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# Assessment of probiotic potential and antagonistic characteristics of *Bifidobacterium bifidum* isolated from infant feces against enteropathogenic *Escherichia coli*

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**Abstract:** Enteropathogenic microorganisms like *Escherichia coli* cause severe intestinal problems by disrupting the gut homeostasis. The live microorganisms, when given in adequate quantities provide several beneficial effects to the host are known as probiotics. One of the pronounced benefits conferred by probiotic is to antagonize the growth of enteropathogens competing for adherence to the intestinal epithelium. *Bifidobacterium* is the major genera of human especially infant are intestinal microbiota. In current study, *Bifidobacterium bifidum* was isolated from the infant stools and probiotic potential was assessed using prescribed tolerance tests against low pH, gastric juices and bile salts. Anti-infectious activity of probiotic *Bifidobacterium bifidum* against enteropathogenic *E. coli* was checked both *in vitro* and *in vivo* using agar well diffusion assay and mice model respectively. Mice feces were evaluated for both *Bifidobacterium bifidum* and *E. coli* counts in all groups and analyzed statistically. *In vitro* results showed *Bifidobacterium bifidum* possess marked antibacterial activity against *E. coli.* There was significant decrease in enteropathogenic *E. coli* burden in the mice group fed with *Bifidobacterium bifidum* before and after challenge. In conclusion, the endogenous *Bifidobacterium bifidum* have excellent probiotic potential and can be used prophylactic and treatment option against enteropathogens.

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**Keywords:** *Escherichia coli; Bifidobacterium bifidum;* probiotics; enteropathogens

## 1. Introduction

Probiotics are viable microorganisms which are useful for the health and well-being of the host when used in proper amount. Natural ways of controlling disease causing organisms and appearance of antimicrobial resistance bacteria has led to the concept of probiotics which control unhealthy fermentation in gastrointestinal tract and produce beneficial effects for health of host by balancing the intestinal microflora [1]. *Lactobacillus* and *Bifidobacterium* are most widely used genera as probiotics which are normal inhabitants of human and animal colon. These both genera have non-pathogenic species and they are beneficial for health of host. Gut of the newborns especially on breast feeding is colonized with *Bifidobacterium species* within days after birth. *Bifidobacteri*um was first isolated from feces of breast-fed infants [2].

Probiotics tolerate conditions of gastrointestinal tract and ultimately colonize in the intestines. Their useful effects are related to prevention of disease-causing microorganisms by colonization in the gut and to achieve this purpose probiotic bacteria must have some special characteristics. Some physical and chemical barriers such as gastric juice, low pH and bile salts in the gastrointestinal tract are present. To be useful for health of host they must overcome these barriers [3]. Throughout the world it has been observed that the children are more infected with enteropathogens. Epithelium of intestines provides the primary defense to the organism as it acts as a barrier against macromolecules and pathogens. The layer of mucus and the microbes residing in the gastrointestinal tract protect the mucosa of gut from the pathogens to adhere and invade [4]. Subsequently, probiotics are proposed for the treatment and prevention of infections of gastrointestinal tract [5]. Due to their several characteristics and properties of health promoting *B. bifidum* have gained a great importance [6]. Many *Bifidobacterium* strains are being used now a days in food products as probiotics [7]. These probiotics must possess several characteristics, the most important is that they must have human origin. Therefore, to understand the health promoting characteristics of *B. bifidum* and for selection as a probiotic, its ingenuous isolation from fecal material is required [8].

Diarrhea remains the persistent cause of death in young children below 5 years, accounting 1.3 billion deaths annually [9]. The leading cause behind these deaths is enteropathogenic *E. coli,* one of the members of diarrheagenic *E. coli* pathotype with high prevalence in community and hospital setting [10]. With every passing year, it has become a major problem due to diversity of its habitat and carrying lethal genes including antibiotic resistance genes and Shiga toxins [11]. As enteropathogenic *E. coli* (EPEC) cause characteristics lesions in intestinal epithelium so it is included in group called as effacing and attaching pathogenic organisms. For the first time in 1955, infantile diarrhea caused by *E. coli* strains were termed as EPEC. Initially EPEC strains were classified on serotype basis but pathogenic properties are used for classification. [12].

