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Enzyme-Based Most Probable Number Method for the Enumeration of *Bifidobacterium* in Dairy Products

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ABSTRACT

An enzyme-based assay in combination with the most probable number (MPN) technique was developed for the enumeration of bifidobacteria. The assay employs the detection of fructose-6-phosphate phosphoketolase (F6PPK) activity as an indicator of the presence of bifidobacteria. The method was validated against viable counts and optimized with respect to selective media in order to quantitatively assess bifidobacteria in dairy products and other probiotic preparations. Several commercial products and homemade fermented milks were analyzed. Counts of bifidobacteria ranged from 10^7 to 10^8 MPN/ml in commercial products and homemade fermented milks. Commercial starters provided by Argentinean industries had between 10^7 and 10^{11} MPN/ml. The results obtained in this study suggest that the combination of F6PPK activity determination and the MPN methodology allows an accurate determination of *Bifidobacterium* in pure cultures, dairy products, and other probiotic preparations.

Bifidobacteria occur among the normal inhabitants of the human intestinal tract, where they are claimed to exert health-promoting effects. This genus has been known to prevent diarrhea caused by pathogenic bacteria, to reduce colon cancer risk and serum cholesterol levels, and to stimulate the immune response *(14, 18, 32, 33)*. Consequently, there has been a rising interest in the manufacturing of products containing bifidobacteria. Moreover, several dairy industries are currently supplementing some products with these microorganisms *(14, 29)*.

To achieve the so-called benefits to the host, it is necessary that bifidobacteria arrive viable to the gut. For these microorganisms, the stomach and duodenum represent a hostile environment to overcome. Although many bifidobacteria survive during the transit through the gastrointestinal tract *(4, 10, 16),* the active peristalsis of some areas would wash out any microorganism unable either to become established as a colonizer or to have a suitable growth rate to avoid dilution. In this respect, the administration of a probiotic in an adequate dose ($>10^6$ bacteria/g) is crucial *(25, 29)*.

Probiotics for gastrointestinal implications containing bifidobacteria vary in the way they are presented in the market, consequently affecting their subsequent performance. These preparations should ensure adequate levels of bifidobacteria, providing their sensitivity to storage conditions and methods of preparation. Hence, the maintenance of viability of bifidobacteria giving an acceptable product shelf life has been a great challenge for dairy industries.

In Japan, the Fermented Milk and Lactic Acid Beverages Association has judged that the minimum count for fresh dairy products should be at least 10^7 viable bifidobacteria/ml *(14)*. The Swiss Food Regulation, the International Standard of FIL/IDF, and the MERCOSUR regulations require that such products contain more than 10^6 CFU of bifidobacteria/g *(13, 17, 29)*. Although several entities recommend a minimum concentration of bifidobacteria to receive the potential health benefits of these microorganisms, there is no recommendation of an official methodology to ensure an accurate enumeration of this genus *(34)*. Since bifidobacteria are generally added to the final fermented product in considerably lower amounts than the other lactic acid bacteria, the estimation of bifidobacteria has always been controversial *(29)*. On this basis, the availability of both sensitive and specific methodologies allowing the enumeration of bifidobacteria present in dairy products is of great importance.

Bifidobacteria are commonly enumerated through viable counts on specific media *(7, 26, 31)*. When mixed cultures are to be analyzed, it is desirable to count on a selective medium in order to reduce the growth of concomitant microorganisms. These interfering microorganisms could inhibit the development of *Bifidobacterium* either by overgrowth or by the liberation of toxic substances. Selective solid media containing vitamins, LiCl, and antibiotics as selective agents have been proposed to count *Bifidobacterium (3, 12, 20, 24, 30, 34)*. Media based on the ability of strains to ferment specific sugars have also been reported, although the selectivity achieved is only partial since not all bifidobacteria have the same fermentation profile, and even some lactobacilli could ferment the selected sugar.

