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Microarray method to monitor 40 intestinal bacterial species in the study of azo dye reduction

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Abstract

Azo dyes are widely used in dye manufacturing, paper printing, textile industries, and as tattoo pigmentation. Since intestinal and skin bacteria can metabolize certain azo dyes to carcinogenic compounds, many researchers have studied the azoreductases of these bacteria. In this study, we used a microarray method to identify the intestinal bacterial species from cultured fecal samples in Brain Heart Infusion (BHI) broth with or without azo dyes that may be involved in azo dye reduction. The microarray was designed to identify 40 bacterial species that are reported in the literature to be predominant in human feces. Results from this study showed 26–30 species are present in the cultured fecal samples. The representative bacteria were then examined for the azo dye reduction activity.

Keywords

Microarray; Human intestinal microflora; Azo dye reduction

1. Introduction

Azo dyes are compounds consisting of a diazotized amine coupled to an amine or a phenol containing one or more azo linkages. There are at least 3000 azo dyes used in food, pharmaceutical, and other industries (Chung et al., 1992). Some azo dyes have also been used in tattoo pigmentation (Baumler et al., 2000). Previously studies have shown that some intestinal and skin bacteria can metabolize certain azo dyes to carcinogenic compounds (Chung et al., 1992; Cerniglia et al., 1982; Rafii et al., 1992). However, only a limited number of bacterial species were studied individually for the presence of azoreductases. Early studies led us to ask several questions: what effects do azo dyes have on the intestinal microflora? How many intestinal bacterial species are involved in azoreduction? Since we cannot use human subjects for the in vivo study, human fecal samples were used to test the azoreductase activity in a complex intestinal bacterial mixture and a microarray method was used to identify the bacterial species present in the cultured fecal samples.

Intestinal bacteria are important for human health because they can digest dietary components, metabolize xenobiotics and drugs, and provide short chain fatty acids and

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vitamins that are later absorbed into the system (Cerniglia et al., 1984; Chadwick et al., 1992). The intestinal population also acts as a barrier against colonization of the gastrointestinal tract by pathogenic bacteria (Vollaard and Clasener, 1994). The human gastrointestinal tract contains over 10^{11} bacterial cells per gram of fecal content consisting of more than 400 bacterial species (Carman et al., 1993; Drasar and Duerden, 1991; Moore and Holdeman, 1974; Cerniglia and Kotarski, 1999; Drasar and Roberts, 1990; Moore and Moore, 1995). The predominant intestinal bacteria are *Bacteroides* spp., *Clostridium* spp., *Eubacterium* spp., *Bifidobacterium* spp., *Fusobactertium* spp., *Ruminococcus* spp., *Enterococcus* spp., and *Peptostreptococcus* spp.

Traditionally, the human intestinal bacteria population was characterized by microscopic, biochemical, physiological, and culture methods (Drasar and Roberts, 1990; Moore and Moore, 1995). Culture methods are time consuming and many intestinal bacteria are not easily cultured or isolated from the complex mixture. In recent years, many molecular methods have been used for identification of intestinal bacteria; however, many of these methods are group-specific for bacteria, but not species-specific (Langendijk et al., 1995; Harmsen et al., 2002; Kageyama and Benno, 2001; Jansen et al., 1999; Matsuki et al., 2002; Sghir et al., 2000). The complex microflora of the human gut is difficult to study with only species-specific primers because of the diversity of this ecosystem (Matsuki et al., 2002). Previously, we developed polymerase chain reaction (PCR) methods for the detection and quantitation of anaerobic bacteria in human and animal feces (Wang et al., 1994, 1996, 1997). A limitation of this methodology was that each bacterial species was assayed separately.

Microarray technology is a powerful tool used for the simultaneous detection of thousands of genes or target DNA sequences on one glass slide (Chizhikov et al., 2001; Wu et al., 2001). Microarrays were initially used for gene expression studies. However, microarrays can also be used for the detection of bacteria or for performing DNA-based typing of specific pathogenic bacterial strains (Chizhikov et al., 2001; Call et al., 2001; Wang et al., 2002a,b). We developed an oligonucleotide microarray method on the species-level for detecting and identifying 40 intestinal bacterial species on one slide (Wang et al., 2003). In this study, we used this microarray method to identify the intestinal bacterial species involved in azo dye reduction.

