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The impact of non-nutritive sweeteners on isolated and purified microbial cultures derived from probiotics

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Abstract

Non-nutritive sweeteners (NNS) are a well-known substitute for table sugar with great implications in the management of various pathologies, mainly in Diabetes or Obesity. Previous studies have successfully shown that these substances are safe for human consumption, but one concern that remains is that of the impact on gut microbiota, mainly due to the complexity and diversity of the microbiome in humans. Keeping in mind that the gut microbiota contains numerous of Lactobacillus and Saccharomyces species, we simulated the microbiome by obtaining isolated microbial cultures of Saccharomyces boulardii and Lactobacillus reuteri, which we obtained two over the counter (OTC) probiotic supplements. We evaluated the bacteriostatic effect of three sweeteners (Sucralose, Saccharin, Stevia), using the successive dilution method and disc-diffusimetry on solid medium. What we sought to determine was a minimum inhibitory concentration (MIC) of each non-nutritive sweetener.

Keywords: non-nutritive sweeteners, artificial sweeteners, microbiome, bacteriostatic, sucralose, saccharin, stevia

INTRODUCTION

Obesity and diabetes are two of the most prevailing health emergencies throughout the world. Since 1975, obesity has nearly tripled and the number of diabetic people quadrupled between 1980 and 2014 [1,2].

If current trends prevail, by 2035 more than half of the global population will be either overweight or obese and it is projected that by 2045, almost 693 million patients will have diabetes [3,4]. Both of these diseases are caused and worsened by an excess of table sugar, which led the food and pharmaceutical industries to discover potential alternatives, such as non-nutritive sweeteners.

The main target involving diabetes is the management of blood glucose, while for obesity the goal is to reduce the caloric content consumed during the day. NNS are greatly used in obesity, due to their insignificant or lack of caloric content, and in diabetes as a consequence of their absent impact on glycemic levels [5].

They are preferred by virtue of their much greater sweeteners compared to table sugar (Table 1) [6].

NNS are relatively new to the human diet and offer a wide range of relative sweetness when used as sweeteners and food additives [7]. Despite their beneficial impact on people's health, one concern remains regarding their impact on the gut microbiota.

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TABLE 1. Comparing the sweetness potency of the most widely-used NNS, compared to table sugar

Sweetener	Sweetness potency (compared to table sugar)	Acceptable daily intake (mg/kg)
Saccharin	200-700x	45
Sucralose	600x	23
Aspartame	200x	75
Stevia	200-400x	9
Acesulfame-K	200x	23

Human health and the gut microbiome are intimately intertwined [8]. The gut microbiome's (GM) microbes coexists with the host in harmony and form an extremely complex ecosystem [9]. The GM makes a crucial contribution in host metabolism, which is heavily involved in food digestion, energy supplementation and immune system development [10]. Commensal bacteria generate substances that influence host immunological responses, support gut barrier integrity, protect against pathogenic microorganisms, and support the growth and regulation of the immune system. Throughout a person's lifespan, the GM might change depending on factors like age, genetics, food, drug use, and host immune condition [11].

Probiotics have been defined as a mixture of micro-organisms with beneficial effect in the prevention and treatment of particular pathological disorders. Probiotics have been around for as long as there have been fermented food consumed by humans. Most probiotic micro-organisms are bacteria [9].

Probiotics are typically recommended to support the immune systems of hosts and aid in recovery from specific disorders. The most effective way to use probiotics and which ones are beneficial for particular disorders is still unclear to the general population and numerous healthcare providers [12].

Saccharomyces boulardii is the only yeast probiotic that has been shown to be efficient in double-blind studies [13]. Many countries utilize this yeast as a preventive and therapeutic treatment for diarrhea and other gastrointestinal (GI) conditions caused by the use of antimicrobial drugs. *S. boulardii* has many characteristics that make it a promising probiotic agent, including the ability to survive along the GI tract, a preferred temperature range of 37°C and the ability to suppress the growth of many microbial pathogens, both *in vitro* and *in vivo* [9].

Lactobacillus reuteri is a symbiotic Lactobacillus species that has been shown to live throughout the GI tract of vertebrates and mammals, including humans and birds. Certain *L. reuteri* strains administered as probiotics have been demonstrated to give broad-spectrum protection from a variety of hosts, including protection from specific viral, bacterial, fungal and protozoal diseases. The most significant benefits of *L. reuteri* are the reduction of hypercholesterolemia caused by high-fat diet, diarrhea and lactose maldigestion. It has been demonstrated that *L. reuteri* significantly inhibits the growth of the *Streprococcus mutans.* The production of reuterin, a compound with broad-spectrum antibacterial activity, is what gives *L. reuteri* its antibacterial properties. Recent years have seen *L. reuteri* have been utilized widely in dairy-based functional foods as a probiotic supplement [14].

