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Selection of Human Isolates of Bifidobacteria for Their Use as Probiotics

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Abstract

An attempt was made to isolate human strains of Bifidobacteria, all together 36, from fecal samples of 15 breast-fed infants ages 1–6 mo. These isolates were checked for their ability to grow in the presence of 1–3% bile, 0.2–0.4% phenol, and low pH (3.0–5.0) in vitro, to evaluate their capacity to grow under hostile and unfavorable conditions of the human digestive tract. Because milk is to be used as a carrier medium, their ability to grow in 10% sterile skim milk was also evaluated. The bifidobacteria count of the cultured milk samples (0, 24, and 48 h) was taken on tryptone yeast extract agar after 48 h of incubation in the presence of 10% CO_2 at 37°C. The changes in pH and developed titratable acidity were also recorded up to 96 h of incubation. The results indicated that all the isolates obtained had reasonable resistance to pH, bile, and phenol and were capable of growing well in milk. Among the 36 isolates, *Bifidobacterium bifidum* (isolates no. 4, 8, and 17) and *B. breve* (isolates no. 25 and 26) were the most tolerant to unfavorable conditions, and they may therefore be recommended for use in fermented milk or baby food formulations as probiotic dietary adjuncts.

Index Entries: Bifidobacteria; growth characteristics in milk; acidity; count; pH; bile; phenol tolerance; probiotics.

Introduction

The human body harbors numerous types of microorganisms, exogenously as well as endogenously. The total number of microbes living on mucous surfaces and skin of the human body is about 1000 times higher than the total cells in the whole body *(1)*. The large intestine in the human body is where the highest numbers of microbes are housed *(2)*. It is believed that the general well-being of humans depends on the number and type of microbes associated with the gastrointestinal (GI) tract, especially those colonizing the large intestine. The intestinal microflora of a human being

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weighs between 1 and 2 kg, which is roughly the weight similar to that of vital organs such as the liver, brain, or lungs. The digestive tract houses an enormous population of about 10^{14} bacteria, comprising approx 500 different types *(3)*.

The digestive tract of the newborn infant is almost sterile at the time of birth; however, it is rapidly colonized by a number of groups of microorganisms *(4–6)*. Within 2 d of birth, the colon contains 10^9–10^{10} bacteria/g of stools, consisting mainly of enterobacteria, staphylococci, and streptococci *(4,5,7)*. Bifidobacteria starts appearing normally around 3 to 4 d of birth, and within 1 wk, the population of bifidobacteria reaches about 10^{10}–10^{11} bacteria/g of stools, accounting for as high as nearly 99% of the fecal microflora *(4)*, whereas the levels of other bacterial flora decline sharply by about 1000-fold *(8–10)*.

With the introduction of solid food in the diet or at weaning, a sudden change occurs in the fecal flora of infants. There is a remarkable proliferation of *Bacteroides*, *Eubacterium*, *Peptostreptococcus*, and *Clostridium*, resulting in considerable decreases in the ratio of bifidobacteria to other microflora *(11)*. The population of bifidobacteria remains fairly constant (10^9–10^{10}) from childhood to adulthood; however, it gradually declines in the elderly to a population of about 10^7–10^8 bacteria/g of stools *(12)*. It is indicated that "old age" or "senility" is partly owing to an increase in counts of *Clostridium* sp. and decreasing bifidobacteria population.

Among colon biota, lactobacilli and bifidobacteria have drawn considerable attention among researchers in last four to five decades. Since their discovery in 1899 by Tisser at Institut Pasteur, Paris *(13)*, bifidobacteria have attracted the attention of researchers because of their probiotic and therapeutic advantages, which are being constantly strengthened by the tremendous inflow of information worldwide *(14–16)*. Some of the important probiotic/therapeutic benefits provided by bifidobacteria include normalization of intestinal microflora and treatment of acute intestinal disorders and chronic diseases of the digestive systems *(17)*; reduction in serum cholesterol *(18)*; increased biologic value of proteins *(19)*; cure of sepsis and GI disease of staphylococcal and *Proteus* origin *(20)*; reduced counts of *Escherichia coli*, *Enterobacter*, and *Proteus* in the intestine *(21,22)*; treatment of lactose-intolerant infants *(23)*; treatment of gastroduodenitis and peptic ulcer *(24)*; and inhibition of reduction of nitrates to nitrites *(25)*.

It can therefore be strongly said that a preparation containing a live probiotic population of bifidobacteria can be very useful for maintaining normal functioning of the digestive tract in infants and adults in general. They play vital roles, specifically in curing several digestive disorders, in restoring normal gut microflora after heavy antibiotic treatment, as well as in supporting drug therapy in patients. However, it is clear that not all the species of the genus *Bifidobacterium* and not all the strains of a particular single species may possess these desirable properties. Hence, it is of utmost importance to isolate, characterize, and carefully select those species/strains of bifidobacteria that are capable of providing maximum benefits in

general, and also those that are required for some highly specific intent. Since the GI tract is the final destination for such preparations, it would be ideal to isolate the test organisms from the gut itself; thus, fecal matter of breast-fed infants is the best source for strains with good probiotic potential.

Materials and Methods

Collection of Isolates

Fecal samples from 15 breast-fed infants (9 from infants born through normal delivery and 6 from infants delivered by cesarean section) ranging in age from 1 wk to 6 mo were collected and analyzed for bifidobacteria count *(26)*.

