Journal of Food Protection, Vol. 63, No. 3, 2000, Pages 322–326
Copyright ©, International Association for Food Protection

An Enzymatic–Colorimetric Assay for the Quantification of *Bifidobacterium*

RODRIGO BIBILONI, PABLO F. PÉREZ, AND GRACIELA L. DE ANTONI*

Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA), Facultad de Ciencias Exactas, Universidad Nacional de La Plata, Calle 47 y 116 (1900) La Plata, Argentina

MS 99-218: Received 28 July 1999/Accepted 1 November 1999

ABSTRACT

An enzymatic–colorimetric assay for the quantification of *Bifidobacterium* was developed. The method, based upon the standard detection of fructose-6-phosphate phosphoketolase activity, was optimized with respect to bacterial cell pretreatment, time of incubation, and substrate concentration. The relationship between bacterial biomass and phosphoketolase activity was linear in a wide spectrum of bacterial densities. Higher sensitivity over the standard method was achieved by using 0.25% Triton X-100 in the reaction mixture to pretreat the bacterial cells. Because autoaggregation is a frequent feature among *Bifidobacterium* strains, this simple and reproducible method offers good advantage over viable plate count and turbidimetric techniques. The methodology can also be applied to the assessment of adherent *Bifidobacterium* strains to human epithelial cells.

Bacteria from the genus *Bifidobacterium* constitute a significant group in the intestinal microflora of humans and may provide considerable health-promoting benefits to the host. They have been claimed to suppress harmful bacteria by controlling pH of the large intestine through the liberation of lactic and acetic acid *(10)*. They may also play an important role in amelioration of diarrhea or constipation *(18)* and immune activation *(19)*. Other physiological effects that have been ascribed to this genus include vitamin production and antitumoral activity *(6)*. Bifidobacteria are also believed to have anticarcinogenic *(9)* and anticholesterolemic properties *(6)*.

Since the first description of bifidobacteria by Tissier early in this century *(17),* taxonomy of this group has caused discussion. Today, this genus can be distinguished from other bacterial groups like lactobacilli, actinomycetes, and anaerobic corynebacteria by a particular metabolic pathway, the bifid shunt *(16),* whose key enzyme is fructose-6-phosphate phosphoketolase (F6PPK). The demonstration of F6PPK activity serves as a taxonomic tool in the identification of the genus. This key enzyme in the glycolytic fermentation cleaves fructose-6-phosphate into acetylphosphate and erythrose-4-phosphate. The acyl part of the acid anhydride can be converted into hydroxamic acid by reaction with hydroxylamine *(7)*. The hydroxamic acid forms, with trivalent iron, a brightly purplish complex that has been used for qualitative identification of phosphoketolase activity *(2, 3, 14, 16)*. We propose here the use of the F6PPK reaction as a tool for quantitative measurement of biomass in *Bifidobacterium* by a microanalysis method. Because some bifidobacterial strains autoaggregate, this simple and reproducible method offers good advantage over viable plate count and turbidimetric techniques.

Adhesion to intestinal epithelia is an important claim in bifidobacterial strains because adherent strains would be capable to resist washout due to intestinal motility and thus have greater probiotic effects. One application of this method would be to assess quantitatively the adherence of *Bifidobacterium* strains to enterocyte-like cells in culture. Several cellular models have been developed to achieve some information about interactions between bacterial and epithelial cells *(1, 12)*. The F6PPK enzyme provides a useful tool because of its absence in human or animal cells that are commonly used as in vitro models for determination of bacterial adherence.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Human bifidobacterial strains *B. breve* CIDCA 532, *B. bifidum* CIDCA 533, *B. bifidum* CIDCA 536, *B. bifidum* CIDCA 537, *B. infantis* CIDCA 538, *B. bifidum* CIDCA 5310, and *B. bifidum* CIDCA 5311 were isolated from healthy infants' feces and identified previously in our laboratory on the basis of sugar fermentation profile and whole cell protein pattern *(5)*. Strain *B. pseudolongum* CIDCA 531 was isolated from a commercial fermented milk product *(11)*. Before enzymatic or adhesion assays were performed, bacteria were grown in anaerobic conditions in tryptone-phytone-yeast broth *(15)* twice consecutively for 48 h at 37°C. Cells were collected by centrifugation at 14,000 × *g* for 10 min, washed twice in 0.05 M phosphate buffer supplemented with 0.05% cysteine (pH 6.5), and resuspended in the same buffer to yield approximately 10 mg/ml (stock bacterial suspension). Fresh stock bacterial suspensions from each strain were prepared each day and kept at room temperature for no longer than 4 h prior to any assay.

