

## Metabolism of Stevioside and Rebaudioside A from *Stevia rebaudiana* Extracts by Human Microflora

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*Stevia rebaudiana* standardized extracts (SSEs) are used as natural sweeteners or dietary supplements in different countries for their content of stevioside or rebaudioside A. These compounds possess up to 250 times the sweetness intensity of sucrose, and they are noncaloric and noncariogenic sweeteners. The aim of this study was to investigate the in vitro transformation of stevioside and rebaudioside A after incubation with human microflora, the influence of these sweeteners on human microbial fecal community and which specific groups metabolize preferentially stevioside and rebaudioside A. The experiments were carried out under strict anaerobic conditions in batch cultures inoculated with mixed fecal bacteria from volunteers. The hydrolysis was monitored by HPLC coupled to photodiode array and mass spectrometric detectors. Isolated bacterial strains from fecal materials incubated in selective broths were added to stevioside and rebaudioside A. These sweeteners were completely hydrolyzed to their aglycon steviol in 10 and 24 h, respectively. Interestingly, the human intestinal microflora was not able to degrade steviol. Furthermore, stevioside and rebaudioside A did not significantly influence the composition of fecal cultures; among the selected intestinal groups, bacteroides were the most efficient in hydrolyzing *Stevia* sweeteners to steviol.

**KEYWORDS:** *Stevia rebaudiana* sweeteners; stevioside; rebaudioside A; human intestinal microflora; steviol; in vitro metabolism; LC-MS analysis

### INTRODUCTION

*Stevia rebaudiana* (Bertoni) (stevia) is a shrub of the family Asteraceae that is indigenous to Paraguay and Brazil, and it is now cultivated also in some regions of Asia, Europe, and Canada. Stevia leaves contain diterpene glycosides, namely, stevioside, rebaudiosides A–F, steviolbioside, and dulcoside A (Figure 1), which are responsible for the typical sweet taste. Stevioside and rebaudioside A are the most abundant, accounting for 3–10% w/w and 1% w/w, respectively, whereas dulcoside A represents only 0.2%. Stevioside and rebaudioside A are up to 250 times sweeter than sucrose solution at 0.4% (1). Stevia leaf extracts are used in Japan, Korea, and certain countries of South America to sweeten soft drinks, soju, soy sauce, yogurt, and other foods, whereas in the United States they are used as dietary supplements. For example, the Japanese and Koreans have used annually in recent years about 200 and 115 tons of stevia extracts, respectively (2).

Stevia sweetener extractives have been suggested to exert beneficial effects on human health, including antihypertensive

(3, 4), antihyperglycemic (5, 6), antioxidant (7), noncariogenic (8), and anti-human rotavirus (9) activities. These sweeteners are also thought to influence glucose metabolism (10, 11) and renal function (12).

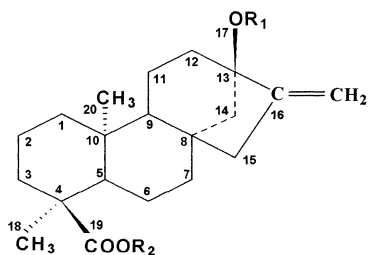
Several toxicological studies were carried out to verify the possible mutagenic and genotoxic effects of stevia extracts on bacterial cells and different mammalian species, and the results were recently reviewed (13–15). These studies and nearly 20 years of use in both Japan and Brazil seem to demonstrate that stevia extracts are safe. However, some aspects have not yet been fully elucidated, and further investigations are required. Specifically, the metabolism of stevioside and rebaudioside A in humans needs to be defined. Indeed, the available data on the fate of these sweeteners were obtained using animal models or in vitro experiments performed mainly with animal fecal microflora. Thus, the distribution of stevioside was studied in rats fed radiolabeled stevioside (16). The highest radioactivity was found after 1 h in the small intestine, whereas after 4 h the radioactivity was mainly concentrated in the cecum. These results were confirmed later by Cardoso et al. (17) using [<sup>131</sup>I]-stevioside injected in Wistar rats. In a more recent study, stevioside was administered to hamsters, and the blood, feces,

