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# Isolation and characterization of probiotic bacteria from the soil samples of the coastal areas of (Gudur division, Nellore Dt.) for utilization in Shrimp farming

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**Abstract**
A study was conducted to isolate and characterize probiotic bacteria from soils of different areas in Nellore district for utilization in Shrimp farming. Conventional, morphological and biochemical tests were conducted to isolate and enumerate the Lactobacillus bacteria from the soil samples. Bacterial growth kinetics, tolerance to various parameters like ammonia, nitrogen, salinity, pH and bile acid were taken into consideration to characterize the isolated bacteria as probiotic. Cholesterol assimilation and viability of bacteria in gastric tract was also studied. Bacterial DNA samples isolated were found to have the V2-V3 sequence of lactobacillus which is of approximately 200bp fragment). All the screened samples were shown to be amplified for V2-V3 region except DZ1 and DZ6.

**Keywords:** Lactobacillus bacteria, Isolation, Characterization, Growth kinetics, Probiotic potential

## 1. Introduction
Aquaculture is the farming of aquatic organisms by intervention in the rearing process to enhance production. This activity allows a selective increase in the production of the species used for human consumption to overcome malnutrition and also to attain good economic growth. Shrimp and prawn cultures are one of the most important practices worldwide. According to FAO [1] the world aquaculture production of shrimp was 43,27,520 tons. One of the main objectives in aquaculture is to decrease the use of antibiotics without decreasing the shrimp production in order to avoid the usual disadvantages such as antimicrobial resistance among pathogenic bacteria. As an alternate biological tool use of probiotics has been practiced to minimize the adverse effects, thereby improving survival and growth rate and enhance the immune capacity [2].

Probiotics are live microorganisms which, when administered in adequate amounts, confer a health benefit on the host [3]. Most probiotic microorganisms belong to the lactic acid bacteria (LAB) viz*. Lactobacillus spp., Enterococcus spp. and Bifidobacterium* [4]. Most of the species of the genus *Lactobacillus* are part of human and animal commensal intestinal flora [5] and consists of a physiologically and genetically diverse group of rod-shaped, Gram-positive, non-pigmented, non-spore forming [6], catalase negative, facultative aerobic to anaerobic LAB [7] with intense applications in fermented food production industry [8]. These microorganisms are also called friendly bacteria and are generally recognized as safe (GRAS) microorganisms [9].

The use of probiotics and other immune stimulants as dietary supplements can enhance innate defense and resistance to pathogens during periods of stress [10, 11]. Probiotics may also improve nutrient availability due to exogenous enzymes secreted into the host intestine or to endogenous enzymes available into the bacterial cells and released when they are lysed by the effect of the acidic environment of host's stomach. Both types of enzymes may increase the digestive activity and degradation of diet compounds of the tested animal, even if not digestible by its own enzymatic machinery. In that vein, gut microbiota helps to convert nutrients into energy, but can also produce some essential nutrients, such as vitamins; hindering the microbial colonization by pathogens either by competitive exclusion for space or through the production of antimicrobial metabolites [12] as bacteriocins produced by some *Lactobacillus* [13] of most importance is to mention the immune modulatory capacity of the microbiota in early response, by activating the immune system of the host [14]. The present study aims at the isolation, characterization of the lactobacillus species from the shrimp

cultured ponds for their probiotic potential under various physical and physiological conditions.

## 2. Materials and Methods

### 2.1. Collection of soil samples

Sixteen (16) soil samples were collected from the brackish water shrimp ponds from Kolanakuduru, Bestapalem, and Tippaguntapalem of Gudur coastal areas, of Nellore district, Andhra Pradesh, India. Geographically these are located in Gudur division at $14^0$-47'N and $79^0$-03'E,.where *L. vannamei* being cultured extensively.

