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Isolation of *Bifidobacterium* from Infant Feces and its Characterization

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Bifidobacteria are the predominant species in the feacal microflora of breast fed infants is due to the bifidigeneic factors. The efficient isolates of Bifidobacteria were selected, based on their probiotic characteristics. Isolate BAL I was able to grow under acidic condition of pH 3.5 and 4.5, resistant against bile salt at 0.5 per cent and resistant to antibiotic Cholaramphenicol (30 mcg), Ampicillin (10 mcg) and Gentamycin (10 mcg). The isolate BAL I was found to show maximum inhibitory activity against *E.coli* (10mm) compared to *Bifidobacterium bifidum* (ATCC 29521) which recorded 8mm of inhibition zone. The integration of bifidobacteria in dairy and poultry products is of interest for reinstalling the intestinal microflora.

Key words: Bifidobacterium, Infant feces, Antibiotics.

The word 'probiotic', from the Greek 'for life', has over the past few years been used in several different ways. Parker (1974) defined probiotics as organisms and substances that contribute to intestinal microbial balance. Fuller (1989)[1], improved the definition, and redefined probiotics as a live microbial feed supplement, which beneficially affect host animal by improving its intestinal balance.

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The definition of probiotic was broadened in the current decade, (Havenaar and Huis in't Veld, 1992)[2] presently; probiotics are defined as "Viable microorganisms (lactic acid and other bacteria, or yeasts applied as dried cells or in a fermented product) that exhibit a beneficial effect on the health of the host upon ingestion by improving the properties of its indigenous microflora".

Bifidobacterium is a genus of Gram-positive, non-motile, often branched anaerobic bacteria. Bifidobacteria are one of the major genera of bacteria that make up the gut flora, the bacteria that reside in the colon. Bifidobacteria aid in digestion, are associated with a lower incidence of allergies[3] and also prevent some forms of tumor growth[4]. Some Bifidobacteria are being used as probiotics. Every human has billions

of bacteria living in their colon and small intestine. Most of the bacteria (95 per cent) are obligate flora that provides benefit in human physiology, while a small percentage can be harmful if they are allowed to propagate. These bacteria are commonly referred to as Probiotics. *Bifidobacterium* categorized as one of these obligate friendly bacteria located within our colon.

Bifidobacterium can exist in several different shapes, including short curved rods, club shaped rods, and bifurcated Y shaped rods (how *Bifidobacterium* got its name). There are over 30 species of *Bifidobacterium* isolated so far.

It is one of the first bacteria to colonize in the large intestine and can be found within the first few days of life in a breast fed newborn infant. Its numbers remain stable throughout an individual's lifespan until old age when they begin to decline. *Bifidobacterium* populations can be influenced by a multitude of factors including stress, diet, and antibiotic usage. *Bifidobacterium* and other friendly flora serve several purposes in our digestive system:

1. They help maintain the immune system outpost that exists only in our GI tract
2. Are mediators in the immune response that begins in the large intestine
3. They also help to maintain normal structure and function of the cells of the large and small intestine

Bifidobacterium provide another layer of protection and reinforce the barrier of the GI tract from the outside world, limiting the passage of potential allergens and pathological organisms into our blood. Normal flora like *Bifidobacterium* also contributes to the availability of vitamins such as Vitamin K, Biotin, Pantothenic acid, and Vitamin B_{12}.

One of the mechanisms that allow *Bifidobacterium* to provide protection against other microorganisms is the fact that it produces lactic acid and acetic acid. These two acids make the environment unsuitable for many different types of bacteria. Another mechanism that *Bifidobacterium* use to provide protection is as simple as overcrowding. They grow so well and in such great numbers that other pathological bacteria do not have any space to grow. This is the reason that antibiotics provide a means for infection in the GI tract because they kill off the healthy protective flora normally present in the colon. *Bifidobacterium* and other friendly flora are anti-microbial, immunomodulating, anti-carcinogenic, anti-diarrhea, anti-allergic, and anti-oxidants. Certain *Bifidobacterium* strains are able to produce substances that compete and prevent pathogenic bacteria from adhering to the receptors on epithelial cells of intestinal surfaces[5].

MATERIAL AND METHODS

Collection of new born baby fecal sample

About ten fresh fecal samples were obtained from the infants in the age group of 2 days to 9 months. Anaerobically all plates were incubated in bell jars flushing of N_2 gas inside and also using lighted candle inside to absorb the O_2, using paraffin wax to seal the jar to avoid air entering in the jar and incubated the plates for a period of 5 days. Bifidobacteria were enumerated using TPY solid medium[6]. The *Bifidobacterium* pure single colony was obtained by cross streak method. Colonies picked from countable plates were selected for gram reaction, morphology and biochemical tests.