One gram of feces was weighed and added aseptically to a 250 mL-conical flask containing 99 mL of sterile peptone water. The contents of the flask were mixed thoroughly to obtain a uniform suspension of the sample. Further dilutions were prepared in sterile tubes containing 9 mL of respective broth medium for different counts.

For enumeration and isolation of bifidobacteria from infant feces, three different media were used: tryptone phytone yeast extract agar (TPYA) *(27)*; neomycin sulfate, paromomycin sulfate, nalidixic acid, lithium chloride agar *(28)*; and lithium chloride sodium propionate agar *(29)*. Suitable dilutions of fecal samples for various counts were prepared in respective broth medium.

A 1-mL quantity of appropriate dilution was added to sterile Petri plates. Respective agar medium (sterile, molten, and cooled to 45–50°C) in 15 to 20-mL quantities was poured into the Petri plates. The contents were mixed thoroughly and quickly before the medium started to solidify. The plates were kept undisturbed until proper solidification. The second layer (4 to 5 mL) of the same medium was poured onto previously poured and solidified agar medium. The plates were incubated in a CO_2 incubator (Model INCO2; Memmert GmbH co.kg, Germany) maintained at 37°C for 48 h with a 10% CO_2 atmosphere. The typical colonies of the bifidobacteria obtained on various media were counted at the end of incubation, and the count was recorded as colony-forming units (CFU) per gram of sample.

Several isolates were picked up from these plates and purified by repeated streaking on TPYA under the incubation conditions as mentioned earlier. These isolates were subjected to primary screening by (1) morphologic examination (Gram staining), (2) catalase test, (3) litmus milk reactions, (4) checking for gas production, and (5) spore formation *(27)*. After primary screening, 36 typical isolates showing Gram-positive reaction; negative catalase; litmus milk reactions such as acid production, coagulation, and reduction; no gas production; and no spore formation were selected for further detailed biochemical characterization *(27)*. The tests performed were indole production, nitrate reduction *(30)*, gelatin liquefaction *(31)*, glycerol fermentation *(32)*, urease production *(33)*, and 16 different carbohydrate fermentation tests *(32)*.

All 36 isolates were unable to produce indole, reduce nitrate, liquefy gelatin, ferment glycerol, and produce urease. Based on carbohydrate fermentation tests, 14 isolates were identified as *B. bifidum* (isolates no. 1–4, 7–9, 12, 15, 17, 18, and 21–23) and 12 were identified as *B. breve* (isolates no. 13, 24–30, 32–34, and 36). The remaining 10 isolates (isolates no. 5, 6, 10, 11, 14, 16, 19, 20, 31, and 35) were placed under the category of *Bifidobacterium* sp. because the carbohydrate fermentation pattern of these isolates matched with more than one bifidobacteria species.

Maintenance of Isolates

The isolates were maintained in sterile TPYA stabs in 18×150 mm tubes *(27)* by giving regular transfers to them and incubating the stabs at 37°C for 48 h with 10% CO_2 atmosphere. After ensuring proper growth at the end of 24–48 h of incubation, the stabs were stored at 5–7°C in a refrigerator.

Activation of Isolates

To perform any test, first of all the isolates were activated by giving three successive transfers in TPY broth tubes (5-mL quantity) at 37°C for 24–48 h under 10% CO_2 atmosphere, and then the same were used to perform the respective test.

Test for Probiotic Potential of Bifidobacteria Isolates

All 36 isolates identified as bifidobacteria were screened for their tolerance to unfavorable conditions, which normally exist in the human digestive tract. The isolates were tested in vitro for (1) tolerance to low pH, (2) ability to grow in the presence of phenol, and (3) ability to grow in the presence of bile salts.

Phenol Tolerance

Four different concentrations of phenol (0.2, 0.3, 0.4, and 0.5% [w/v]) were taken to check the in vitro phenol tolerance of the isolates per the method suggested by Teply *(35)* with some modifications.

The isolates were inoculated to 5 mL of phenol tolerance medium consisting of TPY broth added to phenol crystals and incubated at 37°C for up to 96 h in a CO_2 incubator with 10% CO_2 atmosphere. The tubes were observed for turbidity at 24-h intervals up to 96 h. The isolates, which developed turbidity, were considered capable of growing at that particular level of phenol concentration. The isolates showing no growth after an incubation of 96 h were further checked for their ability to resist phenol concentration by transferring a loopful from the tube of TPY broth containing phenol with no turbidity to normal TPY broth without phenol, and the tubes were incubated at 37°C for a further 96 h. Isolates capable of growing in normal TPY broth within 96 h were considered capable of "surviving" respective phenol concentration, whereas those that were unable to show any sign of growth in normal TPY broth were considered not capable of surviving at the respective phenol concentration.

Bile Tolerance

Bile tolerance of the bifidobacteria isolates was checked per the method given by Clark and Martin *(36)* with some modifications. The detailed procedure is as follows. Four different concentrations of bile salts (1.0, 2.0, 3.0, and 4.0% [w/v]) were taken to check the extent of bile tolerance of the bifidobacteria isolates. The isolates were inoculated to 5 mL of bile tolerance medium. A further procedure to determine growth or tolerance to bile was similar to that described and adopted for phenol tolerance.

pH Tolerance

The pH tolerance of the bifidobacteria isolates was checked by the method described *(34)* with some modifications. Three pH values in the acidic range (5.0, 4.0, and 3.0) were taken to check the tolerance of the isolates to low pH. The isolates were inoculated to 5 mL of TPY broth tubes having different pHs. The pH of normal TPY broth was adjusted with the help of gradual addition of HCl (35%) to the desired pH before sterilization. The rest of the procedure to check ability to grow or to determine tolerance to low pH was the same as that described for phenol tolerance.