### 2.2. Isolation and enumeration of Lactobacillus bacteria:

All soil samples were subjected for serial dilution under extreme sterile conditions using nutrient agar supplemented with 15% sodium chloride. All the prominent bacterial colonies obtained were subjected for pure culture isolation. Further the pure colonies were diluted in 0.85% NaCl and analyzed by spread inoculation. An inoculum (0.1 ml) of each decimal dilution of samples was plated onto the surface of de Man, Rogosa and Sharpe (MRS) agar (Difco, Detroit, MI, USA) which were incubated anaerobically in anaerobic jar (BBL, Gas Pak Plus), for enumeration of *lactobacillus* bacteria, which were incubated under aerobic condition and the bacterial counts were recorded after incubation at 30°C for 48 h. The samples were cultivated under sterile condition in MRS agar plates and they were incubated at $37^0$Cfor 48hrs. Each isolate were cultured in MRS agar again and preserved in refrigerator in order to conserve them. Representatives of bacterial colony types from MRS agar were isolated and identified using conventional morphological and biochemical tests according to Bergey's manual of determinative bacteriology [15].

### 2.3. Identification and characterization of probiotics

The isolates were identified by characterizing them using gram staining, catalase test, carbohydrates fermentation test according to Holt *et al*. [15].

### 2.4. Kinetics of bacterial growth

The growth kinetic of each strain was performed by inoculating 20 µL (1.0 Abs 580 nm) of each isolate in 100 mL TSB or MRS supplemented with 2.5% NaCl. The absorbance (580 nm) was determined at 0, 3, 6, 9, 12, 15, 18, 22 and 24h using Spectrophotometer (ELICO). The results were plotted to identify the phases, especially the log phase [16].

### 2.5. Tolerance to ammonia nitrogen (TAN)

Strains were tested for tolerance to ammonia nitrogen following the technique of Devaraj *et al*., [17]. Ammonia nitrogen concentrations tested were: 0.05, 0.1, 0.5, 1.0, 5.0, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 100, 120, 140,160,180 and 200 µg $L^{-1}$. Twenty microliters of each strain culture were inoculated in Falcon tubes with 10 mL of TSB medium with 2.5% NaCl and incubated at 35°C for 24 h. The medium was used as a control. The absorbance was determined at 580 nm using a Spectrophotometer (ELICO).

### 2.6. Salinity tolerance

Sterile filtered sea water was collected from Blue park hatchery, Ramatheertham, Nellore Dt. From this required salinities like 0,5,10, 15, 20, 25, 30PPT were obtained by diluting with fresh water. All the Falcon tubes with 9 mL of trypticase soy broth and made upto 10 ml with above prepared salinity waters respectively and inoculated with 20 µl of each isolate and incubated at 35ºC for 24 h. Absorbance was read in a spectrophotometer at 550 nm, using sterile medium as a blank.

### 2.7. pH tolerance test

Acidification was measured by selected bacterium investigated at different pH. MRS broths with different pH including 3, 4, 5, 6, 7, 8 and 9 were prepared using HCl 1% and NaOH 1 N and divided in universal bottles [18]. The broths media along with control bottles were autoclaved at 121°C for 15 min and then inoculated with overnight culture of the selected strain in MRS broth followed by incubation at 30°C. Optical density (OD) as growth rate of bacteria was measured by spectrophotometer at 600 nm after 2 h incubation. The viability of the isolates was also controlled by duplicate inoculation on MRS agar [19-21].

### 2.8. Bile acid tolerance

All isolates were investigated for bile salt tolerance following the method of Vinderola *et al*. [22]. Briefly, 0.2 ml of each isolate inoculum suspension ($10^7$-$10^8$ CFU/ml) was added to 10 ml of MRS broth containing different concentrations of bile salt (0.15e0.45% w/v) and MRS broth without bile salt as control and incubated at 37 $^0$C for 24 h. Optical density (OD), was recorded at 560 nm after incubation and compared with the control. Lactobacillus isolates showing resistance more than 50% at 0.3% (w/v) bile salt were considered as bile resistant as this is the minimum range for being a probiotic culture.

$$\% \text{ Resistance} = \frac{\text{Increment of OD in MRS broth with bile salt /pH 2, 3, 5}}{\text{Increment of OD in MRS broth without bile salt /at pH 7}} \times 100$$