In this respect, the aim of this work was to develop a suitable procedure to quantitatively assess the concentration of *Bifidobacterium*. A combination of the most probable

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TABLE 1. *Enumeration of* Bifidobacterium *in different samples and comparison between viable counts and the EB-MPN method*[a]

Samples	Bifidobacteria (CFU/ml)	MPN/ml[b]
Mixed cultures		
CIDCA 5310/CIDCA 332	$3.04 \pm 0.41 \times 10^{7}$[c]	2.30×10^{7} (7.18×10^{6}–7.34×10^{7})
Commercial starters		
Lyophilized starter	1.5×10^{8}[d]	5.70×10^{7}[e] (1.87×10^{6}–1.73×10^{8})
Frozen granulated starter	8.3×10^{10}[d]	5.76×10^{11} (2.44×10^{10}–1.18×10^{12})
Commercial fermented milks		
Purchased at the supermarket[f]	ND	$<10^{1}$
Supplemented with CIDCA 532	$2.35 \pm 0.39 \times 10^{6}$[c]	4.22×10^{6} (1.62×10^{6}–1.10×10^{7})
Provided by a local industry I	4.2×10^{8}[d]	8.69×10^{7} (4.11×10^{7}–1.97×10^{8})
Provided by a local industry II	3.2×10^{7}[d]	7.18×10^{7} (3.40×10^{7}–1.63×10^{8})
Homemade fermented milks		
(*a*) Prepared with CIDCA 333 until pH 4.5 and supplemented with CIDCA 532	$1.10 \pm 0.09 \times 10^{9}$[c]	9.93×10^{8} (5.32×10^{8}–1.85×10^{10})
(*b*) Prepared with the lyophilized starter until pH 4.5 and supplemented with CIDCA 531	$3.34 \pm 0.39 \times 10^{8}$[c]	1.73×10^{8} (8.90×10^{7}–3.31×10^{8})
(*c*) Prepared from fermented milk I	ND	1.23×10^{8} (5.71×10^{7}–2.55×10^{8})
(*d*) Prepared with CIDCA 333 and 532	1.20×10^{8}[c]	1.23×10^{8} (3.80×10^{7}–3.93×10^{8})
(*e*) Prepared with the lyophilized started and CIDCA 531	ND	2.00×10^{8} (7.40×10^{7}–5.38×10^{8})
Probiotic supplement (capsules)		
Pro-Biotics Acidophilus	9.8×10^{8}[d]	3.65×10^{8}[e] (6.68×10^{7}–5.503×10^{8})

[a] In all cases, bile MRS (0.3% wt/vol of bile) was used as a culture medium, and standard deviation was less than 5%. ND, not determined.
[b] Mean value; numbers in parentheses represent the confident interval ($P < 0.99$).
[c] Counts of bifidobacteria on MRS agar were determined before the addition of the lactobacilli.
[d] Counts of bifidobacteria were determined by the manufacturer on MRS agar plus dycloxaciline (0.5% wt/vol) (microorganisms/g of product).
[e] Expressed in MPN/g of product.
[f] Three different products were analyzed.

number (MPN) method in a selective medium with a specific enzymatic detection of bifidobacteria through the fructose-6-phosphate phosphoketolase activity (F6PPK) showed up as an appropriate strategy.

MATERIALS AND METHODS

Cultures. *Bifidobacterium pseudolongum* CIDCA 531; *Bifidobacterium breve* CIDCA 532, CIDCA 5312, CIDCA 5314, and CIDCA 5315; and *Bifidobacterium bifidum* NCC 189 belonging to the NESTLE culture collection (formerly CIDCA 536), CIDCA 537, CIDCA 5310, CIDCA 5311, and CIDCA 5313 were grown in deMan Rogosa Sharpe (MRS) agar (Biokar Diagnostics, Beauvais, France) supplemented with cysteine 0.05% wt/vol for 48 h at 37°C in anaerobic conditions (AnaeroGen, Oxoid Ltd., Basingstoke, UK). *Lactobacillus delbrueckii* subsp. *bulgaricus* CIDCA 331, CIDCA 332, and CIDCA 333 and *L. delbrueckii* subsp. *lactis* CIDCA 132 and CIDCA 1311 were grown overnight in MRS (Biokar) at 37°C in aerobiosis *(8).* Mixed cultures were generated by mixing equal volumes of different cultures containing *B. bifidum* CIDCA 5310 and *L. delbrueckii* subsp. *bulgaricus* CIDCA 332 grown as described above. For more details concerning strains, see references *5* and *9.* A lyophilized starter and a frozen granulated starter were provided by local industries. Five commercial fermented milks were purchased at local supermarkets or provided by local milk industries and immediately analyzed. In addition, several homemade fermented milks were prepared in 100 ml of ultrahigh temperature milk by inoculation of different starters and strains (letters *a* to *e* in Table 1): *(a) L. delbrueckii* subsp. *bulgaricus* CIDCA 333 (1% vol/vol), and when pH was 4.5, the resulting fermented milk was supplemented with a stationary-phase culture of *B. breve* CIDCA 532 (1% vol/vol); *(b)* inoculation of a lyophilized starter provided by a local industry (0.005% wt/vol), and when pH reached 4.5, a stationary-phase culture of *B. pseudolongum* CIDCA 531 was added (1% vol/vol); *(c)* subculture of a fermented milk provided by a local milk industry; *(d) L. delbrueckii* subsp. *bulgaricus* CIDCA 333 simultaneously in-

