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In vivo characterization of probiotic organism isolated from coconut toddy using ornamental fish, Black molly

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ABSTRACT

Toddy which is considered as tropical alcoholic beverage was procured from Uhukottai area of Tiruvallur (Dist), Tamilnadu, India during early morning from the tender fronds of the inflorescence of Coconut (Cocus nucifera) plant and was employed for the probiotics to analyze their antibacterial potency. Six different bacterial colonies were isolated from the toddy, out of which, a single strain had shown good antagonistic properties against selective human pathogens. The strain was further identified by its 16S rRNA sequence analysis and revealed the microbe is phylogenetically related to the members of genus, Bacillus subtilis sub sp. *Further the organism was checked for its intestinal colonization activity by in-vivo analysis using ornamental fish (Black Molly) by microbial plating of intersecting intestine.*

Key words: Toddy, 16S r RNA, *In-vivo* study, Black Molly

INTRODUCTION

Toddy is an alcoholic beverage created from the sap of various species of coconut trees. It is well known by various names in different regions and is common in parts of Asia, Africa the Caribbean and South America. The toddy sap can be consumed in different ways, either in an unfermented form (sweet toddy/ *neera*) or in fermented form (wine/toddy) or it may be consumed in the form of processed *jaggery*. The whole fermentation of toddy is an uncontrolled spontaneous fermentation process occurs initially from the lower part of inflorescence into the pot itself. The young inflorescence is tightly bound with twigs and beaten with a weighted wooden mallet, morning and evening, for a number of days. This method is collectively known as tapping. After this process a clear inflorescence sap is protruded out and to this oozed out thing a clear cut is made to obtain sap in an earthenware pot and from this step onwards fermentation process take place. High quantity of yeast and bacteria makes toddy a milky flocculent appearance called as toddy. Probiotics are the beneficial organism which plays a major role health benefit in the host like inducing the prevention of proliferation pathogens, suppressing the production of virulence factors of pathogens by stimulating immune system of the host [1, 2]. Toddy consist of variety of Gram positive bacteria which includes Bacillus, Lactobacillus, Streptococcus which produce the lactic acid from sugar with a process called fermentation.

A good probiotic culture should pass through the upper digestive tract must be capable of surviving and growing into the intestinal extreme condition and should maintain its viability and activity in the carrier food before consumption [1]. In the present study, collection of toddy was done from various coconut trees from Uthukottai area of Tamilnadu. It seems that the probiotics present in the toddy adhere to the intestine wall and extends its beneficial effect to the host thus it should have a good adhesive property. This adhesive assay for the intestine was performed

in vivo by using ornamental fish Black molly (*Poecilia sphenops*) against *Vibrio* sp. They were also checked mortality rate by dissecting their intestine and also by plating method employing fish tank [3].

MATERIALS AND METHODS

Selection and isolation of probiotics

Around 43 samples of fermented coconut toddy were collected from different areas of Uthukottai, Tamilnadu. The selection was performed by screening their probiotic properties based on possible biological barrier [4] such as bile salt, tolerance to pH at various concentrations which is similar to human intestinal condition. Among the entire isolated organisms, two organisms exhibit good probiotic characters and further one organism was chosen for the present study [2].

16S rRNA genome sequence of isolated organism

From the six isolated organisms, one organism **K3** was taken for genome sequencing using Gen Blast. For the DNA sequencing initially preparation of template DNA is very important thus an isolated test organism in pure form was taken and suspended in 0.5ml of sterilize saline and were centrifuged at 10,000 rpm for 10 minute. After this process, pellet alone was carried for next step by discarding the supernatant. 0.5ml of Insta Gene Matrix (Bio-Rad, USA) was mixed with the pellet, and they were incubated for 56℃ for 30 min and heated at 100℃ for 10 min. After heating, supernatant alone was transferred to PCR.

PCR amplification

1µL of template DNA was mixed with 20 µL of PCR solution using along with the Primer – 518F/800R for the bacteria. 35 amplification cycles was done at 94℃ for 45 sec, 55℃ for 60 sec, and 72℃ for 60 sec. After the amplification, 1400bp was isolated using along with the *E. coli* genomic DNA as positive control. For purification, Montage PCR Clean up Kit (Millipore) was used for removing unincorporated PCR primer & dNTPs from PCR instrument.

Sequencing of 16S rRNA

Sequencing was done by the purified PCR products of approximately 1,400 bp by using the primers (785F 5' GGA TTA GAT ACC CTG GTA 3' and 907R 5' CCG TCA ATT CCT TTR AGT TT 3'[Ma1]) Sequencing was performed by using Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system service provided at Applied Bio Systems, USA.

