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Research Note

Influence of Artificial Sweeteners on the Kinetic and Metabolic Behavior of *Lactobacillus delbrueckii* subsp. *bulgaricus*

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ABSTRACT

The addition of artificial sweeteners to a LAPT (yeast extract, peptone, and tryptone) medium without supplemented sugar increased the growth rate and final biomass of *Lactobacillus delbrueckii* subsp. *bulgaricus* YOP 12 isolated from commercial yogurt. Saccharin and cyclamate were consumed during microorganism growth, while the uptake of aspartame began once the medium was glucose depleted. The pH of the media increased as a consequence of the ammonia released into the media supplemented with the sweeteners. The *L. delbrueckii* subsp. *bulgaricus* strain was able to grow in the presence of saccharin, cyclamate, or aspartame, and at low sweetener concentrations, the microorganism could utilize cyclamate and aspartame as an energy and carbon source.

Food additives play a vital role in the modern food industry and are generally used to provide some very specific and precisely defined sensory characteristics, such as taste, appearance, consistency, or shelf life *(4).* When designing a fermented food, it is necessary to know the tolerance of the specific starter microorganisms to the chemicals used as additives and to consider the modifications that the microorganism produces on these compounds. However, at present, the effects of some additives on the growth of starter bacteria have not been extensively studied *(3, 7, 10, 11),* and further information is still needed.

Artificial sweeteners are widely used as additives in low-calorie fermented foods and beverages. The most common artificial sweeteners include saccharin (*o*-benzoic sulfimide), aspartame (L-aspartyl-L-phenylalanine methylester), and cyclamate (cyclohexylsulfamate). At present, little information is available concerning the effect of artificial sweeteners on the growth of lactic acid bacteria associated with fermented dairy products. Samona and Robinson *(10)* found no effect of aspartame on the growth of bifidobacteria in milk. Vinderola et al. *(12)* reported that acesulfame and aspartame were not inhibitory for lactic acid starters, *Streptococcus salivarius* subsp. *thermophilus, Lactobacillus delbrueckii* subsp. *bulgaricus,* and *Lactococcus lactis* or for the probiotic bacteria *Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus paracasei, Lactobacillus rhamnosus,* or bifidobacteria.

The aim of this study was to evaluate the effect of the broadly used artificial sweeteners saccharin, cyclamate, and aspartame on the kinetic and metabolic behavior of *L. delbrueckii* subsp. *bulgaricus* YOP 12 isolated from commercial yogurt.

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MATERIALS AND METHODS

Microorganism, culture media, and incubation conditions. *L. delbrueckii* subsp. *bulgaricus* YOP 12 was isolated from an argentine yogurt *(6).* The strain was maintained by transference of the organism in sterile reconstituted nonfat dry milk. The cells were grown in LAPT (yeast extract, peptone, and tryptone) medium *(8)* with the following final concentration: yeast extract, 0.5% (Merck, Darmstadt, Germany); peptone, 0.75% (Merck); tryptone, 0.5% (Merck); and Tween 80, 0.05% (Sigma, St. Louis, Mo.); the cells were then incubated at 37°C for 72 h. This medium contained 0.16 g liter^{-1} of glucose as a contaminant of other additives. The artificial sweeteners used in this study were sodium saccharin, sodium cyclamate, and aspartame, purchased from Sigma, at concentrations of 0.5, 1, or 2 g liter^{-1}. The adjustment of pH to 6.5 was carried out with 10 g liter^{-1} NaOH (Merck) before sterilization by autoclaving for 15 min at 121°C.

Sterilized media were inoculated with 1×10^6 cells per ml from overnight cultures of *L. delbrueckii* subsp. *bulgaricus* that were first washed in 0.9% NaCl (Merck). To determine the effect of artificial sweeteners, the microorganism was previously preadapted by culturing twice in LAPT media containing the different sweeteners.