Growth Characteristics of Bifidobacteria in Milk

The activated pure cultures of bifidobacteria isolates were inoculated at the rate of 1% to a 250-mL quantity of 10% sterile reconstituted skim milk. The reconstituted skim milk was prepared from skim milk powder (Sagar), and the milk was sterilized by autoclaving at 121°C for 20 min. The 250-mL quantity of milk was then distributed in five portions of 50 mL each in 100-mL sterile glass beakers. The beakers were incubated at 37°C in a CO_2 incubator with 10% CO_2 atmosphere. The beakers were taken out one by one at regular 24-h intervals, and the fermented milk was analyzed for bifidobacteria count, pH, and titratable acidity.

Bifidobacteria Count

Bifidobacteria count of the fermented milk samples was determined at 0, 24, and 48 h of incubation as per the method given by Scardovi *(27)*. Eleven grams of the sample was weighed aseptically in a 250 mL conical flask containing 99 mL of sterile 1% peptone water. Further dilutions were then prepared from it using 9 mL of sterile 1% peptone water blanks. The appropriate dilutions were plated in sterile Petri dishes and poured into 15–20 mL of sterile molten TPY agar cooled to 45–50°C, using a double-layer technique. The plates were then incubated at 37°C for 48 h in the CO_2 incubator with 10% CO_2 atmosphere. After incubation the plates were taken out and typical colonies were counted. The count was recorded as bifidobacteria count (CFU/g).

pH Measurement

The changes in pH of the samples as a result of growth of bifidobacteria isolates in milk were measured at intervals of 0, 24, 48, 72, and 96 h of

incubation using a Systronics digital pH meter (Model no. 335) at 20°C per the method specified by the Bureau of Indian Standards *(37)*. The uninoculated control tubes were kept throughout the study period for comparison.

Acidity Measurements

The developed titratable acidity expressed as % lactic acid (LA) of the samples as a result of the growth of bifidobacteria isolates in milk was measured at intervals of 0, 24, 48, 72, and 96 h of incubation per the method suggested by the Bureau of Indian Standards *(37)*. Uninoculated tubes were kept as a control to check that there was no undue acid development in the tubes during the incubation period.

Statistical Analysis

The data obtained for changes in bifidobacteria count, acidity, and pH of milk fermented by bifidobacteria isolates were analyzed *(38)* using randomized block design (RBD). The data on bifidobacteria count, pH, and acidity in the milk system were subjected to statistical analyses *(38)* using completely randomized design (CRD) with unequal replications in order to find the differences among the three groups of bifidobacteria isolates.

Results

Tolerance to Adverse Conditions

The bifidobacteria isolates were tested for their tolerance to unfavorable conditions of the digestive tract by subjecting them to growth in vitro under adverse conditions such as different pH values and phenol and bile concentrations.

Tolerance to Low pH

In the case of the ability of the isolates to tolerate acidic and low pH in vitro, it was seen that all the isolates could grow well at pH 5.0 and were able to survive pH 4.0 with the exception of isolates no. 13 and 36, which could grow at pH 5.0, but were unable to survive at pH 4.0.

Bile Tolerance

In the case of bile tolerance, all the isolates except isolates no. 13 and 36 were capable of growing in 1% bile and were capable of surviving in 3% bile concentration in vitro. Whereas isolates no. 13 and 36 could not even survive 1% bile concentration, isolates no. 8, 21, and 25 were able to survive as high as 4% bile concentration.

Phenol Tolerance

As far as the ability of the isolates to tolerate various levels of phenol concentration, it was seen that all the isolates were capable of growing in

0.2% (w/v) phenol and surviving a 0.3% phenol concentration in vitro. Isolates no. 8, 21, 25, and 26 were found to be more tolerant since they were also capable of showing growth in a 0.3% phenol concentration, but none of these could survive a higher level (0.4%) of phenol.

Growth Characteristics of Bifidobacteria in Milk

To check the suitability of the isolates for use in the preparation of fermented milks, their growth characteristics in sterile reconstituted skim milk were studied. The active and pure isolates were grown individually in sterile skim milk, and readings for viable count at 0, 24, and 48 h of incubation and for pH and acidity at 0, 24, 48, 72, and 96 h of incubation were noted.

Bifidobacteria Count

The viable count (log CFU/g) ranged from 5.26 to 7.21 (mean 6.03) at 0 h, 7.84 to 11.04 (8.89) at 24 h, and 8.60 to 11.89 (10.42) at the end of 48 h of incubation. The highest count at the end of 24 h of incubation was recorded by isolate no. 32 (11.04), which rose marginally to 11.23 at the end of 48 h of incubation. After 48 h of incubation, the highest count was recorded by isolate no. 23 (11.89). Only two isolates (25 and 32) showed a count higher than 10^{10} after 24 h, whereas after 48 h of incubation, as many as 30 isolates (except isolates no. 1, 2, 14, 16, 19, 20, and 26) showed a count higher than 10^{10}. Such isolates may be very useful for the preparation of fermented milk products because they can give a very good viable count in a reasonably short incubation period of 24–48 h.

