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Full Length Research Paper

Identification of cultivable *Bifidobacterium* species isolated from breast-fed infants feces in West-Algeria

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Gastrointestinal microflora exerts a high impact on its host mainly by performing a great variety of metabolic activities, protecting the host against colonization by pathogen and stimulating the gut immune system. The integration of bifidobacteria in dairy products is of interest for reinstalling the intestinal microflora. The mother's milk is a prebiotic factor which stimulate the bifidobacteria growth *in vivo*. Results obtained in west Algeria area show that the number of bifidobacteria reach 26 10^9 cfu/g in breast-fed infant feces. The microbiological, fructose–6-phospho -ketolase and the biochemical tests have revealed *Bifidobacterium* strains. The most prevalent isolates belong to the species *Bifidobacterium longum* (75%) and *Bifidobacterium breve* (25%). In order to evaluate the potential use as a starter culture, the occurrence of some selected properties such as growth and survival in fermented milk were investigated. These strains have a fast growth in glucose, galactose and lactose media compared to reference species. All isolates studied are sensitive to the pH 8. The *B. breve* strain support the conservation conditions in pure culture better than in mixed culture with *Streptococcus thermophilus* or *Lactobacillus acidophilus.*

Key words: Lactic acid bacteria, *Bifidobacterium*, probiotic, fermented milk, growth, survival.

INTRODUCTION

Bifidobacterial species are common members of the human gut microflora, comprising up to 3% of the total fecal adults microflora. They are more numerous in the infant gut, where they form up to 91% of the total microflora in breast-fed babies and up to 75% in formula-fed infants. Because of their prevalence in the infants feces suckled at the breast, bifidobacteria are considered to be beneficial bacteria, and they are used in the preparation of probiotic products (Boudraa et al., 1990; Lievin et al., 2000; Requena et al., 2002). The gastrointestinal microflora is influenced by diet, age, environmental conditions and by the host genotype. Tissier (1906) showed that *Bifidobacterium* species were the predominant microflora in breast fed infants and speculated that infant diarrhea could be treated by giving large dose of *bifidobacteria* orally.

The number of food and other dietary products containing live *bifidobacteria* has increased significantly in recent years, due in part to the beneficial effects. Although substantial research efforts are currently in progress to investigate these claims, the available evidence indicates that ingestion of bifidobacteria may promote desirable drugs in gastrointestinal tracts (Isolauri et al., 2000; Kailasapathy and Chin, 2000; Shu et al., 2000; Crittenden et al., 2001; Ishibashi and Yamazaki, 2001).

Recently, the isolation of *Bifidobacterium* species from feces has assumed considerable importance, as a consequence of interest in the potential health-promoting properties of this genus (Arunachalam et al., 2000). Studies involving *Bifidobacterium* species identification

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from human usually require enumeration of these organisms in feces (Silvi et al., 1996). Due to this wide probiotic activity, the *Bifidobacterium* have large industrial and medical importance (Richardson, 1996; Takahashi et al., 2004) and there has been an increased interest in the use of bifidobacteria as feed supplements (Shah, 2000).

Recently, research has focused on identifying new strains of *Bifidobacterium* with health-promoting properties. It is, nevertheless, important to confirm the safety of any newly identified probiotic strains (Martin and Cabbage, 1992; Collins and Gibson, 1999). The aim of this study is to investigate the identification and the growth potential of different *Bifidobacterium* strains presents in the feces of breast- fed infants in Algeria and their possible use as a probiotic cultures starters.

Materials and methods

Strains origin

Bifidobacterium strains used in this study were derived from three sources:

(1) About 30 fresh fecal samples were obtained from newborn infants (aged 2 to 4 months). For each sample, about 1 g of freshly feces was transferred into flasks containing 9 ml of pre-reduced salt solution (NaCl, 0.9 %) containing 0.2% cysteine-HCl and the suspension was homogenized for 2 min. After checking, serial dilutions were made and 0.1 ml of the suspension was inoculated in TPY agar medium (Beerens, 1990). All plates were incubated anaerobically at 37°C for 5 days, using oxoid gas jars and anaerobic gaspak, capable of producing hydrogen and carbon dioxide. Bifidobacteria were enumerated using TPY solid medium. 25 colonies from the highest dilution of each sample were picked at random and inoculated into TPY agar medium. Colonies picked from countable plates were selected for gram reaction, morphology and biochemical tests.

