweetener
 174 (Table 3). In contrast, group 2 consumed more water with Svetia, without differences
 175 between water consumption with Sucrose, Sucralose and Control group, as shown in table
 176 3. When comparing the groups, it can be seen that group 1 consumed more water with
 177 sweetener than group 2, particularly in the sucrose subgroup ($p < 0.004$), as shown in table 3.

Comment [i13]: This is correct if you refer to after intervention. Please complete the text with this detail.

Comment [i14]: Please indicate that this consumption is more in sucrose subgroup, yes but in both periods before and after intervention

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

	Control	Sucrose	Splenda	Svetia	
	Mean \pm SD	Mean \pm SD	Mean \pm SD	Mean \pm SD	<i>p</i> value
	mL	mg/mL	mg/mL	mg/mL	
Water consumption with and without of sweetener					
Initial					
Group 1	47.68 \pm 0.972	101\pm1.32*	31.83\pm0.987*	43.29 \pm 0.896	0.001**

Comment [i15]: May be is better Before intervention

Group 2	61.65±0.481	65.95±0.481*	62.95±1.87	60.1±1.17	0.001**
Final					
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**

Comment [I16]: May be is better After intervention (6 weeks)

181 One-way ANOVA** was performed to determine the differences between the subgroups, it was
 182 considered significant with $p < 0.001$. A Bonferroni *post hoc* test* was performed to observe intra-group
 183 differences.
 184

185 3.4 Food consumption

186 The subgroups of sucrose and sucralose consumed less food ($p < 0.001$), compared to the
 187 Control and Svetia subgroups. At the end of the 6 weeks of supplementation, the mice of
 188 group 1, subgroup of sucrose, further reduced their feed intake ($p < 0.001$). In group 2, at the
 189 beginning they consumed less amount of food in the Sucrose subgroup, although the Svetia
 190 subgroup increased their food consumption. At the end of the treatment, the Splenda
 191 subgroup consumed more food ($p < 0.001$). When comparing group 1 with group 2, it can be
 192 seen that there are no differences ($p < 0.60$) between the groups regarding the amount of
 193 consumption, the differences observed are between the subgroups.
 194
 195

196 **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)	<i>p</i> value
Food consumption					
Initial					
Group 1	32.08±0.02	24.08±0.011*	25.68±0.03*	29.92±0.034	0.001**
Group 2	27.1±0.32	25.6±0.641*	26.52±0.293	29.7±0.641*	0.001**
Final					
Group 1	32.9±0.755	16.07±0.939*	31.12±0.649	32.73±1.5	0.001**
Group 2	29.7±0.641	28±0.641	30±2.77*	27.7±0.320*	0.006**

197 One-way ANOVA** of one factor was performed to determine the differences between the subgroups,
 198 it was considered significant with $p < 0.05$. A Bonferroni *post hoc* test* was performed to observe intra-
 199 group differences.
 200

201

202 **3.5. Percentage of lymphocytes of Peyer's patches**

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

209

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

	Control	Sucrose	Splenda	Svetia	
	Mean \pm SD	Mean \pm SD	Mean \pm SD	Mean \pm SD	ρ Value
	%	%	%	%	
Lymphocytes					
Group 1	28.66 \pm 3.9	30 \pm 4.8	26.1 \pm 4.1	26.48 \pm 4.3	0.238
Group 2	74.37 \pm 4.3	30.62\pm1.5*	43.87 \pm 2.2	49.12 \pm 2.0	0.028**

212 ANOVA** of one factor was performed to determine the differences between the subgroups, it was
213 considered significant with $p < 0.05$. A Bonferroni *post hoc* test * was performed to observe intra-group
214 differences.
215

216

217 **4. Discussion**

218 **4.1. The preference for food and water intake, as well as changes in body**
219 **weight vary in each strain of mice.**

220 In recent years the consumption of products containing both natural and artificial sweeteners
221 have acquired great demand for its low energy intake and its sweetness, which can be found
222 in multiple products. In this study, mice of group 1 and 2 gained weight with Sucrose
223 consumption, compared with the subgroups of Splenda and Svetia. In group 2, the Svetia
224 subgroup had lower weight gain compared to the Sucrose and Splenda subgroups. Group 2
225 had greater weight gain, this may be due to the characteristics of the strain. In addition, mice
226 of group 1 had a greater predilection for the consumption of sweeteners, particularly of

Comment [i17]: The subtitle is very long. I suggest modify this, may be as «Changes in body weight and food and water consumption»

Comment [i18]: These sentences are unnecessary because your study is not related to human consumption of sweeteners. So, I suggest take out this. Alternative redaction suggested could be «The results presented in this study showed that mice of group 1 and 2...»

