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STABILITY OF FREE AND IMMOBILIZED *LACTOBACILLUS ACIDOPHILUS* AND *BIFIDOBACTERIUM LACTIS* IN ACIDIFIED MILK AND OF IMMOBILIZED *B. LACTIS* IN YOGHURT

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ABSTRACT

This study evaluated the stability of *Bifidobacterium lactis* (Bb-12) and of *Lactobacillus acidophilus* (La-05) both free and immobilized in calcium alginate, in milk and in acidified milk (pH 5.0, 4.4 and 3.8). The stability of immobilized *B. lactis* in yoghurt (fermented to pH 4.2), during 28 days of refrigerated storage was also evaluated. The efficiency of two culture media (modified MRS agar and Reinforced Clostridial Agar plus Prussian Blue) for counting of *B. lactis* in yoghurt was determined. Lee's agar was used to count *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* when *B. lactis* were counted in the MRS medium. *B. lactis* and *L. acidophilus* in both free and immobilized forms presented satisfactory rates of survival in milk and acidified milk because the average reduction of the population was only one log cycle after 21 days of storage. The number of viable cells of immobilized *B. lactis* in yoghurt presented a gradual decline throughout the storage period, passing from 10^8 cfu/ml to no count after 28 days of storage. When the cultures were not in equilibrium just the selective medium was efficient in counting *B. lactis* in yoghurt. The results showed that both microorganisms can be added to milk and acidified milk, because their population was only slightly affected during storage. The presence of traditional culture of yoghurt seems to be harmful for survival of immobilized *B. lactis* and the immobilization in calcium alginate failed as an effective barrier to protect the cells in all analysed treatments.

Key words: probiotics, immobilization, alginate, *Lactobacillus acidophilus*, *Bifidobacterium lactis*, yoghurt, milk, *Lactobacillus bulgaricus*

INTRODUCTION

Probiotic supplements contain viable bacteria that beneficially influence health and nutrition when consumed (30). Most commonly they contain *Lactobacillus acidophilus* and *Bifidobacterium*, both of which are part of the normal intestinal microbiota (2). *Lactobacillus acidophilus, L. casei, Bifidobacterium bifidum, B. longum* and *Saccharomyces boulardii* are frequently used as probiotics in products for humans consumption (27), although other species are also recognised as probiotics. Due to their health benefits probiotic bacteria have been increasingly included in yoghurts and fermented milks during the past two decades (22).

Foods containing these microorganisms are sold in many countries, although their survival in foods is doubtful, since some of the strains are extremely sensitive to a series of factors. Also, methods for counting these organisms have not yet been well established, which is an essential requirement to determine their survival in commercial products (12).

The survival of *L. acidophilus* and *Bifidobacterium* spp. in yoghurts has been shown to be a problem, due to their intolerance of acid conditions and the presence of other

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cultures, such as *L. delbrueckii* ssp. *bulgaricus* (17,24). However, microencapsulation or immobilization techniques could provide protection to acid sensitive bifidobacteria and thus increase their survival rate during the shelf life of the yoghurt and during their passage through the gastrointestinal tract (1,5,6,10,23,28).

The method of immobilization by extrusion is the most common approach to make capsules with hydrocolloids. It simply involves preparing a hydrocolloid solution, adding microorganisms to it, and extruding the cell suspension through a syringe needle in the form of droplets to free-fall into a hardening solution or setting bath (15).

The polysaccharide sodium alginate has been most widely used as an immobilizing vehicle (26). It forms a gel when in contact with calcium and multivalent cations (7). Alginate beads (or microparticles) are stable in low pH conditions but swell in weak basic solutions followed by disintegration and erosion (18).

The immobilization of *Lactobacillus bulgaricus* in calcium alginate offered good protection to the organisms during frozen storage and in ice cream (31). Bifidobacteria immobilized in alginate were more resistant to acid pH values in mayonnaise than the free cells (13).

The objectives of this study were to evaluate the stability of *B. lactis* and *L. acidophilus,* both free and immobilized in calcium alginate, in acidified milk, determine the stability of *B. lactis* in yoghurt, and to verify the efficiency of two culture media, one selective and the other differential, in counting *B. lactis* in yoghurt.

