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Research Article

Rejuvenating the Gut of Antibiotic Incited Zebrafish (*Danio Rerio*) by Probiotic Soil Isolates Persuaded by *Subtilisin* as Endogenous Protease Inhibitors

Arockiya Anita Margret[1*], S Aishwarya[2], A Shree Parkavi[1], V Swetha[1], T Suvedha[1] , G Sandhya[1]

[1]*Department of Biotechnology and Bioinformatics, Bharathidasan University, Bishop Heber College, Tiruchirappalli-620017, Tamil Nadu, India,* [2]*Department of Bioinformatics, Stella Maris College, University of Madras, Chennai- 600086, Tamil Nadu, India*

ABSTRACT

Antibiotics are extensively used as therapeutics against various human and veterinary ailments. It is also used to promote livestock growth, which can be hazardous by its dispersion in water bodies and lead to aquatic toxicity. Antibiotic-associated diarrhea (AAD) is a common complication caused due to the adverse effects of antibiotic administration. It is necessary to have a better understanding on consumption of probiotics, possessing natural endogenous protease inhibitors and its mechanism to target protease from gut pathogens. *Subtilisin* are group of proteases originated from *Bacillus* spp and a molecular docking study revealed its inhibitory potential (-1.90 KJ/Mol) against a target protein *Clostridium difficile* toxin. The bacterial isolates from the vegetative soil were identified as *Aneurinibacillus migulanus* and assayed for probiotic efficiency. The production of *subtilisin* from the distinct soil isolate was quantified (0.961 mg/ml and the enzymatic activity with a yield of 241.1 U/ml) and detected by HPLC studies. The probiotic efficacy of the isolates and the validation of *subtilisin* as enhancers were authenticated on antibiotic incited zebra fish models. The LC50 and histopathological studies attested both acute toxicity and restoration level of extracted protein. Hence this study validates the soil isolates as probiotics and *subtilisin* as endogenous protease inhibitors.

Keywords: Antibiotics; Aquatic toxicity; *Clostridium difficile* toxin; *Aneurinibacillus migulanus*; Probiotic; *Subtilisin*

INTRODUCTION

The usage of antibiotics is effusively used in treating various diseases and alternatively, its bioaccumulation is considered as a threat in the ecosystem. In particular, aquatic resources are extensively affected by their application to improve livestock and treating diseases [1]. Antibiotic-associated diarrhoea (AAD) occurs due to the adverse effects of antibiotic administration and considered as a communal impairment that develops severe gastrointestinal diseases [2]. 5%-30% of patients are widely affected with a relative increase in accordance to the elevated rate of antibiotic spectrum [3]. Orally administered antibiotics are more relatively associated with AAD than the parenteral antibiotics. Cephalosporin, clindamycin, and broad-spectrum penicillin, especially amoxicillin/clavulanate in children, increase the relative risk of AAD [4]. The severity of AAD increases when a pathogenesis is mediated with *Clostridium difficile* is globally recognized as the significant nosocomial enteric pathogen [5]. However, in India the prevalence of this infection among hospitalized patients ranges from 4% to 21% and requires tremendous diagnosis and treatment [6].

For ages probiotics (living microorganisms) are supplemented to restore gut health and have been implemented with exceptional immune modulatory properties. Generally, probiotics are given in conjunction with antibiotics for preventing AAD [7]. *Bacillus* spp is attaining much concern in human health and associated with functional food research that can enhance tolerance and survivability in defensive environment like gastrointestinal tract [8]. Besides the general probiotic strains (*Lactobacillus* and *Bifidobacterium* spp.), *Bacillus* spp. has not extended much credential commercially. This may be due to the disordered concepts in shunting the organism between a beneficial probiotic *versus* harmful pathogen.

Correspondence to: Arockiya Anita Margret, Department of Biotechnology and Bioinformatics, Bharathidasan University, Bishop Heber College, Tiruchirappalli-620017, Tamil Nadu, India, Tel: 09159626250; E-mail: anitamargret@gmail.com

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Hence, it is imperative to understand the microbe's mechanism and range of toxicity which would substantiate the productivity in the health and pharmaceutical industry. *Subtilisin* isolated from *Bacilli spp.* is considered to be highly resistive to physiological conditions such as temperature and acidity. The inherent abilities of producing large number of secretary proteins, enzymes, antimicrobial compounds, heighten the organism as probiotic. It is forecasted that B. *subtilis* protease has the ability to process immature *subtilisin* and activate them as an effective antimicrobial agent [9]. These study efforts to focus on the stimulating effect of *subtilisin* to intensify the soil isolates of *bacillus spp.* as efficient probiotics.