Physiological and biochemical tests

The isolates considered to belong to the genus *Bifidobacterium* were identified by the detection of fructose-6-phosphate phosphoketolase (F6PPK) enzyme in cellular extracts as described by Scardovi (1986)[7]. Cells were grown in 10 ml of TPY broth at 37°C for 18 hours and harvested by centrifugation at 5000 rpm for 10 min. The pellet was washed twice with 5 ml of 0.5 g / l phosphate buffer. After centrifugation, the pellet was collected in 1 ml of buffer and disrupted by sonication at 0°C for obtaining crude cells extract. 0.25 ml of reagents (6 mg/ml NaF, 10 mg/ml sodium iodoacetate and 80 mg/ml fructose-6-phosphate) were added to the cells extract.

The reaction was initiated by incubating for 30 min at 37° C and stopped by adding 1.5 ml of hydroxylamine-HCl (13.9 per cent). After 10 min, 1 ml of trichloroacetic acid (15 per cent) and 1 ml of $FeCl_3.6H_2O$ (5per cent) were added. The presence of fructose-6- phosphate phosphoketolase enzyme was revealed by the appearance of red and purple colors[7-8].

Selection of high biomass production isolate

BAL I, BAL II, BAL III, BAL IV were grown on basal medium TPY broth, 20 ml of medium was added to 100 ml vials. Subsequently they were inoculated with an overnight cultures of *Bifidobacterium* isolated from infant feces at 0.20mg cell ml^{-1}. Anaerobiosis was developed. The vials were incubated 37° C the samples were collected at 72 hours the bacterial biomass as separated by centrifugation at 6000 rpm for 15 minutes. The dry cell weight was after 12hours.

Probiotic characteristics

The isolates were selected based on certain criteria *viz*., acid tolerance, bile tolerance, antibiotic resistance activity, antimicrobial activity against pathogenic microorganism[9].

Acid tolerance of BAL I

The high biomass producing isolate BAL I, *Bifidobacterium bifidum* (ATCC 29521) were grown separately, in TPY broth for 16 to 24 hours at 37°C the cells were harvested by centrifugation (4300 x g for 10 min at 4°C), washed three times in sterile saline (0.85 per cent NaCl) and inoculated (1 per cent) into TPY broth acidified with concentrated HCl to pH 3.5.

A non acidified control at pH 6.8 was also maintained and incubated at 37° C anaerobically, for 90 minutes the absorbance at 650 nm was monitored at 15 min interval.

Bile-tolerance of BAL I

The isolate BAL I was used in acid tolerance was tested in bile tolerance using TPY (pH 3.5). Cells were harvested by centrifugation (4300 x g for 10 min at 4°C), washed three times with sterile saline (0.85 per cent NaCl), and 1 per cent inoculated in to TPY broth with 0.5, 1.0, 1.5, 2.0 per cent bile salt (oxgall). A control was maintained without bile salt under anaerobic condition. After inoculation the absorbance was recorded at 610nm at 1 h interval for a period of 3 h from the time of inoculation.

Antibiotic resistance activity

The *Bifidobacterium* isolate BAL I and standard culture were inoculated into TPY broth individually and incubated for 24 hours. About 25 ml of TPY agar was seeded with the *Bifidobaterium* isolate (10^6 cfu ml^{-1}) mixed well, poured into sterile petri plates to solidify the media in an hour. OCTA-discs (eight antibiotics in a single ring) and also separate discs were placed up side down, on the top of the agar plates and maintained at 4° C for 1 hours, the plates were incubated at 37°C the over night. Resistance and inhibition zone around the discs was recorded[10].

Antagonistic activity

The *Bifidobacterium* isolates (BAL I and standard culture) were studied for their antagonistic activity assay. Active cultures of *Bifidobacterium* cells were separately and to get cell free extract. The cell free extract was used for the detection of antimicrobial activity of *Bifidobacterium* isolates against pathogenic organism by paper disc assay. The pathogenic bacterium used as indicator organism for studying the antagonistic activity was *Escherichia coli,* obtained. The antimicrobial activity was determined by measuring the diameter of the inhibition zone around the disc[11].