Growth kinetics. At different incubation times, portions of the cultures were taken, and bacterial growth was measured by an optical density at 600 nm or a dry weight procedure. Specific growth rate was determined by change in optical density.

The dry weight procedure consisted of the following: (i) portions of the culture were taken at particular intervals and centrifuged at 12,000 $\times$ *g* to sediment bacterial cells to the bottom of a vessel; (ii) the cell pellet was then washed to remove contaminating salt and dried in an oven at 100 to 105°C to remove all

water, leaving only the mass of components that make up the population of cells; and (iii) an increase in the dry weight of the cells was correlated with cell growth.

Analytical measurements. The aliquots of the culture supernatants obtained from different incubation times of *L. delbrueckii* subsp. *bulgaricus* were used for analytical determinations.

In all experiments, to determine some possible spontaneous chemical modification of the artificial sweeteners, uninoculated media were utilized as a control. Any significant chemical modification was observed.

Aspartame and saccharin determination. Aspartame and saccharin analyses were performed by reverse-phase high-performance liquid chromatography (HPLC) with an ISCO system liquid chromatograph (Isco, Inc., Lincoln, Neb.) controlled by the PeakSimple Chromatography Software (SRI Instruments, Torrance, Calif.). All separations were performed at 30°C on a ProntoSil 120–5 C8 SH column (150 by 4 mm inside diameter; Alltech Associates, Inc., Deerfield, Ill.). Detection was by a UV detector at 220 nm. Samples were injected in duplicate onto the column after being filtered through a 0.45-μm membrane filter. A mobile phase was used with 20 mmol liter^{-1} KH_2PO_4 (pH 3; Sigma) in acetonitrile (Fluka Chemie AG, Buschs, Switzerland) with an injection volume of 10 μl and a flow rate of 1.2 ml min^{-1}. Temperature was 30°C.

Cyclamate determination. An HPLC isocratic method with precolumn derivatization with trinitrobenzenesulfonic acid (Sigma) and UV detection was used for the quantification of cyclamate and cyclohexylamine (purchased from Fluka) in samples *(2).* Free cyclohexylamine was analyzed in the first run; subsequently, cyclamate was analyzed as cyclohexylamine, after the simple process of oxidation of the sample by means of hydrogen peroxide. The column used was a Waters Spherisorb ODS2 (Alltech), 5 μm, 150 by 4.6 mm, operated at room temperature. The time per run was 15 min. The eluent consisted of 10 mmol liter^{-1} of monoammonium phosphate (Sigma) in acetonitrile (Fluka) (60:40, vol/vol), whose pH was adjusted to 3.5 with a few drops of 4 mol liter^{-1} of phosphoric acid (Merck) and filtered through a 0.45-μm-pore-size filter. Next, both liquids were mixed in a bottle and degassed with a helium flow. The eluent was pumped at a rate of 1.0 ml min^{-1}, and the column effluent was monitored at 335 nm.

A standard curve with a six-point calibration was made up with the concentration of cyclohexylamine in deionized water that ranged from 0.3 to 30 mg liter^{-1}. The calculations were performed by the internal standard method. Before injection, the samples were appropriately diluted.

Determination of pH. The pH of the samples was measured at the time of sampling. This was performed by pipetting 3-ml portions into 5-ml tubes and using a probe electrode standardized against buffer at pH 4.0 and 7.0 connected to an Altronix (TPX-I) thermal pH meter (Altronix Corporation, Brooklyn, N.Y.).

Ammonia determination. The determination of ammonia was based on the Berthelot reaction. Ammonia reacts with alkaline phenol and then with sodium hypochlorite to form indophenol blue. The A_{630} of the reaction product was measured in a CECIL 2021 spectrophotometer (Cecil Instruments Ltd., Cambridge, UK) and was directly proportional to the original ammonia concentration *(1).*

Glucose determination. The glucose concentration was estimated by a commercial glucose oxidase kit (Wiener Laboratories, Rosario, Argentina). Glucose oxidase catalyzes the following reaction:

$$\beta\text{-D-glucose} + O_2 \rightarrow \text{gluconic acid} + H_2O_2$$

Hydrogen peroxide, in the presence of peroxidase and reduced chromogen, causes the production of a brown or blue oxidized chromogen that was measured at 400 nm in a spectrophotometer CECIL 2021 (Cecil).

