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Sorbitol-fermenting bifidobacteria as indicators of diffuse human faecal pollution in estuarine watersheds

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M.W. RHODES AND H. KATOR. 1999. Sorbitol fermenting bifidobacteria were evaluated as indicators of non-point source human faecal pollution to three sub-estuaries with elevated faecal coliform densities. Human-specific bifidobacteria correlated with identifiable human sanitary deficiencies in feeder streams to estuarine creeks in two of three watersheds examined, one rural and one moderately developed. Sorbitol-fermenting bifidobacteria were recovered at densities ranging from 1 to 90 colony-forming-units $100\,ml^{-1}$ in 11 of 258 water samples but were undetected in sediment ($n = 68$) and scat from resident wildlife (deer, muskrat and raccoon, $n = 20$). Failure to detect sorbitol-fermenting bifidobacteria in water samples during the summer months was consistent with laboratory microcosm results showing non-recoverability of *Bifidobacterium adolescentis* after 5–9 d in membrane-filtered estuarine water at 23 and 30 °C, but persistence for 4 weeks at 10 °C. Persistence of sewage-derived bifidobacteria in membrane-filtered freshwater at 15 °C was also observed. Recovery of sorbitol-fermenting bifidobacteria was complicated by high background levels of Gram-positive rods and cocci. Use of propionic acid and reduced pH (pH = 5·0), or use of a two-step resuscitation protocol using non-selective and selective media, did not improve recovery. Although human specific bifidobacteria hold promise as indicators of diffuse faecal contamination, methodological constraints now limit its application to situations of gross contamination, or sampling potential sources during environmental conditions conducive to bifid persistence.

INTRODUCTION

Although sewage treatment facilities continue to be emphasized as the major source of faecal micro-organisms to natural waters, it has become increasingly apparent that diffuse sources, such as septic systems and stormwater run-off containing faecal pollutants from animals and humans, are significant contributors to deteriorated water quality (Crane and Moore 1986; O'Shea and Field 1992; Lévesque *et al*. 1993; Wyer *et al*. 1995; Anon. 1997). In the United States, shellfish harvesting areas are regulated based on faecal coliform densities and shoreline surveys of the surrounding watershed (Anon. 1995a). Thirty-one percent ($2{\cdot}7 \times 10^6$ ha) of United States shellfish growing areas are harvest limited, frequently from deteriorated water quality arising from sources other than wastewater treatment plants (Anon. 1997). In an attempt to identify pollution sources in shellfish areas closed for direct harvesting of shellfish because of elevated faecal coliforms, a number of candidate indicators were evaluated to distinguish human from animal faecal pollution (Kator and Rhodes 1991).

Bifidobacteria are a major component of the human intestinal microbiota, occurring at densities of $10^{9-10}\,g^{-1}$ (Levin and Resnick 1981; Kimura *et al*. 1997). Attributes of this group that promote its candidacy for distinguishing faecal sources are the relatively infrequent occurrence of bifidobacteria in animals (Resnick and Levin 1981b; Mara and Oragui 1983, 1985), generally low recovery from animals (Mara and Oragui 1983), and the existence of 'human' and 'animal' strains (Levin 1977; Levin and Resnick 1981; Gavini *et al*. 1991). Human bifid sorbitol agar (HBSA) was developed by Mara and Oragui (1983) specifically to enumerate human bifidobacteria on the basis of sorbitol fermentation by 'human'

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strains, such as *Bifidobacterium adolescentis* and *B. breve*, that together constitute approximately 58% (Resnick and Levin 1981b) to more than 90% (Mara and Oragui 1983, 1985) of human bifid isolates.

The absence of apparent extra-enteral growth, even in the presence of elevated nutrients, and sensitivity to oxygen are attributes promoting the use of bifids as indicators of faecal pollution in aquatic environments (Evison and Morgan 1978; Carillo *et al.* 1985). HBSA has been used to recover bifids from receiving waters grossly contaminated with sewage effluent (Wheater *et al.* 1979; Resnick and Levin 1981b; Mara and Oragui 1983), or stormwater run-off from human settlements with no, or limited, sanitary facilities (Mara and Oragui 1985; Jagals and Grabow 1996).

