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Quantitative Procedure for Enumeration of Bifidobacteria

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A membrane filter technique has been developed for the enumeration of bifidobacteria in natural aquatic environments. The technique is quantitative, selective, and differential. The medium (YN-6) contains: yeast extract, 2.0 g; agar, 1.5 g; polypeptone peptone, 1.0 g; vitamin-free Casamino Acids, 0.8 g; sodium chloride, 0.32 g; and L-cysteine hydrochloride, 0.003 g; in 100 ml of deionized water. The medium is adjusted to pH 7.0 before autoclaving. Nalidixic acid (80 μg/ml), neomycin sulfate (2.5 μg/ml), and bromcresol green (300 μg/ml) are included as selective and differential agents. After incubation for 48 h at 37°C in an anaerobic environment, Gram-stained smears from green, glistening, smooth entire colonies are examined microscopically for typical bifidobacterial morphology. No significant difference in recoveries was observed when YN-6 was compared with reinforced clostridial agar, using bifidobacteria freshly isolated from feces and raw sewage. Using this technique with aquatic and fecal samples, less than 9% false-positive and 8% false-negative isolates were observed. These results indicated that the medium was able to satisfactorily recover organisms from a variety of situations.

Bifidobacteria are gram-positive, nonspore-forming anaerobic rods which are common to the digestive systems of many higher animals. Bifidobacteria have received attention as potential indicators of human fecal pollution, as mediators of increased host resistance to gastroenteritis in infants (3), because of their unique catabolic pathway (fructose 6-phosphate shunt) (4), and because of their bifurcating pleomorphic cellular morphology (10).

Although Mossel (D. A. A. Mossel, Abstr. 7th Int. Congr. Microbiol., 1958, p. 440), over 20 years ago, must be credited as initially proposing the use of the bifidobacteria as indicators, the significance of these organisms in sanitary microbiology is still unclear. Since then a number of workers (6-8, 12; G. R. Resnick and M. A. Levin, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, Q9, p. 263) have examined and extended the concept of using these organisms as indicators of fecal pollution. In the works of Evison et al. (6-8) and Gyllenberg et al. (12), bifidobacteria were enumerated on the medium of Gyllenberg and Niemela (11). An evaluation of this medium has not appeared in the literature.

Bifidobacteria are candidates for the above role due to the following alleged characteristics: (i) presence in the feces of humans in densities greater than that of *Escherichia coli* by a factor of 10; (ii) their inability to multiply outside the intestinal track; and (iii) survival characteristics which were considered to be similar to those of *E. coli* (12).

This paper presents the formulation and evaluation of YN-6 medium, including a comparison with the medium of Gyllenberg and Niemela (11). An examination of the levels of bifidobacteria in sewage, feces, and septic tanks is also presented.

MATERIALS AND METHODS

Cultures. The bifidobacteria used in development and evaluation of YN-6 were strains recently isolated from human sewage (*Bifidobacterium longum* strains B29, B30, and B22; *B. adolescentis* strains B23 and B306) and human feces (*B. infantis* strains W2 and 30-14). The organisms were isolated from the indicated source on reinforced clostridial agar (RCA; BBL Microbiology Systems, Cockeysville, Md.). The isolates were considered members of the genus *Bifidobacterium* if they met the following criteria: were gram-positive pleomorphic rods; were unable to grow in air at 37°C; produced acetic and lactic acids as the major metabolic by-products of glucose fermentation with acetic acid in greater concentration than lactic acid; were nitrate negative; and possessed the enzyme fructose 6-phosphate phosphoketolase as determined by the method of Schramm et al. (15). Quantitative analysis of the fatty acid by-products of glucose fermentation in peptone-yeast-glucose broth (13) was carried out by gas chromatography on a Perkin-Elmer 900 gas chromatograph (Perkin-Elmer Corp., Norwalk, Conn.)

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with the aid of a Perkin-Elmer model 1 computing integrator (13, 16). Determination of species was accomplished by comparing observed fermentation patterns with those given in the eighth edition of *Bergey's Manual of Determinative Microbiology* (2). For storage up to 7 days, chopped-meat medium (BBL) was used for extended periods of storage; cultures were lyophilized in skin milk and stored at −70°C.

Test suspensions and samples. Environmental sewage samples were collected in sterile containers and transported to the laboratory on ice. Transit time was less than 3 h. Fecal samples were collected in sterile containers and returned within 1 h. All samples were processed immediately upon arrival in the laboratory. Pieces of approximately 1 g in weight were mixed for 30 s in a Waring blender with 100 ml of phosphate-buffered saline (sodium chloride, 0.85 g; dibasic sodium phosphate, 0.25 g; monobasic sodium phosphate, 0.56 g; and 100 ml of deionized water). Bifidobacteria test suspensions were prepared by dilution of 24-h broth cultures of peptone-yeast-glucose broth in phosphate-buffered saline.