RESULTS AND DISCUSSION

Characterization and identification of Bifidobacterial strains and isolates

Attempts were made to isolate *Bifidobacterium* sp. isolated from ten infants feces (samples 1 to 10) aged from two days to 9months. Among the ten infant feces, the *Bifidobacterium* colonies were obtained from four samples only (Table 1). The samples were named as BAL I BAL II, BAL III, and BAL IV respectively.

The stages of the pre-identification based on morphological aspects show that the Bifidobacteria isolates developed on TPY medium were Circular, white slimy Concave colonies without catalase. Similar observations were noted by[7-6]. All the isolates were found to be Gram

Table 1. Isolation of *Bifidobacterium* sp

Sample	Source	×10--^{6} cfu/g
1	Infant feces	-
2	Infant feces	62
3	Infant feces	-
4	Infant feces	-
5	Infant feces	83
6	Infant feces	22
7	Infant feces	-
8	Infant feces	-
9	Infant feces	13
10	Infant feces	-

positive.Y shaped rods and rods were the shape of the isolates in the present study.

Kojima *et al.* (1968)[12] reported that the Bifidobacteria exhibited pleomorphism as observed in the present investigation.

The isolates showed negative results for catalase activity, nitrate reduction, indole production, gelatine liquefaction and gas production from glucose which supported that the obtained isolates belonged to *Bifidobacterium* sp. Mitsuoka (1984)[13] and Gavini *et al.* (1990)[14] reported that any strain belonging to the *Bifidobacterium* genus must be Catalase nagative, Nitrate reductase negative, does not form Indole, does not liquify the gelatine and does not produce gas from glucose. The results of the present investigation are in conformity with these reports (Table 2).The isolates of present study showed positive result, development of reddish - violet color gave positive result for F6PPK enzyme activity test apart from colony morphology and cell shape. Hence it was identified as *Bifidobacterium* sp.

Fermentation of carbohydrates

The isolates were tested for its utilization of different carbohydrate source (Table 3). The result showed that the isolate BAL I able to utilize the carbon sources like Galactose, Sucrose,

Table 2. Morphological and physiological characterization of Bacterial isolates

Characteristics	Strains				
	BAL I	BAL II	BAL III	BAL IV	STD
Colony morphology	Circular, white silmy concave	Circular, dull white silmy concave	Circular, dull white silmy concave	Circular white silmy concave	Circular white silmy concave
Cell morphology	Y shape Rods	Rods	Rods	Y shape Rods	Y shape rods
Gram staining	+	+	+	+	+
F6PPK test	+	+	+	+	+
Gelatin liquefaction	-	-	-	-	-
Catalase activity	-	-	-	-	-
Nitrate reductase Test	-	-	-	-	-
Indole production	-	-	-	-	-
Gas from glucose	-	-	-	-	-
Motility	-	-	-	-	-

+ Growth observed, - No growth; STD- *Bifidobacterium bifidum* (ATCC 29521)

Table 3. Fermentation of carbohydrates

Carbohydrates	Strains				
	BAL I	BAL II	BAL III	BAL IV	STD
Galactose	+	+	+	+	+
Sucrose	+	-	-	-	+
Lactose	+	+	+	+	+
Cellulose	-	-	-	-	-
Starch	-	-	-	+	+
Fructose	+	+	+	+	+
D - Glucose	+	+	+	+	+

+ growth observed, - No growth; STD- *Bifidobacterium bifidum* (ATCC 29521)

Lactose, and Fructose. The isolate BAL II and BAL III were found to utilize the Lactose, Fructose and D-Glucose. BAL IV has utilized Glucose, Lactose, Sucrose, Fructose and D-Glucose. Among these carbon sources Galactose, Lactose, Fructose and D- Glucose were utilized by all the isolates. In our experiment Fructose, Galactose, Lactose were used as carbon source much higher than the other sugars. BAL I utilize fructose, Galactose, Sucrose, Lactose and the results are similar to the findings of Scardovi (1984) (15) who reported that similar carbohydrate utilization pattern in strains *B. infantis.*

Table 4. Survival of BAL I under high acidic conditions

Duration (min)	OD value at 650 nm	
	pH 3.5	pH 6.8
0	0.75	0.73
30	0.89	0.74
60	1.10	0.75
90	1.21	0.76

Table 5. Survival of *Bifidobacterium sp.* in bile salt

Bile salt concentration (oxgall)	Duration (hours)	OD value at 610nm	
		BAL I	STD
0.5%	0	0.06	0.06
	1	0.19	0.10
	2	0.25	0.15
	3	0.31	0.24
1%	0	0.10	0.10
	1	0.11	0.11
	2	0.12	0.12
	3	0.12	0.12
1.5%	0	0.12	0.12
	1	0.15	0.15
	2	0.15	0.15
	3	0.15	0.15
2%	0	0.16	0.16
	1	0.16	0.16
	2	0.16	0.16
	3	0.16	0.16

Selection of efficient isolate for biomass production

Bifidobacterium isolates BAL I, BAL II, BAL III, BAL IV grew well in TPY medium. The high biomass was produced (0.140g) by BAL I at 37°C, incubated for 48 hours (Fig. 1).

