Research Article

Effect of Saccharin Sodium and the Sodium Cyclamate on Human Cells Treated with *Lactobacillus Plantarum* Lp62

Arielle Oliveira Cruz das Neves, Tauá Alves Melo, Maísa Brito Nunes, Carla Cristina Romano[*]

Laboratory of Immunology, Biotechnology and Genetics Center, Soane Nazaré de Andrade Campus, Universidade Estadual de Santa Cruz, Jorge Amado Highway, km 16, Salobrinho District, Ilhéus, Bahia, Brazil

[*]**Corresponding author:** Dra Carla Cristina Romano, Laboratory of Immunology, Biotechnology and Genetics Center, Soane Nazaré de Andrade Campus, Universidade Estadual de Santa Cruz, Jorge Amado Highway, km 16, Salobrinho District, Ilhéus, Bahia, Brazil

Abstract

The commercial sweeteners have been suggested for diabetics and people who need to lose weight. Its use has grown on a global scale, as well as health problems related to it. Despite their discovery over a hundred years, suspicion and uncertainty remain giving rise to numerous discussions on the safety of human health. Thus, this study aimed to evaluate the effect of saccharin and cyclamate sodic on the viability of human cells, as well as the possible protective effect of *Lactobacillus plantarum* LP62 on cells treated with the sweeteners. We found that human cells exposed to high concentrations of sweeteners reduced their viability, with cyclamate being more harmful than saccharin. The results show the efficacy of lactobacilli in reducing the production of inflammatory cytokines in human cells in the presence of a sweetener, especially in peripheral blood mononuclear cells. Thus, we indicate the LP62 as a promising probiotic candidate and emphasize the need for greater scientific investment in this area of knowledge in order to better clarify the effect of sweeteners on human cells and microorganisms and for understanding of the combination thereof.

Introduction

The synthetic sweeteners and non-calories have been widely used by food industries to replace sugar in soft drinks, juices, fruit jellies, jams, sweets and many others. These so-called diet foods are suitable for people who want or need to reduce caloric intake, as well as for individuals with diabetes for whom the restriction of sugar is recommended. In the past two decades, the consumption of this kind of food has grown at a global scale, as well as the health problems associated with high consumption of synthetic sweeteners. Generally, the agencies of health and safety of different countries regulate which sweeteners are allowed, as well as the maximum amount that can be added to food of a specific group [1]. These products are part of the diet of millions of people, with about 28% of the population reporting its use [2].

There is a discussion about the safety of these sugar substitutes for human health. Although some studies show a positive association of the use of sweeteners with the risk of cancer in many organs, including the colon, the issue is still considered under debate [3].

The saccharine, first commercial sweetener discovered, was first synthesized in 1879 by Remsen and Fahlberg. The saccharine of sodium, one of the forms of saccharin, is not metabolized by the human organism and is negative in most tests for genotoxicity, such as the micronucleus test and Ames test. However, in 1987, studies in rats resulted in the formation of bladder tumors, and these results were considered sufficient evidence of carcinogenicity in animals by the International Agency for Research on Cancer (IARC) [4].

In 1999, the agency concluded that the saccharin solution produces urothelial tumors of the bladder in rats by a mechanism not reactive of DNA which involves the formation of a precipitate urinary of calcium containing phosphate, cytotoxicity and cellular proliferation increased. This mechanism is not considered relevant to humans, due to the critical differences between species in the composition of urine [5].

Another sweetener, sodium cyclamate, unlike saccharin so-

dium, is metabolized by the intestinal microbiota in cyclohexylamine, a compound considered toxic to humans. In the United States, the Food and Drug Administration (FDA) has banned the sale of cyclamate in 1969, after lab tests on rats indicate that large amounts of sweetener can cause bladder cancer, a disease to which these animals are particularly susceptible [6]. In a study in mice, conducted by Sasaki and colleagues in 2002 [7], the sodium cyclamate induced a statistically significant increase of DNA damage to cells in the kidney and urinary bladder, as well in the stomach and colon.

In 2015, another study [8] using rats treated with sodium cyclamate for two months affirms that the substance induces changes in morphological endocrine pancreas. The pancreas of the group treated with cyclamate was congested and the islets of Langerhans showed changes in shape and increase of the area that led to an increase in the weight of the pancreas. This result questions the use of sweeteners in the main group of consumers, the diabetic patients. Could a substance named to aid in the treatment of the disease bring damage to the sick organ? The data require further explanation.

Also in 2015, Amin and Almuzafar [9] conducted a survey in rats and concluded that the frequent consumption of saccharin has the effect of inducing metabolic disorders that affect the levels of body weight, glucose and lipids. These results question the effect of saccharin in the maintenance of weight or growth rate. The authors claim that saccharin induces alterations in hepatic and renal function in a dose-dependent manner and becomes more risky in higher doses, due to its ability to induce oxidative stress through the formation of free radicals. In this way, they suggest the need to alert the consumer awareness on the harmful effects of this food additive and mention the concentration of each material added to food particularly when frequently consumed by children.