### 2.9. Tolerance to simulated human gastrointestinal tract

*In vitro* determination of viability under conditions similar to those prevailing in the GIT was performed according to the method of Charteris *et al.*[23] Briefly, simulated gastric and pancreatic juices were prepared by suspending pepsin (3 mg/ml; Sigma) and pancreatin USP (1 mg/ml; Sigma) in sterile sodium chloride solution (0.5%, w/v), and adjusting the pH to 3.0 and 8.0 with hydrochloric acid (3.0 mol/L) and NaOH (1 mol/L), respectively. Portions (0.2 ml) of washed cell suspensions of the *Lactobacillus* strains in phosphate buffered saline (PBS, pH 7.0) were inoculated in 1.0 ml of simulated gastric or pancreatic juice and 0.3 ml NaCl (0.5%, w/v), mixed and incubated at 37 °C. Total viable counts (cfu/ml) were evaluated after incubation for 180 min in cultures tested for gastric transit tolerance, and for 240 min in cultures tested for small intestinal transit tolerance. The initial viable count (cfu/ml) of the washed cell suspension from each probiotic tested was determined prior to the transit tolerance assay, and was used to calculate loss of viability.

### 2.10. Assay of cholesterol assimilation

Freshly prepared sterile MRS broth was supplemented with 0.3% (w/v) oxgall and 0.2% (w/v) sodium thioglycolate. Water–soluble cholesterol (polyoxyethanyl-cholesterylsebacate, Sigma) [24] was filter sterilized and added to the broth at a final concentration of 100 µg/ml. Lactobacillus strains isolated were inoculated (3%, v/v) into 5 ml of the Ch-MRS-Thio broth, and incubated anaerobically at 37°C for 20 h. Un inoculated sterile broth was used as the control. After incubation, cells were removed by

centrifugation (10000 g, 4°C, 10 min) and the remaining cholesterol concentration in the broth was determined using a modified σ-phthaladehyde method of Ruddel and Morris [25]. The observations were compared to a standard curve prepared by using suitable concentrations of cholesterol read at 550 nm, and percent reduction was determined in the spent broth by comparing values with un inoculated control.

### 2.11. Molecular identification lactobacillus species

#### 2.11.1. DNA extraction

Lactic acid bacteria were isolated from all ten soil samples selected. Serial dilutions were made in sterile physiological saline, plated onto MRS agar (Biolab, Biolab Diagnostics, Midrand, South Africa)supplemented with natamycin, and incubated at 30°C for 24–48 h. Colonies were harvested from plates representing $10^3$ CFU/mL and suspended in10 mL of sterile physiological saline. DNA was isolated from 2 mL of cell suspension by using the method of Dellaglio *et al*. [26].

#### 2.11.2. DNA amplification for lactobacillus identification:

The V2–V3 variable region (approx. 200 base pairs)of the 16S rRNA gene in lactic acid bacteria was amplified by using primers 534(5' ATTACCGCGGCTGCTGG 3') and 341FGC (5' GCAGGCCCGCCGCGCGGCGGGCGGGGCGGGGGC ACGGGGGGCCTACGGGAGGCA 3'). The PCR reaction was performed in 50 µL of PCR mixture containing 0.5 mM of primers, 200 mM dNTP (Takara Bio Inc., Shiga, Japan), 0.5 U Taq DNA polymerase (Takara Bio Inc., Shiga, Japan), 1´PCR buffer (TakaraBio Inc., Shiga, Japan) and 10 µL of DNA. The following conditions were used: initial denaturation at 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s and elongation at 72 °C for 8 min. PCR reactions were performed in a Master Cycler TM Thermal Cycler (Eppendroff, Germany). Amplicons were analyzed on 2 % (by mass per volume) agarose gels with ethidium bromide and 0.5´TB buffer. DNA fragments were visualized under UV light (VilberLourmat, Torcy, France).

## 3. Results and Discussion

A total of 8 isolates viz DZ1-DZ8 were isolated on MRS agar and were biochemically characterized. All isolates were shown to be gram positive except DZ7 and DZ8. Four isolates DZ1, DZ5 and DZ6 were positive for catalase test and all the isolates produced gas when fermented with glucose, two of them with lactose and four with xylose. (TABLE.1.).