In-vivo analysis

Analyzing the effect of probiotic adhesive property on infected fish

Probiotic test organism (K3) was pre incubated at 37°C for 24 hours in nutrient broth and pathogenic organism *Vibrio* sp. (obtained from Tamil Nadu Fishery University) was also pre incubated at TCBS broth 37°C for 24 hours [5]. Four fish tanks were taken and they were marked as Tank 1, Tank2, Tank3 and Tank4.

1. Probiotic organism (K3)
2. Pathogenic organism (Vibrio sp.)
3. Probiotic organism (K3) + Pathogenic organism
4. Uninoculated fish tank

} All the fish tanks were grown by *Black molly* fish

In Tank 1, probiotic organism (K3) was added as fish feed soaked into the commercial available fish feed. A test tube containing probiotic organism was taken, into which commercial available fish feed was added and kept for 5 min to soak and they were taken as probiotic feed (Fig 2).

In Tank 2, 0.5ml of pre incubated pathogenic *Vibrio* sp. organism was added into the tank and this tank was considered to be negative control for this study.

In Tank 3, the fish was made to infect by addition of pathogenic *Vibrio* sp. and they were checked for their mortality against the pathogens by using probiotic organisms as a feed and the mortality was confirmed by dissection the intestine of fish. The intestine were squeezed and washed in saline and the saline was added into the respective

selective medium for identifying the viability of probiotics and pathogenic organisms and they were also cross checked by the viability of the number of fish against the pathogens, the last tank 4 was maintained as positive control for the study.

RESULTS AND DISCUSSION

During the isolation probiotics organisms, six bacterial colonies were observed on nutrient agar plate, each individual organism was maintained separately on nutrient agar slant in pure form. Each organism was screened for its probiotic quality by exposing the organism to various human physical barrier conditions such as tolerance to acidic pH, bile salt and sodium chloride [2, 6]. Among this probiotic survival characterization, one organism namely **K3** showed a great tolerance and this organism was proceeded for indentifying them at molecular level characterization and identifying its adhesive property towards the intestine by *in vivo* using ornamental fish intestine and also in the indirect form by taking the water from the fish tank.

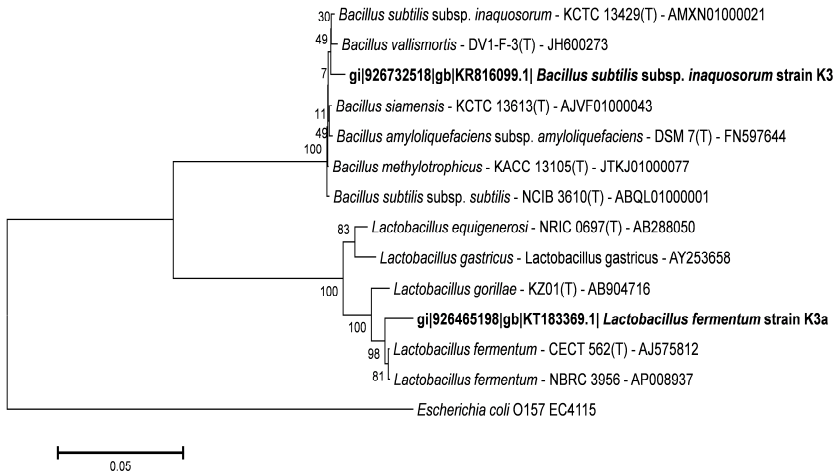
Genome sequence

In the present study the bacterium which was isolated from the natural coconut toddy and showed good probiotics were subjected to identification and classification based upon the genomic sequencing. The DNA was isolated and amplified in PCR and are further characterized by 16s rRNA sequencing by using the primer shown in Table 1. The isolated organism was identified, submitted and latter published in National Council for Bio Informatics (NCBI) under the accession number KR 816099. The Phylogenetic tree was constructed and the organism was identified as *Bacillus subtilis* sub species *inaquosorum* strain as **K3.** Phylogenetic Tree of *Bacillus subtilis* sub sp. *inaquosorum* strain **K3** is given in Fig 1.