Other authors have also related these substances to hepatocellular injuries and alterations in the capacity of antioxidant in the liver [10], tumors in the urine tract [11], changes in lipid profile [12], DNA damage [13], weight gain [14], neurotropic effects [15], changes in the homeostasis of electrolytes and synthesis of neurotransmitters [16], among others. In the 1970s to the present day there are many studies related to the topic. A good part of them, especially those pioneers in the field, use animal models (mainly rats). The difficulty of this model are the physiological differences with the human species, as alleged by IARC (1999) in the classification of saccharin sodium as not carcinogenic to humans although promote urothelial tumors in bladders of rats. In a study conducted by Suez and collaborators in 2014 [17], the authors suggest that the consumption of the artificial sweeteners both in mice and in humans increases the risk of glucose intolerance and that these metabolic effects are mediated by modulation of the composition and function of the microbiota. Hence the need to

intensify the research related to the effect of sweeteners in culture of human cells and their association with microorganisms that may reduce potential harmful effects of the first.

The concept of manipulating the intestinal microbiota using specific microbes to improve the metabolism of the host has gained considerable interest in recent years. Currently, several potential bacterial candidates have been identified, and new mechanisms of action that drives their beneficial effects have been elucidated [18]. The mechanisms of action of these microorganisms include: competitive exclusion of pathogens in the intestinal microbiota, enzyme activity, reduction of secondary carcinogenic bile acids, connection to carcinogens and mutagenic, reduction of DNA damage, suppression of the formation of focuses of aberrant crypts, improvement of the barrier function, increasing the production of short-chain fatty acids and modulation of the immune response [19]. Thus, once the sweeteners may have harmful effects to health, micro-organisms with the potential to mitigate these effects have been sought. Since 2010, some studies conducted at the Universidade Estadual de Santa Cruz (Brazil) have proven the effectiveness of a probiotic strain of a *Lactobacillus plantarum* (*L. Plantarum* LP62) isolated from almonds of cocoa (*Theobroma cacao*) (Genetic and Molecular Research, 2016, in press). Previous studies from our group have proven anti-inflammatory activity of this strain by the negative modulation in the production of IL-8 as well as their resistance to gastrointestinal tract. Thus, the present study aims to evaluate the influence of saccharin and sodium cyclamate on the viability of human cells, as well as the possible protective effect of *Lactobacillus plantarum* LP62 on cells treated with the sweeteners.

Material and Methods

Preparation of Materials

Reactivation of *Lactobacillus plantarum* LP62: The lactobacilli were obtained from the Collection of lactic acid bacteria of the Immunology Laboratory of UESC. Originally the strain was isolated during the fermentation process of fine cocoa and stored in MRS broth + 30 % of glycerol at -80 ⁰C. The reactivation was performed in MRS broth for 18 hours in a stove at 37 ⁰C.

Obtaining sweeteners: saccharin sodium and the sodium cyclamate were purchased commercially in its pure form through the Sigma Chemical Co. (USA). The solutions were aliquoted and stored at -20 ⁰C.

Culture of lineage of tumor cells Ht-29: the cells Ht-29 from the Cell Bank of Rio de Janeiro were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 10% of Fetal Bovine Serum (FBS), 100 Ui/mL penicillin and 100 μg/mL streptomycin. The cells (1×10^6 cells/mL) were maintained in cell culture plaques of 12 and 24 wells and incubated in a humid stove at 37 ˚C and 5% of CO_2. The exchange of cell culture was performed every 48 hours

and the raises every 96 hours.

Culture of peripheral blood mononuclear cells (PBMCs): to obtain mononuclear cells, were collected aseptically from a healthy donor 20 mL of blood, and the anticoagulant used was the heparin. After collection, the sample was kindly transferred for falcon pipe containing 20 mL of Histopaque. It was followed by centrifugation for 30 minutes at 2500 RPM. The "ring" containing the PBMC, formed between the red blood cells and plasma was collected and transferred to another tube. The cells were then washed twice with saline solution, 1800 RPM for 10 minutes, the supernatant was discarded. Then the cells were re-suspended in RPMI 1640 Medium (RPMI), 10% of FBS, 100 IU/mL penicillin and 100 μg/mL streptomycin. Then the count in camera of Neubauer has started, being the concentration set to 1x 10^6 cells/mL. The cells were maintained in culture cell plaques of 24 and 96 wells and incubated in a humid stove at 37 °C and 5% of CO_2. The exchange of cell culture was performed every 48 hours.

Analysis of the viability of human cells ahead sweeteners through the test of 3-bromide 4,5-dimethyl-2-thiazolyl, 2,5-diphenyl-tetrazolium (MTT)

For testing the MTT assay, both the Ht-29 cells as the PBMC were transferred to 96 well plaques, cells Ht-29 were used in a concentration of 1 x 10^5 cells/mL and the mononuclear cells 1 x 10^6 cells/ mL. After 24 hours of membership, they were treated with different concentrations of sweeteners (1, 10, 30 and 50 mM) and kept in stove of CO_2 at 37 ^{0}C. The cell viability was evaluated after 24 hours. For this, the cellular supernatants were removed and 50 μL of the solution of MTT (1 mg/ mL) were added to each well, followed by incubation in the absence of light for 4 hours. Then, the solution was discarded and 100 μL of DMSO were added to each well, the plates were homogenized and shaken by hand for a few minutes. Subsequently, a reading of the absorbance λ = 540 nm was done corresponding to each well using a plate reader (Expert Plus - Asys). The values were expressed in percentages of reduction of MTT in relation to the negative control, where the cells were not exposed to the formulations.