(2) From commercial French fermented milk (Active bifidus), in which three species of lactic acid bacteria were found, *Lactobacillus delbrueckii* subsp. *bulgaricus, streptococcus thermophilus* and *Bifidobacterium* sp. Decimal dilutions are carried out starting from 1 g of fermented milk in reduced peptone water (1 g peptone, 8.5 g NaCl and 0.5 g cysteine-HCl per liter) (Frank et al., 1993). 0.1 ml of dilutions 10^{-4}, 10^{-5} and 10^{-6} were inoculated on TPY medium with 2 mg/l of dicloxacilline for the inhibition of *S. thermophilus* and *L. delbrueckii* (Tamime *et al.*, 1995). All plates were incubated in same conditions as in fecal simples. Pure colonies were studied

(3) Reference strains were obtained from freeze dried pure culture of *Bifidobacterium bifidum (*B6), *Lactobacillus acidophilus* and *Bifidobacterium longum* (B4) from the collection of Institute of Food Research, Cambridge, UK and the Dipartimento Di Scienze E Tecnologie Alimentarie Microbiologiche Sezione Microbiolgia Agaria Alimentare Ecologica Universita Deglia Studidi Milano, Italia.

Culture media

Three culture media were used: (a) TPY medium (trypticase-phytone-yeast) described by Scardovi (1986) for the isolation of Bifidobacteria. (b) M17 medium (Terzaghi and Sandine, 1975) for *Streptococcus*. The pH is adjusted to 6,.7 and the medium are sterilized 20 min at 120°C. acidified MRS medium (De Man et al., 1960) pH 5.5 for *Lactobacillus*. Skim milk was prepared by dissolution of 100 g of skim milk powder in 900 ml of distilled water. The milk is sterilized for 10 min at 110°C.

Long-term conservation of *Bifidobacterium* strains, without appreciable loss of properties, was achieved by maintaining in skim milk containing 0.2% cysteine-HCl with 30% (v/v) glycerol at 20°C. Working cultures were also kept on MRS agar containing 0.2% cysteine- HCl, MRS or M17 agar slant at 4°C and re-streaked every 4 weeks (Samelis et al., 1994; Badis et al., 2004). Details of the incubation conditions will be described for each set of experiments. Morphologically different colonies were picked from the plates and each streaked on a separate plate. The plates were then incubated at 30±1ºC for 48 h and the result colonies examined for purity. The process was repeated until there were no mixed cultures on each plate.

Physiological and biochemical tests

The isolates considering belong to the genus *Bifidobacterium* were identified by the detection of fructose-6-phosphate phosphoketolase (F6PPK) enzyme in cellular extracts as described by Scardovi (1986). Cells were grown in 10 ml of TPY broth at 37°C for 18 h and harvested by centrifugation at 5000 g for 10 min. The pellet was washed twice with 5 ml of 0.5 g/l phosphate cysteine buffer. After centrifugation, the pellet was collected in 1 ml of buffer and disrupted by sonification at 0°C for obtaining crud cells extract. 0.25 ml of reagents (6 mg/ml NaF, 10 mg/ml sodium iodoacetate and 80 mg/ml fructose-6-phosphate) was added to the cells extract. The reaction was started by incubation 30 min at 37°C and stopped by adding 1.5 ml of hydroxylamine-HCl (13.9%). After 10 min, 1 ml of trichloroacetic acid (15%) and 1 ml of $FeCl_3.6H_2O$ (5%) were added. The presence of fructose-6-phosphate phosphoketolase enzyme was revealed by the appearance of red and purple colors (Scardovi, 1986; Meile et al., 2001).

All strains were initially submitted to Gram staining, the catalase test and spore formation. Colonies and cells morphology characteristics on TPY containing 0.2% cysteine-HCl, MRS and M17 agar were also examined. All isolates were tested for further identification as described by Ingrassia et al. (2001). Growth at different temperatures was observed in TPY broth, after incubation for 5 days at 15°C, 37°C and 45°C; 12 days at 4°C and 10°C. Gas production from glucose was determined in MRS broth containing inverted Durham. Hydrolysis of arginine was tested on M16 BPC medium (Saidi et al., 2002). Growth in the presence of 4 and 6.5% NaCl was observed in MRS broth at 30°C for 2 days. The possible growth at pH 3.9 and pH 9 was tested on MRS broth as well as growth in bile salts added to TPY medium to a final concentration of 0.3% (w/v)(Grill et al., 2000). Citrate utilization, in the presence of carbohydrates, was performed on the media of Kihal et al. (1996).