The purpose of the research described in this report was to evaluate sorbitol-fermenting bifidobacteria as source-specific indicators of faecal pollution. Three small sub-estuaries exhibiting excessive levels of faecal coliforms allegedly due to non-point pollution were chosen for this purpose. Bifidobacteria occurrence was examined with respect to temperature, precipitation, proximity to potential faecal sources, sediments, feral animal faeces, and methodological considerations.

MATERIALS AND METHODS

Study sites

Bonum, Taskinas and Timberneck creeks are small ($< 1\,km^2$) Virginia tidal creeks located on the lower Potomac, and middle and lower York rivers, respectively. Sampling sites were selected along a salinity gradient from the mouth of each creek to its headwater, and in feeder streams. Sampling locations were, in part, based on detailed sanitary surveys of the watershed prepared by the Division of Shellfish Sanitation, Office of Water Programs, Virginia Department of Health. Development around Bonum Creek is sparse and consists of only single dwellings. The area is primarily agricultural and some residents maintain small numbers of domestic livestock and fowl. Taskinas Creek mainstem, and feeder stream sites A, B and G, are located within protected areas of the National Estuarine Research Reserve Site (NERRS, National Oceanographic and Atmospheric Administration). Remaining Taskinas feeder stream sites are potentially impacted by farm animals (C–E) and/or were surrounded by residential developments (E–F). Timberneck Creek has the highest density of residences as well as areas of sparsely distributed single family homes and farming operations. All homes in each of the three sub-estuaries are served by on-site sewage facilities.

Sampling procedure

Samples were collected in sterile bottles by grab sampling at 0·3–1·0 m depth except in shallow streams where bottles were immersed immediately beneath the surface. Sampling bottles used to detect bifidobacteria were filled to capacity to exclude air and for Taskinas Creek surveys, sodium bisulphite and cysteine solutions were added to final concentrations of 0·01% and 0·05%, respectively, as quenching agents. Sediment samples were collected either using plastic core liners and extruding the upper 3 cm into sterile plastic bags, or using a spatula to remove surface sediment for transfer to sterile bags. Sediment homogenates were prepared by blending 20 g in 180 ml sterile estuarine (mainstem stations), or phosphate buffer dilution water (Anon. 1995b) (feeder stream stations), for 60–90 s. Animal scat deposits were collected with a clean spatula, placed in sterile plastic bags, and 1 : 10 homogenates (w/v, phosphate buffer dilution water) prepared by blending or stomaching. Ambient water temperature was measured at the time of sampling using either a calibrated thermometer or a Hydrolab H_2O sonde/Surveyor 3 system (Hydrolab Corp., Austin, TX, USA). Salinity was determined using either a temperature-compensated refractometer (American Optical Corp., Keane, NH, USA) or the Hydrolab sonde. Samples were stored at ambient field temperatures in insulated containers during sampling and transport to the laboratory, and were generally processed within 6 h after collection.

Faecal coliform enumeration

Faecal coliforms were enumerated using a five-tube MPN procedure (Anon. 1995b) with lactose broth (Difco) as the presumptive medium and EC medium (Difco) as the confirmatory medium. Verification of the presence of *Escherichia coli* in gassing EC tubes was determined by either biochemical characterization (Anon. 1995b) of isolates from selected tubes, or incorporation of methylumbelliferyl-β-D-glucuronide (Sigma) into EC medium (Feng and Hartman 1982).