Evaluation of the effect of sweeteners in cells Ht-29 treated therapeutically with *Lactobacillus plantarum* LP62

To assess the effect of sweeteners in cells Ht-29 treated with lactobacilli was performed an only experiment with the techniques applied simultaneously. To this end, the cells were initially trypsinized, washed and plaqued on concentration 1 x 10^6 cells/ mL in cultivation cellular plaque with 24 wells. After 24 hours of accession and confluence, they were treated with saccharin and sodium cyclamate at 30 mM, in addition to the micro-organisms (1 x 10^9 CFU/ mL), so we have the following treatments: negative control, saccharin sodium, sodium cyclamate, only lactobacilli, saccharin sodium + lactobacilli and sodium

cyclamate + lactobacilli.

After 24 hours of contact with the formulations previously mentioned, the cell supernatant was collected, part of the sample was serially diluted and plaqued on MRS agar for counting colony forming units (bacterial count), and the rest of the cell supernatant was stored at -20 ^{0}C and analyzed later for production of pro-inflammatory cytokines by ELISA (IL-8 and INF-gamma).

The cells in turn were trypsinized, washed, serially diluted and plaqued on MRS agar for bacterial count of attached bacteria (adhesion assay). The number of total bacteria refers to the sum of the number of CFU in the supernatant with the number of CFU adhered to cells.

Evaluation of the effect of sweeteners in peripheral blood mononuclear cells treated therapeutically with *Lactobacillus plantarum* LP62

To assess the effect of sweeteners on mononuclear cells treated with lactobacilli, the cells were washed and plaqued in 96 well plates with concentration 1 x 10^6 cells/ mL. After 24 hours of accession and confluence, they were treated with saccharin and sodium cyclamate to 30 mM, in addition to the micro-organisms (1 x 10^9 CFU/ mL), so we have the following treatments: negative control, saccharin sodium, sodium cyclamate, only lactobacilli, saccharin sodium + lactobacilli and sodium cyclamate + lactobacilli. After 24 hours of contact with the formulations previously mentioned, the cell supernatant was collected, stored at -20 ^{0}C and analyzed later for production of pro-inflammatory cytokines by ELISA (IL-8 and INF-gamma). The kits were purchased commercially and used according to the manufacturer's recommendation.

Statistical Analysis

The tests were performed in triplicate, and results were expressed on average ± standard deviation. The analysis of variance (value set = 5%) were performed by ANOVA one-*way* non-parametric method and test of Bonferroni, using the GraphPad Prism® version 5.01.

Results

High concentrations of cyclamate and saccharin sodium cause cell death

The results of the assessment of viability of the cells Ht-29 exposed to cyclamate and saccharin sodium are illustrated in Figures 1, 2, respectively. The observation of Figure 1 shows that, with the exception of the treatment SC3 (30 mM), all other differed statistically from the control group. The lower concentrations (SC1-1 mM and SC2 - 10 mM) induced an increase in cell viability to 109.06% and 108.81%, respectively, the intermediary (SC3 - 30 mM) did not generate significant changing while the highest (SC4

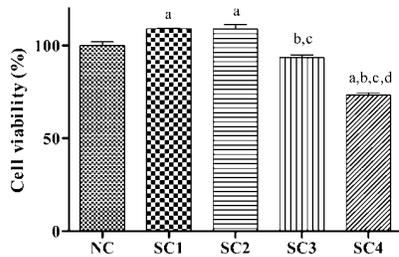- 50 mM) has reduced the viability to 73.27%.

Figure 1: Evaluation of the cells Ht-29 exposed to different concentrations of sodium cyclamate by MTT. NC - negative control, SC1 - sodium cyclamate (1 mM), SC2 - sodium cyclamate (10 mM), SC3 - sodium cyclamate (30 mM), SC4 - sodium cyclamate (50 mM). "a" indicates a significant difference in relation to the negative control, "b" indicates a significant difference in relation to SC1, "c" in relation to SC2 and "d" to SC3.
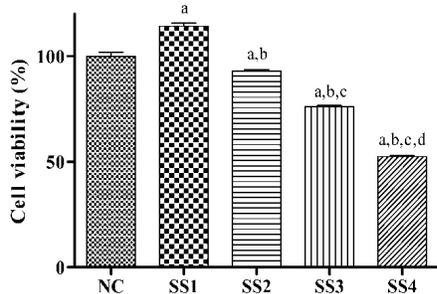

Figure 2: Evaluation of the cells Ht-29 exposed to different concentrations of sodium saccharin by MTT. NC - negative control, SS1 - saccharin sodium (1 mM), SS2 - saccharin sodium (10 mM), SS3 - saccharin sodium (30 mM), SS4 - saccharin sodium (50 mM). "a" indicates a significant difference in relation to the negative control, "b" indicates a significant difference in relation to SS1, "c" in relation to SS2 and "d" to SS3.

