

Selective Medium for Isolation and Enumeration of *Bifidobacterium* spp.

F. J. MUÑO A* AND R. PARES

Departamento de Microbiología, Facultad de Biología, Universidad de Barcelona, Avenida Diagonal, 645, 08028 Barcelona, Spain

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A new method was developed for the isolation and enumeration of *Bifidobacterium* spp. from natural aquatic environments. The method was based on the utilization of a new medium, *Bifidobacterium* iodoacetate medium 25, and resuscitation techniques were used to isolate injured bifidobacteria. The new medium was tested with a nonselective reference medium on sewage and sewage-polluted surface waters. Relatively little colonial growth of any other bacterial genera occurred; when such colonies did grow, *Bifidobacterium* could be easily differentiated by its colonial morphology or, after Gram staining, by its typical bifidobacterial morphology.

For nearly 100 years, indicator bacteria have been used successfully to monitor the bacteriological quality of water. *Escherichia coli* is the most extensively used bacterium because it has proved to be an acceptable indicator under most environmental conditions. Nevertheless, the ability of *E. coli* to regrow in water is now well established and has caused investigators to look for new indicators (4, 8). One of the proposed candidates is *Bifidobacterium* spp., whose potential significance as an indicator of fecal pollution in water has been suggested by several authors since it was first proposed by Mossel (D. A. A. Mossel, Abstr. 7th Int. Congr. Microbiol., p. 440, 1958) over 30 years ago.

All species of *Bifidobacterium* are exclusively of fecal origin, and some of them occur in human feces in proportions exceeding those of *E. coli* (6, 7, 12, 13). Moreover, as *Bifidobacterium* spp. are obligate anaerobes with complex growth requirements, it is unlikely that this genus of organisms could find suitable conditions to grow in water.

Despite these advantages of *Bifidobacterium* spp. to act as potential indicators of fecal pollution in water, there is a lack of available information concerning the extraenteric behavior of *Bifidobacterium* spp. This is mainly due to the absence of a selective medium for the isolation of *Bifidobacterium* spp.

Recently, two selective and differential media have been described for the isolation of *Bifidobacterium* spp.: YN-6 (11) and YN-17 (9). However, as has been reported previously (9), YN-6 medium is inhibitory to some species of *Bifidobacterium* and lacks the desired selectivity to assess a real estimation of the number of viable cells of *Bifidobacterium* spp. (5). On the other hand, YN-17 medium, which is more selective than YN-6, is inhibitory to a part of the viable bifidobacterial population (10). Furthermore, neither medium allows for the ready identification of *Bifidobacterium* colonies. All of the isolated colonies must be studied further in order to differentiate *Bifidobacterium* spp. from other microorganisms (5, 9, 10).

Here we describe a new method for the quantification of the presence of *Bifidobacterium* spp. in aquatic environments. This method is based on the utilization of a new selective and differential medium, *Bifidobacterium* iodoacetate medium 25 (BIM-25), which we describe here. The

method also incorporates resuscitation techniques to allow for the isolation of injured bifidobacteria.

MATERIALS AND METHODS

Natural samples. Raw sewage was collected from a city sewage system. The average most probable numbers of fecal coliforms and fecal streptococci per ml were 3.1×10^5 and 8.2×10^4 , respectively. Besós and Llobregat are two highly polluted, small rivers that flow through densely populated areas north and south of Barcelona, Spain. The mean most probable numbers of fecal coliforms and fecal streptococci per ml were 3.08×10^5 and 4.22×10^4 , respectively, for the Besós River and 8.62×10^4 and 1.32×10^4 , respectively, for the Llobregat River. Polluted seawater was collected from a beach which receives untreated sewage from Barcelona; the mean most probable numbers of fecal coliforms and fecal streptococci per ml were 7.3×10^1 and 1.5×10^1 , respectively. Numbers are mean values that were obtained monthly over a period of 2 years (1985 to 1986).

In all cases, samples were collected in sterile containers and processed within 4 h of collection.