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| Compound       |     | R <sub>1</sub>                            | R <sub>2</sub> |
|----------------|-----|---|----------------|
| Stevioside     | (1) | -glc- <sup>2</sup> -glc                   | -glc           |
| Steviolbioside | (2) | -glc- <sup>2</sup> -glc                   | -H             |
| Rebaudioside A | (3) | glc- <sup>3</sup> -glc- <sup>2</sup> -glc | -glc           |
| Rebaudioside B | (4) | glc- <sup>3</sup> -glc- <sup>2</sup> -glc | -H             |
| Rebaudioside C | (5) | glc- <sup>3</sup> -glc- <sup>2</sup> -rha | -glc           |
| Dulcoside A    | (6) | -glc- <sup>2</sup> -rha                   | -glc           |
| Steviol        | (7) | -H  | -H             |

Figure 1. Chemical structures of the main *S. rebaudiana* sweeteners and their aglycon steviol.

and urine were found to contain steviol and steviol 16,17 $\alpha$ -epoxide (18).

Concerning in vitro studies, the digestive enzymes  $\alpha$ -amylase, pepsin, and pancreatin (19) and hepatic tissue were demonstrated to be unable to hydrolyze stevioside and rebaudioside A (20). In contrast, these sweeteners were proven to be degraded to their aglycon steviol by rat intestinal microflora (19, 21). Conversely, data on the effects of human microflora on stevioside and rebaudioside A are scarce. Therefore, it was considered of interest to investigate the transformation of stevioside and rebaudioside A by human fecal microflora, evaluating both the influence of these sweeteners on the microbial community and also which microbial groups preferentially metabolize these sweet compounds.

## MATERIALS AND METHODS

**Chemicals.** Stevia extracts containing either 85% (w/w) stevioside or 90% (w/w) rebaudioside A and pure steviolbioside were a gift of Specchiasol S.r.l. (Bussolengo, Vr, Italy). Steviol was a gift of Prof. A. D. Kinghorn (University of Illinois at Chicago, Chicago, IL). All reagents used were of HPLC grade (J. T. Baker, Deventer, The Netherlands).

**Human Subjects and Experimental Design.** Eleven healthy volunteers (6 men and 5 women), ages 20–50 years, participated in the study, randomly subdivided in two groups. None had any history of gastrointestinal disease or had taken antibiotics or laxatives for 2 months before the study. This study was approved by the local ethical committee, and volunteers were informed of the requirements and experimental protocols and gave written informed consent. Feces from five subjects were tested with stevioside or glucose, whereas feces from six subjects were successively tested with rebaudioside A or glucose. Stool specimens were collected in the morning, delivered to the laboratory within 1 h after collection to avoid a prolonged contact with atmospheric oxygen, and immediately introduced in an anaerobic cabinet (Forma Scientific, Marietta, OH) under a N<sub>2</sub>/H<sub>2</sub>/CO<sub>2</sub> atmosphere (85:10:5, v/v/v). A subsample of ~6 g was homogenized in 200 mL of incubation medium (22). Forty milligrams of stevioside or rebaudioside A was added to 40 mL of the fecal suspensions under agitation. To supply the same sugar quantity derived from stevioside or rebaudioside A, 27 or 30 mg of glucose, respectively, was also incubated. The fecal cultures were incubated in the anaerobic cabinet at 37 °C for 72 h. At fixed intervals (1 h) and for 48 h, 0.5 mL of fecal suspension was transferred in 0.5 mL of methanol to block bacterial

activities. The last withdrawal was done at 72 h. The methanolic solutions were then centrifuged at 1000g for 1 min, and the supernatant was stored at –20 °C before HPLC analysis. The control solutions (medium, medium plus microflora, medium plus stevioside or rebaudioside A or glucose) were also incubated under the same operative conditions.