Human bifidobacteria enumeration

Quadruplicate 25 ml or triplicate 10 ml and 1 ml aliquots of water samples, and sediment and animal scat dilutions representing 0·01 g and dilutions thereof, were processed by membrane filtration (Anon. 1995b). Filtration rinse and dilution water consisted of either gelatin diluent (Anon. 1968) containing 0·05% cysteine hydrochloride (Anon. 1977), or phosphate-buffered saline prepared using a stock buffer solution (Anon. 1995b) and containing 0·85% NaCl, 0·01% sodium bisulphite and 0·05% cysteine hydrochloride. Filters were incubated anaerobically (Oxoid Anaero*Gen* system, Unipath, Ogdensburg, NY, USA) on HBSA (Mara and Oragui 1983) for 4 h at 30 °C and subsequently incubated for 4–6 d at 37 °C. HBSA plates were examined for yellow raised 'domed' colonies and suspect colonies were examined microscopically for characteristic bifid cellular morphology. Colonies exhibiting 'typical' bifid cellular morphology were

transferred to agar plates to determine their requirement for anaerobic conditions. Counts were expressed as colony forming units 100 ml^{-1}.

Total bifidobacteria enumeration

Bifid sorbitol agar (BSA–PA) was prepared using HBSA without antimicrobial components, modified to contain either 0·5% sodium propionate or propionic acid, and adjusted to pH 5·0 (Beerens 1991). Water samples were filtered and processed as above except that all colony types were screened microscopically for bifid morphology.

Streptococcus bovis enumeration

Modified mBA (mmBA, Oragui and Mara 1984) was used for detection of *Streptococcus bovis*. Replicate volumes were filtered using PBS and filters incubated anaerobically as described above, resuscitated for 4 h at 30 °C, and subsequently incubated for 72 h at 39·5 °C. Selected typical yellow colonies were sub-cultured for purity and cultures characterized according to recommended methods (Knudtson and Hartman 1992; Anon. 1995b). Counts were expressed as colony forming units 100 ml^{-1}.

Microcosm studies

The effect of environmental exposure on recovery of bifidobacteria as described above, and using a two-step resuscitative procedure, was examined for a pure culture and a raw sewage inoculum. A reinforced clostridial broth (RCB) culture of *B. adolescentis* ATCC 15703, or a primary sewage sample, was diluted 500-fold or 10-fold, respectively, into 0·22 μm filtered fresh water from a eutrophic coastal plain lake (Rhodes and Kator 1994) and incubated at 15 °C. At selected time intervals, cell densities were determined by spread-plating or membrane filtration using reinforced clostridial agar (RCA) and BSA–PA. The two-step resuscitation procedure consisted of incubating membranes on RCA for 4 h at 37 °C prior to transferring filters to BSA–PA and incubating at 37 °C for 4–6 d. Mean colony counts were determined from triplicate plates for each dilution.

Persistence of *B. adolescentis* ATCC 15703 was also examined by inoculating duplicate flasks containing 0·22 μm filtered estuarine water of two salinities, 11 and 22 psu (practical salinity units), and incubating at 10, 23 and 30 °C. Cells were enumerated by spread-plating in triplicate onto brain heart infusion agar containing yeast extract (5 g l^{-1}), vitamin K (50 μl l^{-1}) and haemin (5 mg l^{-1}).

Statistical analyses

Correlation of bifidobacteria occurrence and environmental parameters was examined using Fisher's exact test (Zar 1984). Log-transformed count data were used to compare bifidobacteria recovery methods (Student's paired t-test) and to evaluate bifidobacteria persistence as a function of salinity and temperature (one factor ANCOVA) (Zar 1984).

RESULTS

Bacteriological data from mainstem and 26 feeder stream surveys are summarized in Table 1. Faecal coliforms in each sub-estuary were always dominated by *E. coli* and decreased with increasing salinity. Counts were highest (10^{2-3} FC 100 ml^{-1}) in headwaters and feeder streams. Human bifidobacteria were recovered from only 11 of 258 samples (4·3%) and detected only in feeder streams of Bonum and Timberneck creeks. Human bifids were recovered more frequently in the cooler months (Table 2) and never during June to September ($n = 7$). Of the eight sampling dates on which bifids were recovered, temperatures were below 15 °C (six dates) and rainfall occurred during the 3 day period preceding sample collection (four dates). Statistically, there was no significant correlation of human bifidobacteria in feeder streams with either temperatures below 15 °C (Fisher's exact test, $P > 0{\cdot}05$, $n = 18$) or cumulative rainfall over the 3 day period preceding sampling (Fischer's exact test, $P > 0{\cdot}05$, $n = 18$).

