Isolation, Identification Of Human Autochthonous Bifidobacteria And Comparison Of Its Growth On Different Natural Food Products

R Reyed

Citation

Abstract
Microbiological, enzymatical and biochemical tests of carbohydrates fermentation profile were used to identify and classify human indigenous bifidobacteria isolated from human fecal samples. The most prevalent isolates belong to species Bifidobacterium longum, 28 %, Bifidobacterium bifidum 22 % and Bifidobacterium adolescens 17%. Growth of bifidobacteria improved significantly on natural and adjusted carrot extract medium that makes it feasible for routine monitoring. The cell growth reached more than 108 CFU/mL. Our experimental data will be useful for the development of various fermented product.

INTRODUCTION
Human beings harbour incredibly complex and abundant ensemble of microbes. We are in contact with this microflora from birth. Yet, little is known about their influence on normal development and physiology. The intestinal human is more densely populated with microorganisms than any other organ and is a site where the microflora may have a pronounced impact on our biology. Major genus of human microflora includes bacteriodes, Bifidobacteria, coliform, enterobactriace, fusobacteria, and streptococci (Fanaro et al., 2003). The predominance of bifidobacteria in infants at a time when their immune system is not fully developed and the observed antagonistic has stimulated a great deal of interest in the role of bifidobacteria in human health. They have an important role in breaking down dietary carbohydrate and interact directly with the host metabolism (Gibson, 1997), also synthesis and excrete water soluble vitamins, but there are considerable differences in species and strains (Deguchi;1985). These organisms predominate in the colons of breastfed babies; they account for up to 95% of all culturable bacteria and protect against infection. The low colonization rate of bifidobacteria, even in breast-fed infants born in industrialized countries, is probably related to routine hygienic procedures (Hall et al., 1990).

In general, infants with abnormal patterns of microbial colonization appear to be more susceptible to diseases such as colitis, diarrhoea, infant botulism and allergy (Kirjavainen et al., 2001; Millar et al., 2003). They frequently have special nutritional requirements, thus often making their bacteria difficult to isolate and grow in the laboratory. The goal of this work is to investigate the identification and examine the influence of different natural supplementation with additional sources of carbohydrates and nitrogen on the growth rate of human intestinal bifidobacteria.

MATERIAL AND METHODS
STRAINS AND CULTURE CONDITIONS
Fifty eight Bifidobacterial strains were isolated from human stools, ant sized pieces from stool were placed in sterile tube (Ji et al., 1991). For optimal survival of the extremely sensitive anaerobic bacteria, the samples had to be treated within 30 minutes after ejection. Otherwise, the samples were kept in an aerobic jar until analysis (max. 10 hr). The samples were diluted further using reduced physiological salt solution (RPS) (peptone 1 g/l, cystine HCl 2 g/l, and NaCl 9 g/l). Bacterial strain Bifidumbacterium bifidum No.791 kindly obtained from Gut research group, Department of Microbiology, Al-Farabi Kazakh National University, Almaty, Kazakhstan.

Donor : Faeces taken from healthy human volunteers, who had not used antibiotics for the last 6 months aforementioned to the sampling date and none had a history of gastrointestinal disorders.
Liver extract preparations: Liver extract: 500 finely cut beef liver, boiling for 2 hr in 1000 ml of distilled water. The extract was filtrated, sterilized at 110 °C, for 20 minutes (Kenzhebekova, 2003).

Carrot and beet root extracts preparations: They were crushed separately and completely added to 250 ml distilled water boiling for 15 min. The extract filtered through cheese cloth, the filtrate pasteurized at 70 °C for 15 min.

Rice starch preparation: Polished and mashed rice was soaked in distilled water (1:7) and boiled for 15 min at 100 °C. The suspension was filtered through cheese cloth. The filtrate in such a manner had to be adjusted to a pH value of 6.5 and thereafter sterilized at 120 °C for 15 min. (Kenzhebekova, 2003).

Brewer's Yeast autolysate: Brewer's yeast (40 g fresh yeast) added to water and mixed up to completely homogeneous weight, add a little of crystal of methyl-isopropyl phenol or 1-2 ml of chloroform, keep up at 50-52 °C for 72 h. in incubator. After three days autolysate mixed on boiling water path of 20 minutes, then pass through a filter, the filtrate then sterilize at 0.5 atmosphere pressure for 15 min. (Kenzhebekova, 2003).