Carbohydrate fermentation

The carbohydrates fermentation was determined on TPY broth containing bromocresol purple (0.04 g/l) as a pH indicator, and supplemented with 1% of the following carbohydrates: lactose, sucrose, xylose, arabinose, sorbitol, fructose, galactose, mannose, cellobiose, raffinose, melizitose and melibiose. To ensure anaerobic conditions, each tube was supplemented with two drops of sterile liquid paraffin after inoculation (Samelis et al., 1994; Saidi et al., 2002).

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) and the isolates assigned to appropriate species. The isolates belonging to the genus *bifidobacterium* were identified to species level using sugar fermentation and the profiles were compared with the reference strains in standard texts for identification.

Growth and acid production in milk

Skim milk medium was prepared from reconstituted skim milk powder (Régilait imported by Sarl BELL) (100 g/l distilled water) and sterilized by autoclaving in bath at 110°C for 10 min. Sterilized milk was inoculated with active culture (0.2%) of each strain to obtain approximately 10^7 cfu/ml and incubated at 37°C for 24 h. The viable colony-forming units per milliliter were determined each hour. After serial dilution of sample cultures, an aliquot of each dilution was spread on TPY agar for the enumeration of *Bifidobacterium* sp.

Antibiotic resistance

The bifidobateria antibiotic resistance were determined by the diffusion technique in solid TPY medium with pH 7 by the use of antibiotic discs (biomérrieux): neomycin (30 IU), chloramphenicol (30 IU), acid nalidixic (30 µg), vancomycin (30 µg), ampicillin (10 µg), fusidic acid (10 µg), and rifampicin (100 µg). This test was used in the selection of *Bifidobacterium* species.

Measure bacterial growth

The bacterial growth on liquid medium is followed by the measurement of the optical density (D.O.) at 600 nm using a spectrophotometer (digital Spectro 22) (Kot and Bezkorovainy, 1993).

Numeration on solid medium

After vigorous agitation using a vortex during 30 s, the samples are diluted in physiological water containing 0.05% cysteine-HCl. 0.1 ml of adequate dilutions are spread on TPY solid medium. Incubation is made with 37°C for 48 to 72 h. the Petri dishes containing a number of colonies ranging between 30 and 300 are retained for counting (Badis et al., 2004).

Exogenic factors effect

The kinetics of growth of *Bifidobacterium* strains according to the source of carbon (glucose, fructose, galactose and lactose) at 0.1%, in pH (4, 5, 6, 7 and 8) and in temperature of incubation (25°C, 30°C and 37°C) is followed by the measurement of the optical density at 600 nm every 2 h.

Growth in milk medium

The bifidobacteria isolates are maintained in skimmed milk with 0.5% yeast extract and 0.05% cysteine-HCl (Frank et al., 1993). The *Bifidobacterium* and *L. acidophilus* preculture were incubated at 37°C and 42°C for *Streptococcus thermophilus* until coagulation of milk. These precultures are used for the preparation of pure and mixed cultures. The pure culture of each strain was obtained by inoculating sterile milk with a final concentration of 5% of inoculum. After homogenization, the cultures were incubated in the same conditions. To follow the production of acid, growth and pH were determined at regular interval. For the mixed cultures, the strains were inoculated with 5% skim milk and incubated at 42°C (Tamime and Robinson, 1988), while the others were incubated at 37°C**.**

Study of the kinetics of growth

In pure culture, the kinetics of growth of the strains of bifidobacteria was carried out by enumeration on TPY solid medium after 0, 4, 6 and 8 h of incubation. In mixed culture with the lactic bacteria strains, the bifidobacteria are enumerated on TPY medium containing dicloxacilline (2 mg/ml) (Tamime et al., 1995). However, the enumeration of *S. thermophilus* was carried out on M17 medium at 42°C for 48 h in aerobiosis, and *L. acidophilus* on medium MRS acidified with 37°C for 72 h.

Acidity determination

The lactic acid determination produced during the growth in skim milk is measured by the method described by Accolas et al. (1977) using NaOH (N/9) in the presence of phenolphthalein (1%). The acidity developed in milk is also followed by a measurement of pH (Kihal et al., 1996; Alonso-Calleja et al., 2002).

Study of survival in milk

Among the samples incubated in milk, some are taken and preserved at +4°C. Measurements of pH are taken after the 7, 14 and 21 days. This was to determine the rate of the bifidobacteria present in the milk fermented at the time of the conservation (Bizkorovainy, 2001).

