Advances in Infectious Diseases, **2013, 3, 200-204**
http://dx.doi.org/10.4236/aid.2013.33029 Published Online September 2013 (http://www.scirp.org/journal/aid)

Identification of *Bifidobacterium animalis*, *Bifidobacterium adolescentis* and *Bifidobacterium bifidum* from Stool of Children and Detection of Their Antibacterial Properties

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Received April 6th, 2013; revised May 6th, 2013; accepted June 6th, 2013

ABSTRACT

Introduction and Objective: The genus *Bifidobacterium* can generally be found in quantity in the habitats such as human and animal gastrointestinal tract, dental caries, vagina and oral cavity. The aim of this study was to isolate *Bifidobacterium* from stool and determine their inhibitory effect against some pathogens. **Materials and Methods:** 130 samples were collected by wet swabs and kept in sterile tubes containing MRS broth media. And *Bifidobacterium* isolated from stool was enriched in Man-Rogosa-Sharpe medium (MRS) broth and isolated by growing on MRS agar medium and characterized by phenotypic characteristics and PCR technique at genus and species levels. The antimicrobial substance was extracted from ethyl acetate solvent and the antimicrobial activity against some pathogenic bacteria, such as *Salmonella typhi* and *Shigella sonnei*, were investigated. **Results:** Eleven *Bifidobacterium bifidum* and four *Bifidobacterium adolescentis*, which were isolated from fresh stool, were identified by PCR. Antimicrobial substance from MRS broth medium was extracted. This antimicrobial compound showed a potent inhibitory activity against four tested bacteria. These bacteria produced acetic acid and lactic acid as inhibitory substances that were different from bacteriocins. **Conclusion:** Fresh stool may be used as a source of antimicrobial lactic acids bacteria, *Bifidobacterium bifidum* and *adolescentis* as two probiotics can establish themselves in gut and urogenital tract to prevent the human body from adverse effects of pathogens.

Keywords: *Bifidobacterium*; PCR; Antimicrobial Substance; Stool

1. Introduction

Bifidobacteria were first discovered in infant feces by Tissier and named it *Bacillus bifidus* [1]. *Bifidobacteria* are a natural resident of the human and animal gastrointestinal tract, dental caries and vagina, oral cavity, urine and blood [2]. Several *Bifidobacterium* strains are now being used as probiotics by using established criteria which belong to the *Bifidobacterium animalis*, *Bifidobacterium bifidum*, *Bifidobacterium breve*, *Bifidobacterium longum fermentum* and some other species of this genus [2,3]. For human nutrition, probiotics are defined as live microbial food supplements or components of bacteria which have been shown to have beneficial effects on human health [4]. Some of the health benefits which have been claimed for probiotics include the following: Diarrhea Diseases, Radiation induced diarrhea, Inflammatory Bowel Disease, Crohn's disease, Ulcerative colitis, Prevention of colon cancer, Lactose Intolerance, Irritable Bowel Syndrome (IBS), Pouchitis, Constipation, *Helicobacter pylori*, Pancreatitis, Hepatic encephalopathy, Nonalcoholic fatty liver disease (NASH), Allergy, Urogenital infections and HIV, Probiotics in Pregnancy, reduction of serum cholesterol and improvement of the normal microflora [5]. Certain strain of bacteria has been discovered over the years to have probiotic properties, mainly consisting of lactic acid producing bacteria (*Lactobacilli*, *Streptococci*, *Enterococci*, *Lactococci*, *Bifidobacteria*), Bacillus and fungi such as *Saccharomyces* and *Aspergillus* [6]. The key enzyme of hexose catabolism in

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Bifidobacterium is the fructose-6-phosphate phosphoketolase which splits the hexose phosphate to erythrose-4-phosphosphate and acetyl phosphate. Several species, including *Bifidobacterium adolescentis*, are members of the normal intestinal tract and appendix, dental caries and vagina flora of healthy humans. Other species, such as *Bifidobacterium animalis* subsp. *lactis and Bifidobacterium urinalis*, are commonly isolated from dairy products as well as from human urine and they play an important role as probiotics in human and animal nutrition [2]. *Bifidobacterium* produces acids, hydrogen peroxide, bacteriocins and biosurfactants, and thus confers protection of the host. Also *Bifidobacterium* as a natural vaginal flora plays an important role in the reproductive health of woman by maintaining acidic vaginal PH, providing colonization resistance and preventing the growth of pathogens [7-9]. The acidic PH itself act as a natural defense against sexually transmitted disease and AIDS [10,11]. The lactic acid Bacteria (LAB) is one of the most important groups of microorganisms to mankind, being involved in the products (cheese, yogurt and kefir) [12] and stool, blood, urine, dental caries [2]. The aim of this study was to isolate *Bifidobacterium animalis*, *Bifidobacterium adolescentis* and *Bifidobacterium bifidum* from fresh stool by PCR and to examine their antimicrobial activity against some microorganisms such as *Salmonella typhi*, *Shigella dysenteriae*.

