**AENSI Journals**

# Advances in Environmental Biology

ISSN-1995-0756 EISSN-1998-1066

Journal home page: http://www.aensiweb.com/AEB/

# Resistance at Low Ph Values and Bile Tolerance for Selection of Bifidobacterium Strains Isolated from New Born Feces as Potential Probiotic

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**ARTICLE INFO**

***Article history:***
*Received 23 June 2015*
*Accepted 10 August 2015*
*Available online 25 August 2015*

***Keywords:***
*bifidobacteria, bifidobacterium longum, probiotic, lactic acid, acetic acid.*

**ABSTRACT**

With the growing interest in health consciousness and idea of health promotion without associated health risks, the concept of probiotic foods has attracted much attention. Microbe such as *Bifidobacterium* is the most common organisms used as probiotic. In this study, 09 strains of bifidobacteria were isolated from new born feces, and identified using the API 20 A kits. The main phenotypic characteristic of *Bifidobacterium* is the production of lactic and acetic acids with a theoretical molar ratio (acetic:lactic) of 3:2, this organic acids were assessed with HPLC system. The carbohydrate fermentation permitted us to identifier 03 species *b.longum* (04 strains), *b.breve* (03starins) and *b.bifidum* (02 strains). This present work investigated the survival of Bifidobacteria in the gastrointestinal transit under in vitro conditions; tolerate of low ph of the stomach and survive under 0,3, 0,5, and 1% of bile concentration. Results showed a large variation in the viability of all strains in both, low ph and high concentration of bile. The only strain how present a highest rate of surviving is *b.longum* which has been selected for further investigation *in vivo*.



**To Cite This Article:** Kheira Mehdi, Miloud Hadadji, Bachir Benkaddour, Betache Guessas and Mebrouk Kihal., Resistance at Low Ph Values and Bile Tolerance for Selection of Bifidobacterium Strains Isolated from New Born Feces as Potential Probiotic. ***Adv. Environ. Biol., 9(14),*** *122-128, 2015*

## INTRODUCTION

A diverse and complex bacterial population was found in the intestinal microflora of human beings, and approximately 400 types of bacteria have been isolated in the feces of humans. Intestinal contents have a viable microbial count of about $10^{12}$ cfu $g^{-1}$ [1]. The human gut is home to large numbers of microorganisms that occupy mainly the ileum and colon. This complex microbial ecosystem known as the intestinal microbiota adapts to the host, becomes stable, and therefore resists change. The bacterial consortium maintains a large variety of physiological functions that exert both harmful and beneficial effects on human health [2]. *Bifidobacterium* constitutes a major part of the natural microflora of the human intestine, and when present in sufficient numbers, these organisms create a healthy equilibrium between beneficial and potentially harmful microorganisms in the gut [1]. Only a few babies had *Bifidobacterium* in the first day of life, while by day 2, 33% were so colonized with concentrations of $10^{8}$–$10^{10}$ g. By 1 week all had *Bifidobacterium* at concentrations of $10^{10}$–$10^{11}$ g. By 1 year of age, those that were still breast-fed had bacterial colonization with almost exclusive *Bifidobacterium* [3].

*Bifidobacterium* was first isolated in 1899 from the feces of breast-fed infants by Tissier of the Pasteur Institute [1]. These bacteria are Gram positive, nonspore forming, strictly anaerobic, pleomorphic fermentative rods. Optimum growth temperature ranges from 37 to 41°C, with minimum from 25 to 28°C and maximum from 43 to 45°C. The optimum pH is 6.5–7.0 [2]. They do not form aliphatic amines, hydrogen sulfide or nitrites. They produce vitamins, mainly B group, as well as digestive enzymes such as casein phosphatase and lysozyme [3]. Similarly, some *Bifidobacterium* strains produce lipophilicmolecules that have been shown to inhibit the viability of *E. coli*, *Klebsiella pneumonia*, *Yersinia pseudotuberculosis*, *S. aureus*, and *S. Typhimurium* [4]. In catabolizing oligosaccharides, they produce lactic acid and acetic acid and do not produce gas. Up to 30 different species have been reported, isolated from sewage, human and animal feces, rumen of

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cattle, dental caries, and honey bees [5]. *Bifidobacterium* produce strong acids as metabolic end products such as acetate and lactate to lower the pH in the local environment, which provides antibacterial effects [3].