227 Sucrose, and lower for Splenda. Group 2 had a greater predilection for the consumption of
228 water with Svetia. This behavior is derived from the absence or low energy content of
229 Sucralose and Stevia respectively [46, 47], therefore, there was no increase in weight in
230 these groups, compared with the group of Sucrose. It is a fact that drinks with high Sucrose
231 content promote weight gain [48], and is associated with other metabolic disorders that
232 cause states of inflammation and some types of cancer, such as colon cancer [49]. This
233 effect may be due to the fact that carbohydrates interact with receptors of the small intestine
234 that cause secretion of satiety peptides such as the glucagon-like peptide 1 (GLP-1) [50], in
235 addition to gastric distension caused by high water intake with sucrose.

236

237 The preference for water with sucrose in rodents is documented [51, 52], and it has been
238 linked to the discovery of sweet taste receptors T1R3 or gusducin in the intestine [53]. In
239 contrast, in the study conducted by Bello and Hajnal in 2005 with rats, they showed that rats
240 do not like drinks with Sucralose, since the consumption of water without sucralose was
241 similar to the consumption of water with Sucralose [54]. The preference of rodents to
242 sweeteners like Stevia was also studied and it was observed that it has better acceptance
243 compared to other non-caloric sweeteners such as saccharin [55]. This shows that there is
244 variation in the preference between different non-caloric sweeteners and even between
245 species such as mice and rats. Preference also varies between genera; females have a
246 better response to sweetness than males [56].

247

248 In groups 1 and 2, Sucrose subgroups consumed less food, but in group 2, Splenda and
249 Svetia increased food consumption. This situation can be attributed to the energy
250 contribution of each sweetener, sucrose provides greater energy content, which causes a
251 satiety sensation in rodents and inhibits appetite. Groups of non-nutritive sweeteners, which
252 contribute little or very few calories, could cause an increase in appetite [48].

Comment [i19]: This categorical affirmation even if there is a references is not advisable. I suggest the following «This behavior, **probably**»

253 **4.2 Blood glucose** ~~did not change in group 1, but its concentration was lower~~
254 ~~than in group 2.~~

Comment [I20]: I suggest as subtitle: **Blood glucose changes**

255 In group 2, sucralose showed a lower concentration compared to the other subgroups. In the
256 Chang *et.al.* study, in 2010, they evaluated the proximal small bowel exposure to sucralose,
257 applied an intraduodenal glucose infusion in ten healthy subjects, took blood samples at
258 frequent intervals and determined that Sucralose does not modify the glycemic response
259 rate [57]. In addition to Sucralose other artificial sweeteners report a glycemic index similar
260 to Sucrose [58]. In another study conducted by Wang *et.al.* in 2011, they investigated the
261 effect of steviol on insulin resistance and the pro-inflammatory status of adipose tissue in
262 mice fed a high-fat diet; oral administration had no effect on body weight, basal insulin
263 levels, glucose tolerance, and insulin sensitivity improved and decreased secretion of
264 inflammatory cytokines in adipose tissue [59], concluded that the use of Stevia is beneficial
265 and helps control blood glucose levels.

266
267 A study designed to evaluate the effects of stevia on blood glucose concentration and blood
268 pressure (BP) with active treatment of steviol glucoside or placebo for 3 months. There were
269 no changes in systolic/diastolic blood pressure, glucose concentration and glycosylated
270 hemoglobin (HbA1c), therefore, oral stevia is well tolerated and has no pharmacological
271 effect [19].

