Effect of Peptides and Amino Acids Produced by *Lactobacillus casei* in Milk on the Acid Production of Bifidobacteria

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Abstract It was found that the growth of bifidobacteria was greatly stimulated in the mixed incubation with *Lactobacillus casei*. Hence the peptides and free amino acids isolated from the culture filtrate prepared from the incubated milk with *L. casei* were investigated in relation to the stimulation on the acid production of bifidobacteria. *Bifidobacterium breve*, *B. infantis*, and *Lactobacillus casei* were used as test organisms. The preparation of culture filtrate was performed by means of DAHIYA and SPECK, and the removal of free amino acids from culture filtrate by the method of MARKOVITZ and STEINBERG. The main results obtained were as follows: 1. Additions of the culture filtrate containing peptides and amino acids prepared from the incubated milk with *L. casei* and the free amino acids-removed culture filtrate gave appreciable stimulation for the acid production of bifidobacteria over those of control, and the degree of stimulation was maximum with the addition of untreated filtrate at 24 hr of incubation. But little difference was observed in the stimulation between two filtrates after 48 hr of incubation. 2. In the case of the addition of single amino acid or the omission in turn of single amino acid from a mixture of amino acids, the stimulation on the acid production of bifidobacteria was observed to be highest for cystine among 18 amino acids, followed by methionine, lysine, alanine, and histidine. 3. The stimulatory effect by cystine was greater in *B. infantis* than in *B. breve*. Isoleucine or tryptophan gave not only the reduction of acid production of *B. infantis* but also considerably reduced the stimulation by cystine with the increasing amount of isoleucine or tryptophan. 4. Active fractions, having stimulation on the acid production, obtained from gel filtration of the free amino acids-removed culture filtrate with Sephadex G-15 contained five peptides, ranging from 948 to 2,319 in molecular weights. Lys, Met, Ala, Leu and Val of the amino acids detected in the peptides were considered to stimulate the acid production of bifidobacteria.

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When bifidobacteria were incubated with *Lactobacillus casei* in milk, the growth and acid production of bifidobacteria were greatly stimulated¹⁾. Although there seemed to be many factors influencing to the growth and acid production of bifidobacteria, the low molecular weight nitrogenous compounds produced by *L. casei* in milk, particularly peptides and amino acids which from the viewpoint of nitrogen source were considered to be more important for the stimulation were isolated and characterized to gain more information about the roles played by the stimulants such as peptides and amino acids.

Materials and Methods

Microorganisms

The strains of *Lactobacillus casei* (MT-8105), *Bifidobacterium breve* (MT-17),

and *Bifidobacterium infantis* (MT-60) used in this study were kindly presented by the Central Research Laboratory of Morinaga Milk Industry Co., Tokyo.

Media and culture conditions

The stock media and pre-cultures of tested bacteria have been described previously¹⁾.

Determination of acidity and nitrogen

Stimulation effect on the acid production was evaluated by the determination of titratable acidity of incubated milk which was measured by a standard procedure with 0.1 N NaOH using phenolphthalein as an indicator²⁾. Nitrogen was determined by a standard micro-Kjeldahl procedure²⁾.

Measurement of stimulatory activities of culture filtrates, gel-fractions, and amino acids

One ml of the solution containing the test materials such as culture filtrates, gel-fractions, and amino acids was sterilized by passage through Millipore filter (Millipore Corp. Millex-Ha, 0.45 μ m Filter Unit) and then was added to 9 ml of milk (reconstituted non-fat milk containing 11% of solids-not-fat).

The milk inoculated with 1% of active bifidobacteria was incubated at 37°C under anaerobic conditions with the addition of sterilized liquid paraffin. After incubation, the acid production was determined by titration with 0.1 N NaOH.