The results of the bifidobacteria count in skim milk were also statistically analyzed using RBD and were found to vary significantly for different isolates at all the incubation periods. According to the comparison of means, isolates 23, 25, 32, and 36 were at par and gave highest count at the end of 48 h of incubation among all the isolates.

The differences in the viable counts among the three groups of bifidobacteria (Fig. 1, Table 1) were significant ($p < 0.05$) at 0 and 24 h of incubation. However, at 48 h of incubation, the differences were statistically nonsignificant. Apparently, the mean count for the *B. breve* group at all the incubation periods was slightly higher than for the *B. bifidum* group. Statistically, the differences observed were significant ($p < 0.05$) at 0 and 24 h, but not at 48 h.

Changes in Milk pH During Growth

Changes in pH during incubation of isolates up to 96 h in skim milk indicated that the pH values ranged narrowly from 6.50 to 6.67 (mean 6.56) at 0 h. However, as a result of active growth, the viable count values showed wider differences of 5.17–6.13 (5.84) at the end of 24 h, 4.83–5.96 (5.34) at 48 h, 4.61–5.91 (5.09) at 72 h, and 4.33–5.85 (4.92) at 96 h of incubation, respectively. The highest reduction in pH was recorded for isolate no. 25

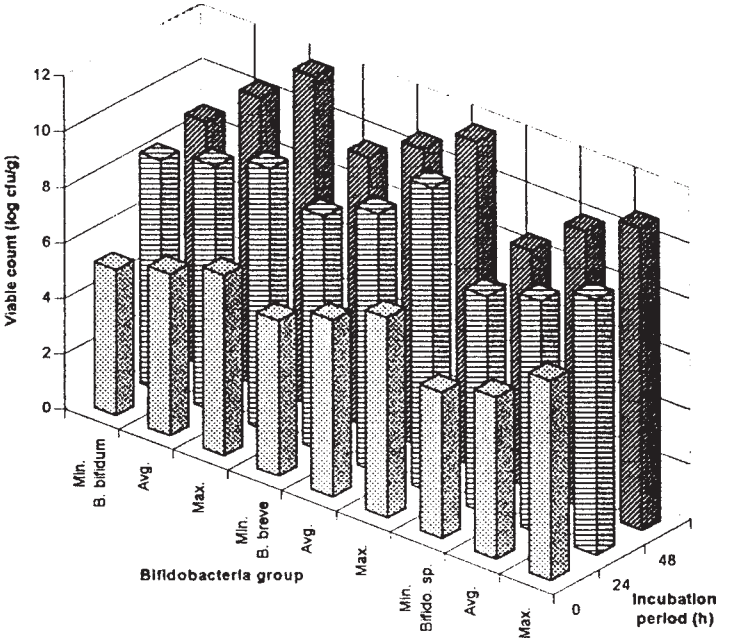

Fig. 1. Comparison of changes in viable count (log CFU/g) by three groups of bifidobacteria during their growth and incubation in sterile skim milk.

Table 1
Statistical Analysis of Data on Comparison of Changes in Viable Count (log CFU/g) by Three Groups of Bifidobacteria During Their Growth and Incubation in Sterile Skim Milk

	Incubation period (h)		
Statistical attribute[a]	0	24	48
Group I (mean)	5.85	8.84	10.38
Group II (mean)	6.39	9.35	10.77
Group III (mean)	5.84	8.40	10.04
SEM	0.1556	0.1591	0.1980
CD (at 0.05)	0.4468	0.4538	NS
CV%	8.86	6.10	6.52

[a]Group I, *B. bifidum* ($n = 14$); group II, *B. breve* ($n = 12$); group III, *Bifidobacterium* sp. ($n = 10$); mean, arithmetic mean (values expressed in log CFU/g); SEM, standard error of mean; CD (at 0.05), critical difference at 5% level; CV%, coefficient of variance.

(5.17) at 24 h, isolate no. 26 (4.83) at 48 h, and isolate no. 4 at both 72 h (4.61) and 96 h (4.33) of incubation. In the case of isolates no. 1, 25–27, 31, 32, and 34, almost a maximum reduction in pH was achieved only at the end of 72 h. For most of the isolates, curdling initiated when pH was reduced below 6.0, usually at the end of 24 h or at the most after 48 h of incubation.