Relationship between F6PPK activity and bacterial biomass. Several dilutions of the stock bacterial suspensions were submitted both to dry weight determinations and enzymatic de-

* Author for correspondence: Fax: +54 (221) 425 4853/424 9287; E-mail: gdantoni@nahuel.biol.unlp.edu.ar.

Downloaded from http://meridian.allenpress.com/jfp/article-pdf/63/3/322/1673318/0362-028x-63_3_322.pdf by guest on 17 December 2021

tection in order to assess linearity of the F6PPK test. Dry weight was determined by placing 1 ml of each diluted bacterial suspension in glass containers and heated at 100°C until constant weight. Determinations were carried out in duplicate. Detection of F6PPK was done by a microanalysis modification of the procedure described by Scardovi and Trovatelli *(16)*. Unless stated otherwise, all chemicals were purchased from Sigma (Sigma Chemical Co., Rockville, Md.). Briefly, 1 ml of each dilution was dispensed in polypropylene tubes (Brinkmann Instruments Inc., Westbury, N.Y.) and submitted to bath sonication (Cole-Palmer Ultrasonic Oscillator 8850, Cole-Palmer, Ill.) at 0°C for 15 min at 50/60 Hz. Ten microliters of fructose-6-phosphate (80 mg/ml) and 20 μl of a solution containing 6 mg/ml NaF and 10 mg/ml Na-iodoacetate were added to 60 μl of the sonicate. After 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 of 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. After centrifugation to clarify the suspensions (3 min at 14,000 × *g*), a 96-well flat-shaped microanalysis plate (Nunc, Roskilde, Denmark) was filled with 250 μl of each supernatant. Absorbance was read in duplicate at 500 nm in an enzyme-linked immunosorbent assay reader (Spectra Rainbow, TECAN, Vienna). Two independent bifidobacterial cultures were used to determine the relationship between F6PPK activity and bacterial biomass. Absorbance at 500 nm was then plotted against dry weight, and a linear regression analysis was applied.

Conditions for the enzymatic test. In order to improve sensitivity, the procedure described for determination of F6PPK activity was studied with regard to bacterial cell pretreatment, incubation time, and substrate concentration.

Bacterial cell pretreatment was studied on equal bacterial suspensions prepared from the stock suspensions. In one set of experiments bacterial suspensions were submitted to sonication in a bath sonicator as described above. In a further set of experiments, cells were disrupted by tip sonication (Soniprep 150, Ultrasonic Disintegrator MSS 150.CX3.1, Sanyo, Japan) five times (30-s periods, 0°C, maximal power) in an ice bath. F6PPK was determined as described in the previous section with and without the addition of Triton X-100 (BDH Chemicals Ltd., Poole, England) (0.25% final concentration) in the reaction mixture. For the control experiments cells were not submitted to sonication and no detergent was added. The development of the reddish violet color was followed by measuring the absorbance at 500 nm at different incubation times. The effect on test sensitivity of different amounts of fructose-6-phosphate was studied by holding the bacterial density constant. For a single bacterial density of 0.8 mg/ml (approximate $OD_{600} = 1$), different amounts of substrate (ranging from 8 mg/ml to 80 mg/ml) were added in the reaction mixture and the procedure was carried on as described previously.

Preparation of Caco-2 cells. Enterocyte-like Caco-2 cells were obtained from the American Type Culture Collection (Rockville, Md.). Cells were grown in RPMI-1640 medium supplemented with L-glutamine (ICN Biomedicals, Costa Mesa, Calif.) and 20% inactivated (30 min, 56°C) fetal bovine serum (Gen S. A., Buenos Aires, Argentina). Streptomycin and penicillin G (Gibco, Life Technologies, Inc., Gaithersburg, Md.) were added to give a final concentration of 100 μg/ml and 50 IU/ml, respectively. Cells were seeded at a concentration of 2×10^5 cells/well in 24-well tissue culture plates (Corning, N.Y.) and incubated at 37°C in a 5% CO_2–95% air atmosphere. Culture medium was changed every 2 days. Cultures were used at late postconfluence (21 days) between 28 and 36 passages.

