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PROOF RESEARCH PAPER

Isolation of Probiotic Bacteria from Guppy *Poeciliareticulata* (Cyprinodontiformes:Poecilidae)

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Abstract

The screening possibility of some *Lactobacillus* sp. bacteria with potential probiotic properties from digestive tract of guppywas investigated in this study.To this end, forty-nine bacterial isolates derived from the digestive tract of the guppy were screened based on the morphological and microscopic characteristics like Catalase test, Gram staining and Spore staining. Then, other examinations for selecting the bacteria with the best potential probiotic properties were done, including: functional antagonist assay, cell surface hydrophobicity, and resistance to acid and bile salts. Additionally,16SrRNA fragment of the isolates with probiotic characteristics depicted 100% sequence identity with *Weissellacibaria*. According to our results, *W. cibaria*has the potential probiotic characteristics to apply in the culture of guppy.

Keywords: Lactobacillus, Probiotic properties, *Weissellacibaria*.

Introduction

Ahopeful preventive practice for preserving animal welfare and a healthy environment is utilization of immunostimulants that can increase production and provide higher profits(Bahi et al., 2017).Nowadays, using eco-friendly disease preventives like probiotics is anaccepted strategy which can increase the production and remove bottlenecks in aquaculture (Dawood et al., 2016). Favorable effects of probiotics on the health of host has been confirmed in different studies. Today, many researchers believe that probiotics are also becoming an essentialsection of the aquaculture practices that can result high production (Nayak, 2010).The important issue for selection of microorganisms as probioticsis that they should be compatible with the host organism. Therefore, ideally the probioticsshould be isolated from the host organism and the region where host organism lives. Many commercially available probiotics are less suitable because they were originated from other regions or countries (Verschuere,Rombaut, Sorgeloos, &Verstraete, 2000).

To screening the probiotic strains, several properties should be investigated, including:inhibition against different pathogens, resistance to acid and bile salts and growth in feces. They should be resistant toprocessing and storage conditions, be safe and also impart benefits(Fuller, 1989; Havenaar& Huis in't Veld, 1992). Routinely, the probiotics LAB strains are Gram-positive, Catalase-negative and non-pigmented. Also, they don't have ability of motion and they don't form spore(Hassan & Frank, 2001), and studies showed that lactic acid producing bacteria such as lactobacilli, streptococci, bifidobacteria, bacillus spp., and fungi like *Sacharomyces cerevisiae*, *Sacharomycesboulaardii* and *Aspergillus oryzae* are the organisms that are used more than others in probiotic preparations (Fuller, 1992). Typically, LAB have attained greatest attention for applying as probiotic, and they have been regarded as favorable probiotics (O'Sullivan, Thornton, O'Sullivan, & Collins, 1992), and there are many studies conducted to investigate the effects of these probiotics on growth performance and other parameters, for example, Pourgholam,Khara, Safari, YazdaniSadati, andAramli (2017) showed that supplementation of *Lactobacillus plantarum* can improve the growth and hematological parameters in Siberian sturgeon (*Acipenserbaerii*).

In this study,isolated bacteria from digestive tract of guppy*Poeciliareticulata* (Peters, 1859), a popular ornamental fish in Iran and other countries, will be investigated to determine its probiotic potential. The isolated bacteria were screened by several morphological and biochemical selections and finally their identity was evaluated using 16SrRNA analysis. Yielded probiotic strains can be applied as an additivein feeding of guppyto improve the

productivity in culture condition.

Methods

Isolation of Presumptive LAB

For isolation of lactic acid bacteria, 20 healthy guppies (with an initial weight of 0.487±0.009 g) were collected from an ornamental fish hatchery (Gorgan, Iran). The fishes were reared in aquariums which weren't previously treated with any chemicals or antibiotics. After 12 h of starvation the animals anaesthetized with 0.01% benzocaine, and each fish was disinfected with alcohol (70%). Thenthe whole digestive tract was extracted, shattered with a scalpeland transferredin a 50 mL conical centrifuge tube containing de Man, Rogosa and Sharpe (MRS) Broth (Merck, Germany), and incubated at 26^0C for72 h. After 72 h, 1 mL of sample was serially diluted. The serial diluted sample was plated over sterilized MRS Agar (Merck, Germany) plates and incubated at 30^0C for 48 h. After incubation, the isolated LAB colonies were identified and subsequently isolated again in MRS Agar medium through cross streaking. Incubation was done as mentioned before. Colonies were again spread on MRS Agar plates for 4 times to ensure purity (Lin, Hwang, Chen, &Tsen, 2006).

