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Evaluation of *in vitro* antagonism and protection against enteropathogenic experimental challenge of different strains of *Bifidobacterium*

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Gastrointestinal microflora highly impacts their host mainly by performing a great variety of metabolic activities, protecting the host from pathogenic colonization. Mother's milk is a prebiotic factor which stimulates bifidobacteria growth *in vivo*. All strains of bifidobacteria were isolated on MRS medium (in addition to 0.05% cystéine HCL and 2 mg/l of nalidixic acid) from different origins (breast-fed infant faeces and yoghurt (bifidus). The strains belong to the following species: *Bifidobacterium longum, B. Bréve, B. bifidum*. We studied the antagonist power of *Bifidobacterium* against enteropathogenes (*S. aureus*, *Escherichia coli*, *P. aeruginosa*, *Salmonella. Sp)*, using agar diffusion method. *In vitro* antagonism test showed that our strains were able to produce antagonistic substances against various pathogenic microorganisms. The activity was completely destroyed by the action of proteolytic enzymes, indicating that the biologically active portion is proteinaceous. These properties suggest that inhibitory substance is considered as "Bacteriocin''; these results emphasize the importance of the antimicrobial activity of *Bifidobacterium* in the dairy industry. Additional tests are needed to determine the exact nature of the inhibitors.

Keywords: Intestinal flora, antagonist activity, antimicrobial substances, organic acids, bacteriocins like, enteropathogenes, inhibiting pathogens.

INTRODUCTION

There is general agreement on the important role of the gastrointestinal (GI) microbiota in the health and well-being status of humans and animals. The concept that certain micro-organisms, when supplied in sufficient quantities conferred direct benefits to the host is defined by the term ‘probiotics’ (Saad et al., 2013). They play an important role in human nutrition. In recent years, there has been a significant increase in research on the characterization and verification of potential health benefits associated with the use of probiotic and prebiotic (Saad et al., 2013). It is generally accepted that probiotic food products should contain a minimal level of viable cells of 10^6 per gram or milliliter of product, although this value is relative since beneficial effect depends on the strain and targeted health benefit (Reimann et al., 2010). The potential mechanisms by which a probiotic agent might exert its protective or therapeutic effect include competition for nutrients or adhesion receptors, production of inhibitory metabolites or antimicrobial agents against pathogens (Ariane et al., 2010).

Bifidobacteria are anaerobic Gram positive bacilli belonging to the dominant gut microbiota in humans and

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Table 1. The reference strains (indicator strains).

Indicator strain	Medium	Reference	Temperature and Incubation time
Bacteria Gram positive			
Staphylococcus aureus		ATCC 6538 IP	30 °C ,18-24 h
Staphylococcus aureus	Chapman	ATCC 29213	
Bacteria Gram negative			
Escherichia coli	GN	ATCC 25922	30 °C ,18-24 h
Escherichia coli		ATCC 8739 IP	
Pseudomonas aeruginosa	Citrimide	ATCC 24853	37 °C, 18-24 h
Pseudomonas aeruginosa	Citrimide	ATCC 27853	
Salmonella .spp S 37	GN		
Enterobacter claoqui 335			37 °C, 18-24 h

ATCC: American Type Culture Collection / IP: Institut of Pasteur

animals. In recent years, bifidobacteria have gained a lot of attention because of their association with numerous health-promoting effects, even though some mechanisms of these beneficial effects remain unexplained (Turroni et al., 2009). Thus, various bifidobacterial strains are currently used as probiotics in functional food products, and selecting new probiotic strains is currently of great interest (FAO/WHO, 2002). These strains must display several characteristics, one of which is that they must be of human origin. Therefore, in the perspective of either understanding the mechanisms of the beneficial effects of bifidobacteria or strain selection for probiotic uses, reliable enumeration and isolation of bifidobacteria from human feces are needed (Ferraris et al., 2010). These bacteria colonize the neonatal intestine from the first week after birth and inhabit the gastrointestinal tract throughout life, where they contribute to human health and well-being (Turroni et al., 2009).

