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Isolation and Characterization of Strains of Bifidobacteria with Probiotic Proprieties *In vitro*

[1]A. Zinedine and [2]M. Faid

[1]Laboratory of Food Toxicology,
National Institute of Health (INH), 27, Avenue Ibn Batouta, P.O. Box 769 Agdal, Rabat, Morocco
[2]Department of Food Engineering and Technology, Hassan II Institute of Agronomy and Veterinary Medicine,
PO Box 6202, Rabat-Institutes, Morocco

Abstract: In this study, twenty six (26) strains of bifidobacteria isolated from different origin were identified and characterized. The identification of isolates was based on phenotypic proprieties, the carbohydrates fermentation profiles and the Fructose-6-phosphoketolase test. Results showed that *B. thermophilum* was the predominant species (53.8%). The other strains were identified as *B. bifidum* (19.2%), *B. longum* (15.3%) and *B. infantis* (11.5%). Identified strains were then screened for their inhibitory effects against pathogenic bacteria by the agar diffusion method. 11 strains of bifidobacteria isolated produced antimicrobial compounds that were active against pathogenic bacteria especially *Listeria monocytogenes* strains. The inhibitory effects remain stable after a heat-treatment at 100°C for 5 min, but decreased after an enzymatic treatment with pronase-E and proteinase-K. Some antimicrobial producing strains tolerated hydrogen peroxide (100-200 μg ml^{-1}), oxbile salts (0.3%) and the lysozyme (0.5 mg ml^{-1}) and could tolerate low pH values and survive to acidity shock (pH = 2). The growth-inhibiting factors produced by bifidobacteria could be identified as bacteriocins-like and would be very beneficial for their application as food preservatives.

Key words: Bifidobacteria · characterization · probiotics · bacteriocin · Morocco

INTRODUCTION

Bifidobacteria, previously known as *Bacillus bifidus*, are a group of microorganisms that were classified into 29 different species in the 9th Edition of Bergey's Manual of Systematic Bacteriology [1]. Nowadays, there are more than 33 species that were identified from the genus *Bifidobacterium* using recent molecular and biochemical methods of which 12 have been associated with the human gastrointestinal tract [2]. Bifidobacteria are Gram positive, non spore-forming, nonmotile, rod-shaped, saccharolytic anaerobes that produce acetic and lactic acids from carbohydrates without the generation of CO_2 [1]. They do not produce ammoniac or H_2S from amino-acides and nitrites from nitrates [3]. Bifidobacteria isolated from humans are known to grew at optimal temperature values between 36 and 38 °C and at optimal pH values ranging from 6.5 to 7, however they are inhibited by temperature values under 25°C and above 45°C [4]. The carbohydrates metabolism by bifidobacteria is different from homofermentative and heterofermentative bacteria. Indeed, fructose-6-phosphoketolase, a typical enzyme of the genus *Bifidobacterium*, is responsible for the degradation of glucose. Determination of this enzyme is a crucial test for the identification of such microorganisms [1]. The secretion of polysaccharides by *B. infantis* strains facilitates the adhesion of theses microorganisms to intestinal epithelial cells that favor the maintaining of the microbial balance necessary for health through their metabolic, trophic and protective activities. According to Servin [5], *B. lactis* DR10 inhibited the binding of *E. coli* O157:H7 to an intestinal cell monolayer and also reduced the invasiveness of this pathogenic strain. Moreover, *Bifidobacterium spp.* CA1 and F9 strains isolated from infant stools, inhibited the entry of *S. enterica* serovar *Typhimurium* into Caco-2 cells. Bifidobacteria are able to synthesize amino-acids (glutamic and aspartic acids), riboflavin and thiamin.

Corresponding Author: Dr. A. Zinedine, Laboratory of Food Toxicology, National Institute of Health (INH), 27, Avenue Ibn Batouta, P.O. Box 769 Agdal, Rabat, Morocco

Teraguchi *et al.* [6] reported also that *Bifidobacterium* species are able to produce vitamin B_2, vitamin B_6 and biotin.

Today, bifidobacteria are a focus of intensive international research for their essential role in fermented food especially for their ability to produce various antimicrobial compounds promoting probiotic properties [7]. In Morocco, up until now, no studies were undertaken on bifidobacteria and their use is still unknown in the food industry in Morocco. Thus, this study aims to isolate and characterize for the first time new strains of bifidobacteria from different origin as well as the study of their probiotic proprieties *in vitro*.