As to the saccharin (Figure 2) all treatments differed statistically from the negative control. Similarly, to what was observed for the cyclamate, the lowest concentration (SS1 - 1 mM) increased the cellular viability (114.22%), while all other reduced (SS2 - 93.06%, SS3 - 77.66% and SS4 - 52.41%). The reduction of viability in the treatments SS2 (10 mM), SS3 (30 mM) and SS4 (50 mM) differed both statistically from the control group and between themselves. The higher the concentration of sweetener, less viability. The result of the feasibility assessment of peripheral blood mononuclear cells exposed to cyclamate and saccharin sodium is illustrated in figures 3 and 4, respectively. The Figure 3 shows that the three lower concentrations of cyclamate (SC1 - 1 mM, SC2 - 10 mM and SC3 - 30 mM) did not alter significantly

the viability of the cells, while the higher concentration (SC 4 - 50 mM) induced death, the viability in this treatment was 52.44%.
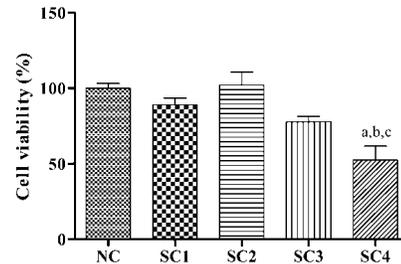

Figure 3: Evaluation of peripheral blood mononuclear cells exposed to different concentrations of sodium cyclamate by MTT. NC - negative control, SC1 - sodium cyclamate (1 mM), SC2 - sodium cyclamate (10 mM), SC3 - sodium cyclamate (30 mM), SC4 - sodium cyclamate (50 mM). "a" indicates a significant difference in relation to the negative control, "b" indicates a significant difference in relation to SC1 and "c" in relation to SC2.

As to the saccharin (Figure 4), there was a reduction of viability in all treatments [SS1 (1 mM) - 72.82%, SS2 (10 mM) - 72.5%, SS3 (30 mM) - 39.6% and SS4 (50 mM) - 28.45%], while the highest concentrations presented a greater damage to cells. The higher the concentration, the lower the cellular viability. Statistically speaking, there was no difference between the treatments SS1 and SS2, as well as there was no difference between SS3 and SS4. The saccharin was more aggressive to the mononuclear cells than cyclamate.
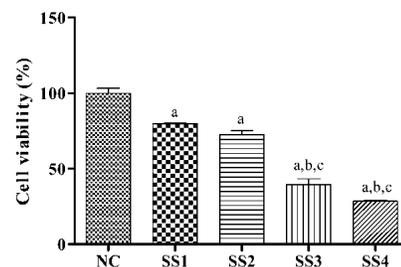

Figure 4: Peripheral blood mononuclear cells exposed to different concentrations of saccharin sodium. NC - negative control, SS1 - saccharin sodium (1 mM), SS2 - saccharin sodium (10 mM), SS3 - saccharin sodium (30 mM), SS4 - saccharin sodium (50 mM). "a" indicates a significant difference in relation to the negative control, "b" indicates a significant difference in relation to SS1 and "c" in relation to SS2.

Saccharin sodium favors adhesion of LP62 to cells Ht-29

The results of the quantification of bacteria in the cellular

supernatant and of the test of tacking are illustrated in Table 1. The statistical analysis of the data shows that the cells treated with the LP62 + sodium saccharin had greater adhesion of lactobacilli (2.75×10^9 CFU/ mL) when compared to groups with LP62 + sodium cyclamate (5.35×10^8 CFU/ mL) or without sweeteners (4.3×10^8 CFU/mL). There was no statistical difference between the number of bacteria adhered in the group treated with cyclamate in relation to control. There was no statistically significant difference between the number of present bacteria in the cellular supernatant or between the number of bacteria on any of the treatments.

Treatments	Bacteria adhered (UFC/mL)	Bacteria in the cellular supernatant (UFC/mL)	Total bacteria (UFC/mL)
Only LP62	$4{,}3 \times 10^8 \pm 0{,}7$	$1{,}05 \times 10^{11} \pm 0$	$1{,}05 \times 10^{11} \pm 0$
LP62 + Sodium cyclamate	$5{,}35 \times 10^8 \pm 0{,}07$	$7{,}45 \times 10^{10} \pm 1{,}34$	$9{,}38 \times 10^{10} \pm 1{,}34$
LP62 + Saccharin sodium	$2{,}75 \times 10^9 \pm 0{,}77^{a,b}$	$9{,}1 \times 10^{10} \pm 1{,}83$	$7{,}50 \times 10^{10} \pm 1{,}76$

Table 1: Quantification of *Lactobacillus plantarum* LP62 (therapeutic test). "a" indicates statistical difference in relation to the control group (only LP62) and "b" indicates a difference in relation to the treatment LP62 + sodium cyclamate. There was no statistically significant difference between the bacteria of supernatant cell nor in the number of total bacteria.