It is also known that the composition of the dominant species of the indigenous bifidobacteria varies in age, with *B. lactis*, *B. longum*, *B. breve* and *B. parvolorum* found in children, which are replaced in the adulthood by *B. adolescentis*, *B. catenulatum*, *B. pseudocatenulatum* and *B. longum* (Ariane et al., 2010). Infection with enteric pathogens continues to be a health problem worldwide, especially in children. Intestinal epithelium provides the first line of defense of the organism, providing an efficient barrier against pathogens and macromolecules. The mucus layer and the resident gut microbiota protect the gut mucosa from adhesion and invasion of pathogen microorganisms (Candela et al., 2010). In this respect, probiotics have been proposed for prevention and treatment of gastrointestinal tract (GIT) infections (Rodrı´guez et al., 2012). In recent years, Bifidobacteria have attracted considerable attention due to their overall beneficial effects on health (Peter et al., 2001); they play a significant role in maintaining the balance of intestinal microflora by correcting intestinal disorders and fighting against diarrhoea and gastro-enteritis (Hamma et al., 2008).

The aim of this study was to identify these *Bifidobacterium*, isolated from different origins and to study their potential and antimicrobial activity against enteropathogenes by using *in vitro* tests.

MATERIALS AND METHODS

Strains' origin

The strains of *Bifidobacterium* used were derived from several samples of commercial French yoghurt (Activia (bifidus); about 40 fresh fecal samples were obtained from newborn infants (their ages less than 05 months)

Lyophilized *B. bifidum* ATCC 15696 (Bbf1) was obtained from the collection of Laval University, Food Science and Nutrition (Québec, Canada, G1VOA6).

Enteropathogenes strains: from the military hospital Collections Regional Oran provided; and from the institute Pasteur of Algeria (Table 1). These pathogenic microorganisms were selected due to their role as pathogens for humans and their presence in the human gut (Arboleya et al., 2011).

Identifications of strains

The identification of bifidobacteria strains was based on determination of morphologic, biochemical and physical characters. All isolates were tested for their Gram reaction; catalase activity using H_2O_2 (Guessas and Kihal, 2004). The examination of biochemical characteristics of enteropathogens was carried out by using API 20E (for pathogenes Gram negative) and API STAPH (for *S. aureus)* (bioMereux, France).

Antibiotic resistance

Culture can also be used to investigate the sensitivity of strains to antibiotics. This sensitivity was tested by the diffusion method (method of discs) (Fleming et al., 1975) on Muller-Hinton medium using different antibiotics (biomérrieux) on which are arranged the disc antibiotics. Then the plates were incubated anaerobically for 24 h at 37°C, using Oxoid anaerobic gas jars and gas paks (Hadadji

and Bensoltane, 2006). Reading of the results is carried out by measuring the diameter of the zone of inhibition that occurred.

In vitro inhibition of pathogen growth

Research interactions between different species of Bifidobacterium and enteric

The ability of the *Bifidobacterium* strains to inhibit the growth of enteropathogenic strains (*Staphylococcus aureus*, *Escherichia coli, Pseudomonas aeruginosa*, *Salmonella .spp S 37, Enterobacter claoqui 335)* was determined using the agar diffusion tests by measuring the diameter of the inhibition zones (Table 1).

Direct method

The antimicrobial activity of our strains was evaluated on solid medium according to the method of Barefoot and Klaenhammer (1986). An agar spot test was used with enteropathogenes strains as sensitive indicator strains (MH medium). Test cultures were spotted onto the surface of the agar. The plates were then incubated for 48 h, under appropriate conditions for each specific pathogen. A clear zone of inhibition around the spot was considered positive (Rodrı´guez et al., 2012; Guessas et al., 2012).

B-indirect method

After centrifugation (8000 tr/ 10 min), supernatants were then stored at−20°C until use in the agar diffusion tests. Overnight (16 h), pathogen cultures were used to inoculate (1% v/v) agar media, 5 mm wells were cut out of the agar and 100 µL of each supernatant was added to the well. Plates were then incubated for 24 to 48 h under appropriate conditions for each specific pathogen (Arboleya et al., 2011).

