Growth of lactic acid bacteria in the presence of sweeteners and an emulsifier

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Introduction

The microbiota is defined as a set of microorganisms inhabiting an ecological niche. Existing in different parts of the human body such as mouth, skin and vagina, the most studied is the intestinal microbiota due to its crucial role in regulating health. Through various methods of identification, it has been established that gut microbiota is composed of both prokaryotes and eukaryotes. The predominating bacteria of the phyla include *Firmicutes, Bacteroidetes, Actinobacteria, Fusobacteria, Verrucomicrobia and Proteobacteria*, the first two being most abundant (1, 2). Research has established the relationship between the gut microbiota and different aspects of health, such as immunomodulation, pathogen control, peristalsis, body weight, behavior and cognitive functions (3, 4), although some are still being studied.

Dysbiosis is defined as any change in the composition of microbiota in relation to those found in healthy individuals. Factors such as antibiotic intake, infections and dietary changes, among others, may lead to this dysbiosis (5). It has been established in journals throughout the past three years, that certain additives used by the food industry may have effects on the composition of the gut microbiota and thus, the health of individuals. Sweeteners and emulsifiers such as aspartame and polysorbate 80, have been studied in the mouse with previous studies indicating that there is no effect on probiotic microorganisms when added to products such as yogurt and ice cream. However, this has been measured in terms of its shelf-life stability (6).

For sweeteners, it has been reported that non-caloric sweeteners (NCS) such as saccharin, sucralose and aspartame induce glucose intolerance in mice when supplied through water, with saccharin inducing the greatest effect. The composition of the feces of different groups was examined by 16S ribosomal RNA sequencing, revealing that those with saccharin intake experienced changes in their microbiota composition (7). The relationship between sweeteners intake, dysbiosis and obesity has been investigated as one of several factors (8). There are also studies on emulsifiers such as carboxymethyl cellulose (CMC) and polysorbate 80 (Tween 80), again in murine models, showing an association between intake of these compounds and the alteration in the microbiota (9). The onset of metabolic syndrome and colitis was observed in mice that were fed emulsifiers when compared to the control group (9).

In vivo experiments have shown that culturing the microorganisms in typical media, where sweeteners and emulsifiers are added at concentrations equivalent to the Acceptable Daily Intake (ADI) and the Estimated Daily Intake (EDI) levels, can provide an insight into the behavior of these conditions.

Methods and Materials

Microorganisms and medium: 9 strains of lactic acid bacteria were used: *Lactobacillus acidophilus* (Danisco), *L. curvatus* NRRL B 4562, *L. helveticus* NRRL B 4526, *L. reuteri* NRRL B 14171*, L. rhamnosus* (I) NRRL B 442, *L. rhamnosus* (II) ATCC 7666, *L. brevis* ATCC 14869*, L. plantarum* ATCC 14917 and *Pediococcus acidilactici* ATCC 8042. They were preserved in Agar Man Rogosa Sharpe (MRS Agar). The inoculum were prepared in test tubes with 10 mL of MRS broth (MRS Broth).

Sweeteners: the following sweeteners were tested: aspartame, stevia, acesulfame K and sucralose at concentrations equivalent to ADI, EDI and an equivalent to aspartame EDI, as shown in Table 1 (10, 11).

Emulsifier: Tween 80, 1% was used.

Table 1. Concentrations of additives (ADI, EDI and EDI equivalent) used in experimentation.

Sweetener (mg/kg)	Aspartame	Stevia	Acesulfame K	Sucralose
ADI	40	4	15	5
EDI	13.3	1.3	0.69	1.6
EDI Asp	13.3	13.3	13.3	13.3

Equipment: Centrifuge Eppendorf 5418, Bioscreen (Growth Curves USA), laboratory incubator (Riossa).

Methodology: The experiment was conducted in two parts: in the first, microorganisms preserved in MRS agar were transferred to test tubes with 10 mL of MRS broth and incubated at 37 °C for 18 hours. After incubation, serial dilutions from 10^{-2} to 10^{-6} were made in saline solution (0.95% NaCl), to spread the last by extension surface in Petri dishes with MRS agar supplemented with sweeteners and emulsifier, as the concentrations shown in table 1, by triple. The control experiment is Agar MRS; the plates were incubated at 37 °C, making observations at 12, 24 and 36 hours to analyze the colony morphology.