272
273 **4.3. In group 1, the lymphocytes of the sucrose group were increased, but**
274 ~~decreased in the subgroups of sucralose and stevia. In contrast, in group 2,~~
275 ~~lymphocytes decrease in the sucrose subgroup.~~

Comment [I21]: I suggest change this subtitle by: **Changes in the lymphocyte percent**

276 Studies on the effect of sweeteners on the immune system of the small intestine and
277 particularly Peyer's patches are still scarce. In the study by Sehar *et.al.*, in 2008, they report
278 that Stevia can stimulate the proliferation of T and B cells, increasing humoral and cellular

279 immunity [41], in lymphocytes from the spleen, in Balb/c mice of both sexes, evaluated
280 viability by stimulating lymphocytes *in vitro* directly with stevioside and did not decrease
281 viability. This study was carried out on lymphocytes purified from Peyer's patches, as a site
282 of first contact with the ingested and absorption sweeteners. In addition, the response
283 between strains was different, in Balb/c mice (group 1) sucrose increased the percentage of
284 lymphocytes from Peyer's patches, and in group CD1 (group 2), sucrose reduced this
285 percentage. Another possible explanation for the decrease is found in the type of study and
286 sweetener used. In *in vitro* studies where the product used not for commercial use
287 (Esvetia/Truvia) if not reactive grade, stevia was administered at different doses, some
288 superior to those used in this work, without differences in the results [60]. These results
289 could be extrapolated to the human being since the metabolism of Stevia is similar between
290 rodents and humans. On the other hand, the consumption of sucrose has been related to a
291 decrease in the phagocytic index in neutrophils [37], which means that the consumption of
292 sucrose can alter the function of the cells and particularly in the Peyer's patches as the first
293 contact site of the sweetener. The effect of Sucralose on the immune response of
294 inflammatory bowel diseases has been observed [61], in chronic inflammatory processes as
295 a consequence of an increase in intestinal permeability [62] which causes immunological
296 reactions against diet antigens and components of the intestinal microbiota [63]. In the study
297 carried out by Abou-Donia *et.al.*, in rats indicated that Splenda has adverse effects such as
298 reduced microbiota, increased fecal pH, and over-expression of proteins that limit the
299 bioavailability of drugs [64]. The cause of the inhibition of the bacteria of the intestine is
300 related to the deterioration of the digestive proteases caused by the consumption of
301 Sucralose [65] that increases the intestinal permeability that causes inflammation of the
302 mucous membranes and that leads to the excessive activation of the lymphocytes, which
303 contributes to the pathogenesis of the Intestinal Inflammatory Disease and the Crohn's
304 disease [66, 67].

305

306 **4. CONCLUSION**

307 It is a fact that sweeteners modify in a greater or lesser proportion the lymphocyte population
308 of Peyer's patches and this variation depends directly on the dose, the frequency, the strain
309 of the rodents and the type of sweetener. In group 1, the Svetia subgroup had little weight
310 gain compared to the subgroups of sucrose and sucralose. In contrast, Group 2 had a
311 greater weight gain, perhaps due to the characteristics of the strain. In addition, mice of
312 group 1 showed a greater predilection for the consumption of sweeteners, particularly of
313 sucrose, and low for sucralose, but with a lower weight compared to group 2. Finally, in
314 group 1, the lymphocytes of the sucrose subgroup increased, with decreased in the
315 subgroups of sucralose and stevia. In contrast, in group 2, the lymphocytes decreased in the
316 sucrose subgroup.

317

318 **COMPETING INTERESTS**

319 Authors have declared that no competing interests exist.

320

321

322 **ETHICAL APPROVAL**

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

326

327 **REFERENCES**

328 1. Fernstrom JD, Navia JL. Workshop Summary. The Journal of Nutrition.
329 2012;142(6):1170S–2S. DOI: 10.3945/jn.111.149823

330 2. Ifland JR, Preuss HG, Marcus MT, Rourke KM, Taylor WC, Burau K, et al. Refined food
331 addiction: A classic substance use disorder. *Med Hypotheses*. 2009;72(5):518–26. DOI:
332 10.1016 / j.mehy.2008.11.035.

333 3. Jones JM, Elam K. Sugars and health: is there an issue?. *J Am Diet Assoc*.
334 2003;103(8):1058-60. DOI: 10.1053/JADA.2003.50563.

