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## Potential Evaluation and Health Fostering Intrinsic Traits of Novel Probiotic Strain *Enterococcus durans* F3 Isolated from the Gut of Fresh Water Fish *Catla catla*

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**Abstract** Over the last few years, marine environment was found to be a source of surplus natural products and microorganisms with new bioactive secondary metabolites of interest which can divulge nutritional and biological impact on the host. This study aims to assess the possible, inherent and functional probiotic properties of a novel probiotic strain *Enterococcus durans* F3 (*E. durans* F3) isolated from the gut of fresh water fish *Catla catla*. Parameters for evaluating and describing the probiotics described in FAD/WHO guidelines were followed. *E. durans* F3 demonstrated affirmative results including simulated bile, acid and gastric juice tolerance with exhibited significant bactericidal effect against pathogens *Staphylococcus aureus*, *Salmonella* Typhi, *Escherichia coli* and *Pseudomonas aeruginosa*. This can be due to the enterocin produced by *E. durans* F3 strain, which was resolute by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gel with amplification of the anticipated fragment of a structural gene; enterocin A, followed by antibiotic susceptibility assessment. Effective antioxidant potentiality against  $\alpha$ -diphenyl- $\alpha$ -picrylhydrazyl free radicals including lipase, bile salt hydrolase activity with auto-aggregation and cell surface hydrophobicity was similarly observed. Results are proving the potentiality of *E. durans* F3, which can also be used as probiotic starter culture in dairy industries for manufacturing new products that imparts health benefits to the host. Finding the potent and novel probiotic strains will also satisfy the current developing market demand for probiotics.

**Keywords** *Enterococcus*, *Catla catla*, probiotics, enterocin, intestinal microbiota

## Introduction

From last several years, probiotics have been continuously used for health benefits

for human and animals due to their beneficial effects like; tolerance to bile and acid, ability to continuously persist in gastrointestinal tract (GIT), reduction of cholesterol level, improvement of intestinal microflora, immune response stimulation (Gill et al., 2001), production of bacteriocins used as an alternatives of antibiotics (Franz et al., 2011), tumoricidal activity of natural killer cells (Matsumoto et al., 2005), capability to produce and improve the bioavailability of nutrients (Monteagudo et al., 2012; Turgis et al., 2013). Enormous amount of research in exploration and investigation is going on, to develop different and innovative nutritional supplementation approaches. In this regard, numerous health stimulating compounds such as probiotics, synbiotics prebiotics, phytobiotics and other functional food supplements have been studied (Adnan et al., 2017a). Varieties of microbial strains are currently used as probiotics in to the market. First and most commonly used are *Lactobacillus* and *Bifidobacterium*, which are indigenous to human GIT (Mombelli and Gismondo, 2000). Other newly lactic acid bacteria (LAB) includes; *Leuconostoc*, *Pedococcus*, *Lactococcus*, *Propionibacterium*, *Streptococcus* and *Enterococcus* (Krasaekoopt et al., 2003; Power et al., 2008; Vandenplas et al., 2007; Vinderola and Reinheimer, 2003). However, it is also robustly believed that neither all probiotic do their activities with the same magnitude nor all probiotics act in the same approach. Therefore, there is an imperative need to search for newer, effective with broad range of health benefits.

Recently, marine sources have established their place in providing abundant resources for human nutrition and health. They also hold a countless varieties of living organisms having plethora of bioactive compounds with different bioactivities, when compared to terrestrial ecosystem (Adnan et al., 2018a; Hill and Fenical, 2010). Various probiotic LAB strains have been also isolated from the GI tract of different fishes (Buntin et al., 2008; Diaz et al., 2013; Ringo and Olsen, 1999), shrimps (Maeda et al., 2014), sponges (Asagabaldan et al., 2017), molluscans (Romalde and Bajra, 2010), and from other organisms (Adnan and Joshi, 2013). Many researchers during the last decade scrutinized diligently the microbial ecology of the GI tract of marine organisms (especially of fishes) (Al-Harbi and Uddin, 2004; Al-Harbi and Uddin, 2005; Hovda et al., 2007; Ringo et al., 2006a; Ringo et al., 2006b; Spanggaard et al., 2000; Yang et al., 2007; Zhou et al., 2009).

However, finding of novel strains with probiotic characteristics, which are superior to those currently in the market will satisfy the demands. Therefore, the purpose of this exploration is to isolate/validate a novel and potent probiotic strain, from the gut of freshwater fish *Catla catla* with a new approach and done for the first time.

## Materials and Methods

### Ethics statement

*Catla catla* fish is commonly and freely available in market all over the world for human consumption. They were purchased from the market just for the isolation of probiotic strain from its gut. In any circumstances, live *Catla catla* fish was not used/harmed for any other experiments throughout this research.

### Isolation and screening of bacterial cultures

*Catla catla* fish gut samples were enriched in MRS broth. Spread plate technique was performed onto MRS agar (Hi-Media®, India) plate with a diluted solution and incubated at 30°C for 48 h. Colonies with yellowish colour (usual for lactobacilli) were selected for further morphological examination. These colonies were stored in 20% glycerol at -20°C for future use. The putative lactobacilli isolates were first selected by confirming the catalase activity and Gram's staining. Only Gram-positive colonies with catalase-negative activity were chosen for further scrutiny.

### Identification of *E. durans* F3 by conventional biochemical methods

Identification of *E. durans* F3 strain was primarily carried out by using conventional biochemical methods which includes, production of acid from glucose, growth at different temperatures, NaCl concentrations, growth at different temperatures, NaCl concentrations, production of acid from glucose, catalase test homo/heterofermentative activity and Gram's reaction. However, *E. durans* F3 was also additionally tested for starch hydrolysis test, phenylalanine test, IMVIC test, urea hydrolysis test, nitrate reduction test, casein hydrolysis test, triple sugar iron tests and 11 different carbohydrate fermentation (glucose, fructose, sucrose, maltose, lactose mannose, mannitol, ribose, xylose, Na gluconate, and inositol), for further identification. All the tests made it possible to identify *E. durans* F3 from other LAB (Harrigan and Margaret, 1976).