By FAO/WHO definition, probiotics are viable microorganisms that confer health benefits to the host once consumed in adequate amounts. Microbes such as *Lactobacillus* and *Bifidobacterium* are the most common organisms used as probiotics [6]. Ingested bifidobacteria can pass through the stomach's acid and the unfriendly bile salts and once in the colon, they bind to the epithelial cells and to the intestinal mucin. Their density in the human GIT varies with age, diet, lifestyle, and activity. *B. longum* is the most frequently reported *Bifidobacterium* as having health benefits [5].

The aim of this study was to test the sensibility of *Bifidobacterium* strains at low pH values and high concentration of bile as a criterion to select probiotic strains.

## MATERIAL AND METHODS

*2.1 Isolation of microorganism:*

Strains were isolated from stool samples of infants breastfed within younger than 5 month (No baby had been on antibiotic treatment prior to faecal sampling). Samples were homogenized and diluted in peptone water supplemented with filter-sterilized cysteine·HCl. A mixture of antibiotics, including 2 g of neomycin sulfate, 3 g of nalidixic acid, 60 g of lithium chloride and 4 g of paromomycin sulfate, were prepared in 1 L of distilled water, filtersterilized with 0.45 μm Millipore filters and stored at 4 °C until used. The mixture (5 mL) was added to 100mL of MRS (de Man, Rogosa and sharpe agar) agar prior to plating (MRS-NNLP) [7, 8, 9].

Neomycin sulphate and nalidixic acid were included as growth inhibitors of Gram-positive and Gram-negative rods, respectively, and also lithium chloride was commonly used as a selective agent in bifidobacterial enumeration [9]. Fitered stérilized L-cysteine hydrochloride was also added at the rate of 0.05% in MRS broth (MRS-Cys) to lower the oxidoreduction potential in culture media and provides better anaerobic conditions for the growth of bifidobacteria, this amino acid is also regarded as an essential nitrogen source for bifidobacteria [10, 11, 12].

Plates were incubated anaerobically using gas pack (EZ campy container system) at 37 °C for 72 h. Typical colonies were selected for the gram coloration, and after purification by successive planting, biochemical tests (catalase, oxidase, nitrate reductase, $CO_2$ formation from glucose and growth in a salt medium) and identification with API 20A kits were realized. The curved, Y shaped or V shaped gram positive strict anaerobic cells which showed characteristic morphological and biochemical of *Bifidobacterium* were tested for the production of acetate and lactate.

*2.2 Determination of organic acids by HPLC:*

Modified MRS was prepared as the formulation of Lactobacilli MRS medium without sodium acetate, which might interfere with the results of the subsequent analysis of organic acids. Modified MRS medium were autoclaved at 121 °C for 15 min, media were supplemented with 0.05% (w/v) cysteine–HCl [13].

Organic acids were determined using method describing in [13, 14, 15] with the following modifications; the 1.5 ml samples were centrifuged at 13,000 ×g for 30 min and the supernatants were filtered through 0.45 μm filters. Samples were diluted with ethanol solution (1:4, v:v) were injected into a SHIMADZU HPLC system equipped with a vacuum degasser (prominence degasser PGU-20AS) gradient pump (prominence liquid chromatograph LC-20AD), and a UV/Vis detector (prominence SPD-20A), Analytes were separated with Column (18C). The mobile solution was ethanol and the flow rate was 0.1 ml/min. The UV detector wavelength was 210 nm. Standard calibration curves were prepared using serial dilutions of lactic and acetic acids.