TABLE 1. *Effect of saccharin and cyclamate on the growth and glucose consumption of* Lactobacillus delbrueckii *subsp.* bulgaricus *in LAPT media*

Sweetener (g liter^{-1})	Specific growth rate (h^{-1})[a]	Final dry weight (g liter^{-1})	Rate of glucose consumption (g liter^{-1} h^{-1})
Saccharin			
0.0	0.005 A[b]	0.057 A	0.015 B
0.5	0.008 A	0.078 A	0.011 A
1.0	0.070 C	0.289 B	0.010 A
2.0	0.050 B	0.289 B	0.010 A
Cyclamate			
0.0	0.005 A	0.057 A	0.015 C
0.5	0.095 B	0.275 B	0.011 B
1.0	0.087 B	0.302 B	0.007 A
2.0	0.088 B	0.318 C	0.008 A

[a] Specific growth rate was determined by change in optical density.

[b] Values not sharing the same letter within a column are significantly different ($P < 0.05$).

Statistical analysis. Experiments were carried out in triplicate. The experimental data were analyzed by a one-way analysis of variance test. Variable means showing statistical significance were compared by Tukey's test (Minitab-12 software, Minitab Inc., State College, Pa.). All statements of significance are based on the 0.05 level of probability.

RESULTS

Table 1 shows the effect of different saccharin and cyclamate concentrations on the growth of *L. delbrueckii* subsp. *bulgaricus* and the rate of the contaminant glucose consumption (0.16 g liter^{-1}) in LAPT media. With the addition of 0.5 g liter^{-1} of saccharin, a slight reduction in the rate of glucose consumption was detected. At 1 g liter^{-1}, the highest increase of bacterial growth rate was observed. The dry weight also increased, reaching similar values with 1 and 2 g liter^{-1} of the sweetener. Added cyclamate significantly increased the specific growth rate, but there was no effect of concentration above 0.5 g liter^{-1}. However, cell dry weight increased with the concentration of cyclamate.

Independently of the sweetener concentration in the medium, the rate of glucose consumption was lower in the presence of the additives.

Figure 1 shows the effect of different aspartame concentrations on the growth of *L. delbrueckii* subsp. *bulgaricus.* During the first 8 h of incubation, the microorganism did not significantly modify either the bacterial growth rate (0.005 h^{-1}) or the specific rate of sugar consumption (0.2 h^{-1}).

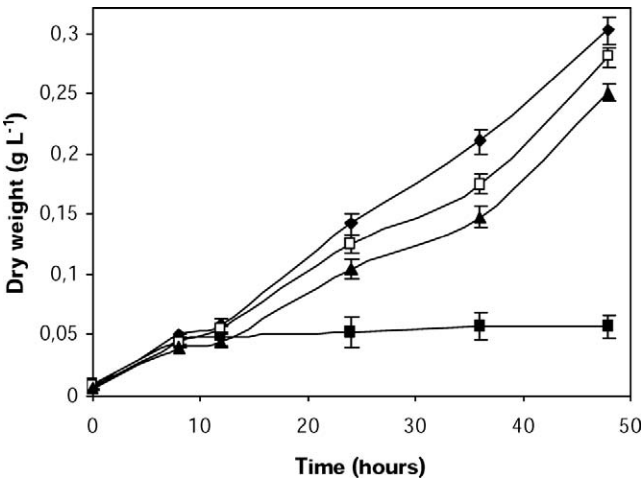

FIGURE 1. *Growth of* L. delbrueckii *subsp.* bulgaricus *in LAPT media without sweetener (■) and supplemented with 0.5 g liter^{-1} (♦), 1 g liter^{-1} (□), and 2 g liter^{-1} (▲) of aspartame. Values for dry weight are the average values of results obtained from three replicates. Vertical bars represent standard deviation.*

After glucose consumption, in all aspartame concentrations, the microorganism showed a higher specific growth rate and final biomass than what was observed during the first hour of growth.