RESULTS

Characteristics of the isolates

All purified isolates were Gram positive bacteria and were presumed to be lactic acid bacteria. All the isolates were negative for catalase, oxidase and nitrate reduction tests. The bifidobacteria colonies on TPY solid medium are punctiform, luisante, white and cream color, regular contour with a diameter of 0.1 to 0.5 mm. The bifidobacteria number in fermented milk is 3×10^7 ufc/ml and 26×10^9 cfu/g in the fecal matter. All the isolates obtained are pleomorphic rods, nonsporulating, strictly anaerobic, gelatinase negative, with no indol production, resisting up to 2% bile salt and positive for the F6PPK test. All these characters confirm the *Bifidobacterium* genus. The majority of the isolates are identified as belonging to *Bifidobacterium*. Further characterizations were performed by analyzing sugar fermentation and antibiotic resistance for the identification of *Bifidobacterium* species.

The strains B2, B3, *Bif5* and B4 fermented the arabinose (Table 1). This property differentiate *B. longum* from the other *Bifidobacterium* species of human origin. Moreover, the strains belonging to this species do not use cellobiose and salicin. Whereas, the B1 strain cannot

Table I. Morphological cultural, physiological and biochemical characteristics of the isolated strains from infant feces (B1, B2, B3, Bif 5) and the reference strains (B4 and B6)

Characteristics	Strains					
	B1	**B2**	**B3**	**Bif5**	**B4**	**B6**
Colony morphology	Circular white slimy	Circular white slimy	Circular white slimy	Circular white slimy	Circular white slimy	Circular white slimy
Cell morphology	Rods	rods	rods	rods	rods	rods
Gram stain reaction	+	+	+	+	+	+
Spores formation	-	-	-	-	-	-
Catalase activity	-	-	-	-	-	-
Oxydase activity	-	-	-	-	-	-
NH_3 from arginine	-	-	-	-	-	-
Gelatin liquefaction	-	-	-	-	-	-
Indole production	-	-	-	-	-	-
Resistance to bile salts	+	+	+	+	+	+
Gas from glucose	-	-	-	-	-	-
Arabinose	-	+	+	+	+	-
Cellobiose	+	-	-	-	-	-
Fructose	+	+	+	+	+	+
Galactose	+	+	+	+	-	+
Glucose	+	+	+	+	+	+
Maltose	+	+	+	+	+	-
Lactose	+	+	+	+	+	+
Inuline	+	-	-	-	-	-
Mannose	+	+	+	+	+	-
Melizitose	+	+	+	+	+	-
Melibiose	+	+	+	+	+	+
Raffinose	+	+	+	+	+	-
Ribose	+	+	+	+	+	-
Salicine	+	-	-	-	-	-
Sorbitol	+	-	-	-	-	-
Sucrose	-	+	-	+	+	+
Trehalose	-	-	-	-	-	-
Xylose	-	+	+	+	+	-

ferment xylose and sucrose. The reference strains B6 is characterized by limiting carbohydrate fermentation. This phenotypical characteristics described by Scardovi (1986)and Tamime et al. (1995) resulted in identification of two *Bifidobacterium* species, *B. breve* B1 and *B. longum* B2, Bif5 and B3 from infant feces. *B. longum* B4 and *B. bifidum* B6 were the reference strains (Table 1).

Exogenic factors effect

Influence of carbon source: A variability was observed in the growth of the isolated strains. The latency phase is more significant in reference strains (B4, B6). In the presence of glucose (Figure 1A), 1 h is necessary for the latency phase in the case of B1, B2, B3 and Bif5 strains. In addition, the maximum growth of B1, Bif5 and B4 strains is reached at 5 h of incubation and the growth rate is 0.5, 0.4 and 0.1, respectively. Whereas, B2, B3 and B6 strains, the maximum growth is obtained after 7 h with a growth rate of 0.4, 0.27 and 0.4 respectively. The growth of the strains on lactose medium is slightly similar to that obtained on galactose medium (Figures 1B and 1C). The maximum growth is reached after 7 h of incubation, with a growth rate of 0.3.

Effect of pH: The results of the kinetics growth of the strains (B1, B2, B3, Bif5 and B6) on TPY medium with different pH (5, 6, 7, and 8) reveal the existence of a