2. Material and Method

In total, 130 samples were collected by wet swabs and kept in sterile tubes containing MRS broth media. The sources of samples were fresh stool. The entire sample tubes where Man-Rogosa-Sharpe medium incubated at 37˚C and 5% CO_2 conditions for 1days, then subcultured on (MRS) agar (Hi-media, India) for 24 h. The colonies were characterized by phenotypical properties including morphology, gram positive staining and absence of catalase, oxidase and motility [13]. The DNA of the bacteria was extracted from single colonies after growing the *Bifidobacterium* on MRS agar under anaerobes conditions overnight as described previously [14]. For preliminary detection of *Bifidobacterium*, the PCR assay was performed using the primers cited in **Table 1**.

The composition of PCR mixture was 50 mM KCl, 10 mM HCL (PH: 8.5), 1.5 µl $MgCl_2$, 12 µl dNTPs, 2 µl of each primer, 0.5 µl of Taq polymerase and 10 µl of DNA template in final volume of 25 µl. The PCR conditions were initial denaturation one cycle of 94˚C for 5 min, followed by 35 cycles of 94˚C for 20 s, annealing of 55˚C for 20 s, extension of 72˚C for 30 s and then a final extension at 72˚C for 5 min for *Bifidobacterium adolescentis* and *Bifidobacterium bifidum* and for *Bifidobacterium animalis* 9 min at 94˚C for initial denaturation and 35 cycles of 30 s at 94˚C for denaturation, 30 s at 62˚C for annealing, 30 s at 72˚C for extension, followed by 10 min at 72˚C for a final extension using a thermocycler (Techgene, UK). The PCRproducts were analyzed on 1% agarose gel. The confirmed species by PCR were tested for antimicrobial properties. Antimicrobial compound was isolated using ethyl acetate solvent from *Bifidobacterium adolescentis* and *Bifidobacterium bifidum* separately. After 4 days incubation, the MRS broth media containing bacteria was mixed with ethyl acetate and agitated with a magnatic stirrer for 1 day. Then the media was allowed to settle for 30 min. following settlement, the solution was separated into two phases, which the supernatant was comprised of the extracted antimicrobial compound. The color of ethyl acetate was turned yellow after agitation. The supernatant then was dried at 45˚C. The quantity of antimicrobial compound was determined as 70 mg.

The Minimal Inhibitory Concentration (MIC) of this antimicrobial substance determined using modified E. test, by incorporating 20 µl of the each extract in paper discs. The final concentration of the extract in disc was estimated as 1.06 mg in total incorporated volume [17].

Table 1. The primers used in this study [15,16].

Species	primer	size
Bifidobacterium spp.	Pbi F1:5'-CCGGAATAGCTCC-3' Pbi R2:5'-GACCATGCACCACCTGTGAAA-3'	(914 bp)
B. animalis	Ban F2:5'-AACCTGCCCTGTG-3' Pbi R1:5'-GCACCACCTGTGAACCG-3'	(925 bp)
B. adolescentis	BIA-1:5'- GGAAAGATTCTATCGGTATGG-3' BIA-2:5'-CTCCCAGTCAAAAGCGGTT-3'	(244 bp)
B. bifidum	BiBIF-1:5'-CCACATGATCGCATGTGATTG-3' BiBIF-2:5'-CCGAAGGCTTGCTCCCAAA-3'	(278 bp)

The standard strains of *B. animalis*, *B. adolescentis* and *B. bifidum* as positive controls were included in each set of PCR amplification; DNA template in a final volume of 25 µl. All the reagents were purchased from Cinnagen Company, Tehran, Iran.