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An intricate environment has been established by the microorganisms of human intestine recognized for its influence on safety and wellbeing of human [13]. Within the intestinal microbiota more than 400 species can be recognized which may achieve almost as above as $10^{12}$ g/L population level in the large intestine [14]. For ability to protect against diarrhea and several diseases *Bifidobacterium* microflora has been predominantly studied. Being the principal gastrointestinal microorganism in animals and humans, *Bifidobacteria* have attracted considerable consideration in current years [15]. These bacteria inhabit the neonatal intestine from the first week after birth and reside the gastrointestinal tract throughout life, where they contribute to human health and safety [6]. Keeping in view the context, the present study was designed to assess the probiotic and antagonistic characteristics of *B. bifidum* isolated from infant feces against enteropathogenic *Escherichia coli* (EPEC).

## 2. Materials and Methods

### 2.1. Ethical Approval

This study was approved by The Institutional Bioethics and Biosafety Committee of the University of Agriculture, Faisalabad (D/No. 1472/ORIC). This study was conducted in accordance with the Declaration of Helsinki.

### 2.2. Sample collection and Isolation

Infant stool samples were collected from different hospitals of Pakistan using a sterile container. After the collection, the samples were transported to Probiotics and Food Microbiology laboratory at Institute of Microbiology using cold chain and maintained at 4˚C until further processing. Samples were homogenized followed by 10 fold serial dilution [16] and 0.1ml of the final dilution of each sample was inoculated on de Man, Rogosa and Sharpe (MRS) (Sigma-Aldrich) agar plates containing 0.05% L-cysteine (w/v), a selective medium for Bifidobacterium and incubated for 48 h at 37˚C under microaerophilic/anaerobic conditions using anaerobic atmosphere generation bags (Anaerogen, Oxoid). Diarrheagenic *E. coli* (EPEC) ATCC 43887 and other species of *Pseudomonas*, *Salmonella* and *Staphylococcus* were procured from Institute of Microbiology, University of Agriculture Faisalabad, Pakistan.

### 2.3. Phenotypic characterization

Microscopy following Gram's staining was performed to check the size, shape and Gram's reaction. Biochemical characteristics were done using list of biochemical reactions [17].

### 2.4. Determination of Probiotic Properties

#### 2.4.1. Resistance at low pH

Bacterial colonies were grown in selective broth i.e. MRS media at 37˚C for 24 hours using anaerobic conditions. Bacterial cells were collected using normal saline

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(0.9% w/v) solution, up to the point of optical density was 2 at 600 nm ($OD_{600nm}$). Bacterial cells were suspended in various buffers of different pH i.e. glycine-HCL pH 2.0; glycine- pH 3.0 and sodium phosphate pH 7.0 as a control. These preparations were incubated at 37˚C for 12 hours. After incubation, viable counts were determined by plating 10-fold serial dilutions on MRS agar plates. Resistance to low pH was determined by the percentage survival after the incubation at low pH [18]

2.4.2. Bile Salt Hydrolase Activity (BSH)

Bacterial culture was inoculated on selective broth supplemented with different concentrations of bile salts (0.5% & 1%) and 0.2% (w/v) glycodeoxycholic acid (GDCA, Sigma) and 0.37 g/L of $CaCl_2$ and incubated at 37˚C for 72 hours without agitation in anaerobic condition. After incubation viable count was determined following spread plate method. The presence of glare around colonies indicates the BSH activity. Resistance to bile was determined by the survival percentage of viable counts [19]. MRS agar plates without supplementation were used a negative control.