MATERIAL AND METHODS

Isolation of microorganisms: Strains were isolated from different origin (fecal matter of newborns, bovine meat and traditional fermented milks). Each sample was kept in a sterile bag containing peptone water (0.1%) added to 0.25% L-cysteine (Sigma, St. Louis, Mo., USA). Samples were then homogenized and diluted in peptone water with L-cysteine. Each sample was spread on MRS agar (Biokar, Diagnostics, France) containing 0.25% L-cysteine and incubated at 37°C for 48h in an anaerobic chamber (MAC500; Down Whitley Scientific, West Yorkshire, UK) containing an atmosphere of 85% N_2, 10% H_2 and 5% CO_2. Different references strains of bifidobacteria (*B. longum* ATCC 15707, *B. longum* ATCC 15708, *B. animalis* ATCC 27536, *B. bifidium* ATCC 5696, *B. adolescentis* ATCC 15704, *B. pseudolongum* ATCC 25526 and *B. thermophilum* ATCC 25866) was used to test the quality of the medium used. All theses references species were given by STELA Center, Laval University, Canada.

Identification of isolates

Phenotypic characterization: Each strain isolated was cultured on MRS_C agar (MRS agar added to 0.25% of L-cysteine) (Sigma, St. Louis, Mo., USA). Inoculated plates were incubated at 37°C for 24 to 48h. Strains were observed with a microscop (1000 x) after coloration with methylene blue. Only strains with the "Y" phenotypic form were selected for the identification [8].

B The selected colonies were purified by repeated streaking on MRS_C agar and identified by the API 50 CH kit (Biomérieux, France) according to the guidelines of the manufacturer. Prior to use, strains were activated in MRS_C broth at 37°C for 24 h. After centrifugation at 6000 t min^{-1} for 5min, cells were washed twice with PBS (phosphate saline buffer, Sigma) and then suspended in 2 ml of PBS added to 0.25% L-cysteine. API 50 CH kits were inoculated by the strain tested. API kits, covered with mineral oil, were incubated at 37°C for 24 to 48 h in anaerobic conditions. After incubation, the acidification of wells leads to a change of the coloration from yellow to purple.

Phosphoketolase assay: The principle of this assay was based on the ability of studied strains to transform fructose-6-phosphate into acethyl-phosphate and erythrose-phosphate by the enzyme phosphoketolase [1]. For this, strains studied were grown in MRS_C broth fors 24 h at 37°C, the culture was centrifuged for 6000 t min^{-1} for 5min and the cells was washed twice with a solution containing (PBS buffer 0.05% adjusted to pH 6.5 and added to 0.25% L-cysteine). Cells were then suspended in 2 ml of a lysozyme solution (10 mg ml^{-1}) and incubated at 37°C for 3 h. After pre-treatment, 0.25 ml of a solution containing sodium fluoride (NaF, 3 mg ml^{-1}) and potassium or sodium iodoacetate (5 mg ml^{-1}) in water was added. To that, 0.25 ml of sodium fructose-6-phosphate (80 mg ml^{-1} in water) was added, the solution was vortexed and then incubated at 37°C for 30 min. Following incubation, 1.5 ml of a solution of hydroxylamine-HCl (13 g/100ml in water, adjusted to pH 6.5) was added and allowed to incubate at room temperature for 10 min. 3 ml of a solution of trichloroacetic acid (TCA at 15% w/v), 1.0 ml of 4N HCl and 1.0 ml of ferric chloride (FeCl, 6 H2O, 5% w/v in 0.1 N HCl) were added and incubated at room temperature for 10 min. A reddish-violet color develops immediately with the addition of ferric chloride if the culture contains phosphoketolase activity. The intensity of the colour formed may vary depending on the bifidobacterial strain used. The reaction mix without phosphoketolase activity develops a light yellow colour.