335 4. Tran C, Tappy L. Sucrose, glucose, fructose consumption: what are the impacts on
336 metabolic health?. *Rev Med Suisse*. 2012;8(331):513, 5-8.

337 5. Cardello HM, Da Silva MA, Damasio MH. Measurement of the relative sweetness of stevia
338 extract, aspartame and cyclamate/saccharin blend as compared to sucrose at different
339 concentrations. *Plant Foods Hum Nutr*. 1999;54(2):119-30.

340 6. Food and Drug Administration agency. No Calories Sweet. FDA. 2011;1. Accessed 20
341 May 2019. Available: http://www.fda.gov/fdac/features/2006/406_sweeteners.html

342 7. Tandel KR. Sugar substitutes: Health controversy over perceived benefits. *J Pharmacol*
343 *Pharmacother*. 2011;2(4):236-43. DOI: 10.4103 / 0976-500X.85936.

344 8. Mennella JA, Pepino MY, Reed DR. Genetic and environmental determinants of bitter
345 perception and sweet preferences. *Pediatrics*. 2005;115(2):e216-22. DOI: 10.1542 /
346 peds.2004-1582.

347 9. Margolskee RF. Molecular mechanisms of bitter and sweet taste transduction. *J Biol*
348 *Chem*. 2002;277(1):1-4. DOI: 10.1074 / jbc.R100054200.

349 10. Bellisle F, Drewnowski A. Intense sweeteners, energy intake and the control of body
350 weight. *Eur J Clin Nutr*. 2007;61(6):691-700. DOI: 10.1038 / sj.ejcn.1602649.

351 11. Garcia-Almeida JM, Casado Fdez GM, Garcia Aleman J. A current and global review of
352 sweeteners. Regulatory aspects. *Nutr Hosp*. 2013;28(Suppl 4):17–31. DOI: 10.3305 /
353 nh.2013.28.sup4.6793.

354 12. Schiffman SS, Rother KI. Sucralose, A Synthetic Organochlorine Sweetener: Overview
355 Of Biological Issues. *J Toxicol Environ Health Part B*. 2013;16(7):399–451. DOI: 10.1080 /
356 10937404.2013.842523.

- 357 13. Davis EA. Functionality of sugars: physicochemical interactions in foods. *Am J Clin Nutr.*
358 1995 Jul;62(Suppl 1):170S-7S. DOI: 10.1093 / ajcn / 62.1.170S.
- 359 14. Rosales-Gómez CA, Martínez-Carrillo BE, Reséndiz-Albor AA, Ramírez-Durán N,
360 Valdés-Ramos R, Mondragón-Velásquez T, et. al. Chronic consumption of sweeteners and
361 its effect on glycaemia, cytokines, hormones and lymphocytes of GALT in CD1 mice.
362 *Biomed Res Int.* 2018;2018:1345282. DOI: 10.1155/2018/1345282. eCollection 2018.
- 363 15. Popkin BM, Nielsen SJ. The sweetening of the world's diet. *Obes Res.*
364 2003;11(11):1325-32. DOI: 10.1038 / oby.2003.179.
- 365 16. Mehnert H. Sugar substitutes in the diabetic diet. *Int Z Vitam Ernahrungsforsch Beih.*
366 1976;15:295-324.
- 367 17. Ikeda T. Sugar substitutes: reasons and indications for their use. *Int Dent J.*
368 1982;32(1):33-43.
- 369 18. Arnold DL. Two-generation saccharin bioassays. *Environ Health Perspect.* 1983;50:27-
370 36. DOI: 10.1289 / ehp.835027.
- 371 19. Barriocanal LA, Palacios M, Benitez G, Benitez S, Jimenez JT, Jimenez N, et al.
372 Apparent lack of pharmacological effect of steviol glycosides used as sweeteners in humans.
373 A pilot study of repeated exposures in some normotensive and hypotensive individuals and
374 in Type 1 and Type 2 diabetics. *Regul Toxicol Pharmacol.* 2008;51(1):37-41.
375 DOI: 10.1016 / j.yrtph.2008.02.006.
- 376 20. Chan P, Xu DY, Liu JC, Chen YJ, Tomlinson B, Huang WP, et al. The effect of stevioside
377 on blood pressure and plasma catecholamines in spontaneously hypertensive rats. *Life Sci.*
378 1998;63(19):1679-84.
- 379 21. Chatsudthipong V, Muanprasat C. Stevioside and related compounds: therapeutic
380 benefits beyond sweetness. *Pharmacol Ther.* 2009;121(1):41-54.
381 DOI: 10.1016 / j.pharmthera.2008.09.007.