2. Materials and methods

2.1. Source of bacterial strains and culture conditions

Reference strains for 40 human intestinal bacterial species (Table 1) were obtained from American Type Culture Collection (ATCC). Anaerobic bacteria were cultured at 35 °C in either prereduced anaerobically sterilized (PRAS) Brain Heart Infusion (BIH) broth supplemented with vitamin K and hemin (Remel, Lenexa, KS, USA), inoculated under an oxygen-free cannula using 85% nitrogen, 10% hydrogen and 5% carbon dioxide, or on PRAS brucella blood agar plates supplemented with vitamin K and hemin (Remel).

2.2. Microarray method

The assay was previously described by Wang et al. (2003). Briefly, the method was developed to detect and identify 40 common intestinal bacterial species. These 40 species were reported to be predominant in the human gastrointestinal tract, primarily using on culture methods (Carman et al., 1993; Drasar and Duerden, 1991; Moore and Holdeman, 1974; Cerniglia and Kotarski, 1999; Drasar and Roberts, 1990; Moore and Moore, 1995). Three 40-mer oligos specific for each bacterial species (a total of 120 probes) were designed based on a comparison of the 16S rRNA gene sequences available in the GenBank database. The oligo DNA-array was made on epoxy slides (MWG-biotech, High Point, NC, USA). The 120 oligonucleotide probe numbers and the corresponding bacterial species are listed in Table 1. Using two universal primers (Amp-F and Amp-R), the 16S rRNA gene from all bacterial DNA isolated from fecal samples was amplified and labeled with Cyanine5-dCTP by PCR.

Method for PCR amplification of cyanine5 (CY5)-labeled 16S rDNA: 25 μl of PCR mixture was made by combining 15.6μl of water; 2.5 μl of 10 × BSA-buffer (1 ml 10 × buffer is composed of 0.5 ml 1M Tris–HCl, pH 8.5, 0.2 ml 1M KCl, 30μl of 1M $mgCl_2$, 0.27 ml of water, BSA 5 mg); 2.3μl of dNTP (2.5mM each of dATP, dTTP, dGTP, and 1.7mM of dCTP, Invitrogen, Carlsbad, CA); 1.2 μl of 1mM of CY5-dCTP (Perkin-Elmer Life and Analytical Sciences, Boston, MA, USA); 1.2 μl of primers Amp-F and Amp-R (50 ng/μl each); 0.3 μl of Taq DNA polymerase (5 unit/μl, Invitrogen, Carlsbad, CA); and 2 μl of bacterial DNA or fecal DNA (1–10 ng/μl). The Amp-F and Amp-R primer sequences are GAGAGTTTGATYCTGGCTCAG and AAGGAGGTGATCCARCCGCA, respectively (Y is C or T; R is A or G). PCR was performed in a 9700 GeneAmp PCR System (Perkin-Elmer Life and Analytical Sciences, Boston, MA, USA), using thin-walled 0.2 ml tubes. The amplification conditions were incubation at 95 °C for 3 min, then 35 cycles of 95 °C for 10 s, 53 °C for 10 s, and 72 °C for 70 s, followed by one cycle at 72 °C for 4 min and a cool down to 4 °C.

The PCR product was purified with a Centri-Spin column (Princeton Separations, Adelphia, NJ, USA) following the manufacturer instructions. The purified CY5-labeled PCR products were dried by Speed-Vac centrifugation (Savant, Farmingdale, NY, USA) and dissolved in 13 μl of hybridization buffer (MWG-biotech). The tube was heated for 3 min in a boiling water bath, then immediately placed into ice-water for 2 min. The solution was collected by brief centrifugation and applied onto the microarray. A small glass cover slip that was autoclaved and dried was used to cover the hybridization solution on the array area. The slide was placed into a hybridization chamber (Corning Inc., Corning, NY, USA) and then immersed in a water bath for hybridization overnight at 42 °C. After hybridization, the cover slip was removed by washing the slides for 5 min with 0.5 × SSC, 0.1% SDS. The slides were then washed for 5 min with 0.1 × SSC, 0.1% SDS, followed by a 5 min wash with 0.1 × SSC only. All of the washing steps were conducted at room temperature. The slides were dried by centrifugation for 1 min at 3000 × *g* in an IEC clinical centrifuge with IEC CAT 801 rotor (International Equipment Company, Needham Heights, MA, USA) and scanned with the ScanArray Express Microarray Scanner (Packard BioScience-Perkin-Elmer, USA). Potential cross-reactivity problems for the species-specific probes were resolved by

designing three probes for each species. Positive reactions for at least two out of the three probes for a species were required to consider an identification of the species as positive.