Research on the nature of the inhibitor

Inhibition due to acids

To eliminate the effect of acids, our strains were cultured on MRScys (medium buffer, pH 7) containing 0.25% glucose to minimize acidification buffered, and acid produced by the strains is neutralized; only the antimicrobial substance it produced expresses its action on the pathogenic strains (Ruiz-Barba et al., 1994; Scillinger et al., 1996).

Inhibition due to hydrogen peroxide

To avoid the effect of hydrogen peroxide in the inhibition of pathogenic strains, the supernatant cultures of our strains were treated with 1 mg / ml catalase. The supernatant was filtered (0.22 µm), tested by the sink method on different pathogenic strains and was then stored at −20°C until use in the agar diffusion tests. Plates were then incubated for 24 h under appropriate conditions for each specific pathogen.

Searching the protein nature of the antimicrobial substance

To know if this substance belongs to bacteriocins, it should have a protein nature; the culture filtrate of our strains is treated by different enzymes. Thus 1 ml of the culture filtrate is treated with 1 mg / ml of trypsin, α- chymotrypsin or pepsine. The filtrate is treated by the enzymes and sterilized by filtration (0.22 mm) and supernatants were then stored at−20°C until use in the agar diffusion tests. The action of the filtrate is tested by the method of agar wells and incubated at 37°C / 24 to 48 h (Alvarado et al., 2005).

Confirmation of the presence of bacteriocin

The Bifidobacteria can produce inhibitory substances. To ensure the presence of bacteriocin, *Bifidobacterium* (of 16 h) was cultivated in 50 ml MRS cys broth and after incubation, the tubes were centrifuged at 8000 rev/min. To eliminate the effect of organic acids such as lactic and acetic acids, the supernatant was neutralized (pH = 7) (NaOH 5 N).

We prepared tubes containing 10 ml of broth medium inoculated with the strain (*B. bifidum* with *S. aureus* ATCC 29213 and *B. longum* (B3) with *E. coli* ATCC 8739). Tubes were then incubated for 24 h under appropriate conditions for each specific pathogen. Bacterial growth is monitored by measuring the optical density of every two hours. At the 6th hour after the incubation the supernatant is added to one of the two tubes (Labioui et al., 2005).

Study of the kinetics of growth in mixed culture (with pathogenic strains)

The study of the 03 kinetics of growth with 03 strains of *Bifidobacterium* showed a strong antibacterial activity in the presence of pathogenic strains in mixed culture with different strains. Overnight (1 6h), cultures were used for inoculation (3% v/v) (03 tubes of 100 ml of skim milk); the first tubes received only Bifiobacteria; the second, pathogens *P. auerogenosae* or *S. aueus* and *E. coli; and* the third tubes contained the mixed culture *(Bifidobacter*ium with pathogenic strains). Every 2 h, the enumeration of *Bifidobacterium was* realized on MRScys medium with pH 6.8; *P.auerogenosae* was realized on medium Citrimide; *S.aueus* on Chapman medium and *E. coli* on medium GN. Tubes were then incubated for 24 h under appropriate conditions for each specific pathogen.

RESULT

Identification of strains

All pure cultures of *Bifidobacterium* obtained from MRS solid medium containing 0.05% cysteine-HCl, nalidixic acid 2 mg/ml and lithium chloride (LiCl) 3 mg/ml) were utilized for bifidobacteria (Tamime et al., 1995). They are pleomorphic rods and Gram positive, catalase negative nonsporulating and strictly anaerobic, gelatinase negative, with no indol production, resisting up to different concentrations of bile salt (2- 3%). The majority of the isolates are identified as belonging to the genus, *Bifidobacterium* (*Bifidobacterium.longum, B. Breve, B. bifidum*).

Antibiotic resistance

Most strains isolated, are very susceptible to Gram positive spectrum antibiotics (macrolid erythromycin, Spiramycin), lincomycin, teicoplanin, broad-spectrum antibiotics (rifampicin and chloramphenicol) and beta-lactams (peni-

Table 2. Spectrum of antimicrobial activity of *Bifidobacterium* strains by the method of diffusion.