MATERIALS AND METHODS

Isolation and molecular identification of the soil isolates

The soil sample was collected from the garden of Bishop Heber College Tiruchirappalli Tamil Nadu, India. Preliminary identification of the soil isolates was based on the Bergey's Manual of Systematic Bacteriology [10] and confirmed by 16S rRNA amplification.

The sequencing was performed as described by [11] using Gene Amp PCR System 9700, Applied Biosystem. (Primers - forward: 16S-RS-F CAGGCCTAACACATGCAAGTC and reverse 16S-RS-R GGGCGGWGTGTACAAGGC). The 16S rDNA sequence was deposited in the NCBI Gene Bank nucleotide sequence database under accession numbers GQ280054.1 and LC110197.2.

Probiotic assessment

The probiotic properties of the isolated strain were determined by analysing its tolerance to sodium chloride and low-pH. The tests were performed in accordance to the protocol of [12].

Extraction and characterisation of crude subtilisin

The crude protease extraction was processed in accordance to [13]. *Subtilisin* isolated from *Bacillus sp.* is considered to be extracellular; the intracellular constituents are also validated in this study. Thus, the supernatant was used as an enzyme source and quantitated for the protease activity and protein estimation. Lowry's method [14] was adopted by using BSA as the standard and the biochemical *assay* (Folin reaction) determined the total level of *protein.* The enzyme activity studies were based on the conventional Anson method [15]. The activity of protease was measured spectrophotometrically at 280 nm by the detection of aromatic amino-acids released from casein as a substrate.

Hplc analysis

Detection of *subtilisin* was validated with HPLC run (Perkin Elmer Corp.Norwalk,U.SA) equipped with UV detector. The test sample (crude extract) was applied to C-18 column with an injection volume of 10 ml. 59% of Acetonitrile and 0.1% Trifluoroacetic acid was used as an eluent at the flow rate of 1 ml min−1 and the absorption was measured at 220 nm.

Acute toxicity tests using Zebra fish *(Danio rerio)*

The crude protein extract was tested against adult zebrafish with a test time of 96 hours. The test was carried out according to Organization for Economic Co-operation and Development (OECD) Test No. 236; 2013.The wild-type characteristics of the fishes were assayed as described by Singh and Nüsslein-Volhard (2015).

Rearing and maintenance of experimental animals

Adult zebra fishes were obtained from a commercial dealer and 10–15 nos. were kept in 5 litre acrylic tank with the following conditions; 28.5°C, with a 14/10 h light/dark cycle. The zebra fish were 2-3 months old, weighed 0.5 ± 0.2 g and their mean length was 30 ± 4 mm. The animals were fed three times per day, with commercially available dry fish food. The fishes were allowed to acclimatize to the laboratory conditions for two weeks before the experiment. Fishes weighing close to 0.4 g were selected for the experiment.

Determination of LC50

Different concentrations of 100,250,500, 750, and 1000 µg/mL of the crude extract were tested in order to obtain the median lethal concentration (LC50). Each concentration was performed by triplicate, using 10 animals per assay. The exposition time with the different solutions was 96 h.

Assessment of weight gain

The Effects of antibiotic treatment on weight gain and mortality rate with (different concentrations 100,250,500 750 1000µl of crude protein)and without the administration of protein extract was studied on adult zebra fish (at a ratio of 2% of body weight/ twice per day) by following the protocol of [16] and their weights were measured prior and after the exposure .0.05 g of Amoxicillin was weighted and mixed with 100 g of feed components (5 g/kg diet) .After a week of acclimatization, the animals were randomly divided into three groups as follows: control, Amoxicillin diet, Amoxicillin treated with different concentrations of crude protein extract. The weight of the fish in each tank was recorded after a week's progress of the experiment, and the expanse of faecal matter was observed.

Monitoring of mortality and histopathological examination

Mortality was monitored continuously, and the fish were considered dead when the movement of the operculum and response to mechanical stimulation could no longer be detected. The statistical differences between the treatment and control groups at the same time point were analyzed using the least significant difference (LSD) test and $p<0.05$ was regarded as statistically significant.