2.3. Human fecal culture for the azo dye reduction study

Brain Heart Infusion broth (100 ml) with a stirrer in a 500 ml beaker was autoclaved and placed into an anaerobic hood for prereducing anaerobically. Fresh human fecal sample (10 g) was added into this 100 ml BHI broth, and then stirred until completely mixed in the anaerobic hood. Five milliliter each of the mixed BHI-fecal sample was aliquoted into 15 ml sterile tubes, then 0.17 ml of sterile azo dyes (10mM of Direct Blue 15, 50mM of Orange II or 50mM of Methyl Red) or 0.17 ml of control (distilled water) was added into different tubes. The tubes were incubated at 37 °C in an anaerobic hood for 16 h without shaking. One milliliter of the upper phase from each tube were transferred into 1.5 ml tubes and centrifuged at 14,000 × *g* for 10 min. The supernatant was used to measure the azo dye reduction as described below. The pellet was washed with 1ml of 0.85% NaCl and used for isolation of the genomic DNA by using the Easy-DNA kit (Invitrogen, Carlsbad, CA, USA) as described in the earlier publications (Wang et al., 2002b, 2003). The DNA was used for PCR amplification and labeling with Cynine5-dCTP. The labeled PCR products were used to hybridize with the oligo-array on epoxy slides.

For azo dye reduction determination, 0.2 ml each of the supernatant was diluted with 1.8 ml water (1:10). However, for the Methyl Red detection, 0.2 ml of the supernatant was diluted with 1.6 ml water and 0.2 ml of 100% acetic acid. The samples were measured using appropriate wavelengths in a Hewlett-Packard 8453 UV-Visible Spectrophotometer (A_{601} for Direct Blue 15; A_{482} for Orange II; A_{430} for Methyl Red) with 1:10 water diluted supernatant from BHI-feces-water as a blank.

2.4. Examination of azo dye reductive activity by different bacterial pure cultures

Seventeen bacterial species that were strong positive in microarray test were examined for azo dye reduction activity by pure culture of ATCC strains. Each species was inoculated into 5ml BHI broth supplemented with vitamin K and hemin (*Ruminococcus* and *Eubacterium* spp. were cultured with additional Rumen Fluid supplements) under an oxygen-free cannula (85% N_2, 10% H_2 and 5% CO_2). The cultures were incubated at 37 °C for 17 h or 48 h until visible growth of the bacteria were observed. An azo dye solution of Direct Blue 15 (3.3 mg/ml in water), sterilized by using a 0.2 μm syringe filter (Gelman Sciences, Ann Arbor, MI, USA), was added for 0.5 ml each into the culture tubes under anaerobic conditions. The cultures were incubated at 37 °C for 24 h. One ml of the mixture from each tube was transferred into 1.5 ml tubes and centrifuged at 14,000 × *g* for 10 min. The supernatant was diluted (1:10) and assayed for azo dye reduction by measuring the A_{601} using 1:10 diluted PRAS BHI broth as a blank.

3. Results and discussion

After 16 h incubation of the mixtures of the BHI-fecal sample-azo dyes, the blue, orange, or red color from three different azo dyes (Direct Blue 15, Orange II and Methyl Red) completely disappeared. Table 2 shows the results of azo dye reduction. The concentration

of the individual dye was totally reduced, but the fecal sample had some background absorbance for Orange II and Methyl Red samples. To determine which bacterial species in the fecal culture were the major sources of the azoreductase, two methods were used in this study: first, we used the microarray method to determine which bacterial species were present in the cultured fecal samples. Then, we examined the potential of azo dye reduction activity using pure cultures of the selected bacterial species that showed strong positive microarray results.