Survival of BAL I under high acid conditions

The isolate BAL I and standard culture (*Bifidobacterium bifidum* - ATCC 29521) were tested for tolerance to hydrochloric acid at pH of 3.5, for 90 minutes from 0.75 to 1.21 (Table 4, Fig. 2). The growth of the BAL I isolates in the acidified TPY broth of pH 3.5, the turbidity was increased the result were monitored by absorbance at 650 nm at 15 min interval. HCl resistance may play a more important role during passage of the bacteria through the stomach (16). Therefore the strains were tested for tolerance to hydrochloric acid. The result of this study showed that BAL I and *Bifidobacterium bifidum* (ATCC 29521) were able to grow at pH 3.5. This is similar to the findings of Chung *et al.,* (1999)[17].

Survival of *Bifidobacterium sp.* in bile salt

Bifidobacteria tolerant to low pH of the stomach and bile produced in small intestine are preferred[18].

Table 6. Antibiotic sensitivity assay of *Bifidobacetrium* sp.

Antibiotic	Symbol	Quantity	BAL I	STD
Chloramphenicol	C	30mcg	R	R
Ampicillin	A	10mcg	R	MS
Tetracycline	T	30mcg	MR	MS
Gentamycin	G	10mcg	R	S
Kanamicin	K	30mcg	MR	S
Co-trimoxazole	Co	25mcg	S	S
Amikacin	Ak	30mcg	S	R
Streptomycin	S	25mcg	S	R

