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Antimicrobial Susceptibility and Plasmid Profile of Bifidobacteria Isolated from Infant Stool

Yazid A.M[1*], M. Shuhaimi[1], A.M. Ali[2], A.R. Raha[2], R.R. Ernie Eileen[2], H. Rowina[2] and N.A.N. Abdullah[3]

[1]Department of Food Technology, [2]Department of Biotechnology, Universiti Putra Malaysia, 43400 UPM Serdang, Malaysia, [3]Paediatric Institute, Hospital Kuala Lumpur, Jalan Pahang, 50586 Kuala Lumpur, Malaysia.

Abstract

A total of 8 bifidobacteria strains were isolated from faeces of breast-fed infants. They were identified as *Bifidobacterium breve* (2 strains) and *B. infantis* (6 strains). Almost all strains were resistant to amino glycoside antibiotics, but sensitive to chloramphenicol, nitrofurantoin and erythromycin. Susceptibility to other antimicrobial was varied. Four strains shown to have plasmids DNA with band size 50, 8 and 2.3 kb.

Introduction

Bifidobacteria are unique group of bacteria. They are non-motile, non-spore forming, and Gram-positive with the vegetative cells branching in y or v shapes during proliferation (Mitsuoka, 1984). These organisms play a significant role in controlling acidity of the large intestinal tract. Bifidobacteria are known to hydrolyse indigestible complex carbohydrate such as lactulose, into acetic and lactic acids. These acids are able to inhibit the growth of many potential pathogens and other putrefactive bacteria (Rasic, 1983). Likewise, bifidobacteria are believed to have anti-carcinogenic (Mitsuoka, 1982) and anti-cholesterolemic (Hata *et al*., 1982) properties.

Until recently, controversy exists as to which genus bifidobacteria should be assigned. Since the first description of these bacteria, they have been assigned to several genera: *Bacillus*, *Bacteroides*, *Tisseria*, *Nicordia*, *Lactobacillus*, *Actinomyces*, *Bacterium* and *Corynebacterium* (Mitsuoka, 1984). Orla-Jensen (1924) suggested that these bacteria be recognised as a separate genera, but this change was not made until the 8th edition of Bergey's manual (Rogosa, 1974). Until then, bifidobacteria were grouped with the genus *Lactobacillus*. At the initial stage, only one species was assigned to the genus *Bifidobacterium*. Nonetheless, both Reuter (1971) and Mitsuoka (1984) expanded the list to 28 species as described in Bergey's manual.

In general, there is a lack of homology between the various species of bifidobacteria (Scardovi *et al*., 1971). In this paper, we identified *Bifidobacterium* strains isolated from the faeces of breast-fed infants by phenotypic characteristics such as carbohydrate fermentation pattern, gelatine liquefaction test and nitrate reduction test. In addition, antimicrobial susceptibility test and plasmid isolation was performed.

Materials and Methods

Isolation and characterization of bifidobacteria: Fresh faecal specimens were collected from 4 breast-fed infants aged between 1 to 5 months. Bifidobacteria were isolated as previously described (Beerens, 1991). The weight of each sample was taken and tenfold dilutions were carried out using 0.1 % peptone water. The bifidobacteria isolates were characterized and identified phenotypically according to the method and procedure described by Scardovi (1986). The strains were identified according to the procedures described by Sgorbati *et al*. (1982).

Antimicrobial Sensitivity test: A suspension of 10^8 cell/ml was used as inoculum. Sensitivity testing was done by Stokes disc diffusion technique (Stokes and Ridgeway, 1980) using TPY agar (Scardovi, 1986) as a plating medium. Standard discs of 17 antimicrobial agents (Beckton Dickinson, USA) were seeded in the plates and incubated anaerobically at 37°C for 48 h.