2.4.3. Resistance to simulated gastric juice

Simulated gastric juice (SGJ) was prepared by suspending pepsin in sterile saline and adjusted to pH 2.0 with concentrated HCl. The tolerance of the tested bacteria to simulated gastric was recorded [20].

2.4.4. Antibacterial activity- In vitro assay

Antagonistic activity of the isolated *Bifidobacterium bifidum* against enteropathogenic *E. coli* (EPEC) and other selected bacteria was determined by the agar well diffusion method [21]. Briefly nutrient agar medium was seeded with an overnight 1% (v/v) culture of target EPEC and poured into a sterile petri plate and allowed to solidify at room temperature. Wells were made in the solidified agar using a sterile metal borer and filled with supernatant of *Bifidobacterium bifidum* (100 µL/well) prepared from overnight fresh cultures. Same protocol was done for other bacterial species. The plates were allowed for diffusion and incubated at 37˚C for 24 h. Inhibitory zone was measured and recorded [21].

*2.5.* Preparation of infant milk and the fermented milk

Milk powder was dissolved in distilled water. Two different volumes were taken; one was fermented with isolated *B. bifidum* and other one was prepared by adding enteropathogenic *E. coli* inoculum. Both preparations were incubated at 37 ºC [22].

2.5.1. *In vivo* studies in mice as an experimental model

The trials were carried out on mice of the same species and of the same age. Mice were kept separately in metal cages in animal house facility at Institute of Microbiology. The mice were fed on basal diet ad libitum. Total four groups (three experimental and one control group) were made; each consisting of three mice. In control group mice did not

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receive milk inoculated with *B. bifidum* and *E. coli*. In group 1 mice received milk inoculated with *E. coli* during the 1st week of trials. While in group 2 the mice received milk inoculated with *B. bifidum* during the 1st week and fermented milk with *E. coli* during the 2nd week. Whereas in group 3, mice received milk inoculated with *E. coli* during the 1st week then received fermented milk with *B. bifidum* [23].

*2.6.* Fecal analysis

Feces were collected at regular intervals after treatment during the period of the fermented milk treatment. *B. bifidum* and *E. coli* were counted during and after treatments for a period of two weeks and morbidity was recorded.

## 3. RESULTS

*3.1*. Isolation and identification of *Bifidobacterium bifidum*

On MRS agar plates colonies were white, creamy and convex with even margins. Based on the morphological characteristics, the presumptive *Bifidobacterium* were selected and picked from plates of MRS agar. Microscopy results illustrated a typical morphology of *Bifidobacterium* appearing as paired long and short rods or small chains. Biochemical characterization confirmed the presence of *B. bifidum* (Table 1).

3.2. Probiotic characteristics of isolated strains

Probiotic evaluation requires extensive *in vitro* and *in vivo* investigation. Many *in vitro* models can simulate with good approximation the strains surviving abilities in the GIT and confer a health benefit to the host. Such tests include investigations of the resistance to gastric acidity, bile salts, tolerance to gastric juice etc.

3.3. Resistance of *B. bifidum* at acidic pH

One of the main properties of probiotic bacteria is its survival capacity in different digestive conditions. Tolerance to acidic environment by isolated *B. bifidum* was determined. Tolerance level was significantly variable at different pH values. Test results were collected after six replications. The selected strain does not lose his viability when exposed to various pH of 2.0, 3.0 and 7.0. Mean value of CFU/mL at pH 7 was $2.57\times10^5$ while mean value of CFU/mL at pH 2 was $2.71\times10^5$. It was concluded that *B. bifidum* show tolerance to acidic environment for a period.

3.4. Bile salts hydrolase activity of *B. bifidum*

To reach the colon in viable state, strains must cope with conjugated bile salts stress in the upper small intestine [24]. Tolerance to bile salts by isolated *B. bifidum* was tested by plate count method after 72 hours of incubation in several replications. Mean value was calculated and compared with control (no bile salts). Mean value of CFU/mL for control was recorded as $1.04\times10^6$ and for bile salts as $1.13\times10^6$. This study

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demonstrated that *B. bifidum* can hydrolyze the conjugated bile salts and hence, can survive in the presence of bile salts.