Figure 2a through 2c shows that, independently of the sweetener added, a pH decrease was observed during the first 8 h of growth. This behavior correlated with the glucose consumption at the beginning of growth. The pH increased in direct relation to the ammonia released in the media with the sweeteners from 8 h of growth onward.

Table 2 shows the sweetener concentrations as a function of the incubation time during *L. delbrueckii* subsp. *bulgaricus* YOP 12 growth in modified LAPT media. With 0.5 g liter^{-1} of saccharin, no significant modification in its concentration was observed. At higher concentrations, saccharin was consumed from the beginning of microorganism growth. Cyclamate concentration decreased at 48 h of incubation independently of the initial concentration. Aspartame was not consumed during the first 8 h. At 48 h of incubation, a consumption of 0.18, 0.51, and 1.08 g liter^{-1} was detected in the presence of 0.5, 1, and 2 g liter^{-1}, respectively.

DISCUSSION

The results indicate that *L. delbrueckii* subsp. *bulgaricus* can utilize cyclamate and aspartame at concentrations normally present in yogurt (0.5 g liter^{-1}) when glucose, an easily fermentable sugar, is low or absent in the culture media. However, saccharin at the same concentration was not able to be utilized. The higher biomass and growth rate observed in the presence of the sweeteners suggested that they can be utilized as an energy and carbon source. The profile of sweetener depletion was correlated with the pH increase, suggesting that the ammonia was released during the metabolism of the noncaloric sweeteners. The higher pH as a consequence of the metabolism of the sweetener by the microorganism (reaching values between pH 7 and 8, as shown in Fig. 2) may be responsible for the lower