Plasmid isolation: Bifidobacteria strains were propagated in 3.0 ml TPY broth (Scardovi, 1986) and incubated at 37°C for 24 h. The plasmid DNA was isolated according to the method developed by Anderson and Mckay (1983), with some modifications. The cells were harvested by centrifugation at 6000 rpm for 10 min. Pelleted cells were washed once in 1 ml sterile distilled water and centrifuged at 6000 rpm for 5 min, resuspended in 200 µl of solution A (20 mM glucose, 10 mM Tris pH 8.0, 10 mM EDTA and 50 mM NaCl). Lysozyme was added at 5 mg/ml to the suspension and incubated for 30 min at 37°C. A volume (400 µl) of solution B (1% SDS, 0.2 M NaOH) was added to the cell suspension and kept at room temperature for 5 min. Subsequently, 300 µl of 3 M sodium acetate, pH 5.2, was added and put on ice for 5 min. Cells were pelleted

and the clear supernatant was transferred to a new tube. 400 µl of phenol was added to the cells and mixed gently. Thereafter, 400 µl of chloroform isoamyl alcohol mixture (24:1) was added and vortexed for 10 seconds. A two-phase mixture was obtained by centrifugation at 10000 rpm for 10 min and the clear upper layer was transferred to a new tube. The DNA was precipitated by adding 2 volumes of cold ethanol (96%) and kept at -20°C, overnight. The DNA was pelleted by centrifugation at 12000 rpm for 10 min, at 4°C. Pelleted DNA was washed in 70% ethanol and then air-dried. The DNA pellet was then dissolved in 20 µl TE added with 1 µl of RNase (10 mg/ml) and analysed by agarose gel electrophoresis (Meyers *et al*., 1976).

Results and Discussion

A total of 8 strains of bifidobacteria were isolated from the faecal samples of 4 infants (Table 1). All the strains were unable to liquefy gelatine and showed negative result for the nitrate reduction test. These suggested that all the strains tested were lacking in proteolytic enzyme and did not require oxygen for their metabolism (Mitsuoka, 1984). The isolates were identified as follows: *B. infantis* (6 strains) and *B. breve* (2 strains). The results of the present study were in close agreement with initial description of *B. breve* and *B. infantis* by Scardovi (1986), both species fermented ribose but not xylose. Two isolates were identified as *B. breve* since both fermented mannitol and sorbitol (Scardovi, 1986). The isolates that did not ferment melibiose, raffinose and amygdalin were identified as *B. breve* strain G48 and those that ferment melibiose, raffinose and amygdalin as *B. breve* strain F100. Six strains were identified as *B. infantis* since they were unable to ferment sorbitol. The melibiose negative strains resembling *B. infantis* D36 and 745 were also isolated. Strains that were able to ferment both melibiose and amygdalin were designated as *B. infantis* Z80, F66 and F91. Isolate with melibiose-positive, raffinose and amygdalin-negative, was regarded as *B. infantis* strain F112.

The results of the antimicrobial susceptibility tests were shown in Table 2. In general, most of the strains tested were resistant to amino glycoside antibiotics (streptomycin, neomycin, kanamycin and gentamicin), nalidixic acid, polymyxin B, oxytetracycline and sulfisoxazole. However, strains G48 and F100 were susceptible to streptomycin, strain F91 was susceptible to neomycin and, strains G48 and F100 were susceptible to gentamicin. All strains were susceptible to bacitracin, chloramphenicol, nitrofurantoin and erythromycin. Strain F91 was susceptible to all the antibiotics except penicillin and ampicillin.

In general, the results of the present study were in agreement with earlier investigation by Miller and Finegold (1967). They analysed 480 strains of bifidobacteria, representing 15 species for antimicrobial susceptibility. They observed that most strains were resistant to polymyxin B, nalidixic acid, kanamycin, gentamicin and metronidazole. Misra and Kuila (1992) investigated the effects of various levels of antibiotics against a selection of *B. bifidum strains* and observed that all the strains were resistant to 1 µg ampicillin, 100ug carbenicillin, 30 µg cephaloridine, 30 µg nalidixic acid and 10 units of penicillin. Similar observations were made by Lim *et al*. (1993). They observed 37 strains of bifidobacteria and showed wide variations in their susceptibility to nitrofurantoin and tetracycline. The minimum inhibitory concentrations of those antibiotics were 1.56 to 50.0 µg/ml and 0.39 to 50 µg/ml, respectively. They also observed that all the *Bifidobacterium* strains tested were resistant to 200 µg/ml of nalidixic acid, and only one strain of *B. bifidum* and 6 strains of *B. longum* were inhibited by 200 µg/ml polymyxin B sulphate.