Strains test / indicator strain	Bbf1	B2	B3	BV	B4	RBL8
Staphylococcus aureus 6538	14	17	9	12	5	6
Staphylococcus aureus 29213	28.5	5	10	0	10	8
Escherichia coli 25922	14	6	8	0.6	0	10
E.coli 8739	12	15	10	-	6	5
*Pseudomonas aeruginosa*24853	11	10	3	9	14	3
*Pseudomonas aeruginosa*27853	14	22	10	0	10	2
Salmonella .spp S 37	0	11	14.5	0	-	-
Enterobacter claoqui 335	0	2.3	1.6	0	2	0

Zone of Inhibition (mm) on MRS pH (6.8).

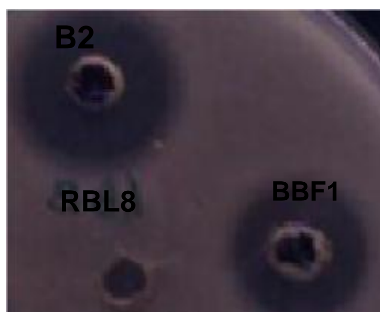

Figure 1. Action of catalase activity by the method of diffusion Bifidobacterium with *E.coli* ATCC 8739.

cillin, ampicillin, amoxicillin, piperacillin, Oxacillin). Variability has been seen in their susceptibility to tetracycline, clindamycin and to cephalosporin, Trimethoprime-Sulfamethoxazol and imipenem. In contrast, all isolated species are resistant to metronidazol, Gram-negative spectrum antibiotics (fusidic acid, nalidixic acid) and aminoglycosid (neomycin, gentamicin, kanamycin and streptomycin), vancomycin and cefoxitin, paramomycin, gentamicin, streptomycin.

Research of interactions between different species of *Bifidobacterium* and pathogenic strains

The results were expressed in mm, by measuring the distance between the limited colony bacteria and the beginning of the zone of non-inhibition of the indicator strain. Strains with a clear zone of lateral extension are greater than 0.5 mm, considered producing antibacterial substances (Fleming et al., 1975). The stain Bbf1 showed a strong activity against *Staphylococcus aureus* (ATCC 29213). However, there is no zone of inhibition of Bbf1, RBL8 with strain of *Enterobacter claoqui*, *Pseudomonas aeruginosa* (27853), *Salmonella .spp S37* and *Enterobacter claoqui* (Table 2).

Research on the nature of the inhibitor inhibition due to acids

We studied the ability of *Bifidobacterium* strains to inhibit 08 of enteropathogenic strains. On MRScys medium (pH 6.8), these bacteria were inhibited by our strains of *Bifidobacterium* tested. In contrast, on MRScys medium (buffer pH 7), only strain B3 showed a strong antibacterial activity against *Staphylococcus aureus*, but the other strains showed a very low activity or no activity against the entero*pathogens* (Figure 1).

Inhibition due to hydrogen peroxide

The antibacterial substances contained in the culture extract of BV, B2 were not inactivated in the presence of this enzyme that lacks hydrogen peroxide. In contrast, the substance contained in the antibacterial strain (RBL8) was inactivated in the presence of catalase, which excludes an inhibition by hydrogen peroxide (Figure 2a, b).

Action of proteolytic enzymes on bacteriocin activity after the strain

The antibacterial substances of Bbf1 and B3 strain, with *S. aureus* ATCC 29213 and *E. Coli ATCC* 8739 respectivelyy were inactivated by the proteolytic enzyme; no inhibition zone was detected after treatment with these enzymes (α-Chymotrypsine or Trypsine, Pepsin). So the antibacterial substance is, therefore, a substance of a proteinaceous nature. These properties of the bacteriocin are very important to determine, but it would be interesting to confirm these results *in vitro* (Table 3).