Incubation. Anaerobic incubation conditions were achieved in a soft-type glove box maintained at 37°C (Coy Laboratory, Ann Arbor, Mich.). The suitability of the BBL GasPak as an alternate system for use in providing anaerobic culture conditions in growing bifidobacteria on YN-6 was examined. Samples of raw sewage and septage were assayed on YN-6 medium, using five replicate plates per dilution. The plates were incubated at 37°C in a glove box or in a BBL GasPak. Mean colony counts obtained from plates incubated in the glove box were compared with those in the GasPak.

Medium formulation. YN-6 medium contains (in grams per liter): yeast extract, 20; peptone, 10; Casamino Acids (Difco Laboratories, Detroit, Mich.), 8; sodium chloride, 3.2; and bromcresol green, 0.3. The mixture must be boiled for 10 min and cooled to ambient temperature before adding cysteine hydrochloride (0.4 g) and nalidixic acid (80 mg). Although it is recognized that lactose could hydrolyze under these conditions, increased and more uniform recoveries were obtained by incorporating the boiling procedure. No evidence of a pH shift was observed. pH is adjusted to 6.9 with 10 N sodium hydroxide before adding 15 g of agar and autoclaving (15 min, 121°C). The medium is cooled to 60°C, and 1 ml of neomycin stock solution (2.5 mg/ml) is added. Then 4-ml volumes are dispersed into 50-mm petri dishes with tight lids. The prepared medium is best stored in the dark at 4°C. The ingredients indicated were chosen for either their growth-promoting capacity or their ability to confer a selective advantage. The concentration of each component was varied individually to determine the optimal level for recovery of bifidobacteria. YN-6-based medium or RCA spread plates were used to determine 100% recovery. In all cases, data from three replicate plates per dilution were compared with the controls, and concentrations providing over 90% recovery (95% confidence limits) were selected for incorporation in the final medium. Casamino Acids and polypeptone peptone (BBL) were chosen as the sources of amino acids. Yeast extract (Oxoid Ltd., London) was included to satisfy vitamin requirements. The fermentable carbohydrate source, lactose, was selected for recovery of the greater number of species of human origin. Neomycin sulfate (ICN Life Sciences Group, Cleveland, Ohio) and nalidixic acid (Calbiochem-Behring Corp., La Jolla, Calif.) were included as selective agents to inhibit growth of gram-positive and gram-negative rods, respectively. Bromcresol green (Fisher Scientific Co., Pittsburgh, Pa.) is present as a selective agent as well as an indicator of pH. If lactose is fermented, the colony will be green against a blue filter; cysteine hydrochloride acts as a reducing agent. The medium, once prepared, is dispensed in 4-ml volumes in 50-mm tight-lid petri dishes and can be stored at 4°C in the dark for up to 7 days.

Membrane filtration. Standard membrane filtration procedures were used for preparing samples (1). The glass filtering apparatus was sterilized between samples by exposure to ultraviolet light for 5 min. A minimum sample volume of 10 ml was filtered, diluted in phosphate-buffered saline as required. A hot probe was used to pierce the covers of the petri dishes to facilitate exchange of gases over the filters. The inverted plates were incubated for 48 h at 37°C under anaerobic conditions. Plates should be placed under anaerobic conditions within 1 h after filtering.

Identification of isolates. Colonies on YN-6 are presumptively positive if they are 1 to 2 mm in diameter, green (light-dark), circular, entire, convex or pulvinate, smooth, butyrous, and opaque and if they display typical bifid morphology upon Gram staining. A dissecting microscope (×20 magnification) must be used for observing colonial morphology. Confirmation of colonies as bifidobacteria was done as diagramed in Fig. 1.

Counting range. The counting range of YN-6 medium was determined by assaying dilutions of a test suspension of *B. adolescentis* (ATCC 15707) in triplicate. The set of plates with a mean observed colony count of approximately 60 colonies was designated as the reference count from which the expected count for other dilution was computed.

Evaluation of membrane filters. Membrane filters were evaluated for their suitability for use in recovery of bifidobacteria on YN-6. Six different filters from five companies were compared by determining their quantitative and qualitative abilities for the recovery of bifidobacteria from septic tank samples. The counts of Gelman GN-6 filters were arbitrarily designated as 100% recovery. Two experiments were conducted with five replicates at each dilution filtered onto: Gelman GN-6, Millipore HA (certified), Millipore HC, Sartorium, Nuclepore, and Schleicher & Schuell filters (Table 1).