The second experiment involved getting kinetic data for microbial growth in MRS broth with sweeteners and emulsifier at concentrations equivalent to EDI for each one and 13.3 mg/kg in general (Table 1) with MRS broth as control. Inoculates were prepared as mentioned and after leaving overnight the biomass was centrifuged at 5000 rpm for 5 minutes to remove traces of culture medium. Subsequently the cell pellets were re-suspended in saline solution to a final concentration of 1 x 10^2 cell/mL. 50 µL of this suspension were added to 340 µL of MRS broth with 10 µL of additives solution in Bioscreen incubation plates under aseptic conditions. Triple experiments were done and microbial growth was followed until stationary phase. Samples of final culture were inoculated in MRS agar to observe the colony morphology.

Results and Discussion

The specific growth rate (µ) for each microorganism exposed to the tested additives were calculated. Tables 2 and 3 show the values µ (1/h) with equivalent concentrations of EDI aspartame and each additive EDI.

Table 2. Growth rate in the presence of additives in concentrations of 13.3 mg / kg (equivalent EDI)

Additive	Aspartame	Acesulfame K	Sucralose	Stevia	Tween 80	MRS
	13.3 g/kg	13.3 g/kg	13.3 g/kg	13.3 g/kg	1%	
Strain	μ [1/ h]	μ [1/ h]	μ [1/ h]	μ [1/ h]	μ [1/ h]	μ [1/ h]
L. acidophilus	0.144	0.114	0.127	0.125	0.110	0.125
L. curvatus	0.109	0.097	0.080	0.114	0.078	0.082
L. helveticus	0.073	0.103	0.088	0.080	0.091	0.098
L. rhamnosus I	0.128	0.112	0.095	0.128	0.108	0.128
L. brevis	0.089	0.094	0.058	0.069	0.067	0.106
L. plantarum	0.121	0.119	0.085	0.115	0.043	0.072
L. rhamnosus II	0.075	0.094	0.092	0.068	0.078	0.086
P. acidilactici	0.075	0.062	0.079	0.085	0.058	0.072
L. reuteri	0.089	0.120	0.124	0.115	0.056	0.106

Table 3. Growth rate in the presence of additives (EDI)

Additive	Aspartame	Acesulfame K	Sucralose	Stevia	Tween 80	MRS
	13.3 mg/kg	0.69 mg/kg	1.6 mg/kg	1.3 mg/kg	1%	
Strain	μ [1/ h]	μ [1/ h]	μ [1/ h]	μ [1/ h]	μ [1/ h]	μ [1/ h]
L. acidophilus	0.179	0.134	0.148	0.135	0.154	0.142
L. curvatus	0.115	0.124	0.133	0.128	0.059	0.097
L. helveticus	0.107	0.115	0.112	0.115	0.109	0.110
L.rhamnosus I	0.102	0.123	0.112	0.146	0.098	0.106
L. brevis	0.188	0.133	0.168	0.142	0.074	0.124
L. plantarum	0.094	0.122	0.119	0.108	0.074	0.062
L. rhamnosus II	0.140	0.126	0.130	0.134	0.114	0.126
P. acidilactici	0.063	0.062	0.068	0.065	0.061	0.069
L. reuteri	0.099	0.111	0.124	0.104	0.094	0.107

Aliquots of each microorganism were spread in MRS agar to investigate any changes in the morphology and colony growth after having been in contact with the additives, and to compare with the development of lactic acid bacteria in sweetener concentration media equivalent to the ADI.

Overall, microorganisms presented the typical morphology of lactic acid bacteria, according to Bergey's Manual (12), with colonies of 2-4 mm diameter, circular, raised, convex, regular full edge, smooth surface, opaque white. No significant changes were noted, except for five bacteria, as shown in Table 4.

Table 4. Morphological changes of bacteria under different culture conditions

Additive	Asp ADI	Ste ADI	Suc ADI	Asp EDI		Ste EDI		Ac K EDI		Tween 80 1%	
Strain	CC*	CC		CC	G*	CC	G	CC	G	CC	G
L helveticus	++	++	Small colonies						--		No
L. plantarum	+++			-	-	-	-	-	-		
L. rhamnosus										halo	
L. curvatus										+++	
L. reuteri	halo	halo	halo			-					

* CC = Colony color; G = Growth

As shown in Tables 2 and 3 the specific growth rate of microorganisms is affected differently depending on the additive and its concentration; in the case of equivalent EDI (13.3 mg / kg) *L. brevis* and *L. reuteri* grow slower under addition of Stevia and Tween 80, respectively. In contrast, this does not occur with every EDI, indicating a relationship between the concentration and the measured effect.

For morphology, although little change is observed in general, there are notable differences in the colony color and the growth of some bacteria. This depended on the culture conditions, whereby if the sweetener or emulsifier was added to MRS agar or whether the microorganisms were grown in MRS broth with additive and seeded in Agar MRS, as reported in table 4.

Other aspects such as the additive impact on probiotic activity of strains need to be further studied.

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