**Table 1:** Biochemical characterization of isolated Bacterial strains

| Parameter/Strain | DZ 1 | DZ 2 | DZ 3 | DZ 4 | DZ 5 | DZ 6 | DZ 7 | DZ 8 |
|---|---|---|---|---|---|---|---|---|
| Grams staining | + | + | + | + | + | + | - | - |
| Catalase | + | - | - | - | + | + | - | - |
| Fermentation with glucose | + | + | + | + | + | + | + | + |
| With lactose | - | + | - | - | - | + | - | - |
| Xylose | + | - | - | + | + | + | - | - |

### 3.1. Growth kinetics

All the 8 isolates were subjected for growth kinetic studies to identify the phases especially the log phase. All the isolates were shown the maximum growth from 6hrs to 18hrs (log phase from 6-18hrs) and they reached their stationery phase after 18hrs (Fig.1.).

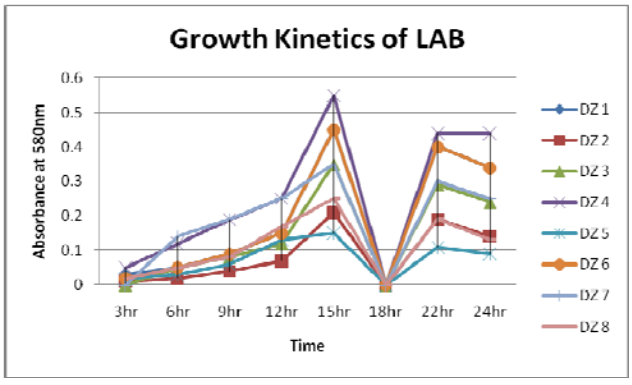


**Fig 1:** Growth kinetics of different Lactobacillus strains isolated from soil samples.

### 3.2. pH and Salinity tolerance

The effect of acidity on the viability of all isolates was assessed by their growth in different incubation pH in MRS broth. All the 8 isolates selected were incubated in different pH MRS broths for 2hr. Among the isolates DZ4 reported high tolerance in all the pH concentrations. (Fig.2.). Resistance to low pH is one of the major selection criteria for probiotic strains [27], because high acidity in the stomach, high concentration of bile components in small intestine of host can influence the probiotic strains selection especially when the probiotic is to show its effect in the gut of shrimp [28]. In the present study all the six isolates were resistant to low pH for 1hr and 6hrs time duration. Jatindra *et al*. [29] selected 55 acid tolerant strains of LAB in PBS buffer PH-2.5 for 3hrs [30] also reported the *Lactobacillus rhamnous* strain survival at pH-3.0 after 2hrs period.

All the selected eight isolates were incubated at different salinities of 0,5,10,20, 25 and 30 for 24hr. High bacterial tolerance to salinity was observed up to 20% but growth in all the strains considerably decreased 25 to 30%. Among all the isolates maximum tolerance to different salinities was conferred by DZ4 whereas the minimum tolerance was reported in DZ7. (Fig.3.)