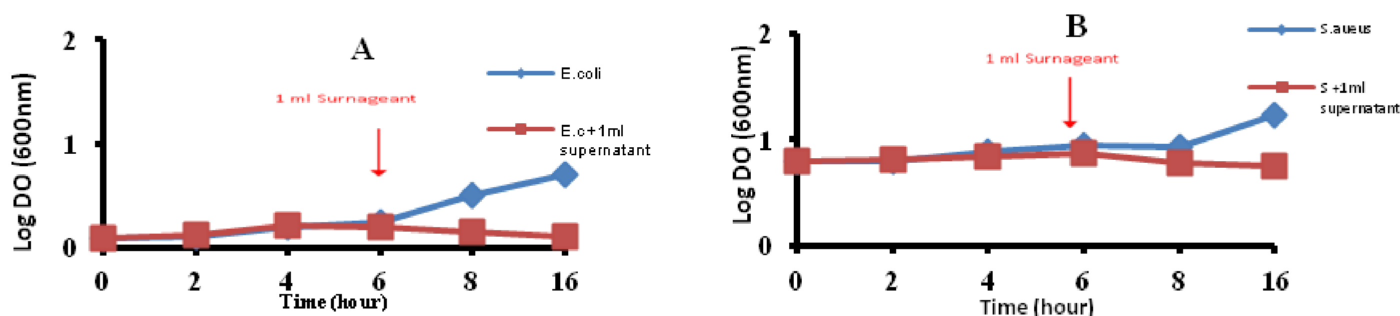

Figure 2. (A) the growth of *E. coli* ATCC 8739 in presence of bactériocin like product B3 and (B) the growth of *S. aureus* ATCC 29213 in presence of bactériocin like product Bbf1.

Table 3. The action of proteolytic enzymes on bacteriocin activity of reference strain Bbf1 and strain B3 with 02 enteropathogenes (*S. aureus* ATCC 29213 *and E. Coli ATCC* 8739 respectively).

Treatment	Diameter of inhibition (mm)	
Enzymes protéolytic	*E. Coli* B3	*S. aureus* Bbf1
α-Chymotrypsin	-	-
Trypsin	-	-
Pepsin	-	-

Search of bacteriocin

After incubation at 37°C for 16 h, we calculated growth of bacteria using the density values (Log DO) to know bacteriocin and its effect on growth of *E. coli* in the presence of 1 ml supernatant of B3 and *S. aureus* in the presence of 1 ml supernatant of strains Bbf1(after 6th hours) (Figure 2A, and B). The results are translated by a curve which showed a decrease of these bacteria, once we add the 1 ml supernatant of Bifidobacteria. These results are explained by the presence of bacteriocins produced by *Bifidobacteruim*.

Study of the kinetics of growth in mixed culture (with pathogenic strains)

In culture mixture of Bbf1 with *P. aeruginosa,* the number of viable cells per ml declined from 7.55 log CFU/ml to 5.5 log CFU/ml after 18 h of incubation (Figure 3A), and below 1 log CFU/ml, after 48 h. The inhibition of *P. aerugenosa* by B3 strain decreases than inhibition by Bbf1.

The acidity produced by *P. aerugenosa* strain in skimmed milk expressed as Dornic degree, showed a production of 40°D after 8 h of culture. The production increased to reach 56°D after 24 h of culture and decreased after 48 h; on the other hand, in mixed culture, the inhibition of a growing culture of *P. aerugenosa* by strain Bbf1 in milk was examined and compared with the growth of *P. aerugenosa* (Figure 3B).

The inhibition of *E. coli* by B2 strain, in culture mixed reached 0 cells per ml after 48 h of incubation in skimmed milk. However, Bbf1 and B3 decrease from 7 UFC/ml, and 7.8 UFC/ml respectably to 2 and 3 UFC/ml. In single culture *of E. coli*, growth increases from 6.99 log CFU/ml to 7.1 log CFU/ml after 24 h, and decreases to 6.8 log CFU/ml after 48 h of incubation (Figure 4A). The acidity was produced by *E. coli* in skimmed milk; the production of acidity increased to reach 20°D after 4 h to 60°D after 48 h of culture (Figure 4B).

In culture mixture of B3 with *S. aueus,* the number of viable cells per mm declined from 6 log CFU/ml to 4 after 18 h of incubation (Figure 5A) and below 0.8 log CFU/ml cells per ml after 48 h; and cells did not re-grow within 48 h. The acidity produced by B3 strain in skimmed milk is evaluated by the amount of acid released and expressed as Dornic degree shows a production of 40°D after 8 h of culture. The production increased to reach 59°D after 24 h of culture.

