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The acid, bile tolerance and antimicrobial property of *Lactobacillus acidophilus* NIT

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ABSTRACT

The characteristics of potential probotic *Lactobacillus acidophilus* NIT isolated from infant feces were evaluated in vitro. The strain was examined for resistance to pH 2–4 and 1–3% bile, adhesion to Caco-2 cells, and antimicrobial activities against enteric pathogenic bacteria. *L. acidophilus* NIT was shown tolerance property to bile, acid and strong antimicrobial activity against tested enteropathogens by the well-diffusion method. Furthermore, good adhesion and significant potential for decreasing adhesion of pathogens to Caco-2 cells were observed in this experiment. These results showed that *L. acidophilus* NIT may be useful for improving probiotic formulae with respect to protection against enteric pathogenic infection.

1. Introduction

Specific strains of *Lactobacillus*, *Bifidobacterium* and also, some *Propionibacterium* strains have been introduced as probiotics in food products due to their growing evidence of health benefits (Alvarez-Olmos & Oberhelman, 2001; Guarner & Malagelada, 2003; Shanahan, 2002). Lactobacilli are natural inhabitants of healthy human intestinal tract and also have a long history of use in foods and fermented products. It is desirable that these bacteria have suitable general aspects (origin, identity, safety, and acid and bile resistance), technical aspects (growth properties in vitro and during processing), and functional and beneficial features (Holzapfel & Schillinger, 2002). Previous work has shown *Lactobacillus* probiotic strains can possess inhibitory activity toward the growth of pathogenic bacteria, resistance to acid and bile, adherence to the intestinal epithelial cells and positive effects on the host health (Finlay & Falkow, 1989; Gorbach, 1996; Jacobsen et al., 1999).

Selection of suitable probiotic candidates is the principal basis for improving the bio-therapeutic action and functional properties of probiotic foods and pharmaceutical products. The essential characteristics for *Lactobacillus* to be used as probiotics during manufacturing include the following: (i) recognition as safe (GRAS; generally recognized as safe); (ii) viability during processing and storage; (iii) antagonistic effect against pathogens; (iv) tolerance to bile acid challenge, and (v) adherence to the intestinal epithelium of the host among others (Begley, Gahan, & Hill, 2005; Lin, Hwang, Chen, & Tsen, 2006; MacFarlane & Cummings, 2002; Vesterlund, Paltta, Karp, & Ouwehand, 2005).

In order to survive in and colonize the gastrointestinal tract, probiotic bacteria should express high tolerance to acid and bile and have the ability to adhere to intestinal surfaces (Kirjavainen, Ouwehand, Isolauri, & Salminen, 1998; Lee & Salminen, 1995). Survival ability and temporary colonization of the human gastrointestinal tract have been demonstrated for some lactic acid bacteria (Alander et al., 1997; Johansson et al., 1998). However, in vivo testing is expensive and time consuming and requires approval by ethical committees. Therefore, reliable in vitro methods for selection of promising strains are required. Enterocyte-like Caco-2 cells (Pinto et al., 1983) have been successfully used for in vitro studies on the mechanism of cellular adhesion of nonpathogenic lactobacilli (Greene & Klaenhammer, 1994; Tuomola & Salminen, 1998). Also, Caco-2 cells have been used to examine the antimicrobial activity of lactobacilli (Hudault, Liévin, Bernet-Camard, & Servin, 1997) against pathogenic bacteria. Although many potentially probiotic strains are currently available for commercial use either in the form of fermented foods or pure culture in powdered, tablet or capsule form, these bacteria may not have above characteristics. It therefore appears appropriate to isolate and characterize *Lactobacillus* strains with specific and well-characterized activities, not only to improve the quality and functional properties of probiotic products but also to advance both applied and fundamental science in the area of probiotics.

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The objective of the present study is to characterize the potential probiotic *Lactobacillus acidophilus* NIT originally isolated in infant feces. The acid, bile tolerance and antimicrobial property of this strain were evaluated. Enterocyte-like Caco-2 cells were used for testing *L. acidophilus* adhesion properties and the abilities to inhibit the adhesion of pathogens. Reference pathogens strains were obtained from CCTCC (China Center for Type Culture Collection, Wuhan, China) and DSM (Deutsche Sammlung von Mikroorganismen).