To study the influence of the stevioside, rebaudioside A, and glucose on the human fecal microbial community, qualitative microbiological analyses were carried out on the controls (medium plus fecal suspension) and the test cultures (medium plus stevioside or rebaudioside A or glucose plus fecal suspension) at time 0 and 24 h after incubation.

Finally, to evaluate which microbial groups were involved in the metabolism of stevioside and rebaudioside A, the whole colonies grown on different selective media were separated and suspended in the incubation medium with added stevioside and rebaudioside A (1 mg/mL). The cultures of selected microbial groups, obtained after 24 h of anaerobic incubation, were analyzed by LC-DAD-MS to monitor the biotransformation of stevioside and rebaudioside A.

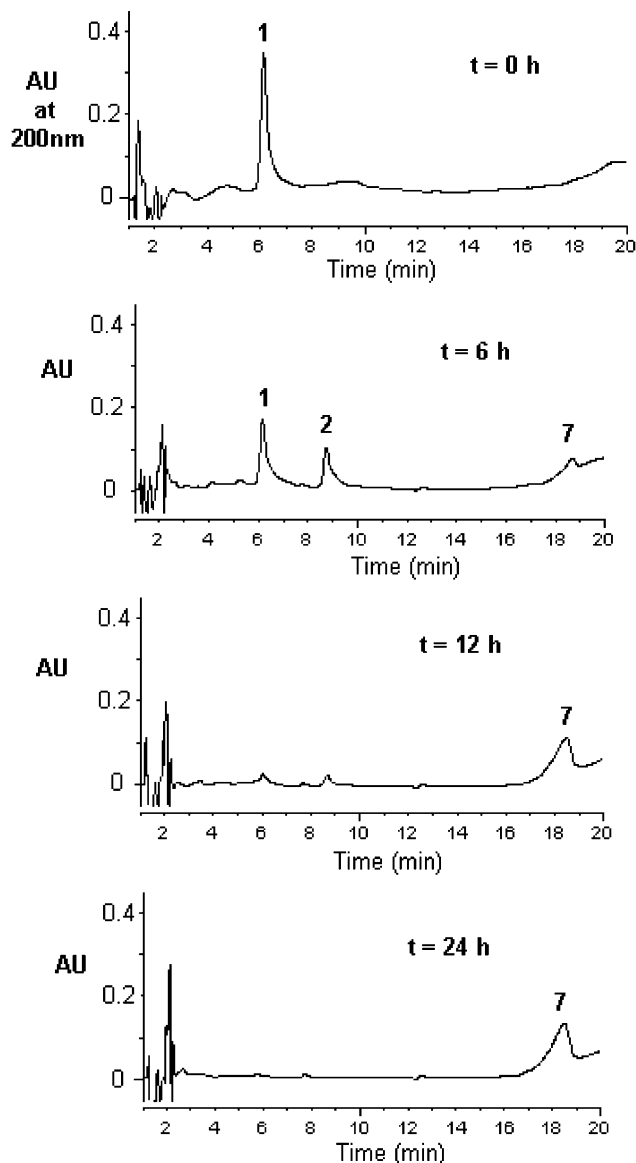
**Bacteriological Analyses.** Samples from fecal suspensions were serially diluted 10-fold in a pre-reduced dilution blank (23). Dilutions were plated on blood agar (23) for total anaerobes, on Difco tryptic soy agar for total facultative anaerobes, on kanamycin–vancomycin blood agar medium (24) for Bacteroidaceae, on Beerens medium (25) for bifidobacteria, on sulfite–polymixin–milk agar (26) for clostridia, on Lamvab medium (27) for lactobacilli, on Difco KF *Streptococcus* agar for enterococci, and on Difco McConkey agar for coliforms. All media used for cultivation of anaerobe microorganisms were pre-reduced in the anaerobic cabinet for 48 h before use. Plates were incubated aerobically as appropriate at 37 °C. Microorganisms were classified for the genus level on the basis of Gram reaction, spore formation, cell morphology, and fermentation end-product (23). Microbial counts were reported to the wet weight of the fecal inoculum and determined as log colony-forming units (CFU) per gram.