Isolation of stimulants

1) Preparation of culture filtrate

The procedure of DAHIYA and SPECK³⁾ was used for the preparation of culture filtrate. After three successive subcultures, the cells of *L. casei* were centrifuged, washed, and suspended in the sterilized phosphate buffer (pH 6.8). The suspension was added (3%, v/v) into 500 ml of sterilized reconstituted non-fat milk (10% solids). After incubating at 37°C for 18 hr when milk reached to ca. pH 4.6, the cells and casein were removed by centrifugation. The supernatant was condensed to 100 ml, and 400 ml of a cold acetone-ethanol mixture (3 : 1) was added. The precipitate was allowed to develop in a refrigerator for 4 hr with occasional shaking, and then the supernatant was obtained through filter-paper. The filtrate was evaporated to dryness under vacuum with rotary evaporator, and its volume was made again to 100 ml after adjusting to pH 7.0. The filtrate was passed through an ion exchange resin (Amberlite IRC 120) in the H⁺ cycle. The column was washed with distilled water until the washings showed no lactose by the Molisch test. The material held on the column was eluted with 4% aqueous ammonia solution, and the ammonia solution of eluate was removed by drying under vacuum. The eluate was concentrated under vacuum to near dryness, and the volume was made to 20 ml by the addition of distilled water.

2) Removal of the free amino acids from the culture filtrate

The filtrate prepared by the method mentioned above was separated into two portions. One portion was treated with ninhydrin to remove free amino acids by the method of MARKOVITZ and STEINBERG⁴⁾, and the other portion was left intact. Namely,

Stimulation on Acid Production of Bifidobacteria

0.5 g of ninhydrin was added to the filtrate which was adjusted to pH 2.5 with citrate buffer, and the mixture was heated for 10 min in a boiling water bath. The reaction mixture was rapidly cooled, filtered, extracted with diethyl ether to remove unreacted ninhydrin, and freeze dried. In the case of measurement of the stimulation by culture filtrate, the freeze dried compounds were diluted with distilled water to the same volume of culture filtrate before the ninhydrin treatment.

3) Fractionation of the free amino acids-removed culture filtrate with Sephadex G-15

The free amino acids-removed culture filtrate was applied to a column (2×70 cm) of Sephadex G-15. The column was eluted with distilled water at a flow rate of 2 ml/min at room temperature (15°C). The positive biuret reaction of fractions were detected by the method of MATHESON and TATTRIE⁵⁾, using ninhydrin reagent.

4) Isolation of peptides by low-voltage electrophoresis and paper chromatography

The active fractions from 3) were mapped by means of electrophoresis chromatography. Low-voltage electrophoresis⁶⁾ was performed at 500 V (25 V/cm) for 45 min with chromatography paper (No. 51, Toyo Roshi, Tokyo) in an Electrophoretic apparatus (SJ-1060 A, Atto Co., Tokyo). Pyridine-acetic acid-water (20 : 0.8 : 180, pH 6.5) was used as an electrophoresis buffer. The solvent for ascending paper chromatography in the second dimension was propanol-water (7 : 3). Peptides were detected after spraying the dried papers with ninhydrin-cupric nitrate reagent⁷⁾ and heating for 15-20 min in an oven at 90°C.

5) Amino acid analysis

Determination of the amino acids in acid hydrolysates of the peptides was made with an amino acid analyzer (KLA-5, Hitachi, Tokyo) after the peptides were hydrolysed using the method of MOORE and STEIN⁸⁾.

Results and Discussion

1. The stimulation for the acid production of bifidobacteria by the culture-filtrates from *L. casei*

The content of the free amino acids in the culture filtrate obtained from the incubated milk with *L. casei* was found to be about 3 times more than that originally present in milk (Table 1). On the other hand, about 97% amino acids and 15% of peptides (data not shown) contained in the filtrate were lost by the treatment with ninhydrin.

When comparing the free amino acid composition of the culture filtrate with that of skim milk, it was interesting to note that the concentrations of glycine and in particular cystine in the culture filtrate were slightly lower than those of skim milk in spite of the copious quantities of low molecular weight nitrogenous compounds produced by *L. casei*. This finding seems to be of particular importance for the improvement of growth of bifidobacteria from the viewpoint of supplying cystine.