### Identification by 16S rRNA molecular method

Further confirmation of *E. durans* F3 was carried out by 16S rRNA gene sequencing method. Genomic DNA was isolated from the overnight grown cells on minimal medium by NaCl-cTAB method (William et al., 2012) and quantification was done as per the method described by Sambrook et al., (1982). Later, quality of the extracted DNA (10 µL) was assessed by dissolving in Tris buffer with pH 8 (30 µL) and OD was taken at 260/280 nm. A pair of universal primer 27f (5'AGAGTTTGATCMTGGCTCAG3') and 1492r (5'CGGTTACCTTGTTACGACTT3') was used for carrying out the amplification of 16S rRNA gene. PCR reaction mixture contained 1x ReadyMix™ Taq PCR reaction mix (Sigma-Aldrich®, India) (10 µL), forward primer (1 µL), reverse primer (1 µL), genomic DNA template (2 µL; 50 ng/µL), and nuclease free water (6 µL). The reaction was carried out in Applied BiosystemsVeriti® thermal cyclers with program adjusted as: initialization at 95°C for 4 min, denaturation at 95°C for 30 sec (35 cycles), annealing at 54°C for 30 sec, and extension at 72°C for 1 min, followed by final elongation step at 72°C for 5 min with hold at 4°C for ∞ time. Agarose gel (1%) was used for detecting the amplified PCR products with ethidium bromide as a fluorescent dye for visualizing under UV light. GenElute™ PCR Clean-up kit (Sigma- Aldrich®, India) was used for further purification of amplified PCR product. Eurofins Genomics India Pvt Ltd., Bangalore, sequenced the purified PCR product. Basic Local Alignment Search Tool (BLAST) was then used for sequence match analysis and sequences were submitted to NCBI's GenBank database.

### Phylogenetic analysis

Clustal-W, which is a part of Molecular Evolutionary Genetics Analysis (MEGA 7.0) tool was used for carrying out the pairwise and multiple sequence alignment of 16S rRNA gene sequences of *E. durans*. Phylogenetic analysis was performed using the Neighbor-Joining approach in MEGA 7.0 and p-distance method was used for computing the evolutionary distances (Nei and Kumar, 2000). Taxa analyzed with evolution history was represented by bootstrap consensus tree inferred from 1,000 replicates.

### Probiotic potential of *E. durans* F3

#### Acid tolerance

*E. durans* F3 strain was grown in MRS broth (HiMedia®, India) for overnight at 30°C. It was sub-cultured into fresh MRS broth and incubated till the culture was grown up to 0.6 OD at 600 nm. One milliliter of culture was added into various tubes containing 10 mL of sterile MRS broth with different pH range (2.0, 3.0, and 4.0), while pH 7.0 was used as a control. After exposure to acidic conditions, viable cell counts were calculated for 0, 2, 4, and 6 h at 37°C. Survival percentage of *E. durans*

F3 to different pH values was then calculated as percentage:

$$\text{Survival (\%)} = \text{final (CFU/mL)} / \text{control (CFU/mL)} \times 100.$$

#### Ability to survive under simulated gastrointestinal tract conditions

According to Huang and Adams, (2004), *in vitro* simulated gastrointestinal tract (GIT) conditions were prepared. Similarly, gastric juice was prepared by mixing 1 mL of sodium chloride (0.7%) (Sigma-Aldrich®, India) and pepsin (3 mg) (Sigma-Aldrich®, India), with a final pH in between 2 or 3. Whereas, simulated intestinal juice was prepared by mixing pancreatin (1 mg) (Merck®), 1 mL of sodium chloride (0.7%) (Sigma-Aldrich®, India), 1.5% of bile salts (Sigma-Aldrich®, India) with final pH 8.

*E. durans* F3 was grown (0.6–0.7 OD at 600 nm) into fresh MRS broth by inoculating the log phase culture, followed by centrifugation of cell culture (8,000 rpm, 10 min, 4°C). Washing of cell pellet was then performed by resuspending the cells in sterile phosphate buffered saline (PBS) (pH 7.0). Resuspended cells (0.5 mL) were added into 2.0 mL of simulated gastric or intestinal juices with incubation at 37°C for 6 h. Survival cell count were calculated at 0 (initial time) and 2, 4, and 6 h by spreading the 0.1 mL of cell suspension on MRS agar plates with incubation at 37°C. Following formula was used to calculate the survival percentage of *E. durans* F3 under simulated gastric juices:

$$\text{Survival (\%)} = \text{final (CFU/mL)} / \text{control (CFU/mL)} \times 100.$$

#### Bile salt tolerance

According to the method of Vinderola and Reinheimer, (2003), *E. durans* F3 was tested for its ability to survive in the presence of bile salts. Log phase culture of *E. durans* F3 was inoculated (2% v/v) into MRS broth supplemented with 0.2%, 0.5%, 1.0%, 1.5%, 2%, and 2.5% (w/v) of bile salt (Hi-Media®, India) and control culture (without bile salts) with incubation at 37°C for 24 h. Percentage of growth was then calculated after OD was measured at 560 nm.

#### Antagonistic activity

Antagonistic effects of *E. durans* F3 were evaluated by using agar cup/well diffusion method on Muller Hinton agar (MHA) (Hi-Media®, India) against different pathogenic organisms like *P. aeruginosa*, *S. aureus*, *S. Typhi* and *E. coli*. Test cultures were grown in a tube of nutrient broth at 37°C with adjusted turbidity to match 0.5 McFarland standards. Overnight grown culture of *E. durans* F3 was then centrifuged (8,000 rpm, 10 min, 4°C). Collected supernatant was then adjusted for pH 6.7–7.0 with 2 min incubation in water bath at 90°C. 0.2 mm size of syringe filter was used to filter sterilized the neutralized supernatant and inoculated into the wells made on the plates. Plates were incubated for 24 h at 37°C to observe the zone of inhibition. Sterile MRS broth was used as a negative control, while chloramphenicol antibiotic solution (100 µg/mL) was used as a positive control.