The target bacteria were some clinically isolated pathogens including *Shigella dysenteiae*. The standard strains which were included in the study *Salmonella typhi* (*PTCC* 1609), staphylococcus aureus (PTCC 1112), and Bacillus cereus (PTCC 1247) (Persian type culture collection (PTCC)) obtained from collection center of fungi and bacteria, Tehran, Iran. The experiments were repeated 3 times and the results were constant in all tests.

The E. test and ANOVA variance were used for data analysis by application of SPSS software (SPSS Inc. No.15, Chicago, IL, USA).

3. Results

All of the preliminary isolated *Bifidobacterium* were subjected to PCR for confirmation of specific primers and species—specific primers. Using species-specific, 11 of them were confirmed *Bifidobacterium bifidum* (**Figure 1**) and 6 were *Bifidobacterium adolescentis* (**Figure 2**). The confirmed strains by PCR were tested for their antimicrobial properties.

The activity of antimicrobial substances was tested against target pathogens after adjustment of pH at 7 using 5M NaOH. The antimicrobial compounds showed potent inhibitory activity against two tested bacteria *Salmonella typhi* and *shigella dysenteiae*. The MICs of two selected antimicrobial compounds obtained from *Bifidobacterium bifidum* were determined using modified E. test [17] (**Figures 3** and **4**).

The important point of this study was isolation the *Bifidobacterium* species from fresh stool. In the other hand we know that these bacteria are probiotic and can protect our body against the pathogenic bacteria. The obtained results revealed that the mentioned stools have many *Bifidobacterium* and *Lactobacillus* and these bacteria produce antibiotic in addition the organic acids and hydrogen peroxide. The antimicrobial compounds isolated from *Bifidobacterium adolescentis* and *Bifidobacterium bifidum* showed activity against some pathogenic bacteria such as *Salmonella typhi* and *Shigella dysenteriae*. The obtained MICs were between 25 - 400 µl ML (**Table 2**). The considerable point in this study was extraction of a compound with antimicrobial activity which was only dissolved in ethyl acetate but was non-dissolvable in water solvents.

4. Discussion

Bifidobacterium can inhibit the growth and attachment of pathogens to epithelial cells. These organisms are produced compounds as hydrogen peroxide and bacteriocin-like that can kill the pathogenic microorganisms in human body [9,18,19]. In a study that was performed by Shuhaimi *et al.* [20] *Bifidobacterium infantis G*4 isolated

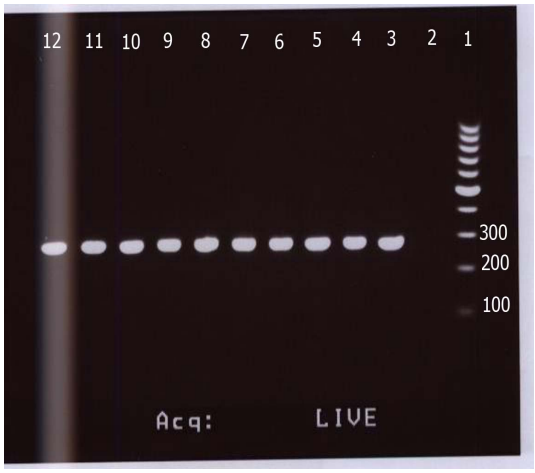

Figure 1. Agarose gel of PCR products amplified (278 bp) by species-specific primers of *B. bifidum*, 1: marker 100 bp, 2: Control negative, 3: control positive, 4 - 12: positive isolates.

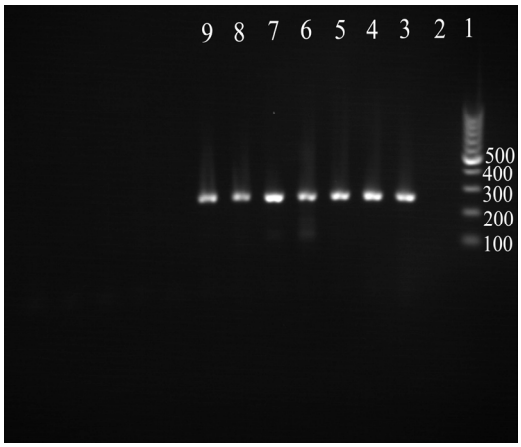

Figure 2. Agarose gel of PCR products (244 bp) amplified by species-specific primers of *B. adolescentis*, 1: marker 100 bp, 2: Control negative, 3: control positive, 4 - 9: positive isolates.

