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Assessment of Bifidobacteria as Indicators of Human Fecal Pollution

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The distribution of bifidobacteria in the environment has been examined by using YN-6 medium. Although feces of humans, chickens, cows, dogs, pigs, horses, cats, sheep, beavers, goats, and turkeys were examined, bifidobacteria were isolated only from the feces of humans and swine. The frequency and distribution of component species of human fecal isolates were as in isolates from raw sewage. *Bifidobacterium longum* and *B. adolescentis* were most often isolated and in the highest densities. The levels of bifidobacteria in raw sewage were in the range of 10^6 organisms/100 ml, and the effect of primary and secondary sewage treatment on the number of viable organisms present was not significant. High densities of bifidobacteria were found in all samples from septic tanks. It was found that bifidobacteria did not survive as well as *Escherichia coli* in either fresh or marine waters. The ratio of bifidobacteria to *E. coli* is an indication of the age and of the effectiveness of treatment of sewage effluent.

The use of bacterial indicators for estimating water quality is a well-established practice. Mossel in 1958 (D. A. A. Mossel, Abstr. 7th Int. Congr. Microbiol., 1958, p. 440) proposed bifidobacteria as potential indicators of fecal contamination. The lack of a suitable medium for selectively enumerating these organisms and the difficulty with anaerobic methodology resulted in a paucity of data which made it impossible to evaluate his proposal.

The characteristics of an ideal indicator of fecal pollution have been discussed by Bonde (2) and more recently by Cabelli (4). Briefly stated, an indicator organism must be: (i) present in sufficient density to allow detection; (ii) easy to enumerate; (iii) present in a constant ratio to a pathogen(s); (iv) have survival properties similar to those of the pathogen(s); (v) unable to multiply in extraenteral environments; and (vi) released into the environment solely in the feces of warm-blooded animals. In addition, when using bacterial indicators to assess the magnitude of sewage input to receiving waters or the distance of the sampling site from the source, the survival characteristics of the bacterial indicator must be known.

The survival characteristics of bifidobacteria have been investigated by Gyllenberg et al. (10) and reported as similar to those of *Escherichia coli*. Results of preliminary environmental sampling have been reported by Evison et al. (6-8). With the recent availability of a medium capable of quantitative and selective recovery of bifidobacteria (YN-6), a survey of the distribution of bifidobacteria in organisms of sanitary significance and a preliminary survey of environmental samples has been conducted (I. G. Resnick and M. A. Levin, Abstr. Ann. Meet. Am. Soc. Microbiol. 1977, Q19, p. 263).

This report examines the distribution of bifidobacteria in the environment, the effects of various methods of waste treatment, and survival of these microorganisms in receiving waters. The application of the YN-6 technique to the problem of determining the type and source of pollution in a river is demonstrated and discussed.

MATERIALS AND METHODS

Fecal samples. Samples from 22 healthy adults, 23 chickens, 15 cows, 14 dogs, 11 pigs, 6 horses, 4 cats, 4 sheep, 4 beavers, 2 goats, and 2 turkeys were examined. Specimens were collected and transported in sterile containers. Freshly obtained feces (less than 6 h from time of defecation) were diluted 1/100 (wt/vol) in phosphate-buffered saline (PBS; sodium chloride, 0.85 g; dibasic sodium phosphate, 0.25 g; monobasic sodium phosphate, 0.056 g; and 100 ml of deionized water) and homogenized for 30 s in a Waring blender. Appropriate dilutions of the homogenate were assayed on YN-6 medium. After 48 h of anaerobic incubation at 37°C, 10 suitably isolated typical colonies were picked from each filter. The isolates were considered bifidobacteria if they: required strict anaerobic conditions for growth; displayed typical Gram stain morphology; were nitrate

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negative, catalase negative, and nonmotile; fermented lactose without producing gas; and released acetic and lactic acids (ratio of 1:1) as the major metabolic by-products of glucose fermentation in peptone-yeast-glucose broth with minor amounts of ethanol or succinic acid. The species designations of the isolates identified as bifidobacteria are based on a compilation of the fermentation patterns given in the eighth edition of *Bergey's Manual of Determinative Bacteriology* (3) and on species recently proposed by Scardovi and Zani (16), Scardovi and Crociani (14), and Scardovi and Trovatelli (15) (Table 1). The enumeration of fecal coliforms was accomplished by filtering appropriate dilutions of fecal homogenate on mTEC medium (5).