#### Preparation and assay of crude enterocin

Enterocin was partially purified from overnight grown culture of *E. durans* F3. 100 mL of cell free extract was precipitated by the addition of ammonium sulphate (0%–90%) and centrifuged at 10,000 rpm for 10 min at 4°C. Pellet was dissolved in the PBS (pH 7.0) and dialyzed using dialysis membrane. It was then further purified through a gel filtration chromatography

packed with sephadex G-100 column (Sigma-Aldrich®, India) pre-equilibrated with PBS (pH 7.0). The fraction were collected and concentrated in a lyophilizer. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) system was then used to determine the molecular weight of the purified enterocin using molecular weight marker (Merck®, India) according to the method of Laemmli (1970). SDS gel was further stained with Commassie Brilliant Blue R-250, followed by destaining i.e. overnight washing with a mixture of acetic acid-methyl alcohol-water (5:5:1 v/v).

### Genetic identification of enterocins produced by *E. durans* F3

*E. durans* F3 was screened for the presence of genes (*entA*, *entB*, and *entP*) encoding enterocins by PCR. Specific PCR primers were synthesized on the basis of published sequences for the amplification of these genes (Cintas et al., 2000). PCR was run with 1x final concentration of ReadyMix™ Taq PCR reaction mix (Sigma-Aldrich®, India) under following condition: initialization at 95°C for 4 min, denaturation at 95°C for 1 min (35 cycles), annealing at 50°C for 1 min, and extension at 74°C for 1 min, followed by final elongation step at 74°C for 5 min with hold at 4°C for infinity time. Agarose gel (1%) was used for detecting the amplified PCR products with ethidium bromide as a fluorescent dye for visualizing under UV light.

### Assessment of antibiotic susceptibility

Antibiotic susceptibility test was determined semi-quantitatively using the agar overlay diffusion method of National Committee for Clinical Laboratory Standards (1993).

### Antioxidative activity against $\alpha$ -diphenyl- $\alpha$ -picrylhydrazyl (DPPH) free radicals

Antioxidant activity of cell free extract and intake cell extract of *E. durans* F3 was measured against  $\alpha$ -diphenyl- $\alpha$ -picrylhydrazyl (DPPH) in terms of radical scavenging ability (Adnan et al., 2018b). For cell free extract, 100  $\mu$ L of the supernatant, which was used earlier during antibacterial activity, was added in a tube containing 2 mL of  $6 \times 10^{-5}$  DPPH solution in ethanol (Sigma-Aldrich®, India). For intake cell extract, log phase culture was harvested by centrifugation (6,000 rpm, 10 min, 4°C). Cell pellet was then washed and resuspended in PBS (pH 7.0). 100 intact cells were separately mixed with 2 mL of  $6 \times 10^{-5}$  DPPH solution in ethanol (Sigma-Aldrich®, India), followed by 30 min incubation in dark. After the incubation period, reduction of DPPH free radicals was measured at 517 nm. One milligram per milliliter of L-ascorbic acid (Sigma-Aldrich®, India) was used as a positive control, DPPH solution (lacking sample) was used as a negative control and ethanol was used as a blank. Following formula was used to calculate the percentage inhibition of DPPH radicals:

$$\text{DPPH scavenging activity (\%)} = (A_0 - A_1) / A_0 \times 100$$

Where,  $A_0$ =absorbance of the control

$A_1$ =absorbance of the sample

### Screening for lipase and bile salt hydrolase enzyme (Bsh) activity

Presence of the lipase enzyme which can catabolize lipid (fat & oil) from the medium was carried out on Spirit Blue Agar (SBA) (Hi-Media®, India) medium. Using sterile cork borer, 6 mm size of wells were made on the SBA plates. They were then filled with the 100 mL of fresh culture, followed by incubation for 24–48 h at 37°C. Visualization of clear blue colour halos around the wells denotes the presence of lipase.

MRS agar plates supplemented with 0.5% bile salts like sodium glycocholate (GCA), sodium deoxycholate (DCA) and 0.04% CaCl<sub>2</sub> was used to assess the Bsh activity of *E. durans* F3 (Kumar et al, 2010). After the incubation of plates for 24 to 48 h at 37°C, presence of precipitated bile salts around the colonies with a silvery shining was observed to confirm the Bsh activity.

### Haemolytic and gelatinase activity

*E. durans* F3 strain was tested for pathogenicity via hemolytic and gelatinase activity. Hemolytic activity was tested on blood agar plates supplemented with 5% human blood (Hi-Media<sup>®</sup>, India). Hemolytic reaction was recorded after 24–48 h by observing the  $\alpha$ ,  $\beta$  and  $\gamma$ -hemolysis.

Gelatinase activity was determined using the fresh culture of *E. durans* F3, which was streaked on to the nutrient gelatin agar plate, followed by incubation for 48 h at 37°C. After the period of incubation, plates were flooded with the saturated ammonium sulphate solution. Presence of gelatinase was confirmed by a clear zone around the colonies.

### Determination of cell surface hydrophobicity

*E. durans* F3 adhesion ability to hydrocarbon was determined by the modified method of Vinderola and Reinheimer (2003). Overnight grown culture of *E. durans* F3 was centrifuged (6,000 rpm, 10 min, 4°C). Cell pellet was then washed and resuspended in phosphate buffer saline (pH 7.0). Absorbance of cell pellet was adjusted to 0.6 OD at 600 nm. *E. durans* F3 cell suspension (2.0 mL) and xylene or n-hexadecane (1.0 mL) (Sigma-Aldrich<sup>®</sup>, India) were mixed by vortexing and incubated for 1 h at 37°C for phase separation. After incubation period, aqueous phase was taken out and absorbance was measured at 600 nm. Percentage cell hydrophobicity was calculated as percent decrease ( $\Delta\text{Abs} \times 100$ ) in the absorbance of the aqueous phase after mixing and phase separations relative to that of original suspension ( $\text{Abs}_{\text{initial}}$ ) as following formula:

$$\text{Surface hydrophobicity (\%)} = 100 \times (\text{Abs}_{\text{initial}} - \text{Abs}_{\text{final}}) / \text{Abs}_{\text{initial}} = \Delta\text{Abs} / \text{Abs}_{\text{initial}} \times 100.$$