MATERIALS AND METHODS

Cultures

Lactobacillus acidophilus (La-05) and *Bifidobacterium lactis* (Bb-12) (Chr. Hansen, Valinhos, Brazil), in the DVS (direct vat set) form, pure and freeze dried, were maintained at -18°C in the proportion of 1g per 150 ml in a sterile solution of 12% reconstituted skim milk. These milks were thawed for inoculation of acidified milks and yoghurts and for preparation of the calcium alginate beads.

Before use a mixed commercial culture of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus,* from Danisco Cultor Niebüll GmbH (Niebüll, Germany), was replicated twice in 12% reconstituted sterile skim milk, at 45ºC for 3 hours.

Culture counts

In the absence of other cultures, *L. acidophilus* and *B. lactis* were counted in DeMan Rogosa and Sharp (MRS) agar (Oxoid) by the pour plate technique. A 2% solution of sodium citrate was used to prepare serial dilutions. One ml of each dilution was pour plated in MRS agar. After solidification, the plates were inverted and incubated at 37ºC for 72 h in jars with the Anaerogen (Oxoid) system for generating anaerobic atmosphere. Plating was carried out in duplicate.

For quantitative measurements of the number of viable cells of immobilized bacteria, it was necessary to solubilize the alginate beads to liberate the microorganisms. This was made in 2% sterile sodium citrate solution, using the Stomacher 400 (Seward, London, UK), at medium velocity and room temperature, for 2 minutes.

Immobilization process

The immobilization of *L. acidophilus* and *B. lactis* in calcium alginate was carried out according to Fávaro-Trindade and Grosso (6).

Evaluation of the stability of immobilized *L. acidophilus* and *B. lactis* in acidified milk

Model systems were elaborated to study the viability of incorporating *L. acidophilus* and *B. lactis* into acid foods. For this, milk (pH 6.4) previously standardised at 15% solids and sterilised in retort at 121ºC for 8 minutes was used as the standard. Other samples of milk in the same conditions were acidified by the addition of 4N lactic acid in order to achieve pH values of 5.0, 4.4 and 3.8. The calcium alginate beads containing *L. acidophilus* or *B. lactis* were added to these acidified milks at a rate of 5% in relation of the milk volume. The cultures of *L. acidophilus* or *B. lactis*, dissolved in milk as stated early, were added to these acidified milks at a rate of 5% in relation of the milk volume. All samples were stored at 7ºC for 28 days in a B.O.D. incubator (model TE 390, Tecnal, Piracicaba, Brazil). Counts were made after 0, 7, 14, 21 and 28 days. This experiment was repeated three times for each pH.

Evaluation of the stability of immobilized *B. lactis* in yoghurt

Yoghurt was prepared from whole milk powder, reconstituted at 15% solids, and sterilized in retort at 121ºC for 8 minutes. Fermentation was carried out at 45ºC with a 2% inoculum of a mixed culture of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*) until a pH of 4.2 was reached. Afterwards, the yoghurt was blended for 5 minutes. The blended product was transferred to plastic cups and the calcium alginate beads containing *B. lactis* added at a rate of 5% in relation of the yoghurt volume. The contents were gently mixed with a spatula and the cups capped and stored at 7ºC in a B.O.D. incubator (model TE 390, Tecnal, Piracicaba, Brazil) for 28 days. With the purpose of determining the stability of the immobilized *B. lactis* in the yoghurt, counts of this organism and of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* were made after 0, 7, 14, 21 and 28 days of storage. This experiment was repeated twice.

Evaluation of the culture media employed for counting immobilized *B. lactis* in yoghurt

L. acidophilus and *Bifidobacterium* spp, the most commonly used probiotic group, are fastidious organisms and in the

laboratory they are grown on complex media such as MRS broth or Reinforced Clostridial Medium (11). Thus, two media were evaluated for the counting of *B. lactis* in the presence of yoghurt cultures, one selective and the other differential.