The LP62 was able to reduce the production of pro-inflammatory cytokines in cells Ht-29 exposed to sweeteners

The result of the dosages of IL-8 and INF-gamma of cells Ht-29 exposed to sweeteners and the LP62 are illustrated in Figures 5,6, respectively. According to Figure 5, the production of IL-8 by cells treated only with saccharin sodium (0.55 ng/mL) was higher when compared to the control group (0.18 ng/mL), and the cyclamate has not increased nor decreased its production. The group treated with lactobacilli presented an increase in the production of this compound (0.71 ng/mL). The two groups treated with sweeteners + LP62 did not show a statistically significant difference compared to the negative control, including the group treated with saccharin + LP62 (0.29 ng/mL) was able to reduce the production of interleukin in comparison to the group treated only with saccharin.
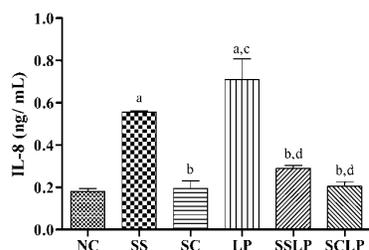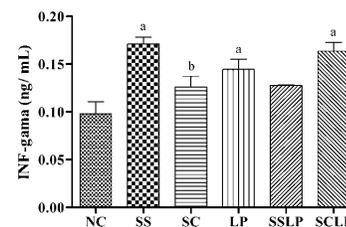

Figure 6: Dosage of IFN-gamma of Ht cells-29 treated with sodium cyclamate, saccharin sodium and *Lactobacillus plantarum* LP62 by ELISA. NC - negative control, SS - saccharin sodium, SC - sodium cyclamate, LP - *Lactobacillus plantarum* LP62, SSLP - saccharin sodium + LP62 and SCLP - sodium cyclamate + LP62. "a" indicates no statistical difference in relation to the negative control and "b" difference compared to the group treated with saccharin sodium. r = 0.991.

The dosage of INF-gamma (Figure 6) shows that the saccharin sodium, the LP62 and the group treated with cyclamate + LP62 significantly increased the production of this cytokine in comparison to the control (0.17; 0.14; 0.16 and 0.09 ng/mL, respectively). Similarly, to what was observed for IL-8, although the group treated with sodium saccharin has increased production of INF-gamma, when adding the LP62 the dosage decreases (0.12 ng/mL) to the point of not differ statistically from the control.

The LP62 reduced the production of pro-inflammatory cytokines in cells exposed to cyclamate and to saccharin sodium

The results of the determination of IL-8 and INF-gamma by peripheral blood mononuclear cells treated with sodium saccharin, cyclamate sodium and *L. Plantarum* LP62 are illustrated in Figures 7,8, respectively. The results show that both cyclamate and saccharin increased IL-8 production (12.5 and 8.25 ng/mL, respectively)

Figure 5: Dosage of IL-8 of cells Ht-29 treated with sodium cyclamate, saccharin sodium and *Lactobacillus plantarum* LP62 by ELISA. NC - negative control, SS - saccharin sodium, SC - sodium cyclamate, LP - *Lactobacillus plantarum* LP62, SSLP - saccharin sodium + LP62 and SCLP - sodium cyclamate + LP62. "a" indicates no statistical difference in relation to the negative control, "b" difference compared to the group treated with sodium saccharin, "c" in relation to the group with sodium cyclamate and "d" to the group treated with the LP62. r = 0.995.

significantly in comparison to the negative control (0.67 ng/mL). In the presence of LP62, associated or not to sweeteners, there was no statistically significant difference compared to the control group nor among themselves. The LP62 was effective in reducing the production of this cytokine in question in the groups treated with both saccharin and cyclamates for 0.44 and 0.65 ng/mL, respectively.
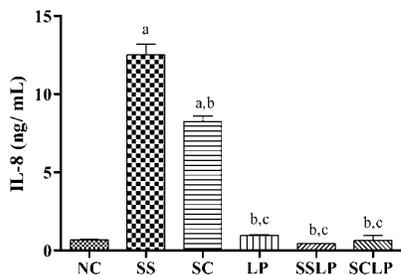

Figure 7: Production of IL-8 by peripheral blood mononuclear cells treated with sodium cyclamate, saccharin sodium and *Lactobacillus plantarum* LP62. CN - negative control, SS - saccharin sodium, SC - sodium cyclamate, LP - *Lactobacillus plantarum* LP62, SSLP - saccharin sodium + LP62 and SCLP - sodium cyclamate + LP62. "a" indicates no statistical difference in relation to the negative control, "b" difference compared to the group treated with saccharin sodium and "c" in relation to the group with sodium cyclamate. r = 0.995.
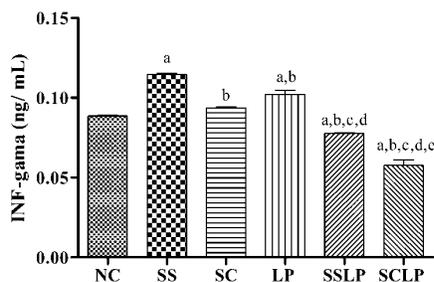