### Cell aggregation assay

*E. durans* F3 cell aggregation assay was performed by modified method of Kos et al., (2003). Supernatant was collected by centrifugating (6,000 rpm, 10 min, 4°C) the freshly grown culture of *E. durans* F3 (Step 1). Washing of cell pellet was then performed by resuspending in PBS (pH 7.0) and absorbance was adjusted to 0.6 OD at 600 nm ( $\text{Abs}_{\text{initial}}$ ). Suspension was then centrifuged again and the pellet was dissolved in equal volume of supernatant from step 1, followed by incubation for 2 h at 37°C. At the end of incubation, 2 mL of the upper layer was taken and absorbance ( $\text{Abs}_{\text{final}}$ ) was measured at 600 nm by using broth as a blank. The percentage difference between initial and final absorbance as an index of cellular autoaggregation can be calculated as follows:

$$\text{Aggregation (\%)} = 100 \times (\text{Abs}_{\text{initial}} - \text{Abs}_{\text{final}}) / \text{Abs}_{\text{initial}} = \Delta\text{Abs} / \text{Abs}_{\text{initial}} \times 100.$$

### Potentiality of *E. durans* F3 as yoghurt culture

*E. durans* F3 ability to coagulate the milk was carried out against three varieties of milk obtained from the market (Whole, semi-skimmed and skimmed). 10 mL of milk were filled in all the tubes and autoclaved. Tubes were then inoculated with 1 mL of overnight grown culture of *E. durans* F3, followed by incubation for 24–72 h at 37°C.

## Statistical analysis

Statistical analysis was conducted using the GraphPad Prism software (Version 7.03), and results are presented as mean values from three replicate experiments, while error bars represent SD of the mean values.

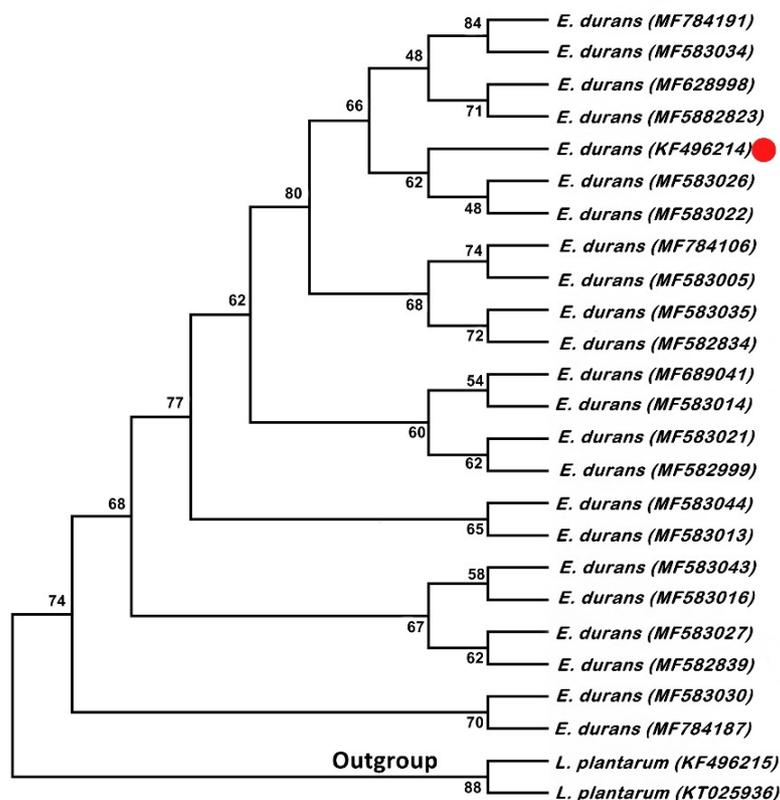
## Results

### Isolation and identification of *E. durans* F3 from fish gut

A LAB strain named as *E. durans* F3 belongs to the genus *Enterococcus* was isolated from the gut of *Catla catla* on MRS (de Man, Rogosa and Sharpe) agar plate supplemented with BCP (bromocresol purple) as pH indicator on the basis of morphological, biochemical and 16S rRNA sequencing method (Fig. 1). Colonies of *E. durans* F3 were appeared as small, circular with entire margin. It was facultatively anaerobic, catalase negative, able to ferment glucose, can produce ammonia from arginine, can grow at 45°C and 6.5% NaCl concentration and Gram-positive cocci in shape. However, the sugar fermentation profile and other various biochemical tests results are represented in Table 1. Nucleotide sequences were deposited to NCBI with accession numbers KF496214 after the successful identification.

### Phylogenetic analysis

16S rRNA gene sequences of *E. durans* with *L. plantarum* as outgroup. P-distance method was used for computing the evolutionary distances. Twenty five nucleotide sequences were involved in the analysis and positions containing missing data



**Fig. 1.** Phylogenetic relationship according to the nucleotide sequences of the 16S rRNA fragment of the *Enterococcus durans* F3 strain with other *E. durans* strains identified with their GenBank accession numbers. Tree is supported by bootstrap values.

**Table 1. Biochemical characterization of *E. durans* F3 isolate**

Characteristics		Isolate F3
Colony appearance		Small, circular, with entire margin
Gram's nature		Gram positive cocci
Catalase test		-
Grow at 45°C		+
Growth at 6.5% NaCl concentration		+
Ammonia production		+
Sugar profile		
Glucose	Gas	-
	Acid	+
Fructose	Gas	-
	Acid	+
Sucrose	Gas	-
	Acid	+
Maltose	Gas	-
	Acid	+
Lactose	Gas	-
	Acid	+
Mannose	Gas	-
	Acid	+
Mannitol	Gas	-
	Acid	-
Ribose	Gas	-
	Acid	+
Na gluconate	Gas	-
	Acid	-
Xylose	Gas	-
	Acid	-
Inositol	Gas	-
	Acid	-

+, positive; -, negative.

and gaps were excluded with having the 636 positions in the final data set. Fig. 1 shows the Neighbor-Joining tree. Ratio of replicate trees is shown above the branches in which the associated taxa clustered together in the bootstrap test. Although, these results confirm that the *E. durans* F3 strain unequivocally belongs to the clade of genus *Enterococcus*.