The of intestinal segments of sacrificed fishes were investigated for histopathological assay. Thin section of 7 µm thickness was prepared from paraffin blocks and the changes were examined microscopically.

Molecular docking

The 3D structure of *Clostridium difficile* Toxin a (TcdA) and *Subtilisin* were downloaded from PDB with specific ID numbers (ID-4R04; 1a1y). The target protein structure was docked against the subtilisin using PATCH/FIRE DOCK web server to check its interaction and binding.

RESULTS

Molecular identification of the bacterial isolate

The colony isolates were initially identified by preliminary studies (Table 1) and later endorsed by 16s RNA sequencing (Figure 1 and Figure 2). The processed sequences were submitted in public data base of NCBI (Gen Bank Id: MT509984.1; MT509985.1).

Table 1: Morphological and biochemical attributes of isolated bacteria

Colony morphology	
Configuration	Circular
Margin	Wavy
Elevation	Flat
Surface	Smooth
Texture	Rough
Pigment	White-Creamy
Opacity	Opaque
Gram's staining	+
Cell shape	Rod
Biochemical Tests	
Catalase	+
Citrate	+
Gelatin hydrolysis	+
Gram staining	+
Indole	_
Methyl red	+
Nitrate reduction	+
Voges Proskauer	_
Triple sugar agar	+
Casein hydrolysis	+
Starch	+
Glucose	+
Sucrose	_
Lactose	_

Legend: '+': Presence; '–': Absence

The DNA sequence was aligned and further analysed for its homology which was performed by using BLAST tool. The NCBI homology BLAST showed a 99.87% similarity to Aneurinibacillus migulanus BJ-44 and Aneurinibacillus sp. YR247 (Figure 1).

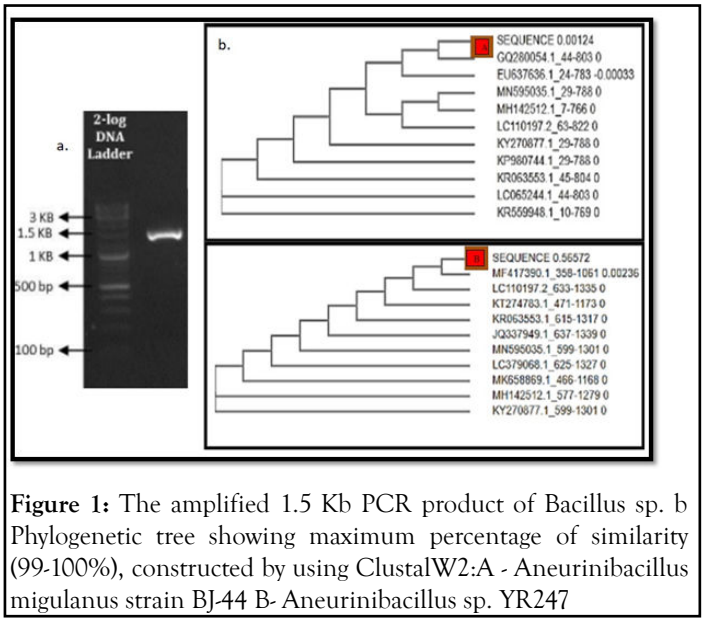

Figure 1: The amplified 1.5 Kb PCR product of Bacillus sp. b Phylogenetic tree showing maximum percentage of similarity (99-100%), constructed by using ClustalW2:A - Aneurinibacillus migulanus strain BJ-44 B- Aneurinibacillus sp. YR247

Evaluating the probiotic attribute of the soil isolates

In vitro evaluation of the probiotic characteristics based on gastrointestinal tract tolerance was determined at low pH and high salt concentration. The results were quantitated based on survival rate of the bacterial isolate as visible growth (Table 2). The soil sample procured from garden, survived in low-pH (2.5-2hrs) and high salt (8% -4hrs) conditions.

Table 2: Tolerance of soil isolates towards Acidity and Salinity

Pararameter	Ph2.5		Ph 3		Ph 7.0		3.5 0% Na cl		6.5 0% Na cl		8% Nacl	
Garden soil isolate	2hr	4hr	2hr	4hr	2hr	4hr	2hr	4hr	2hr	4hr	2hr	4hr
	+	-	++	+	+++	+++	++	+++	++	++	++	+++

Legend: '+': Tolerant; '-': Non tolerant

Extraction and characterisation of *Subtilisin*

Anson's method deduces the enzyme activity where, one unit of enzyme activity is defined as the amount of enzyme required to liberate 1 µmol of tyrosine per min under the defined assay conditions.

