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Acid Adaptation of Bifidobacteria Isolated from Infant Stools to Simulated pH of Human Stomach

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Abstract

Bifidobacterium isolates obtained from infants stools were assessed for their ability to survive in HCl solutions adjusted to pH levels of 1.0, 2.0, and 3.0. Five isolates (*Bifidobacterium breve* C20, *B. infantis* D22, *B. infantis* F91, *B. infantis* G4 and *B. infantis* Z45) out of 28 were able to survive at pH 1.0 after 0 h exposure. Survival was not detected after 1 h exposure to this pH level. At pH 2.0, only five isolates (*B. infantis* D22, *B. infantis* F66, *B. infantis* F34, *B. breve* F100 and *B. infantis* G4) survived after 1 h exposure. Four isolates (*B. infantis* D22, *B. infantis* F117, *B. infantis* F34 and *B. infantis* Z45) were able to survive at pH 3.0 during the 3 h incubation period. At pH 6.5 (control), essentially all isolates of bifidobacteria survived during 3 h incubation.

Introduction

Metchnikoff (1907) the Russian biologist, popularized the hypothesis that probiotic bacteria were capable of increasing length of life. He supported the theory with observations of Bulgarians who ate yoghurt regularly and showed remarkable longevity. Lactic acid producing bacteria are common components of probiotics. They are popular choice because of the historical belief that these bacteria are desirable members of the intestinal microflora. Lactic acid bacteria have long been used in the manufacture of dairy foods and are thus generally regarded as safe. However in recent years, the use of bifidobacteria as probiotic micro-organisms emerges drastically (Tannock, 1997). Bifidobacteria are the predominant bacteria in the stools of breast-fed infants (Bullen *et al*., 1976). They were first isolated. Recently, bifidobacteria have been classified into 30 species including nine species found in human (Ishibashi *et al*., 1997).

The advantages of using bifidobacteria as dietary adjunct are that they produced mild sour taste and less bitter taste than other starter culture. The beneficial effects of probiotics may be exerted by the present of their whole cells in the colon. Therefore, an essential determinant in the choice of a probiotic organism is its ability to survive in the environment in which it is intended to act. One important characteristic is the ability of the bacteria to survive through the human stomach and digestive system and take up residence in the large intestine (Kim, 1988). In order for that to occur, viable cells of bifidobacteria must be able to survive through the extreme of pH encountered in the digestive system of humans.

Therefore the present study was conducted to determine the ability of *Bifidobacterium* spp. isolated from infant stools to resist low pH.

Materials and Methods

Maintenance of Viable Cells: Twenty eight isolates of *Bifidobacterium* were obtained from our collection (Shuhaimi *et al*., 1999) and 1 strain of *Lactobacillus acidophilus* was isolated from commercial yoghurt drink. All bifidobacteria isolates were maintained and propagated in TPY broth (Scardovi, 1986) and *Lactobacillus* strain was maintained and propagated in MRS broth. The cultures were transferred every 18 hours using 1 percent inoculum. Cultures were incubated anaerobically at 37°C in an anaerobic jar (BBL, Rockville).

Preparation of HCl Solutions: Solutions of 37 percent hydrochloric acid (HCl) in double distilled water were adjusted to pH levels of 1.0, 2.0 and 3.0 by using 1M NaOH. Sterile double distilled water (pH 6.5) was used as a control. The solutions were prepared in 100 ml volumes using Kimax screw cap bottles, sterilised by autoclaving at 121°C for 15 min, and stored at room temperature until needed.

Enumeration of Bifidobacteria in pH Solutions: Stored solutions were thoroughly mixed and 9.0 ml of each pH solution was transferred into sterile universal bottles. 1.0 ml of stock culture containing approximately 10_9 cfu/ml of bifidobacteria was then transferred into each of four pH solutions (pH 1.0, 2.0, 3.0 and control). This procedure was repeated for each species of *Bifidobacterium. Bifidobacterium* spp. were then plated immediately (time 0 h) on TPY agar using a spread plate method (Scardovi, 1986) to obtain initial numbers. Serial dilutions were made using Ringer solution (E. Merck, Germany). The pH solutions containing bifidobacteria were then incubated anaerobically at 37°C, followed by intermittent plating after 1, 2 and 3 hours to simulate survival of bifidobacteria under pH

Table 1: Survival of *Bifidobacterium* spp. Isolated from Infant Stools in Solution pH 6.5 as Determined by Viable Counts[1]