3.5. Tolerance to gastric juice

Gastric juice is also an important stress that must face probiotics to remain viable and have a good probiotic effect. Tolerance to gastric juice by isolated *B. bifidum* was tested after 90 minutes of incubation and mean value was calculated and compared with control (no gastric juice). Mean value of CFU/mL for control was recorded as $2.14\times10^7$ and for gastric juice as $2.53\times10^7$. It was concluded that *B. bifidum* show tolerance to gastric juice.

3.6. Antibacterial activity

The ability to eliminate pathogenic strains in the host is an important characteristic that probiotic strains should possess in the maintenance of a balanced gut microflora. Antibacterial activity against enteropathogenic *E. coli* and some other bacteria was determined using the well diffusion method. *B. bifidum* exhibited varying degrees of inhibitory activity against *Escherichia coli*, *Staphylococcus* aureus, *Salmonella enterica* and *Pseudomonas spp.* as presented in Table 2. *B. bifidum* showed very strong inhibitory activity against reference strains of enteropathogenic *E. coli*. Strong and moderate inhibitory activity was recorded against *Salmonella enterica* and *Staphylococcus aureus* respectively while there was no inhibitory activity against *Pseudomonas* spp.

3.7. Microbial analysis of *in vivo* mice experimental model

3.7.1. Viable bacterial count (VBC)

Feces of mice were statistically analyzed with altered treatments of *B. bifidum* and *E. coli* for viable bacterial count presented in Table 3. It was suggested by the statistical results that the viable bacterial count of the fecal samples of mice was significantly different ($P<0.05$) amongst all groups under study. The statistical results indicated that the viable bacterial count of the mice fecal samples differ significantly ($P<0.05$) among all groups (Table 3).

3.8. Post-mortem analysis

After dissecting the mice, observations of gastrointestinal tract particularly small intestines as presented in Figure 1.

## 4. Discussion

Bifidobacteria are the normal inhabitant of intestinal tract. In the present study, *Bifidobacterium bidium* was isolated from infant feces. High number of *B. bifidum* found in stools of breast-fed infants, identified and confirmed through phenotypic and

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biochemical charaterization. A typical morphology was shown by *B. bifidum* as they appeared as paired long and short rods or small chains and bifid (Y or V shaped) morphology. The results were in accordance with the findings of [25,26].

Probiotic evaluation requires extensive *in vitro* and *in vivo* investigation. Many *in vitro* models can simulate with good approximation the strains surviving abilities in the GIT and confer a health benefit to the host. Acid and bile tolerance represent basic *in vitro* selection criteria for probiotics [27,28]. The former can reach values as low as pH 1.5 and a good probiotic source should withstand at least pH 3.0 [29]. In current study, selected probiotic strains demonstrated good resistance against low pH and bile salts which partially guarantee their survival in the gastrointestinal tract. As already pointed out by various workers in the field *in vitro* studies can only partially mimic the actual in situ conditions in the gut ecosystem [30]. Yet, such *in vitro* systems remain powerful tools especially for screening numerous samples.

Bacterial probiotic investigators have previously determined a testing concentration of 0.3% or 0.15% of bile salts (w/v) [29,31,32], thus, the striking feature if this study was that strains were tested with 0.5% and 1% of bile salts. The results conferred a differential behaviour of *B. bifidum* from other strains isolated previously [32-34]. Nonetheless, the tested concentration of 0.5 % bile salts is within the range of the physiological concentrations of bile and has been used previously by other researchers to mimic the small intestine environment [35]. Various Bifidobacterium strains from human and non-human origins showed tolerance to such concentration [36-38] whereas fewer strains showed tolerance to 1% [28]. This ability to survive in higher bile salt concentration may suggest its use in specific condotions.

