Effect of prebiotics on bifidobacterial species isolated from infant faeces

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The present study was conducted to evaluate the effect of prebiotics on the growth of bifidobacterial species isolated from the faeces of breast fed infants. Identification of isolates to the genus level was based on phenotypic characteristics like the unique pleomorphic morphology and carbohydrates fermentation profile. Molecular tools like species specific mPCR primers were used for confirmation. A total of 4 isolates were identified namely \textit{B. longum} (IB$_{10}$ and IB$_{12}$), \textit{B. breve} (IB$_{39}$) and \textit{B. bifidum} (IB$_{42}$). Addition of 4 % WPC was used to enhance the growth of \textit{Bifidobacteria} in bovine whey. Prebiotics like inulin and honey at 0.4 and 3 %, respectively were adjudged as the optimum level for exerting maximum prebiotic activity on all the four bifidobacterial isolates.

**Keywords:** Prebiotics, Bifidobacterial, Infant, Bacteria

**IPC Int. Cl.**: C12M, C12N, A01K 1/01, B62B 7/00, B62B 9/00

Bifidobacterial species are common members of the infant gut where they form up to 91 % of the total microflora in breast-fed babies and up to 75 % in formula fed infants. Tissier’s discovery of \textit{Bifidobacteria} in breast-fed infants played a key role in establishing the concept that specific bacteria take part in maintaining health. There are about 29 bifidobacterial species that have been identified and among them eleven species have been isolated from infant faeces. The most frequently isolated \textit{Bifidobacterium} species in infant faeces are \textit{B. bifidum}, \textit{B. longum}, \textit{B. infantis} and \textit{B. breve}. These organisms which are gram positive, non motile, non spore forming, anaerobic, pleomorphic rods play a significant role as probiotics in controlling the pH of the large intestine through production of lactic and acetic acid thereby restricting the growth of many potential pathogens and putrefactive bacteria. Until recently the isolates of \textit{Bifidobacterium} have been identified to the genus level by using gram staining, morphological observations and end product analysis of glucose metabolism as indices. Since the conventional method of identification methods require a large amount of time and labour molecular techniques are being widely employed. Recent research has led to rapid advances in the application of molecular techniques based on 16S rDNA and 23S rDNA gene sequences to study the diversity of \textit{Bifidobacteria} in ecosystem.

Whey is a biological source of most valuable proteins and is rich in minerals and vitamins especially Vitamin B$_2$. It is an important source of lactose, serum proteins and soluble vitamins, which makes this product to be considered as a functional food. Whey-dominant formulas promote better growth of \textit{Bifidobacterial} species than casein-dominant formulas. Colonic foods, which encourage the growth of favourable bacteria, are referred as prebiotics. They have been defined as non digestible food ingredients that beneficially affect the host by selectively stimulating the growth or activity of one or a limited number of bacteria in the colon and this improve host health. Oligosaccharides such as lactulose, galacto oligosaccharides, inulin, fructo oligosaccharides (FOS) and other food carbohydrates are some of the examples of prebiotics. Breast milk contains prebiotic oligosaccharides that are fermented by colonic \textit{Lactobacilli} and \textit{Bifidobacteria} stimulating their growth and activity. This helps to develop and mature the intestinal immune function. Prebiotics like FOS and inulin occur naturally in a variety of fruits, vegetables, grains, Jerusalem artichoke, onions, garlic, wheat, honey, tomatoes, asparagus, barley, and chicory.

In the present study prebiotics were used to study their effect on the growth of \textit{Bifidobacterial} species identified from infant faeces.
Materials and methods
Forty six fresh faecal samples collected from clinically healthy newborn infants of both the sexes born through normal delivery in the age group from 3 to 90 days in and around Madhavaram, North Chennai, Tamil Nadu, India were examined. About 1gm of freshly voided infant faecal samples collected in sterile sample vials containing Yoshioka broth, were plated on Yoshioka agar and incubated at 39°C for 48 hrs under anaerobic condition using Anaero gas pack (Hi media cat.no. LE002F). Presumptive individual colonies were selected for Gram’s staining, biochemical test and molecular identification using multiplex PCR specific primers. The data obtained were analyzed statistically.

Phenotypic identification
Phenotypic identification of bifidobacterial species was done by Grams staining and viewed using Nikon Model YS100 Binocular Microscope. ANAERO23 Test kit of LA CHE MA MIKRO-LA-TEST (Ref: 10003396 PLIVA LACHEMA Diagnostics, Czechoslovakia) was used for the biochemical characterization of Bifidobacterial species.