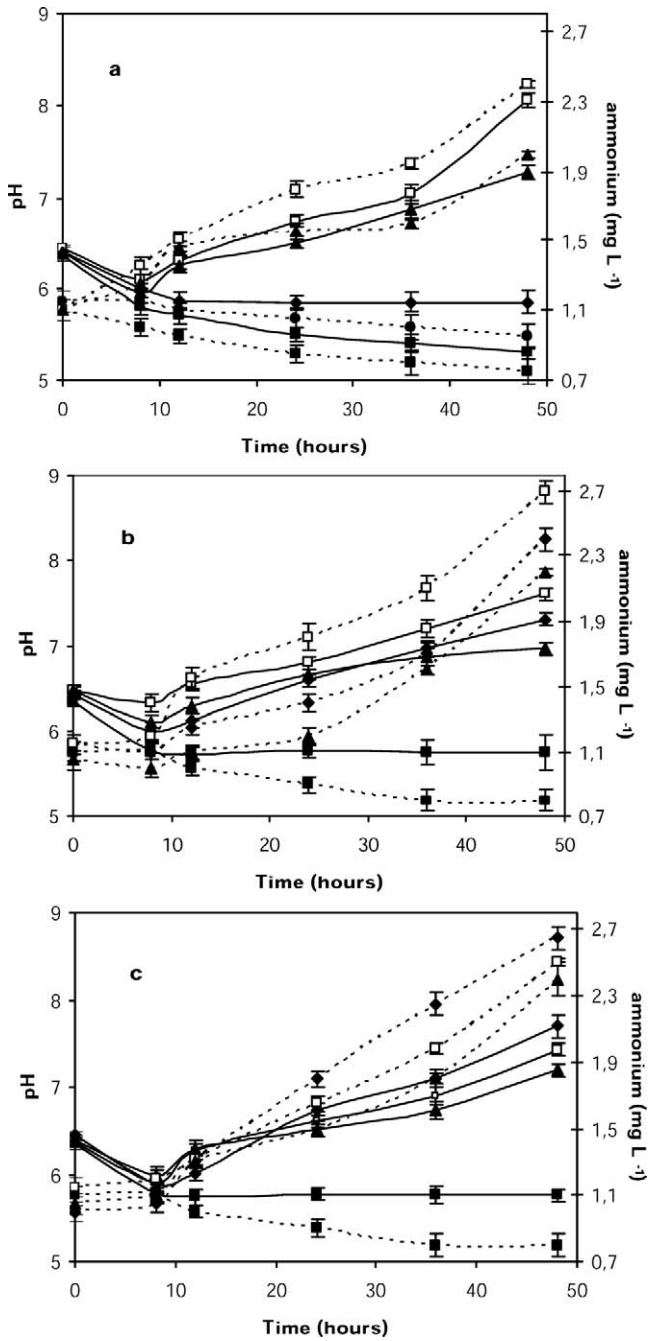

FIGURE 2. *pH (———) and ammonium production (– – –) during the growth of* L. delbrueckii *subsp.* bulgaricus *in LAPT media without sweetener (■) and supplemented with 0.5 g liter^{-1} (♦), 1 g liter^{-1} (□) and 2 g liter^{-1} (▲) of saccharin (a), cyclamate (b), and aspartame (c). Values for pH and ammonium production are the average values of results obtained from three replicates. Vertical bars represent standard deviation.*

stimulation of the growth rate observed with 1 and 2 g liter^{-1} of artificial sweeteners (Fig. 1).

Cyclamate (cyclamic acid and its salt) could be metabolized to cyclohexylamine, and from this compound, the microorganism would release ammonia into the media. Renwick et al. *(9)* reported that cyclamate is metabolized by gut bacteria to cyclohexylamine. Little is known about the degradation of cyclohexylamine by microorganisms. Iwaki et al. *(5)* isolated a strain of the genus *Brevibacterium*

TABLE 2. *Sweetener utilization by* Lactobacillus delbrueckii *subsp.* bulgaricus *in modified LAPT media*

Artificial sweetener (g liter^{-1})	Residual sweetener in the culture supernatant (g liter^{-1})			
	Incubation time (h):			
	0	8	24	48
Saccharin				
0.5	0.52 A[a]	0.54 A	0.52 A	0.50 A
1.0	0.95 D	0.40 C	0.24 B	0.14 A
2.0	1.83 D	0.93 C	0.62 B	0.46 A
Cyclamate				
0.5	0.49 D	0.31 C	0.24 B	0.14 A
1.0	1.00 C	0.39 B	0.31 B	0.24 A
2.0	1.76 C	1.61 C	1.40 B	0.97 A
Aspartame				
0.5	0.58 B	0.52 B	0.48 B	0.40 A
1.0	1.16 C	1.16 C	0.97 B	0.65 A
2.0	1.74 C	1.74 C	1.26 B	0.66 A

[a] Values not sharing the same letter within a row are significantly different ($P < 0.05$).

that degraded cyclohexylamine via cyclohexanone together with NH_3 formation.

Aspartame began to be consumed by *L. delbrueckii* subsp. *bulgaricus* YOP 12 once the media was glucose depleted, perhaps as consequence of a diauxic phenomenon. We think that aspartame could be transformed by the esterase that hydrolyzed the methyl ester, which yields a dipeptide and methanol, and by the peptidase, which would typically break the peptide bond between the aspartic acid and phenylalanine moieties. So, the amino acids produced can be used as carbon and nitrogen sources, while the ammonia released into the environment produces an increase in pH. Different results were reported by Vinderola et al. *(12).* These authors indicated that acesulfame and aspartame, at the concentration normally used in fermented dairy drinks (0.3 g liter^{-1}), were not inhibitory for lactic acid starters or probiotic bacteria. The highest aspartame concentration tested (1.2 g liter^{-1}) was inhibitory only for three lactic acid bacteria strains and one probiotic strain. Likewise, Samona and Robinson *(10),* working with aspartame (0.4 and 0.8 g liter^{-1}), found no effect on the growth of bifidobacteria in milk.