### Probiotic activities of *E. durans* F3

#### Ability to survive under acid, bile salt and simulated gastrointestinal tract conditions

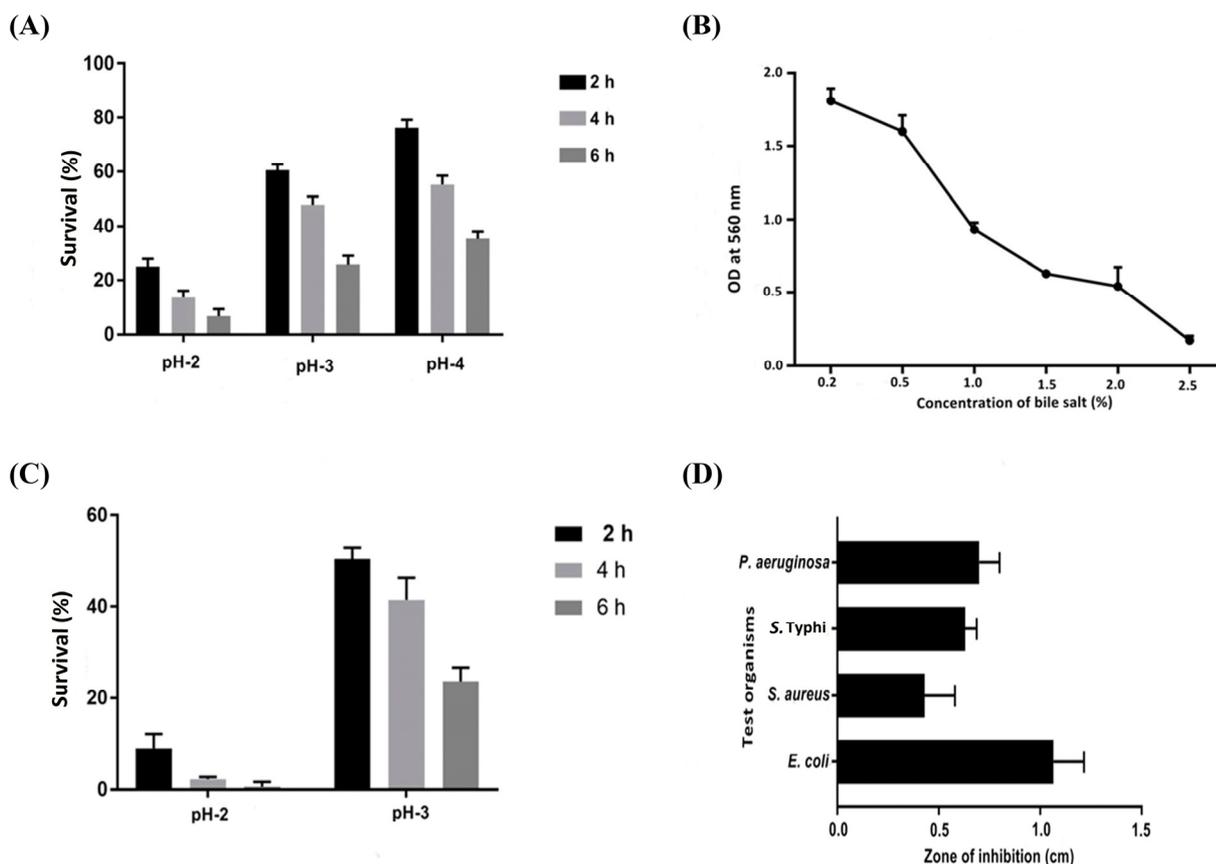
The most anticipated characteristic necessary for probiotics is their ability to survive in the presence of bile salts under

acidic conditions. *E. durans* F3 demonstrated high tolerance to acidic environments (Fig. 2A), excluding pH 2, where 25% of the viability was present after 2 h. No significant differences were observed at pH 3 and pH 4 when compared to control (pH 7). Incubation time was range from 0 to 6 h.

The bile tolerance of *E. durans* F3 was investigated from 0.2%–2.5% of bile salts (Fig. 2B). Outcome of the test revealed that *E. durans* F3 was able to grow up to 2.0% of bile salt concentration in a medium. However, under simulated gastric juice conditions at pH 2, *E. durans* F3 was survived only at time 0, and after 2 h, cell viability was not occurred (Fig. 2C). Moreover, no significant difference in cell viability was observed under simulated gastric juice condition with pH 3 when compared with control. Similar values of cell viability were observed after 6 h under simulated intestinal juice conditions and control demonstrating that *E. durans* F3 was able to survive under simulated intestinal juice at pH 8.

### Antagonistic activity, molecular mass determination and enterocin production

Array of pathogenic bacteria were tested against Cell free extract of *E. durans* F3 using the agar cup diffusion assay. *In vitro* tests under neutralized pH were achieved and results confirmed the inhibitory activity of *E. durans* F3 in the form of zone of inhibition. The strain was considered to be active, as it inhibited/supressed the growth of one or more pathogenic strains (*E. coli*, *P. aeruginosa*, *S. Typhi* and *S. aureus*) (Fig. 2D).



**Fig. 2.** (A) Bar graph showing acid tolerance response of *Enterococcus durans* F3 at different pH. (B) Bile salt tolerance of *E. durans* F3 against different bile salt concentration. (C) Bar graph showing ability of *E. durans* F3 to survive under simulated gastric juice conditions. (D) Bar graph showing antibacterial activity of *E. durans* F3 against *Escherichia coli*, *Staphylococcus aureus*, *Salmonella Typhi*, and *Pseudomonas aeruginosa*. Error bars represent SD of the mean values of results from three replicate experiments.

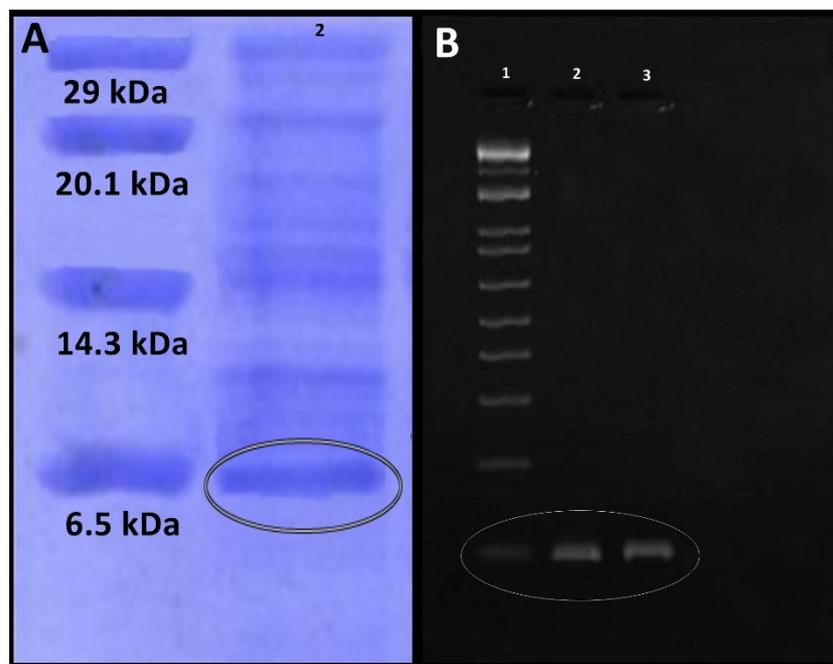
Purification of enterocin produced by *E. durans* F3 was carried out by ammonium sulphate precipitation, which was then further purified by gel filtration chromatography packed with Sephadex G-100 column. Fractions containing enterocin activity was detected by well diffusion assay. Following pre-concentration by lyophilizer, SDS-PAGE was used for testing the purity and mass of the active fraction. Protein band with an approximate molecular mass of 6.5 kDa was observed. This result suggests that the enterocin produced by *E. durans* F3 has an approximate molecular mass of 6.5 kDa (Fig. 3A).