The selective medium was MRS – deMan, Rogosa and Sharpe agar (Oxoid, Hampshire, UK) supplemented with: 0.5% of a 10% solution of L-cysteine hydrochloride (Synth, São Paulo, Brazil), 0.5% of a dicloxacillin solution (10 mg/100 ml water) (Sigma, Louis, USA) and 1% of a 10% solution of lithium chloride (Vetec, São Paulo, Brazil) (4,9), modified by addition of 0.01% aniline blue (Nuclear, São Paulo, Brazil). A 2% solution of sodium citrate was used to prepare serial dilutions from 10^1 to 10^8. The spread plate technique was used and after the medium solidification, the plates were inverted and incubated at 37ºC for 72 h in anaerobic jars with the Anaerobac (Probac, São Paulo, Brazil) system.

Lee's agar (20) was used to count *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* when *B. lactis* was counted in the selective medium. In this case the spread plate technique was used and the plates were inverted and incubated at 37ºC for 48 hours in jars containing the Microaerobac (Probac, São Paulo, Brazil) microaerophyllic generating system.

The differential medium was RCPB, composed of RCA – Reinforced Clostridial Agar (Oxoid, Hampshire, UK) with added 0.03% Prussian Blue (Aldrich, USA) (25) and spread plate technique. A 2% solution of sodium citrate was used to prepare serial dilutions from 10^1 to 10^8. The plates were inverted and incubated at 37ºC for 72h under anaerobiosis in jars with the Anaerobac (Probac, São Paulo, Brazil) system.

Statistical analysis

A means difference analysis was used to check for significant differences between the values obtained according to Tukey's Test, with the help of software STATISTICA 6.0.

RESULTS AND DISCUSSION

Evaluation of the stability of *L. acidophilus* and of *B. lactis* in free and immobilized forms in acidified milk

Free *L. acidophilus* showed good stability in acidified milk: the viable count did not change at pH 6.4 and 5 and was reduced by only one log cycle after 14 days of storage at pH 4.4 and 3.8 (Table 1). The population of immobilized *L. acidophilus* was reduced by one log cycle after 14 days of refrigerated storage at all pHs values (Table 2). This result is in agreement with that reported by Laroia and Martin (17), in which *L. acidophilus* survived in great numbers in a frozen fermented product with pH values varying from 3.9 to 4.6, and with that reported by Gilliland and Speck (8), where *L. acidophilus* remained viable in milk acidified with lactic acid.

The survival of free *B. lactis* in acidified milks was considered satisfactory, since a reduction of only one log cycle was registered after 21 days of storage at initial pH of 5.0, and between 7 and 14 days at pH 4.4.

The pH 3.8 was more harmful to the free *B. lactis* only after 28 days of refrigerated storage**,** showing a reduction of two log cycles. The immobilized cells of *B. lactis* suffered a reduction of only one log cycle after 14 days of refrigerated storage, at pH value 3.8 (Table 4).

This result differs from those of Laroia and Martin (17), in which *B. lactis* failed to survive in products with pH 3.9 to 4.6. However, according to Lankaputhra *et al.* (16), the resistance during refrigerated storage in acid products varies according to the species of *Bifidobacterium*. These researchers tested the viability of 9 species of *Bifidobacterium* in acidified milk at pH 4.3, 4.1, 3.9 and 3.7, stored under refrigeration for 42 days, and showed that only three species, *B. infantis* 1912, *B. longum* 1941 and *B. pseudolongum* 20099, were capable of surviving well. The remaining species (*B. bifidum* 1900 and 1901, *B. adolescentis* 1920, *B. breve* 1930, *B. longum* 20097 and *B. thermophilum* 20210) were destroyed by the low pH or presence of H_2O_2.

Table 1. Population of free *L. acidophilus* incorporated into milk (pH 6.4) and into acidified milk at pH 5.0; 4.4 and 3.8 (cfu/ml).

	pH			
Time (days)	6.4	5.0	4.4	3.8
0	9.8×10^{7a}	3.1×10^{7a}	3.1×10^{7a}	8.2×10^{7a}
7	8.3×10^{7a}	3.8×10^{7a}	3.4×10^{7a}	6.0×10^{7a}
14	4.7×10^{7a}	3.5×10^{7a}	6.1×10^{7a}	7.3×10^{7a}
21	1.1×10^{7a}	2.4×10^{7a}	1.1×10^{6b}	3.0×10^{6b}
28	1.0×10^{7a}	2.0×10^{7a}	3.5×10^{6b}	1.6×10^{6b}

Different letters in the same column indicate a significant difference between the means ($p<0.05$).