As shown in Table 1, this phenomena may be due to the fact that *L. casei*, like other bacteria, hydrolyses predominantly casein, which has the low level of cystine

Table 1. Free amino acid composition of skim milk before and after incubation with *L. casei* for 24 hr at 37°C

Amino acid (mg/100 ml)	<i>L. casei</i>		Skim milk (no inoculum)
	UTN	TN	
Lys	0.758	0.013	0.075
His	0.093	0.012	0.066
Arg	0.060	c	0.066
Asp	0.072	c	0.117
Thr	0.031	c	0.121
Ser	0.032	c	0.124
Glu	0.471	c	1.518
Pro	0.558	c	0.048
Gly	0.142	c	0.402
Ala	0.501	0.014	0.230
Cys	0.048	c	0.068
Val	0.830	0.024	0.154
Met	0.282	0.016	0.053
Ile	0.538	0.009	0.068
Leu	3.179	0.016	0.067
Tyr	0.512	0.090	0.077
Phe	1.034	0.090	0.053
Total	9.141	0.284	3.307

UTN: untreated filtrate with ninhydrin. TN: treated filtrate with ninhydrin. c: trace.

compared with that of whey protein, rather than whey protein when the milk was incubated with *L. casei*. In the case of evaluating milk as a cystine source, this is an essential point to which more attention be paid in mixed incubations with bifidobacteria and *L. casei*.

MILLS and THOMAS⁹⁾ reported that the free amino acids initially present in milk were an important nitrogen source for growth of *Streptococcus cremoris* at low cell densities, and peptide originally present in milk supplied an almost constant proportion of nitrogen for growth. With regard to the stimulation on the acid production by bifidobacteria, additions of both ninhydrin-treated (nitrogen content : 0.045 mg/ml) and untreated (nitrogen content: 0.060 mg/ml) filtrates to the milk inoculated with bifidobacteria gave appreciable stimulation for the acid production of bifidobacteria over those of control (water added), and the degree of stimulation was maximum with the addition of untreated filtrate in *B. breve* and *B. infantis* at 24 hr of incubation (Fig. 1). Accordingly, the finding that untreated filtrate showed higher stimulation for acid production by bifidobacteria as compared with that of treated filtrate is considered to be due to the difference in the contents of free amino acids between untreated and treated filtrates. Namely, the ratio of the content of amino acid in ninhydrin treated filtrate to that of untreated filtrate was only 3% as shown in Table

Stimulation on Acid Production of Bifidobacteria

Fig. 1. Effect of the culture filtrates produced by *L. casei* on the acid production of *B. breve* and *B. infantis*. —○—, untreated filtrate with ninhydrin; —△—, treated filtrate with ninhydrin; —●—, control.

1. But after 48 hr of incubation, little difference in the stimulation on the acid production by bifidobacteria was observed both in the milks added with the treated filtrate and with the untreated one. Therefore, it is presumed that the free amino acids were utilized during the early stage of incubation (24 hr), and then peptides became available during the prolonged incubation (48 hr).

2. The effect of the amino acids added on the acid production of bifidobacteria

In order to gain more knowledge about the stimulatory effect of amino acids on the acid production of *B. breve* and *B. infantis* in milk, single amino acid or a mixture of amino acids was added to milk. The amount of each amino acid added was 5 mg/100 ml and for cystine was 0.625 mg/100 ml. As shown in Table 2, addition of cystine gave the remarkable stimulation on the acid production of *B. breve* (approx. 68%) and *B. infantis* (109%) over that of control. On the contrary, omission test with cystine resulted in the decrease of acid production both in *B. breve* (38%) and in *B. infantis* (60%), respectively, as compared with that of control. Therefore, the effect of cystine added on the acid production of *B. infantis* seemed to be greater than that of *B. breve*.

The single and omission tests with methionine on the acid production of *B. breve* showed 40% of increase and 35% of decrease compared to control, respectively. But in *B. infantis*, 30% of increase and 20% of decrease were observed with methionine. Lysine or alanine for *B. breve* and histidine for *B. infantis* showed higher rate of acid production than control by over 10%. The other amino acids tested showed little changes on acid production of *B. breve* and *B. infantis* except isoleucine and tryptophan for *B. infantis*. Namely, addition of isoleucine and tryptophan decreased the acid production of *B. infantis* by 5% for isoleucine and 4% for tryptophan, respectively, compared with that of control. While the removal of isoleucine or tryptophan from a mixture of 18 amino acids resulted in an increase of acid production by 7% for isoleucine or tryptophan, respectively. In view of these results, isoleucine and tryptophan were considered to be the only two amino acids who showed the inhibitory