Test organisms	Viable bacteria (log_{10} CFU/ml)[2]			
	0 h	1h	2h	3h
B. breve A50	7.643	7.352	7.851	7.653
B. infantis C17	7.782	7.686	7.716	7.829
B. infantis C15	7.58	7.574	7.58	7.544
B. infantis C83	7.556	7.574	7.491	7.491
B. breve C20	7.322	7.458	7.406	7.415
B. breve Cu1	7.643	7.667	6.52	5.74
B. infantis C25	7.13	7.06	7.11	7.09
B. breve D19	7.389	7.371	7.322	7.312
B. infantis D36	7.58	7.431	7.332	7.279
B. infantis D22	7.161	7.14	7.061	7.025
B. breve D73	7.316	6.922	6.903	6.942
B. breve F58	7.029	7.255	7.137	7.243
B. infantis F66	6.848	6.884	6.916	6.785
B. infantis F117	7.107	7.245	7.26	7.286
B. infantis F69	7.736	7.447	7	7.041
B. infantis F34	7.447	7.591	7.667	7.568
B. infantis F91	7.498	7.47	7.484	7.462
B. breve F100	6.47	7.279	6.653	6.973
B. infantis F112	7.512	7.47	6.96	6.966
B. breve F64	7.097	7.12	7.021	7.19
B. breve F81	6.041	6.255	6.204	6.398
B. infantis G4	7.519	7.591	7.556	7.648
B. breve G48	7.342	7.423	7.477	7.568
B. *infantis* G41	7.049	7.033	7.025	7.017
B. infantis Z37	7.8	7.623	7.447	7.512
B. infantis Z45	7.538	7.653	7.658	7.439
B. infantis Z47	7.663	7.525	7.613	7.279
B. infantis Z80	7.531	7.505	7.455	7.716
B. breve Z86	8.146	7.544	7.602	7.677
L. acidophilus	6.961	7.11	7.057	6.978

[1]Counts determined by spread plating of 0.1 ml sample on TPY agar, incubated anaerobically at 37°C for two days
[2]Experiments were conducted in duplicate

Table 2: Survival of *Bifidobacterium* spp. Isolated from Infant Stools in HCl Solution pH 1.0 as Determined by Viable Counts[1]

Test organisms	Viable bacteria (log_{10} CFU/ml)[2]			
	0 h	1h	2h	3h
B. breve A50	-	-	-	-
B. infantis C17	-	-	-	-
B. infantis C15	-	-	-	-
B. infantis C83	-	-	-	-
B. breve C20	3.30	-	-	-
B. breve Cu1	-	-	-	-
B. infantis C25	-	-	-	-
B. breve D19	-	-	-	-
B. infantis D36	-	-	-	-
B. infantis D22	2.653	-	-	-
B. breve D73	-	-	-	-
B. breve F58	-	-	-	-
B. infantis F66	-	-	-	-
B. infantis F117	-	-	-	-
B. infantis F69	-	-	-	-
B. infantis F34	-	-	-	-
B. infantis F91	3.574	-	-	-
B. breve F100	-	-	-	-
B. infantis F112	-	-	-	-
B. breve F64	-	-	-	-
B. breve F81	-	-	-	-
B. infantis G4	5.724	-	-	-
B. breve G48	-	-	-	-
B. infantis G41	-	-	-	-
B. infantis Z37	-	-	-	-
B. infantis Z45	1.698	-	-	-
B. infantis Z47	-	-	-	-
B. infantis Z80	-	-	-	-
B. breve Z86	-	-	-	-
L. acidophilus	-	-	-	-

[1]Counts determined by spread plating of 0.1 ml sample on TPY agar, incubated anaerobically at 37°C for two days
[2]Experiments were conducted in duplicate

conditions resembling human stomach (Clark and Martin, 1993). Enumeration of bifidobacteria after each storage interval was accomplished as described above using TPY agar. The plates were incubated anaerobically at 37°C for 48 h. Following incubation, colony forming units were counted and recorded. The results were mean of two experiments.

Results

Twenty eight isolates of bifidobacteria and 1 strain of *Lactobacillus acidophilus* were examined for their survival in hydrochloric acid solutions adjusted to pH 1.0, 2.0, 3.0 and in sterile double distilled water pH 6.5 (control). The initial numbers of bifidobacteria varied between 10^6 to 10^8 cfu/ml. Table 1 showed the results after exposure to double distilled water control (pH 6.5). Almost all the isolates examined were able to survive and maintain their number of viable cells from 10^6 cfu/ml to 10^8 cfu/ml after 3 hours exposure. Table 2 contains data for survival of bifidobacteria to pH 1.0. Five isolates (*B. breve* C20, *B. infantis* D22, *B. infantis* F91, *B. infantis* G4 and *B. infantis* Z45) out of 28 could survive just after plated immediately (0 h) to obtain initial numbers. Among these, B. "Mantis G4 showed the highest viability (10^5 cfu/ml). After 1 hour incubation no viable cells could be detected.