Inhibitory activity assay: The antibacterial activity of bifidobacteria strains was studied using the agar diffusion test [9]. Strains of bifidobacteria were grown on MRS_C agar (adjusted to pH 7 with a solution of $NaHCO_3$ (2g l^{-1})) for 24h at 37°C. The culture was centrifuged at 6500 t min^{-1} and the supernatant was recuperated, filtered with a Millipore filter (0.22 µm). Pathogenic bacteria tested were grown on TSA medium (Biokar, France). Then, wells were hollowed out in the TSA medium and a volume of 80 µl of the supernatant was dropped in each well and incubated at 4°C for 30 min to facilitate the liquid diffusion in the agar medium. Finally, Petri dishes were incubated at 37°C for 24h. The antimicrobial activity was determined by measuring the clear zone around the wells. A diameter of 1.5 mm or greater around the culture was considered as

a significant inhibition. Microorganisms used in the inhibition test included *Listeria monocytogenes* (3 strains), *Escherichia coli* (2 strains), *Salmonella typhimurium* (2 strains) and *Staphylococcus aureus* (2 strains). All theses strains were from the collection of the Microbiology and Biotechnology Department, IAV Hassan II (Morocco). The stability of the inhibitory effect of bifidobacteria was studied by a heat-treatment of the supernatant at 100 °C for 5 min and by an enzymatic treatment with pronase-E and proteinase-K. Determination of the inhibitory activity of strains was the same as described above.

Resistance to H_2O_2: Strains studied were cultivated in MRS_C for 24 at 37°C. Each strain cultivated was diluted (1/100) in tubes containing MRS_C broth. Different concentrations of H_2O_2 (50, 100, 200 and 300 μg l^{-1}) were prepared. For each concentration, 200 μl were transferred to sterile Micro-dishes then added to 20 μl of the diluted culture of strain. Micro-dishes were incubated for 18 h at 37°C. The results were reported by measuring the Optical Density (OD) at 650 nm using a spectrophotometer (Specord 200, Analytikjena, Germany) for the determination of the Minimal Inhibitory Concentration (MIC) of H_2O_2 for each strain.

Growth at different pH values: The principle of this test is similar to H_2O_2 test. Diluted cultures of strains were adjusted to different pH values (3.5, 4, 4.4, 4.6, 4.8, 5 and 6.6) using HCl (5N). For each culture with a determined pH value, 200 μl were transferred to sterile micro-dishes. Micro-plates were incubated for 18h at 37°C and the cell growth was determined by measuring the optical density at 650 nm. The resistance of bifidobacteria to the acidity shock was also evaluated by the enumeration of cells after incubation of strains studied in MRS_C adjusted to pH = 2 with HCl (5N) at 0, 90 and 180 min.

Resistance to lysozyme and oxbile salts: Strains isolated were activated in MRS_C. Different dilutions of cultures were prepared in peptone water. 1 ml of each dilution was streaked on MRS agar containing sterilized bovine bile acids (0.3% w/v) or lysozyme (0.5 mg ml^{-1}). The resistance of strains was evaluated by enumerating the colonies on Petri dishes after incubation for 24h at 37°C.

RESULTS AND DISCUSSION

Strains were considered to be members of the genus *Bifidobacterium* on the basis of fructose-6-phosphate phosphoketolase production (the reddish-violet color was formed by bifidobacterial cells) [1] and the following characteristics: Anaerobic growth; Gram-positive; catalase negative; negative for nitrate reduction; indole not produced; acidification of glucose without gas production. Carbohydrates fermentation profiles of isolated strains are shown in Table 1. As shown, Among 26 strains isolated, 14 strains (53.8%) were identified as *B. thermophilum*. The other strains isolated were identified as *B. bifidum* (19.2%), *B. longum* (15.3%), while 3 strains (11.5%) were classified as *B. infantis*. Bifidobacteria are natural microorganisms of the intestinal tract of newborns nourished with breast milk. The major species originated from newborns fecal matter are *B. infantis* and *B. bifidum* [10]. According to Tamine *et al.* [11], *B. addolescentis* and *B. longum* are originated from adults. In this survey, all strains of *B. thermophilum, B. bifidum* and *B. infantis* were isolated from newborns. *B. longum* were originated from foods (meat and fermented milk).