**Chromatographic Conditions.** An Alliance 2695 (Waters, Milford, MA) equipped with a model 2996 (Waters) photodiode array detector was used. Stevioside, rebaudioside A, and their metabolites were separated on a 250 × 4.6 mm i.d., 5  $\mu$ m, C<sub>18</sub> Symmetry column (Waters) at a flow rate of 1.5 mL/min. The elution was carried on by a linear gradient using water (A) and acetonitrile (B) as eluents, as follows: 0–10 min, 30–40% B; 10–20 min, 40–80% B; held for 5 min, 80% B. The column was thermostated at 30 °C and 50  $\mu$ L of sample injected. The chromatograms were acquired in the range of 195–350 nm and integrated at 200 nm.

**Mass Spectrometry.** A Hewlett-Packard 5989A single-quadrupole instrument equipped with an electrospray interface (HP 59987A) and connected to a model 1090 HPLC (Hewlett-Packard) was used. Nitrogen was the nebulizing gas at a pressure of 50 psi and a temperature of 300 °C. Mass acquisitions were carried out in the scan mode from  $m/z$  100 to 1200 and in selected ion monitoring (SIM). The analytical column was a 150 × 2.1 mm i.d. C<sub>18</sub> Symmetry (Waters), and the flow rate was 0.4 mL/min. The injection volume was 20  $\mu$ L. Stevia glycoside  $m/z$  signals were acquired in positive ion mode and steviol signals in negative ion mode.

**Calibration Curves.** Stevia glycosides and steviol were dissolved in methanol (0.5 mg/mL), and the resulting stock solutions were stored at –20 °C. Aliquots of these solutions were used to prepare the working solutions in the range of 1–100  $\mu$ g/mL.

**Statistical Analyses.** Statistical analyses were performed on a personal computer using the Statistica software (StatSoft, Tulsa, OK). A repeated-measures ANOVA design with treatment as independent factor was used to investigate the effect of stevioside and rebaudioside A on intestinal human bacterial and compared with the effect of glucose. For stevioside and rebaudioside A metabolism the treatments were as follows: only medium; medium plus stevioside or rebaudioside A plus fecal suspension; medium plus stevioside or rebaudioside A; medium plus fecal suspension; medium plus fecal suspension plus glucose. *P* values <0.05 were considered to be significant.

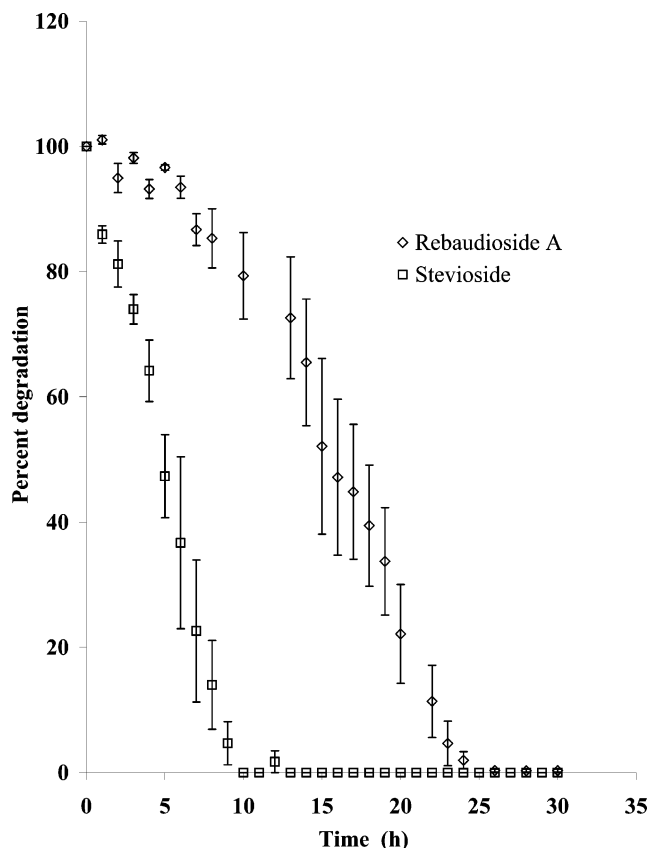


**Figure 2.** Typical HPLC chromatograms obtained from stevioside incubated with human fecal suspension: (1) stevioside; (2) steviolbioside; (7) steviol.