During the course of the field studies, a method was added for enumeration of total bifids. Thus, samples from the Taskinas Creek watershed were also analysed for total bifidobacteria as described by modifying the procedure to detect sorbitol-fermenting bifids. As parallel enumerations using BSA–PA prepared with sodium propionate or propionic acid showed similar results for sewage samples (Student's t, $P > 0{\cdot}05$, $n = 7$), this medium was prepared with propionate which is less toxic and corrosive than the acid.

Prior to field use of modified BSA–PA, the potential effect of a resuscitation step to improve bifidobacteria recovery was evaluated by exposing bifidobacteria in sewage, or a pure culture of *B. adolescentis*, to membrane (0·22 μm)-filtered fresh water at 15 °C. After 2–4 weeks of exposure, bifidobacteria counts had declined by three to four orders of magnitude but were still recoverable. Recovery was not increased using the two-step resuscitation method described ($P > 0{\cdot}05$, $n = 8$) (Table 3), although cells appeared stressed because of lower counts on spread plates where cells were directly exposed to the selective medium.

Use of BSA–PA yielded infrequent recoveries of bifids in water samples from Taskinas Creek (5/29) and its feeder streams (4/56). Positive samples containing 1–12 bifids 100 ml^{-1} were recovered on only three occasions, each fol-

Table 1 Occurrence of human bifidobacteria in three sub-estuaries and associated feeder streams

Subestuary	Location	Salinity (psu)	Mean faecal coliform MPN 100 ml^{-1}	Human bifid occurrence*
Bonum	Downstream	12	4 (11)†	0/11
	Midstream	10	38 (11)	0/11
	Upstream	7	130 (10)	0/10
	Feeder streams:			
	A	0	210 (11)	0/10
	B	<1	82 (11)	1/10
	C	0	480 (7)	2/8
	D	0	140 (11)	3/10
	E	<1	110 (11)	2/11
Taskinas	Downstream	11	61 (10)	0/8
	Midstream	6	570 (10)	0/10
	Upstream	2	760 (17)	0/17
	Feeder streams:			
	A	0	240 (8)	0/7
	B	0	160 (8)	0/7
	C	0	710 (8)	0/7
	D	0	320 (8)	0/8
	E	0	180 (8)	0/8
	F	0	700 (8)	0/8
	G	0	210 (8)	0/8
Timberneck	Downstream	19	4 (9)	0/9
	Midstream	18	61 (29)	0/29
	Upstream	17	120 (8)	0/8
	Feeder streams:			
	A	0	340 (6)	1/6
	B	<1	280 (6)	0/6
	C1	0	200 (6)	1/6
	C2	14	590 (4)	0/4
	D	0	360 (6)	0/6
	E	0	1500 (6)	1/6
	F	0	2200 (5)	0/5
	G	0	110 (4)	0/4
Total no. water samples			265	258

* Sorbitol-fermenting bifidobacteria enumerated on human bifid sorbitol agar (Mara and Oragui 1983).
† Number of samples in parentheses.

lowing rainfall (3 day cumulative rainfall = 0·8–3·0 cm) and at water temperatures of 4–17 °C.

Sediments collected from 30 estuarine and 38 feeder stream samples from Timberneck Creek were negative for human bifidobacteria. Deer, muskrat and raccoon scat samples were also negative for sorbitol-fermenting bifids but bifidobacteria were recovered on BSA–PA from muskrat faeces (Table 4).