B. thermophilum was described as one of the most common species in ruminants. This species appears to be particularly resistant. It is able to survive or multiply at a relatively high temperature (46°C) and was shown to be highly tolerant to oxygen. It could survive also for several days in a contaminated environment [12]. In practice, this organism has been found at a level of 10^8/g in cow faeces obtained from cowshed. It has been also isolated from meat by Beerens [13]. The Contamination of turkey MDM (Mechanically Deboned Meat) samples with *B. thermophilum* was also reported [12]. In general, four species had been reported in literature for the use as probiotic cultures in dairy products: *B. longum*, *B. infantis*, *B. breve* and *B. bifidum*. Recent experience revealed that *B. animalis* had been applied in fermented dairy products instead of *B. longum* due to the fact that this species is somewhat less sensitive against acidification [14].

The inhibitory test showed that 6 strains of *B. themophilum* (UL73, UL77, UL92, UL95, UL100, UL101) inhibited the growth of strains of *L. monocytogens*, *E. coli*, *S. typhimurium* and *S. aureus* used. Two strains of *B. infantis* (ULa and ULc) and two strains of *B. longum* (ULg and ULi) were active against *E. coli* and *S. typhimurium.* One strain of *B. bifidum* ULb was active against *L. monocytogens* only. The diameters of the inhibition zones ranged from 1 to 3.4 (Table 2).

The antibacterial compounds produced by bifidobacteria could resist after a heat-treatment at 100°C for 5 min. For all strains of *B. themophilum*, *B. longum*, *B. infantis* and *B. bifidum* that were able to inhibit the

Table 1: Carbohydrates fermentation by isolates of bifidobacteria

	Carbohydrates									
Strain code	Gly	Ado	Fru	Man	Malt	Raf	Xyl	Tag	Glu	Identified species
UL68	-	-	+	+	+	-	-	+	-	*B. bifidum*
UL70	-	-	+	+	+	-	-	+	-	*B. bifidum*
UL73	-	-	+	+	+	+	-	+	-	*B. thermophilum*
UL77	-	-	+	-	+	+	-	+	-	*B. thermophilum*
UL78	-	-	+	+	+	+	-	+	-	*B. thermophilum*
UL80	-	-	+	+	+	+	-	+	-	*B. thermophilum*
UL81	+	-	+	-	+	-	-	+	-	*B. bifidum*
UL82	-	-	+	+	+	+	-	+	-	*B. thermophilum*
UL83	-	-	+	+	+	+	-	+	-	*B. thermophilum*
UL84	-	-	+	+	+	+	-	+	-	*B. thermophilum*
UL85	-	-	+	+	+	+	-	+	+	*B. thermophilum*
UL91	-	-	+	+	+	+	-	+	-	*B. thermophilum*
UL92	+	-	+	-	+	-	-	+	-	*B. thermophilum*
UL95	-	-	+	-	+	+	-	+	-	*B. thermophilum*
UL100	-	-	+	+	+	+	-	+	-	*B. thermophilum*
UL101	-	-	+	-	+	+	-	+	-	*B. thermophilum*
UL103	+	-	+	-	+	+	-	+	-	*B. thermophilum*
ULa	-	-	+	-	-	-	-	-	-	*B. infantis*
ULb	-	-	+	+	+	-	-	+	-	*B. bifidum*
ULc	-	-	-	-	-	-	-	-	-	*B. infantis*
ULd	-	-	-	-	-	-	-	-	-	*B. infantis*
ULe	-	-	+	+	+	-	-	+	+	*B. longum*
ULf	-	-	+	+	+	-	-	+	+	*B. bifidum*
ULg	-	-	+	+	+	-	-	+	+	*B. longum*
ULh	-	-	+	+	+	-	-	+	+	*B. longum*
ULi	-	-	+	+	+	-	-	+	+	*B. longum*

+ : Positive reaction ; - : Negative reaction; Gly: Glycerol; Ado : Adonitol ; Fru : D-Fructose ; Man : Mannitol ; Malt : Maltose: Raf: Raffinose; Xyl: Xylitol; Tag: D-Tagatose; Glu: Gluconate

growth of pathogenic bacteria, there was a disappearance of the inhibitory effect after treatment with pronase-E and proteinase-K. Theses findings indicated that the growth-inhibiting factors produced by theses strains are naturally proteins and could be identified as bacteriocins-like.