## RESULTS

**Figure 2** shows typical HPLC chromatograms obtained from stevioside incubated with human fecal suspension. The identities of stevioside, steviolbioside, and steviol were confirmed by cochromatography, on-line UV spectra comparison, and mass spectrometric analyses. The LC-MS spectrum in negative ion mode of the peak with  $t_R$  18 min gave an ion with  $m/z$  317.1 corresponding to steviol (data not shown). Similar mass spectra in positive ion mode were obtained for stevioside ( $t_R$  6.1) and steviolbioside ( $t_R$  9.2), thus confirming their identities.

The stability of the sweeteners was evaluated by incubating stevioside, rebaudioside A, and glucose for 24 h under the same experimental conditions but in the absence of the fecal suspension. The recovery of stevioside and rebaudioside A was >95%. The controls, namely, the medium and the medium plus fecal suspension, did not produce compounds overlapping with stevioside and rebaudioside A metabolites, and the time course of stevioside and rebaudioside A degradation by human microflora is shown in **Figure 3**. The plotted results are mean values



**Figure 3.** Stevioside and rebaudioside A degradation curves. The results are mean values with standard deviations of the different fecal samples.

with standard deviations from double injections of the different fecal samples.

**Table 1** summarizes the results obtained after growing some isolated fecal bacterial strains in the presence or absence of stevioside and glucose, rebaudioside A and glucose, and glucose, respectively. The quantitative data are expressed as log CFU/g of dried feces. The isolated fecal bacterial groups were incubated with stevioside or rebaudioside A, and the results obtained are reported in **Table 2**.

## DISCUSSION

The analytical results obtained by incubating stevioside with human intestinal microflora show that this compound was completely degraded to its aglycon steviol in ~10 h. Steviolbioside concentration peaked after 2–4 h of incubation, and after this time, it decreased rapidly to zero. Steviol was detected only after 3–4 h of incubation, and subsequently its concentration increased rapidly. These results suggest that stevioside is initially hydrolyzed to steviolbioside, and then this intermediate is rapidly metabolized to steviol. Moreover, it seems that the  $\beta$  1:19 bond is rapidly hydrolyzed to produce steviolbioside and a glucose moiety, whereas the  $\alpha$  1:13 linkage appears to be more resistant to microflora hydrolysis. Rebaudioside A was also completely metabolized to steviol by human microflora, but a longer time was required (24 h). Indeed, after an initial lag phase of ~6–7 h, rebaudioside A was hydrolyzed to steviolbioside ( $C_{max}$  = 12–15 h), and this was rapidly converted to steviol.

Steviol, as the final stevioside and rebaudioside A metabolite, remained unchanged during a 72 h incubation with human microflora, indicating that bacterial enzymes are not able to cleave the steviol structure. These results are partially in

**Table 1.** Quali-quantitative Values (Log<sub>10</sub> Colony-Forming Units per Gram of Dried Feces) of Some Isolated Fecal Bacterial Strains Grown in the Presence or Not of Stevioside (*n* = 5) or Rebaudioside A (*n* = 6)