Adhesion assays. Before any adhesion assays were performed, Caco-2 monolayers were washed twice with sterile phosphate-buffered saline (6.8 g/liter PO_4HK_2, 8.6 g/liter NaCl, pH 7.0) at room temperature. Then, 0.5 ml of bifidobacterial suspension containing 0.70 ± 0.06 mg or 0.35 ± 0.04 mg of bacteria was added to each well of the tissue culture plate. The strains used in this experiment were *B. breve* CIDCA 532, *B. bifidum* CIDCA 536, *B. bifidum* CIDCA 537, *B. infantis* CIDCA 538, and *B. bifidum* CIDCA 5310. After incubation for 1 h at 37°C, monolayers were washed three times with PBS to remove nonadherent bacteria. Monolayers were lysed with distilled water and the material from each well was collected by centrifugation at 14,000 × *g* for 10 min. Supernatants were discarded and pellets resuspended in the reaction mixture supplemented with 0.25% Triton X-100 to perform the F6PPK reaction to measure adherent bacteria. Wells containing Caco-2 monolayers only were used as controls. Adhesion experiments were conducted in duplicate over two different cell passages with two independent bacterial cultures. For a single culture plate, adherence of each strain was determined in triplicate. Percent adhesion was related to the total F6PPK activity added per well and calculated as follows:

$$\% \text{ adhesion} = (\text{mean } OD_{500} \text{ of adherent bacteria} / \text{mean } OD_{500} \text{ total added bacteria}) \times 100.$$

Four serial dilutions of the suspensions added to the wells were submitted to the F6PPK reaction in order to assess linearity for that range of bacterial densities, because the above equation is valid only in the linear portion of the curve.

Statistical analysis. Linearity of the F6PPK reaction was assessed by linear regression analysis. Adherence of bifidobacterial strains was compared by one-way analysis of variance using a standard computerized statistical program (Systat 5.0, Systat, Inc., Evanston, Ill.).

RESULTS

Relationship between F6PPK activity and bacterial biomass. Bacterial F6PPK activity produced a linear increase in absorbance with increasing cell biomass for all the strains tested (Fig. 1). The degree of enzymatic activity showed variations between species, even at strain levels among the same species. The slope of the regression curve for each strain reflected those differences. Linearity of the enzymatic assay was found to extend approximately up to 1 mg for most of the strains, although for strain *B. breve* CIDCA 532 linearity was not found over 0.2 mg of biomass. The purple reddish color that developed was stable only for 2 h. No color development was detected in the control tubes without bacteria. Reproducibility studies performed using independent cultures of strains *B. bifidum* CIDCA 5310 and *B. bifidum* CIDCA 537 showed no significant day-to-day variation in the enzymatic activity curves ($P > 0.1$).

Effect of different bacterial pretreatments on assay sensitivity. The effect of addition of Triton X-100 and two sonication conditions on the assay sensitivity was studied. The above factors were studied in strains *B. bifidum* CIDCA 536, *B. bifidum* CIDCA 537, and *B. bifidum* CIDCA 5310 (Fig. 2). Bath sonication did not produce any changes

Downloaded from http://meridian.allenpress.com/jfp/article-pdf/63/3/322/1673318/0362-028x-63_3_322.pdf by guest on 17 December 2021