Analyses of environmental samples for either bifid group using HBSA or BSA–PA were problematic because of high densities of non-target bacterial colonies. Interfering background densities ranged from 10^{2-3} 100 ml^{-1} water to 10^{3-4} and 10^{4-6} g^{-1} sediment and animal scat, respectively. A variety of non-target colonies developed that included both bacilli and cocci. Scat samples with high numbers of *Strep. bovis* on mmBA (Oragui and Mara 1984) had similar recoveries of non-target coccoid bacteria on HBSA and BSA–PA that were identified as *Strep. bovis*.

Microcosm studies demonstrated that *B. adolescentis* per-

Table 2 Seasonal meteorological conditions during independent feeder stream surveys when human bifidobacteria were recovered

Location	Survey month	Bifid detection frequency	Temperature (°C)	Cumulative rainfall (cm) 3 days prior to sampling
Bonum	February	1/3	5	0
	February	2/6	4	4·8
	April	1/7	14	0·5
	May	2/8	18	2·8
	November	1/8	10	0·0
	February	1/8	12	0·0
Timberneck	February	2/5	10	0·0
	May	1/6	22	0·8

Table 3 Comparison of methods for enumerating bifidobacteria with or without resuscitation from membrane filtered (0·22 μm) fresh water inoculated with either sewage or a pure culture of *Bifidobacterium adolescentis* and incubated in vitro at 15 °C*

		cfu ml^{-1} on BSA–PA		
			Membrane filtration	
Bifid inoculum	Exposure time (days)	Spread-plating	Resuscitated	Not resuscitated
Sewage	0	2·0E04	2·7E04	2·5E04
	1	1·8E04	1·7E04	1·6E04
	6	9·0E01	2·2E02	1·5E02
	20	nd†	1·4E01	9·3E00
	29	nd	1·1E01	8·3E00
Pure culture	0	1·0E05	2·1E05	2·2E05
	7	6·8E02	2·5E04	6·7E04
	16	$<$1·0E01	1·0E01	2·8E02

* Bifidobacteria enumerated on BSA–PA by direct spread-plating or by membrane filtration with and without resuscitation on RCA for 4 h at 37 °C prior to transfer to BSA–PA. Triplicate plate counts prepared for each dilution.
† nd, Not determined.

sisted in filtered estuarine water for 4 weeks at 10 °C but was unrecoverable after 5–9 d at 23 or 30 °C (Fig. 1). Persistence at both 11 and 22 psu was significantly affected by temperature (ANCOVA, $P < 0{\cdot}001$). Although survival appeared to be greater at 11 than at 22 psu, these differences were not significant (ANCOVA, $P > 0{\cdot}05$).

DISCUSSION

Use of bifidobacteria as indicators of human faecal pollution has been validated in field studies involving frank and significant inputs (Wheater *et al.* 1979; Levin and Resnick 1981; Resnick and Levin 1981b; Mara and Oragui 1983, 1985; Carillo *et al.* 1985; Jagals and Grabow 1996) but has not been evaluated under conditions where pollution originates from diffuse or non-point sources. In the present study, detection of sorbitol-fermenting bifids in feeder streams leading to estuarine systems was interpreted as indicative of human faecal contamination. The National Shellfish Sanitation Program (Anon. 1995a) requires that detailed sanitary surveys be conducted of the watershed surrounding shellfish harvesting areas. During surveys of both Bonum and Timberneck creek watersheds, Virginia Department of Health personnel identified violations that included malfunctioning septic systems,

Table 4 Densities of faecal indicator bacteria in feral animal faeces from the Taskinas Reserve watershed and tidal creek