Samples from two sewage treatment plants (STP; University of Rhode Island and Narragansett) of raw, primary, secondary, and chlorinated sewage were examined. The university STP receives domestic wastes from a population composed primarily of adults. The Narragansett STP also handles primarily domestic wastes, but there is some industrial input. Samples of raw sewage were also obtained from the Warwick STP. This system handles a large volume of industrial and domestic wastes.

All samples of sewage were transported in sterile containers at ambient temperature and processed within 3 h. Sodium thiosulfate (0.05 g/600 ml) was added to chlorinated effluents at the time of sampling. The samples were diluted with PBS and assayed on YN-6 and mTEC media. For those samples in which the frequency of the various species of bifidobacteria present was determined, isolation and identification were carried out as indicated for fecal samples. Laboratory experiments were conducted to further characterize the sensitivity of bifidobacteria to chlorine. Volumes of postsecondary sewage were chlorinated with a solution of sodium hypochlorite and shaken for 15 min. The residual chlorine concentration was then measured by the *N,N*-dimethyl-*p*-phenylene diamine (DPD) colorimetric method (1), and sufficient chlorine was added to obtain a free chlorine residual. Four trials were conducted at 22°C with pH ranging from 5.9 to 6.8.

The levels of bifidobacteria, *E. coli*, and fecal streptococci were determined before and after chlorination on YN-6, mTEC, and ME (12) media, respectively.

Septic tank samples were transported, processed, and evaluated as were the sewage samples.

Survival experiments. An inoculum of bifidobacteria was added to a sterile holding medium yielding a final dilution of 1:100 to stimulate in situ dilution. The holding flasks were kept at various temperatures, chosen as representative of those encountered in the environment. The numbers of viable bifidobacteria present were determined at various times after inoculation on YN-6 medium. The inocula consisted of either pure cultures of bifidobacteria grown on peptone-yeast-glucose broth or raw sewage obtained from the Narragansett STP. When pure cultures of bifidobacteria were used as inocula, reinforced clostridial agar (Difco Laboratories, Detroit, Mich.) spread plates as well as YN-6 were used to determine the number of viable cells present with time. The holding menstra used were: (i) PBS (autoclaved); (ii) fresh waters, obtained from a stream, and (iii) marine waters, obtained from Narragansett Bay (salinity, 32 parts per thousand, wt/vol [32 g/kg]). The fresh and marine waters were filter sterilized by using membrane filters with a pore size of 45 μm.

Environmental samples from various natural situations in New England were examined. Samples were taken below the surface of the waters in sterile Nalgene bottles, transported at 4°C, and processed as described (1) in less than 3 h from the time of collection. The number of bifidobacteria present were determined on YN-6 and, in some instances, on the

TABLE 1. *Species of the genus Bifidobacterium*[a]

Species	Fermentation										
	Arabinose	Xylose	Ribose	Gluconate	Cellobiose	Lactose	Mannitol	Melezitose	Salicin	Starch	Trehalose
bifidum	−	−	−	−	+	+	−	−	−	−	
adolescentis	+	+	+	+	+	+	v[b]	v	+	v	
catenulatum	+	+	+	+	+	+	−	−	−	−	
angulatum	+	+	+	−	−	+	−	−	+	+	
dentium	+	+	+	+	+	+	+	+	+	+	
infantis	−	−	+	−	v	+	−	−	v	v	
liberorum[c]	−	+	+	−	v	+	−	v	v	v	
lactentis[c]	−	−	−	−	v	+	+	−	−	v	
breve	−	−	+	−	+	+	+	v	v	v	
parvulorum[c]	−	−	+	−	v	+	+	−	−	+	
longum	+	+	+	−	−	+	−	+	−	v	
pseudolongum	+	+	+	−	v	v	−	v	v	+	
suis	+	+	−	−	−	+	−	−	−	−	
asteroides	+	+	+	+	+	−	−	−	+	−	
indicum	−	−	+	+	+	−	−	−	+	−	
coryneforme	+	+	+	+	+	−	−	−	+	−	
magnum	+	+	+	−	−	+	−	−	−	−	

[a] A compilation of the fermentation patterns presented in the eighth edition of *Bergey's Manual of Determinative* bacteriology (3), with data of Scardovi and Zani (16) and Scardovi and Crociani (14).

[b] Variable.

[c] Uncertain species designation.

medium of Gyllenberg et al. *E. coli* was quantitated on mTEC medium.