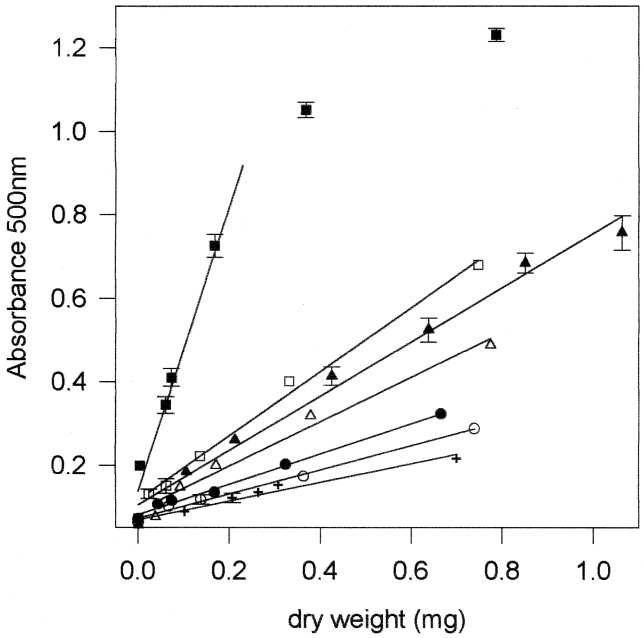

FIGURE 1. *Bacterial F6PPK activity of different bifidobacterial strains as a function of biomass.* B. pseudolongum *CIDCA 531 (○),* B. breve *CIDCA 532 (■),* B. bifidum *CIDCA 533 (+),* B. bifidum *CIDCA 536 (●),* B. bifidum *CIDCA 537 (△),* B. bifidum *CIDCA 5310 (▲),* B. bifidum *CIDCA 5311 (□). Each data point represents the average of duplicates ± standard error. Error bars were omitted if bars were smaller than symbols.*

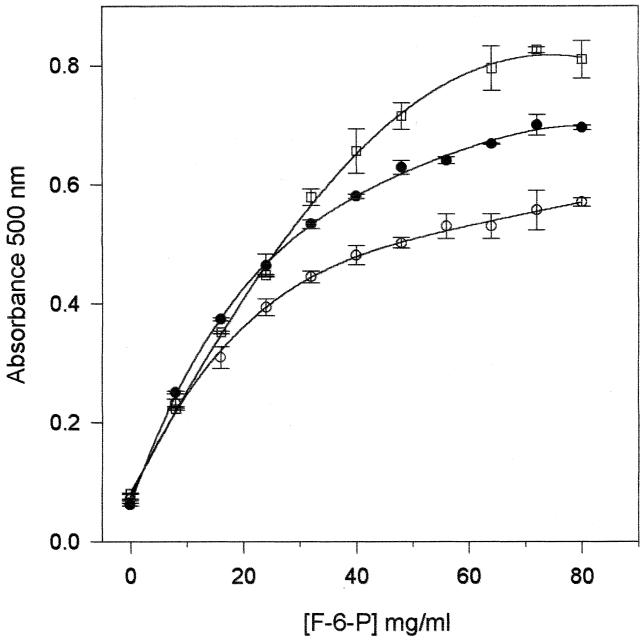

FIGURE 3. *F6PPK assay as a function of fructose-6-phosphate concentration. Key:* ○, B. bifidum *CIDCA 536;* ●, B. bifidum *CIDCA 537;* □, B. bifidum *CIDCA 5310. The data represent mean values of duplicates ± standard error.*

in the sensitivity for the three tested strains: the curves obtained were similar to the controls (no sonication and no detergent added). A more drastic sonication generated curves with slightly better sensitivity, more evident with strain CIDCA 537 (Fig. 2B) than on the other two tested (Fig. 2A and 2C). For all the strains, an improvement in sensitivity was achieved through the addition of Triton X-100 to the reaction mixture. A combination of sonication and the addition of Triton X-100 showed that the effect of sonication was not necessary to improve color development.

Effect of fructose-6-phosphate concentration on enzymatic activity. The same three strains were tested for their enzymatic activity at different substrate concentrations. Absorbance of the reaction product at 500 nm increased with concentration of fructose-6-phosphate until saturation was reached (Fig. 3). Strain CIDCA 5310 yielded higher enzymatic activity at the same substrate concentration, and CIDCA 536 exhibited the lowest values. Saturation for strain CIDCA 5310 was found above 65 mg/ml of fructose-6-phosphate. Strain CIDCA 537 approached saturation at 55 mg/ml, whereas strain CIDCA 536 did so at 45 mg/ml.