oculated with *B. breve* CIDCA 532 (1% wt/vol each); and *(e)* simultaneous inoculation of the lyophilized starter provided by a local industry (0.005% wt/vol) and *B. pseudolongum* CIDCA 531 (1% vol/vol). In all cases, inoculated milks were incubated overnight in aerobic conditions at 37°C until the desired pH was reached. Capsules of 0.5 g Pro-Biotics Acidophilus Dietary Supplement (Nutrition Now, Inc., Vancouver, Wash.) were dissolved in 0.1% tryptone for further quantification.

Optimization of a selective medium for the quantification of *Bifidobacterium*. The selection of the culture medium for the enumeration of bifidobacteria in the presence of other lactic acid bacteria was performed as follows: several pure strains of lactobacilli and bifidobacteria were inoculated in the putative selective media and in the media reported by other researchers *(34)*. Nalidixic neomicin lithium (NNL)-MRS, Oxgall gentamicin (OG)-MRS, and lithium propionate (LP)-MRS were used as selective media, according to Vinderola and Reinheimer *(34)*. In all cases, MRS was used as a basal medium to which different selective compounds were added. The selective compounds used were the following: nalidixic acid (Sigma Chemical Co., Rockville, Md.) 0.0015% wt/vol, neomycine sulfate (Britania S.A., Buenos Aires, Argentina) 0.01% wt/vol, and lithium chloride (Sigma) 0.3% wt/vol for NNL-MRS; ox bile (Merck & Co., Rahway, N.J.) 0.02% wt/vol and gentamicin (Britania) 0.003 wt/vol for OG-MRS; and lithium chloride and sodium propionate (both Sigma) 0.3% wt/vol for RP-MRS. Bile MRS was also used, by testing different concentrations of ox bile (0.15, 0.3, 0.6, and 0.9% wt/vol). Finally, a full synthetic medium containing ammonium acetate (Sigma) 0.4% wt/vol, biotin (Sigma) 8×10^{-7} wt/vol, calcium pantothenate (Sigma) 8×10^{-4} wt/vol, cystine (Sigma) 0.04% wt/vol, lactose (Sigma) 7% wt/vol, K_2PO_4H (Sigma) 0.5% wt/vol, sodium acetate (Sigma) 5% wt/vol, ascorbic acid (Sigma) 0.1 wt/vol, and 1 ml of the following salts: $FeSO_4 \cdot 7H_2O$, 0.2% wt/vol; $MgSO_4 \cdot 7H_2O$, 4% wt/vol; and $MnSO_4 \cdot 2H_2O$, 0.135% wt/vol; NaCl 0.2% wt/vol was tested *(23)*.

After a suitable incubation time, tubes were inspected for bacterial growth. When indicated, inhibitors were prepared separately and sterilized by filtration (0.45-μm pore size, Millipore) prior to the supplementation. Unless stated otherwise, all chemicals were purchased from Sigma.

Estimation of *Bifidobacterium*. *Bifidobacterium* was quantified by a five-tube MPN method. Test tubes were filled with 3.6 ml of bile MRS (containing ox bile 0.3% wt/vol). Then, 0.4 ml of appropriate decimal dilutions of the samples to be analyzed was inoculated to each tube. Each experiment was designed with five tubes for each dilution and evaluated in duplicates. After incubation for 48 h at 37°C in anaerobic conditions, tubes were inspected for the presence of bacterial growth. Samples scored as positive in bile MRS, based on observed turbidity, were then washed with 50 mM K_2HPO_4 (pH 7.0) *(1)* and distributed in microtitration plates in order to assay the F6PPK activity. After the enzymatic test, the MPN (mean and confidence interval) was calculated by using the MPN calculator, version 4.04 *(15)*. The program enables the user to enter the number of replicates and dilutions, volumes used, and dilution factors to generate an MPN with confidence intervals.