The microarray method was used to determine the bacterial species in original fecal and cultured fecal samples with or without azo dyes. The fecal cultures were incubated without shaking; therefore the upper phase should contain most of the actively growing bacteria. Fig. 1 shows the microarray test results. To read this figure, one should compare Table 1 for probe number and the corresponding bacterial species. For example, since three separate probes were used for each bacterial species, so four different species are distributed in the 12 spaces across top row, with species number 1–4 planted on the top row. Table 3 is the result read from Fig. 1. Comparing un-cultured or cultured fecal sample, the only difference is that after culture, *Escherichia coli* and *Enterococcus faecalis* became weakly positive from negative. This indicated that these two species were enriched under these culture conditions and present in lower numbers in the original fecal sample. We calculated the dilution factor as described below: 10 g feces was placed into 100 ml medium (1:11), then 1ml was used for isolation of DNA. The DNA (total 200 μl) was 1:10 diluted and 2μl of the dilution was used for PCR-microaray (1:1000). So the total dilution folds are 1.1×10^4. The minimum cell number for positive microarray result is approximately 10 cells (Wang et al., 2003). Therefore, if the original cell number for certain bacterial species in feces are more than 1.1×10^5 per gram fecal sample (wet weight), that species can give a positive microarray result. The predominant anaerobic bacterial species in feces are more than 10^9 cells per gram feces (Carman et al., 1993; Drasar and Duerden, 1991; Moore and Holdeman, 1974), hence many bacterial species gave very strong positive microarray test results. However, the initial published literature based the predominant intestinal bacterial species on-culture results, therefore easy to grow bacterial species may be reported as predominant but their actual cell numbers in fecal sample might be low. The microarray method should be a more accurate representation of the predominant fecal microflora.

It is possible that Direct Blue 15 and Orange II may have some toxicity to some bacterial species, because the microarray test results showed only 26 and 27 positive species, respectively, but the culture-control and culture with Methyl Red have 30 species positive each (Table 3). However, this did not affect the overall azo dye reduction because the colors from these two dyes were also completely disappeared.

To determine what species are the major sources of azoreductase activity, 17 selected bacterial species that showed strong positive microarray results were examined for azo dye reduction activity by pure culture of the selected ATCC strains incubated with Direct Blue 15 (Table 4). Among these 17 species, *Clostridium perfringens, Clostridium clostridioforme, Enterococcus faecalis, Ruminococcus obeum,* and *Bifidobacterium adolescentis* showed the highest azo dye reduction activity. In a separate study, we have cloned, over-expressed, and purified the azoreductase from *E. faecalis* (unpublished data), which confirms that this

bacterial species may play an important role for the azo reduction in the gastrointestinal tract.

Bacteria can lose or gain genetic materials mostly via plasmids. The azoreductase genes are not located on plasmids but are chromosomal. Isolates from fecal samples were difficult to identify that behave identical to an ATCC strain, even at the species level. So, it is valid at least partly to demonstrate biological activity in a mix of wild bacterial strains found in a clinical fecal samples then switch to pure ATCC cultures. The experimental results demonstrated which species are most prevalent in G–I tract and most likely have the azoreductase gene. Then separate studies can be continued to clone, isolate or characterize the azoreductase gene. One example is that we have already cloned, characterized and purified the azoreductase from *E. faecalis* ATCC 19433.

This study is an example of the application of microarray method for detection and identification of bacteria in azo dye research. Actually, the microarray method we developed has many more potential applications; for example, the method could be used for examining intestinal bacterial species in various patients clinically treated for intestinal diseases and for experimental animal studies to determine the effect of food additives, antimicrobial residues, and xenobiotics on the intestinal microflora.