Table 2. Stimulation on acid production (%) of *B. breve* and *B. infantis* by addition of single amino acid or omission in turn of single amino acid from a mixture of amino acids

Amino acid	Single test		Amino acid	Omission test	
	<i>B. breve</i>	<i>B. infantis</i>		<i>B. breve</i>	<i>B. infantis</i>
Control	100(0.342%)	100(0.251%)	18 amino acids	100(0.963%)	100(0.793%)
+Lys	114	106	–Lys	80	75
+His	107	110	–His	90	65
+Arg	105	104	–Arg	98	70
+Asp	104	101	–Asp	98	90
+Thr	103	100	–Thr	98	88
+Ser	103	104	–Ser	99	77
+Glu	103	104	–Glu	98	100
+Pro	103	101	–Pro	99	91
+Gly	103	104	–Gly	99	95
+Ala	114	104	–Ala	88	86
+Cys	168	209	–Cys	62	40
+Val	106	104	–Val	98	96
+Met	140	103	–Met	65	79
+Ile	106	95	–Ile	86	107
+Leu	105	104	–Leu	90	70
+Tyr	100	104	–Tyr	99	89
+Phe	101	104	–Phe	98	100
+Try	101	96	–Try	98	107

Incubation was performed at 37°C for 24 hr. Each amino acid added was 5 mg/100 ml medium except cystine (0.625 mg/100 ml).

activity on the acid production of *B. infantis* when added separately to the milk.

Furthermore, in the case of *B. infantis*, it was of interest to note that the stimulation effect of added cystine on the acid production was reduced with the increasing amount of isoleucine or tryptophan added. For example, when the amount of isoleucine added was increased from 1.0 mg to 10.0 mg, stimulatory effect by cystine was reduced from 91% to 87% as shown in Table 3.

Table 3. Effect of addition of isoleucine or tryptophan on acid production of *B. infantis* in skim milk added with cystine

Amino acid added (mg/100 ml)	Titrateable acidity (%)	Rate (%)	Amino acid added (mg/100 ml)	Titrateable acidity (%)	Rate (%)
Control	0.529	100	Control	0.529	100
(Cys: 0.625 mg)			(Cys: 0.625 mg)		
Cys+Ile			Cys+Try		
1.0 mg	0.482	91	1.0 mg	0.512	97
2.5 mg	0.474	90	2.5 mg	0.502	95
5.0 mg	0.468	89	5.0 mg	0.500	94
10.0 mg	0.460	87	10.0 mg	0.486	92

Incubation was performed at 37°C for 24 hr.

Stimulation on Acid Production of Bifidobacteria

According to the results of the minimal nutritional requirements of *Lactobacillus bifidus* reported by HASSINEN *et al.*¹⁰⁾, it was found that cystine (cysteine) was the only amino acid required for the growth of the strains of *L. bifidus*, and also the requirement for cystine was specific as evidenced by the inactivity of the compounds of closely related chemical structure such as homocysteine and β -mercaptoethylamine. In the present study, it was recognized that cystine was the most effective among 18 amino acids tested for acid production of *B. breve* and *B. infantis* in milk, but the mechanism of cystine contribution for the growth of bifidobacteria has not yet been elucidated.

MADA¹¹⁾ reported that omission test with aspartic acid showed 50% reduction of acid production by bifidobacteria. On the contrary, the reduction rate was only 2% and 10% for *B. breve* and *B. infantis*, respectively, in the omission test with aspartic acid as indicated in Table 2. Thus, the suggestion made by MADA was not supported by the results obtained in the present investigation.

3. Purification of peptides and its stimulatory effect on acid production of bifidobacteria

1) Sephadex column chromatography

As Fig. 2 indicated, two peaks indicating positive reaction with ninhydrin were found in the fractions obtained from gel-filtration of the amino acids-removed culture filtrate on Sephadex G-15. Namely, one peak was from tube no. 16 to 22 and the other from tube no. 23 to 34. Moreover, the fractions from tube no. 17 to 20 (A) and from tube no. 27 to 30 (B) were collected as test material, respectively.

Fig. 2. Elution profile of the amino acids-removed culture filtrate on a Sephadex G-15 column (2×70 cm). Elution was performed with distilled water. —○—, absorbance at 280 nm. ---●---, absorbance at 570 nm.