The medium of Gyllenberg and Niemela (11) was compared with YN-6 for recovery from raw sewage and freshwater samples. The number of bifidobacteria recovered was noted as well as the number of false-positive presumptive colonies.

RESULTS

The results of the procedure were established by comparing the recoveries of bifidobacteria on YN-6 with those obtained with RCA spread plates incubated under similar conditions (Table 2). As can be seen, an overall recovery of 91.7% was achieved with strains representative of those

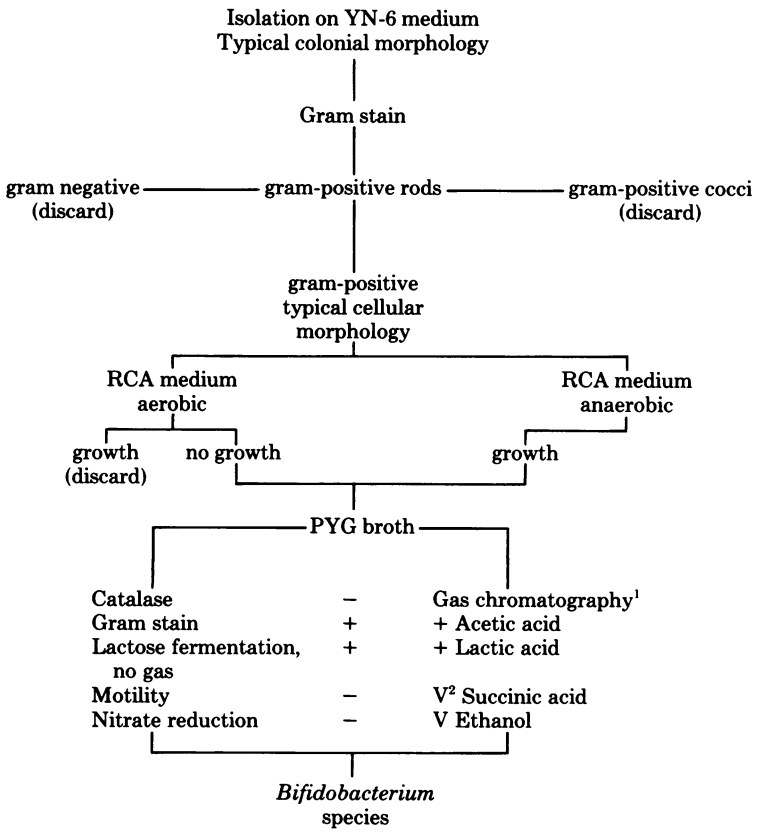

FIG. 1. *Identification of genus Bifidobacterium. Footnotes: 1, ratio of acetic to lactic acid must exceed 1:1; 2, variable (may or may not be present).*

TABLE 1. *Evaluation of membrane filters*

Filter type[a]	Lot no.	% Recovery[b]	False-positive[c]	False-negative[d]	Background[e]
Gelman GN-6	81891	100 ± 5	5	0	8.4
Millipore HA (certified)	C8J622777	76 ± 10	0	0	32
Millipore HC	C7M20059	64 ± 15[f]	−6	100[f]	35
Sartorius	13756	111 ± 5	5	0	ND[g]
Nuclepore	N040CPR	0			
Schleicher & Schuell	70/4	18 ± 1	15	0	0

[a] YN-6 medium was used to assay septic tank samples. The Millipore HC filter had a designated pore size of 0.7 μm; all others were designated as having 0.45-μm pore sizes.

[b] Two experiments were conducted with five replicate plates used at each dilution. Gelman GN-6 was arbitrarily selected as a standard. Values indicate 95% confidence limits.

[c] Percentage of typical colonies which could not be confirmed as bifidobacteria.

[d] Percentage of atypical colonies which were confirmed as bifidobacteria.

[e] Expressed as percentage of typical colonies.

[f] Plates were extremely difficult to count due to unusual colonial morphology. No clear differentiation between typical and atypical colonies was possible.

[g] No data.

species most often isolated from the feces of humans.