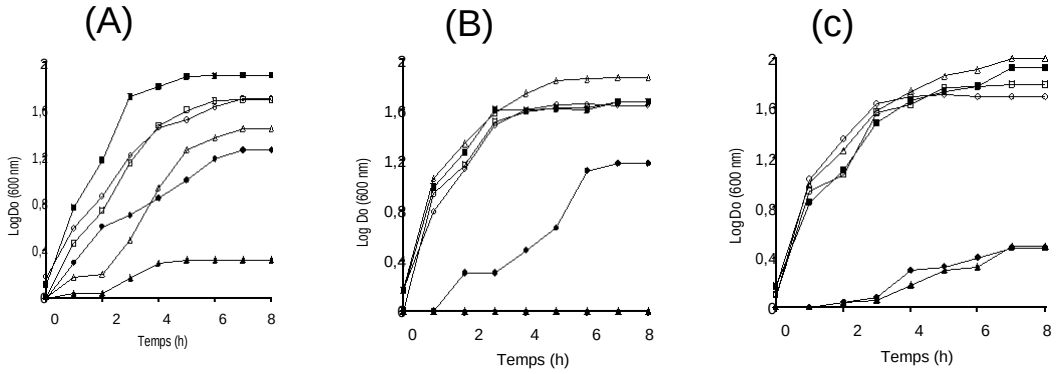

Figure 1. Growth of *Bf. breve* B1( ), *Bf. longum* Bif5 ( ), *Bf. longum* B2 (∆), *Bf. longum* B3 (O) and *Bf. longum* B4 ( ) and *Bf. bifidum* B6 ( ) on TPY glucose (A), TPY lactose (B) and TPY galactose (C).

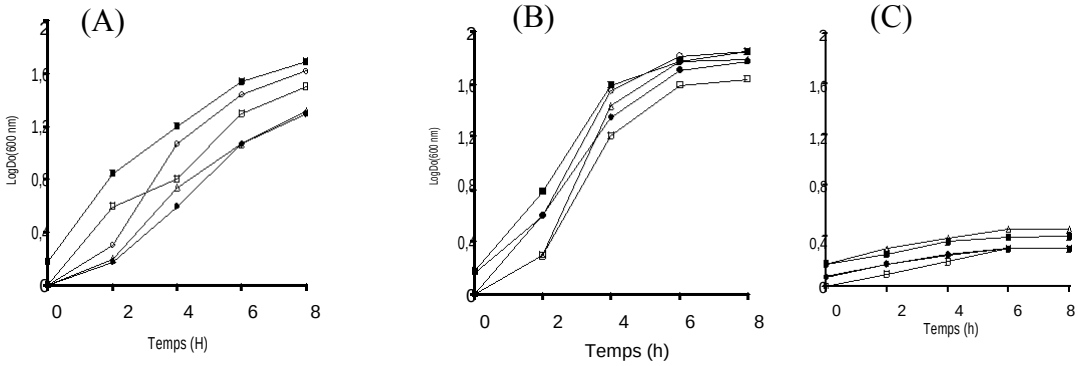

Figure 2. Growth of *Bf. breve* B1( ), *Bf. longum* Bif5 ( ), *Bf. longum* B2 (∆), *Bf. longum* B3 (O) and *Bf. bifidum* B6 ( ) on medium TPY with pH 5 (A), pH 6 (B) and pH 8 (C).

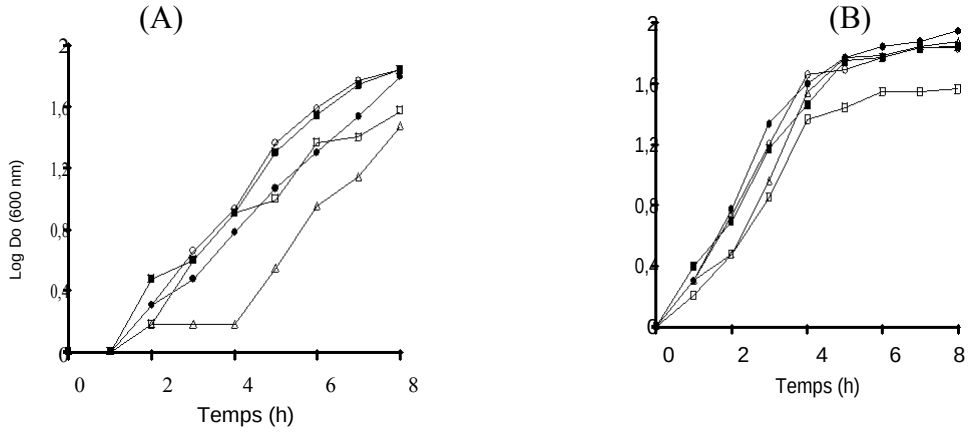

Figure 3. Growth of *Bf. breve* B1( ), *Bf. longum* Bif5 ( ), *Bf. longum* B2 (∆), *Bf. longum* B3 (O) and *Bf. bifidum* B6( ) on medium TPY AT 25°C (A) and 45°C (B).