L. delbrueckii subsp. *bulgaricus* YOP 12 was able to grow in the presence of artificial sweeteners. The higher biomass and growth rate observed in the presence of low cyclamate and aspartame concentrations suggested that they can be utilized as an energy and carbon source. During growth, the microorganism produces changes in the media as a consequence of the ammonium release. This research is useful in showing that the organism is capable of productively metabolizing noncaloric sweeteners.

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REFERENCES

1. Allen, S. E., H. M. Grimshaw, J. A. Parkinson, and C. Quarmby. 1974. Inorganic constituents: nitrogen, p. 184–206. *In* S. E. Allen (ed.), Chemical analysis of ecological materials. Blackwell Scientific Publication, London.
2. Casals, I., M. Reixach, J. Amat, M. Fuentes, and L. Serra-Majem. 1996. Quantification of cyclamate and cyclohexylamine in urine samples using high-performance liquid chromatography with trinitrobenzenesulfonic acid pre-column derivatization. *J. Chromatogr. A* 750:397–402.
3. Gomes, A. M. P., M. G. M. Teixeira, and F. X. Malcata. 1998. Viability of *Bifidobacterium lactis* and *Lactobacillus acidophilus* in milk: sodium chloride concentration and storage temperature. *J. Food Process. Preserv.* 22:221–240.
4. Guilliland, S. E. 1998. Fermented milks and probiotics, p. 195–212. *In* E. Harth and J. L. Steel (ed.), Applied dairy microbiology. Marcel Dekker Inc., New York.
5. Iwaki, H., M. Shimizu, T. Tokuyama, and Y. Hasegawa. 1999. Biodegradation of cyclohexylamine by *Brevibacterium oxydans* IH-35A. *Appl. Environ. Microbiol.* 65:2232–2234.
6. Manca de Nadra, M. C., R. R. Raya, A. Pesce de Ruíz Holgado, and G. Oliver. 1986. Threonine aldolase in *Lactobacillus bulgaricus* ATCC 11842 and YOP 12. *Milchwissenschaft* 41:630–632.
7. Rada,V., and J. Dlabal. 1998. Susceptibility of bifidobacteria to nisin. *Lett. Appl. Microbiol.* 26:123–125.
8. Raibaud, P., M. Caulet, J. V. Galpin, and G. Mocquot. 1961. Studies on the bacterial flora of the alimentary tract of pigs. II. Streptococci: selective enumeration and differentiation of the dominant group. *J. Appl. Bacteriol.* 24:285–291.
9. Renwick, A. G., J. P. Thompson, M. O. Shaughnessy, and E. J. Walter. 2004. The metabolism of cyclamate to cyclohexylamine in humans during long-term administration. *Toxicol. Appl. Pharmacol.* 196:367–380.
10. Samona, A., and R. K. Robinson. 1993. Effect of sweetening agents on the growth and survival of *Bifidobacterium* spp. *J. Dairy Sci.* 76(Suppl. 1):120.
11. Vachon, H., and Z. Ustunol. 1998. Effect of sweetener type on lactic and acetic acid production by lactic acid bacteria and bifidobacteria. *J. Dairy Sci.* 82(Suppl. 1):7.
12. Vinderola, C. G., G. A. Costa, S. Regenhardt, and J. A. Reinheimer. 2002. Influence of compounds associated with fermented dairy products on the growth of lactic acid starter and probiotic bacteria. *Int. Dairy J.* 12:579–589.