Table 2. Population of immobilized *L. acidophilus* incorporated into milk (pH 6.4) and into acidified milk at pH 5.0; 4.4 and 3.8 (cfu/ml).

	pH			
Time (days)	6.4	5.0	4.4	3.8
0	1.3×10^{7a}	1.8×10^{7a}	8.4×10^{6a}	7.5×10^{6a}
7	1.1×10^{7a}	1.1×10^{7a}	8.9×10^{6a}	6.1×10^{6a}
14	6.2×10^{7a}	6.4×10^{7a}	6.7×10^{6a}	2.4×10^{6a}
21	1.5×10^{6b}	2.5×10^{6b}	1.3×10^{5b}	3.8×10^{5b}
28	1.8×10^{6b}	1.1×10^{6b}	4.5×10^{5b}	1.2×10^{5b}

Different letters in the same column indicate a significant difference between the means ($p<0.05$).

Table 3. Population of free *B. lactis* in milk (pH 6.4) and in milk acidified to pH 5.0; 4.4 and 3.8 (cfu/ml).

	pH			
Time (days)	6.4	5.0	4.4	3.8
0	8.4×10^{7a}	5.4×10^{7a}	3.2×10^{7a}	8.9×10^{7a}
7	7.0×10^{7a}	5.6×10^{7a}	6.0×10^{7a}	9.0×10^{7a}
14	7.7×10^{7a}	4.1×10^{7a}	4.4×10^{6b}	6.3×10^{6b}
21	3.6×10^{7a}	3.5×10^{7a}	9.0×10^{6b}	3.2×10^{6b}
28	2.0×10^{7a}	2.7×10^{6b}	2.7×10^{6b}	1.0×10^{5c}

Different letters in the same column indicate a significant difference between the means ($p<0.05$).

Table 4. Population of immobilized *B. lactis* in milk (pH 6.4) and in milk acidified to pH 5.0; 4.4 and 3.8 (cfu/ml).

	pH			
Time (days)	6.4	5.0	4.4	3.8
0	2.5×10^{7a}	3.5×10^{7a}	2.4×10^{7a}	1.1×10^{7a}
7	1.5×10^{7a}	3.1×10^{7a}	1.9×10^{7a}	1.1×10^{7a}
14	1.6×10^{7a}	3.3×10^{7a}	5.9×10^{7a}	4.2×10^{6b}
21	4.0×10^{7a}	5.2×10^{6b}	1.9×10^{6b}	2.4×10^{6b}
28	9.9×10^{6b}	3.2×10^{6b}	2.9×10^{6b}	2.0×10^{6b}

Different letters in the same column indicate a significant difference between the means ($p<0.05$).

Evaluation of the stability of immobilized *B. lactis* in yoghurt

One of the requirements for microorganisms to be used for therapeutic purposes is that they remain viable in the food used as a vehicle for their consumption (14). *B. lactis* immobilized in calcium alginate was incorporated into high acid yoghurt (pH 4.2) and a gradual decline in the number of viable cells was shown throughout the storage period (Table 5).