variability. However at pH 5 (Figure 2A), the strains B3, B2 and B6 have a latency phase of 2 h, which is absent in B1 and Bif5. The maximum growth is obtained after 8 h of incubation. The calculated growth rates are slightly similar for all strains, 0.4. In pH 6 the exponential growth phase was obtained in the interval time of 2 to 4 h. The growth rate was 0.24 in (B3) and 0.35 in (B6). However, at pH 8, (Figure 2 C) the growth of the strains is too low

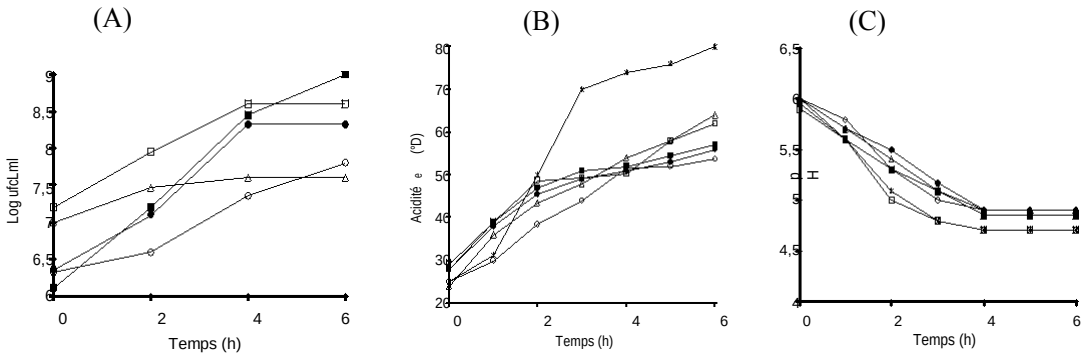

Figure 4. Kinetics of growth (A), produced acidity (B) and pH evolution(C) of *Bf. breve* B1( ), *Bf. longum* Bif5 ( ), *Bf. longum* B2 (∆), *Bf. longum* B3 (O), *Bf. bifidum* B6( ) and *Sc. thermophilus* (∗) in mixed culture in skim milk with *Lb. acidophilus*.

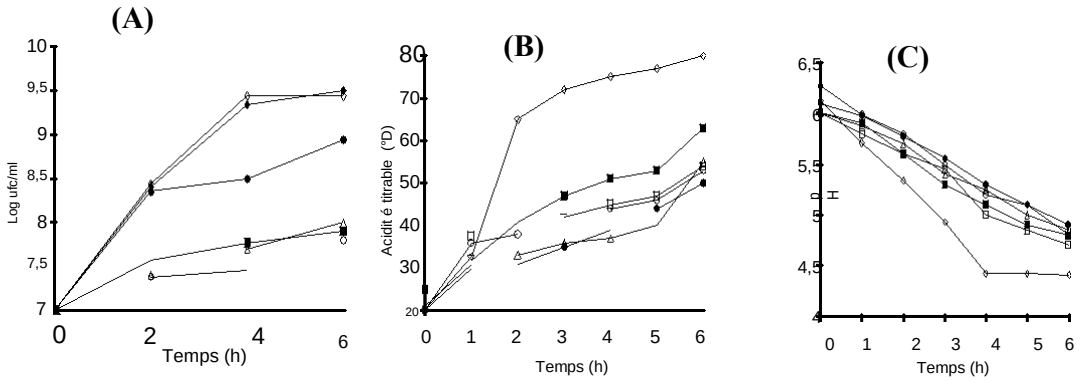

Figure 5. Kinetics of growth (A), produced acidity (B) and pH evolution(C) of *Lb. bulgaricus* ( ), *Bf. breve* B1 ( ), *Bf. longum* Bif5 ( ), *Bf. longum* B2 (∆), *Bf. longum* B3 (O) et *Bf. bifidum* B6 ( ) in mixed culture in skim milk with *Sc. thermophilus.*

comparatively with the others pH. The calculated growth rate was 0.03 in (B3 and Bif5) and was 0.1 in (Bif5, B3 and B6). In addition, in pH 4 the growth of the strains was completely inhibited.

Temperature effect

The growth kinetics results of the strains B1, B2, B3, Bif5 and B6 under the influence of the incubation temperature are illustrated in the Figures 3A and B. A similar behaviour of the incubated strains at 25 and 30°C was observed. A longer latency phase of growth was observed in the strain B2 (Figure 3A) . At 25°C, the growth rate reached 0.3 in (B1, Bif5, B6) and 0.35 in (B2 and B3). However, all strains incubated at 25°C were characterized by a longer exponential phase. Whereas, similar growth were observed at 30°C and 37°C for all strains.