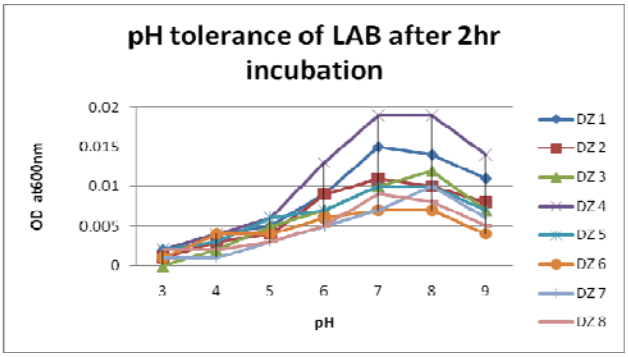


**Fig 2:** pH tolerance of different *Lactobacillus* strains isolated from soil samples.

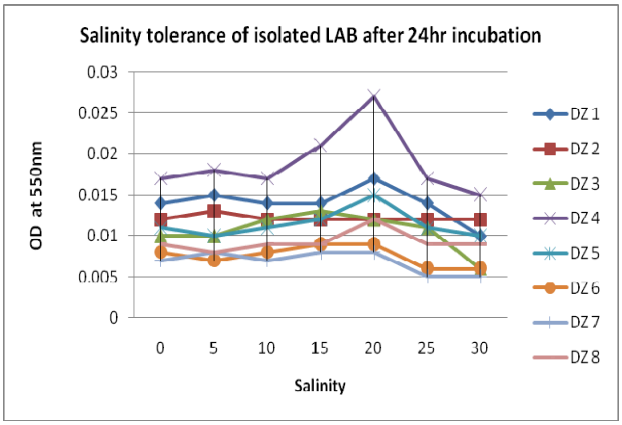


**Fig 3:** The salinity tolerance of different *Lactobacillus* strains isolated from soil samples.

### 3.3. Bile tolerance

Bile salt tolerance is one of the criteria to select any microbial strain to be used as a probiotic culture. The presence of bile in the intestine affects the viability of *Lactobacillus.* All eight isolates were grown in different concentrations of bile salt and the results showed that only three isolates were resistant to bile salt. Maximum resistance was conferred by DZ4 (0.017, 0.014, 0.014, 0.012, 0.012) and minimum was conferred by DZ3 (0.01,0.01,0.009,0.003,0.002) respectively at 0.15%, 0.2%, 0.25%, 0.3% and 0.35% (w/v) of bile salt.(Fig.4.) Resistance against bile salt and survival in gastric juices at pH 3 and pH 8 is next important criteria for colonization and metabolic activity [31]. The mean bile salt concentration of human or animal gastrointestinal tract is about 0.3% so it is considered as high enough and critical to screen for resistant bacteria strain [32]. According to Maragkoudakis *et al*. [33] all isolated strains tolerated 0.3% bile salts concentration in 4hrs. The results of the present study showed that all six isolates were resistant to low pH.

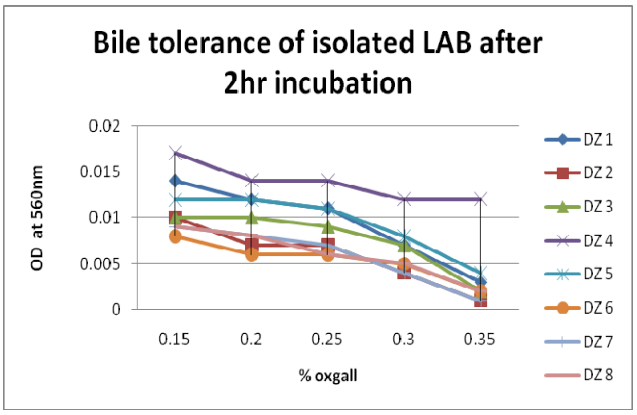


**Fig 4:** Bile tolerance conferred by isolated *Lactobacillus* strains from soil samples.

### 3.4. Survival rate in gastric and intestinal juices

The survival of *Lactobacillus* in low pH of stomach is important for bearing the intestinal acid stress. Among the resistant isolates at both pH 3 and pH 8 of gastro intestinal juices the maximum survival rate was reported with DZ4 (at pH-3, 73% and at pH-8, 89% respectively. Whereas the minimum survival rate was reported with DZ6 at pH-3 i.e.,50% and with DZ7 at pH-8 i.e., 62.5%. (Fig.5.)