R – Resistant S – Susceptible MS – Moderately Susceptible

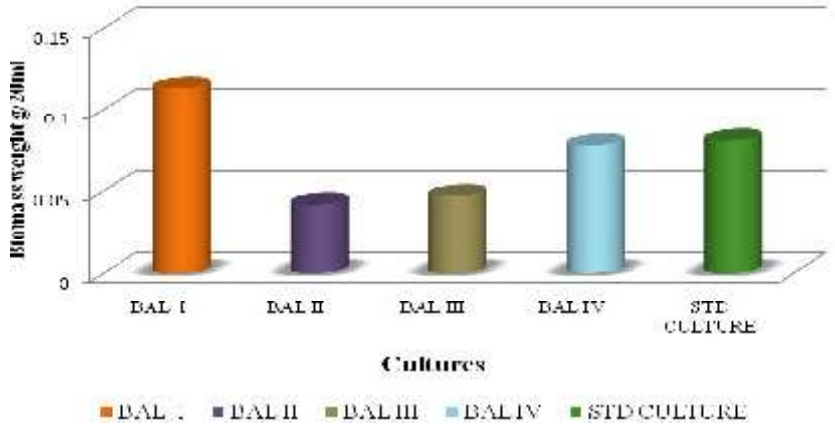

Fig. 1. Selection of high biomass production isolate

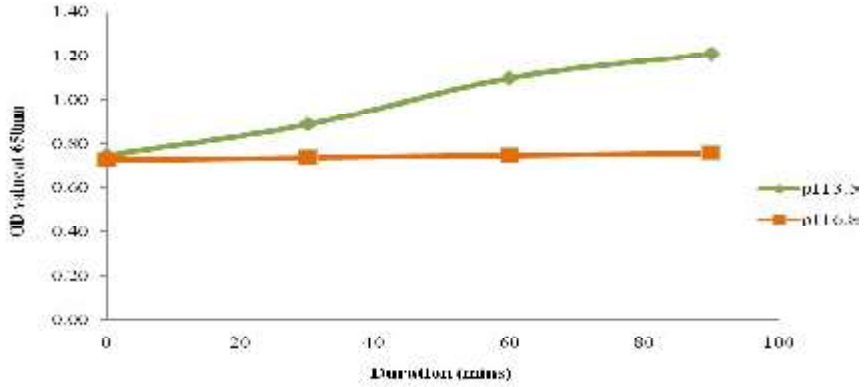

Fig. 2. Survival of *Bifidobacetrium* sp in acid conditions

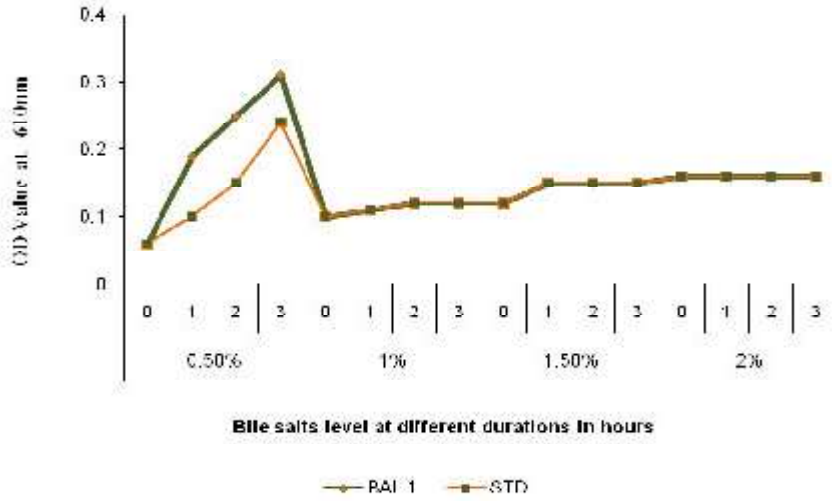

Fig. 3. Survival of *Bifidobacterium* sp. in bile salt

Table 7. Antagonistic activity of *Bifidobacterium* sp. against *E.coli*

Isolates	Inhibition zone (diameter)
BAL I	+(10mm)
STD	+ (8mm)

STD- *Bifidobacterium bifidum* (ATCC 29521)

Isolate BAL I showed increasing trend in growth the OD value increased from 0.75 to 1.21 at 0.5 per cent level of bile salt as the incubation period increased (Table 5, Fig. 3). The increase in OD value which indicates that the isolates can grow in bile salt medium, which shows that the isolate can tolerate bile salt at 0.5 per cent. There is no growth in 1.0, 1.5 and 2.0 per cent level. The growth was monitored and summarized data presented in Table 10 figure. Similar report was made by Chung *et al.,*(1999)[16] who found the strains SI 31 and HI 30 grew considerably better in the presence of 0.15 per cent bile salt. BAL I found to be better than the standard isolate *Bifidobacterium bifidum* (ATCC 29521). It is reasonable to assume that a strain must be able to grow well in the presence of bile to remove cholesterol from an environment, since bile must be present for its activity to occur.

Antibiotic resistance activity

The isolate BAL I was found to be resistant to Chloramphenicol (30 mcg), Ampicillin (10 mcg). Gentamycin (10mcg). The standard strain *Bifidobacterium bifidum* (ATCC 29521) resistant to Chloramphenicol (30 mcg), Amikacin (30 mcg) and Streptomycin (25 mcg). BAL I was susceptible to Co – trimoxazole (25 mcg), Amikacin (30 mcg) and Streptomycin (25 mcg) (table 6). The reason behind the study was to find out the influence of antibiotics on the suppression of *Bifidobacterium* already present in the intestine. Maria Rosaria *et al.* (2007) (19) who reported the Bifidobacetria was intrinsically resistant to Kanamycin, Gentamicin, Streptomycin, Polymyxin B, Nalidixic acid, Paromomycin, Neomycin.

Antagonistic activity of the isolate BAL I

The antagonistic activity of the BAL I was compared with *Bifidobacterium bifidum* (ATCC 29521) against *E.coli.* The results revealed that the isolate BAL I was found to show maximum inhibitory activity against *E.coli* (10mm) compared with standard culture which recorded 8mm inhibition zone (Table 7). The antagonistic activity of the isolate over pathogenic microorganisms is often based on the production of substances that inhibit (or) inactivate the spoilage microorganisms. *Bifidobacterium* secreting antimicrobial substances was reported by McNaught and MacFie (2001)[20]. Biûdobacteria have the ability to produce organic acids and other antimicrobial compounds such as proteinaceous compounds called bacteriocins[21] which are responsible to inhibit the *E.coli*.