The identification of isolated LAB candidates were conducted based on morphological and microscopic characteristics like Catalase test, Gram staining and Spore staining (Johnson & Christine, 1995). The bacteria that were negative in Catalase test and Spore staining and positive in Gram staining were selected as potential probiotic LAB.Then,antagonist assay, cell surface hydrophobicity test and the test of resistance to acid and bile salts were done to determine the bacteria with potential probiotic properties among identified LAB.

Selection of LAB with Potential Probiotic Properties

In vitro Functional Antagonist Assay

The antagonism towards bacterial pathogens was evaluated according to Ramos, Thorsen, Schwan, and Jespersen(2013).*Escherichia coli*(ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853) were applied as pathogens in this assay (pathogens were prepared from Persian Type Culture Collection, Iranian Research Organization for Science and Technology). The pathogen strains were reactivated, cultured and maintained in Tryptic Soy Agar (TSA, Biolife, Italiana) for 24 h at 37^0C. Then the incubation of the pathogen strains and LABs at 37^0C for 24 hwere done in Nutrient Broth (Merck, Germany) and MRS Broth, respectively. For antagonist assay the plates were filled with TSA with 2% (v/v) of the Broth medium containing pathogen (melted and then cooled down to temperature between 40 and 45^0C), making 5 mm diameter holes in the plate with a sterile holemaker. Then, centrifugation of overnight cultures of all selected LAB isolates were done at 10,000 g, at 4^0C in 5 min. After filter-sterilization of the supernatant of each sample (0.22µm, Millipore) and pH adjustment to 7.0,30 µl of each one were added to the wells (performed in triplicate). Finally, the diameters of the halos of inhibition were measured after the incubation at 37^0C during 24 h, and were recorded in mm.The LAB isolates that formed greater halo diameter were selected fornext assay.

Investigation of Cell Surface Hydrophobicity

Investigation of bacterial cell surface hydrophobicity was done by measuring microbial adhesion to hydrocarbons (MATH) (Kotzamanidis, Kourelis, Litopoulou-Tzanetaki, Tzanetakis, &Yiangou, 2010). After washing the bacterial cells in PBS twice, resuspension in PBS was done to get an optical density between 0.6-0.7 at 600 nm (A_0) which was contained about 10^8 CFU/mL of bacteria. One milliliter of *n*-hexadecane was added to 3 mL of the washed cell suspension. After a 10 min preincubation at 23^0C, the two-phase system was vortexed for 2 min and then left to stand for 20 min at 23^0C. After removing the aqueous phase, measurement of absorbance at 600 nm (A_1) was done. Finally, the formula: H% = (1- A1/A0) × 100 was applied to calculate the percentage of cell surface hydrophobicity.The bacteria with greater H% were selected for next assays.

Resistance to Acid and Bile Salts

The resistance of isolates to bile salts and in a low pH environment was tested according to the method applied previously by Tulumoglu, Kaya, andSimsek(2014) with some modification. One mL of sample was taken from the 18 h of overnight culture and harvested by centrifugation (10,000 g, 5 min, 4^0C). Resuspension of cells in PBS solutions with pH 2.5separately was performed after washing them with PBS at pH 7.2 three times. Assessment of resistance was performed by means of reflecting the time spent by food in stomachwith viable colony counts enumerated after incubation at 37^0C for 0, 1, and 2 h. Finally, the calculation of survival rate as log 10 values of colony-forming units per mLwas done (CFU mL^{-1}).

Tolerance of isolates to bile salts was assessed by MRS Broth enriched with oxgall (0.3%, w/v) (Merck, Germany). After incubation for 0, 2, 4, and 6 h, the viable colonies were countedin appropriate dilutions using the pour plate method.Finally, calculation of the survival rate was done as described before.

Finally, the isolates with probiotic characteristics were assayed by molecular identification.