Fig. 3 showed that stimulation for the acid production of *B. breve* and *B. infantis* was observed in the milk, to which the different peaks (A and B) were added separately, after 24 hr of incubation, and also the stimulation was higher in B than in A. Accordingly, it seemed to indicate that the fractions contained in A and B possessed growth stimulants for bifidobacteria such as peptides.

Fig. 3. Acid production of *B. breve* and *B. infantis* by the addition of fractions from chromatography on a Sephadex G-15 column. Fractions added contained 7.5 $\mu\text{g/ml}$ of nitrogen. — Δ —, fraction of tube no. 17-20. — $\circ$ —, fraction of tube no. 27-30. — $\bullet$ —, control.

2) Low-voltage electrophoresis and paper chromatography of peptides

Five distinct zones of peptide indicating positive biuret reaction were detected both in A and B, using low-voltage electrophoresis and paper chromatography, respectively. By means of electrophoresis, the four ninhydrin positive zones from 1 to 4, all migrating toward anode, were separated equally in A and B. The three of these four zones (II, III and IV) were not separated further into additional zones even after paper chromatography. On the other hand, zone 1 was separated further into zones I_1 and I_2 by means of paper chromatography, as R_f values in Table 4 indicated. However, as shown in Fig. 4, the main components were judged to be zone I_1 and zone I_2 in A, and zones: II, III and IV in B, mainly based on the quantitative viewpoint of the peptide. Therefore, zones I_1 , and I_2 , and zones II, III, and IV were obtained from A and B, respectively, in larger quantities by preparative low-voltage electrophoresis and paper chromatography for the further study concerning the amino acid composition. But the homogeneity of each of the peptides obtained was not ascertained due to the distinctive difference in R_f value of each peptide.

Table 4. Migration distances^a and R_f values^b of zones separated by low-voltage electrophoresis and paper chromatography

Zone	R_f	Migration distance (cm)
I_1	0.40	3.5
I_2	0.64	3.5
II	0.46	5.8
III	0.54	10.2
IV	0.50	12.5

^aLow-voltage electrophoresis, pH 6.5, 500 V (25 V/cm), 45 min. ^bSolvent, propanol-water (7:3).

Stimulation on Acid Production of Bifidobacteria

Fig. 4. Representation of a two-dimensional chromatogram of the peptides from Sephadex G-15 column (A: fractions of tube no. 17-20; B: fractions of tube no. 27-30); electrophoresis performed at pH 6.5 in pyridine-acetic acid-water (20:0.8:180), 500 V (25 V/cm), 45 min followed by chromatography in propanol-water (7:3). ○: trace.

3) Amino acid composition of peptides and its effect on the acid production

The amino acid composition of each of the five peptides was presented in Table 5. Each of the peptides differed not only in amino acid composition but also the ratio of the acids in the peptides varied. When the five peptides were hydrolysed into

Table 5. Amino acid composition of peptides I₁, I₂, II, III, and IV^a from culture filtrate produced by *L. casei*

Amino acid	Peptides				
	I ₁	I ₂	II	III	IV
Lys	1.0 ^b	c	2.4	1.2	c
His	c	c	1.0 ^b	c	c
Arg	1.0	c	1.1	c	c
Asp	1.1	1.3	1.2	1.4	1.9
Thr	1.2	c	1.2	1.0 ^b	1.0 ^b
Ser	1.2	1.3	2.4	1.0	1.2
Glu	2.0	3.3	2.1	2.3	2.0
Pro	1.0	c	1.3	c	c
Gly	1.5	1.3	2.2	2.8	2.2
Ala	1.3	1.3	2.3	1.3	1.9
Cys 1/2	c	c	c	c	c
Val	1.3	1.0 ^b	1.3	1.0	1.1
Met	1.2	c	c	c	c
Ile	1.2	c	1.1	c	c
Leu	1.2	1.1	2.4	1.1	1.0
Tyr	1.1	c	1.2	c	c
Phe	1.2	c	1.1	c	c
Approx. mol. wet.	1,913	948	2,319	1,162	1,163