RESULTS

Of the fecal samples examined, only those of human and swine origin yielded bifidobacteria on YN-6 medium. Table 2 gives the concentrations and frequencies of the observed species. *B. longum* and *B. adolescentis* were the most common species isolated from human feces (concentrations of 2.0×10^6 to 2.0×10^9 and 1.0×10^8 to 6.0×10^9 organisms/g of feces, respectively). In general, the distribution and frequency of species isolated from raw sewage were similar to those of human feces (Table 3). However, significantly more *B. bifidum* were isolated from sewage than from feces. A possible explanation is that since *B. bifidum* is isolated more often from the feces of children than of adults (13), it was present in the sewage samples but was not a major component of the fecal samples of the adults tested. Table 4 shows the relationship between bifidobacteria and *E. coli* from STPs and septic tanks. Although the ratios of bifidobacteria to *E. coli* (B/E ratios) varied from 55.5 to 0.79, there was no apparent effect of the treatment process except for chlorination on density of bifidobacteria or on the B/E ratio. We found that after the chlorination process, no viable organisms could be detected on YN-6 medium. This was not unexpected considering the strong oxidative effect of chlorine and the anaerobic nature of bifidobacteria. From the laboratory experiments (four trials) on chlorination of batch cultures, it was determined that a residual chlorine concentration as low as 0.2 ppm, wt/vol (0.2 mg/liter), after 15 min of contact time reduced the density of bifidobacteria from 7.9×10^4/100 ml to less than 10/100 ml.

TABLE 2. *Quantitation of bifidobacteria in feces of 22 healthy adults and 11 adult swine on YN-6 medium*

Source and species	Range (organisms/g)	Arithmetic mean (organisms $\times 10^8$/g)
Adults		
B. longum	2×10^6–2×10^9	28.83
B. adolescentis	1×10^8–6×10^9	38.67
B. breve	3×10^6–3×10^8	1.54
B. bifidum	1.3×10^8	0.59
B. thermophilum	3×10^6	0.01
B. infantis	3×10^6	0.01
Swine		
B. thermophilum	1×10^6	0.010
B. longum	1×10^5–1×10^6	0.005
B. pseudolongum	2×10^5	0.002
B. adolescentis	1×10^5	0.001

TABLE 3. *Frequency of isolation of component species of bifidobacteria from feces of 22 healthy adults and raw sewage*

Species	% of total isolates	
	Human feces	Raw sewage
B. longum	41.4	39.6
B. adolescentis	55.5	35.4
B. breve	2.2	2.5
B. infantis	0.01	Not found
B. thermophilum	0.01	6.1
B. bifidum	0.59	10.0

TABLE 4. *Relationship between bifidobacteria and E. coli in sewage*

Location	Organisms/100 ml ($\times 10^3$)		B/E ratio
	Bifidobacteria[a]	*E. coli*[b]	
University STP			
Raw	83.3	1.5	55.5
Primary	36.5	2.1	17.1
Secondary	98	8.7	11.2
Narragansett STP			
Raw	150	29	5.2
Primary	250	72	3.4
Secondary	210	70	3.0
Warwick STP, raw	2.3	2.9	0.79
Septic tanks			
1	55,000	5,300	10.4
2	99,000	12,000	8.3
3	2,300	150	15.3
4	6,600	<10	
5	11,400	260	43.8
Cespool	33,000	3,000	11.0

[a] Enumerated on YN-6 medium.
[b] Obtained by the mTEC procedure of Dufour et al. (5).

Table 4 also illustrates the ability of bifidobacteria to persist in septic tanks and cesspools. A mean density of bifidobacteria of $10^{4.88}$/ml was found, with a mean B/E ratio of 17.8.

Bifidobacteria were found to have survival properties differing from those reported by Gyllenberg and Niemela (9). Figures 1 and 2 show the survival characteristics of *B. longum* (B7) at 40 and 20°C, *B. longum* (B1) at 8 and 20°C, and *B. adolescentis* (30-6) at 22°C in PBS expressed as percentage of recovery of viable cells on reinforced clostridial agar. Although there was variability in the T_{90} values for these organisms (*B. longum* B7, T_{90} = 20 h at 20°C; *B. longum* B1, T90 = 3.5 days at 22°C), the decrease in viability was approximately 26 times faster than that of *E. coli* (17).