Survival of bifidobacteria at pH 2.0 is shown in Table 3. Results showed that 20 isolates survived just during the time it took to. plate the sample (0 h) to obtain initial numbers. Five isolates were able to survive after 1 hour incubation. *B. infantis* D22 was decreased in viability of about 3 log cycles whereas, *B. infantis* F34 and *B. breve* F100 decreased 2 log cycles. *B. infantis* F66 and *B. infantis* G4 showed very little change in numbers from the initial inoculum and no viable cells could be detected after 2 h incubation for all the isolates examined at this pH level.

The tolerance to pH 3.0 for all the isolates tested were better than in pH 1.0 and pH 2.0. Almost all the isolates tested were able to survive after withdrawn immediately (0 h) from this pH solution, except for *B. infantis* D36 and

Table 3: Survival of *Bifidobacterium* spp. Isolated from Infant Stools in HCl Solution pH 2.0 as Determined by Viable Counts[1]

Test organisms	Viable bacteria (log_{10} CFU/ml)[2]			
	0 h	1h	2h	3h
B. breve A50	-	-	-	-
B. infantis C17	3.230	-	-	-
B. infantis C15	5.720	-	-	-
B. infantis C83	-	-	-	-
B. breve C20	4.161	-	-	-
B. breve C11	2.978	-	-	-
B. infantis C25	-	-	-	-
B. breve D19	4.170	-	-	-
B. frifantis D36	-	-	-	-
B. infantis D22	5.628	2.875	-	-
B. breve 073	1 .699	-	-	-
B. breve F58	-	-	-	-
B. infantis F66	5.477	5.954	-	-
B. infantis F117	5.628	-	-	-
B. infantis F69	4.317	-	-	-
B. infantis F34	4.316	2.778	-	-
B. infantis F91	6.512	-	-	-
B. breve F100	6.146	4.653	-	-
B. infantis Fl 12	-	-	-	-
B. breve F64	1.699	-	-	-
B. breve F81	2.544	-	-	-
B. infantis G4	5.740	6.041	-	-
B. breve G48	-	-	-	-
B. infantis G41	-	-	-	-
B. infantis Z37	-	-	-	-
B. infantis Z45	4.582	-	-	-
B: infantis Z47	6.544	-	-	-
B. infantis Z80	4.097	-	-	-
B. breve Z86	4.12	-	-	-
L. acidophilus	2.204	-	-	-

[1]Counts determined by spread plating of 0.1 ml sample on TPY agar, incubated anaerobically at 37°C for two days
[2]Experiments were conducted in duplicate

Table 4: Survival of. *Bifidobacterium* spp. isolated from Infant Stools in HCl Solution' pH 3.0 as Determined b Viable Counts[1]

Test organisms	Viable bacteria (log_{10} CFU/ml)[2]			
	0 h	1h	2h	3h
B. breve A50	4.728	-	-	-
B. infantis C 17	7.618	-	-	-
B. infaniis C15	6.518	-	-	-
B. infantis C83	6.279	2.0	-	-
B. breve C20	6.653	4.580	-	-
B. breve C11	7.326	3.0	-	-
B. infantis C25	7.045	5.740	5.832	-
B. breve D19	6.628	4.312	2.301	-
B. infantis D36	-	-	-	-
B. infantis D22	6.580	5.929	5.544	4.919
B. breve D73	4.544	3.0	-	-
B. breve F58	-	-	-	-
B. infantis F66	6.342	4.602	-	-
B. infantis F1 17	7.061	5.52	33041	3.061
B. infantis F69	4.470	-	-	-
B. infantis F34	7.653	4.544	3.708	2.477
B. infantis F91	7.352	4.919	2.653	-
B. breve F100	6.4.15.	3.699	-	-
B. infantis F112	6.681	-	-	-
B. breve F64	6.813	-	-	-
B. breve F81	5.431	-	-	-
B. Infantis G4	6.352	7.44	5.720	-
B. breve G48	6.176	2.477	-	-
B. infantis G41	3.097	-	-	-
B. infantis Z37	5.613	3.230	-	-
B. infantis Z45	7.408	6.217	4.681	3.389
B. infantis Z47	7.120	5.398	-	-
B. Infantis Z80	6.796	4.518	-	-
B. breve Z86	4.748	-	-	-
L. acidophilus	3.110	-	-	-