Molecular Identification

DNA Extraction and PCR Amplification

Bacterial genomic DNA was extracted following the instructions of the Yektatajhiz Nucleic Acid Purification kit (Cat No: YT9030, Tehran, Iran) and dissolved in TE buffer (1 mM EDTA, 10 mMTris-HCl, pH 8).The quality of DNA was analyzed by 1.2% TAE agarose gel electrophoresis. The genomic DNA was applied in the PCR reaction as a templateto amplify the 16S rRNAtarget regions.Twouniversal primers,27F (27F 5′- AGAGTTTGATCCTGGCTCA -3′) and1492R (5′- GGTTACCTTGTTACGACTT - 3′) wereusedtoamplifythe16SrRNA fragment. PCR amplification was performed using a Mastercycler® Eppendorf gradient thermal cycler (Brinkmann Instruments, Inc.) (Pfanebecker&Fröhlich, 2008).PCR programincluded a primary denaturation at 95°C for 5 min, 33 cycles of 95°C for 30 s, 59°C for 45 s, 72°C for 1 min and final extension at 72°C for10 min. Amplification of the nearly1409 bp intended fragment wasverified by electrophoresis on 2% 1.2% TAE agarose gel. After visualization by ethidium bromide (10 mg/ml) the most intense products were sent to Bioneer company, Korea for sequencing.

Sequence Analysis

The Chromasprogram version 2.6.4 was used to edit and evaluate the sequencing results. Sequence identity was assayed with BLAST program (basic local alignment search tool), and also sequences were deposited to the GenBank16S rRNA database with accession numbers MF445196.1 and MF445195.1.MEGAprogram (version 05) was applied to analyze multiple sequence alignments and molecular phylogeny (Tamura, 2016) with the method of Neighbor-Joining (Saitou &Nei, 1987). The method of Maximum Composite Likelihood was also used to compute the evolutionary distances (with 1,000 bootstrap replicates)(Tamura, 2016). The analysis involved 19 nucleotide sequences. All siteswith gaps and missing data were excluded. The final datasetincludes a total of 1274 sites.

Results

Isolation of presumptive LAB

49 bacterial isolates were collected from the digestive tract of guppies using serial-dilution plating method in MRS Agar. All presumptive LAB isolates were positive in Gram staining, 3 were cocci, 17 were bacilli, and 29 were bacilli cocci. Also, all of these isolates were negative in Catalase test and Spore staining (Table 1).So, all of isolates were selected for second test.

Probiotic Characteristics Assay of LAB

***In Vitro* Functional Antagonist Assay**

The effect of all isolates on *E. coli* were similar, but 6 bacterial isolates (L29, L30, L37, L46, L48, and L49) producing maximum inhibition zones against indicator strain*Pseudomonasaeruginosa*were selected for further identification and probiotic characterization. (Table 2).

Cell Surface Hydrophobicity Test

Cell surface hydrophobicity (H) of L29, L30, L37, L46, L48, and L49 isolates were ranged between 1.4 and 38.3. Maximum H was obtained from L29 and L30 isolates. (Table 3)

Resistance to Acid and Bile Salts

The exposure of 2.5 pH for 2 h did not effect on the viability of L29 and L30 isolates considerably.Also, both of isolates could survive at 0.3% oxgall and could maintain their viability. The relative survival of L29 and L30 after 6 h were 7.00 and 6.30 log CFU mL^{-1}, respectively. (Table 4)

Molecular Identification

16SrRNA region of the L29 and L30 isolates was amplifiedsuccessfully. Sequence similarity search against the GeneBank database indicated that both isolates depicted 100% similarity with *Weissellacibaria*.The optimal tree based on 1400bp fragment of the 16SrRNAwith the sum of branch length = 0.62518751 is shown in the Figure 1. The generated phylogenetictree grouped the both isolates in one cluster with *Weissellacibaria*. According to the distance matrix, the maximum and minimum evolutionary distance was found between our isolates and *Oenococcusoeni and Weissellacibaria* respectively (Figure 2).