Several reports have revealed that some intestinal lactobacilli and bifidobacteria produce antimicrobial substances that are active against pathogenic bacteria [5, 15, 16]. The inhibition of pathogens by bifidobacteria could also be due to the pH decrease. Scardovi [17] reported that *Bifidobacterium* species did not produce butyric and propionic acids, but produce lactic and acetic acids. Such acids are responsible for the decrease of pH in intestines and the inhibition of the growth of pathogenic bacteria [18]. However, previous studies have reported the ability of strains of bifidobacteria to produce antibacterial compounds [7, 19, 20]. Indeed, Anand *et al.* [21] demonstrated that bifidin is a bacteriocin produced by *B. bifidum* NCDO 1452. Bifidin was able to inhibit the growth of *E. coli*, *Bacillus cereus*, *S. aureus* and *Pseudomonas fluorescens*. Yildrin and Johnson [7] have isolated and purified from *B. bifidum* NCFB 1454 a bacteriocin (bifidocin B) that was active against strains of *Listeria spp*., *Bacillus spp*. and *Enterococcus spp*. The bacteriocin isolated was sensible to pronase-E and proteinase-k, resistant to different organic solvents (N-hexane, methanol and acetone) and remain active after storage for three months at -70°C and after exposition to pH values ranging from 2 to 10 [7].

The evaluation of probiotic proprieties of strains isolated is represented in Table 3. As shown, the MIC of H_2O_2 ranged from 100 to 200 μg ml^{-1} for all strains studied. The resistance of bifdobacteria to hydrogen peroxide is important since H_2O_2 is produced by certain

Table 2: Diameters (mm) of the inhibition zones produced by bifidobacteria strains against pathogenic bacteria

	Pathogenic bacteria								
	L. monocytogens			*E. coli*		*S. aureus*		*S. typhimurium*	
	1	2	3	4	5	6	7	8	9
B. bifidum UL68	-	-	-	-	-	-	nd	-	-
B. bifidum UL70	-	-	nd	-	-	-	-	-	-
B. thermophilum UL73	3.4	3.0	2.4	1.6	1.5	2.1	2.3	2.4	2.0
B. thermophilum UL77	2.4	2.5	2.1	1.4	1.0	1.5	1.2	2.1	2.5
B. thermophilum UL78	-	-	-	-	-	-	-	nd	-
B. thermophilum UL80	-	-	-	-	nd	nd	-	-	-
B. bifidum UL81	-	-	-	-	-	-	-	-	-
B. thermophilum UL82	-	-	-	-	-	-	-	-	-
B. thermophilum UL83	-	-	nd	nd	-	-	-	-	-
B. thermophilum UL84	-	-	-	-	-	-	-	-	-
B. thermophilum UL85	-	-	-	-	-	-	-	-	-
B. thermophilum UL91	-	-	-	-	-	-	-	-	-
B. thermophilum UL92	3.1	2.0	3.2	2.3	1.8	1.6	2.5	2.8	1.8
B. thermophilum UL95	2.5	1.5	2.0	1.8	nd	2.4	2.2	1.1	1.0
B. thermophilum UL100	3.0	2.8	nd	1.4	2.0	1.9	nd	1.6	2.6
B. thermophilum UL101	2.0	2.1	1.8	2.4	2.5	2.3	2.5	2.4	2.2
B. thermophilum UL103	-	-	-	-	-	-	-	nd	-
B. infantis ULa	-	-	-	1.8	1.9	-	nd	1.9	2.0
B. bifidum ULb	1.8	1.6	2.0	-	-	-	-	-	-
B. infantis ULc	-	-	-	1.5	1.8	-	-	2.6	2.1
B. infantis ULd	-	-	-	-	nd	-	nd	-	-
B. longum ULe	-	-	-	-	-	-	nd	-	-
B. bifidum Ulf	-	-	-	-	nd	-	-	-	-
B. longum ULg	-	-	-	3.4	3.2	-	-	1.9	1.7
B. longum ULh	-	-	-	-	-	nd	-	-	-
B. longum ULi	-	-	-	2.5	2.7	-	-	2.0	1.6

-: No inhibition zone, nd: not determined

microorganisms such as acid lactic bacteria (*Lactobacillus spp*. and *Streptococcus spp*.) during the fermentation of foods [22]. The test of the tolerance of strains isolated to low pH values showed that 5 strains of *B. thermophilum* (UL77, UL92, UL95, UL100, UL101) were able to grow at a minimal pH value of 4. The ability of strains of bifidobacteria isolated to survive at low pH values is very important since this propriety is an advantage for theses microorganisms to incorporate fermented foods with acid lactic bacteria with high acidifying proprieties.