[1]Counts determined by spread plating of 0.1 ml sample on TPY agar, incubated anaerobically at 37°C for two days
[2]Experiments were conducted in duplicate

B. breve F58 (Table 4). Eighteen isolates were able to survive after 1 hour incubation. The viability of *B. infantis* G4 almost not changed from the initial numbers. After 2 hours incubation only 8 isolates survived whereas the others not. Among these only 4 isolates (*B. infantis* D22, *B. infantis* F117, *B. infantis* F34 and *B. infantis* Z45) can survive after 3 hours incubation. The viability of *B. infantis* D22 was decreased about 2 log cycles. *B. infantis* F117 and *B. infantis* Z45 were decreased about 4 log cycles and *B. infantis* F34 was decreased about 5 log cycles. *Lactobacillus acidophilus* strain examined was unable to grow at pH 1.0 and can only survive at pH 2.0 and 3.0 just after exposure (0 h) to these pH conditions.

Discussion

Several studies have been published on the effect of low pH on the survival of lactic acid producing micro-organisms (Berrada *et al*., 1991; Pochart *et al*., 1992). Work was conducted by Pochart *et al*. (1992) on the growth of bifidobacteria in fermented cow milk adjusted to pH 1.0, 2.0 and 3.0. They reported that at pH 1.0, there was virtually no survival after one hour incubation. At pH 2.0, a significant decrease in viability occurred after one hour incubation. They also observed that, there was no significant decrease in number of bifidobacteria incubated for three hours at pH 3.0. The results of the present study were in close agreement with their results for pH 1.0 and 2.0, even though the strains used were not similar. Besides, these experiments were conducted in acidified distilled water instead of acidified fermented cow's milk. In this respect also, almost all the isolates were less resistant to pH 3.0 from those of theirs. The results also showed that the sensitivity of *Bifidobacterium* to low pH was isolate specific. In fact, Berrada *et al*. (1991) observed strains specificity of bifidobacteria to low pH. They found that two strains of *Bifidobacterium* contained in commercial fermented milk show different sensitivity to low pH. One strain of *Bifidobacterium* had decreased viability from approximately 600,00 cfu/g to 100 cfu/g after only five

minutes. Other strains decreased slightly but still exceeded 10,000,000 cfu/g after 90 minutes incubation.

The present study, as well as those of other researchers (Berrada *et al*., 1991, Pochart *et al*., 1992) demonstrate the need to identify pH tolerant strains of bifidobacteria prior to their use as probiotic preparation. Pochart *et al*. (1992) also investigated the ability of strain *Bifidobacterium* spp. to survive through the upper gastrointestinal tract wheh ingested in fermented milk. After ingestion of 10.0 ± 0.5 $\log_{10}$, bifidobacteria in 400 g fermented milk, ileal flow of bifidobacteria increased significantly and reached a maximum of 8.8 ± 0.2 $\log_{10}$, bifidobacteria/h 1.7 ± 0.4 h after ingestion of fermented milk. The average number of bifidobacteria recovered from the terminal ileum during the 8 h after fermented-milk ingestion was 9.0 ± 0.1 log 10 and constituted 23.5 ± 10.4 percent of the number ingested. These results indicate that in healthy adults, Bifidobacterium spp survive transit through the gastrointestinal tract when ingested in fermented milk. Many factors seem to affect the survival of bifidobacteria during gastric transit. Ganong (1977) stated that the rate of food passage through the stomach depends upon its composition, primarily fat content and the type of foods. Liquid foods leave the stomach much more rapidly than solid foods. Another aspect to be considered is the influence of food substances on pH of the stomach. Skim milk raised the pH of gastric juice *in vitro* therefore, it offered some protective effect to the bacteria (Conway *et al*., 1987). Consequently, the carrier foods play a significant role on the survival of bifidobacteria. 80 percent of gastric emptying were within 90 minutes for two strains of bifidobacteria in fermented milk (Berrada *et al*., 1991).

In conclusion, the present study demonstrated that none of the Bifidobacterium isolates possessed the same degree of sensitivity to low pH. Thus, choosing a correct Bifidobacterium isolate resistant to certain degree of acidity is important for the organism to exert beneficial effect to the host.

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