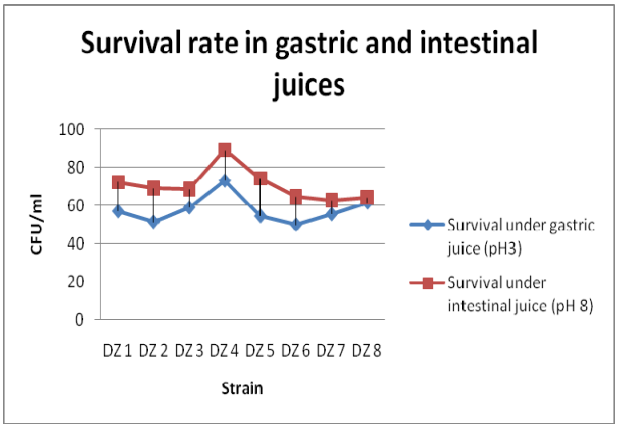


**Fig 5:** Graph showing the survival rate of isolated *Lactobacillus* strains in gastric and intestinal juices.

### 3.5. Cholesterol assimilation

The isolated DZ4 showed maximum resistance with bile salt at 0.15 concentrations (w/v) with salinity at 20, with pH at 7 to 8 and at an incubation period of 15hrs showed more growth and shown to be positive when tested for all biochemical parameters and was selected for further identification and characterization. (Fig.6.) A good probiotic bacterium should have cholesterol reduction efficiency. M.Bilige *et al*. [34] isolated 30 *lactobacillus* strains, MG2-1 have high cholesterol removal rate (51.74±0.04%). According to Nag pal *et al*. [35] probiotic have many health biological properties; one of them was anti-cholesterol assimilation because elevated levels of certain blood lipids are a greater risk for cardiovascular disease. Lavanya *et al*. [36] found that *L.brevis*has ability to reduce the cholesterol level up to 80% in 24hrs. Another report showed lactic acid bacteria can reduce the serum cholesterol level up to 50% in the presence of bile salt in 48 hrs. [37].

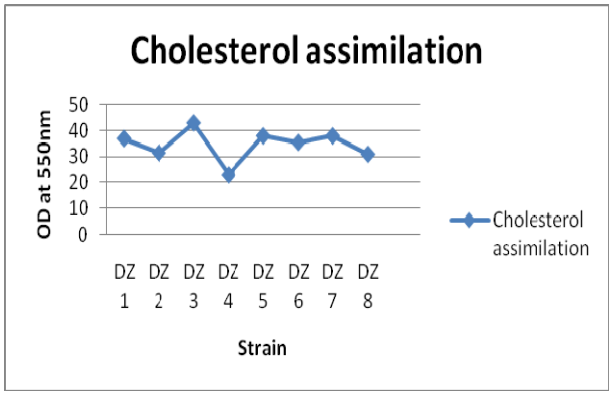


**Fig 6:** Cholesterol reduction efficiency of isolated *Lactobacillus* strains

### 3.6. Molecular Identification of LAB

All the eight bacterial samples were subjected for molecular characterization. Of the 16s rRNA gene using the specified primers and conditions mentioned in the materials and methods section. Bacterial DNA samples isolated were found to have the V2-V3 sequence of *lactobacillus* which is of approximately 200 bp fragment (Fig.7). All the screened samples were shown to be amplified for V2-V3 region except DZ1 and DZ6. They failed to amplify the V2-V3 regions. Similar results were obtained by *L. plantarum L. fermentum, L. sakei* by Svetoslav *et al*. [38] during the evaluation of

*Enterococcus mundtii* ST4V (a potential probiotic and bacteriocin-producing strain), during its survival in commercial boza.

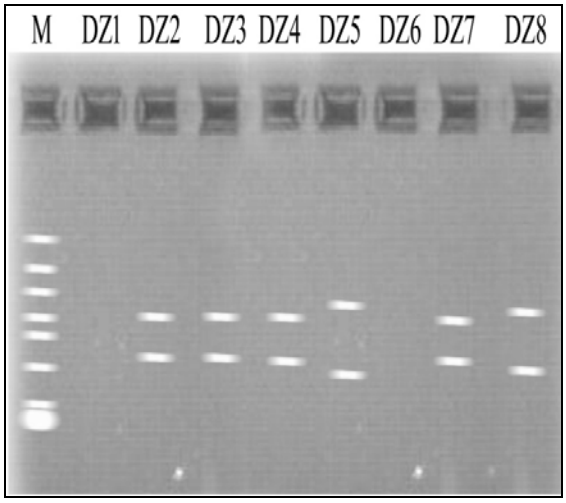


**Fig 7:** Molecular identification of LAB

**Summary**

The massive use of antimicrobials for disease control and growth promotion in animals increases the selective pressure exerted on the microbial world and encourages the natural emergence of bacterial resistance. Several alternative strategies to the use of antimicrobials in disease control have been proposed and have already been applied very successfully in aquaculture. The introduction of microbial control practices by means of probiotics may have a beneficial effect on the cultures in hatcheries. In this context we have selected coastal areas of Gudur division where the white shrimp (*Litopenaeus vannamei*) culture is prevalent, for isolation of soil samples to identify the bacteria that have probiotic potential. Present study reveals that the soil of above coastal areas are rich in Lactic acid bacteria, which act as natural probiotics. Various biochemical and molecular tests conducted on the selected 16 soil samples confirms that DZ4 strain fulfill all the qualities that probiotic must have. Hence DZ4 was selected for further investigation to control diseases and promote growth and immune capacity in shrimp culture ponds.

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