Discussion

Recently, aquaculture has accompanied by considerable advances in production of different aquatic species like ornamental fishes (Kesacordi-Watson, Kaspar, Lategan, & Gibson,2008). Guppy as a native fish to South America (north eastern) is a famous ornamental fish in the world, even though the production of this fish has increased recentlybecause of high demand and pressure on wild resources (Karayücel, Orhan, &Karayücel,2008). In Iran, guppy is a famous fish for export, so improvement of its growth and immune system is very important.It was shown that probiotics canimprovethe growth paramrtersand immune system of different species in aquaculture. Therefore,we aimed to use some isolated probiotics (*Lactobacillus*sp) from digestive tract of guppyto improve its culture condition. Many studies reported the isolation of bacterial strains with

Table 1 Catalase test, Gram staining and Spore staining results of lactic acid bacteria isolated from the gut of guppy

Strains	Morphology	Catalase	Gram	Spore	Strains	Morphology	Catalase	Gram	Spore
L1	bacillus coccus	-	+	-	L26	bacillus coccus	-	+	-
L2	bacillus	-	+	-	L27	bacillus	-	+	-
L3	coccus	-	+	-	L28	coccus	-	+	-
L4	bacillus coccus	-	+	-	L29	bacillus coccus	-	+	-
L5	bacillus	-	+	-	L30	bacillus coccus	-	+	-
L6	bacillus	-	+	-	L31	bacillus	-	+	-
L7	bacillus coccus	-	+	-	L32	bacillus	-	+	-
L8	bacillus	-	+	-	L33	bacillus coccus	-	+	-
L9	bacillus	-	+	-	L34	bacillus coccus	-	+	-
L10	bacillus	-	+	-	L35	bacillus coccus	-	+	-
L11	bacillus coccus	-	+	-	L36	bacillus coccus	-	+	-
L12	bacillus coccus	-	+	-	L37	coccus	-	+	-
L13	bacillus	-	+	-	L38	bacillus	-	+	-
L14	bacillus	-	+	-	L39	bacillus coccus	-	+	-
L15	bacillus	-	+	-	L40	bacillus coccus	-	+	-
L16	bacillus coccus	-	+	-	L41	bacillus coccus	-	+	-
L17	bacillus	-	+	-	L42	bacillus coccus	-	+	-
L18	bacillus coccus	-	+	-	L43	bacillus coccus	-	+	-
L19	bacillus	-	+	-	L44	bacillus coccus	-	+	-
L20	bacillus coccus	-	+	-	L45	bacillus coccus	-	+	-
L21	bacillus coccus	-	+	-	L46	bacillus coccus	-	+	-
L22	bacillus coccus	-	+	-	L47	bacillus	-	+	-
L23	bacillus coccus	-	+	-	L48	bacillus coccus	-	+	-
L24	bacillus	-	+	-	L49	bacillus coccus	-	+	-
L25	bacillus coccus	-	+	-					

Table 2. Antagonist activity of LAB isolated from the intestine of guppy against *Pseudomonas aeruginosa* (ATCC 27853) (Determined by the average diameter of the inhibition zone formed around the wells)

Strains	Halo diameter of neuter supernatant (mm)	Strains	Halo diameter of neuter supernatant (mm)	Strains	Halo diameter of neuter supernatant (mm)
L1	-a	L18	-	L35	15.17
L2	-	L19	-	L36	15.56
L3	-	L20	-	L37	16.28
L4	-	L21	-	L38	14.38
L5	-	L22	-	L39	16.10
L6	-	L23	-	L40	17.79
L7	-	L24	-	L41	15.34
L8	-	L25	16.58	L42	17.73
L9	-	L26	-	L43	16.13
L10	-	L27	-	L44	14.97
L11	-	L28	-	L45	14.76
L12	-	L29	19.69	L46	16.11
L13	-	L30	19.88	L47	15.77
L14	-	L31	16.36	L48	16.56
L15	-	L32	-	L49	17.45
L16	-	L33	15.76		
L17	-	L34	14.23		

[a] No inhibition zone formed around these wells

Table 3. Results of cell surface hydrophobicity test

	L29	L30	L37	L46	L48	L49
H%	32.8	38.3	0.7	1.4	6.3	3.2

Table 4. The viability (log $CFUmL^{-1}$) of isolates within GI conditions

Factors	L29	L30
Tolerance to acid after 2 h	8.17	7.30
Tolerance to bile salts after 6 h	7.00	6.30