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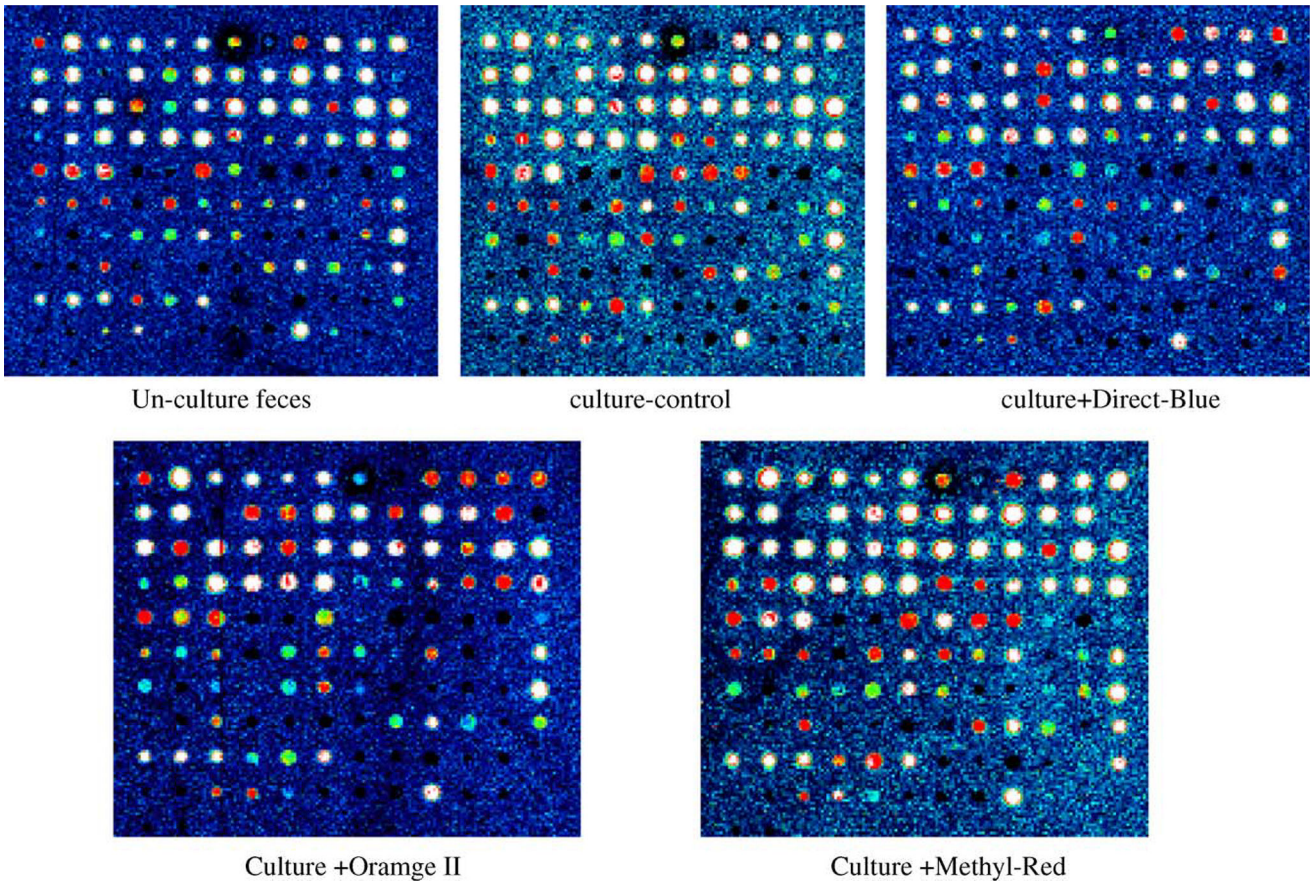

Fig. 1.

Microarray test results for cultured feces with azo dyes and the same control fecal sample without culture (un-culture feces) or after culture (culture-control). The position of the oligo probes on the array from top-left to the right-bottom are 1–120, 12 per lane total 10 lanes. The probe number and the corresponding bacterial species are listed in Table 1. The positive signal intensities from strongest to weakest are white, red, yellow, green and blue (see web version for color picture).

Table 1

Bacteria and the probe numbers in the microarray

Number	Bacterial species and strain	Probe number
1	*B. thetaiotaomicron* ATCC 29148	1, 2, 3
2	*B. vulgatus* ATCC 8482	4, 5, 6
3	*B. fragilis* ATCC 23745	7, 8, 9
4	*B. distasonis* ATCC 8503	10, 11, 12
5	*C. clostridioforme* ATCC 29084	13, 14, 15
6	*C. leptum* ATCC 29065	16, 17, 18
7	*F. prausnitzii* ATCC 27768	19, 20, 21
8	*P. productus* ATCC 27340	22, 23, 24
9	*R. obeum* ATCC 29174	25, 26, 27
10	*R. bromii* ATCC 27255	28, 29, 30
11	*R. callidus* ATCC 27760	31, 32, 33
12	*R. albus* ATCC 27210	34, 35, 36
13	*B. longum* ATCC 15707	37, 38, 39
14	*B. adolescentis* ATCC 15703	40, 41, 42
15	*B. infantis* ATCC 15697	43, 44, 45
16	*E. biforme* ATCC 27806	46, 47, 48
17	*E. aerofaciens* ATCC 25986	49, 50, 51
18	*L. acidophilus* ATCC 4356	52, 53, 54
19	*E. coli* ATCC 25922	55, 56, 57
20	*E. faecium* ATCC 19434	58, 59, 60
21	*B. uniformis* ATCC 8492	61, 62, 63
22	*B. ovatus* ATCC 8483	64, 65, 66
23	*B. caccae* ATCC 43185	67, 68, 69
24	*C. perfringens* ATCC 13124	70, 71, 72
25	*C. butyricum* ATCC 19398	73, 74, 75
26	*C. ramosum* ATCC 25582	76, 77, 78
27	*C. difficile* ATCC 9689	79, 80, 81
28	*C. indolis* ATCC 25771	82, 83, 84
29	*F. russii* ATCC 25533	85, 86, 87
30	*F. nucleatum* ATCC 25586	88, 89, 90
31	*B. catenulatum* ATCC 27539	91, 92, 93
32	*B. angulatum* ATCC 27535	94, 95, 96
33	*E. rectale* ATCC 33656	97, 98, 99
34	*E. eligens* ATCC 27750	100, 101, 102
35	*E. limosum* ATCC 8486	103, 104, 105
36	*E. lentum* ATCC 25553	106, 107, 108
37	*L. fermentum* ATCC 9338	109, 110, 111
38	*E. faecalis* ATCC 27274	112, 113, 114
39	*P. magnus* ATCC 14955	115, 116, 117