Media: After 10³ fold serial dilution of 1 g faeces, the sample were added to liver cystiene Lactose (LCL) agar (Rasic, 1984). Briefly, faeces were obtained and transported immediately to the laboratory in anaerobic RPS, for isolation and counts of viable cells number of bifidobacteria. LCL solid, medium was used that contains: Liver extract 1000 ml; Lactose 10 g/l; cystiene -HCl 500 mg/l; Agar; 750 mg/l; peptone 10g/l. NaCl ; 5g /l. Growth of bifidobacteria: 1 ml of culture broth was serially diluted with sterile reduced physiological salt solution (RPS) and 0.1 mL of the diluted solution was plated on the agar plate containing previous medium to which 20 g /L agar was added previously. The plates were incubated anaerobically at 37 °C for 72 hr and colony forming units / mL of sample (CFU/ mL) was counted. Colonies with the curved, Y- or V-shaped gram positive severe anaerobic cells were picked. Bifidobacteria strains were stored on MRS-agar medium and subcultured at 37 °C for 48 hr in MRS broth, and 0.05% L-cysteine-HCl was added for Bifidobacterium cultures.

METHODS

The substrates based on carrot extract, Lactose 10 g/l. cystiene -HCl 500 mg/l; Agar; 750 mg/l; NaCl ; 5g/l. and peptone 10.0 g/l were prepared as followed:

(A) Natural medium without pH adjustment: M1: Carrot extract with rice starch (10 % v/v), initial pH 6.3, M2: Carrot extract with brewer's yeast autolysate (10 % v/v), initial pH 6.0, M3: Carrot extract with a mixture of rice starch and brewer's autolysate (10% v/v), Initial pH 6.4, M4: Carrot extract initial pH 6.5, M5: Liver extract (LCLagar) initial pH 7.

(B) Natural Medium with adjusted pH M1: Carrot extract with rice starch (5% v/v), initial pH 7, M2 Carrot extract with brewer's yeast autolysate (5 % v/v)., initial pH 7, M3: Carrot extract with a mixture of rice starch and brewer's autolysate (5% v/v) Initial pH 7, M4: Carrot extract initial pH 7, M5: Liver extract (LCL-agar) initial PH 7.

Enzyme test: The method of the F6PPK detection described by Scardovi (1986) was used for the identification of bifidobacteria. bifidobacteria were cultivated an aerobically in an anaerobic chamber in 20 ml Liver Cysteine lactose Agar (LCL- agar) broth at 37°C for 48 hrs. The cells were harvested by centrifugation at 14 000 × g for 3 min. The pellet was washed twice with a phosphate buffer solution 1:(0.05 M phosphate buffer pH 6.5 plus cysteine 500 mg/l) and the cells were suspended in 1 ml of buffer. The cells were disrupted by sonication, in ice for 2 min. The sonicate was mixed with 0.25 ml each of solutions 2 (6 mg NaF and 10 mg Na iodoacetate in 1 ml distilled H2O) and 7 fructose-6-phosphate 80 mg/l distilled H2O. After 30 minutes of incubation at 37°C, the reaction was terminated by the addition of 1.5 ml of solution 3 (hydroxylamine HCl, 13.9 g/100 ml of water, freshly neutralized with NaOH to pH 6.5). The mixture was kept for 10 minutes at room temperature and then 1 ml each of solutions 4 (TCA 15% (w/v) in water) and 5 (4 M HCl) were added. Finally, 1 ml of the color-developing solution 6[FeCl3.6H2O 5% (w/v) in 0.1 M HCl] was added. The development of a reddish-violet color immediately after shaking the tube indicated the presence of fructoso-6- phosphate phosphoketolase. This enzyme is distinctive for bifidobacteria. The result was negative if the color remained yellow. Biochemical profiling of carbohydrate fermentation reaction characteristics for bifidobacterial spp. The ability of bifidobacterial sp. isolated strains to ferment 14 carbohydrate was determined (Scardovi, 1984). The carbohydrates fermentation was determined on MRS broth containing bromocresol purple (0.016 %) as a pH indicator, and supplemented with 1% of the following carbohydrates: Lactose, L- Arabinose , Xylose,
Mannose, Cellobiose, Salicin, Sorbitol, Mannitol, D-Ribose, Salicin, Starch, Trehalose, Raffinose, Lactulose. To ensure anaerobic conditions, each tube was supplemented with two drops of sterile liquid paraffin after inoculation (Samelis, et al., 1994; Norris, 1950).