Sucralose is an artificial sweetener, found in 1976. This NNS was created by replacing three of the hydroxyl groups on the sucrose molecule with three chloride atoms. Sucralose has a pleasant sweet taste, is 450-650 times sweeter than sucrose and has a quality and time intensity profile that is quite similar to sucrose. It works reasonably well with other nutritional and artificial sweeteners. It is extremely soluble in water and stable throughout a wide pH and temperature range. When kept at high temperature, it does release hydrochloric acid and cause some sort of discoloration. Despite being created from sugar, sucralose has no calories since it is not metabolized by the body and is not recognized as a sugar. While 11-27% of the sucralose that is consumed is absorbed, the majority of it is directly expelled in the feces. The kidneys primarily remove the quantity received from the GI tract from the bloodstream and discard it in the urine. Food and Drug Administration (FDA) approved that sucralose is safe for human consumption and US Food and Drug Administration (USFDA) authorized sucralose for use as a universal sweetener [15].

Saccharin is an artificial sweetener and it was discovered in 1878. It is an NNS that has an unappealing metallic or bitter aftertaste. The original molecule is only sporadic soluble in water, so this sweetener is typically used as the sodium or calcium salt. Is 300 times sweeter than sucrose. At typical dosage, no study has ever demonstrated a direct causal link between saccharin consumption and health issues in people [15].

Stevia rebaudiana is a natural herb. Stevia, a non-artificial sweetener, has no calories, but it is 200-300 times sweeter than sucrose, thanks to steviol glycoside [16]. Since the human body cannot metabolize these sweet glycosides, stevia contains no calories. Stevia is a great sweetener for cooking and baking because, unlike artificial sweeteners, the sweet glycoside does not degrade in heat. According to studies, stevia tends to bring down high blood pressure. Additionally, diabetes patients' nutritional state is greatly improved [15].

MATERIALS AND METHODS

1. Materials

Reagents: ethanol 96%; sterile 0,9% saline solution

Growth medium: Sabouraud Glucose broth; MRS Agar; Mueller Hinton broth.

Probiotics: Protectis® (*Lactobacillus reuteri* DSM 1793; Bulardi® (Saccharomyces boulardii DBVPG 6763).

Sweeteners: Huxol Indulcitor lichid ® (saccharine 1,2%); Dulce de stevie Dacia Plant ® (stevia 3%); FlavDrops MyProtein® (0,05% sucralose).

Equipment: Petri Dishes, 96-well plates, Micropipettes, Laboratory flasks, tubes and tablewear, Inoculation loops, Biosafety cabinet, Laboratory incubators.

2. Methods

2.1. Preparing the microbial cultures

Saccharomyces boulardii

In order to create the isolated microbial cultures, at first we realized a probiotic suspension using the content of one probiotic capsule, which was later suspended in a tube on x mL of sterile 0.9% saline solution. As a next step, we used a sterile inoculation loop to inseminate the readily prepared *S. boulardii* suspension on a Petri dish provided with Sabouraud medium, which is a well-known growth medium for fungi. The Petri dish was incubated for 24 h at 37°C.

After the microbial growth took place, we took a small portion of the growing culture, which we inseminated again following the same steps, in order to obtain in the and an isolated and purified *S. boulardii* culture.

Lactobacillus reuteri

For the *L. reuteri* species, we followed the same steps we used for *S. boulardii,* the only differences being the culture medium, which in this case was MRS Agar and the incubation period, which took place for 48 hours in anaerobiosis.

2.2. Preparing the microbial suspension

The microbial suspension was obtained by collecting a small portion of the microbial culture, which was later suspended in sterile 0.9% saline solution, in order to obtain a microbial suspension with density adjusted to 1.5 × 108 CFU/mL according to the 0.5 McFarland nephelometric standard.

2.3. Serial dilution method

Saccharomyces boulardii

For the serial dilution method we used two 96-well polystyrene microtiter plates, one for each microbial species. In the first step, we pipetted 90 µL of growth medium in each well. As illustrated in Figure 1, in the second stage of the method we pipetted in duplicate 90 µL of each sweetener in the first column of the plate. We then extracted 90 µL of the formed solution found in the first well and pipetted the corresponding 90 µL into the second well. The same process was repeated until the 10th column was reached, thus obtaining serial dilutions of sweetener concentrations. The last two columns, 11th and 12th, were reserved for the control and blank respectively.