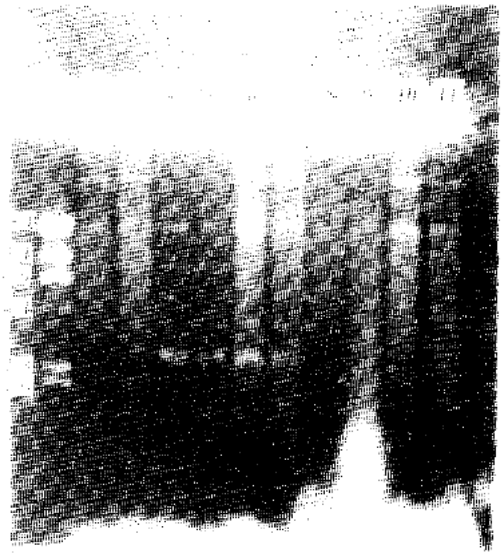

Fig. 1: Plasmid profiles of *Bifidobacterium* species
Lane 2 and 3, *B. breve* G48; Lane 4 and 5, *B. breve* F100; Lane 6 and 7, *B. infantis* 745; Lane 8 and 9, *B. infantis* F91; Lane 10 and 11, *B. infantis* Z80; Lane 1, Marker A/ Hind111 (bands identified top to bottom; 23.1, 9.4, 6.6, 4.4, 2.3, and 2.0 kb)

Antimicrobial susceptibility of intestinal microorganisms is important consideration because the administration of antimicrobial substances can alter the intestinal microbial balance and resulted in the suppression of certain beneficial bacterial groups, including bifidobacteria. The altered microbial balance may results in intestinal disorders (Kobayashi *et al*., 1973). The susceptibility of bifidobacteria to various antimicrobial agents are of interest not only in searching for selective agents for enumeration of viable cells in products containing bifidobacteria, but also in understanding the alteration of normal intestinal

Table 1: Phenotypic characteristics of *Bifidobacterium* species[a]

Phenospecies	*Bifidobacterium infantis*				*Bifidobacterium breve*			
Strain	D36	Z45	780	F112	F66	F91	G48	F100
Acid from:								
Arabinose	-	-	-	-	-	-	-	-
Xylose	-	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-	-
Ribose	+	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+
Cellobiose	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+
Trehalose	+	+	+	+	+	s	+	+
Melibiose	-	-	+	+	+	+	-	+
Raffinose	-	-	+	+	+	+	-	+
Melezitose	+	+	+	-	+	+	+	+
Mannitol	+	+	+	+	+	+	+	+
Sorbitol	-	-	-	-	-	-	+	+
Esculin	+	+	+	+	+	+	+	+
Salicin	+	+	+	+	+	+	+	+
Amygd aline	-	-	+	+	+	-	-	+