2. Materials and methods

2.1. Bacterial strains and culture condition

L. acidophilus NIT used as potential probiotic was originally derived from infant feces. This strain was conserved on Key Laboratory for Molecular Design and Nutrition Engineering, Ningbo Institute of Technology, Zhejiang University. Bacterial pathogens used were *Escherichia coli* CCTCC AB 206316, *Salmonella typhimurium* CCTCC M 90030, *Clostridium histolyticum* DSM 627, *Bacteroides vulgatus* DSM 1447, *Clostridium difficile* DSM 1296. All bacterial pathogens were reactivated in LB broth (Luria–Bertani media). *L. acidophilus* NIT was cultured in MRS broth, as detailed by De Man, Rogosa and Sharpe (Oxoid Ltd., Hampshire, United Kingdom). All *L. acidophilus* and pathogens were cultured at 37 °C under anaerobic conditions (10% H_2, 10% CO_2, and 80% N_2; Concept 400 anaerobic chamber, Ruskinn Technology, Leeds, UK). Pure cultures were stored at −80 °C in growth medium, supplemented with glycerol (15%, v/v, final concentration).

2.2. Acid tolerance

The method was according to Claire, Glenn, and Robert (2006) with some modifications. Broth culture was performed by transferring 100 μl reactive culture into a fresh 10 ml broth. The cultures were incubated anaerobically for 24 h. The culture was then swirled gently to unsettle the cells from the bottom and 1 ml removed to a 1.5 ml microcentrifuge tube following aspiration. Then the tubes were centrifuged for 5 min at 10,000*g* to produce a pellet, and the cells were resuspended in 10 ml glycine–HCl buffer at pH 2, 3 or 4. Samples from the glycine–HCl buffers were taken at 1, 2 and 3 h after the pellet was resuspended and subjected to serial dilutions. About 20 μl above sample solution was spread onto the surface of MRS agar plates and the colony counts used for calculation of the CFU ml^{-1}. The results were used to construct survival curves for each of the bacteria. The experiment was determined in three independent experiments, and each assay was performed in triplicate to calculate intra-assay variation.

2.3. Bile tolerance

The method was adapted from that described by Pereira and Gibson (2002). Cultures were revived as outlined previously and inoculated into anaerobic sealed tubes containing 10 ml MRS Broth (Oxoid). The tubes were incubated at 37 °C and grown overnight. About 100 μl was then inoculated into fresh tubes of MRS broth or MRS broth containing 1–3% bile (oxgall, Sigma). The pH of all broth was adjusted to 6.5. The bacteria growth was measured by MRS agar clone count with taking 20 μl culture for every 2 h in anaerobic chamber. The sample was removed from the tubes and subjected to serial dilutions in half-strength peptone water (Oxoid) with 0.5 g l^{-1} Cysteine–HCl (Sigma), and aliquots spread onto MRS agar plates to calculate the CFU ml^{-1}. The experiment was determined in three independent experiments, and each assay was performed in triplicate to calculate intra-assay variation.

2.4. Antimicrobial profile

For detection of antimicrobial activity, a modification of the 'well-diffusion' test described by Hechard, Dherbomez, Cenatiempo, and Lettllier (1990) was used. Pathogens were inoculated in liquid nutrient agars at 37 °C and cultured for 12 h. The agar was then left to set in regular microbiological plates and an 8 mm diameter well was punched in the agar. The selected probiotic *L. acidophilus* was cultured overnight in MRS broth before 'well-diffusion' assay. An aliquot of the cultures was set aside and the remainder centrifuged. To eliminate the possible inhibition action of hydrogen peroxide and lactic acid, the supernatant was filtered through a filter, adjusted to pH 6.0 and added a sterile solution of catalase (1000 U ml^{-1}, Sigma). The remaining cells were resuspended to their original volume with fresh MRS broth. Three different suspensions (original overnight culture, treated supernatant, resuspended broth) were filled into the agar well separately. The agar was then cultured overnight at 37 °C under anaerobic conditions. The diameter of the inhibition zone surrounding the wells was then measured. The diameter of the zone was scored as follows: as the diameter of the well is 8 mm, 8 mm equals no inhibition (–), diameter between 0 and 3 mm (weak, +), diameter between 3 and 6 mm (good, ++) and diameter larger than 6 mm (strong, +++). All experiments were performed in three independent experiments, and each assay was performed in triplicate to calculate intra-assay variation.