The activity of *B. bifidum* to enteropathogenic *E. coli* was determined using the well diffusion method. The result illustrated a significant zone of inhibition against the EPEC as compared to other strains of bacteria selcted in the study. Jin et al., disseminated similar results proving variable zone of inhibition against selected pathogenic strains of bacteria [21].

Feces of mice were statistically analyzed with altered treatments of *B*. *bifidum* and *E. coli* for viable bacterial count. It was suggested by the statistical results that the viable bacterial count of the fecal samples of mice was significantly different ($P<0.05$) amongst all groups under study. The statistical results indicated that the viable bacterial count of the mice fecal samples differ significantly ($P<0.05$) among all groups. In the current studies the significant differences in the counts of *B. bifidum* and *E. coli* were observed that were in accordance with the studies by Fliss et al [23]. Upon microbiological analysis *B. bifidum* counts were significantly increased during the period of offering milk fermented with *B. bifidum* suggested that it has resisted passage of gastro intestinal tract throgh adhesion and colonization confering the probiotic potential. Our study also illustrated that *B. bifidum* administration to mice cause significant decline in the enteropathogenic *E. coli*

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in feces of mice suggesting the antipathogenic activity of *B. bifidum* against EPEC. Same results were obtained in the findings of a study by [22]. Macroscopic evaluation of small intestinal contents of all groups of mice showed differences in color, texture, shape and odour of the carcass and these results were in accordance with the findings of [22].

## 5. Conclusions

In conclusion, the isolated human *B. bifidum* exhibited high acid and bile tolerance and antibacterial activity against enteropathogenic *E. coli*. These strains suggest the great potentiality of probiotics in controlling infection by enteropathogenic *E. coli* in humans. The combination multiple strains in the presence of gut microflora challenged with other enteric pathogens should be further investigated.

## 6. Acknowledgements

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## 7. Author contributions

All authors contributed to data analysis, preparing, and drafting the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work. Conceptualization Dr. Muhammad Ashraf and Dr. Rizwan Aslam; Methodology, Rehana Kanwal and Sadia Munir; Investigation, Dr. Sultan Ali; Resources, Dr. Muhammad Ashraf; Data Curation, Dr. Iahtasham Khan and Mubarik Ali; Writing – Original Draft Preparation, Rehana Kanwal and Sadia Munir; Writing – Review & Editing, Ghazanfar Abbas; Visualization, Ali Abbas; Supervision, Ghazanfar Abbas; Project Administration, Muhammad Ashraf; Funding Acquisition, Ihtasham Khan."

## 8. Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

**Table 1.** Cultural, Morphological and Biochemical characteristics of the isolates.

| **Test parameters** | **Sample number** | | | **Reference strain** |
|---|---|---|---|---|
| | **FS1** | **FS2** | **FS3** | ***(Bifidobacterium bifidum)*** |
| MRS agar plates (colonies) | White circular, entire margin | Creamy, small circular, entire margins | White, small | Circular, white, creamy with entire margins |

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| Morphological examination | Gram-positive rods, chains | Gram-positive rods | Gram-positive rods and Gram-positive short chain | Gram-positive rods |
|---|---|---|---|---|
| Indole production test | - | - | - | - |
| Nitrate reduction test | - | - | - | - |
| Catalase test | - | - | - | - |
| Oxidase test | - | - | - | - |
| Gelatin liquification test | - | - | - | - |
| Lactose fermentation | + | + | + | + |
| Sucrose fermentation | - | + | - | + |
| Sorbitol fermentation | - | - | + | - |

**Notes:** (-) = negative; (+) = Positive

**Table 2.** Antagonistic effect of isolated *Bifidobacterium bifidum* against pathogenic bacteria.

| **Bacteria** | **Zone of inhibition (mm) ±SD** |
|---|---|
| *Escherichia coli* | 14.6 ±0.58 |
| *Staphylococcus aureus* | 7.3± 0.58 |
| *Salmonella enterica* | 13± 1 |
| *Pseudomonas spp.* | N/L |