In an effort to simulate the in situ conditions of bifidobacteria in receiving waters, experiments were carried out with raw sewage as the inocula and sterile fresh and marine waters as the holding fluid. Figures 3 and 4 show the survival curves obtained. From these curves, the

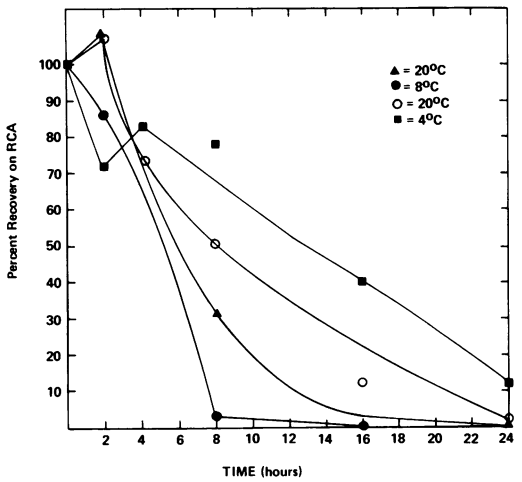

FIG. 1. *Survival of B. longum (B7) in PBS at 20 and 8°C and B. adolescentis at 20 and 4°C.*

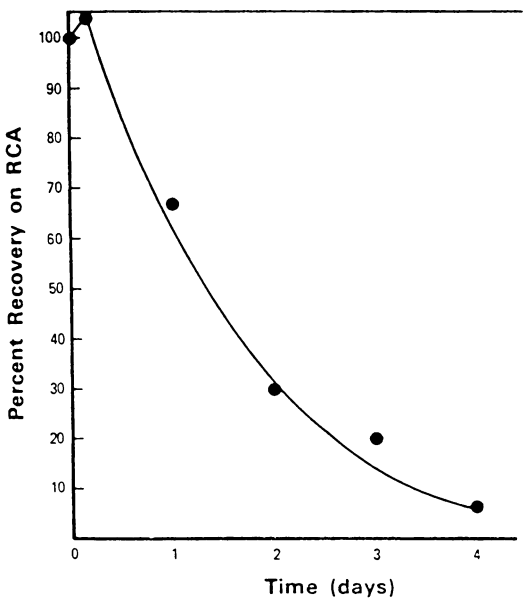

FIG. 2. *Survival of B. adolescentis (30-6) in PBS at 22°C.*

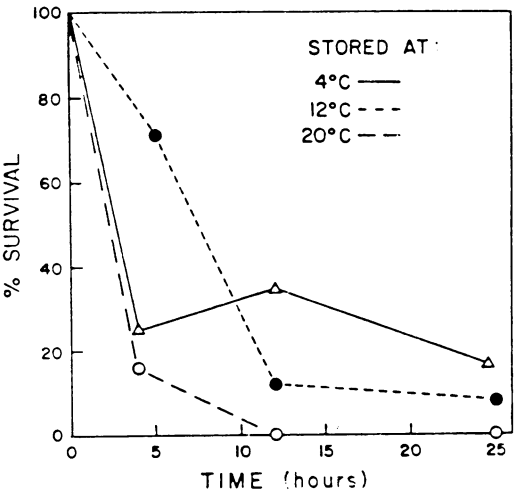

FIG. 3. *Survival of bifidobacteria from a raw sewage inoculum in filter-sterilized fresh water stored at 4, 12, and 20°C.*

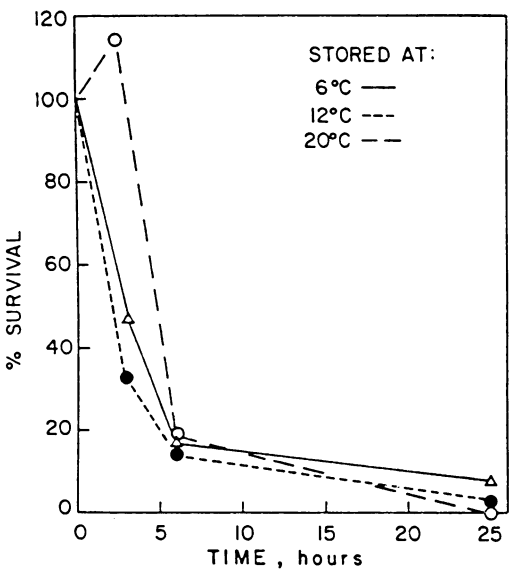

FIG. 4. *Survival of bifidobacteria from a raw sewage inoculum in filter-sterilized marine water (32‰) stored at 6, 12, and 20°C.*

calculated T_{90} values of bifidobacteria suspended in fresh and marine waters at 20°C were 5 and 10 h, respectively.