Study of the adherence of *Bifidobacterium* to Caco-2 cells. Adherence studies performed on Caco-2 cells

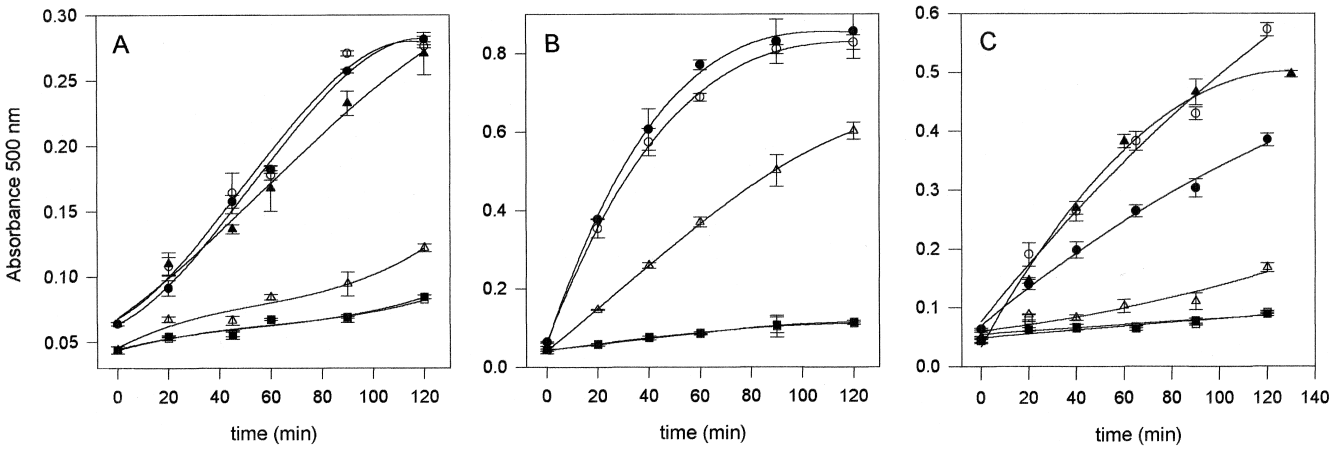

FIGURE 2. *Effect of Triton X-100 and sonication on the quantification of F6PPK in strains (A)* B. bifidum *CIDCA 536, (B)* B. bifidum *CIDCA 537, and (C)* B. bifidum *CIDCA 5310. Treatments performed to bacteria were:* □, *no treatment;* ■, *bath sonication;* ○, *bath sonication with Triton X-100;* ●, *only Triton X-100;* △, *sonication with tip-sonicator;* ▲, *sonication with tip sonication and addition of Triton X-100. Determinations were carried out in duplicate. Each data point represents the mean ± standard error.*

Downloaded from http://meridian.allenpress.com/jfp/article-pdf/63/3/322/1673318/0362-028x-63_3_322.pdf by guest on 17 December 2021

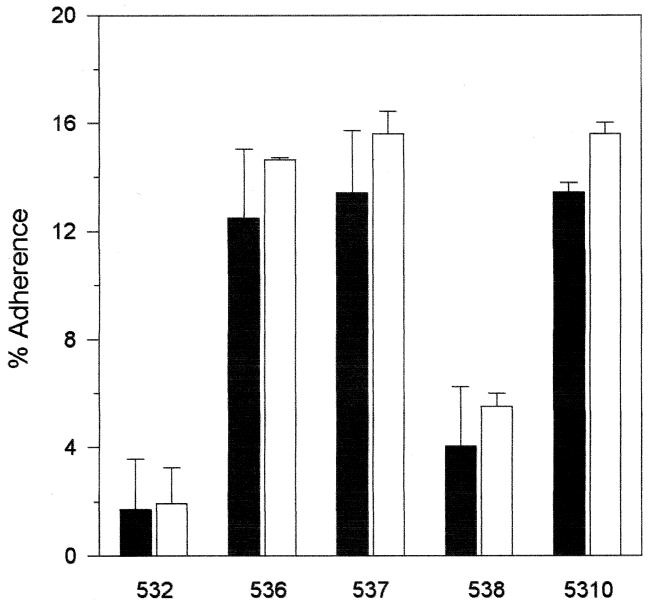

FIGURE 4. *Adhesion of human bifidobacterial strains to human intestinal epithelial Caco-2 cells in culture. Strains were assayed at two densities. (■) 0.17 ± 0.02 mg and (□) 0.35 ± 0.03 mg. Each experimental data point is the average of triplicates of two different cell passages and two independent bacterial cultures.*

showed that the ability of bifidobacterial strains to adhere to enterocyte-like cells in culture was variable (Fig. 4). Whereas some strains adhered effectively to Caco-2 cells, others showed little adhesion. Percent adhesion was the same for all strains at both bacterial densities tested, indicating that adherent bacteria increased with concentration. These data suggest that monolayers would not be saturated with bifidobacteria. A higher sensitivity can be achieved using 6-well tissue culture plates (data not shown).