	Mean indicator count g^{-1} faeces			
			Bifidobacterium spp.	
Animal*	*Escherichia coli*	*Streptococcus bovis*	BSA–PA	HBSA
Deer	$\leqslant 10^2$ ($n = 2$)†	1×10^6 ($n = 10$)	I‡ ($n = 10$)	I ($n = 10$)
Muskrat	$3{\cdot}4 \times 10^5$ ($n = 6$)	$< 1 \times 10^3$ ($n = 5$)	nd§	$< 1 \times 10^4$¶ ($n = 5$)
Raccoon	1×10^9 ($n = 3$)	$> 1 \times 10^6$ ($n = 5$)	I ($n = 5$)	I ($n = 5$)

* Deer, *Odocoileus virginianus*; muskrat, *Ondatra zibethicus*; and raccoon, *Procyon lotor*.
† Number of independent samples in parentheses.
‡ Interference by high densities of non-target colonies.
§ Not determined.
¶ Mean of 10^4 non-sorbitol-fermenting bifidobacteria were recovered on HBSA.

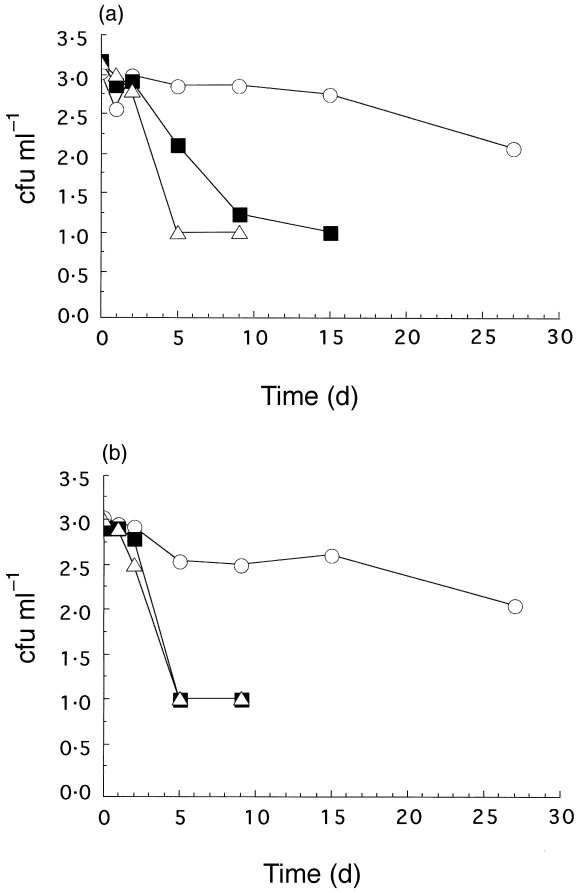

Fig. 1 Survival of *Bifidobacterium adolescentis* in membrane-filtered estuarine water as a function of temperature and salinity. (a) 11 psu; (b) 22 psu. (○), 10 °C; (■), 23 °C; (△), 30 °C

full latrines or lack of facilities adjacent to implicated streams. In contrast, no sanitary system violations were noted proximate to Taskinas Creek. Diffuse pollution from individual wastewater treatment systems allegedly contributes to 32% ($0{\cdot}9 \times 10^6$ ha) of shellfish harvest area limitations in the United States (Anon. 1997). Although the sorbitol-fermenting bifidobacteria are a promising tool for detecting human faecal pollution and possibly for identifying source location, its seasonal persistence characteristics and media specificity problems argue against routine use for detecting small diffuse inputs.

In the present study, low frequency of detection and non-recovery from warm waters and estuarine sites implied that bifid persistence is limited in the aquatic environment. Field data indicating a better likelihood of bifid recovery at cool water temperatures was supported by microcosm experiments showing enhanced bifid persistence at low temperature. Survival at 23 and 30 °C was markedly reduced compared with that at 10 °C, and bifid persistence at the latter temperature continued for weeks. In contrast, Levin and Resnick (1981) reported rapid die-off measured in hours for bifidobacteria in sterile fresh and marine water. These observations may be method-related because the recovery medium, YN-6 (Levin and Resnick 1981), is inhibitory to some bifids (Mara and Oragui 1983). Enhanced survival at lower temperatures is in agreement with previous findings for fresh and estuarine waters (Kator and Rhodes 1991). However, the extended persistence observed under *in vitro* conditions would be reduced in the natural environment where additional factors (predation, antimicrobial substances, sunlight etc.) could contribute to die-off (Kator and Rhodes 1991).