Figure 8: Production of IFN-gamma by peripheral blood mononuclear cells treated with sodium cyclamate, saccharin sodium and *Lactobacillus plantarum* LP62. NC - negative control, SS - saccharin sodium, SC - sodium cyclamate, LP - *Lactobacillus plantarum* LP62, SSLP - saccharin sodium + LP62 and SCLP - sodium cyclamate + LP62. "a" indicates no statistical difference in relation to the negative control and "b" difference compared to the group treated with sodium saccharin, "c" to the group treated with cyclamate, "d" to LP62 and "e" to the group treated with sodium saccharin + LP62. r = 0.991.

About the INF-gamma, saccharin sodium and the LP62 increased the production of this cytokine in relation to negative control. There was no significant difference in the group treated

with sodium cyclamate. Since the cells treated with sweeteners associated to LP62 showed a statistically significant reduction in the production of this cytokine, both not only compared to the control group, but also to cells treated with sweeteners only. Thus, the Sodium Saccharin increased the production of IL-8 and INF-gamma, while cyclamate only of IL-8. The LP62 reduced the production of IL-8, but increased INF-gamma. In association with saccharin, lactobacillus reduced the production of both cytokines when compared to cells in which there were no association with Lactobacillus. Finally, the LP62 associated with cyclamate was able to reduce the production of IL-8 and INF-gamma both in comparison with the control and to the group in which there was no association.

Discussion

The study evaluated the viability of cells treated with different concentrations of saccharin and sodium cyclamate. The two substances were harmful to cells when used in high concentrations. Both in cells Ht-29 as in peripheral blood mononuclear the cyclamate has reduced the viability only in highest concentration (50 mM) (Figures 1,3), however, the saccharin showed this same effect from 10 mM for Ht-29 (Figure 2) and 1 mM, which was the lowest concentration evaluated, to mononuclear cells (Figure 4). The results also indicate that human cells are more tolerant to the presence of cyclamate then saccharin. In the test of the MTT, the increased of cell viability compared to the negative control indicates cell growth, i.e., lower generation time and consequent greater replication of cells. Thus, low concentrations of both sweeteners induced proliferation of cells Ht-29, as if functioned as a nutritional supplement. However, higher concentrations were able to kill these cells or at least slow down their growth.

In the case of peripheral blood cells, the situation is a little different, because these cells have a too low rate of replication, so the assessment of viability by MTT for cells that are not tumor, since the reduction of viability, what we observe is not the difference in the viability by reducing the growth/death and yes, almost exclusively by death. In this way, we can conclude that the saccharin sodium in all tested concentrations (1, 10, 30 and 50 mM) and the sodium cyclamate at 50 mM caused death of mononuclear cells. Therefore, comparing the peripheral blood cells to the Ht-29, it is possible to observe that the initial ones are more sensitive to sweeteners than the lineage. We also observed that considering that it is an experiment with non-tumor human cells and non-immortalized (PBMC) the standard deviation is greater. The data corroborate the results found by Eyk [3], who examined five artificial sweeteners, among them the saccharin and cyclamate sodium for 24, 48 and 72 hours. Eyk relates that after 24 hours of exposure to cyclamate there was growth of cells Ht-29 for low concentrations and death only in the highest concentration. In the same way with saccharin that in 24 hours showed a reduction

in cell viability in almost all concentrations in a dose-dependent manner.

A study conducted by Garland and colleagues [20] reports that cells AY-27, a lineage of epithelial cells processed in the bladder of rat after 24 hours of exposure to sodium saccharin showed reduction in cell viability in a dose-dependent manner, although in a bland way when compared to the present study. We observed then two main behaviors of sweeteners: induction of cell growth and death. The induction of growth was observed in cells Ht-29 exposed to low concentrations of sweeteners, here is a fact of great importance since these cells are tumor, so there is a possibility of studied sweeteners to contribute to the maintenance and growth of malignant tumors in humans. This induction was not observed in non-tumor cells; however, we must consider that is characteristic of this cell type a growth rate of almost zero. The other behavior that we observe is the death, noticed in both tumor cell types when treated with high concentrations of sweeteners, at this point, despite the promotion of the death of the cells Ht-29, we cannot speak of anti-tumor specific effect of cyclamate and/or saccharin sodium because the substances promoted death both of tumor cells (Ht-29) and not tumor ones (PBMC). We cannot say either the mechanism by which occurred the death of both cell types was the same as the cells that have different physiological patterns. We emphasize the need for a more detailed study in this aspect in order to clarify the possibility of sweeteners to favor or not the maintenance of malignant tumors in humans as well as their possible antitumor activity.