The specificity of the method was determined by biochemical confirmation of the presumptive positive colonies (as described in Materials and Methods) on YN-6 from filtered samples of sewage, human feces, and freshwater environmental samples. Of 240 presumptive positive colonies

TABLE 2. *Recovery of bifidobacteria species on YN-6, using fresh cultures*

Environmental isolate	% Recovery[a]
B. longum (B29)	88
B. longum (B30)	88
B. adolescentis (23)	66
B. adolescentis (B306)	100
B. infantis (30-14)	100
B. infantis (W-4)	100
B. longum (B22)	100
Avg	91.7

[a] Expressed as [(organisms/milliliter on YN-6)/(organisms/milliliter on RCA spread plate)] × 100. An average of three replicate filters or plates was used.

tested, 92% of 221 colonies were confirmed as bifidobacteria (Table 3). Of 99 presumptive negative colonies tested, only 8% were confirmed as bifidobacteria (false-negatives).

The selectivity of the YN-6 method varies with the nature of the sample under consideration. In samples of sewage, feces, and septage, the density of bifidobacteria is high relative to the other organisms present. In natural aquatic environments, the presence of environmental organisms in high densities hinders the selective recovery of bifidobacteria on YN-6. This often results in the presence of colonies of gram-positive facultative anaerobic streptococci. In this event, the differential qualities of the medium must be relied on.

The effective counting range of the medium was between 20 and 120 colonies per plate (Fig. 2). It must be kept in mind that the effective counting range will vary with the nature of the sample. If there are a large number of background organisms present, the number of bifidobacteria which can be accurately counted will be diminished.

Examination of the mean plate counts of organisms grown on YN-6 in an anaerobic glove box versus incubation in the BBL GasPak system indicated that the two methods of achieving anaerobic conditions are equivalent in recovery of bifidobacteria (Table 4).

D^2 analysis was performed on 21 environmental samples of various types (5). The D^2 values were distributed within the $P = 0.25$ and $P = 0.975$ control limits, as expected when sampling populations of randomly distributed particles (Fig. 3). We can conclude from this analysis that the organisms in the samples examined were randomly distributed and that there was no apparent deviation from the precision required to achieve this distribution of D^2 values around the $P = 0.5$ control line. It should be pointed out that the distribution of D^2 values around the $P = 0.5$ control line is not as expected by chance alone. This is possible due to some aspect of YN-6 which limits the plate-to-plate variability by selecting against defective cells.

TABLE 3. *Confirmation of presumptive positive colonies*

Sample source	No. of presumptive positives[a]	No. of confirmed positives[b]	% Confirmed
Raw sewage[c]	92	75	82
Human feces[d]	95	93	98
Environmental isolates[e]	53	53	100
Total	240	221	92

[a] Positive colonial morphology and Gram strain.
[b] Biochemical and gas chromatograph confirmation.
[c] Obtained from the sewage treatment plant operated by the University of Rhode Island at West Kingston and from the East Greenwich, R.I., municipal sewage treatment plant.
[d] From two people.
[e] From samples obtained in Rhode Island estuarine, Coventry River, and Washington (Lake). All samples were filtered in triplicate. All colonies from a selected (on basis of colonies/filter for ease of isolation) filter were evaluated. At least one filter sample was included.

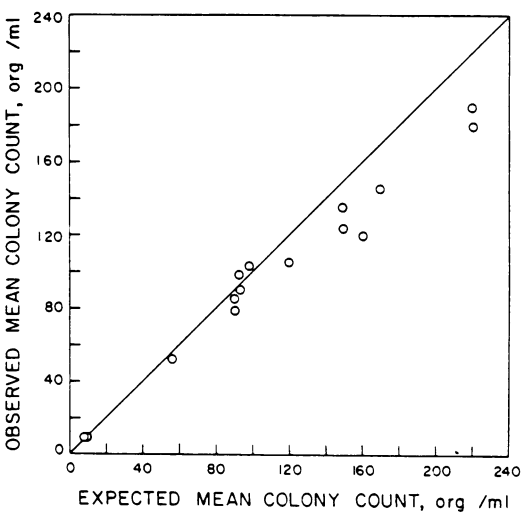

FIG. 2. *Estimation of the effective counting range for colonies of bifidobacteria on YN-6. The expected mean colony count, from environmental samples plated in triplicate, of a suitable dilution (yielding 20 to 80 colonies/plate) of the same sample. Solid line is line of equivalence.*

The precision of the YN-6 method was further evaluated by the computation and graphic representation (Fig. 4) of the percentage of precision versus the mean colony counts per dilution of the 21 samples for which D^2 analyses was performed. The expected percentage of precision

TABLE 4. *Incubation of YN-6 medium in an anaerobic glove box versus the GasPak*

Sample source	Mean recovery[a] (10^4 organisms/ml)		Relative % recovery[b]
	Glove box	GasPak	
Raw sewage	5.0	4.8	104
Raw sewage	14	13	107
Septic tank contents	47	55	85

[a] Mean of five replicate filters.