from infant stool, and was tested for their antibacterial activity, antimicrobial susceptibility and adherence properties to human colon carcinoma HT29 cell lines. The isolate was observed to be effective in inhibiting the growth of pathogens namely *Salmonella enterica* ssp. *enterica* serovar Enteritis, *vibrio cholera*, *Escherichia coli*, *Bacillus cereus*, *Pseudomonas aeruginosa and Listeria monocytogenes*. S. Kozhakhmetov [21] reported detection of *Bifidobacterium spp*. (*Bifidobacterium adolescentis* 180, *Bifidobacterium breve* 204, *Bifidobacterium breve* 584, *B. breve* 587) to be effective in inhibiting the growth of pathogens namely *E. coli*, *P. mirabilis*, *P. mirabilis*, *S. aureus*, *Salmonella enterica* subs. *enterica* serovar Typhimurium, *Bacillus spp. A*.

In a study, *Bifidobacterium longum* (NCFB 2259) was

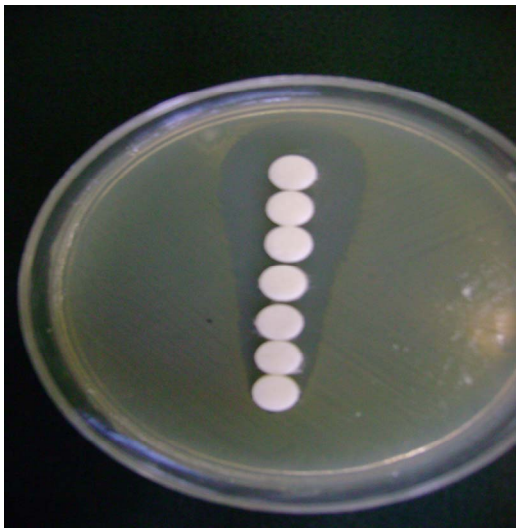

Figure 3. E test representing MIC of antimicrobial compound obtained from *B. bifidum* against *Shigella dysenteriea.*

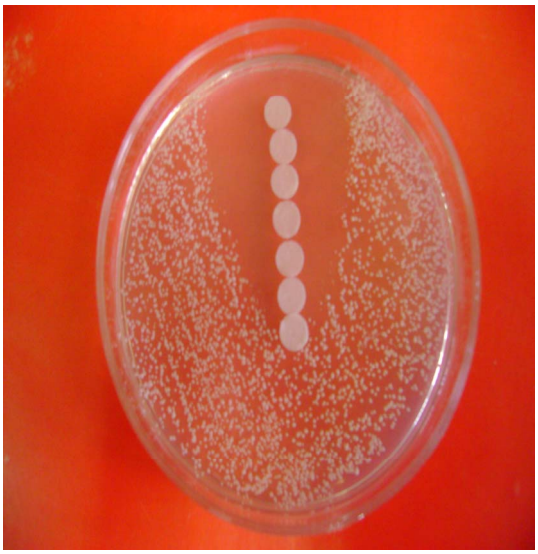

Figure 4. E test representing MIC of antimicrobial compound obtained from *B. bifidum* against *Salmonella typhi.*

effective in inhibiting the growth of pathogens *E. coli* O157:H7, one of the leading causes of bacterial food borne diseases [22]. It is expected that the antimicrobial compound of *B. adolescentis* was not much different from active compounds *Bifidobacterium bifidum*. The MIC values for gram negative and gram positive indicated that gram positive and negative bacillus were more sensitive to *bifidobacterium* antimicrobial compound than gram positive cocci.

5. Conclusion

The objectives of this study showed that about 13% of healthy peoples in Ahvaz city—Iran can be supported from rectal pathogens by *Bifidobacterium* probiotics but others are at risk of being attacked by harmful microbes. The food containing probiotics may be colonized by the *Bifidobacterium* and Lactobacillus species in the rectum through oral—fecal track.

Table 2. MIC of antimicrobial compound obtained from *B. bifidum* and *B. adolescentis* against pathogenic bacteria using E. test.

Bacteria	MIC ($\mu g \cdot mL^{-1}$)	
	B. bifidum	*B. adolescentis*
Salmonella typhi	200	200
Shiglla dysentriae	200	200
Bacillus cereus	120	140
Staphylococcus aureus	180	220

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