As for the dosage of cytokines, saccharin sodium increased the production of pro-inflammatory cytokines both in cells Ht-29 and in peripheral blood ones (Figures 5-8). It was more harmful than the cyclamate, since it increased just IL-8 in cells of peripheral blood, but maintained the levels of INF-gamma for both cell types. At this point we observed the importance of the presence of LP62, since when cells were treated with saccharin associated with Lactobacillus the effect of the sweetener was abrogated. The explanation of the fact may arise from the test of adherence of cells Ht-29 (Table 1), where we see that the lactobacilli showed higher rate of adherence to cells exposed to saccharin. In this group (saccharin + LP62) the percentage of bacterial accession was greater, being about five times higher than the group treated with cyclamate + LP62 and six times higher than the treated only with lactobacilli. As there was no increase in the number of total bacteria in the treatment in question in relation to others, we consider that the saccharin did not induce bacterial growth superior than the others, but increased the percentage of bacteria attached to eukaryotic cells. Thus, the higher bacterial adhesion proved to be a factor of great importance, being able to reduce the production of pro-inflammatory cytokines.

Several authors have cited the capacity of adhesion of probiotics on intestinal cells as a necessary mechanism to ensure its functionality [21-24]. It is believed that the ability of accession to the layer of mucus is vital in achieving certain probiotic properties [25]. This characteristic is considered a positive factor and beneficial for probiotic candidates.

Other microorganisms show this same property, as reported by Noh and collaborators [26], the authors evaluating the effect of acid lipoteichoic extracted from a strain of *Lactobacillus plantarum* reported that this important component of the cell wall of *Lactobacillus* and from other gram-positive bacteria exerted an anti-inflammatory process in intestinal epithelial human cells by blocking the production of IL-8. In the same year, Kainulainen and collaborators [27], evaluating the effect of a strain of *Lactobacillus acidophilus*, isolated from the jejunum of healthy dogs, reported that the same adheres to intestinal cells of the human lineage (Caco-2 and Ht-29) and present anti-inflammatory activity since it was able to reduce the production of IL-8 in cells Ht-29 stimulated with LPS. There was some disagreement between the therapeutic trial of Ht-29 cells and the mononuclear ones. The mononuclear cells responded better to the presence of LP62 than the lineage, the result of the dosage of IL-8 was quite promising, the presence of both sweeteners has stimulated the production of interleukin, however, the addition of lactobacillus reduced these values in a way that did not differ from control. In the case of INF-gamma, the addition of LP62 to cells treated with sweeteners has reduced the production of factor to levels lower than the control.

It´s remarkable the fact that the LP62, when used without sweeteners, raises the dosage of cytokines. Lactobacillus increased the production of INF-gamma in the two cell types and of IL-8 in the HT-29. It is important to notice that despite the increase of IL-8 in cells Ht-29, this does not occur in mononuclear cells. We believe that the clarification of the fact is based on the intrinsic physiology of the cell, being the Ht-29 a tumor cell associated with a strong inflammatory power. Gackowska and colaborators [28] also studied the effect of probiotics on peripheral blood cells, however without association with the sweeteners. The three microorganisms evaluated in the study - *Lactobacillus acidophilus*, *Lactobacillus delbrueckii* subsp. *Bulgaricus* and *Bifidobacterium bifidum* - were good inducers of cytokine production, the *L. Acidophilus* was the strongest inductor of INF-gamma, similar fact to that observed in the present study. Despite not being a desirable characteristic for probiotics, this does not exclude it. We must consider the anti-inflammatory capacity of LP62 in the presence of sweeteners, which reduced IL-8 and INF-gamma as in the Ht-29 cells as for the mononuclear cells treated with saccharin, as well as in the mononuclear cells treated with cyclamate.

So, we make clear the need for further studies aiming to elucidate the real capacity of *Lactobacillus plantarum* LP62 to be used as a component of probiotic food for human populations, ensuring its safety and effectiveness. We also emphasize the need for more research regarding the effect of sweeteners on human

cells, especially regarding the toxicity and ability pro or anti-tumor of the same.

Acknowledgments

References

1. Bergamo AB, Silva JAF, Jesus DP (2011) Simultaneous determination of aspartame, cyclamate, saccharin and acesulfame-K in soft drinks and tabletop sweetener formulations by capillary electrophoresis with capacitively coupled contactless conductivity detection. Food Chemistry 124: 1714-1717.

2. Shankar P, Ahuja S, Sriram K (2013) Non-nutritive sweeteners: Review and update. Nutrition 29: 1293-1299.

3. van Eyk AD (2015) The effect of five artificial sweeteners on Caco-2, HT-29 and HEK-293 cells. Drug Chem Toxicol 38: 318-327.

4. Whysner J, Williams GM (1996) Saccharin mechanistic data and risk assessment: urine composition, enhanced cell proliferation, and tumor promotion. Pharmacol Ther 71: 225-252.

5. International Agency for Research on Cancer (1999) IARC Monographs on the evaluation of carcinogenic risks to humans. Some chemicals that cause tumours of the kidney or urinary bladder in rodents and some other substances 73.

6. Ansari ARM, Mulla SJ, Pramod GJ (2015) Review on artificial sweeteners used in formulation of sugar free syrups. International Journal of Advances in Pharmaceutics 4: 5-9.