RESULTS AND DISCUSSION

The results obtained for morphological, physiological and biochemical tests were compared with those in standard texts for Identification (Scardovi, et al., 1969; Miyake, et al., 1998, Ingrassia et al., 2001) Bifidobacterium strains producing acetate and lactate as by-products of fermentation were identified according to Bergey's Manual of Systematic Bacteriology (Scardovi, 1986). All isolates were Gram positive bacteria and were supposed to be lactic acid bacteria, negative for catalase, oxidase and nitrate reduction tests, gelatinase negative, with no indol production, producing acetate and lactate as by-products of fermentation (Scardovi, 1986). The detection of fructose-6-phosphate phosphoketolase (F6PPK-test) is used for the identification of Bifidobacterium sp. (Scardovi 1986). By light microscopy, bifidobacteria have certain morphological features which are unique which, taken in distinguishing these organisms from others (Smart et al., 1993).

Figure 1

Table 1: Fermentation of carbohydrates pattern of various kinds bifidobacteria

<table>
<thead>
<tr>
<th>Bifidobacterium spp.</th>
<th>Fermentation patterns of bifidobacteria isolated in this study from human fecal</th>
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<tr>
<td></td>
<td>Lactose</td>
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<tr>
<td>B. longum</td>
<td>+</td>
</tr>
<tr>
<td>B. adolescentis</td>
<td>+</td>
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<tr>
<td>B. bifidum</td>
<td>+</td>
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Bifidobacteria, are classified according (Table 1,2 ) to their ability to breakdown and utilize lactose, lactulose, L-Arabinose, Mannose, this kind of bifidobacteria belong to B. longum 28 % , and another kind of bifidobacteria belong to B. adolescentis 17 % which able to grow on Cellobiose, Sorbitol and Mannitol, and bifidobacteria that ferment only lactose, lactulose, Cellobiose (changeable) related to B. bifidum 22 %. Bifidobacterial cultures which were identified into B.longum and B.adolescent exist in intestines of adults. Contrary B. bifidum found basically in children (Werner, 1966). The key reaction in the fermentation of glucose by genus Bifidobacterium appeared to be a phosphoketolase cleavage of fructose-6- phosphate into acetylphosphate and erythrose-4- phosphate. Pentosephosphates are then formed by the action of transaldolase and transketolase Xylulose-5-phosphate phosphoketolase splits pentosephosphates into acetylphosphate and glyceraldehyde-3-phosphate. Through reactions which also occur in glycolysis, lactate is formed from glyceraldehyde-3-phosphate. This is the characteristic and key enzyme of the “bifid shunt” that characterizes the genus of bifidobacteria, although there are actually three subtypes of F6PPK in bifidobacteria (Biavati, 1982).

Figure 2

Table 2: Taxonomic profile of isolated Human stool Bifidobacteria

<table>
<thead>
<tr>
<th>Bifidobacterial isolates</th>
<th>Numbers of strains</th>
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<tr>
<td>Bifidobacterium longum</td>
<td>24</td>
</tr>
<tr>
<td>Bifidobacterium bifidum</td>
<td>19</td>
</tr>
<tr>
<td>Bifidobacterium adolescentis</td>
<td>15</td>
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</table>

Growth of bifidobacteria on various natural vegetable media with natural pH. Fig.(1) shows the pHs of the various natural media. Considerable variations of pHs were found after the time course of fermentation (72 hr). Which were in relation to log (cfu / mL). M.4 final pH 4.1 / 9.5 (log cfu /ml), but M.(1) final pH 5.5 with 5.3 log (cfu / mL). Various degrees of growth were obtained depending on media investigated and the bacterial strains. On the other side, when cultured in natural medium (Fig. 1) the number of bifidobacteria was increased in carrot extract medium decreasing respectively M.4 > M.3 >M.2 >M.5 >M.1. In case of adjusted pH medium. M.4 > M.3 > M.1 > M.2 > M5. (Fig. 2) Carrot extract medium (M.4) in natural and adjusted pH medium gave relatively higher growth rate which expressed in colony forming unit (log cfu /ml), this is due to the presence of precursor of co-enzyme A- panthein phosphate, and the other is an undefined thermostable and water soluble compound. (Gomes et al., 1999) Their growth associated with the decreasing in pH level during fermentation, this is due to the synthesis of acids during the process of fermentation , which is the main distinction and physiological behavior for genus of bifidobacteria, the molar ration of acetic acid and lactic acid range from 1.5 to 1 (Scardovi,1986 ).The synthesis of acid causes changes of pH and the number of colony forming unit (log cfu /ml), one study , shows that the fermentation of glucose by Bifidus pathway gives rise to acetic acid and lactic acid ( Vlkova et al., 2000). It was noticed that the biochemical activity of the bifidobacteria culture in used substrates which are expressed
as different velocity of fermentation and different simulative
effect of added juices. These differences might be caused by
distinct nutritional necessities and abilities of substrate
utilization. the good growth of Bifidobacterium culture in
natural and pH adjusted medium which showed resistance to
acids would be therefore functional for products which need
higher number of cells with higher potential of the intestinal
colonization.