The inhibition of a growing culture of *S. aureus* by strain B3 in milk was examined and compared with the growth of *Staphylococcus aureus* in milk (Figure 5B). A decrease in *S. aureus* count after 8 h was noted; on the other hand, in single culture, the growth of *S. aureus* increased after the same period of time. After 24 h, the decrease in *S. aureus* growth was considerable and continued until only two bacteria were counted after 48 h.

DISCUSSION

Bifidobacteria species are common members of the human gut microflora, comprising up to 3% of the total fecal adults microflora (Hadadji et al., 2005). The results of analyses identified six strains of *Bifidobacterium* belonging to the following species: *B. bifidum, B. longum, B.Bréve.* We have isolated and identified strains of *Bifidobacterium* from infant feces and yoghurt, on MRS medium containing 0.5% cysteine-HCl. Our study also showed a cellular polymorphism (Mahmoudi, et al., 2013). Cells forming colonies are Gram positive, characterized by various forms, with Y or V shape, but often bifid forms that are typical of bifidobacteria. All isolates were catalase and oxidase negative (Leahy et al., 2005).

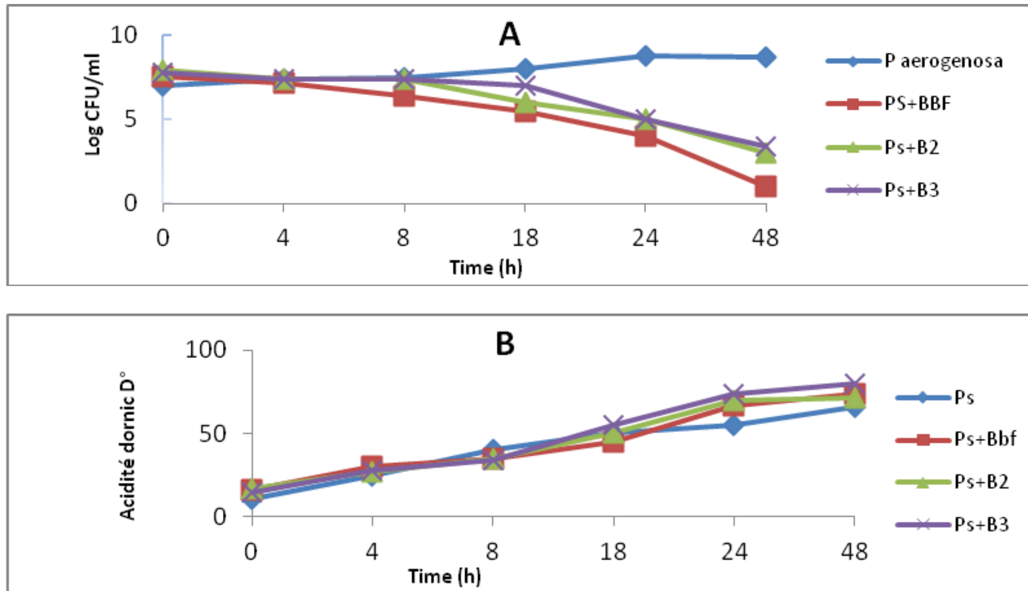

Figure 3. Growth of bifidobacteria (Log CFU/ml) (A), the kinetic of acidification (B), during the growth of the strains *Bf. breve(B2)*, *Bf. bifidum* (Bbf1), *Bf. longum* (B3) in single culture and culture Mixed and *P. aerugenosa* (ATCC 27853 27853), in skim milk at 37°C.