7. Sasaki YF, Kawaguchi S, Kamaya A, Ohshita M, Kabasawa K, et al. (2002) The comet assay with 8 mouse organs: results with 39 currently used food additives. Mutation Research 519: 103-119.

8. Kundi H, Butt SA, Shabnam H (2015) Variation in the area of islets of langerhans in sodium cyclamate treated rats. Pak Armed Forces Med J 65: 656-659.

9. Amin KA, Almuzafar HM (2015) Alterations in lipid profile, oxidative stress and hepatic function in rat fed with saccharin and methyl-salicylates. Int J Clin Exp Med 8: 6133-6144.

10. Abhilash M, Paul MVS, Varghese MV, Nair RH (2011) Effect of long term intake of aspartame on antioxidant defense status in liver. Food and Chemical Toxicology 49: 1203-1207.

11. Andreatta MM, Muñoz SE, Lantieri MJ, Eynard AR, Navarro A (2008) Artificial sweetener consumption and urinary tract tumors in Cordoba, Argentina. Preventive Medicine 47: 136-139.

12. Figlewicz DP, Ioannou G, Jay JB, Kittleson S, Savard C, et al. (2009) Effect of moderate intake of sweeteners on metabolic health in the rat. Physiology & Behavior 98: 618-624.

13. Bandyopadhyay A, Ghoshal S, Mukherjee A (2008) Genotoxicity Testing of Low-Calorie Sweeteners: Aspartame, Acesulfame-K, and Saccharin. Drug Chem Toxicol 31: 447-457.

14. Feijó FM, Ballard CR, Foletto KC, Batista BAM, Neves AM, et al. (2013) Saccharin and aspartame, compared with sucrose, induce greater weight gain in adult Wistar rats, at similar total caloric intake levels. Appetite 60: 203-207.

15. Villareal LMA, Cruz RAM, Ples MB, Vitor II RJS (2016) Neurotropic effects of aspartame, stevia and sucralose on memory retention and on the histology of the hippocampus of the ICR mice (*Mus musculus*). Asian Pacific Journal of Tropical Biomedicine 6: 114-118.

16. Abhilash M, Alex M, Mathews VV, Nair RH (2014) Chronic effect of aspartame on ionic homeostasis and monoamine neurotransmitters in the rat brain. International Journal of Toxicology 33: 332-341.

17. Suez, J. Korem T, Zilberman-Schapira G, Segal E, Elinav E (2015) Non-caloric artificial sweeteners and the microbiome: findings and challenges. Gut Microbes 6: 149-155.

18. Cani PD, Hul MV (2015) Novel opportunities for next-generation probiotics targeting metabolic syndrome. Current Opinion in Biotechnology 32: 21-27.

19. Chong ESL (2014) A potential role of probiotics in colorectal cancer prevention: review of possible mechanisms of action. World J Microbiol Biotechnol 30: 351-374.

20. Garland EM, Parr JM, Williamson DS, Cohen SM (1988) In vitro cytotoxicity of the sodium, potassium and calcium salts of saccharin, sodium ascorbate, sodium citrate and sodium chloride. Toxicol In Vitro 3: 201-205.

21. Santos RB, Barbosa LPJL, Barbosa FHF (2011) Probióticos: microrganismos funcionais. Ciência equatorial 1: 26-38.

22. Wang X, Wu Q, Deng K, Wei Q, Hu P, et al. (2015) A novel method for screening of potential probiotics for high adhesion capability. J. Dairy Sci 98: 4310-4317.

23. Jensen H, Ross S, Jonsson H, Rud I, Grimmer S, et al. (2014) Role of Lactobacillus reuteri cell and mucusbinding protein A (CmbA) in adhesion to intestinal epithelial cells and mucus *in vitro*. Microbiology 160: 671-681.

24. Ranadheera CS, Evans CA, Adams MC, Baines SK (2014) Effect of dairy probiotic combinations on *in vitro* gastrointestinal tolerance, intestinal epithelial cell adhesion and cytokine secretion. Journal of functional foods 8: 18-25.

25. Juge N (2012) Microbial adhesins to gastrointestinal mucus. Trends in Microbiology 20: 30-39.

26. Noh SY, Kang SS, Yun CH, Han SH (2015) Lipoteichoic acid from *Lactobacillus plantarum* inhibits Pam2CSK4-induced IL-8 production in human intestinal epithelial cells. Molecular Immunology 64: 183-189.

27. Kainulainen V, Tang Y, Spillman T, Kilpinen S, Reunanen J, et al. (2015) The canine isolate Lactobacillus acidophilus LAB20 adheres to intestinal epithelium and attenuates LPS-induced IL-8 secretion of enterocytes in vitro. BMC Microbiol 15: 4.

28. Gackowska L, Michalkiewicz J, Krotkiewski M, Helmin-Basa A, Kubiszewska I, et al. (2006) Combined effect of different lactic acid bacteria strains on the mode of cytokines pattern expression in human peripheral blood mononuclear cells. J Physiol Pharmacol 57: 13-21.