Molecular identification of bifidobacterial species
Genomic DNA was extracted from the 24 hrs presumptive bifidobacterial culture using Hi yield genomic DNA mini kit (Cat. No. YGB 100/YGB 300) from Real Biotech Corporation, India.

Multiplex PCR
Multiplex PCR with 5 primer sets designed was used for the detection of bifidobacterial species.

Whey was obtained by heat coagulating cow milk at with 1.5 % citric acid and then pasteurized (72°C for 15 seconds). Whey protein concentrate -70 was purchased from Mahaan Proteins Limited, New Delhi. Inulin from Vilco Industries, Mumbai was used as a prebiotic in this study. Agmark grade honey marketed by Himalayan Herbals India Ltd., was used as a source of prebiotic in the study. Individual tubes of Yoshika broth containing one percent inoculum of Bifidobacterium cultures supplemented with whey protein, inulin and honey, respectively was incubated anaerobically overnight at 39°C to study their prebiotic effect.

Results
Phenotypic identification
The Staining results and illustrations in Fig. 1 show Gram positive pleomorphic patterns. Out of 46 infant faecal samples, collected 6 showed biochemical characteristics typical of bifidobacterial species using Anaero–23 test kit. The results obtained were compared with those in standard tests for identification and the isolates were assigned to appropriate species. Thus, six isolates of bifidobacterial species were identified by their ability to ferment carbohydrates.

Molecular identification of bifidobacterial species
Multiplex polymerase chain reaction
Fig. 2 illustrates the use of Multiplex PCR for detection of species using species specific primer of the required product size. The present usage of multiplex PCR is based on the work of Dong et al. (2000). The multiplex primers included B. bifidum, B. breve, B. infantis, B. longum and B. adolescentis. Isolates IB10 and IB12 presented a product size of 467 bp, IB39 and IB42 presented a product size of 991bp and 1180 bp, respectively.

Effect of Whey Protein Concentrate (WPC to Yoshika broth) on the growth of bifidobacterial species
Table 1 shows the effect of addition of whey protein concentrate (to Yoshika broth) on the viability
of bifidobacterial species. Statistical analysis revealed a highly significant difference (P ≤ 0.01) in the viable count between control isolates and treated isolates. Enhanced count was noticed with 4 and 6% WPC but there was no significant difference between them.

**Effect of varying levels of inulin (to Yoshika broth) on the growth of bifidobacterial species**

The findings in the present study (Table 2) showed an enhanced viable count with the addition of inulin. From the table it is revealed that no significant difference (P ≥ 0.05) was noticed in the viable counts between 0.4, 0.6 and 0.8 levels of inulin. Among all the isolates, IB_{10} showed a highly significant (P ≤ 0.01) count.

Table 1—Effect of varying levels of whey protein concentrates on the growth of bifidobacterial species

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Control</th>
<th>2%</th>
<th>4%</th>
<th>6%</th>
<th>F Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IB_{10}</td>
<td>11.756±0.023</td>
<td>11.996±0.101</td>
<td>12.225±0.053</td>
<td>12.264±0.053</td>
<td>13.52**</td>
</tr>
<tr>
<td>IB_{12}</td>
<td>10.809±0.027</td>
<td>10.884±0.022</td>
<td>11.230±0.053</td>
<td>11.250±0.050</td>
<td>33.28**</td>
</tr>
<tr>
<td>IB_{39}</td>
<td>10.673±0.029</td>
<td>10.780±0.079</td>
<td>10.883±0.070</td>
<td>10.893±0.069</td>
<td>2.56NS</td>
</tr>
<tr>
<td>IB_{42}</td>
<td>10.650±0.043</td>
<td>10.835±0.056</td>
<td>11.055±0.044</td>
<td>11.085±0.032</td>
<td>20.26**</td>
</tr>
<tr>
<td>F Value</td>
<td>218.09**</td>
<td>67.03**</td>
<td>102.81**</td>
<td>132.87**</td>
<td></td>
</tr>
</tbody>
</table>

Average of six trials #log cfu/mlNS- No significant difference  
Small case superscripts show difference between replicates  
Capital case superscript shows difference between treatments

**Effect of varying levels of honey (to Yoshika broth) on the growth of bifidobacterial species**

From the Table 3, it is evident that isolates IB_{10}, IB_{12}, IB_{39} and IB_{42} showed a significant increase in viable count with 3 and 5% inclusion levels of honey when compared to 1% and control. The present study revealed no significant difference (P ≥ 0.05) in the viable count of isolates between 3 and 5% of honey in all isolates. Isolate IB_{10} shows maximum viable counts of 12.530±0.036 and 12.580±0.033 (log_{10} cfu/ml) with inclusion levels of 3 and 5% honey respectively followed by isolate IB_{12}. The isolates IB_{39} and IB_{42} showed no significant difference among them in the increasing level of inclusion of honey.