2.5. Adhesion assay

A monolayer of Caco-2 cells was used to determine the adhesion of lactobacilli. Cell growth and adherence assays were performed as previously described Coconier, Klaenhammer, Kernéis, Bernet, and Servin (1992) and Jacobsen et al. (1999). Enterocyte-like Caco-2 cells were cultured in a monolayer with DMEM (Gibco) containing 20% (v/v) fetal calf serum (FCS; Gibco), antibiotics (100 U ml^{-1} penicillin, 100 mg ml^{-1} streptomycin; Gibco) at 37 °C in a 10% CO_2–90% air atmosphere. For the bacterial adhesion assay, Caco-2 cells were seeded on glass cover slips placed in six-well tissue culture plates (9.6 cm^2 per well, Falcon, Becton Dickinson Company) for Gram staining. Overnight cultures of bacteria were appropriately diluted (10×) with DMEM to give a bacterial concentration of approximately 10^8 cells ml^{-1}, and 120 μl was used to inoculate the Caco-2 cells. After incubation for 1 h at 37 °C, all of the dishes were washed four times with PBS (pH, 7.2) to release unbound bacteria. The cells were then fixed with 3 ml of methanol and incubated for 5–10 min at room temperature. After removal of the methanol, the cells were stained with 3 ml of Giemsa stain solution (1:20) (Sigma) and left to incubate for 30 min. The dishes were washed until no color was observed in the washing solution, dried in an incubator at 37 °C overnight, and examined microscopically (magnification, ×100) under oil immersion. Each adherence assay was conducted in duplicate over three successive passages (8–13 cell passages) of intestinal cells. For each monolayer on a glass coverslip, the number of adherent bacteria was counted in 20 random microscopic areas.

2.6. Inhibition of pathogens adhesion to Caco-2 cells

To study the effect of *Lactobacillus* treatment on the pathogens interaction with enterocyte-like cells, Caco-2 cells were used. The cell infection assay was conducted as previously reported Coconier, Lievin, Hemery, and Servin (1998). Overnight (18 h) MRS culture and pathogens in LB broth culture were separately harvested by centrifugation and washed twice with PBS. Bacteria were diluted in antibiotic-free DMEM (pH 7.3, 25 °C) and 0.5 ml/well of the bacterial suspension was added to 24-well tissue culture plates. The

ability of lactobacilli to inhibit five pathogenic strains adhesion (10^7 CFU per well) to Caco-2 cells was evaluated by simultaneous addition of 10^8 CFU per well of tested lactobacilli isolate. Plates were incubated for 1 h at 37 °C, washed three times with sterile PBS. The cell-associated pathogens (extracellular plus intracellular bacteria) were lysed with 1% (v/v) Triton X-100 (Sigma) in deionized water for 5 min. This concentration of Triton X-100 did not affect bacterial viability for at least 30 min (data not shown). Appropriate dilutions of the lysate were plated on LB agar to determine the number of viable cell-associated bacteria by bacterial colony counts. The inhibition test was determined in three independent experiments, and each assay was performed in triplicate to calculate intra-assay variation.

2.7. Statistical analysis

Tests for the statistical significance of differences were compared by Student's *t*-test for all experiments. All statistics were performed using SPSS 11.0 software (SPSS, Inc., Chicago, IL, USA).

3. Results

3.1. Resistance to acid

The acid tolerance profile of *L. acidophilus* NIT was tested in different pH values (Fig. 1). At the lowest pH 2, the numbers of bacteria had decreased to an undetectable level after 2 h, however within 1 h treatment there still had more than 20% survival. At pH 3, the surviving percentage was greater than at pH 2 in the whole process. After 3 h interaction, the viable rate of *L. acidophilus* NIT was achieved to about 10%. When the test pH was increased to pH 4, a high survival was observed. But, with increasing the incubation time, the number of viable bacteria was decreased.