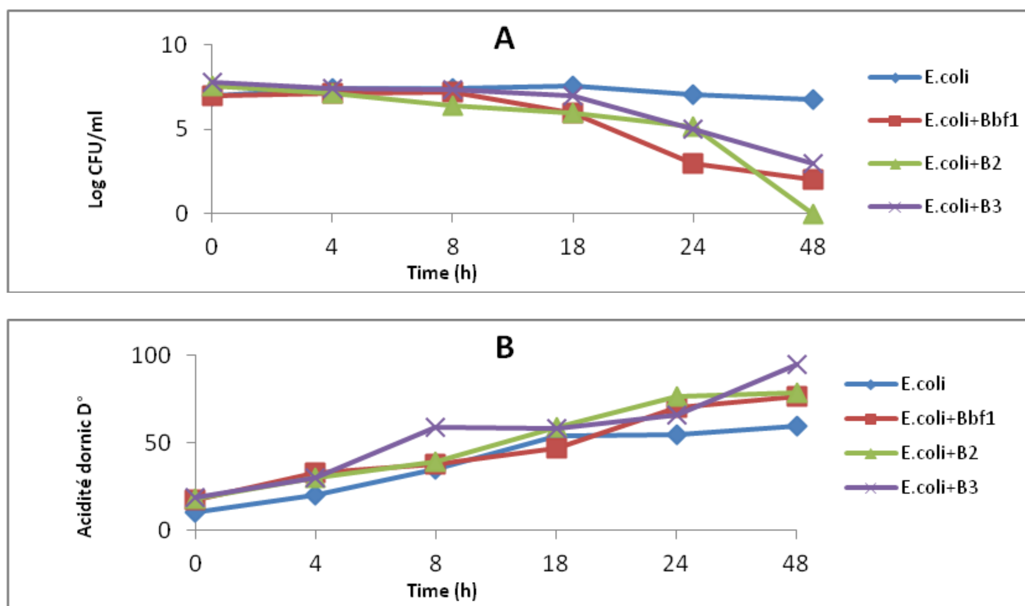

Figure 4. Growth of bifidobacteria (Log CFU/ml) (A), the kinetic of acidification (B), during the growth of the strains *Bf. breve(B2)*, *Bf. bifidum* (Bbf1), *Bf. longum* (B3) in single culture and culture Mixed and *E. coli* ATCC 8739), in skim milk at 37°C.

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 and resisted up to different concentrations of bile salt (2- 3%). The adaptive ability to resist bile salt appears to be highly variable within members of the Bifidobacterium genus (Waddington et al., 2010; Cronin et al., 2011). Strains isolated from different origins are resistant to nalidixic acid, neomycin, rifampicin, streptomycin and vancomycin. These antibiotics are used as selective agents in synthetic media for the isolation and enumeration of bifidobacteria (Ventura, 2004). This criterion is used as antibiotic selectable marker. Sensitivity of bifidobacteria to chloramphenicol has been reported by

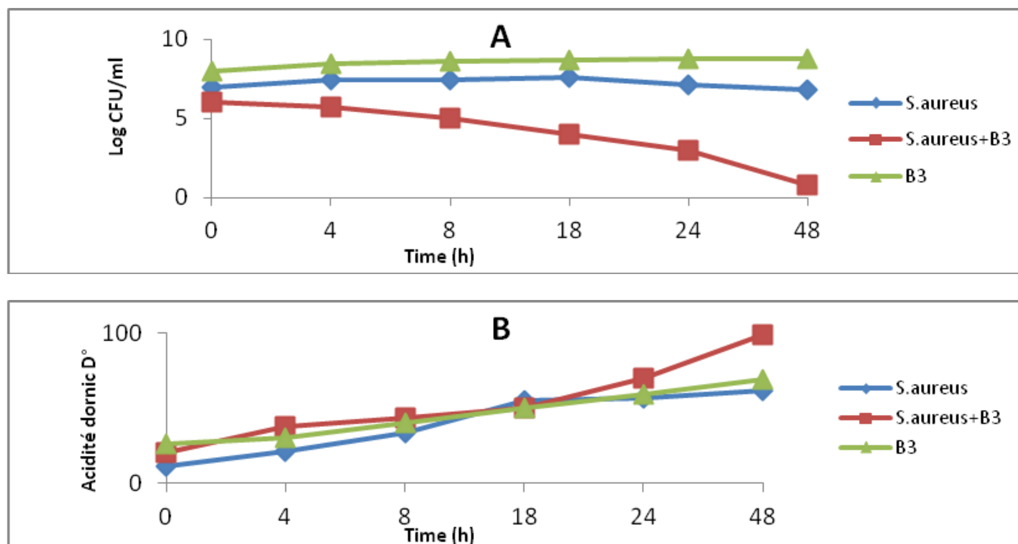

Figure 5. Growth of bifidobacteria (Log CFU/ml) (A), the kinetic of acidification (B), during the growth of the strains *Bf.longum* (B3) in single culture and culture Mixed and *S. aureus* ATCC 29213, in skim milk at 37°C.

several authors (Scardovi, 1986; Delcenserie et al., 2002).