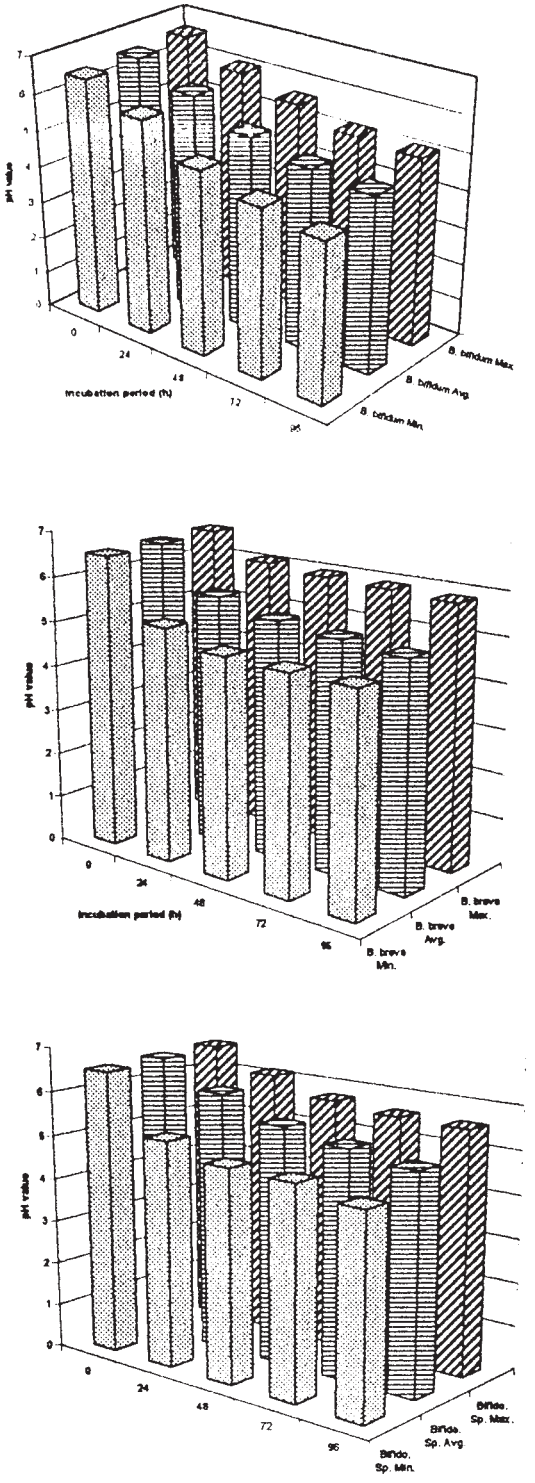

Fig. 2. Comparison of changes in pH by three groups of bifidobacteria during their growth and incubation in sterile skim milk.

Table 2
Statistical Analysis of Data on Comparison of Changes in pH by Three Groups of Bifidobacteria During Their Growth and Incubation in Sterile Skim Milk

	Incubation period (h)				
Statistical attribute[a]	0	24	48	72	96
Group I (mean)	6.57	5.99	5.33	4.96	4.78
Group II (mean)	6.56	5.57	5.26	5.12	5.01
Group III (mean)	6.56	5.96	5.45	5.22	5.01
SEM	0.0111	0.0722	0.0845	0.0769	0.0791
CD	NS	0.2073	NS	NS	NS
CV%	0.5826	4.24	5.43	5.20	5.51

[a]Group I, *B. bifidum* ($n = 14$); group II, *B. breve* ($n = 12$); group III, *Bifidobacterium* sp. ($n = 10$); mean, arithmetic mean (values expressed as pH units); SEM, standard error of mean; CD (at 0.05), critical difference at 5% level; CV%, coefficient of variance.

All the isolates except no. 36 showed firm curdling without whey separation after 48 h of incubation.

Analysis of the data for pH with RBD indicated that the variation observed in pH values among different isolates was significant ($p < 0.05$), when all 36 isolates were compared. However, isolates no. 1, 3, 4, 8, 12, 24–27, 31, 32, and 34 were statistically at par and were ranked first on the basis of their ability to reduce pH.

On analyzing the data using CRD with unequal replications, it was found that at 0 h the differences in mean pH of the three groups of bifidobacteria isolates were nonsignificant. This was an expected trend since they were inoculated at an equal rate in the same type of milk medium (Fig. 2, Table 2). At 24 h of incubation, owing to varying rates of acid production, the differences were statistically significant ($p < 0.05$), even though it was observed that at the end of 48 h of incubation, the *B. breve* group had a lower mean pH compared with the *B. bifidum* group. However, after 72 h of incubation, the latter overgrew the former and showed a lower pH (mean value 4.78) at the end of 96 h of incubation. Nevertheless, the differences in pH, at 48, 72, and 96 h, for the three groups again were statistically nonsignificant.

Development of Acidity During Growth in Milk

The changes in acidity (% lactic acid) owing to growth of the bifidobacteria isolates in skim milk up to 96 h were studied. The values for acidity ranged from 0.17 to 0.20 (mean 0.18) just after inoculation (i.e., at 0 h), 0.21 to 0.62 (0.31) at the end of 24 h, 0.36 to 0.69 (0.49) at 48 h, 0.41 to 1.21 (0.66) at 72 h, and 0.49 to 1.32 (0.76) at 96 h of incubation, respectively. The results of statistical analysis indicated significant ($p < 0.05$) differences in acidity of the isolates. Isolates no. 4, 25, and 26 were statistically at par and were ranked first for achieving highest acidity. Even after 72 and 96 h