[b] Expressed as (mean counts with glove box/mean counts with GasPak) × 100.

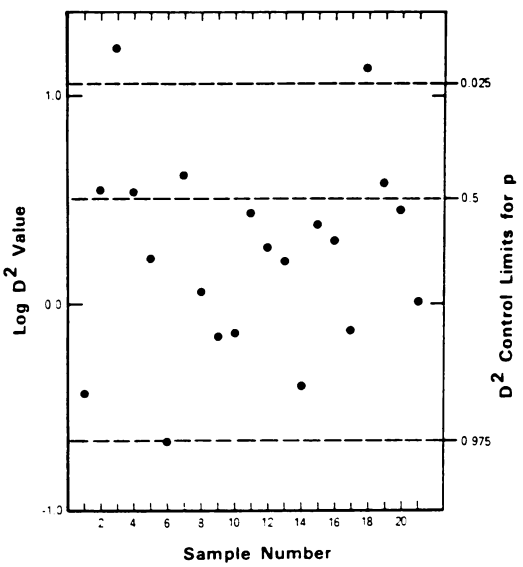

FIG. 3. *D^2 analyses of 21 samples of diverse origin analyzed on YN-6 medium.*

was computed as $2S/\bar{X}n \times 100$, where S is the standard deviation, $\bar{X}$ is the mean plate count, and n is the number of replicate plates per dilution.

The expected percentage of precision when five replicate plates were used was acceptable. The four points which lie above the line of expected percentage of precision in Fig. 4 are still within the 25% limits expected for a mean count of 20 organisms per plate.

Table 1 gives the results of the comparison of various filter types on YN-6 medium. As can be seen, Gelman GN-6, Millipore HA (certified), and Sartorius (47-mm membrane, 0.45-μm pore size, white gridded) filters are suitable for use with YN-6 medium. Although the recovery on Millipore HA filters was low (76%), there were no false-positive colonies detected.

DISCUSSION

The medium YN-6 was developed for the purpose of enumerating bifidobacteria of sanitary significance in natural aquatic environments. A comparison of YN-6 with the medium of Gyllenberg and Niemela (11) demonstrated the superiority of the YN-6 method for the selective recovery of bifidobacteria. Evaluation of the medium from a quantitative viewpoint was favorable. The method is amenable to execution in water quality laboratories. The major shortcoming of this method is the limited ability to repress gram-positive facultative cocci. Many antibiotics and dyes have been screened without success in identifying an agent sufficiently selective in inhibitory action.

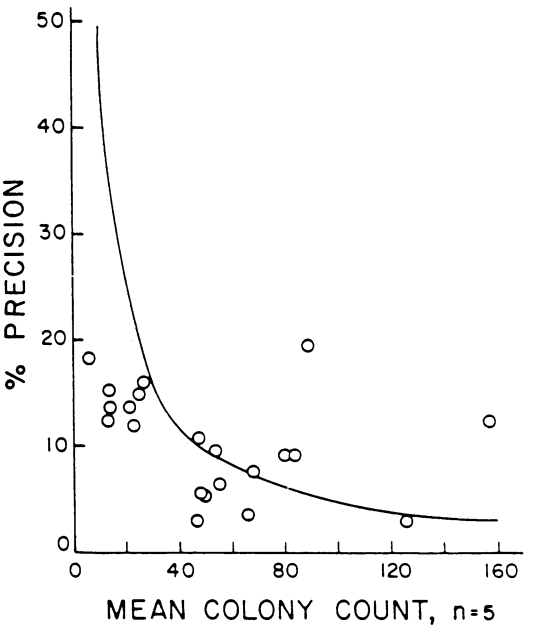

FIG. 4. *Percentage of error in plate counts for 22 samples of diverse origin analyzed on YN-6 medium versus the mean plate count* (○). *The solid line indicates the expected error for $n = 5$ plates per dilution.*

In the course of confirming presumptive positives, it was noted that Gram stain characteristics paralleled biochemical findings (Table 4). Therefore, Gram stain reactions of isolated colonies on YN-6 medium can be considered a verification test. The typical appearance of organisms which have been grown on YN-6 medium and Gram stained is as follows: gram-variable thick pleomorphic rods which may exhibit branching, bulbous clubs, coryneforms, buds, spheroids, and bifurcated Y and V forms. After the technician becomes familiar with the method, it is not necessary to Gram stain presumptive colonies. Identification of colonies by their typical morphology resulted in 90% accuracy.

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