| fecal bacteria  | t0 h <sup>a</sup> | blank <sup>b</sup> | rebaudioside A <sup>e</sup> | t0 h <sup>a</sup> | blank <sup>b</sup> | stevioside <sup>e</sup>     |
|-----------------|-------------------|--------------------|-----------------------------|-------------------|--------------------|-----------------------------|
| total aerobes   | 7.80 ± 0.78       | 8.38 ± 0.68        | 7.65 <sup>c,d</sup> ± 0.85  | 8.76 ± 1.84       | 8.69 ± 1.86        | 8.18 ± 2.05                 |
| total anaerobes | 10.10 ± 0.60      | 10.37 ± 0.46       | 10.17 ± 0.75                | 10.36 ± 0.37      | 10.21 ± 0.40       | 9.51 <sup>c,d</sup> ± 0.57  |
| bacteroidaceae  | 10.58 ± 0.28      | 10.63 ± 0.11       | 10.50 ± 0.20                | 10.85 ± 0.39      | 10.56 ± 0.34       | 10.09 <sup>c,d</sup> ± 0.34 |
| bifidobacteria  | 8.37 ± 1.99       | 8.36 ± 2.01        | 8.29 <sup>c,d</sup> ± 1.74  | 9.10 ± 0.69       | 8.77 ± 0.80        | 8.77 ± 0.50                 |
| clostridia      | 8.01 ± 0.23       | 7.94 ± 0.72        | 7.88 ± 0.82                 | 7.04 ± 1.08       | 6.86 ± 0.97        | 6.94 ± 0.65                 |
| coliforms       | 7.80 ± 0.63       | 8.19 ± 0.51        | 7.50 <sup>d</sup> ± 0.87    | 7.44 ± 1.36       | 8.38 ± 1.79        | 7.30 ± 1.64                 |
| enterococci     | 6.78 ± 0.58       | 6.51 ± 0.82        | 6.20 <sup>c</sup> ± 0.84    | 7.25 ± 1.60       | 7.17 ± 1.45        | 6.90 ± 1.50                 |
| lactobacilli    | 6.57 ± 1.97       | 6.44 ± 1.99        | 5.90 ± 1.92                 | 7.07 ± 1.34       | 6.68 ± 1.43        | 6.31 <sup>c</sup> ± 1.40    |

<sup>a</sup> t0 h = microflora at time zero. <sup>b</sup> Blank: (media + fecal suspension) after 24 h of incubation time. <sup>c</sup> Rebaudioside A or stevioside vs t0 h, *p* < 0.05. <sup>d</sup> Rebaudioside A or stevioside vs blank, *p* < 0.05. <sup>e</sup> Rebaudioside A or stevioside: (media + fecal suspension + rebaudioside A or stevioside) after 24 h of incubation time.

**Table 2.** Stevioside (*n* = 6) or Rebaudioside A (*n* = 6) Hydrolysis by Isolated Human Fecal Bacterial Groups

| sample             | incubation time (h) | %               |                 |                 |                 |                 |                 |
|--------------------|---------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                    |                     | St <sup>a</sup> | Sv <sup>a</sup> | Sb <sup>a</sup> | Ra <sup>a</sup> | Sv <sup>a</sup> | Sb <sup>a</sup> |
| blank <sup>b</sup> | 24                  | 100.02 ± 0.68   | 0.00            | 0.00            | 102.10 ± 0.81   | 0.00            | 0.00            |
|                    | 48                  | 100.11 ± 0.21   | 0.00            | 0.00            | 99.87 ± 0.99    | 0.00            | 0.00            |
| bacteroidaceae     | 24                  | 60.94 ± 2.47    | 14.52 ± 1.89    | 9.48 ± 3.11     | 82.63 ± 6.56    | 16.78 ± 4.41    | 6.18 ± 2.65     |
|                    | 48                  | 25.97 ± 3.89    | 61.60 ± 6.83    | 18.01 ± 4.34    | 0.00            | 73.41 ± 4.89    | 30.85 ± 3.16    |
| bifidobacteria     | 24                  | 103.00 ± 0.11   | 0.00            | 0.00            | 96.53 ± 0.11    | 0.00            | 0.00            |
|                    | 48                  | 103.62 ± 0.21   | 0.00            | 0.00            | 100.24 ± 1.51   | 0.00            | 0.00            |
| clostridia         | 24                  | 99.89 ± 1.23    | 0.00            | 0.00            | 102.81 ± 0.49   | 0.00            | 0.00            |
|                    | 48                  | 100.34 ± 1.64   | 0.00            | 0.00            | 101.67 ± 1.21   | 0.00            | 0.00            |
| coliforms          | 24                  | 103.47 ± 0.98   | 0.00            | 0.00            | 99.60 ± 0.31    | 0.00            | 0.00            |
|                    | 48                  | 90.97 ± 1.14    | 0.00            | 0.00            | 102.48 ± 2.11   | 0.00            | 0.00            |
| enterococci        | 24                  | 104.56 ± 1.34   | 0.00            | 0.00            | 103.42 ± 1.11   | 0.00            | 0.00            |
|                    | 48                  | 103.65 ± 0.97   | 0.00            | 0.00            | 104.23 ± 1.81   | 0.00            | 0.00            |
| lactobacilli       | 24                  | 99.46 ± 1.69    | 0.00            | 0.00            | 101.04 ± 0.92   | 0.00            | 0.00            |
|                    | 48                  | 101.25 ± 0.79   | 0.00            | 0.00            | 102.55 ± 1.32   | 0.00            | 0.00            |