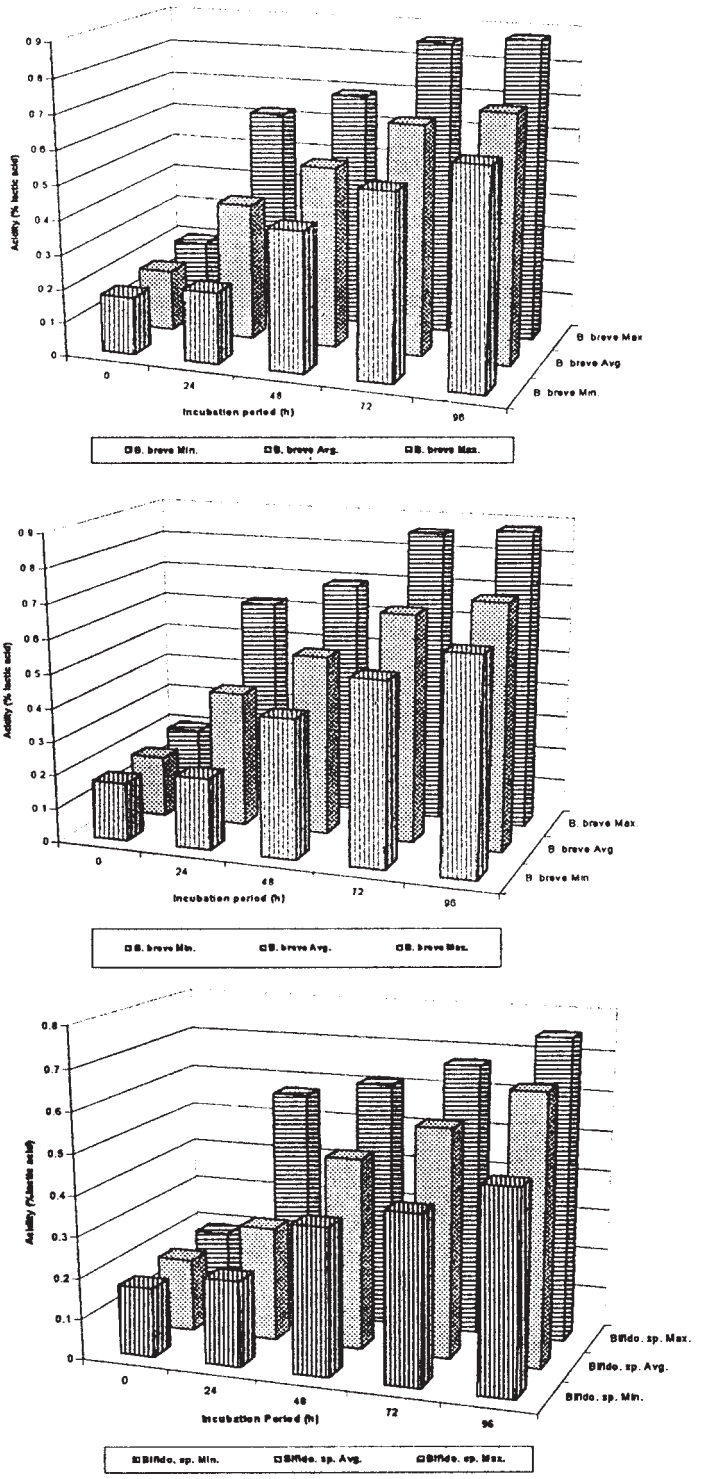

Fig 3. Comparison of rate of acid production (% lactic acid) by three groups of bifidobacteria during their growth and incubation in sterile skim milk.

Table 3
Statistical Analysis of Data on Comparison of Rate of Acid Production (% lactic acid) by Three Groups of Bifidobacteria During Their Growth and Incubation in Sterile Skim Milk

	Incubation period (h)				
Statistical attribute[a]	0	24	48	72	96
Group I	0.18	0.26	0.49	0.72	0.85
Group II	0.18	0.40	0.53	0.67	0.72
Group III	0.18	0.28	0.47	0.56	0.66
SEM	0.0025	0.0316	0.0242	0.0384	0.0347
CD	NS	0.0908	NS	0.1103	0.0996
CV%	4.79	34.80	16.80	20.07	15.76

[a]Group I, *B. bifidum* (n = 14); group II, *B. breve* (n = 12); group III, *Bifidobacterium* sp. (n = 10); mean, arithmetic mean (values expressed as % lactic acid); SEM, standard error of mean; CD (at 0.05), critical difference at 5% level; CV%, coefficient of variance.

of incubation, all the isolates except no. 4 showed acidity values not exceeding 1%. The highest level of acidity was achieved by isolate no. 32 (0.62) after 24 h, isolate no. 26 (0.69) after 48 h, and isolate no. 4 after both 72 h (1.21) and 96 h (1.32) of incubation. In the case of isolates no. 31, 32, and 34, the maximum level of acid production was achieved within 48 h of incubation.

Regarding the rate of acid production at different time intervals by the three groups (Fig. 3, Table 3), it was observed that the differences in the titratable acidity at 0 h of incubation were statistically nonsignificant. This was expected since the rate of inoculation as well as the type of milk medium used was the same for all the isolates. However, after 24 h of incubation, the differences among the three groups were found to be significant ($p < 0.05$), with the *B. breve* group exhibiting the highest level of acidity (mean value of 0.38% LA). After 48 h of incubation, the differences observed were again nonsignificant. This trend is similar to the one described for pH. However, at the end of 72 h of incubation, the differences recorded were significant ($p < 0.05$) with the *B. bifidum* group (mean value of 0.72% LA) overtaking the *B. breve* group (mean value of 0.67% LA). The same trend also continued for the incubation period of 96 h, with mean acidity values of 0.85% LA for the *B. bifidum* group and 0.72% LA for the *B. breve* group, respectively.

From the data obtained for pH as well as acidity, it can be clearly seen that overall, almost all the isolates reflected a sudden shift in the acidity values during the incubation period from 48 to 72 h. Most of the isolates reached their lowest pH and/or corresponding highest acidity at the end of 72 h of incubation. After 72 h, there were little changes observed in acidity as well as pH values for most of the isolates. The viable counts (log CFU/g) of bifidobacteria obtained at the end of the 72-h period of incubation were not taken, but the results of even 48 h showed an average

count of 10.42. Hence, 48–72 h may be considered the ideal incubation period for most of the isolates as far as viable count and acid development are concerned.