According to Laroia and Martin (17) and to Martin and Chou (21), the low pH of fermented products is harmful to some species/strains of *Bifidobacterium*. However, the present study was shown that free and immobilized *B. lactis* incorporated into acidified milks (pH 4.4 and 3.8) presented a much higher survival rate than in yoghurt at similar pH values, since the maximum reduction in viable count was two log cycles after 28 days of storage (Tables 3 and 4), whilst in yoghurt the whole population was destroyed in the same period (Table 5). Thus, the survival of the free and immobilized *B. lactis* in yoghurt may have been affected by other factors, such as inhibitory substances produced by the yoghurt culture or an excess of dissolved oxygen. The alginate matrix failed to function as a barrier to these factors. This result was different from that reported by Khalil and Mansour (13), in which immobilization was effective in the protection of *Bifidobacterium bifidum* and *B. infantis* cells immobilized in alginate and incorporated into mayonnaise (pH 4,4). Immobilization in alginate also improved the survival of *Lactobacillus bulgaricus* in a milk based dessert (31). Lee and Heo (19) showed that *B. longum* encapsulated in Ca-alginate spheres survived to simulated gastroenteric juice (pH 1.55) significantly better than free cells. This study has indicated that survival of alginate immobilized bacteria decreased with the decrease of sphere size (diameters 1-2,6 mm) and increased with the increase of alginate concentration (1-3%).

The populations of *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* suffered reductions of two and one log cycles, respectively, after 21 days of storage (Tables 5 and 6). This result confirms the predominance of *S. thermophilus* during

Table 5. Counts of immobilized *B. lactis* (in supplemented MRS medium), *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* (in Lee's agar) in yoghurt at pH 4.2, during 28 days of storage at 7ºC (cfu/ml).

Storage (days)	*B. lactis*	*S. thermophilus*	*L. bulgaricus*
0	7.2×10^{8a}	8.1×10^{8a}	9.5×10^{8a}
7	8.6×10^{6b}	5.0×10^{8a}	6.7×10^{8a}
14	1.9×10^{5c}	6.9×10^{8a}	4.6×10^{7b}
21	5.4×10^{3d}	8.4×10^{7b}	3.0×10^{7b}
28	-	5.1×10^{7b}	1.8×10^{6c}

- There was no count at the lowest dilution plated (10^{-1}); Different letters in the same column indicate a significant difference between the means ($p<0.05$).

Table 6. Counts of immobilized *B. lactis*. *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* (all in RCPB medium) in yoghurt at pH 4.2, during 28 days of storage at 7ºC (cfu/ml).

Storage (days)	*B. lactis*	*S. thermophilus*	*L. bulgaricus*
0	6.0×10^{7a}	9.1×10^{7a}	8.0×10^{7a}
7	2.9×10^{6b}	3.4×10^{7a}	6.3×10^{7a}
14	-	6.0×10^{7a}	5.9×10^{7a}
21	-	9.6×10^{6b}	1.1×10^{7a}
28	-	1.8×10^{6b}	8.7×10^{5b}

- No count was possible; Different letters in the same column indicate a significant difference between the means ($p<0.05$).

the refrigerated storage of yoghurt prepared with addition of *B. lactis*, as reported by Rybka and Kailaspathy (29).

Evaluation of culture media for the counting of *B. lactis* in yoghurt

S. thermophilus, *L. delbrueckii* ssp. *bulgaricus* and *B. lactis* were easily differentiated in RCPB by the distinct morphologies of their colonies. *L. delbrueckii* ssp. *bulgaricus* grew forming colonies with diameters of 2 to 3 mm, each with a small white clearly defined centre surrounded by a relatively large blue halo; *S. thermophilus* grew forming colonies with white centres (less clearly defined than those of *L. delbrueckii* ssp. *bulgaricus*) and a blue halo with a diameter of about 1 mm; *B. lactis* formed very small cylindrical white colonies (approximately 0.5 mm in diameter). This result was similar to that reported by Ongoo and Fleet (25), with the difference that these authors obtained larger colonies for *S. thermophilus* than for *L. delbrueckii* ssp. *bulgaricus.* This difference could have been due to the use of strains from different companies, so from different origins.

After 7 days of storage (Table 6) counting of *B. lactis* in RCPB became impossible, because the colonies of *S. thermophilus and L. delbrueckii* ssp. *bulgaricus* dominated the whole plate, since their numbers were much higher than that of *B. lactis*.