Enzyme units were measured using tyrosine (0-100 µmole) as standard and Bacillus sp showed higher enzymatic activity (241.1 U/ml) on casein induced nutrient agar medium.

Subsequently in accordance to coefficient factor the concentration of protein was found to be 0.961 mg/ml. The HPLC chromatogram (Figure 2) shows seven separated fractions of the crude extract where a definite peak with an increased height and area was found at a retention time 3.5 min.

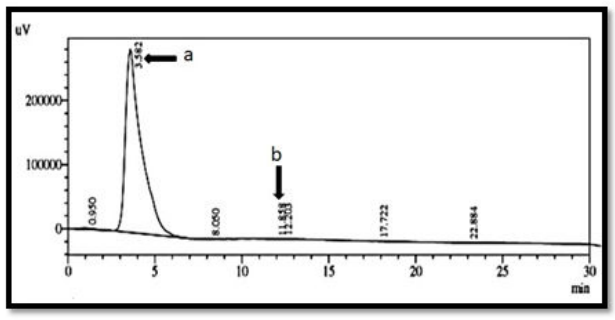

Figure 2: Chromatogram of Intracellular Derived Crude Protease Extract a. Transient region of Protease Activity b. Prosubtilisin.

This study presents a chromatogram which has similar retention peaks ranging from 11–17 min and a distinct pro-*subtilisin* peak was inferred at 11.858 min.

Effect of crude protease extract on antibiotic induced zebra fish

The acute toxicity of the isolated crude protein extract was assessed through an *in vivo* LC50 study. The estimated LC50 value was calculated as 616.59 µg/mL (Figure 3).

An acute toxicity study was performed on zebra fish for 96 hours (Table 3). The influence of antibody was experimented on zebra fish and the body weight of six groups was detected. The results showed that antibiotic treated groups with crude extract had higher weight gain than the control group.

Although a very minor differences were found in the weight advancement a continuous improvement in the body mass was observed. The experiment summarized an effective partaking of the extracted protease in the highest concentration significantly with contributing increased body weight than the control group ($p<0.05$). (Figure 3). Therefore, this study illustrates the therapeutic significance of the intracellular protease extract as a potent to act against AAD.

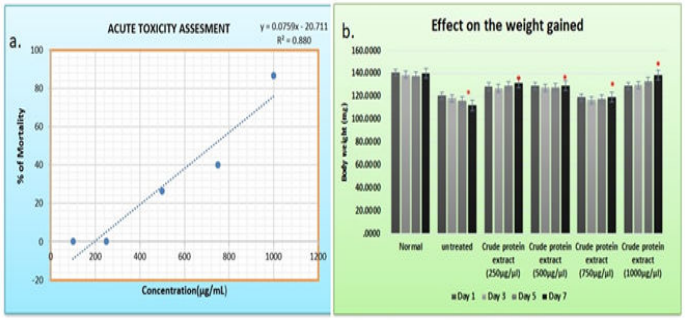

Figure 3: Determination of LC 50 Values

Table 3: Percentage of animal death with the varied concentrations of extracted protease on D. rerio

Concentration of the crude protein extract	100 µg/mL	250 µg/mL	500 µg/mL	750 µg/mL	1000 µg/mL	Control
No of animal deaths	0	0	1	2	4	0
Percentage (%)	0	0	20	40	80	0

The mortality rate of zebra fish relatively with the antibiotic induced was relatively low.

The histopathology studies compared the normal gut sections with those of the treated and untreated groups. Figure 4 depicts the typical intestinal structure which comprises of villi (V) that is surrounded with muscle layer (ML), Mucus secreting goblet cells (GC) and enterocytes (EC).

When exposed to antibiotics the entire intestine is distorted the Figure 4 shows damage occurred in intestinal mucosa which hinders the cellular development. Vacuolization (VE vacuolization of the enterocyte) is a predominant feature in the antibiotic induced groups that is accompanied by inflammation and edema.

This ultimately leads to cell necrosis (N) and is substantiated with hyperplasia of goblet cells (HGC) and detachment of the epithelium (DEL).

The treated with group with crude protein extract showed a high degree of restoration (Figure 4). There was a prominent feature of villi with distinguished goblet cells and the enterocytes.