At last, we pipetted 10 µL of previously prepared microbial suspension in each well, except for the wells

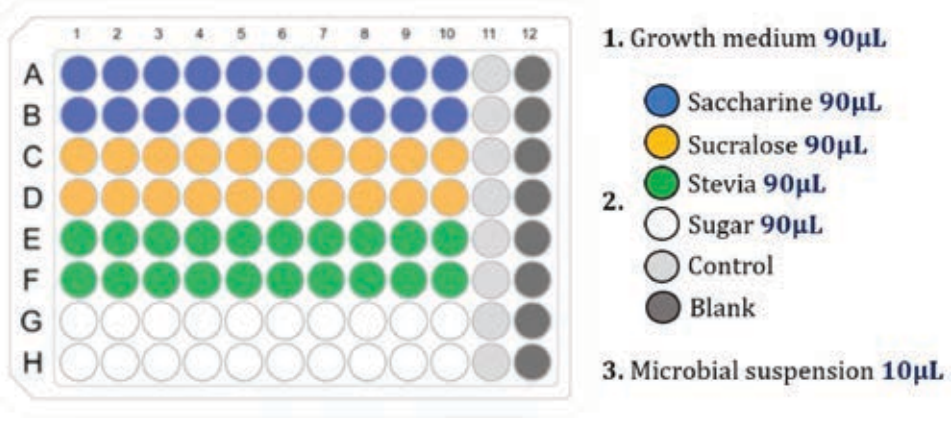

FIGURE 1. Serial dilution method using 96-well plates

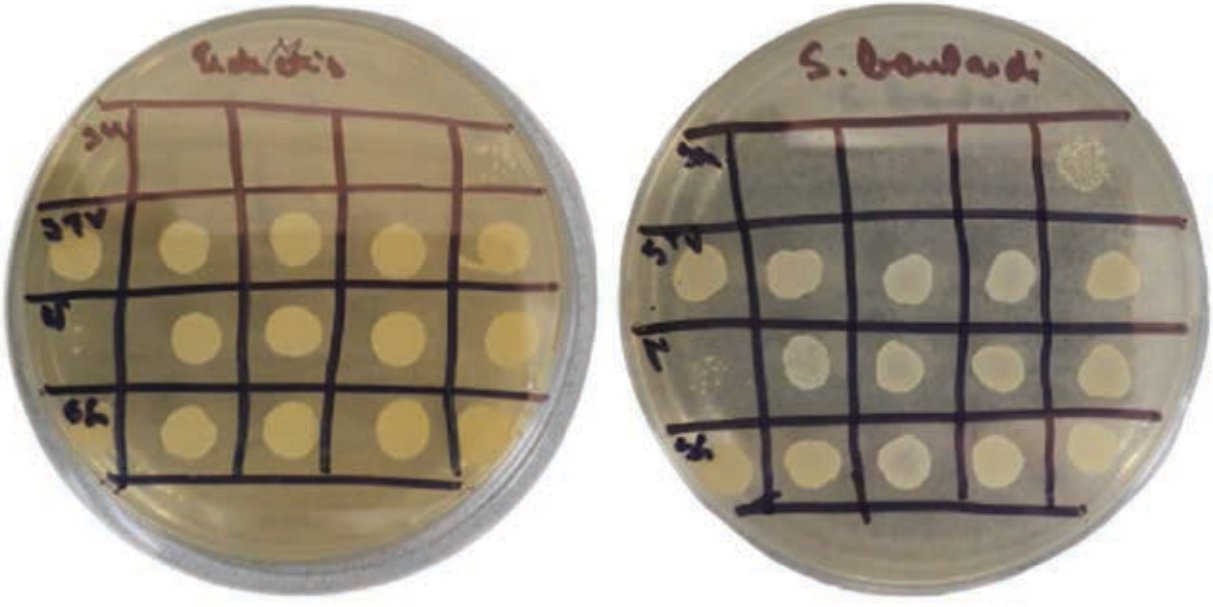

FIGURE 2. Petri dishes provided with Mueller Hinton broth, after incubation

corresponding to the 11th column. The plates were then further incubated under the conditions applied for the Petri dishes [17].

2.4. The disc-diffusimetric method

For the disc-diffusimetric method we used a Petri dish provided with Mueller Hinton broth. The Mueller Hinton medium culture is a well-known medium used for antimicrobial susceptibility tests (AST) [18].