REFERENCES

1. Fuller, R., A review: Probiotics in man and animals. *J. Appl. Bact.,* 1989; **66**: 365-378.
2. Havenaar, R. and J. H. J. Huis in't Veld., 'Probiotics; A General Review' in The Lactic Acid Bacteria in Health and Disease, *Wood, B., ed*, Elsevier, Barking 1992; 151-170.
3. Bjorksten, B., E. Sepp, K. Julge, T. Voor and M. Mikelsaar. Allergy development and the intestinal microflora during the first year of life. *Journal of Allergy and Clinical Immunology*, 2001; **108**(4): 516-520.
4. Guarner, F. and J. R. Malagelada. Gut flora in health and disease. *The Lancet,* 2003; **361**(9356): 512-519.
5. Ahmad, C., P. Natascha, C. Wei. and Z. Hao. Review:Antimicrobial proteinaceous compounds obtained from bifidobacteria: From production to their application. *International Journal of Food Microbiology* xxx (2008) xxx–xxx**.** Article in press 2008.
6. Beerens, H. Year elective and selective insulation medium for *Bifidobacterium* sp. *Lett. Appl. Microbiol.,* 1990; **11:** 155-157.
7. Scardovi, V. Genus *Bifidobacterium* Orla-Jensen 1924, 472AL, p. 1418- 1434. In P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology (volume 2). Williams and Wilkins, Baltimore 1986.
8. Meile, L., M.R. Lukas, T.A. Geissmann, M. Herenisperger and M. Teuber. Characterization of the d-xylulose 5 phosphate/d-fructose 6-phosphate phosphokelolase gene(xfp) from *Bifidobacterium lactis. J. Bacterial*., 2001; 2929-2936.
9. Chou, L. S and B. Weimer. Isolation and

characterization for acid and bile tolerant isolates from strains of *Lactobacillus acidophilus*. *J. Dairy Sci.,* 1999; **5:** 365-372.

10. Pal, V., M. Jamuna. and K. Jeevaratanam. Isolation and characterization of bacteriocin producing lactic acid bacteria from a south Indian special dosa batter. *Journal of Culture Collections,* 2005; **4**(1): 53-60.
11. Bhunia, A. K., M. C. Johnson and B. Ray. Influences of growth condition on the production of a bacteriocin, pediocin ACH by *Pediococcus acidolacti* H. *Applied and Environmental Microbiology,* 1984; **57:** 1265-1267.
12. Kojima, M., S. Suda, S. Hotta, K. Hamada. Introduction of pleomorphism in *Lactobacillus bifidus. J. Bacteriol.,* 1968; **95:** 710-711.
13. Mitsuoka, T. Taxonomy and Ecology of Bifidobacteria. *Bifidobacteria Will Microflora,* 1984; **3:** 11-28.
14. Gavini, F, A. M. Pourcher, D. J. Bahaka Freney, C. Romond, D. Izard.The *Bifidobacterium* kind Critical classification, identification, aspects. *Méd. Badly. Repugnant***,** 1990; **20:** 53-62.
15. Scardovi, V. *Bifidobacterium* Orla-Jensen, 1984; 1924, 472, 1418-1434. In N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. I. The Williams & Wilkins Co., Baltimore.
16. Conway, P. L., S. L. Gorbach and B. R. Goldin.. Survival of lactic acid bacteria in the human stomach and adhesion to intestional cells. *J. Dairy Sci.,* 1987; **70**: 1-12.
17. Chung, H. S., Y. B. Kim, S. L. Chun, G. E. Ji. Screening and selection of acid and bile salt resistant *Bifiodbaceteria*. *Int. J. Microbiol.,* 1999; **47:** 25-32.
18. Clark, P. A., J. H. Martin. Selection of *Bifidobacteria* for use as dietary adjuncts in cultured dairy foods: III – tolerance to simulated bile concentration of human small intestines. *Cult. Dairy Prod. J.,* 1994; **29:** 18-21.
19. Maria Rosaria D'Aimmo, Monica Modesto, Bruno Biavati. Antibiotic resistance of lactic acid bacteria and *Bifidobacterium* sp. isolated from dairy and pharmaceutical products. *Int. J. of Food Microbiol.,* 2007; **115:** 35-42.
20. McNaught, C. E. and J. Mac Fie., Probiotics in clinical practice: a critical review of the evidence. *Nutr. Res.,* 2001; **21:** 343-353.
21. Ibrahim, S. A., M. M. Salameh. Simple and rapid method for screening antimicrobial activities of *Biûdobacterium* species of human isolates. *Journal of Rapid Methods and Automation in Microbiology,* 2001; **9:** 52-63.