### Genetic identification of enterocins produced by *E. durans* F3

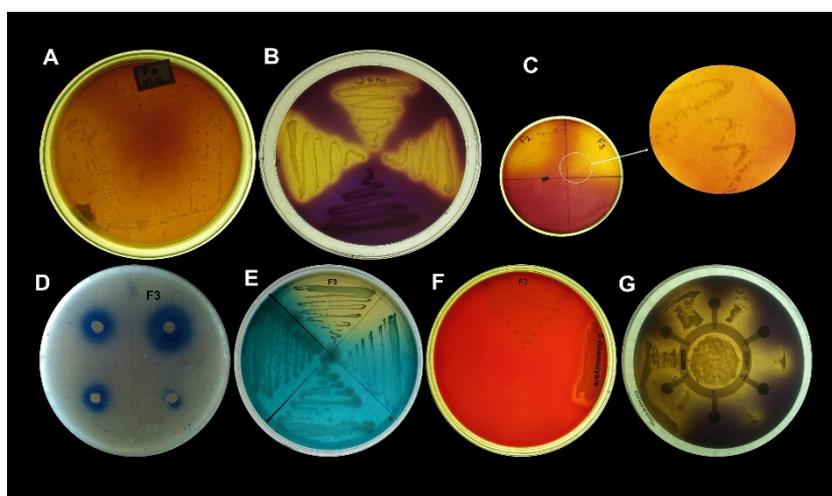
Further screening of *E. durans* F3 strain was done for confirming the presence of known genes which encodes class II enterocins A, B and P using PCR. Total isolated DNA from the enterocin producing *E. durans* F3 strain was subjected to amplification with primers to the enterocin genes. *E. durans* F3 showed bands corresponding to the *entA* (nearly at 100 bp) (Fig. 3B). No reaction took place with the enterocin B and P primers, which suggested that Bacteriocins produced by *E. durans* F3 are similar to enterocin A.

### Assessment of antibiotic susceptibility and antioxidant activity

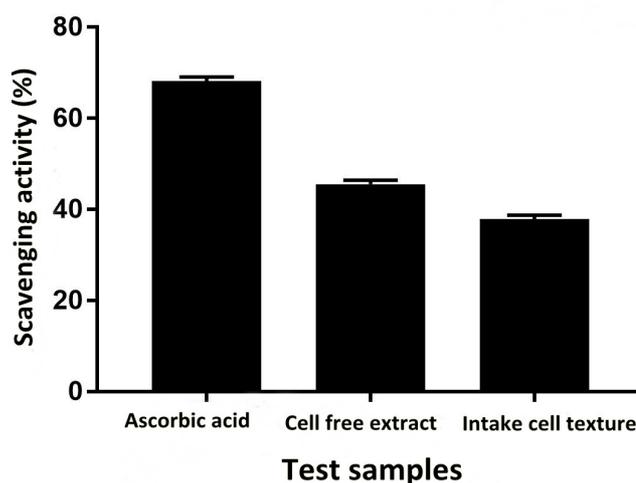
Sensitivity and resistance level of *E. durans* F3 was tested using antibiotic susceptibility test against different standard antibiotics. Fig. 4G shows the complete antibiotic susceptibility profile. *E. durans* F3 was found to be susceptible to all tested antibiotics (vancomycin, ciprofloxacin, gentamycin, linezolid, streptomycin and penicillin). The zone of inhibition around the antibiotic discs in Fig. 4G illustrates the commendable susceptibility of *E. durans* F3. Antioxidant potential of *E. durans* F3 cell free extract and intake cell extract was evaluated against  $\alpha$ -diphenyl- $\alpha$ -picrylhydrazyl (DPPH) free radicals in comparison to ascorbic acid. Cell free extract was better DPPH scavengers compared to intake cell extract (Fig. 5). The higher cell free extract antioxidant activity of *E. durans* F3 suggests that it can assist well as a probiotic aide in scavenging free radicals.



**Fig. 3. (A) SDS-PAGE of enterocin from *Enterococcus durans* F3.** Lane 1 (Standard: molecular size indicated below the band in kDa). Lane 2, partial purified enterocin with approximate size of 6.55 kDa. (B) Amplification of DNA of *E. durans* F3 with specific enterocin primers yielded a 100 bp fragment (characteristic for enterocin A). Lane 1, DNA marker; Lane 2 & 3, *E. durans* F3 amplified fragment.



**Fig. 4.** (A) Growth of *Enterococcus durans* F3 on MRS+BCP plate, (B) Growth of *E. durans* F3 on MRS+CaCO<sub>3</sub> plate, (C) Bile salt hydrolase activity of *E. durans* F3 MRS-sodium deoxycholate (DCA)-sodium glycocholate (GCA) agar plate, (D) Zone of lipid hydrolysis on spirit blue agar plate, (E) HHD medium plate showing homofermentative *E. durans* F3 with green colour colony, (F) Gamma-hemolytic activity by *E. durans* F3 on blood agar plate, (G) Assessment of antibiotic susceptibility of *E. durans* F3 against different antibiotics.