Table 1: Primer used for sequencing

518F	5' CCAGCAGCCGCGGTAATACG 3'
800R	5' TACCAGGGTATCTAATCC 3'

Fig 1: Phylogenetic Tree of *Bacillus subtilis* sub sp. *In aquosorum* strain K3



Adhesive property – *In vivo* study

A probiotic should have the good tolerance level against the human physiological barrier and also should have the adhesive property into the intestine for establishing its beneficiary towards the host. For analyzing the adhesive property of probiotics, fish feeds soaked into pre inoculated broth and provided for fish culture (Fig 2). The ornamental fishes were made to infect by using a pathogenic *Vibrio* sp. and the mortality against the pathogen was confirmed based upon the viability of the number of fish in the tank, this showed the probiotic organism K3 have an antimicrobial property against pathogen (Table 2).

The adhesive property of the probiotic organism was confirmed by intersecting the live fish intestine which withstood from the vibriosis (Fig 3 a, b). The intestine was squeezed into the saline and cultured. When compared

with the negative control, probiotic had the largest number, and they minimized the growth of number of pathogens based on antimicrobial activity (Fig 4 a, b; Table 3 a, b).

Fig 2: Fish feeds soaked into pre inoculated broth

Fig 3: Dissection of live fish intestine for vibriosis analysis

a **b**

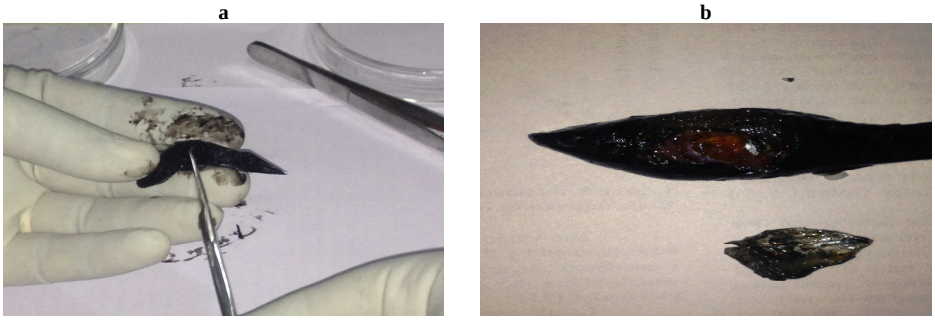


Fig 4: Intersecting the live fish intestine which withstands from the vibriosis

a **b**

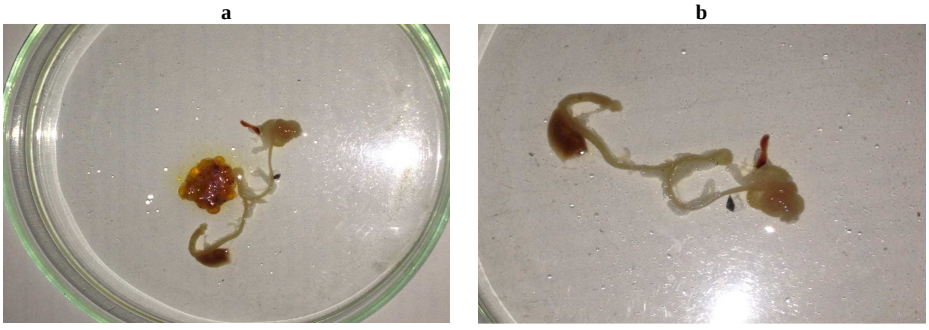


Table 2: Mortality rate of fish and the number of days survived

Fish	Organism	No. of days survived
Black molly	*Bacillus* sp. (K3)	10
	Vibrio sp. (Pathogen)	3
	Bacillus sp. + *Vibrio sp.*	7

Table 3a: Microbial count of intersected intestine with pathogen alone

Source	*Vibrio sp.* (Pathogen)
Intestine	3.8×10^4 CFU/ml
Water	1.0×10^{10} CFU/ml

Table 3b: Microbial count of intersected intestine with pathogen and probiotics together

Source	*Vibrio sp.* (Pathogen)	*Bacillus sp.* (K3)
Intestine	7×10^1 CFU/ml	8×10^3 CFU/ml
Water	17×10^5 CFU/ml	82×10^4 CFU/ml

CONCLUSION

In the present study, the bacterium was isolated from coconut toddy, tested for its probiotic characterics, identified as specific strain at genus/species levels and also checked for its adhesive property towards the fish intestine by *in-vivo*. The isolated strain was identified as *Bacillus subtilis* sub sp. *inaquosorum* strain **K3.** The *Bacillus subtilis* strain was found to have good adhesive property towards the intestine and also exhibited good antimicrobial activity against the pathogens by reducing the number of organisms in the culture.

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