3.2. Resistance to bile

The bile resistance of *L. acidophilus* NIT was evaluated by supplement with bile (oxgall). Fig. 2 shows growth curves in MRS broth or MRS broth containing 1%, 2%, 3% bile. The maximal viable count could rise to 10^9 CFU ml^{-1}, when *L. acidophilus* was incubated in MRS broth without bile. In contrast, the growth in MRS broth containing different amount of bile was inhibited. With the increase of bile, the growth was obviously decreased. However, it still had more than 10^5 CFU ml^{-1} viable bacteria in MRS broth with 2% bile after 15 h incubation.

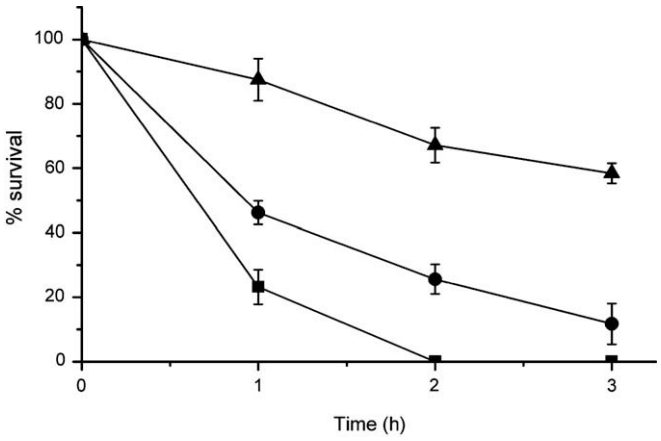

Fig. 1. Survival of *Lactobacillus acidophilus* NIT in the presence of acid. (■) pH 2, (●) pH 3 and (▲) pH 4. Error bars are ±SE.

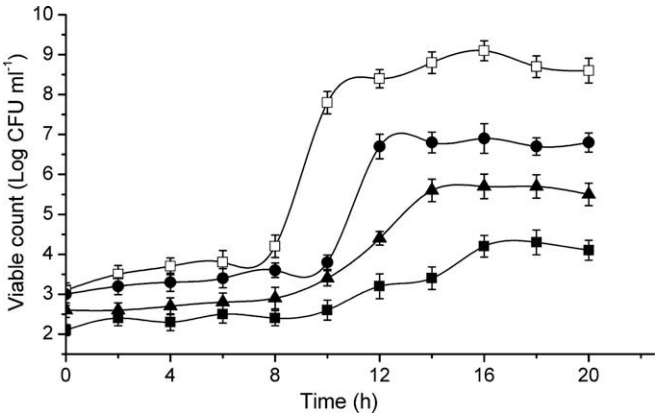

Fig. 2. Growth of *Lactobacillus acidophilus* NIT in standard and bile-containing media. The viable count of bacteria in MRS or MRS supplemented with bile (oxgall). (□) Control group, (●) 1% bile (▲) 2% bile and (■) 3% bile.

3.3. Antimicrobial profile

The inhibition of *L. acidophilus* NIT against bacterial pathogens was shown in Table 1. Overnight culture and pretreated supernatant were able to inhibit all the examined pathogens, while resuspended culture did not. Overnight culture of *L. acidophilus* was able to inhibit more pathogens than the supernatant and/or resuspended broth. The overnight culture had a strong inhibition to all the pathogens except *C. histolyticum*. When the supernatant was neutralized and treated with catalase, it still showed inhibition activity to all pathogens. However, the resuspended bacteria in fresh MRS broth had no inhibition to *C. difficile* and *C. histolyticum*.

3.4. Adhesion to Caco-2 cells

All probiotic and pathogen strains tested were able to adhere to Caco-2 cells (Fig. 3). *L. acidophilus* showed the strongest adhesion ability (121 adhesive bacteria), while the least adhesive strain was pathogen *B. vulgatus* (54 adhesive bacteria). Among the pathogens, *E. coli* with a result of 112 adhesive bacteria was the strongest strain. The number of adhesive bacteria of *C. difficile*, *Chistolyticum* and *S. typhimurium* was 64, 95 and 67, respectively.

3.5. Inhibition of pathogens adhesion

In the presence of *L. acidophilus* NIT, the inhibition of pathogens adhesion was shown in Table 2. All the adherent pathogens were obviously reduced by co-culture with *L. acidophilus*. The reduction of *E. coli* and *S. typhimurium* adhesion to Caco-2 cells was more than 50% added with *L. acidophilus* 10^8 CFU per well.