*2.3 Determination of tolerance to the gastro-intestinal conditions:*

*2.3.1 Growth at different Ph values:*

Strains were grown overnight at 37C° under anaerobic condition, an aliquot of 1ml of this culture were transferred to 9ml of MRS-Cys broth adjusted at differente Ph values (1,2,3and 4,) using HCL 1N, MRS-Cys broth at ph6,8 were used as a control.

The pH in human stomach ranges from 1 during fasting, to 4.5 after a meal, and food ingestion can take up to 3 h [16], Culture were than incubated anaerobicly at 37C°, One milliliter from each pH solution was taken immediately (0 h) and after 60, 120, 180 and 240mn. Serial dilutions were prepared, Appropriate dilutions were plated in MRS agar and incubated anaerobicly. Enumerations of viable cells were also determined by measuring the optical density with spectrophotometer (UV/vis) at 620nm.

*2.3.2 Resistance to bile salts:*

The mean intestinal bile concentration is believed to be 0.3% (w/v) and the transit time of food through the small intestine is generally suggested between 1 and 4h [16].

An concentration of bile salts was prepared with distilled water and filer-sterilized with 0,45 Millipore filter. This solution was added to MRS-Cys broth at a final concentration of 0,3 0,5 and 1%. Test strains were cultured and treated similarly to the previously described procedures, except that the acid was replaced by 0,3, 0,5 and 1% of bile salts. Incubation and enumeration of viable cells were also realized under the same condition. All tests were carried out in triplicate.

## RESULTS AND DISCUSSION

Colonies formed by *Bifidobacterium* on MRS-NPPL medium are smooth, convex, cream or white, glistening, and of soft consistency. Their contour is regular with a diameter of 0.1 to 0.5 mm. All purified isolates were Gram positive and present negative results for (catalase, oxidase, nitrate reductase, $CO_2$ formation from glucose and growth in a salt medium) and positive result for the citrate permease. Table 1 present the results obtained after identification with API 20A kits system.

From this result obtained, strains are presumed to be a member of the genus *Bifidobacterium*, the carbohydrate fermentation profil permitted to identifier 03 species according to the encyclopedia of dairy science [1] ; *bifidobacterium breve* (BV1, BV2, BV3), *bifidobacterium bifidum* (B1, B2) and *bifidobacterium longum* (L1, L2, L3, L4).

*3.1 Production of organic acids:*

Organic acids were identified according to their retention times compared with standard solutions of lactic and acetic acids. During fermentation in modified MRS medium, approximately 3:2 mol of acetic/lactic acid were produced (fig 01), observations that concur with the theoretical amounts reported by Scardovi [7, 14], this results permitted to classifier strains as being of bifidobacteria.

*3.2 Growth at different ph values:*

The results obtained were represented by survival rate (table 2) according to the following equation: Survival percentage % = log ufc $t_x$h / log ufc $t_0$h * 100

X: different time of incubation

The acid tolerance (survival at different Ph valious) showed that none of the strains survived at Ph 1 for any time of incubation. At ph 2 each strain demonstrated a rapid decrease in viability compared to Ph 3 and 4.

The only strain surviving at ph 2 is L2 how shown a small decrease between incubation after 1 and 4h, for all the other strains we noticed a wide variation between 1 and 4h of incubation at the same Ph. Another strain that show a high rate of survival, in addition to the strains L2, is L4. Both of these strains were able to keep a stable survival rate for phs 3 and 4 and for all incubation time.We also noticed significantly decreased after 2h of incubation at Phs 3 and 4 for BV1, BV3, B2, and B.

The result is in agreement with previous studies found by Takahashi who reported that most of the studied strains exhibited survival rates of less than 1%, with the exception of one strain of *B. longum* that showed a survival rate of 25% after incubation at pH 3.0 for 2 h [17].

In the study of Silvia et al the résistance of *B. longum* was higher than that of the reference strain *B. lactis* Bb12 [18]. Valérie et al also reported that *B. longum* and *B. breve* harbored the best tolerance to acid stresses [19].

Contrary to Matsumoto et al indicated that the acid tolerance of *B. longum* was weak; these strains died or decreased after exposure to pH 5 for 3 h [20].