Symbols: + , positive reaction; s, delayed reaction; -, negative reaction

Table 2: Antimicrobial susceptibility of *Bifidobacterium* species

Antibiotic	Zone of inhibition of *Bifidobacterium* species (mm)*							
	D36	G48	Z45	Z80	F112	F66	F91	F100
Bacitracin (10 μg/ml)	22 (s)	35 (s)	22 (s)	28 (s)	29 (s)	20 (s)	40 (s)	33 (s)
Nialidixic acid (30μg/ml)	(R)	(R)	(R)	(R)	(R)	(R)	(R)	(R)
Streptomycin (10μg/ml)	(R)	15 (s)	(R)	(R)	(R)	(R)	(R)	15 (s)
Ampicillin (10μg/ml)	30 (s)	35 (s)	20 (s)	32 (s)	35 (5)	25 (s)	(R)	36 (s)
Chloramphenicol (30μg/ml)	20 (s)	33 (s)	23 (s)	32 (s)	35 (s)	17 (i)	30 (s)	32 (s)
Cefamandole (30μg/ml)	15 (s)	30 (s)	13 (0	22 (s)	25 (s)	16 (s)	30 (s)	23 (s)
Nitrofurantoin (300μg/ml)	25 (s)	20 (s)	19 (s)	35 (s)	38 (s)	22 (s)	32 (s)	18 (s)
Penicillin (10μg/ml)	20 (s)	35 (s)	15 (s)	35 (s)	36 (s)	27 (s)	(R)	40 (s)
Tetracycline (30μg/ml)	12 (r)	30 (s)	11 (r)	12 (r)	14 (r)	12 (r)	17 (i)	30 (s)
Polymyxin B (300μg/ml)	(R)	(R)	(R)	10 (1)	10 (i)	(R)	(R)	(R)
Neomycin (30μg/ml)	7 (r)	12 (r)	8 (r)	10 (r)	8 (r)	8 (r)	20 (s)	15 (i)
Kanamycin (30μg/ml)	9 (r)	15 (i)	8 (r)	7 (r)	(R)	8 (r)	(R)	15 (i)
Novobiocin (30μg/ml)	12 (r)	30 (s)	19 (s)	34 (s)	29 (s)	19 (s)	16 (r)	27 (s)
Oxytetracycline (30μg/ml)	9 (r)	24 (s)	9 (r)	15 (i)	11 (r)	9 (r)	24 (s)	20 (s)
Sentamycin (10μg/ml)	8 (r)	19 (s)	8 (r)	10 (r)	(R)	10 (r)	(R)	20 (s)
Sulfisoxazole (1.0μg/ml)	(R)	(R)	(R)	20 (s)	1 (s)	(R)	(R)	(R)
Erythromycin (15μg/ml)	20 (s)	35 (s)	19 (s)	30 (s)	30 (s)	18 (s)	30 (s)	34 (s)

*Diameter of zone of inhibition was compared to the standard zone diameter interpretative chart provided by the supplier. Degree of susceptibility: (s) susceptible, (i) intermediate, (r) resistant, (R) no zone of inhibition (resistant)

microflora when antimicrobial agents were taken. Bifidobacteria are the most prevalent bacteria in the intestine of breast-fed infant. Their presence could prevent colonisation of the gut by potential pathogens.

Important phenotypic traits such as antibiotic-resistance, toxin and bacteriocin production, modification of bacterial virulence and increased metabolic capabilities of a microorganism are mostly rendered by plasmids (Falkow, 1975; Broda, 1979). Plasmids are autonomously replicating DNA, which are extra-chromosomally situated in the microorganisms.

Davies *et al*. (1981) stated that plasmids could also provide

a means of strain typing or molecular fingerprinting. In this study, we have examined 8 bacterial isolates belonging to the genus *Bifidobacterium* for the presence of plasmid DNA. The results showed different size bands within each of the 4 strains harbouring detectable plasmids (Fig. 1). The 23 kb band present in each of the 5 strains isolated are residual DNA of chromosomal origin. Since plasmid profiles may have taxonomic value as well as providing useful ecological and evolutionary information. Two strains of *B. breve* (G48 and F100) harbouring detectable plasmids were also shown (Fig. 1). Strain G48 has a high molecular weight plasmid of about 50 kb whilst strain F100 contained 2 detectable plasmids of 8 and 2.3 kb. However, only two of the three *B. infantis* strains isolated contained plasmids. Strain Z45 habours two plasmids sized 50 kb and 2.3 kb, respectively. On the other hand, strain Z80 contained only one plasmid of 50 kb. From these observations, two plasmids of 50 and 2.3 kb seemed to predominate in both *B. infantis* and *B. breve*. They indicated linkage between the presence of a 50 kb plasmid in *B. infantis* and *B. breve* with resistance towards neomycin. Other antibiotic resistant genes that may be carried on this plasmid are tetracycline, kanamycin and gentamicin. The results also indicated a possible relationship between the resistance towards kanamycin with the 2.3 kb plasmid haboured by *B. infantis* and *B. breve*.

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