Table 1
The antimicrobial activity of *Lactobacillus acidophilus* against pathogens

	Lactobacillus acidophilus NIT		
	Overnight	Supernatant	Resuspended
Salmonella typhimurium CCTCC M 90030	+++	+	+
Clostridium histolyticum DSM 627	++	+	–
Escherichia coli CCTCC AB 206316	+++	+	+
Bacteroides vulgatus DSM 1447	+++	+	+
Clostridium difficile DSM 1296	+++	+	–

Note: –, No inhibition; +, zone of inhibition between 0 and 3 mm diameter (weak); ++, zone of inhibition between 3 and 6 mm diameter (good); +++, zone of inhibition larger than 6 mm diameter (strong); Overnight, the overnight culture broth containing bacteria and supernatant; Supernatant, supernatant adjusted to pH 6 and added with and treated with catalase. Resuspended, resuspended bacteria in fresh MRS broth. Results are medians of three experiments.

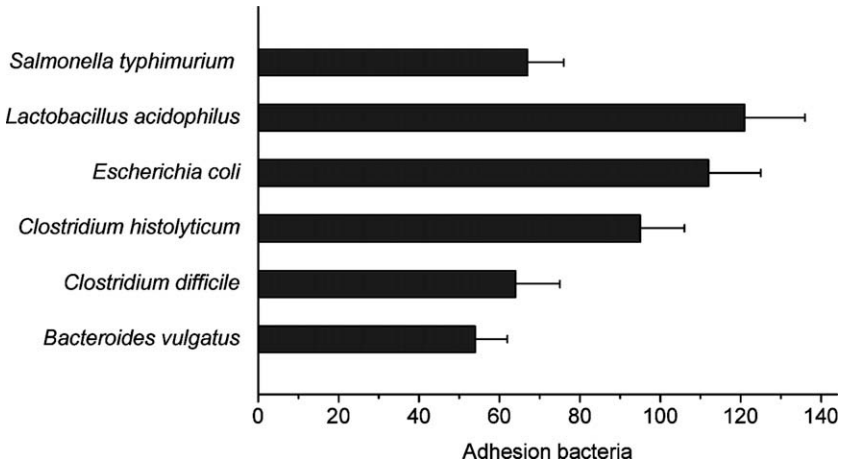

Fig. 3. Adhesion to Caco-2 cells of *L. acidophilus* and pathogen strains tested. Results were shown as number of adhering lactobacilli in 20 microscopic areas ± the standard deviation.

Table 2
Inhibition of pathogens adhesion by co-culture with *L. acidophilus* NIT

	Adhesive pathogen count (10^5 CFU ml^{-1})	
	Untreated	Co-cultured
Salmonella typhimurium	4.4 ± 0.3	1.9 ± 0.2
Clostridium histolyticum	6.3 ± 0.4	4.1 ± 0.2
Escherichia coli	8.5 ± 0.2	3.2 ± 0.4
Bacteroides vulgatus	3.5 ± 0.3	3.4 ± 0.3
Clostridium difficile	4.2 ± 0.2	1.5 ± 0.2

Note: *L. acidophilus* was added at a concentration of 10^8 CFU per well, and the pathogens at 10^7 CFU per well separately. Values are means ±SE.

4. Discussion

With the great interest on health-oriented nutritional habits, the food industry is requested to provide more and more functional foods containing healthful components. By the recently adopted definition that probiotics are live micro-organisms which when administered in adequate amounts confer a health benefit on the host (FAO/WHO., 2001), probiotic bacteria seem to be such components. However, to sustain a certain strain as a probiotic, a group of requirements should be fulfilled (Collins, Thornton, & Sullivan, 1998) and probiotic activities demonstrated in well-designed human studies. This paper presents results of some preselective studies on *L. acidophilus* NIT.

In order to exert positive health effects, the lactobacilli should resist the stressful conditions of the stomach and upper intestine that contain bile (Chou & Weimer, 1999). Acidity is believed to be the most detrimental factor affecting growth and viability of lactobacilli, because their growth was down significantly below pH 4.5 (Lankaputhra & Shah, 1995; Lankaputhra, Shah, & Britz, 1996). In the present study, it was observed that *L. acidophilus* NIT had certain resistance ability to acid and bile. Therefore, this stain may be expected to survive acidic conditions that exist in fermented food products or stomach and intestinal juice, which may contribute to increased shelf life.