In similar studies, Collado et al has found that the most resistant strain is *B .breve;* with the highest ability to develop an acid-tolerance response which allowed their greater survival in otherwise lethal conditions [21].

In similar studies, other researchers have found that the most resistant strain is *b.breve;* and that there is a possibility of relationship between the predominance of this species in infants and its higher ability to survive the gastrointestinal conditions [18].

*3.3 Bile salts tolerance:*

Once bacteria reach the small intestinal tract, their ability to survive depends on their resistance to bile. As bile entering the duodenal section of the small intestine has been reported to reduce the survival of bacteria [22].

Table 3 shows the survival rate % of the tested *bifidobacterium* strains of bile tolerance in proximal human intestinal condition. Out of 09 strains studied we noticed that the bile tolerance decreased with increased bile concentration.

Our results are in agreement with similar studies presented by Clark and Martin how showed that *B. longum* exhibited the best tolerance to 2.0 or 4.0% of bile concentration [23]. In the study of Silvia et al *B. longum* IPLA 20003 showed the highest survival with a 0.84% whereas the reference strain *B. lactis* Bb12 survived only a 0.0004% after bile juice challenge [18].

Valerie et al also indicated *that B. longum* and *B. breve* species showed the highest interest in terms of potential selection of probiotics from human origin [19]. In contrast Ibrahim and korovainy reported that *B. infantis* had the best survival rates followed by *B. bifidum, B. breve and B. longum* when exposed to bile salt at concentrations ranging from 0 to 3 g/ l [24].

*Conclusion:*

Tolerance to gastrointestinal transit (acidic pH in the stomach and bile in the small intestine) could be applicable to the direct selection of potentially probiotic bifidobacterial strains.

**Table 1:** Strains identification with API 20A kits system.

| tests | Reaction/enzymes | results | | Strains results | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | positive | negative | L1 | L2 | L3 | L4 | BV 1 | BV 2 | BV 3 | B1 | B2 |
| IND | indol formation | Red | Yellow | - | - | - | - | - | - | - | - | - |
| URE | urease | red | Yellow | - | - | - | - | - | - | - | - | - |
| GLU | Acidification glucose | Yellow | Purple | + | + | + | + | + | + | + | + | + |
| MAN | Acidification manitol | | | - | - | - | - | - | + | - | - | - |
| LAC | Acidification lactose | | | + | + | + | + | + | + | + | + | + |
| SAC | Acidification saccharose | | | + | + | + | + | + | + | + | + | + |
| MAL | Acidification maltose | | | + | + | + | + | + | + | + | - | - |
| SAL | Acidification salicin | | | + | + | + | + | - | - | - | + | + |
| XYL | Acidification xylose | | | + | + | + | + | - | - | - | - | - |
| ARA | Acidification arabinose | | | + | + | + | + | - | - | - | - | - |
| GLE | Hydrolysis gelatin | Diffusion of black pigment | No diffusion of pigment | - | - | - | - | - | - | - | - | - |
| ESC | Hydrolysis esculin | | | +/- | +/- | +/- | +/- | +/- | +/- | +/- | +/- | +/- |
| GLY | Acidification glycerol | Yellow | Purple | - | - | - | - | - | - | - | - | - |
| GEL | Acidification cellobiose | | | - | - | - | - | - | - | - | - | - |
| MNE | Acidification mannose | | | - | - | - | - | + | + | - | - | - |
| MLZ | Acidification melezitose | | | + | + | - | + | - | - | - | - | - |
| RAF | Acidification rafinose | | | + | + | + | + | + | + | + | - | - |
| SOR | Acidification sorbitol | | | - | - | - | - | + | + | + | - | - |
| RHA | Acidification rhamnose | | | - | - | - | - | - | - | - | - | - |
| TRE | Acidification trehalose | | | - | - | - | - | - | - | - | - | - |