Enzymatic test. Detection of F6PPK activity was done by a microanalysis test as described previously *(1)*. Unless stated otherwise, all chemicals were purchased from Sigma. Briefly, samples were centrifuged 10 min at 4,000 × *g* at room temperature, supernatants were discarded, and washed pellets were submitted to the enzymatic essay. A reaction mixture containing 10 μl of fructose-6-phosphate (80 mg/ml), 20 μl of a solution containing 6 mg/m NaF and 10 mg/ml Na-iodoacetate, 60 μl phosphate-buffered saline supplemented with 0.05% cysteine and 0.25% final concentration Triton X-100 (BDH Chemicals Ltd., Poole, UK) was added to each tube. After a 30-min incubation at 37°C, the reaction was stopped with 70 μl of 13.9% wt/vol hydroxylamine-HCl, freshly neutralized with NaOH to give a pH of 6.5. After 10 min at room temperature, 40 μl of 15% wt/vol trichloroacetic acid and 40 μl 4 M HCl were added. Color development was achieved by adding 40 μl of 50 mg/ml $FeCl_3 \cdot 6H_2O$ in 0.1 M HCl. A purple color was considered positive; otherwise, the suspension remained yellow. The color became more evident after standing, which allowed particles to settle. For those controversial tubes, samples were centrifuged 2 min at 14,000 × *g*, and absorbance of the supernatant was then read at 500 nm in an enzyme-linked inmunosorbent assay reader (Spectra Rainbow; Tecan, Vienna). All readings over the reading recorded for the blank were considered as positive.

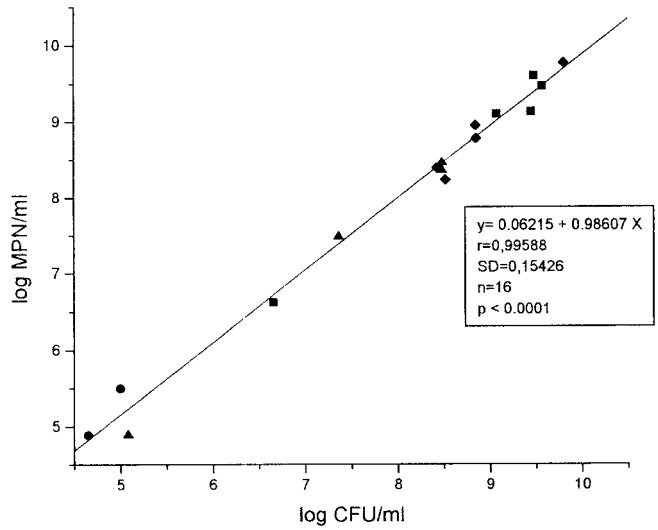

FIGURE 1. *Comparative quantification of different strains of* Bifidobacterium *by the EB-MPN and counts in solid MRS. Each point corresponds to a different culture of strains CIDCA 531* (♦), *CIDCA 532* (■), *CIDCA 5310* (▲), *and CIDCA 5313* (●).

RESULTS

Applicability of the enzyme-based MPN method. Strains *B. pseudolongum* CIDCA 531, *B. breve* CIDCA 532, and *B. bifidum* CIDCA 5310 and CIDCA 5313 were enumerated using appropriate dilutions of cultures grown in MRS broth by the enzyme-based–MPN (EB-MPN) method. Those tubes with positive growth were inspected for enzymatic activity by the F6PPK test. The results obtained by the EB-MPN were compared to counts of the same strains on MRS plates (Fig. 1). The resulting plot showed a correlation coefficient (r) of 0.99588. This strong positive correlation suggested the use of the EB-MPN as an accurate tool for the enumeration of *Bifidobacterium*.

