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% cysteine 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 enteropathogens (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, enteropathogens, 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...
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. parvulorum* 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 (Rodríguez 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 enteropathogens 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).*

*Enteropathogens 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 H₂O₂ (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*) (bioMérieux, 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 (biomérieux) 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 (Hadjadji

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**Table 1. The reference strains (indicator strains).**

<table>
<thead>
<tr>
<th>Indicator strain</th>
<th>Medium</th>
<th>Reference</th>
<th>Temperature and Incubation time</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacteria Gram positive</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td></td>
<td>ATCC 6538 IP</td>
<td>30 °C, 18-24 h</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>Chapman</td>
<td>ATCC 29213</td>
<td></td>
</tr>
<tr>
<td><strong>Bacteria Gram negative</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>GN</td>
<td>ATCC 25922</td>
<td>30 °C, 18-24 h</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td></td>
<td>ATCC 8739 IP</td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>Citrimide</td>
<td>ATCC 24853</td>
<td>37 °C, 18-24 h</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>Citrimide</td>
<td>ATCC 27853</td>
<td></td>
</tr>
<tr>
<td><em>Salmonella</em> spp S 37</td>
<td>GN</td>
<td></td>
<td>37 °C, 18-24 h</td>
</tr>
<tr>
<td><em>Enterobacter cloacae</em></td>
<td></td>
<td>335</td>
<td></td>
</tr>
</tbody>
</table>

ATCC: American Type Culture Collection / IP: Institut of Pasteur
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 cloacae 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 enteropathogen 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 (Rodríguez et al., 2012; Gueas et al., 2012).

**B-indirect method**

After centrifugation (8000 tr/min for 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 (16 h), cultures were used for inoculation (3% v/v) (03 tubes of 100 ml of skim milk); the first tubes received only Bifidobacteria; the second, pathogens *P. aerogenosae or S. aureus* and *E. coli*; and the third tubes contained the mixed culture (*Bifidobacterium* with pathogenic strains). Every 2 h, the enumeration of *Bifidobacterium* was realized on MRS cys medium with pH 6.8; *P. aerogenosae* was realized on medium Citrimide; *S. aureus* 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 (Tamine 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, telcoplanin, broad-spectrum antibiotics (rifampicin and chloramphenicol) and beta-lactams (peni-
Table 2. Spectrum of antimicrobial activity of *Bifidobacterium* strains by the method of diffusion.

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

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

cillin, ampicillin, amoxicillin, piperacillin, Oxacillin). Variability has been seen in their susceptibility to tetracycline, clindamycin and to cephalosporin, Trimethoprim-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 were 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 cloaqui, Pseudomonas aeruginosa* (27853), *Salmonella*.spp S37 and *Enterobacter cloaqui* (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 enteropathogens (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 respectively 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).
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 respectively 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. aureus*, 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).
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 liquefy 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 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. aerogenosa* (ATCC 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.
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 (De Vuyst 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, De Vuyst 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 Salmonella, enterobacter, P. aerugenosa). Other strains tested were inhibited in MRSscy (pH no buffer 6.8), by the Bifidobacterium. However, Salmonella enterica, Enterobacter cloaqui were not inhibited by Bbf1, BV RBL8 strains. In MRScsy (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 antipathogens 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 H2O2 (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 com-
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 (Desmazaud, 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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