High levels of background bacteria were problematic for

detection of bifidobacteria in water, faecal and sediment samples. Although colonial morphology facilitates distinguishing bifids from other bacteria that grow on media designed for detection of bifidobacteria, i.e. bacteroides, clostridia, eubacteria, lactococci, lactobacilli, enterococci, streptococci (Resnick and Levin 1981a; Shah 1989; Hartemink *et al*. 1996; Silvi *et al*. 1996), the possibility remains that high densities of non-target bacteria could prevent growth and/or detection of bifids. When detected, bifid densities in water samples were at much lower concentrations than the background microbiota. Beerens agar (1990) is reported to be comparatively selective and elective as tested with pure cultures of a variety of intestinal bacterial genera or human and rat faeces (Silvi *et al*. 1996). In this study, HBSA (Mara and Oragui 1983) was modified as proposed by Beerens (1990) by substituting a combination of low pH and propionic acid as selective agents. It was anticipated that if the modified medium, BSA–PA, was successful in inhibiting background growth, membranes could be neutralized on a buffer-saturated adsorbent pad prior to transfer to HBSA for *in situ* determination of sorbitol fermentation. However, the non-selectivity of the medium precluded use of this two-step approach.

Munoa and Pares (1988) demonstrated that the inability of *Bifidobacterium* spp. to produce colonies on a selective medium was the result of sub-lethal injury following exposure to sea water. A two-layer recovery procedure was developed incorporating resuscitation on a non-selective medium followed by an overlay of a selective agar. Previously, it had been noted that enumeration techniques could exacerbate sub-lethal stress and produce reduced counts of *B. adolescentis* (Kator and Rhodes 1991). However, in the present study, a two-step procedure using a resuscitation step on a non-selective agar before exposure of membranes to BSA–PA did not enhance recovery. Additional studies are needed to evaluate other procedural modifications, e.g. reduction of oxygen toxicity, that could improve recovery of stressed cells.

Although bifidobacteria occur in some domestic and feral animal faeces (Evison and Morgan 1978; Mara and Oragui 1983, 1985; Beerens 1991), sorbitol-fermenting bifids are reported to occur almost exclusively in humans with the exception of pig isolates (Resnick and Levin 1981b) that do not to grow on HBSA (Mara and Oragui 1983). Sorbitol-fermenting bifids were not recovered from numerous faecal samples of feral deer, raccoon and muskrat, major components of wild animal populations in the eastern coastal United States. Unequivocal exclusion of sorbitol-fermenting bifids from the microbiota of these animals is not possible because of the high levels of background growth encountered.

Observations of bifidobacteria accumulation in sediments subject to chronic faecal pollution (Wheater *et al*. 1979) suggest that particle association and reduced oxygen could promote bifid survival. Considering the relatively small and transient loading sources, physical processes contributing to indicator reduction (dilution, adsorption and sedimentation), and limited persistence characteristics, the absence of bifidobacteria in sediments and its infrequent recovery at low densities in water samples is not surprising.

If the sorbitol-fermenting bifidobacteria are to have utility as indicators of human faecal contamination in aquatic environments impacted by diffuse pollution sources, the sensitivity and selectivity of contemporary detection methods must be improved. New methods involving gene probes (Yamamoto *et al*. 1992; Yaeshima *et al*. 1996; Kaneko and Kurihara 1997), colorimetric detection of bifid colonies based on enzymatic activities (Chevalier *et al*. 1991; Roy and Ward 1992; Hartemink *et al*. 1996) and new selective agars (Hartemink *et al*. 1996; Pacher and Kneifel 1996) may hold promise with respect to environmental samples.

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