When the supplemented MRS medium was used, *B. lactis* grew in adequate numbers, forming small brilliant blue colonies with diameters of approx. 0.5 mm. The area immediately around the colonies were more blue than the rest of the medium. *L. delbrueckii* ssp. *bulgaricus* failed to grow in any of the dilutions plated and *S. thermophilus* showed limited growth, in numbers much lower than expected for the 10^{-1} to 10^{-3} dilutions, forming small light blue colonies with diameters of approx. 0.2 mm. The blue color of the colonies was much lighter than *B. lactis,* and there was no surrounding blue area, as observed for *B. lactis.*

In the plates containing mixtures of the three microorganisms, the supplemented MRS was efficient as a selective medium for the counting of *B. lactis*, since *L. delbrueckii* ssp. *bulgaricus* did not grow, while *S. thermophilus* showed only limited growth. It was possible to count the viable cells of *B. lactis* even when this number was several log cycles lower than that of the other cultures present. In Lee's agar, *B. lactis* failed to grow under the conditions used, and *S. thermophilus and L. delbrueckii* ssp. *bulgaricus* could be distinguished from each other by a difference in the colour of the colonies, the former forming bright yellow colonies and the latter cream coloured colonies.

According to Dave and Shah (3), some media containing antibiotics inhibit the growth of *Bifidobacterium* spp., the count therefore not being representative of the true number of viable cells present in the product analysed. However, this problem was not observed in this work, when the supplemented MRS medium, which contained antibiotic, was used.

CONCLUSIONS

One of the requirements for microorganisms to be used as dietary adjuncts is the need to maintain viability and activity in the carrier food before consumption. In this study, free and immobilized *B. lactis* and *L. acidophilus* presented a good survival rate in milk and acidified milk. On the other hand, the survival of immobilized *B. lactis* in yoghurt was considered to be unsatisfactory, that is, immobilization in calcium alginate failed as an effective barrier to protect the cells.

Both RCPB and supplemented MRS were efficient media for counting *B. lactis* in yoghurt whilst the cultures were in equilibrium; when *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* prevailed, the differential medium (RCPB) did not allow for the counting of *B. lactis,* the selective medium (MRS) being more efficient.

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RESUMO

Estabilidade de *Lactobacillus acidophilus* e *Bifidobacterium lactis* nas formas livre e imobilizada em leite acidificado e de *B. lactis* imobilizado em iogurte

Este trabalho avaliou a estabilidade de *Bifidobacterium lactis* (Bb-12) e de *Lactobacillus acidophilus* (La-05) nas formas livre e imobilizada em alginato de cálcio, em leite e leite acidificado (pHs 5.0, 4.4 e 3.8), e a estabilidade de *B. lactis* imobilizado em iogurte (fermentado até pH 4.2), durante 28 dias de estocagem refrigerada. Também foi estudada a eficiência de dois meios de cultura (ágar MRS modificado e Reinforced Clostridial Agar, acrescido de Prussian Blue) para enumerar *B. lactis* em iogurte. Ágar Lee foi usado para enumeração de *Streptococcus thermo*philus e *Lactobacillus delbrueckii* ssp. *bulgaricus* quando *B. lactis* era enumerado no meio MRS. Ambos os microrganismos, nas formas livre e imobilizada, apresentaram uma taxa de sobrevivência adequada nos leites acidificados, uma vez que houve redução de apenas um ciclo log, após 21 dias de estocagem refrigerada. O número de células viáveis de *B. lactis* imobilizado mostrou um declínio gradual durante o período de armazenamento do iogurte, passando de 10^{8} ufc/ml até não ter mais contagem na diluição 10^{-1}. Quando as culturas não estavam em equilíbrio, o meio MRS modificado foi mais eficiente para a contagem de *B. lactis* em iogurte. Em vista destes resultados pode-se concluir que ambos os microrganismos podem ser incorporados em leite e leite

acidificados, haja visto que a redução na população foi pequena durante o período de armazenagem estudado. A presença da cultura tradicional de iogurte parece ter afetado negativamente a sobrevivência de *B. lactis* e a imobilização não proveu proteção às células, em nenhum dos tratamentos estudados.

Palavras-chave: probióticos, imobilização, alginato, sobrevivência, *Lactobacillus acidophilus*, *Bifidobacterium lactis*, yoghurt, milk, *Lactobacillus bulgaricus*

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