**Figure 3**
Figure 1: Final pH and growth of human culture
bifidobacteria in different natural media

**Figure 4**
Figure 2: Final pH and growth of human culture
bifidobacteria in different natural media adjusted pH

M.1: Carrot extract with rice starch (10% v/v), initial pH 6.3
M.2: Carrot extract with yeast autolysate (10% v/v), initial
pH 6.0
M.3: Carrot extract (5%) with a mixture of rice starch +
yeast autolysate (5 v/v), Initial pH 6.5
M.4: Carrot extract, initial pH 7.0
M.5: Liver extract (LCL-agar) initial PH 7.0 (Control)

Bifidobacteria in various media were incubated in an
aerobically at 37 °C for 72 hr.

M.1: Carrot extract with rice starch (10% v/v), Initial pH 7
M.2: Carrot extract with yeast autolysate (10% v/v), Initial
pH 7 M.3: Carrot extract (5% v/v) with a mixture of rice
starch and yeast autolysate (5% v/v) Initial pH 7 M.4 Carrot
extract (10% v/v) Initial pH 7 M.5: Liver extract (LCL-
agar) Initial PH 7 (control). Bifidobacteria in various media
were incubated in an aerobically at 37 °C for 72 hr.

An understanding the phenomena of the lowering of pH or
by anther meaning the resistance of Bifidobacteria would be
advantageous for the future management of probiotics
Bifidobacterium but our selected strain awaits further
research. The reduction in growth of bifidobacteria in
another media may be due to the lack of easily available
nutrients like sugar or amino acids, also the most examined
ingredient in this study are mot appropriate for the
fermentation of bifidobacteria , in case of rice starch to be
used as a natural media for fermentation, amylase needs to
be added before or during fermentation or this culture lack
the amylase which is necessary for saccharification of the
rice starchy. This result suggests that some component in
beetroot, yeast extract and rice starch are responsible for
poor growth of bifidobacteria. Through sensory evaluation
it was observed that M.1, M.2, M.3, M.5, produced
unfavorable flavor during fermentation. Therefore M.4 in
natural and adjusted pH media were considered to be
suitable for growth of bifidobacteria that consequently
favorable fermented vegetable products.

Bifidobacteria play an important role in the fermentation of
carbohydrates in the colon, and oligofructose, and raffinose
have been implicated as bifidogenic factors (Gibson, 1994).
Despite the research to improve bifidobacterial
concentrations in the intestine, molecular genetic research
with these organisms has been very limited. Only a small
number of genes and two plasmids of members of the genus
Bifidobacterium have been cloned and characterized (Meile,
2001; Tanaka, 2000). Any fermentable dietary component
that arrives undigested in the colon has the potential to act as
a prebiotic. To the present day almost all prebiotics have
been carbohydrates. These range from small sugar alcohols
and disaccharides, to oligosaccharides and large
polysaccharides (all with a variety of sugar compositions and
glycosidic linkages) Such a diverse range of chemical
structures would be expected to provide an equally diverse
range of effects on the colonic microflora (Crittenden. 1999).
The ability to efficiently utilize such a variety of substrates
indicates that bifidobacteria possess an array of glycosidases,
making them nutritionally versatile and allowing them to
adapt and compete in an environment with changing
nutritional conditions (Crittenden. 1999). The rate at which
an organism can grow on a particular carbon source
influences its ability to compete with other bacteria in the
colon and, therefore, determines whether a particular
substrate can be used as a prebiotic (Hopkins, 1998). The
detection of fructose-6-phosphate phosphoketolase (F6PPK-
test) is used for the identification of Bifidobacterium sp. The
innovative method is time consuming and therefore it was
modified more than a few times lately. The examined intestinal isolates used bifidobacteria which are grow well in low pH and in Carrot, lactose, Cystine–HCl, peptone, NaCl and agar that increase the number of viable cells as well as accelerate the fermentation. This is significant from an economic point of view, because of the shortened time of fermentation, lower priced constituent and time consuming. It could be used for analysis in environmental Microbiology, dairy products and food microbiology.

References


References


Author Information

Reyed M. Reyed
Department of Microbiology, Faculty of Biology, Kazakh National University Named Al-Farabi