We studied the interaction of *Bifidobacterium* strains with eight enteropathogenic strains. Studies report inhibition of a large number of pathogens by bifidobacteria *in vitro* or *in vivo*, including *E. coli*, Salmonella (DeVuyst et al., 2004; Servin et al., 2004). According to the study of Ariane et al. (2010), the strains of *bifidobacterium* showed the largest inhibition zone against *S. typhi* 19430, and the smallest one was against *E. faecalis* 19433; and Bifido A did not show antagonistic activity against *E. coli* 4238. However, DeVuyst and Marka (2006) tested 10 strains of bifidobacteria belonging to different species of *E. coli* 1845 and *Salmonella enterica ser typhimurium* SL 1344, and noticed that the strong antibacterial activities are due to the acidity. These results are similar to those found by Arboleya et al. (2011), that *St. aureus* and *C. difficile* were not inhibited by any of the bifidobacterial supernatants whilst no inhibition was observed for any of the strains in which the pH had been neutralized. This indicates that most likely the inhibition was due to the production of organic acids. The acid is the major factor of the antimicrobial multifactorial mechanism of Bifidobacterium against harmful intestinal bacteria (Rodrı´guez et al., 2012).

Our strains tested showed a variability of behavior towards enteropathogenic bacteria (*E. coli, S. aureus* and *Salemonella*, *enterobacter, P. aerugenosa*). Other strains tested were inhibited in MRScyc (pH no buffer 6.8), by the *Bifidobacterium*. However, *Salmonella enterica, Enterobacter claoqui* were not inhibited by Bbf1, BV RBL8 strains. In MRScys (pH buffer), the inhibition of *S. aureus* by B3 strain may not be due to the production of organic acids (acetic acid, lactic acid). So we can say that it is due to bacteriocin production; however acidity plays a combined role in this inhibition.

The antibacterial activity of strains may be due to the production of several antibacterial agents. The substance found in antipathogenes current culture of the strain RBL8 was not inactivated in the presence of catalase, which excludes inhibition by hydrogen peroxide; however none of the strains of *Bifidobacterium* tested showed the ability to produce H_2O_2 (Rodrı´guez et al., 2012). The antimicrobial activity of B3 and Bbf1 was completely destroyed by the action of proteolytic enzymes; this suggests that the biologically active part of the bacteriocin is the proteinaceous nature. These properties of the bacteriocin are very important to determine, but it would be interesting to confirm these results *in vitro* (Shu and Gill, 2001; Tsai et al., 2008). So we note the double action of Bbf1 either by acidifying action and/or a bacteriocin production. Being aware of bacteriocin and its effect on growth of *E. coli*, *S. aureus* decreased after adding the supernatant. These results explain the presence of bacteriocins like produced by B3 and Bf1. These substances were characterized by other researchers as molecules of proteinaceous nature (Delvis-Broughton et al., 1990). *B. bifidum* can naturally produce more than one bacteriocin. Similar results were found by Carmen et al. (2000), which confirm the presence of bacteriocin inhibitory *H. pylori*. One bacteriocin produced by *Bifidobacterium bifidum* NCFB 1454 has a molecular weight of 4.4 kDa and has been shown to be active against a wide range of Gram-positive bacteria (De Vuyst, 2006; Morni., 2007).

In mixed culture, the growth of 03 enteropathogens with different strains of *Bifidobacterium* (Bbf1, B2 and B3) on skimmed milk was seen. After 4 h of incubation, the growth of strains tested began to decrease; however, no growth was detected after 48 h of incubation. The compa-

rison between the graphs and the pH of the acid shows that acidity increases with decreasing pH; the production of organic acids affects the inhibition of the growth of pathogenic strains that cannot survive in low pH (Desmazeaud, 1983).

Several clinical trials and *in vitro* studies have provided significant results supporting the health benefits of probiotics, particularly for the treatment of diarrhoea in children and elderly people, stimulating immunity, enhancing the intestinal barrier, the remission of intestinal inflammation and for the irritable bowel syndrome.

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