***Bifidobacterium* enumeration.** *Bifidobacterium* spp. were recovered from water samples by using a new selective and differential medium, BIM-25. Its composition is as follows, in grams per liter: reinforced clostridial agar (RCA; 11564; BBL Microbiology Systems, Cockeysville, Md.), 51; nalidixic acid, 0.02; polymyxin B sulfate, 0.0085; kanamycin sulfate, 0.05; iodoacetic acid (sodium salt; I-6375; Sigma Chemical Co., St. Louis, Mo.), 0.025; and 2,3,5-triphenyl-tetrazolium chloride (TTC; 8380; Merck & Co., Inc., Rahway, N.J.), 0.025. The RCA basal medium lacked thermosensitive components and was autoclaved and allowed to cool to 55 to 60°C. Filter-sterilized antibiotics, iodoacetate, and TTC were then added.

In order to improve the recovery of injured bifidobacteria, we incorporated the resuscitative technique of solid medium repair (2) into the enumeration procedure. By this procedure, enumerations were made as follows. River and sewage samples were diluted in phosphate-buffered saline, and portions (0.1 ml) were surface plated onto 5 ml of RCA (BBL) and incubated at 37°C for 5 h under anaerobic conditions (GasPak; BBL). Thereafter, 20 ml of BIM-25 was carefully overlaid onto the plates, which were incubated for 5 days under the conditions described above. Appropriate volumes

* Corresponding author.

of seawater samples were filtered through 0.45- μ m-pore-size membrane filters (Millipore Corp., Bedford, Mass.). Filters were first incubated on RCA for 5 h and then transferred to BIM-25. In all cases, enumerations of *Bifidobacterium* spp. were made in parallel on RCA. This rich, nonselective medium served as a reference, as reported previously (11).

Other selective media used in this study were YN-6 (11) and YN-17 (9). The plates containing these media were incubated for only 2 days, as indicated previously (9, 11). Samples were inoculated in triplicate on all media used in this study.

***Bifidobacterium* identification.** From each sample, plates containing 20 to 100 colonies were studied. The colonies were identified as members of the genus *Bifidobacterium* by the following criteria: (i) they were gram positive (or variable) pleomorphic rods; (ii) they were unable to grow under aerobic conditions; and (iii) they showed fructose-6-phosphate-phosphoketolase (F6PPK) activity, as determined by the method described by Scardovi (13).

Preparation of injured cultures of *Bifidobacterium* spp. Experiments with 10 *Bifidobacterium* strains were done in order to determine the effectiveness of the resuscitative techniques described above. Injured cultures were obtained as follows. Stationary-phase bifidobacterial cultures grown in TPY broth (13) for 20 h were harvested by centrifugation at $3,000 \times g$, washed twice in sterile one-quarter-strength Ringer solution (Difco Laboratories, Detroit, Mich.), and suspended at concentrations of approximately 10^6 cells per ml in 500-ml-capacity flasks containing 250 ml of filter-sterilized (pore size, 0.22 μ m) Besós River water or seawater. The flasks were kept at 14°C in the dark for 3 days. Injured cells were those that did not grow on BIM-25 but that did grow on RCA.

***Bifidobacterium* strains.** The *Bifidobacterium* strains used in these experiments were mainly river and sewage isolates (10). We classified them as *Bifidobacterium breve* C2 and ALLA21; *Bifidobacterium adolescentis* 40; and *Bifidobacterium longum* 1, 3BS, and 4BS by the carbohydrate fermentation test described by Mara and Oragui (9). We were not able to distinguish between some strains of *Bifidobacterium infantis* and *Bifidobacterium longum* by this method, and we denoted these strains as the *Bifidobacterium* "infantis-longum" group described by Scardovi (13). Three strains were classified under this nomenclature as 38, 1BS, and 7BS. Other bifidobacteria used were *Bifidobacterium bifidum* ATCC 15696, *Bifidobacterium adolescentis* ATCC 15703, and *Bifidobacterium breve* ATCC 15700.

Statistical analysis. Natural logarithmic transformations of the observed counts were obtained. Differences in the mean recoveries on the various media were compared by using the Student *t* test (1).