**Table 3:** Mean **±** (SEM) values of *B. bifidum* count ($10^9$ CFU/mL) and *E. coli* count ($10^9$ CFU/mL) in different groups before and after treatment in 14 days trial.

| Group | Mean ± SEM values of *B. bifidum* count Before treatment | Mean ± SEM values of *B. bifidum* count After treatment | Mean ± SEM values of *E. coli* count Before treatment | Mean ± SEM values of *E. coli* count After treatment |
|---|---|---|---|---|

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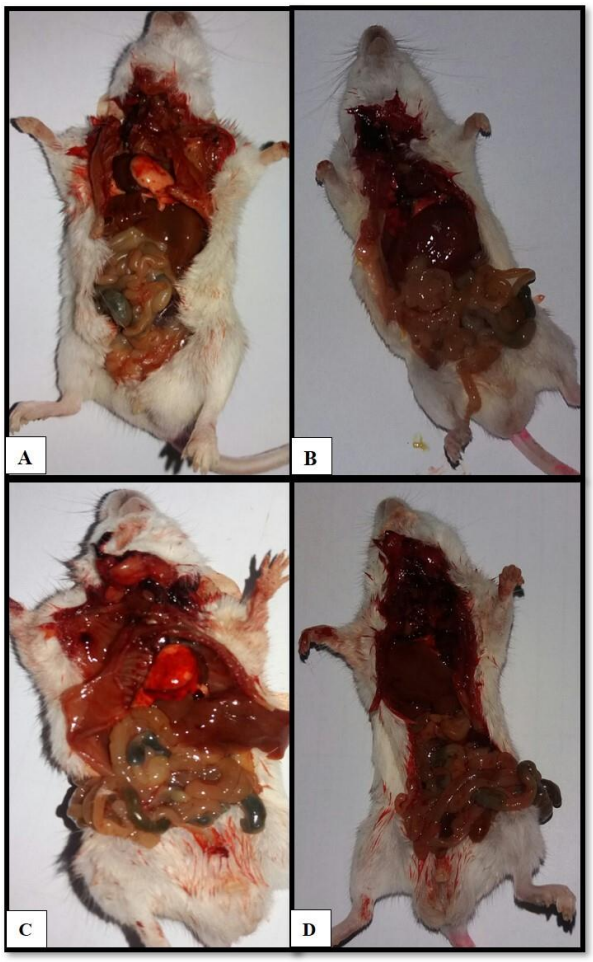
| | | | | |
|---|---|---|---|---|
| Control | $1.15\pm0.02^{BC}$ | $0.94\pm0.03^{C}$ | $28.2\pm0.26^{B}$ | $251.67\pm1.52^{B}$ |
| Group 1 | $1.12\pm1.00^{B}$ | $0.90\pm0.01^{C}$ | $2.56\pm0.15^{C}$ | $291.33\pm3.21^{A}$ |
| Group 2 | $1.19\pm0.06^{A}$ | $211.33\pm1.52^{B}$ | $28.567\pm0.20^{AB}$ | $3.43\pm5.13^{C}$ |
| Group 3 | $1.2100\pm0.02^{B}$ | $290.33\pm1.52^{A}$ | $28.867\pm0.15^{A}$ | $0.20\pm2.08^{C}$ |

**Notes:** Means sharing different superscripts are statistically significantly different at ($P<0.05$)

**Figure 1: Characteristic morbidity lesions in different groups of mice.**




**Notes: Control group (A):** Samples presented uniformity in size as well as intestinal lining was also in good condition**, Group 1 (B):** Dark brownish color was presented by the small intestines which accompanied by foul odor while dissecting the mice. Shrinkage of the contests of small intestine was also present, **Group 2 (C):** Contents of small

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intestine presented almost the same characteristics as those observed in the control group**)** and **Group 3 (D):** In this group the changings in the shape and color as well as contractions were less as compared to those in 1st and 2nd groups.

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