- 382 22. Koyama E, Kitazawa K, Ohori Y, Izawa O, Kakegawa K, Fujino A, et al. In vitro
383 metabolism of the glycosidic sweeteners, stevia mixture and enzymatically modified stevia in
384 human intestinal microflora. *Food Chem Toxicol.* 2003;41(3):359-74.
- 385 23. Geuns JM. Stevioside. *Phytochemistry.* 2003;64(5):913-21.
- 386 24. Renwick AG. The intake of intense sweeteners - an update review. *Food Addit Contam.*
387 2006;23(4):327-38. DOI: 10.1080 / 02652030500442532.
- 388 25. Duffy, Valerie B, Sigman-Grant, Madeleine et.al. Position of the American Dietetic
389 Association: use of nutritive and nonnutritive sweeteners. *J Am Diet Assoc.*
390 2004;104(2):255-75. DOI: 10.1016 / j.jada.2003.12.001
- 391 26. Ford HE, Peters V, Martin NM, Sleeth ML, Ghatei MA, Frost GS, et al. Effects of oral
392 ingestion of sucralose on gut hormone response and appetite in healthy normal-weight
393 subjects. *Eur J Clin Nutr.* 2011;65(4):508-13. DOI: 10.1038/ejcn.2010.291.
- 394 27. Baird IM, Shepard NW, Merritt RJ, Hildick-Smith G. Repeated dose study of sucralose
395 tolerance in human subjects. *Food Chem Toxicol.* 2000;38(Suppl 2): S123-9.
- 396 28. Murphy KT, Walport M. *Inmunobiología de Janeway.* 7th ed: McGRAW-HILL:
397 Interamericana editores; 2009.
- 398 29. Mackie RI, Sghir A, Gaskins HR. Developmental microbial ecology of the neonatal
399 gastrointestinal tract. *Am J Clin Nutr.* 1999;69(5):1035S-45S. DOI: 10.1093 / ajcn /
400 69.5.1035s.
- 401 30. David A. Hughes LGD, Adrienne Bendich. *Diet and Human Immune Function.* 1st ed:
402 Humana Press;2004.
- 403 31. Aguilera Montilla N. PBea. Mucosal immune system: A brief review. *Immunol.*
404 2004;23:207-16. DOI: 10.1371 / journal.pbio.1001397.
- 405 32. Brandtzaeg P, Kiyono H, Pabst R, Russell MW. Terminology: nomenclature of mucosa-
406 associated lymphoid tissue. *Mucosal Immunol.* 2008;1(1):31-7. DOI: 10.1038 / mi.2007.9.
- 407 33. Forchielli ML, Walker WA. The role of gut-associated lymphoid tissues and mucosal
408 defence. *Br J Nutr.* 2005 Apr;93 (Suppl 1):S41-8.

409 34. Farstad IN, Halstensen TS, Lien B, Kilshaw PJ, Lazarovits AI, Brandtzaeg P. Distribution
410 of beta 7 integrins in human intestinal mucosa and organized gut-associated lymphoid
411 tissue. *Immunology*. 1996;89(2):227-37. DOI: 10.1046/j.1365-2567.1996.d01-727.x.

412 35. Lefrancois L. Development, trafficking, and function of memory T-cell subsets. *Immunol*
413 *Rev*. 2006;211:93-103. DOI: 10.1111 / j.0105-2896.2006.00393.x.

414 36. Neutra MR, Pringault E, Kraehenbuhl JP. Antigen sampling across epithelial barriers and
415 induction of mucosal immune responses. *Annu Rev Immunol*. 1996;14:275-300. DOI:
416 10.1146/annurev.immunol.14.1.275.