^aPeptides were acid hydrolyzed 24 hr at 110°C. ^bCalculated by dividing the micromolar values by the micromolar value of the amino acid present in least abundance. ^cIndicates a negligible quantity.

their constitutive amino acids, Lys, Met and Ala of the amino acids detected in them were considered to be most effective for the stimulation on the acid production of bifidobacteria, as shown in Table 2. Lys was contained in peptides I₁, II and III, while Met in peptide I₁ only. Seven amino acids found common in all peptides were as follows: Asp, Ser, Glu, Gly, Ala, Val, and Leu. Table 2 also indicated that Ala of these seven amino acids showed the considerable stimulation on the acid production of bifidobacteria, followed by Leu and Val when added separately to milk. On the other hand, considerable reduction of acid production of *B. infantis* were observed when Ser or Asp was removed in omission test.

Therefore, it may be reasonable to conclude that some of the amino acids (Lys, Met, Ala, Leu, and Val) mentioned above contribute to stimulate the acid production of bifidobacteria with the smaller peptides produced by peptidases of bifidobacteria from the added peptides. Molecular weight of each peptide was presumed to be about 1,913 (I₁), 948 (I₂), 2,319 (II), 1,162 (III), and 1,163 (IV), respectively, by the amino acid composition.

It has long been recognized that peptides are of great nutritional value for many types of bacteria, and bacteria usually hydrolyse peptides and use the amino acids either directly for protein synthesis, or after further breakdown, as sources of nitrogen, carbon, or sulphur. On the other hand, there are many factors affecting the peptides transportation into bacterial cell. In terms of size of peptides, in general, peptides with more than 5 or 6 amino acid residues are not taken up by bacteria. Namely, as MILLS and THOMAS⁹⁾ reported that the upper size limit for peptide transport through lactic streptococcal membranes was considered to be five or six residues, it may be impossible for the five peptides detected in the filtrate to be utilized directly by bifidobacteria as the nitrogen source when considering their molecular weights. However, it may be presumed that some of the smaller peptides derived from the decomposition of the peptides may be taken up directly into the bifidobacteria and utilized for their growth and acid production, although the data is lacking in this regard.

Accordingly, extracellular and intracellular peptidases of bifidobacteria may be considered to play an important role in the utilization of the peptides for their growth in the mixed incubation with *L. casei*.

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Stimulation on Acid Production of Bifidobacteria

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Lactobacillus casei の生産するペプチドおよび アミノ酸がビフィズス菌の生酸性に及ぼす影響

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ビフィズス菌を *L. casei* と混合培養した時, ビフィズス菌の発育性が著しく改善された。従ってこの原因を究明するために窒素源供給の観点から, *L. casei* により牛乳中で形成される低分子窒素化合物特にペプチドおよび遊離アミノ酸を分離し, それらがビフィズス菌の生酸性への効果について検討した。使用した菌種は *Bifidobacterium breve*, *B. infantis* および *L. casei* であった。培養液の調製は DAHIYE ら, 遊離アミノ酸の除去は MARKOVITZ らの方法によった。主要な結果は次の通りである。

1. *L. casei* の 24 時間培養の培養液およびその液から遊離アミノ酸を除去したものをそれぞれ牛乳に添加し, ビフィズス菌の生酸性への影響について検討した結果, 両培養液とも対照に比して生酸性促進の効果が認められ, その促進効果は前者の培養液の方がより大であった。

2. 牛乳に各種アミノ酸の単独または混合添加, さらに除去試験の結果, ビフィズス菌の生酸性を著しく促進したアミノ酸は, cystine で, その効果は *B. breve* より *B. infantis* の場合に大であった。Isoleucine および tryptophan は *B. infantis* の酸生産を阻害したが, cystine と isoleucine あるいは tryptophan との混合添加では, isoleucine または tryptophan の添加量の増加によって, cystine の促進効果は著しく阻害された。

3. *L. casei* による培養液を Sephadex G-15 でゲル濾過し, 生酸性促進効果を示す画分から, 濾紙電気泳動により 5 種のペプチドを単離したがそれらの分子量の範囲は 948 から 2,319 であった。なおそれらのペプチド中に見出された Lys, Met, Ala, Leu と Val がビフィズス菌の生酸性を促進するものと推察された。

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