Discussion

Tolerance to Adverse Conditions

Low pH

A probiotic culture should possess the ability to survive passage through the mouth, stomach, small intestine, and large intestine. The conditions likely to be encountered during transit include peristaltic motion, a low pH of between 1.0 and 3.0 of the stomach, a low oxidation-reduction potential, an action of digestive enzymes, a high bile salt concentration, the presence of phenolic compounds, and so on. Survival of some strains of bifidobacteria through the stomach band small intestine has been reported *(39,40)*.

It is also important to know the transit time for the bifidobacteria and the exact low pH in the GI tract. Berrada et al. *(39)* reported 80% stomach emptying within 90 min for two fermented dairy products containing bifidobacteria. The rate of food passage through the stomach depends on its composition, primarily fat content *(41,42)*. If the fermented dairy product was consumed along with other food items, or with a meal, the length of time spent in the stomach might increase, which could influence the survival of bifidobacteria. Regarding the influence of the food substances on pH of the stomach, dairy products offer a protective environment for bifidobacteria during gastric passage. Convey et al. *(43)* reported that skim milk raised the pH of gastric juice in vitro. Martini et al. *(44)* reported an increase in gastric pH (>2.7) for 3 h following ingestion of milk or yogurt.

Several researchers have reported tolerances of bifidobacteria under low pH, under either simulated pH of human stomach (1.0–3.0) or low pH of fermented dairy products around 4.0. Hence, the present study was conducted to determine the survival of bifidobacteria in the pH range of 1.0–4.0.

In an earlier study by Khedkar *(45)*, in vitro pH tolerance of 17 bifidobacteria isolates (*B. adolescentis*) obtained from feces of breast-fed infants indicated that the isolates were capable of growing at pH 5.0 and surviving at pH 4.0. The results of our study are in agreement with these earlier observations. The similarity may be owing to the fact that both studies were carried out in the same region (Anand, Gujarat, India), and, hence, the properties of bifidobacteria isolates in the intestinal tract of the infants may be similar because of diet, weaning practices, and other environmental factors.

Hoier *(46)* studied acid tolerance of *Bifidobacterium* Bb-12 and *L. acidophilus* La-5 in MRS nutrient solution adjusted to pH 1.0–4.0 with HCl with 2- and 12-h incubation periods and found that Bb-12 had a higher pH tolerance than La-5. Both species survived 100% at pH 3.0 and 4.0.

Clark et al. *(34)* examined the tolerances of four strains of bifidobacteria under simulated pH of human stomachs. These strains of bifidobacteria—*B. infantis*, *B. adolescentis*, *B. bifidum*, and *B. longum*—were suspended in sterile HCl solution of pH 1.0, 2.0, and 3.0. The survival was examined every hour up to 4 h. The results indicated that *B. infantis*, *B. adolescentis*, and *B. longum* can be successfully used as dietary adjuncts since they are able to survive in vitro pH levels normally encountered in the human stomach. Clark et al. *(34)* also found that *B. longum* could survive even at pH 1.0. Apart from their study, a few recent reports on survival of bifidobacteria at low pH are also available *(47–50)*.

Bivanti et al. *(51)* reported that of 110 strains of bifidobacteria, isolated predominantly from human feces, 10 were acid resistant (skim milk acidified to pH 4.0) after 15 d of storage at 4°C and 3 after 30 d of storage. The 13 resistant strains belonged to *B. bifidum*, *B. infantis*, *B. breve*, *B. longum*, and *B. pseudocatenulatum*. This is also one of the important technologic features required for probiotic culture to be used for product making.

Bile

Several reports, including two from India, are available regarding the ability of bifidobacteria to grow in vitro in the presence of or to withstand varying levels of bile salts. Misra and Kuila *(52)*, when testing *B. bifidum* isolates from breast-fed infant stools along with standard strains of *B. bifidum* NCDO 1452, NCDO 1453, NCDO 1454, and NDRI, observed that only strains BX, BXVII, NCDO 1454, NCDO 1452, and NDRI could survive in a 0.5% solution of bile salts. Khedkar *(45)* found that 17 fecal isolates from breast-fed infants of *B. adolescentis* could grow up to only 0.5% bile and could survive up to 4.0% bile.

Holcomb et al. *(53)* tested the resistance of *B. bifidum* to bile and found that it resists very well up to 0.45% bile concentration. Hoier *(46)* found that *Bifidobacterium* Bb-12 when tested for bile tolerance in milk yeast medium containing 0.5–2.0% ox bile at 37°C for 2 and 24 h exposure was not inhibited by bile except at the highest concentration. Ibrahim and Bezkorovainy *(54)* evaluated the tolerance of *B. infantis*, *B. bifidum*, *B. breve*, and *B. longum* in the presence of 0.0–0.6% bile and observed that the number of *B. infantis* declined significantly in the presence of bile salt but that some cells survived in 0.3% bile after 48 h. *B. infantis* thus had the best survival rate followed by *B. bifidum*, *B. breve*, and *B. longum*. Clark and Martin *(36)* also reported the bile tolerance of *B. longum*, *B. infantis*, *B. adolescentis*, and *B. bifidum* using bile concentrations of 2 and 4% with plating after exposure for 0 and 12 h at respective bile concentrations. It was observed that only *B. longum* could survive both 2 and 4% bile concentrations. The other strains of bifidobacteria could not survive even in 2% bile concentration. Sanders et al. *(55)* found that most of the bifidobacteria were resistant to 1.0–3.0% bile concentration. Kim et al. *(47)* isolated 200 bifidobacteria from healthy Korean people. Of these 20 were examined for acid and bile tolerance and one strain, *B. longum* A_2, showed potential for use as a probiotic culture.