417 37. Sanchez A, Reeser JL, Lau HS, Yahiku PY, Willard RE, McMillan PJ, et al. Role of
418 sugars in human neutrophilic phagocytosis. *Am J Clin Nutr*. 1973;26(11):1180-4. DOI:
419 10.1093 / ajcn / 26.11.1180.

420 38. Goldsmith LA. Acute and subchronic toxicity of sucralose. *Food Chem Toxicol*. 2000;38
421 (Suppl 2):S53-69.

422 39. Berry C, Brusick D, Cohen SM, Hardisty JF, Grotz VL, Williams GM. Sucralose Non-
423 Carcinogenicity: A Review of the Scientific and Regulatory Rationale. *Nutr Cancer*.
424 2016;68(8):1247–1261. DOI:10.1080/01635581.2016.1224366.

425 40. Mortensen A. Sweeteners permitted in the European Union: Safety aspects
426 *Scandinavian Journal of food & Nutrition*. 2006; 50(30): 104-116. DOI:
427 10.1080/17482970600982719.

428 41. Sehar I, Kaul A, Bani S, Pal HC, Saxena AK. Immune up regulatory response of a non-
429 caloric natural sweetener, stevioside. *Chem Biol Interact*. 2008;173(2):115-21.
430 DOI: 10.1016 / j.cbi.2008.01.008.

431 42. Chaiwat Boonkaewwan CT, and Molvibha Vongsakul. Anti-Inflammatory and
432 Immunomodulatory Activities of Stevioside and Its Metabolite Steviol on THP-1 Cells. *J Agric*
433 *Food Chem*. 2006;54:785-9. DOI: 10.1021/jf0523465.

- 434 43. Boonkaewwan C, Ao M, Toskulkao C, Rao MC. Specific immunomodulatory and
435 secretory activities of stevioside and steviol in intestinal cells. *J Agric Food Chem.*
436 2008;56(10):3777-84. DOI: 10.1021 / jf072681o.
- 437 44. Norma Oficial Mexicana. Especificaciones Técnicas para la producción, cuidado y uso
438 de los animales de laboratorio. NOM-062-ZOO-1999, 1999. Spanish.
- 439 45. Dalle-Donne I, Aldini G, Carini M, Colombo R, Rossi R, Milzani A. Protein carbonylation,
440 cellular dysfunction, and disease progression. *J Cell Mol Med.* 2006;10(2):389–406.
- 441 46. Thomas JEG, Michael J. Stevia: It's Not Just About Calories. *Open Obesity Journal.*
442 2010;2:101-9.
- 443 47. Moreno-Martínez MGR, Sánchez-González DJ. Efecto de los edulcorantes no nutritivos
444 (aspartame y sucralosa) en el peso de las ratas. Estudio prospectivo, controlado,
445 aleatorizado, doble ciego. *Revista de Sanidad Militar.* 2011;65(4):168-75. Spanish.
- 446 48. Drewnowski A, Bellisle F. Liquid calories, sugar, and body weight. *Am J Clin Nutr.*
447 2007;85(3):651-61. DOI: 10.1093 / ajcn / 85.3.651.
- 448 49. Dragsted LO, Daneshvar B, Vogel U, Autrup HN, Wallin H, Risom L, et al. A sucrose-rich
449 diet induces mutations in the rat colon. *Cancer Res.* 2002;62(15):4339-45.
- 450 50. Feinle C, O'Donovan D, Horowitz M. Carbohydrate and Satiety. *Nutrition Reviews.*
451 2002;60(6):155-69. DOI: 10.1093 / ajcn / 61.4.960S.
- 452 51. Constantino CF, Salas G, G Tovar C, Duran-de-Bazua C, Gracia I, Macias L, et. al.
453 Effects on Body Mass of Laboratory Rats after Ingestion of Drinking Water with Sucrose,
454 Fructose, Aspartame, and Sucralose Additives. *The Open Obesity Journal.* 2010;2:116-24.
455 DOI: 10.2174 / 1876823701002010116
- 456 52. Martínez A, Madrid JA, López-Espinoza A, Vivanco P. Consumo de soluciones
457 endulzadas en octodones (Octodón-degú). *Acta Comportamental.* 2009;17:141-53.
458 Spanish. Se encuentra en: <http://www.revistas.unam.mx/index.php/acom/article/view/18145>

459 53. Margolskee RF, Dyer J, Kokrashvili Z, Salmon KS, Ilegems E, Daly K, et al. T1R3 and
460 gustducin in gut sense sugars to regulate expression of Na⁺-glucose cotransporter 1. Proc
461 Natl Acad Sci U S A. 2007;104(38):15075-80. DOI: 10.1073 / pnas.0706678104.