The resistance of strains isolated to the acidity shock showed that only *B. thermophilum* UL92 and *B. thermophilum* UL101 could tolerate a pH value (pH = 2). After 180 min, their numbers reached 2×10^6 and 7.2×10^2 cfu ml^{-1}, respectively. While the numbers of all strains decreased to levels below 10^0 cfu ml^{+-} after their exposition to pH 2 for 180 min. The resistance of bifidobacteria to high values of acidity is important since theses microorganisms could be used in formulations or be added to some foods and should resist to the acidity of the stomach during their gastric transit [23].

Concerning the ability of strains used to tolerate the lysozyme (0.5 mg ml^{-1}), results showed that *B. thermophilum* UL77 had the weak percentage of mortality (4.2%). where, *B. thermophilum* UL100 and *B. thermophilum* UL 101 showed high percentages of mortality of about 50.3 and 45% respectively. Strains of *B. infantis* ULa, *B. bifidum* ULb, *B. infantis* ULc, *B. longum* ULg and *B. longum* ULi were more sensible to the lysozyme with mortality frequencies between 65.6 and 95%. These findings are in agreement with data reported by Heine *et al.* [24] on the resistance of some bifidobacteria species to concentrations of the lysozyme

Table 3: Tolerance of antimicrobial producing strains of bifidobacteria to the acidity, hydrogen peroxide, lysozyme and bile salts

Strains	Minimal growth pH for	Tolerance to the shock acidity (cfu ml^{-1}) 0 min	90 min	180 min	MIC of H_2O_2 ($\mu g\ ml^{-1}$)	Mortality (%) Lysozyme (0.5 mg ml^{-1})	bile salts (0.3%)
B. thermophilum UL73	>5	3.2×10^8	5.1×10^5	$<10^2$	100-200	19.3	11.2
B. thermophilum UL77	<4	2.3×10^8	10^4	$<10^2$	100-200	4.2	73.4
B. thermophilum UL92	<4	8.0×10^8	2.4×10^7	$2\ 10^6$	100-200	15.8	21.1
B. thermophilum UL95	4	1.7×10^8	10^4	$<10^2$	100-200	19.2	87.0
B. thermophilum UL100	<4	5.1×10^8	$<10^2$	$<10^2$	100-200	50.3	48.4
B. thermophilum UL101	<4	6.4×10^8	2.7×10^5	$7.2\ 10^2$	100-200	45.0	49.7
B. infantis ULa	>5	5.0×10^8	$<10^2$	$<10^2$	100-200	80.5	70.5
B. bifidum ULb	>5	6.5×10^8	$<10^2$	$<10^2$	100-200	65.6	80.8
B. infantis ULc	>5	4.7×10^8	$<10^2$	$<10^2$	100-200	77.8	88.7
B. longum ULg	>5	6.7×10^8	$<10^2$	$<10^2$	100-200	72.5	95.5
B. longum ULi	>5	4.1×10^8	$<10^2$	$<10^2$	100-200	95.0	76.8

below 1 mg ml^{-1}. As shown in Table 3, *B. thermophilum* UL73, *B. thermophilum* UL92, *B. thermophilum* UL100 and *B. thermophilum* UL101 showed high percentages of resistance to bile salts (0.3%) of about 11.2, 21.1, 48.4 and 49.7% respectively. Theses findings indicated that theses strains could be considered as probiotics since their viability is over 50%.

CONCLUSION

As far as we know, this is the first report that describes the isolation and characterization of strains of bifidobacteria from different origin in Morocco. *B. thermophilum* was the predominant species isolated. The other strains isolated were *B. bifidum*, *B. longum* and *B. infantis*. Some isolated strains were able to inhibit the growth of pathogenic bacteria and the antibacterial compounds produced could be identified as bacteriocins-like. Strains isolated showed also some probiotic proprieties which suggests their possible use in the food industry. Indeed, a process for the incorporation of theses strains in fermented foods is under investigation by our research group. However, more studies are needed to complete the isolation and the characterization of new strains of bifidobacteria that could be beneficial for the human health.

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