As demonstrated in Figure 2, we divided each plate in 5 areas for each sweetener, corresponding to the first 5 wells found on the 96-well polystyrene microtiter plates from the successive dilution method. We chose to limit ourselves to the first 5 wells, the reason being that at a first glance, there was an obvious growth after this threshold, more specifically the growth of the microbial species was not affected from the 6th well forward.

Since our goal is to determine the MIC, it would be unreasonable to include the other wells, besides the first 5 wells, in the disc-diffusimetric method.

After dividing the plates, we pipetted in each spot 10 µL of the solution found in the corresponding well. After pipetting, we incubated the plates for 24h at 37°C [17].

RESULTS AND DISCUSSIONS

Saccharomyces boulardii

In identifying the MIC, we observed that in the case of sucralose, a growth inhibition took place at the 5th dilution, at a concentration of 0.031 µg/mL respectively. Saccharine also showed an inhibitory effect on the microbial proliferation, but in this case this was noticed only at the highest concentration, at 12000 µg/mL.

On the other hand, Stevia did not impact the microbial proliferation in a negative manner, its behavior being comparable to that of table sugar.

Lactobacillus reuteri

All three sweeteners impacted the *L. reuteri* species comparable to the *S. boulardii.* Sucralose showed a MIC at the 5th dilution, while Saccharin inhibited the proliferation at the highest concentration. The only difference was a more noticeable growth inhibition of the two sweeteners, compared to the inhibitory effect observed on *S. boulardii*. Sucralose behaved in the same manner, with a lack of inhibitory effect over the microbial proliferation.

Deniņa et al. showed an inhibitory effect on the microbial proliferation of six L. reuteri strains. The study used stevioside and rebaudioside A – the most abundant gligosides found in Stevia rebaudiana Bertoni leaves – in different concentrations, ranging from 0.2 to 2.6 g/L, the inhibitory effect being pronounced for two of the six strains [14].

On the other side of the spectrum, Kunova et al. investigated a potentially beneficial, prebiotic-like behavior of steviol glycosides over eight strains of

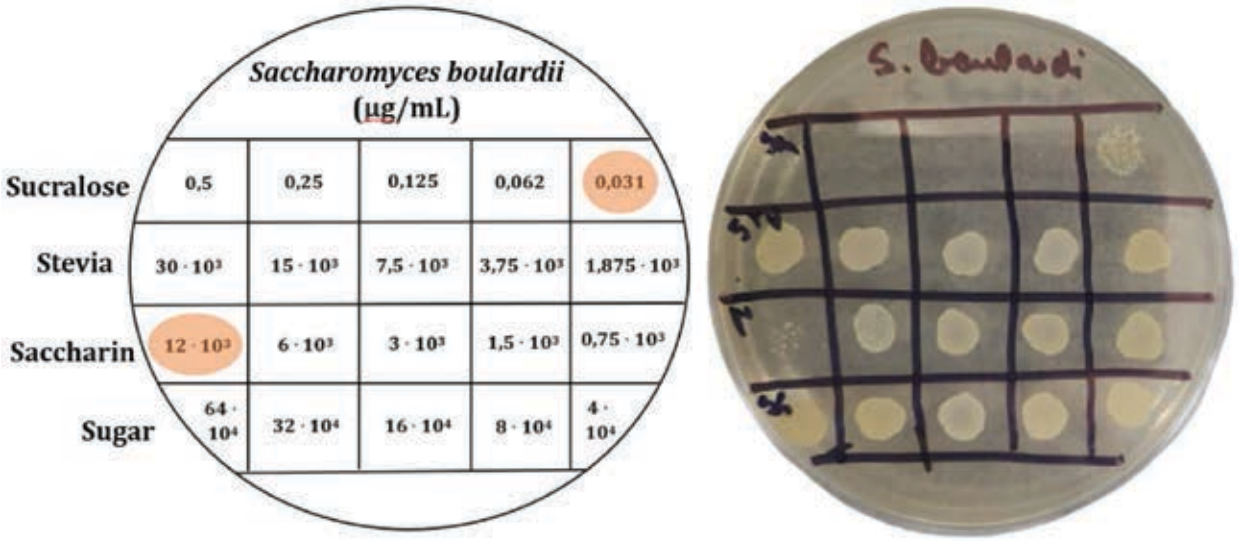

FIGURE 3. The minimum inhibitory concentration of each sweetener for the* S. boulardii *species