RESULTS

Evaluation of the media YN-6 and YN-17. During the initial studies, enumerations of *Bifidobacterium* spp. from river water samples were carried out with the selective media YN-6 and YN-17. In these analyses, RCA was used as the reference medium (Table 1). YN-6 and YN-17 showed a low degree of selectivity and recovered a significantly lower number of bifidobacteria than did the reference medium. These results are in agreement with the findings we reported previously (10) and indicate that YN-6 and YN-17 are toxic to a part of the bifidobacteria in river water.

Development of the new selective medium BIM-25. The need for a new medium for the isolation of *Bifidobacterium*

TABLE 1. Comparison of selective recoveries of *Bifidobacterium* spp. by several isolation media^a

Medium	No. of <i>Bifidobacterium</i> recovered/ml	No. of total organisms/ml	Selectivity ^b	Accuracy ^c
RCA	1.5×10^4	1.8×10^5	8.11	100
YN-6	3.7×10^{1d}	6.4×10^3	0.57	0.24
YN-17	6.6×10^{2d}	2.2×10^3	30.00	4.40

^a Geometric mean values from a total of eight Besós River water samples.

^b Expressed as [(mean number of *Bifidobacterium* colonies)/(mean number of total colonies)] $\times 100$.

^c Expressed as [(mean number of *Bifidobacterium* colonies on the test medium)/(mean number of *Bifidobacterium* colonies on RCA)] $\times 100$.

^d Significantly lower bacterial counts compared with RCA ($P < 0.05$).

spp. that was superior to YN-6 and YN-17 led us to develop BIM-25. In initial experiments, in which several river water samples were analyzed, we showed that incorporation of RCA medium with the antibiotics used in YN-17 medium produced a new medium (RCA-17) that was much less toxic to *Bifidobacterium* spp. than YN-17 and that had the same degree of selectivity as YN-17 (data not shown). To develop this new medium, RCA-17 was used as the basal medium and some selective and differential compounds were tested. Iodoacetate (25 mg/liter) and TTC (25 mg/liter) were definitively incorporated, and the resulting medium was named BIM-25. Moreover, incubation of the plates was extended from 2 to 5 days, thus increasing the differences in size between *Bifidobacterium* colonies and colonies of other bacteria capable of growing on BIM-25. The results obtained for the isolation of *Bifidobacterium* spp. from Besós River water samples are shown in Table 2. BIM-25 was more selective than RCA-17 but was also somewhat toxic to some of the bifidobacteria. In experiments carried out with pure cultures of 12 *Bifidobacterium* strains (data not shown), BIM-25 inhibited only two strains of *B. adolescentis* (see above). These two strains grew well if the concentration of iodoacetate was reduced by half, but this resulted in a decrease of selectivity.

Role of metabolic stress in the enumeration of *Bifidobacterium* spp. It is now well established that indicator bacteria can become injured on exposure to the aquatic environment, resulting in discrepancies in CFU between nonselective and selective media (2, 3). For this reason, we decided to investigate whether *Bifidobacterium* spp. would lose their ability to produce colonies on BIM-25 when they were stressed. An inoculum of *B. breve* ALLA21 was placed in filter-sterilized Besós River water and phosphate-buffered saline. Periodically, viable counts were made in parallel on the following media: RCA, BIM-25, and BIM-50 (BIM-50 is

TABLE 2. Comparison of selective recoveries of *Bifidobacterium* spp. by several media^a

Medium	No. of <i>Bifidobacterium</i> recovered/ml	No. of total organisms/ml	Selectivity ^b	Accuracy ^c
RCA	7.0×10^3	8.7×10^4	8.03	100
RCA-17	4.6×10^{3d}	1.3×10^4	35.38	65.71
BIM-25	4.2×10^{3d}	5.3×10^3	79.13	59.65

^a Geometric mean values from a total of seven samples.

^b Expressed as [(mean number of *Bifidobacterium* colonies)/(mean number of total colonies)] $\times 100$.

^c Expressed as [(mean number of *Bifidobacterium* colonies on the test medium)/(mean number of *Bifidobacterium* colonies on RCA)] $\times 100$.

^d Significantly lower bacterial counts compared with RCA ($P < 0.05$).