Table 5 gives the results of environmental sampling of two rivers with identified sewage outfalls and one river with a nonpoint source. The density of bifidobacteria dropped rapidly with increasing distance from the outfall, as did the B/E ratio. It is clear from these data that bifidobacteria were present in human and swine feces in sufficient density in sewage to be useful as indicators. The count of 220,000 × 10^3 organisms/100 ml along the Pawtuxet River was due to the ineffective treatment of sewage at an ATP under construction. The B/E ratio at the first outfall on the Pawtuxet River provides an example of a unique application of bifidobacteria determinations. Although an *E. coli* density of 5,200 organisms/100 ml was apparently associated with a fecal input, the proximity and magnitude of the source could not be determined. If the *E. coli* density is considered along with that of the bifidobacteria (22,000 organisms/100 ml), we can feel confident that we are close to a fresh, unchlorinated source of human fecal pollution.

TABLE 5. *Relationship between bifidobacteria and E. coli in river water*

Location	Distance from outfall (km)	Organisms/100 ml		B/E ratio
		Bifidobacteria[a]	*E. coli*[b]	
Pawtuxet River	0	210	52	4.0
	1.2	60	340	0.2
	8.8	<2	10.0	<0.2
	0	229,000	100,000	2.3
	1.6	140	350	0.4
Thames River[c]	0.8	6,900	470	14.7
	0	130,000	9,000	14.4
	0.8	760	1,060	0.7
Seekonk River	Nonpoint source	133	900	0.1
		630	900	0.7

[a,b] See Table 4.

[c] One sample above and one below the outfall.

This is of extreme interest, since this first outfall was in the center of a small cluster of old buildings with no STP in the vicinity. Samples from closer points could determine the exact source of pollution and permit appropriate corrective action.

DISCUSSION

It is clear that bifidobacteria satisfy, in a limited sense, the criteria for a bacterial indicator of human fecal pollution. Bifidobacteria are present in animal feces, sewage, and septic tanks in sufficient densities to allow detection. Of the fecal samples examined, only those of human and swine origin yielded bifidobacteria on YN-6 medium. In most cases, the possibility of contribution of bifidobacteria by swine feces can be ruled out by a survey of the area. Although bifidobacteria have been reported from the rumen of cows, they are most likely absent from bovine feces due to digestion of the bacteria in the abomasum (true stomach). This specificity of YN-6 medium is attributed to its formulation and evaluation with fresh isolates of human strains. Furthermore, bifidobacteria species present at concentrations 10-fold lower than the concentrations of either the predominant strains of bifidobacteria or the background organisms isolated on YN-6 will not be detected.

The YN-6 method for enumeration of bifidobacteria is relatively inexpensive and easy to perform. However, the medium is some situations is not selective enough, which results in overcrowding by interfering organisms. The lowest concentrations of bifidobacteria which can be detected are dependent upon the levels of gram-positive facultative cocci present. In feces, this is approximately 10^3 organisms/g. Due to differential survival characteristics of bifidobacteria and the interfering organisms, this becomes a problem when sampling at a great distance from the outfall.

The relatively short survival time of these organisms is presently a problem in terms of maintenance of a constant ratio of indicator to pathogen. The transit time before samples are filtered must be kept to a minimum (3 h), since even then only 60 to 70% of the population can be recovered. Based on the data in Fig. 1 through 3, the temperature of storage during transit has little effect on the viable count.

In attempting to compare these data with those obtained by Gyllenberg et al. (10), two significant factors must be taken into consideration. The inocula used in this study consisted of raw sewage which had been exposed to the conditions prevailing in the sewage-collecting system for the time required to travel from each contributing residence to the plant, and thus the organisms were already stressed to stimulate the in situ occurrences, whereas Gyllenberg et al. (10) used fresh feces as inocula. In addition, Gyllenberg et al. were using the medium of Gyllenberg and Niemela (9), which in our laboratory has been found to be nonselective, yielding many false-positive colonies (facultative anaerobic gram-positive rods).

The field data presented in Table 5 support the laboratory data presented on the survival of bifidobacteria in fresh water relative to *E. coli*. If the densities of bifidobacteria at sampling points past the point source are multiplied by a factor of 26 (the approximate difference in T_{90} values for bifidobacteria and *E. coli*), the B/E ratios calculated with these adjusted bifidobacteria densities approximate the B/E ratio at the point source. This would indicate that the differential die-off rates between *E. coli* and bifidobacteria observed in the laboratory parallel those in freshwater environments.

From the data presented, the use of bifidobacteria enumerated on YN-6 medium as an indicator of unchlorinated human fecal pollution of recent origin can be recommended. The enumeration of bifidobacteria can be of value in detecting septic tank leaks, raw sewage inputs, and crossover of storm runoff and sewage lines. The B/E ratio is an indication of the age of the fecal contamination.

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