Optimization of the selective culture media for bifidobacteria. Several media described by many authors *(3, 12, 13, 19–21, 30, 31, 34)* were assayed on our own collection of bifidobacteria and lactobacilli and on other reference strains (see "Materials and Methods"). All tested strains were able to grow in these liquid media with the

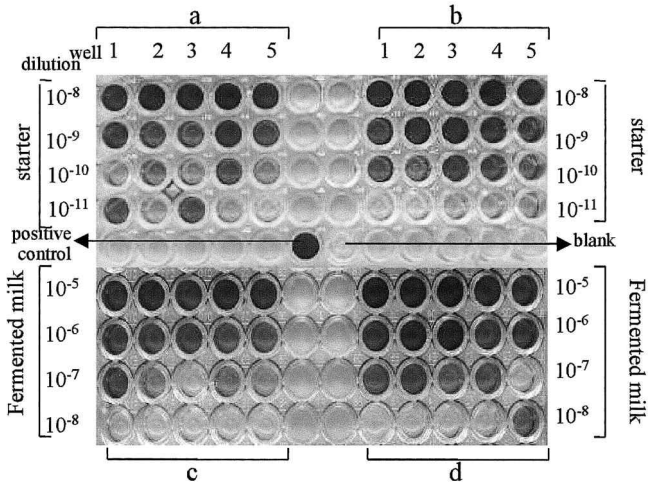

FIGURE 2. *Results obtained by the EB-MPN method of dairy products provided by a local milk industry (starter—a and b, and a commercial fermented milk—c and d). Blank was performed by adding all phosphoketolase reagents except bacterial suspensions. Positive control was performed by testing a pure strain of* Bifidobacterium. *Each experiment was conducted in duplicate.*

exception of *L. delbrueckii* subsp. *bulgaricus* CIDCA 332. Since most lactobacilli strains could grow on those media, we assayed bile as an inhibitory agent. Increasing concentrations of bile (0.3, 0.6, and 0.9% wt/vol) were studied in order to detect the minimum concentration able to inhibit the growth of lactobacilli. After a 24-h incubation, no growth of lactobacilli CIDCA 331, 332, and 333 was observed; however, the growth of *Bifidobacterium* was slightly poor at all the concentrations tested. Extending the time of incubation to 48 h, tubes corresponding to bifidobacteria showed higher optical density. Lactobacilli were not able to grow even after 48 h of incubation. At 0.6 and 0.9% wt/vol bile, all bifidobacteria-tested strains were able to grow, although they developed poorly in comparison to bile 0.3% wt/vol. Consequently, we chose bile 0.3% wt/vol and 48 h of incubation as the standard conditions for the EB-MPN. The medium was named modified-bile MRS broth (MB-MRS).

Enumeration of bifidobacteria with the EB-MPN method. Table 1 and Figure 2 depict the results obtained for the enumeration of bifidobacteria by the EB-MPN assay using MB-MRS in several samples: pure strains, mixed cultures, commercial starters, fermented milks (commercial as well as homemade), and other probiotic preparations. Values of bifidobacteria ranging from 10^5 to 10^9 MPN/ml were found in pure cultures. Viable counts for those samples corresponded to the same range of concentration. For those samples prepared by mixing equal volumes of pure cultures of *B. bifidum* CIDCA 5310 and *L. delbrueckii* subsp. *bulgaricus* CIDCA 332, bifidobacteria values calculated by the EB-MPN method correlated to the viable counts.

Starters and fermented milks provided by local industries were also analyzed. Counts of *Bifidobacterium* ranged from 10^7 to 10^{11} MPN/ml for the starters and between 10^7 and 10^8 MPN/ml for the fermented milks before being distributed to the supermarkets. Our results matched the specifications on the labels. Commercial fermented milks purchased at the supermarket had less than 10 MPN *Bifidobacterium*/ml. Only when a commercial fermented milk was supplemented with a bifidobacterial strain was it possible to count significant levels.

Several fermented milks were prepared in our lab with different strains of lactobacilli and *B. pseudolongum* CIDCA 531. Also, the lyophilized starter from a local industry was grown in milk until pH 4.2, then strain CIDCA 531 was added. In both experiments, counts of bifidobacteria were approximately 10^8 MPN/ml. These results were comparable to those obtained for the commercial products before being distributed to the supermarkets.

One commercial probiotic preparation in the form of multistrain capsules was analyzed. Bifidobacteria levels were around 10^8 MPN/g of product, matching the level claimed by the manufacturer.