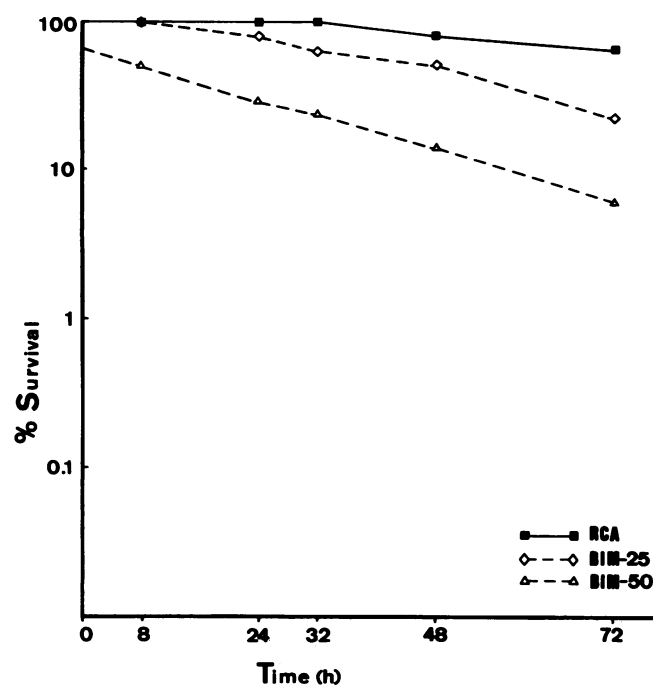


FIG. 1. Survival of *B. breve* ALLA21 in phosphate-buffered saline, as determined with three different enumeration media.

the same as BIM-25, but it contains 50 mg of iodoacetate per liter). These results are reported as survival curves in Fig. 1 and 2. The cells of this strain became progressively incapable of growing on BIM-25 and BIM-50, even though they were able to grow on RCA. It was apparent that the BIM-25 medium could not serve as a selective medium for the

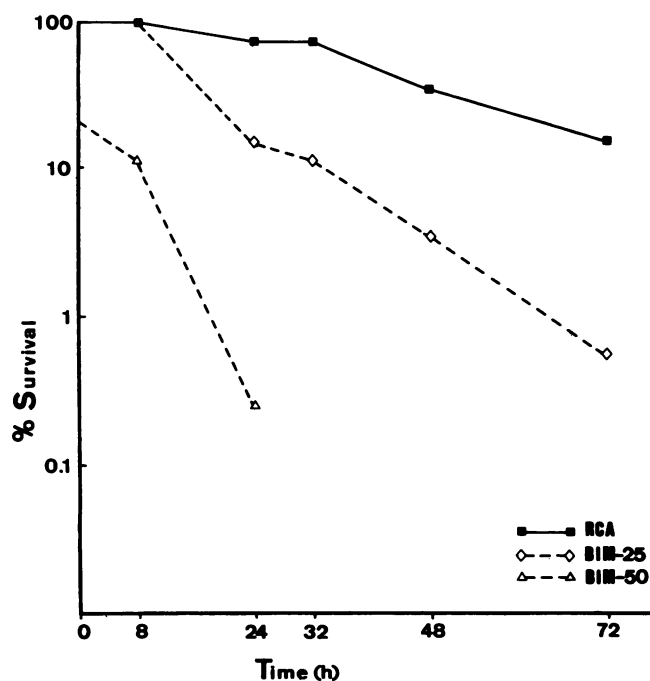


FIG. 2. Survival of *B. breve* ALLA21 in filter-sterilized river water, as determined with three different enumeration media.

TABLE 3. Effectiveness of the injury repair technique for the enumeration of artificially stressed *Bifidobacterium* spp.

<i>Bifidobacterium</i> strain	<i>Bifidobacterium</i> recoveries (% of reference medium) from ^a :			
	Fresh water		Seawater	
	BIM-25	BIM-25 ^b	BIM-25	BIM-25 ^b
<i>B. breve</i>				
ATCC 15700	6.2	96.2	33.3	97.3
ALLA21	46.4	96.1	18.3	99.2
C2	14.5	100	23.4	96.8
<i>B. bifidum</i> ATCC 15696	0.5	100	0.4	98.6
<i>B. longum</i>				
3BS	5.5	85.0	7.3	100
4BS	7.1	92.8	10.8	96.3
1	7.1	100	28.4	98.8
<i>B. "infantis-longum"</i> group				
1BS	10.0	100	18.3	100
7BS	0.3	86.7	13.2	89.6
38	2.6	100	1.8	100

^a Expressed as [(colonies on the test medium)/(colonies on RCA medium)] × 100.