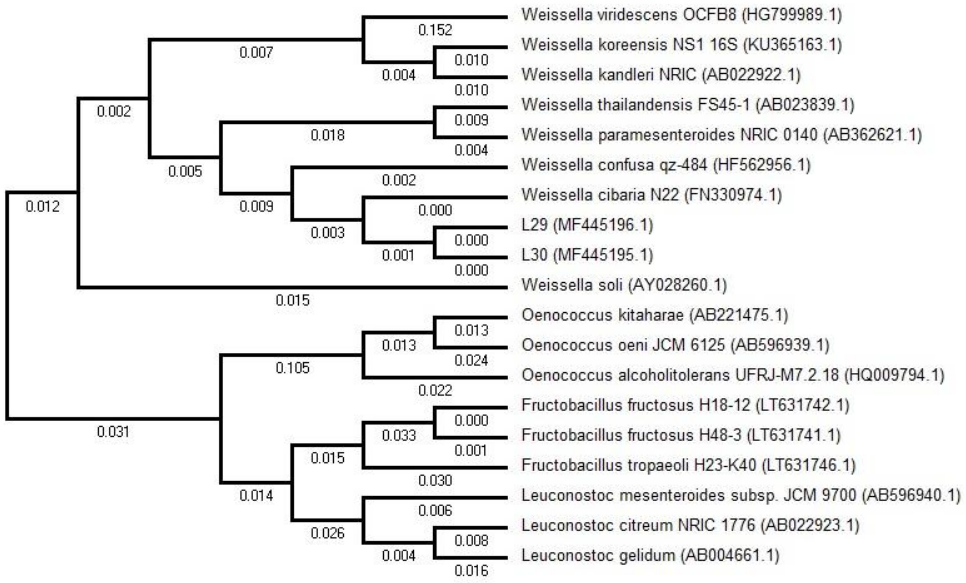

Figure 1. Molecular phylogenetic tree of L29, L30 isolates and other microorganisms presented in the GeneBank database constructed with 1409bp of 16S rRNA fragment using neighbor-joining method. *Oenococcus spp, Fructobacillus spp and Leuconostoc spp* are used as outgroup.

	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	*F. tropaeoli*	0.000																		
2	*F. fructosus* H18-12	0.064																		
3	*F. fructosus* H48-3	0.063	0.001																	
4	*L. mesenteroides*	0.068	0.088	0.087																
5	*L. citreum*	0.086	0.091	0.091	0.021															
6	*L. gelidum*	0.083	0.096	0.096	0.021	0.024														
7	*O. kitaharae*	0.209	0.208	0.209	0.180	0.173	0.178													
8	*O. oeni*	0.214	0.212	0.213	0.185	0.179	0.190	0.038												
9	*O. alcoholitolerans*	0.192	0.194	0.195	0.167	0.163	0.168	0.051	0.057											
10	*W. koreensis*	0.135	0.129	0.130	0.121	0.121	0.133	0.191	0.200	0.181										
11	*W. kandleri*	0.132	0.127	0.128	0.119	0.119	0.132	0.193	0.206	0.180	0.019									
12	*W. soli*	0.121	0.116	0.117	0.106	0.106	0.118	0.189	0.209	0.181	0.033	0.031								
13	*W. confusa*	0.121	0.128	0.129	0.109	0.115	0.126	0.185	0.205	0.200	0.043	0.041	0.035							
14	*W. cibaria*	0.118	0.124	0.126	0.108	0.112	0.123	0.192	0.210	0.196	0.041	0.039	0.033	0.005						
15	*W. thailandensis*	0.131	0.135	0.134	0.118	0.126	0.133	0.207	0.216	0.210	0.058	0.059	0.052	0.036	0.041					
16	*W. paramesenteroides*	0.127	0.129	0.128	0.109	0.116	0.124	0.207	0.213	0.206	0.052	0.054	0.046	0.031	0.036	0.014				
17	*W. viridescens*	0.257	0.257	0.259	0.256	0.270	0.282	0.324	0.333	0.336	0.163	0.170	0.187	0.175	0.174	0.187	0.188			
18	L29 (MF445196.1)	0.119	0.126	0.127	0.109	0.113	0.124	0.193	0.209	0.195	0.040	0.038	0.034	0.006	0.001	0.042	0.035	0.175		
19	L30 (MF445195.1)	0.119	0.126	0.127	0.109	0.113	0.124	0.193	0.209	0.195	0.040	0.038	0.034	0.006	0.001	0.042	0.035	0.175	0.000	0.000