DISCUSSION

Cell-associated phosphoketolase activity has commonly been used for a qualitative determination of *Bifidobacterium,* and its quantitative application has recently been reported *(1).* In the present study, we proposed the measurement of F6PPK activity in combination with the widely known MPN method to enumerate *Bifidobacterium.* Providing that F6PPK has never been described in other lactic acid bacteria, a positive reaction could be considered a distinctive confirmation of *Bifidobacterium.* Moreover, the use of a selective medium to reduce the growth of the accompanying starter bacteria would improve the sensitivity in the enumeration of bifidobacteria.

Several selective solid media have been proposed for the assessment of bifidobacteria in different samples. Some of them, including the media recommended by the International Dairy Federation *(13),* are based on the ability of the strains to ferment specific sugars. However, this strategy fails to allow the growth of many bifidobacteria, considering their sugar fermentation patterns. Besides, it is possible that some lactobacilli could ferment the selected sugars, especially *L. delbrueckii* subsp. *lactis,* which ferments a wide spectrum of sugars *(8).* Other authors recommend the supplementation with antibiotics, vitamins, and growth factors to a basal medium to achieve selectivity for bifidobacteria. However, the inhibitory power of the selective solutions may affect the accompanying strains in a different way *(20).*

Vinderola and Reinheimer *(34)* proposed the use of bile 0.15% wt/vol as an inhibitory agent to supplement MRS agar for the enumeration of bifidobacteria occurring with lactobacilli and streptococci, though not all the strains were inhibited by this agent in liquid medium. Previous studies have demonstrated that lactobacilli and bifidobacteria have differential sensitivity to bile *(4, 10, 16).* Consequently, we decided to evaluate the use of bile as a selective agent. The results obtained in our study indicated that bile 0.3% was a suitable concentration to inhibit the growth of other lactic acid bacteria; hence, that was the concentration employed for the selective liquid medium in the EB-MPN.

The EB-MPN presents certain advantages in comparison to viable count techniques employing selective or differential media: a more objective result when colonies are not different enough or sufficiently separated. Taking into account that viable counts are tedious and require long times of incubation, the EB-MPN represents a more rapid and convenient analytical procedure suitable for automation. In addition, the detection of *Bifidobacterium* through the measurement of F6PPK activity is still considered specific for this genus. The results obtained in this study for the EB-MPN strongly correlated with viable counts for bifidobacteria (Fig. 1).

Manipulation of probiotic bacteria during the preparation of the products highly affects their survival and product performance. Unfortunately, precautions during processing are not always observed, consequently yielding a lower number of viable cells than those specified. In our study, commercial fermented milks purchased at the supermarket had low amounts of bifidobacteria, whereas those products in the form of capsules matched the specifications on the label. In a recent publication *(11),* only 2 of 13 probiotic products tested had levels of bacteria claimed by the manufacturer. Moreover, some products fail to have the organisms that are listed on the label *(6).* Storage of bifidobacteria is still problematic both for industry and research laboratories due to the high sensitivity of these microorganisms to oxygen and low pH *(2, 27, 28, 35).* In this respect, and considering that the cold chain may be interrupted during distribution with the consequent decrease of pH, low counts of bifidobacteria are expected for shelf products on the market. The previous hypothesis was confirmed when fermented milks were prepared in our laboratory or provided by a milk industry before being distributed to the supermarkets. In this case, values of approximately 10^7 to 10^8 bifidobacteria MPN/ml were obtained. Similar results were obtained when different strains of bifidobacteria were added to commercial fermented milks (Table 1). EB-MPN was suitable for the detection of bifidobacteria, not only when added at the end of the production but also when these microorganisms were grown together with the other bacteria of the starter, thus indicating that bifidobacteria damaged by low pH and oxygen can be recovered when they are grown together with the other microorganisms from the starter. This methodology allows the detection of a very low number of *Bifidobacterium,* since only one bacterium could reach a concentration of at least 10^5 CFU/ml after incubation in MRS for 48 h at 37°C.

The EB-MPN has also a direct application in quality control in dairy industries and can be used to follow the different steps in the elaboration of dairy products. This method can detect values of bifidobacteria recommended by the Swiss Food Regulation, the International Standard of FIL/IDF, and the MERCOSUR regulations or even lower. Also, by means of absorbance readings, it is possible to eliminate controversial results. Considering that the count of bifidobacteria may also be problematic due to the autoaggregating properties of some strains *(10, 22),* the EB-MPN could be an interesting alternative to test bifidobacteria levels over viable counts.

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