**Table 2:** Tolerance to low ph of the stomach expressed as % of survival.

| strains | pH | 1h | 2h | 3h | 4h |
|---|---|---|---|---|---|
| L1 | 2 | 52 | 34,5 | 14 | 0 |
| | 3 | 55,12 | 50 | 31 | >10 |
| | 4 | 58,75 | 52,8 | 33,8 | >10 |
| L2 | 2 | 70,72 | 68,57 | 60,73 | 52,73 |
| | 3 | 73,37 | 68,91 | 65,37 | 58,37 |
| | 4 | 77,70 | 72,10 | 69,8 | 60,8 |
| L3 | 2 | 39,16 | 20,2 | 10,75 | >1 |
| | 3 | 40,83 | 29,8 | 17,77 | >10 |
| | 4 | 44,16 | 33,2 | 25,75 | 12,10 |
| L4 | 2 | 42,5 | 23 | >10 | >1 |
| | 3 | 65,4 | 60,5 | 49,8 | 38,5 |
| | 4 | 74,9 | 60 | 54,2 | 46 |
| BV1 | 2 | 12,56 | 6 | 0 | 0 |
| | 3 | 36,70 | 26,23 | 16,70 | 0 |
| | 4 | 38,11 | 30,2 | 20 | 0 |
| BV2 | 2 | 31,4 | 16,8 | 5,4 | 0 |
| | 3 | 34,4 | 29,2 | 10,6 | 0 |
| | 4 | 41 | 38,3 | 18 | >1 |
| BV3 | 2 | 20,4 | 12 | >1 | 0 |
| | 3 | 26,6 | 22 | >10 | >1 |
| | 4 | 32 | 23,4 | >10 | >1 |
| B1 | 2 | 33,8 | 19,9 | >1 | 0 |
| | 3 | 35,2 | 20 | >1 | 0 |
| | 4 | 40 | 29,9 | >10 | 0 |
| B2 | 2 | 49,8 | 23 | 09 | 0 |
| | 3 | 62,6 | 49 | 22,3 | 0 |
| | 4 | 74,6 | 62,2 | 44,1 | >1 |

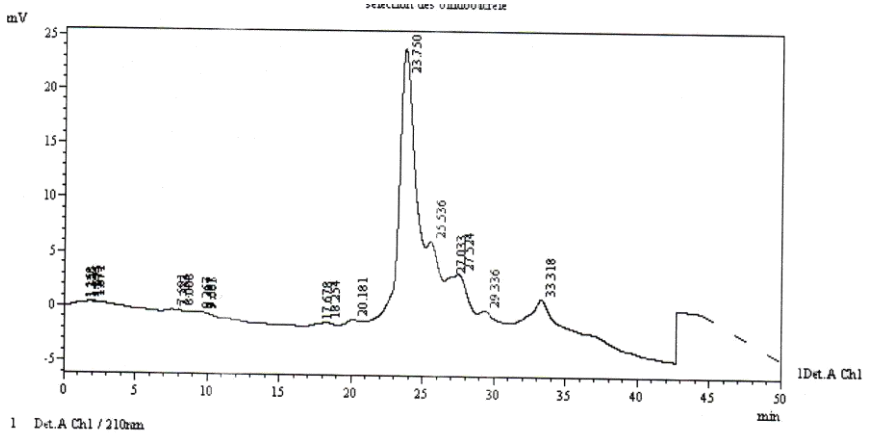
**Table 3:** Tolerance to bile salts expressed as % of survival.