Figure 2. Estimates of pairwise genetic distances between the specimens under the maximum composite likelihood model.

probiotic potential from different sources. For example, Mouriño et al.(2016) isolated 41 bacterial strains from the hybrid South American catfish (*Pseudoplatystomareticulatum* × *Pseudoplatystomacorruscans*) and in another study by Cota-Gastélum et al. (2013) 37 bacterial ctrains were isolated from *Oreochromisniloticus*. The first step of our study to select the isolates with probiotic potential was *in vitro* functional antagonist assay. In thisassay, six bacterial isolates demonstrated inhibition zones in the range of approximately 14 to 20 mm against the indicator strain.Similarly,Balakrishna (2014) evaluated an*in vitro* antagonism of isolated bacteria from guppy against pathogen bacteria, and according to the results, the inhibitory activities of 4 strains of the 46 isolates were moderate to strong.Also,in the study by Mouriñoet al. (2016) on the hybrid South American catfish, 10 of 41 LAB isolates demonstrated inhibition zones of more than 10 mm.

The second step to select bacteria with probiotic potential was investigation of hydrophobicity. As showed in the results, only two isolates, L29 and L30,

were highly hydrophobic (hydrophobicity value of 32.8 and 38.3 %) while the other isolates were non-hydrophobic. High hydrophobicity is an important feature as it indicates that the isolates can adhere to the guppy's intestine.One of the important characteristics of bacterial strains which are used as probiotics is the ability of adhesion to epithelial cells and mucosal surface because colonization of pathogens will be reduced or prevented (Vine et al., 2004). Also, the process of adhesion is complex, and involves contact between the bacterial cell membrane and interacting surfaces. Several studies have been conducted to investigatethe composition, structure and forces of interaction related to bacterial adhesion to intestinal epithelial cells and mucus (Del, Busetto, Vignola, Sgorbati, &Palenzona,1998; Pérez, Minnaard, Disalvo, & De Antoni,1998). The mechanism of adhesion is not studied here, and remains unclear.

The third important criterion to select bacteria with probiotic potential is resistance to acid and bile salts because theyshouldtolerate bile acids (Joborn, Olsson, Westerdahl, Conway, &Kjelleberg,1997), and to resist to the digestion process in digestive tract (Strompfova, Laukova, &Ouwehand, 2004). Probiotic bacteria must remain alive after the passage through the stomach where gastric acid is secreted represent as a primary defense mechanism (Chou & Weimer, 1999). According to our*in vitro*study, the L29 and L30 isolatesdisplayed a resistance to acidic conditions and contact with bile salts.The results show that both L29 and L30 are highly resistant to the low pH of the stomach and bile salts.

Molecular Identification of the two LAB isolates (L29 and L30) revealed 100% identity with*W. cibaria*which has been reported from Persian sturgeon (*Acipenserpersicus*) and the hybrid South American catfish (*Pseudoplatystomareticulatum* × *Pseudoplatystomacorruscans*) (Soltani et al., 2013; Mouriño et al, 2016).This species was earlierconsideredas a species of Lactobacillussp.,and has been isolated from humans and fermented foods. This Gram-positive facultative anaerobic LAB is used as a potential probiotic agent. This LAB secretes large amounts of hydrogen peroxide (Björkroth et al., 2002; Kang, Kim, Chung, Lee, & Oh,2006), and also it can secrete a bacteriocin which acts against Gram-positive bacteria (Srionnual, Yanagida, Lin, Hsiao, & Chen,2007).

This investigation has demonstrated that *W. cibaria* has the potential as probiotic for guppy, so this LAB can be introduced as a new probiotic in the culture of guppy.However, the effect of isolated probiotic on growth should be studied in the future *in vivo*investigations. The main benefit of application of these probiotic strains would be limiting the appearance of bacteria which cause different diseases in freshwater aquaculture systems asprobiotic therapy can effectively manage many problems in aquaculture that are related to disease outbreaks, nutritional status and water quality. (Balakrishna, 2014).

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