Number	Bacterial species and strain	Probe number
40	*R. gnavus* ATCC 291492	118, 119, 120

Table 2

Azo dye reduction of cultured fecal sample, $n = 1$ (the remained absorbance was background of fecal sample at this wavelengths)

	Before culture (0 h)	After culture (16 h)
A_{601} for Direct-Blue 15	1.364	0.051
A_{482} for Orange II	1.912	0.120
A_{430} for Methyl Red	1.596	0.216

Table 3

Microarray test results read from Fig. 1

Bacterial species	Un-culture feces	Culture-control	Culture + Direct-Blue	Culture + Orange II	Culture + Methyl Red
B. thetaiotaomicron	+	+	+	+	+
B. vulgatus	+	+	+	+	+
B. fragilis	+	+	+	+	+
B. distasonis	+	+	+	+	+
B. uniformis	+	+	+	+	+
B. ovatus	+	+	+	+	+
B. caccae	+	+	+	+	+
C. clostridioforme	+	+	+	+	+
C. leptum	+	+	+	+	+
C. perfringens	+	+	+	−	+
C. butyricum	+	+	−	+	+
C. ramosum	+	+	+	+	+
C. difficile	−	−	−	−	−
C. indolis	+	+	−	−	+
R. obeum	+	+	+	+	+
R. bromii	+	+	+	+	+
R. callidus	+	+	+	+	+
R. albus	+	+	+	+	+
R. gnavus	−	−	−	−	−
B. longum	+	+	+	+	+
B. adolescentis	+	+	+	+	+
B. infantis	+	+	+	+	+
B. catenulatum	+	+	+	+	+
B. angulatum	+	+	+	+	+
E. biforme	+	+	+	+	+
E. aerofaciens	+	+	+	+	+
E. rectale	+	+	+	+	+
E. eligens	+	+	+	+	+

Bacterial species	Un-culture feces	Culture-control	Culture + Direct-Blue	Culture + Orange II	Culture + Methyl Red
E. limosum	–	–	–	–	–
E. lentum	–	–	–	–	–
F. prausnitzii	+	+	+	+	+
F. russii	–	–	–	–	–
F. nucleatum	–	–	–	–	–
P. productus	+	+	+	+	+
P. magnus	–	–	–	–	–
L. acidophilus	–	–	–	–	–
L. fermentum	–	–	–	–	–
E. faecium	–	–	–	–	–
E. faecalis	–	+	–	+	+
E. coli	–	+	–	–	+
Total (+)	28	30	26	27	30

Table 4

Azo dye (Direct Blue 15) reduction activity of 17 bacterial species in pure culture

Bacterial species	Remained percentage after azo dye (Direct Blue 15) reduction (%)
C. perfringens ATCC 3624	2.3
C. clostridioforme ATCC 25537	4.1
C. leptum ATCC 29065	49.5
C. ramosum ATCC 25582	68.6
B. vulgatus ATCC 8482	56.2
B. distasonis ATCC 8503	70.7
B. thetaiotaomicron ATCC 29148	62.2
B. uniformis ATCC 8503	75.3
B. longum ATCC 15707	70.6
B. adolescentis ATCC 15703	15.6
E. biforme ATCC 27806	62.1
E. aerofaciens ATCC 25986	70.8
E. eligens ATCC 27750	75.6
P. productus ATCC 27340	72.8
R. obeum ATCC 29174	8.2
R. bromii ATCC 27255	49.8
E. faecalis ATCC 19433	7.7