As a functional probiotic, anti-pathogen activity is one of important properties. The antimicrobial ability of *L. acidophilus* NIT against some enteropathogens was assayed in this study. The overnight *L. acidophilus* culture showed strong inhibition action to selected pathogens (Table 1). Meanwhile, treated supernatant without peroxide and lactic acid also had week anti-pathogen activity. It was suggested that *L. acidophilus* NIT produced bacteriocins to inhibit the test pathogens. Some authors have reported that production of bacteriocins by lactobacilli is relatively common, which may contribute to their colonization of habitats and their competitive edge over other bacteria (Garriga, Hugas, Aymerich, & Monfort, 1993). The antimicrobial activity of lactic acid bacteria may be due to a number of factors. Among these are decreased pH levels, competition for substrates and the production of substances with a bactericidal or bacteriostatic action, including bacteriocins (Parente & Ricciardi, 1999). In fact, the drop in pH arising from the production of lactic acid can be enough to inhibit certain strains. This is because the non-dissociated form of lactic acid triggers a lowering of the internal pH of the cell that causes a collapse in the electrochemical proton gradient in sensitive bacteria, hence having a bacteriostatic or bactericidal effect (O'KeeVe & Hill, 1999).

Although antagonistic effects are essential, the ability of probiotic bacteria to adhere to the intestinal epithelium is a prerequisite for probiotic micro-organisms to be effective. Thus, the ability to adhere to epithelial cells and mucosal surfaces has been suggested to be an important property of many probiotic bacterial strains (Collado, Gueimonde, Hernandez, Sanz, & Salminen, 2005; Ouwehand, Isolauri, Kirjavainen, & Salminen, 1999). The binding ability of our isolates was evaluated using the human colon carcinoma cell line Caco-2 as a cellular model. Caco-2 has been used frequently because it exhibits, in vitro, the characteristics of a mature enterocyte (Pinto et al., 1983). Because association with, and invasion of, the cultured cell lines have been reported to mimic the in vivo conditions of adhesion and infection of pathogenic bacteria (Kerneis et al., 1992; Mounier, Vasselon, Hellio, Lesourd, & Sansonetti, 1992). In this study, we demonstrated that *L. acidophilus* NIT had a strong adhesion property to Caco-2 cells (Fig. 3). Some studies demonstrated that the ability of some bacterial strains to adhere and colonize the intestinal cell in vivo or the cultured intestinal cell in vitro is similar (Finlay & Falkow, 1989). However, it must be considered that Caco-2 cells were only used as a model to study adhesion because of the different morphological, physicochemical and environmental conditions surrounding the epithelial cells in both types of experiences.

Recent studies have explained the role of lactobacilli in the prevention and treatment of gastrointestinal disorders (Coconier et al., 1998; Hudault et al., 1997). One of the important factors is the competitive inhibition of enteropathogen attachment to epithelial cells by lactobacilli. So, we investigated the competitive inhibition of adherence of pathogenic bacteria to Caco-2 cells by adhering *L. acidophilus* cells. The *L. acidophilus* can strongly inhibit the adhesion of most selected pathogens (Table 2). Interestingly, there was

no obvious effect on the adhesion of *B. vulgates* ($P > 0.05$). However, the mechanisms by which lactobacilli inhibit pathogen adhesion to human cell lines in vitro are not fully understood. Steric hindrance rather than blockage of specific receptors may be involved (Bernet, Brassart, Neeser, & Servin, 1993). Additional experiments are needed to determine the precise mechanism of inhibition observed in our study.

In conclusion, *L. acidophilus* NIT isolated from infant feces in this study presented interesting probiotic characteristics, especially greater resistance to acid and bile conditions, as well as good adhesion capacity to Caco-2 cells. This stain also showed greater enteropathogen growth inhibiting activity and interference with pathogens adhesion to Caco-2 cells. These characteristics may enable them to establish themselves in the intestinal tract and to compete with other bacterial groups. Further studies are needed to characterize the antimicrobial factors, and assay in vivo.

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