Growth in milk

The milk inoculated with the pure *Bifidobacterium* sp. strains coagulates between 6 to 8 h of incubation. In addition, the strains Bif5 and B2 develop a coagulum with viscous consistency contrary to the strains B1, B3, B6 which form a clot with low consistency. The strains in mixed cultures form a thick and quite firm curd.

Growth in pure cultures

These results show that the five strains B1, B2, B3, Bif5 and B6 acidify milk less than the other strains of lactic bacteria *S. thermophilus, L. bulgaricus, L. acidophilus*. A light similarity was observed between the acidification profiles of different bifidobacteria strains, on the basis of the pH evolution and titrable acidity developed after 4, 6 and 8 h of incubation (Figure not shown).

After 7 h of incubation, acquired titrable acidity reached 42°D in Bif5. In the same way the maximum rate of acidification reach values of 11, 9.78, 9.77, 8.56 and 7.33 mM/h in Bif5, B1, B2, B3 and B6, respectively. After that, the cultures were reduced. This reduction is proportional to acidity produced and the pH values, which was varied between 4.8 and 5.6. However, the growth of B1, B2, B3, Bif5 and B6 progress slowly. The growth rate varied between 0.1 in (B6) and 0.24 in (B1 and B2). After 3 h of growth *L. acidophilus* produces an acidity of 40°D, *S. thermophilus* 46.5 and *L. bulgaricus* 50°D.

The maximum acidification rate calculated for *S. thermophilus* and *L. acidophilus* was similar 12.22 mM/h and it was 14.67 mM/h for *L. Bulgaricus.* The pH values observed was between 4.7 and 5. The enumeration realized reveals on average number of 10^8 ufc/ml. In addition, the growth rate calculated varies between 0.7 for *S. thermophilus* and 0.8 for *L. acidophilus*.

Growth in mixed cultures

The mixed cultures of strains were more active than the pure one. After 3 h of incubation, the strains produce more lactic acid (30°D) (Figures 4 and 5). On the other hand, the acidity produced in mixed culture with *L. acidophilus* reached values *w*hich vary between 50 and 55°D. The maximum rate acidification is slightly similar in all strains of bifidobacteria. It reached on average 9 mM/h. However, the weakest acidifying activity is noted for the strain B6 with a rate of acidification of 7.33 mM/h. In addition, the final pH reaches a value of 4.9. The bifidobacteria number obtained was 24 x 10^8, 28 x 10^8 and 23 x 10^8 ufc/ml, with a growth rate of 0.29, 0.3 and 0.4, respectively, in Bif5, B1 and B6 strains.

S. thermophilus and *L. acidophilus* coagulate milk in 2 h of incubation. Final acidity reached 72°D, with acidification rate of 41.67 mM/h. This acidifying activity is also correlated with a significant growth of the strain *L. acidophilus.* In the same way, the number of *L. acidophilus* in log ufc/ml obtained in all the cultures varies according to the strain used. The evolution of titrable acidity in the mixed cultures of *S. thermophilus* and the strains of bifidobacteria also reveals the existence of a significant acidifying activity. After 6 h, the acidity reaches a values ranging between 40°D (B2) and 54°D (B1). We observed that the level of growth of the strains of bifidobacteria increases slightly. In addition, the growth rate of the strains B1 and B2 increase slightly. Association between *S. thermophilus* and *L. bulgaricus* is very active; after 4 hours acidity and the pH respectively reached 76°D and 4.4, with an acidification rate of 39.43 mM/h. The number of *L. bulgaricus* and *S. thermophilus* reaches 29 x 10^8 and 10^9 ufc/ml, respectively. The results obtained mention several points on the nature and the quality of associations carried out. The bifidobacteria strains are more active with *L. acidophilus* than with *S. thermophilus* and this on the basis of titrable acidity and the calculated growth rate.

DISCUSSION

Numeration revealed the presence of a very significant number of bifidobacteria in the feces of breast fed infants 26 x 10^9 ufc/g. Similar count was found by Beerens et al. (1980), Romond et al. (1980), Scardovi (1986), Beerens (1990) and Kaufman et al. (1997) . The bifidobacteria predominance species in the feacal microflora of breast fed infants was exclusively due to the bifidigenes factors released from the mother's milk (Modler et al., 1990). The enumeration of the bifidobacteria from French fermented milk (Bifidus Actif) showed a count of x 10^7 ufc/ml. These results agree with the data reported by Tamime et al. (1995) and indicate that the incorporation of bifidobacteria can be controlled microbiologically (Rolfe, 2000). The TPY medium was practically a selective medium for the isolation of bifidobacteria species. Romond et al. (1980) and Silvi et al. (1996) suggested the use of sterile milk supplemented with Bacto-liver (0.5%) and cysteine (0.001%) for enhanced growth of *Bifidobacterium* species in milk. The use of antibiotics in TPY medium was employed to isolate and count the bifidobacteria species in fermented milk (Tamime et al., 1995; Franck et al., 1993).