462 54. Bello NT, Hajnal A. Male rats show an indifference-avoidance response for increasing
463 concentrations of the artificial sweetener sucralose. Nutrition Research. 2005;25:693-9. DOI:
464 10.1016/j.nutres.2005.07.003.

465 55. Sclafani A, Bahrani M, Zukerman S, Ackroff K. Stevia and saccharin preferences in rats
466 and mice. Chem Senses. 2010;35(5):433-43. DOI: 10.1093 / chemse / bjq033.

467 56. Valenstein Valenstein ES. Selection of nutritive and nonnutritive solutions under different
468 conditions of need. J Comp Physiol Psychol. 1967;63:429-433.

469 57. Ma J, Chang J, Checklin HL, Young RL, Jones KL, Horowitz M, et al. Effect of the
470 artificial sweetener, sucralose, on small intestinal glucose absorption in healthy human
471 subjects. Br J Nutr. 2010;104(6):803-6. DOI: 10.1017 / S0007114510001327.

472 58. Ferland A, Brassard P, Poirier P. Is aspartame really safer in reducing the risk of
473 hypoglycemia during exercise in patients with type 2 diabetes? Diabetes Care.
474 2007;30(7):e59. DOI: 10.2337 / dc06-1888.

475 59. Wang Z, Xue L, Guo C, Han B, Pan C, Zhao S, et al. Stevioside ameliorates high-fat
476 diet-induced insulin resistance and adipose tissue inflammation by downregulating the NF-
477 kappaB pathway. Biochem Biophys Res Commun. 2012;417(4):1280-5.
478 DOI: 10.1016 / j.bbrc.2011.12.130.

479 60. Koyama E, Sakai N, Ohori Y, Kitazawa K, Izawa O, Kakegawa K, et. al. Absorption and
480 metabolism of glycosidic sweeteners of stevia mixture and their aglycone, stevion, in rats
481 and humans. Food Chem Toxicol. 2003;41(6):875-83.

482 61. Garcia D, Ramos AJ, Sanchis V, Marin S. Effect of Equisetum arvense and Stevia
483 rebaudiana extracts on growth and mycotoxin production by Aspergillus flavus and Fusarium
484 verticillioides in maize seeds as affected by water activity. Int J Food Microbiol. 2012;153(1-
485 2):21-7. DOI: 10.1016 / j.ijfoodmicro.2011.10.010.

- 486 62. Kau AL, Ahern PP, Griffin NW, Goodman AL, Gordon JI. Human nutrition, the gut
487 microbiome and the immune system. *Nature*. 2011;474(7351):327-36. DOI: 10.1038 /
488 nature10213.
- 489 63. Qin X. What made Canada become a country with the highest incidence of inflammatory
490 bowel disease: could sucralose be the culprit? *Can J Gastroenterol*. 2011;25(9):511.
- 491 64. Abou-Donia MB, El-Masry EM, Abdel-Rahman AA, McLendon RE, Schiffman SS.
492 Splenda alters gut microflora and increases intestinal p-glycoprotein and cytochrome p-450
493 in male rats. *J Toxicol Environ Health A*. 2008;71(21):1415-29. DOI: 10.1080 /
494 15287390802328630.
- 495 65. Podolsky DK. The current future understanding of inflammatory bowel disease. *Best
496 Pract Res Clin Gastroenterol*. 2002;16(6):933-43.
- 497 66. Cabarrocas J, Savidge TC, Liblau RS. Role of enteric glial cells in inflammatory bowel
498 disease. *Glia*. 2003;41(1):81-93. DOI: 10.1002 / glia.10169.
- 499 67. Qin X. Etiology of inflammatory bowel disease: a unified hypothesis. *World J
500 Gastroenterol*. 2012;18(15):1708-22. DOI: 10.3748 / wjg.v18.i15.1708.