^b Incubation on BIM-25 was preceded by a 5-h incubation period on RCA.

isolation of the bifidobacteria, unless injury could be repaired. This could be accomplished, perhaps, by incorporating a resuscitative step into the enumeration technique. With this aim, we tried the overlaying and membrane filter transfer techniques, as described above. A 5-h period of incubation on RCA was determined to be sufficient for resuscitation by a series of experiments carried out with injured cultures of *B. breve* ALLA21 and was finally tested with injured cultures of 10 *Bifidobacterium* strains. The results are shown in Table 3. All the viable bifidobacteria (as determined by counts on RCA) were recovered on BIM-25 medium only if definitive incubation on the selective medium was preceded by a 5-h resuscitative incubation on RCA. This step produced injury repair in all the strains tested.

Enumeration of *Bifidobacterium* spp. in natural waters with BIM-25 medium and the injury repair technique. On the basis of their typical cellular appearance and because of the high degree of selectivity shown by growth on BIM-25 medium, we reasoned that it would be possible to identify *Bifidobacterium* spp. by studying only the cellular morphology of the isolates, without determining the presence of F6PPK in each colony. We studied the correspondence between cellular morphology and F6PPK activity in colonies formed by rods obtained from analyses of sewage and river water samples. These samples yielded a total of 425 colonies, 219 of which were gram-positive cocci and 206 of which were gram-positive rods. Of the latter colonies, 183 resembled *Bifidobacterium* spp. by their cellular morphology (with branching and bifurcations) and possessed detectable F6PPK activity. They were thus identified as members of the genus *Bifidobacterium*. The other 23 colonies were formed by rods without branching or bifurcations, and none of them possessed any detectable enzyme (F6PPK) activity. These results confirmed that cellular morphology is a confirmatory criterion for the identification of *Bifidobacterium* spp. Therefore, we used this criterion to identify this microorganism in a series of analyses carried out with isolates from sewage, river, and marine water samples. The results are shown in

TABLE 4. Recovery of *Bifidobacterium* spp. from water with BIM-25 agar medium after incorporation of the injury repair technique^a

Sample	No. of <i>Bifidobacterium</i> spp. recovered/ml from:		No. of total organisms recovered/ml from BIM-25	Selectivity ^b	Accuracy ^c
	RCA	BIM-25			
Sewage	1.32×10^5	1.28×10^5	1.32×10^5	96.9	97.7
Besós River	1.19×10^4	1.16×10^4	2.40×10^4	48.3	97.5
Llobregat River	3.90×10^2	3.60×10^2	1.20×10^2	30.0	92.3
Seawater	0.58	0.48 ^d	2.92	16.5	82.7

^a Geometric mean values from a total of 12 samples.

^b Expressed as [(mean number of *Bifidobacterium* colonies)/(mean number of total colonies)] × 100.

^c Expressed as [(mean number of *Bifidobacterium* colonies on the test medium)/(mean number of *Bifidobacterium* colonies on RCA)] × 100.

^d Significantly lower bacterial counts compared with RCA ($P < 0.05$).

Table 4. The degree of selectivity of the medium was variable, depending on the sample source. On the contrary, accuracy was much more consistent and high. Count differences on RCA were only significant for marine water samples. Finally, all red colonies with a diameter of less than 2 mm were formed by gram-positive cocci that grew well under aerobic conditions. Colonies that were white with a diameter that clearly exceeded 2 mm were always *Bifidobacterium* spp. Pink colonies were formed by cocci, bifidobacteria, and other rods. No gram-negative bacteria were isolated on BIM-25.