**Fig. 5.** Bar graph showing antioxidant activity of *Enterococcus durans* F3 against DPPH free radicals. Error bars represent SD of the mean values of results from three replicate experiments. DPPH,  $\alpha$ -Diphenyl- $\alpha$ -Picrylhydrazyl.

### Screening for lipase and bile salt hydrolase enzyme (Bsh) activity

Lipolytic activity of *E. durans* F3 was observed on SBA medium containing emulsified lipid and spirit blue dye. Blue colour halos around the well was observed. This demonstrates the ability of *E. durans* F3 to produce lipolytic enzyme indicating lipolysis (Fig. 4D).

Bsh activity is also considered as one of the important factor in selection of probiotics, which found to reduce serum cholesterol. Presence of precipitated bile salts around the colony of *E. durans* F3 on MRS agar plate supplemented with DCA and GCA with a shine confirmed its ability to hydrolyze DCA and GCA via the production of Bsh enzyme (Fig. 4C).

### Cell aggregation and surface hydrophobicity assay

Adhesion of probiotic strains depends on cell surface properties like hydrophobicity and extracellular protein profiles,

which also varies among probiotic strains. Therefore, adhesion ability of *E. durans* F3 was measured by cell aggregation and hydrophobicity assays using two hydrocarbons (xylene and n-hexadecane). Percent cell surface hydrophobicity of *E. durans* F3 was found to be 38.7% in the presence of xylene and 34.4% in case of n-hexadecane. To evaluate the cell aggregation potential of *E. durans* F3, the cellular auto-aggregation was measured. *E. durans* F3 results showed higher capability to self-aggregate (Fig. 6).

### Hemolysis and production of gelatinase

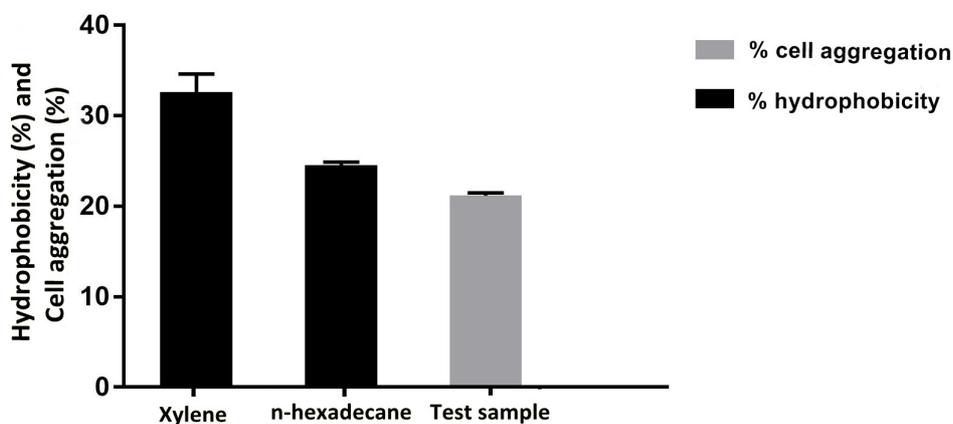
Even after 48 h of incubation on blood agar plates, *E. durans* F3 did not exhibited any hemolytic effect, green zone ( $\alpha$ -hemolysis) or inhibition zone ( $\beta$ -hemolysis), which is considered as  $\gamma$ -hemolysis. However, *E. durans* F3 was also found to be negative for gelatinase activity. Therefore, it is considered as non-pathogenic (Fig. 4F).

### Curd formation ability of *E. durans* F3

Significant curd formation was seen by *E. durans* F3 after 72 h in all three types of milk (skimmed, semi-skimmed and full fat). This test indicates the use of *E. durans* F3 as a starter culture in dairy industries for producing yoghurts or other dairy products.

## Discussion

Large number of probiotic strains has been isolated mainly from the terrestrial sources successfully, with the ability to provide health benefits for a long time. Whereas, marine environment is the least explored on the planet, especially in concern of microorganisms, which are estimated to exceed 10 million species in such habitats (Grassle and Maciolek, 1992). Marine environments cover many ranges of habitats with very low to extreme high temperatures,  $-32^{\circ}\text{C}$  to  $400^{\circ}\text{C}$ . Such kinds of habitats are the home of a wide diversity of microorganisms, which are acclimatized to the high temperatures, high pressure, and acidity (Gerday and Glansdorff, 2007; Merkel et al., 2013; Vetriani et al., 2004). Similarly, countless marine organisms reside in such habitat, which is believed to contain a unique microbial flora. Isolation of bacterial communities from such sources might be very helpful in identifying a potential probiotics with health benefits.



**Fig. 6.** Bar graph showing relationship between hydrophobicity (%) and auto-aggregation ability (%). Error bars represent SD of the mean values of results from three replicate experiments.

In the present study, *E. durans* F3 exhibited desired features to be a potent probiotic strain. Various *Enterococcus* species (*E. faecium*, *E. durans*, and *E. faecalis*) have been commonly isolated from processed foods, which might be associated to various environmental factors like extreme salinity, heat resistance and other harsh conditions (Giraffa, 2003; Martin-Platero et al., 2009). Even though, there have been studies reported on the utilization of *Enterococcus* species as probiotics, very few reports on their isolation, especially of *E. durans* from the digestive tract of aquatic animals (Sarra et al., 2013). This study is the first one, which describes the potentiality of *E. durans* as a potent probiotic recovered from the gut of fish with wide-ranging experimental evidences. *E. durans* F3 was identified on the basis of morphological, biochemical as well as 16S rRNA sequencing method.

With a vision to reveal the probable usage of the *E. durans* F3 as a potent probiotic, it was subjected to detailed categorisation in its probiotic profiles. In this sense, it has to survive under acidic conditions, in presence of bile salt in the GI tract. Generally, pH of the stomach ranges from 2.5 to 3.5 (Huang and Adams, 2004) and increase up to 6.5 after ingestion of food (Johnson, 1977). Thus, to remain viable under such extreme environment after oral administration, probiotic cultures faces major physiological challenges. Results in this study is proving the potentiality of *E. durans* F3 for tolerating acid and bile with survival under pH 3.0 and 4.0 when exposed. Even under the pH 2.0, 25% of the viability after 2 h and 2.0% concentrations of bile salts was seen. Further confirmation of bile salt tolerance was established by screening the Bsh enzyme, which can hydrolyze bile salts DCA and GCA. However, the acid tolerance ability of probiotic bacteria depends on the association of the cytoplasmic membrane and pH profile of H<sup>+</sup>-ATPase. Yet, it also depends on the type of bacterium, incubation conditions and organization of development of medium (Madureira et al., 2008).