DISCUSSION

The assay developed in the present study shows the possibility of using F6PPK activity as a tool for quantitative measurement of *Bifidobacterium.* By using Triton X-100 as permeabilizing agent, color development was improved, allowing the quantification of low bacterial quantities. Under the described conditions, a clear dose–response effect was observed. The phosphoketolase assay detected as few as 0.05 mg of bacteria and showed a linear relationship in terms of absorbance per mg up to 1 mg for most of the strains tested, probably because substrate was depleted above this amount of bacteria. Higher amounts of bacteria should be submitted to dilution for better quantification.

The assay has certain promising aspects that include its simplicity and minimal requirement for expensive chemicals or equipment. However, it has some limitations to be considered during the experimental design. It cannot be applied to quantification of bacteria that do not have considerable phosphoketolase activity under assay culture conditions (e.g., strain CIDCA 531 and CIDCA 533 in Fig. 1). The slope of the curves varied considerably from strain to strain, a finding that may indicate differences in specific cellular phosphoketolase activities or cell permeability in individual strains. With regard to strain-to-strain variability of the enzymatic activity, similar results were found by de Vries *(3)* working with bifidobacterial strains from different sources. Because the specific enzymatic activity may vary among different strains of *Bifidobacterium,* a standard curve should be generated for each strain. Preliminary studies had shown that extending the incubation time for more than 2 h did not generate a significant increase in enzymatic activity. Because of the 96-well format, this method allows several determinations to be performed simultaneously.

The tendency of some strains for macro- or microaggregation may be overcome by this technique. Compared to viable count techniques, it presents certain advantages: a more objective result when colonies are not sufficiently separated and a more rapid and convenient analytical procedure suitable for automation (viable counting is tedious, difficult to automate, and requires long times of incubation). In addition, the magnitude of the agitation or vortexing to prepare dilutions can disrupt the aggregates of bacteria in an uneven manner, generating an important dispersion among replicates.

The assay could also be applied to the quantification of bifidobacterial cells in the presence of other bacteria. Although F6PPK activity has been recently demonstrated for some strains of *Gardnerella vaginalis (4),* a positive reaction is still considered to be a distinctive feature for *Bifidobacterium.* At least, no other known lactic acid bacteria other than bifidobacteria have this enzyme *(13).* In our laboratory, suspensions prepared with bacteria belonging to the genera *Streptococcus* and *Lactobacillus* did not lead to color development (data not shown).

Regarding its use for quantitatively assessing the adherence of bifidobacterial strains to epithelial cells, the method shows good advantage over the other two mostly used techniques: light microscopy and radiolabeling. It has a higher reproducibility than the microscopic method due to a more objective result eliminating operator bias because the selection of representative field and counting of bacteria involve some personal judgement. The measurement of bacterial attachment by radioactive labeling is limited by the fact that labeling bacteria requires a special labeling procedure, specific equipment, and an isolated room to prevent contamination. The bioluminescent assay proposed by Ludwicka et al. *(8)* cannot be applied because eukaryotic cells may interfere with their ATP production. A higher sensitivity can be achieved using 6-well tissue culture plates. Adherence studies performed with the F6PPK reaction showed a good correlation with microscopic observations and radiolabeling studies on adhesion of the same strains to Caco-2 cells *(11)* (unpublished observations).

We conclude that cell-associated phosphoketolase activity assay is a rapid, sensitive, and noncontaminating method for quantification of bifidobacterial species with a direct application on the assessment of adherent strains to enterocyte-like cells in culture.

ACKNOWLEDGMENTS

This work was supported by Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (CICPBA), Agencia Nacional de

Downloaded from http://meridian.allenpress.com/jfp/article-pdf/63/3/322/1673318/0362-028x-63_3_322.pdf by guest on 17 December 2021

Promoción Científica y Tecnológica (ANPCyT), and Universidad Nacional de La Plata (UNLP).