The stages of the pre- identification based on morphological aspects show that the bifidobacteria develop on TPY medium small colonies without catalase and oxydase activities, indicating that the strains are strictly anaerobic. The cells are Gram positive and bifide form (Scardovi, 1986; Beerens, 1990). The bifidobacteria pleomorphism observed is often associated with the composition of the medium culture (Kojima et al., 1968).

The Fructose-6-pyrophosphate kinase test (Scardovi, 1986; Orban and Petterson, 2000) is positive with all the pre-identified strains, confirms their membership of the *Bifidobacterium* genus. Mitsuoka (1984) and Gavini et al. (1990) reported that any strain belonging to the *Bifidobacterium* genus must be nitrate reductase negative, does not form indol, does not have a ureasic activity and does not liquify the gelatine. Our selected strains correlated with these conditions.

The bifidobacteria have very significant resistance against some groups of antibiotics like the aminosides and the beta–lactamines groups (Kurmann, 1983). This resistance was observed in our strains. The sensitivity to chloramphenicol was announced by several authors (Scardovi, 1986; Kurmann, 1984; Mitsuoka, 1984). This antibioresistance criterion is used as a marker for bifidobacteria selection (Silvi et al., 1996). The bifidobacteria are also resistant to bile salts (Ferrari et al., 1980; Charteris et al., 2000; Tanaka et al., 2000) which was noted in our strains. The resistance to bile salts was used as criterion for the selection of probiotic strains

(Grill et al., 2000). The biochemical characterization of the strains allow us to identify two species of *Bifidobacterium* the first strain was *B. longum* encounter in the feces and fermented milk (Bifidus Actif), and the second strain was *B. breve* isolated from infants feces. The species *B. longum* dominates the intestinal flora of breast fed infants and it is incorporated in the production of fermented milk in French (Tamime et al., 1995). The species *B. breve* is famous also by these probiotic properties and the majority of acidified therapeutic milk contains this species (He et al., 2001).

The optimization of growth conditions of the bifidobacteria constitutes an essential point in their biomass production. Therefore, the study of certain parameters determining the growth kinetics (such as the temperature and the source of carbon) can provide much information on the physiology of the bacterium. The optimal pH of growth of the diverse strains of bifidobacteria was determined by several authors (Rasic and Kurmann, 1983; Beerens, 1990). Infact, germs acidophiles develop better at pH 5 and 6. The results obtained show that the optimum of growth of our strains is pH 6. The growth of strains was completely inhibited on TPY medium in pH 4. According to some authors the low pH (acid) influenced the latency phase by affecting the activity of certain enzymes like β-galactosidase. These effects were observed with *B. longum* (Marisa et al., 1994), *B. bifidum* (Passerat and Desmaison, 1995) and *B. Breve* and *B. infantis* (Wang and Gibson, 1993). On the other hand, the enzymatic activity seems to be very significant at pH 5.8 and 6.0 (Bouhnik et al., 1992; Passerat and Desmaison, 1995). However the basic pH (8) decrease the level of the growth phase. These observations indicate that the bifidobacteria prefer the neutral or slightly acid medium (Biavati et al., 1992). The inhibiting effect of the pH on the microbial growth acts on three levels: the enzymatic activity, the membrane permeability and bioavailability of some nutrients which depends on ionic balance. The temperature of incubation was a significant parameter which defines the bifidobacteria growth. The bifidobacteria of human origin are mésophiles with optimal growth at 36 to 41°C. The temperature also affects the composition of the cell wall fatty acids (Biavati et al., 1992), which disturb membrane transport and cellular metabolism (Rasic and Kurmann, 1983.

The effect of the carbon source shows that the metabolic activity in the bifidobacteria was clearly supported by the presence of an adequate carbon source with glucose as a good carbon source (Tamime et al., 1995; Hughes and Hoover 1995). Fructose is used to stimulate the bifidobacteria growth *in vitro* (Gibson and Wang, 1994) and *in vivo* (Gibson and Roberfroid, 1995; Gibson et al., 1995; Bouhnik et al., 1999; Perrin et al., 2000). All the strains showed an aptitude of growth on medium TPY galactose.

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