DISCUSSION

We reported here the development of a new medium for the isolation of *Bifidobacterium* spp. from natural waters. This medium, BIM-25, had two important advantages over the previously described YN-17 and YN-6 media (9, 11): (i) its considerable superiority in the selective isolation of *Bifidobacterium* spp. and (ii) its minimal degree of toxicity, which allowed the recovery of *Bifidobacterium* spp. similar to that found in nonselective medium such as RCA. From the experiments carried out with several *Bifidobacterium* spp., we conclude that, when stressed by adverse environmental conditions, the organisms may become unable to grow on BIM-25 medium. This problem was overcome by incorporating resuscitative incubation on RCA into the enumeration procedure.

We also showed that iodoacetate and TTC decisively helped to select and differentiate, respectively, *Bifidobacterium* colonies on BIM-25 medium. Iodoacetate, which inhibits glyceraldehyde-3-phosphate dehydrogenase, drastically reduced the growth of nonbifidobacterial contaminant colonies. At experimental concentrations, all *Bifidobacterium* spp. tested except *B. adolescentis* grew well on BIM-25 plates. The two *B. adolescentis* strains tested grew only if the quantity of iodoacetate was reduced by half. On the other hand, TTC differentiated significantly between *Bifidobacterium* spp. and other bacterial colonies on BIM-25. When Gram morphology and F6PPK activity were analyzed, a high correlation was established between large white colonies and the genus *Bifidobacterium*.

BIM-25 medium can be used successfully as a selective medium for the isolation of *Bifidobacterium* spp. from natural waters. Therefore, this medium could contribute to the future investigation of the extraenteric behavior of this

genus, which has long been proposed as an indicator of fecal contamination of water.

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LITERATURE CITED

1. American Public Health Association. 1985. Standard methods for the examination of water and wastewater, 16th ed., p. 839–847. American Public Health Association, Washington, D.C.
2. Andrew, M. H. S., and A. D. Russell. 1984. The revival of injured microbes, p. 329–369. Academic Press, Inc. (London) Ltd., London.
3. Bissonnette, G. K., J. J. Jezeski, G. A. McFeters, and D. G. Stuart. 1975. Influence of environmental stress on enumeration of indicator bacteria from natural waters. *Appl. Microbiol.* 29:186–194.
4. Cabelli, V. J. 1978. Obligate anaerobic bacterial indicators, p. 171–200. In G. Berg (ed.), *Indicators of viruses in water and food*. Ann Arbor Science Publishers, Inc., Ann Arbor, Mich.
5. Carrillo, M., E. Estrada, and T. C. Hazen. 1985. Survival and enumeration of fecal indicators *Bifidobacterium adolescentis* and *Escherichia coli* in a tropical rain forest watershed. *Appl. Environ. Microbiol.* 50:468–476.
6. Evison, L. M., and A. James. 1975. *Bifidobacterium* as an indicator of faecal pollution in water. *Prog. Water Technol.* 7:57–66.
7. Evison, L. M., and S. Morgan. 1978. Further studies on bifidobacteria as indicators of faecal pollution in water. *Prog. Water Technol.* 10:341–350.
8. Levin, M. A. 1977. Bifidobacteria as water quality indicators, p. 131–138. In A. W. Hoadley and B. J. Dutka (ed.), *Bacterial indicators/health hazards associated with water*. American Society for Testing and Materials, Philadelphia.
9. Mara, D. D., and J. I. Oragui. 1983. Sorbitol-fermenting bifidobacteria as specific indicators of human faecal pollution. *J. Appl. Bacteriol.* 55:349–357.
10. Muñoz, F. J., J. Vives-Rego, and R. Parés. 1985. Detección, aislamiento y caracterización de *Bifidobacterium* en aguas libres superficiales. *Microbiol. Españ.* 38:1–9.
11. Resnick, I. G., and M. A. Levin. 1981. Quantitative procedure for enumeration of bifidobacteria. *Appl. Environ. Microbiol.* 42:427–432.
12. Resnick, I. G., and M. A. Levin. 1981. Assessment of bifidobacteria as indicators of human fecal pollution. *Appl. Environ. Microbiol.* 42:433–438.
13. Scardovi, V. 1981. The genus *Bifidobacterium*, p. 1951–1961. In M. P. Star (ed.), *The prokaryotes*. Springer-Verlag KG, Berlin.