<sup>a</sup> St, stevioside; Sv, steviol; Sb, steviolbioside; Ra, rebaudioside A. <sup>b</sup> Blank: media + St or Ra. St: *n* = 5. Ra: *n* = 6.

agreement with those described by Hutapea et al. (19). These authors incubated stevioside with mouse, rat, hamster, and human intestinal microflora and found steviol 16,17 $\alpha$ -epoxide in the mouse and human samples after 4 and 2 days of incubation time, respectively. Surprisingly, extending the incubation time with the human microflora promoted a complete conversion of this compound to steviol.

The results described in the present study differ from those reported by Hutapea et al. (19). In fact, according to our LC-MS data no steviol epoxide derivatives were found after incubation of stevioside or rebaudioside A samples with human intestinal microflora from different volunteers. A possible explanation for this discrepancy may be the lower specificity of the LC-UV approach applied (19) as compared with the LC-MS used in the present study.

The influence of stevioside and rebaudioside A on intestinal microflora was tested in anaerobic conditions in batch cultures inoculated with fecal samples from healthy human subjects. Bacterial groups studied in mixed cultures represented the predominant populations in the human colon. In control cultures, the counts of the tested microbial groups after 24 h of anaerobic incubation were maintained at the inoculum level, indicating that the incubation medium (salt solution) did not significantly stimulate the growth of any microbial tested groups and that it was effective in maintaining the fecal microbial balance.

Results on the fecal microbial composition in the presence of stevioside or rebaudioside A were substantially similar to those recorded in the control and glucose cultures (Table 1).

Specifically, stevioside exerted a weak inhibition against anaerobic bacteria. In contrast, rebaudioside A showed a weak inhibitory activity on aerobic bacteria and particularly on

coliforms (Table 1). These effects became significant (*p* < 0.05) when data from 24 h incubations of rebaudioside A and glucose were compared.

A high interindividual variability was observed in the counts of the subdominant microbial groups (total aerobes, clostridia, coliforms, enterococci, and lactobacilli); however, these data are in agreement with those previously reported (24). In addition, among the cultures of coliforms, bifidobacteria, enterococci, and bacteroides only the bacteroides (the most relevant of the intestinal groups) were able to hydrolyze stevioside and rebaudioside A (Table 2). On the other hand, bifidobacteria and lactobacilli were not involved in this metabolic activity.

In conclusion, the human fecal microflora was found to completely hydrolyze stevioside and rebaudioside A to their common aglycon steviol in 10 and 24 h, respectively, but it did not degrade steviol. Moreover, the incubation of stevioside or rebaudioside A with human intestinal microflora from different volunteers did not confirm the presence of steviol epoxide derivatives. Stevioside and rebaudioside A did not influence significantly the human intestinal microflora composition; however, it seems that stevioside possesses a slight inhibitory effect on total aerobic bacteria, whereas rebaudioside A influences the proliferation of total aerobes and coliforms. Conversely, only bacteroides among the selected microbial groups were able to hydrolyze the two natural sweeteners tested.

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