Table 2—Effect of varying levels of inulin on the growth of bifidobacterial species

<table>
<thead>
<tr>
<th>Isolate code</th>
<th>Control</th>
<th>0.2%</th>
<th>0.4%</th>
<th>0.6%</th>
<th>0.8%</th>
<th>F Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IB_{10}</td>
<td>11.756±0.023</td>
<td>11.991±0.057</td>
<td>12.360±0.076</td>
<td>12.363±0.077</td>
<td>12.392±0.077</td>
<td>18.80**</td>
</tr>
<tr>
<td>IB_{12}</td>
<td>10.809±0.027</td>
<td>10.984±0.072</td>
<td>11.334±0.060</td>
<td>11.357±0.047</td>
<td>11.391±0.063</td>
<td>21.65**</td>
</tr>
<tr>
<td>IB_{39}</td>
<td>10.673±0.029</td>
<td>10.860±0.080</td>
<td>11.188±0.051</td>
<td>11.197±0.051</td>
<td>11.225±0.061</td>
<td>18.20**</td>
</tr>
<tr>
<td>IB_{42}</td>
<td>10.650±0.043</td>
<td>10.748±0.055</td>
<td>11.142±0.057</td>
<td>11.176±0.057</td>
<td>11.176±0.055</td>
<td>20.68**</td>
</tr>
<tr>
<td>F Value</td>
<td>218.09**</td>
<td>72.07**</td>
<td>82.28**</td>
<td>91.20**</td>
<td>74.85**</td>
<td></td>
</tr>
</tbody>
</table>

Table shows the respective mean ± SE values of the effect of inulin (to Yoshika broth) on the viable count of bifidobacterial species (log_{10} cfu/ml)

**Effect of varying levels of honey on the growth of bifidobacterial species**

Table 3—Effect of varying levels of honey on the growth of bifidobacterial species

<table>
<thead>
<tr>
<th>Isolate code</th>
<th>Control</th>
<th>1%</th>
<th>3%</th>
<th>5%</th>
<th>F value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IB_{10}</td>
<td>11.756±0.023</td>
<td>11.910±0.018</td>
<td>12.530±0.036</td>
<td>12.580±0.033</td>
<td>217.00**</td>
</tr>
<tr>
<td>IB_{12}</td>
<td>10.809±0.027</td>
<td>11.102±0.045</td>
<td>11.268±0.020</td>
<td>11.277±0.016</td>
<td>54.00**</td>
</tr>
<tr>
<td>IB_{39}</td>
<td>10.673±0.029</td>
<td>10.716±0.040</td>
<td>10.876±0.080</td>
<td>10.977±0.113</td>
<td>3.68**</td>
</tr>
<tr>
<td>IB_{42}</td>
<td>10.650±0.043</td>
<td>10.738±0.543</td>
<td>11.250±0.085</td>
<td>11.100±0.068</td>
<td>12.20**</td>
</tr>
<tr>
<td>F Value</td>
<td>218.09**</td>
<td>177.00**</td>
<td>145.09**</td>
<td>117.56**</td>
<td></td>
</tr>
</tbody>
</table>

Table shows the respective mean ± SE values of the effect of honey at 1, 3 and 5% inclusion levels on the viable count of bifidobacterial species (log_{10} cfu/ml)

Average of six trials #log_{10} cfu/ml, Small case superscripts show difference between replicates  
Capital case superscript shows difference between treatments.
Discussion

The cell morphology in the present study depict various cell morphology patterns and is concurrent with the findings of many workers who described the morphology of *Bifidobacteria* as club shaped or with spatulated extremities, star like arrangement or disposed in "V" or palisade arrangement. The present study also affirmed the pleomorphic gram positive nature of *Bifidobacteria* uniform to branched, bifurcated ‘Y’ and ‘V’ forms, spatulate or club shape.