REFERENCES

1. Chauvière, G., M. H. Coconnier, S. Kernéis, A. Darfeuille-Michaud, B. Joly, and A. L. Servin. 1992. Competitive exclusion of diarrheagenic *Escherichia coli* (ETEC) from human enterocyte-like Caco-2 cells by heat-killed *Lactobacillus.* FEMS Microbiol. Lett. 91:213–218.
2. Chevalier, P., D. Roy, and P. Ward. 1989. Detection of *Bifidobacterium* species by enzymatic methods. J. Appl. Bacteriol. 68:619–624.
3. de Vries, W., and A. H. Stouthamer. 1967. Pathway of glucose fermentation in relation to the taxonomy of Bifidobacteria. J. Bacteriol. 93:574–576.
4. Gavini, F., M. Van Esbroeck, J. P. Touzel, A. Fourment, and H. Goossens. Detection of fructose-6-phosphate phosphoketolase (f6PPk), a key enzyme of the bifid shunt, in *Gardnerella vaginalis*. 1996. Anaerobe 2:191–193.
5. Gómez Zavaglia, A., G. Kociubinski, P. Pérez, and G. De Antoni. 1998. Isolation and characterization of *Bifidobacterium* strains for probiotic formulation. J. Food Prot. 61:865–873.
6. Ishibashi, N., and S. Shimamura. 1993. Bifidobacteria: research and development in Japan. Food Technol. 47:126–135.
7. Lipmann, F., and L. C. Tuttle. 1945. A specific micromethod for the determination of acyl-phosphates. J. Biol. Chem. 159:21–28.
8. Ludwicka, A., L. M. Switalski, A. Lundin, G. Pulverer, and T. Wadström. 1985. Bioluminescent assay for measurement of bacterial attachment to polyethylene. J. Microbiol. Methods 4:169–177.
9. Mitsuoka, T. 1982. Recent trends in research on intestinal bifidobacteria. Bifid. Microflora 1:3–7.
10. Modler, H. W., R. C. McKellar, and M. Yaguchi. 1990. Bifidobacteria and bifidogenic factors. Can. Inst. Food Sci. Technol. J. 23:29–41.
11. Pérez, P. F., J. Minnaard, E. A. Disalvo, and G. L. De Antoni. 1998. Surface properties of bifidobacterial strains of human origin. Appl. Environ. Microbiol. 64:21–26.
12. Pinto, M., S. Robine-Leon, M. D. Appay, M. Kedinger, N. Triadou, E. Dussaulx, B. Lacroix, P. Simon-Assman, K. Haffen, J. Fogh, and A. Zweibaum. 1983. Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. Biol. Cell. 47:323–330.
13. Pot, B., W. Ludwig, K. Kersters, and K. H. Schleifer. 1994. Taxonomy of lactic acid bacteria, p. 13–91. *In* L. De Vuyst and E. J. Vandamme (ed.), Bacteriocins of lactic acid bacteria. Blackie Academic and Professional, London.
14. Rada, V. 1997. Detection of *Bifidobacterium* species by enzymatic methods and antimicrobial susceptibility testing. Biotechnol. Technol. 11:909–912.
15. Scardovi, V. 1986. Genus *Bifidobacterium,* p. 1418–1434. *In* P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology. Williams & Wilkins, Baltimore.
16. Scardovi, V., and L. D. Trovatelli. 1965. The fructose-6-phosphate shunt as peculiar pattern of hexose degradation in the genus *Bifidobacterium.* Ann. Microbiol. 15:19–23.
17. Tissier, H. 1900. Recherche sur la flore intestinale des nourrisons etat normal et pathologique. Ph.D. thesis, University of Medicine, Paris.
18. Tojo, M., T. Oikawa, T. Morikawa, N. Yamashita, S. Iwata, Y. Satoh, J. Hanada, and R. Tanaka. 1987. The effects of *Bifidobacterium breve* administration on *Campylobacter enteritis.* Acta Paediatr. Jpn. 29:160–167.
19. Ueda, K. 1986. Immunity provided by colonized enteric bacteria. Bifid. Microflora 5:67–72.

Downloaded from http://meridian.allenpress.com/jfp/article-pdf/63/3/322/1673318/0362-028x-63_3_322.pdf by guest on 17 December 2021