Chung et al. *(49)* screened and selected acid and bile-resistant bifidobacteria from human fecal origin. Al-saleh *(56)* reported that *B. longum* and *B. bifidum* were more resistant to bile as compared with four other species. Wang et al. *(50)* found that 10 of 90 isolates of bifidobacteria from pig origin were tolerant to 0.8–1.0% bile in BS broth at pH 3.5. When incubated at 37°C for 2 h, the survival rate of the isolates was more than 40%.

In in vivo studies, Pochart et al. *(40)* fed milks fermented with bifidobacteria to humans. Samples from the GI tract were taken every hour for 8 h using a gastric tube. They observed that the numbers enumerated at 1-h intervals were significantly lower than the initial numbers ingested.

The maximum concentration of bile during the first hour of digestion of food in the gut can be up to 2% *(57)*. Bile concentration can then decrease to 0.5% by the end of the second hour. Hence, it is necessary to know that the candidate probiotic cultures are able to withstand the bile concentration in this range. Regarding passage of foods in the GI tract, it was observed that certain foods such as cultured dairy products, which are easily digestible, could pass through the GI tract within 12 h *(58)*.

The isolates obtained in our study were subjected to more rigorous incubation conditions in comparison with some of the previous studies and seem to be slightly more resistant to bile concentration as compared with the isolates reported earlier *(35,45)*. This could be an added advantage while using some of these isolates as a dietary adjunct for human consumption.

Phenol

A previous report *(45)* also indicated that all 17 isolates of *B. adolescentis* were capable of growing in 0.2% phenol. Of a total of 17 isolates, 8 could survive 0.3% phenol, 7 could survive 0.4% phenol, and 2 could survive even 0.5% phenol. The phenol tolerance of the isolates in the present study was found slightly lower in this regard.

Aromatic compounds such as phenol, cresol, indole, and skatol and ammonia are the fecal putrefactive products and are found to be associated with the GI tract. Therefore, to survive and multiply in the GI tract microenvironment, the probiotic cultures must be able to survive the aforementioned phenol concentrations. Probiotics are reported to grow and resist up to 0.5–0.6% phenol *(59,60)*.

Some of the most desirable and important properties to look for in a culture when considering it and validating its use as probiotic include the capacity to survive in the medium in which the culture is suspended; the ability to survive passage through the GI tract, especially the stomach (low pH) and small intestine (bile and phenol tolerance); the capability of adhering on the wall of the large intestine; nontoxic and nonpathogenic nature; the capability of producing organic acids, bacteriocins, or antibiotic-like substances and other inhibitory substances to control growth and proliferation of undesirable intestinal pathogens and putrefactive microflora; and the ability to establish and survive for a long period in the intestinal tract of the host *(61,62)*.

Growth Characteristics of Bifidobacteria in Milk

Bifidobacteria Count

Growth characteristics of *B. infantis* strain ATCC 27920 in milk *(63)* indicated that the culture was able to achieve a viable count of 8.86 log CFU/mL, after 48 h of incubation. The growth pattern in milk of three human strains of *B. adolescentis* (NUB, TUB, and Hb_1) isolated from infant feces *(45)* showed average counts of 8.84, 8.43, and 8.68 log CFU/mL, respectively, at the end of 48 h of incubation. The isolates obtained in the present study could achieve better levels of viable count, as far as growth in reconstituted sterile skim milk (10% [w/v]) was concerned.

pH

It was reported that *B. infantis* strain ATCC 27920 could reduce the pH of milk to 4.81 after 60 h of incubation *(63)*. However, in the case of three strains of *B. adolescentis* (NUB, TUB, and Hb1) after 48 h of incubation, the drop in pH value reported was 6.28, 6.70, and 6.68 *(45)*.

Acidity

Rapid acidification and growth are some of the important desirable technologic characteristics required for the probiotic strains in milk media. A study of the growth pattern of different bifidobacteria species in milk *(64)* revealed acidity levels of 0.71 (*B. adolescentis*), 0.78 (*B. infantis*), 0.73 (*B. bifidum*), and 0.68 (*B. longum*) at the end of 24 h of incubation. In the case of *B. infantis* strain ATCC 27920, it was found that the acidity increased to 1.37 after 60 h of incubation *(63)*. Misra and Kuila *(52)* reported the acid production in the range of 0.41–0.95% as lactic acid for the fecal isolates and standard strains of *B. bifidum*. By contrast, a low level of acidity for the three strains of *B. adolescentis* (NUB, TUB, and Hb_1) of 0.302, 0.258, and 0.225, respectively, after 96 h of incubation was reported *(45)*. Regarding rate of acid production, the isolates obtained in the present study were found to perform reasonably well.

Conclusion

The present study revealed that all the *Bifidobacteria* isolates obtained from breast-fed infants had reasonable resistance to pH, bile, and phenol and were capable of growing well in milk. Among the 36 isolates, *B. bifidum* (isolate nos. 4, 8, and 17) and *B. breve* (isolates no. 25 and 26) were the most tolerant to unfavorable conditions, and they may be recommended for use in fermented milk or baby food formulations as probiotic dietary adjuncts.

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