| strains | Bile concentration | 1h | 2h | 3h |
|---|---|---|---|---|
| | 0,3 | 42 | 34,5 | 29,8 |
| | 0,5 | 35,12 | 30 | 21,8 |
| L1 | 1 | 28,75 | 12,8 | >01 |
| | 0,3 | 50,72 | 48,57 | 44,73 |
| L2 | 0,5 | 43,37 | 38,91 | 35,37 |
| | 1 | 27,70 | 22,10 | 19,8 |
| | 0,3 | 39,16 | 36,2 | 30,75 |
| L3 | 0,5 | 40,83 | 31,8 | 27,77 |
| | 1 | 34,16 | 13,2 | >10 |
| | 0,3 | 32,5 | 23 | 19,7 |
| L4 | 0,5 | 25,4 | 13,5 | >10 |
| | 1 | 14,9 | 6 | >01 |
| | 0,3 | 22,56 | 19,6 | 14,5 |
| BV1 | 0,5 | 19,70 | 14,23 | >10 |
| | 1 | 13,11 | >10 | >01 |
| | 0,3 | 31,4 | 26,8 | 15,4 |
| BV2 | 0,5 | 24,4 | 19,2 | >10 |
| | 1 | 21 | 12,3 | >01 |
| | 0,3 | 20,4 | 12 | >10 |
| BV3 | 0,5 | 16,6 | >10 | >01 |
| | 1 | 12 | >01 | >01 |
| | 0,3 | 18,8 | 16,9 | 12,8 |
| B1 | 0,5 | 15,2 | 10,7 | >10 |
| | 1 | 10,8 | >10 | >01 |
| | 0,3 | 29,8 | 23,2 | 19 |
| B2 | 0,5 | 22,6 | 19,6 | 12,3 |
| | 1 | 14,6 | >10 | >01 |

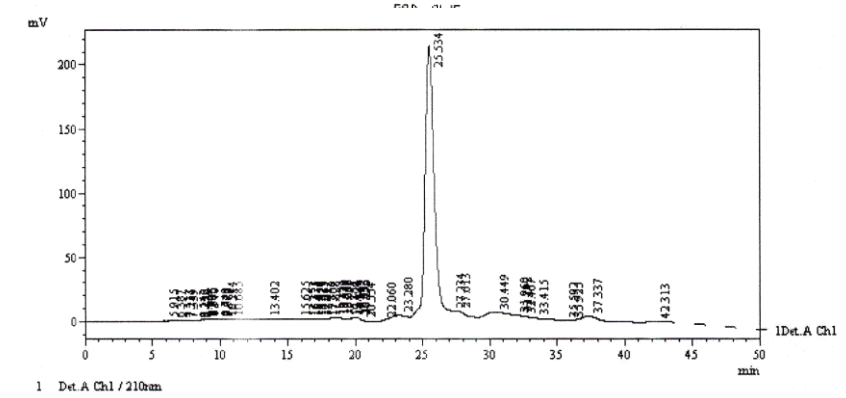
The results obtained during this study indicate that tolerance toward bile salts and acid Phs was variable for all the strains tested.

Combining the results of both, acid and bile tolerance, *B. longum* appears to display the highest ability to survive during gastrointestinal transit. These strains are being further investigated for other probiotic characteristics and *in vivo* studies.

**A**




**B**



**C**

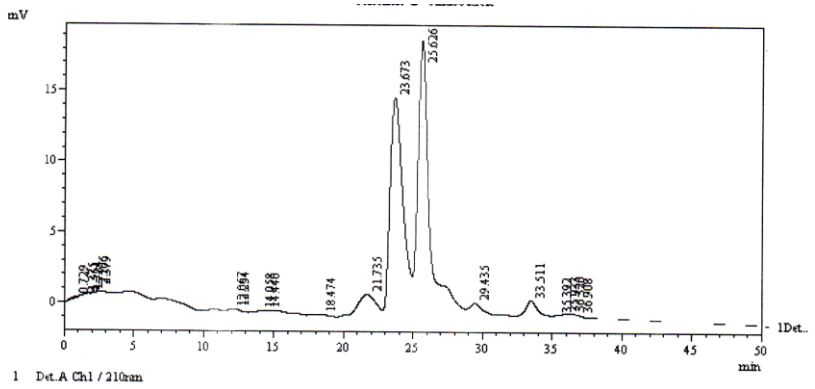



**Fig. 1: D**etermination of organic acids by high performance liquid chromatography (HPLC); **A:** retention time of lactic acid. **B:** retention time of acetic acid. **C:** the fermentation profile of the strains.

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