After passage from the gastric barrier, probiotic bacteria have to survive the second barrier; small intestine to transit through the GI tract. Though, pH of small intestine is in favorable range but existence of bile salts and pancreatin may inhibit the growth. *E. durans* F3 was found to be remain viable in the presence of intestinal juice containing pancreatin at pH 8.0 and simulated gastric juice containing pepsin at pH 3.0. Thus, its survival to such simulated gastrointestinal conditions makes it as an future alternate source for probiotic based food products.

*E. durans* F3 also showed the ability to deter the growth of pathogens like *S. aureus*, *S. Typhi*, *P. aeruginosa* and *E. coli*. Its antibacterial activity can be said due to the production of enterocin. *Enterococcus* species are unique in that sense, as they can produce wide-ranging and structurally diverse enterocins; enterocins L50A, L50B (Cintas et al., 1998) and enterocin Q (Cintas et al., 2000) that are totally different from the lactic acid producing bacteriocins (Franz et al., 2007). Production of structurally diversified enterocins permits them to live in a widespread ecological niches and increase their use in food preservation (Foulquie Moreno et al., 2006). In the present study, SDS-PAGE gel was used for determining the molecular size of the enterocin produced by *E. durans* F3 strain. One antimicrobial peptide band with a molecular size of about 6.5 kDa was observed and verified by amplifying the expected fragment, which contains the structural gene of enterocin A.

Antioxidant activity of *E. durans* F3 was also observed against DPPH free radicals. Both cell free and intake cell extract of *E. durans* F3 were found to possess antioxidant activity. Free radicals reactive nitrogen species and reactive oxygen species are induced inside the body, which can trigger a number of human diseases via altering lipids, proteins, and DNA (Lobo et al., 2010). Hence, *E. durans* F3 can act as an external and additional source of antioxidants, which can contribute in controlling the oxidative stress. Performing antibiotic susceptibility test, which is an essential precondition for a strain to be a probiotic, tested further for potentiality of *E. durans* F3. Strains having resistance traits can impart adverse consequences to human health as they can transfer antibiotic resistance gene in to intestinal pathogens. *E. durans* F3 was found to be sensitive to vancomycin, ciprofloxacin, gentamycin, linezolid, streptomycin and penicillin.

Adhesion and aggregation is a step required by a potent probiotic for colonization to the intestinal epithelial cells and mucosal surface. It is likely to be a requirement for the competitive omission of enteropathogens into the gut by forming biofilms or modulating the immune response of the host (Adnan et al., 2017b; Alander et al., 1997; Forestier et al., 2001; Lee et al., 2003; Plant and Conway, 2002). Significant relationship between aggregation and cell surface hydrophobicity was shown by *E. durans* F3. This relationship indicating its capability of creating an antibacterial environment in the gut, by excreting enterocin or its like substances for inhibiting the growth of pathogenic bacteria, while adhered to epithelial cells.

Evaluation of hemolytic action is also considered as one of the safety viewpoints in selecting the probiotic strains as per the guidelines from FAO/WHO. Results in this study confirmed the non-appearance of hemolytic activity. Furthermore, *E. durans* F3 was also able to produce lipase enzyme which helps the functioning of body in various ways; absorption of minerals and vitamins, boosting immune system, maintaining the optimum levels of pancreatic enzymes, avoiding excess weight gain and controlling obesity (Edward, 2011). Curd formation ability of *E. durans* F3 is also suggested in this study, due to its homofermentative nature. This makes it an impeccable starter culture for producing fermented/probiotic products imparting health benefits in dairy industries, which may also help in modifying the texture and enhancing the flavor of the final product under controlled conditions. In summary, the conclusion and outcomes of this study claim that, *E. durans* F3 demonstrated desirable probiotic properties *in vitro*.

This study emphasizes the desirable *in vitro* probiotic benefits of *E. durans* F3. Antagonistic effects of substances produced by *E. durans* F3 on a vast range of pathogenic bacteria indicated its important role in food preservation and human health. It can be used for the production of various kinds of novel pharmaceutical products, and functional foods. Use of *E. durans* F3 is not only confined to the above mentioned applications, instead it can also be used in animal farming as a probiotic application in different livestock production systems like poultry, pig and ruminant nutrition. This can enhance the growth rate, feed intake, feed efficiency, carcass yield, carcass quality, nutrient digestibility and controlling of enteric pathogens. Moreover, to reduce the worldwide concern of antibiotic resistance in livestock, *E. durans* F3 will stand as a promising and efficient alternative. Various benefits have been observed in animals fed with different LAB-probiotics (Vieco-Saiz et al., 2019). Similarly, production of *E. durans* F3 originated probiotics for livestock feed possibly will fight and help in preventing bacterial diseases. It may also help in weight gain in affected animals, enhancing the quality of the products of animal farming, improve aquaculture water quality. *E. durans* F3 can also combine with other probiotic species, probiotics or enzymes for its use as animal feed in livestock production. In terms of future design, recombinant *E. durans* F3-probiotics may offer additional advantages as *L. plantarum* NC8, which can produce a recombinant dendritic cell-targeting peptide with activity against H9N2 avian influenza virus in chickens (Shi et al., 2016; Wang et al., 2017). Considering all of these indicated properties/activities, it can be a new promising probiotic strain.

## Conflict of Interest

The authors declare no competing interests.

## Author Contributions

Conceptualization: Alshammari E, Adnan M, Kumar P. Data curation: Alshammari E, Patel M, Sachidanandan M. Formal analysis: Alshammari E, Patel M, Sachidanandan M. Methodology: Adnan M, Kumar P. Software: Patel M. Validation: Adnan M, Patel M, Kumar P. Investigation: Alshammari E, Sachidanandan M. Writing - original draft: Adnan M, Kumar P,

Patel M. Writing - review & editing: Alshammari E, Patel M, Sachidanandan M, Kumar P, Adnan M.

## Ethics Approval

This article does not require IRB/IACUC approval because there are no human and animal participants.

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