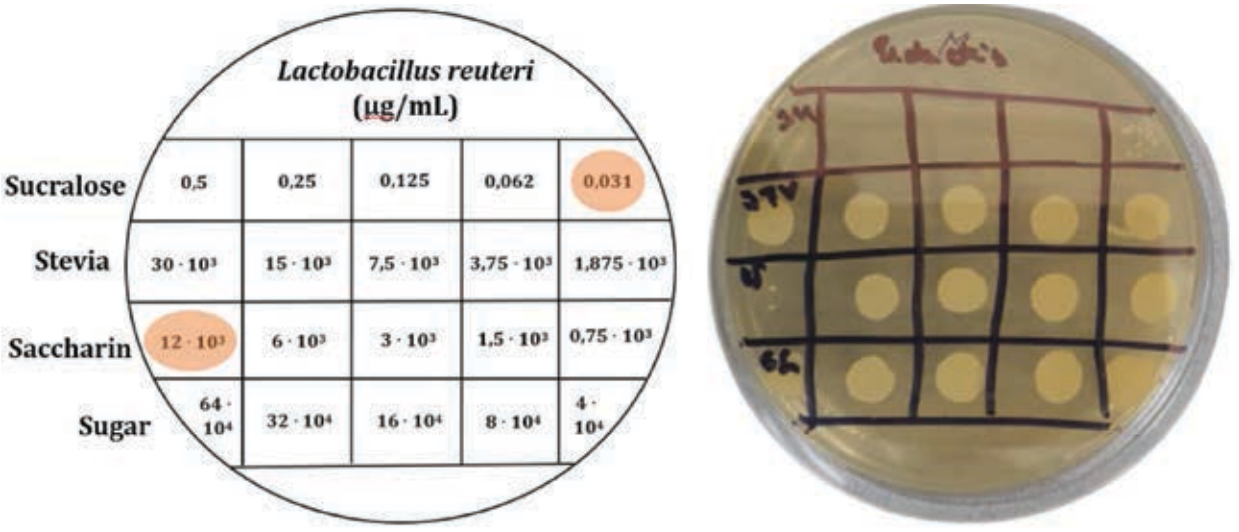

FIGURE 4. The minimum inhibitory concentration of each sweetener for the* L. reuteri *species

bifidobacteria and seven strains of lactobacilli. Although neither glycoside fulfilled the criteria for consideration as prebiotics, they did not alter the proliferation of the strains [19].

Sucralose was shown to inhibit in vitro the proliferation of two strains of gut bacteria, *Escherichia coli* and *Enterobacter aerogenes*. According to Corder et al., the growth of *E. coli* was inhibited to a higher degree compared to the *Enterobacter aerogenes* species. Moreover, chronic exposure to sucralose tends to induce an adaptation of *E.coli* regarding the inhibitory effect of sucralose. When both species were tested in co-culture, *Enterobacter aerogenes* was able to rapidly and completely out-compete *E. coli*, this being one of the hypotheses of sucralose altering the gut microbiota, more specifically by inhibiting various species to a different degree [20].

In a study conducted by Pfeffer et al., a concentration of 0.5% saccharin was shown to have a marginally inhibitory effect on the activity in caecum content of Cara rats, saccharin inhibiting the glucose fermentation by the populated bacteria. On the other hand, Naim et al. has shown an almost 40% growth inhibition of saccharine, inhibiting the growth of 3 Lactobacillus strains and 3 *E. coli* strains [21].

In another *in vitro* study, Wang et al. tested the impact of sucralose, saccharin and stevia on two *E. coli* strains. The results indicated a growth inhibition over 90% in

the case of sucralose and saccharin on both strains, while stevia induced an inhibition of 83% on one strain, while not having an impact on the other strain [22].

CONCLUSIONS

Our research has shown that at the concentration declared by the producers, a bacteriostatic effect was observed in both saccharine and sucralose, the latter having a milder inhibitory effect, while stevia was shown to not negatively impact the proliferation of either microbial species tested.

The available in vitro studies on isolated microbial cultures are scarce and they tend to have contradictory results, while all of them reach the consensus that sweeteners indeed alter the gut microbiota, in a different manner depending on the subject.

The main limitations of our study are the sweeteners and microbial species. We did not use purified sweetener solutions, but instead we opted to use the form of sweetener that is found on the market shelves and used by the entire population. We also did not have an isolated strain, but instead we chose to use the contents of two probiotics that are largely used by numerous people and realized isolated and purified microbial cultures.

Despite these limitations, our study delivered useful information that was already demonstrated in the literature, thus, our findings further strengthen the belief that artificial NNS alter the gut microbiome by having a bacteriostatic effect over some species, while natural NNS tend to have a milder inhibitory effect on the microbiota.

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