The fermentative characters of IB₁, IB₃₉, IB₁₈, IB₄₂, IB₁₀, IB₁₂, corresponded to the fermentative patterns of *B. breve, B. breve, B. bifidum, B. bifidum,* and *B. longum, B. longum* respectively described for *Bifidobacterium* species. *B. longum* fermented glucose, maltose, fructose, galactose, lactose, melizitose, sucrose, raffinose, and arabinose and showed negative reaction with nitrate, indole, salicin, mannitol, rhamnose, cellobiose and sorbitol. *B.breve* fermented glucose, maltose, fructose, galactose, lactose, sucrose, salicin, esculin and raffinose. But it did not react with melezitose, nitrate, rhamnose and arabinose. *B.bifidum* fermented glucose, fructose and galactose and did not ferment maltose, trehalose, mannitol, mannose, salicin, raffinose and xylose.

**Molecular identification of bifidobacterial species**

**Multiplex polymerase chain reaction**

The findings in the present study corroborate with the product size of the reference strains. The results thus confirmed 4 isolates IB₁₀ and IB₁₂ belong to *B. longum*, IB₃₉ and IB₄₂ belong to *B. breve* and *B. bifidum*, respectively. However, isolates IB₁ & IB₁₈ did not correspond to any of the primers and hence was not included for further study.

**Effect of Whey Protein Concentrate (WPC) on the growth of bifidobacterial species**

The result in Table 1 is in accordance to the work reported that addition of whey proteins may enhance the viability of probiotics, probably due to their buffering property. Thus enhanced *bifidobacterial* count was due to fortification with condensed WPC. The increased count proves that certain strains of *Bifidobacteria* grow very slowly in milk and require preformed simple protein and free amino acids for initiation of their growth. Since there was no significant difference between 4 and 6 %, addition of 4 % WPC was used to enhance the growth of *Bifidobacteria* in bovine whey.

**Effect of varying levels of honey on the growth of bifidobacterial species**

As per the European Commission Directive 2006/141/EC, fructo oligosaccharides (FOS) and galacto oligosaccharides (GOS) may be added to infant formula and shall not exceed 0.8 gm/100ml.

The results in Table 2 is in agreement to the work reported that a daily supplementation with 9 gm/day of inulin increased the level of *Bifidobacteria*. Human milk contains a variety of complex oligosaccharides which may be responsible for promoting the growth of *Bifidobacteria*. In the present study addition of 0.4 % of inulin was sufficient to enhance the viable count than with 1 %.

**Effect of varying levels of honey on the growth of bifidobacterial species**

The results in Table 3 shows enhanced bifidobacterial growth with 3 & 5 % honey and is in accordance to research that honey aided in enhanced growth rate of *Bifidobacterium* compared to other sweeteners. The enhanced growth and activity of *Bifidobacteria* could be due to the unique carbohydrate composition and complex mixture of oligosaccharides present in honey. Similar findings were also reported on *B. longum, B. adolescentis, B. breve, B. bifidum, and B. infantis,* that were cultured in medium supplemented with 5 % (w/v) honey, FOS, GOS and inulin. Reports also suggest that honey can act as a potential prebiotic, increasing the populations of *Bifidobacteria* and *Lactobacilli*. In present study the viable count of *B. longum* (IB₁₀) increased from 11.756 ± 0.023 to 12.530 ± 0.036(log₁₀ cfu/ml) in the presence of 3 % honey which was more than the addition of 5 % wild Tualang honey and common honey to skimmed milk inoculated with *B. longum*. Among the isolates IB₁₀ showed enhanced viable count with honey.

**Conclusion**

Bifidobacteria have been in the spotlight of scientific research during the past two decades. This attention is due to health promoting effects of these organisms in humans and because of these effects bifidobacteria are often used in probiotic preparations or as target organisms for prebiotic substrates. Innovative technologies like the use of commonly available prebiotics can be used to improve the viability of these beneficial bacteria to prolong their health promoting effects. Preliminary research is being demonstrated for their potential effects in the reduction of several chronic diseases including colon cancer and ulcerative colitis. Thus further ensuing research in the mode of action of prebiotics would pave way for symbiotic food as an aided alternate therapy for gastrointestinal disorders.
References


22. Ustunol Z & Gandhi H, Growth and viability of commercial Bifidobacterium spp. on honey sweetened skim milk, J Food Protect, 64( 2001) 1775-1779.