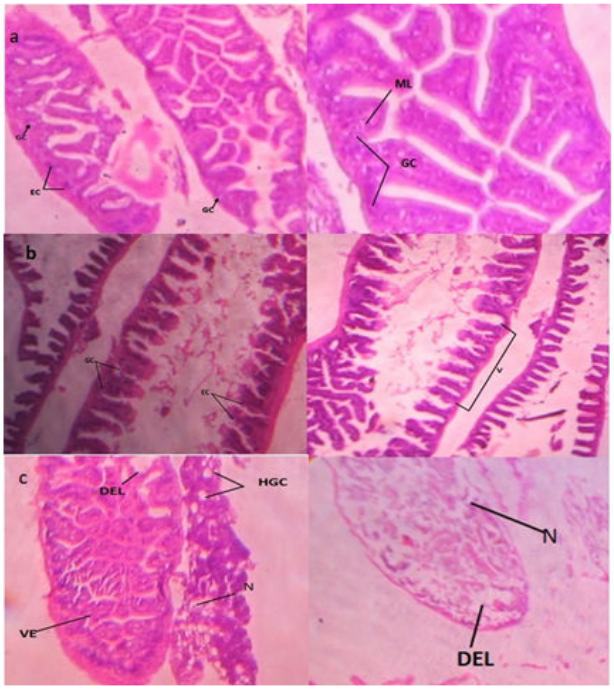

Figure 4: Histopathological studies to compare the normal antibiotic induced and treated intestine tissues a. Healthy (Normal) tissue, where EC, enterocytes; GC, goblet cells; ML, muscle layer. b. Antibiotic induced and treated intestine tissue GC, goblet cells; EC, enterocytes; V, Villi c. Antibiotic induced HGC, hyperplasia of goblet cells; VE, vacuolization of the enterocytes; N, necrosis; DEL, detachment of the epithelium (H&E, ×40).

Protein – protein docking

The molecular docking studies were carried out between the 3D model of *subtilisin* against AAD causing pathogenic toxin [*Clostridium difficile* Toxin A (TcdA)]. Here, the protein *subtilisin* was considered as ligand and its interaction was found to be -1.90 KJ/Mol, after minimization. The docking was performed by the webserver Patch Dock and Fire Dock. The initial process was performed using Patch dock the obtained results were then refined in fire dock. According to the position and number of amino acids the docking positions were configured and Figure 5 shows the docked pictorial representation which indicates the inhibitory potential of serine protease.

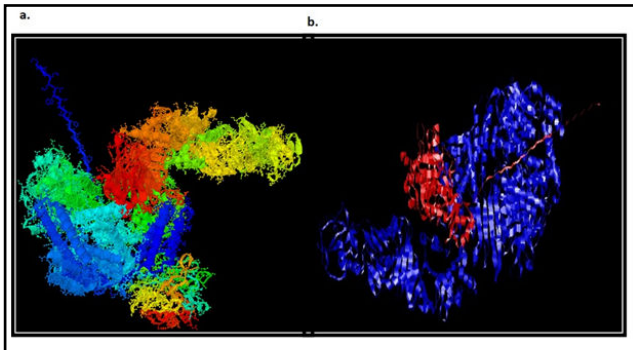

Figure 5: Pictrorial representation of the docked proteins a. The best docked pose b. differentiating the proteins as target (Blue) and ligand (Red).

DISCUSSION

The present study was carried out to evaluate the production of *subtilisin* from a soil borne *Bacillus* species. The species identification of bacterial isolates was authenticated by molecular characterization studies such as 16S rRNA amplification and sequencing [17]. The *soil* isolate depicted a sequence (>99%) similarity with *Aneurinibacillus migulanus* which is commonly called as *Bacillus brevis*. It is reported that *Bacillus* species is one of the dominant bacterial species in soil [18] experiencing much genetic diversity. The heterogeneity of *Bacillus brevis* has been indicated by the wide varied range of growth temperatures [19]. This has resulted in numerous classifications [20]. A profound phenotypic heterogeneity of this strain has out come to the presence of genetically unrelated strains [21]. The soil isolate retained the viability when exposed to low acidic environment (pH values of 2–3) and exhibited high sodium chloride tolerance.

The ability of the isolate to survive and grow in the high concentration of salt and extreme pH can confer it to be a potential probiotic that can endure the harsh gastric conditions. As per the report of [22], *Lactobacillus* isolates had tolerance to acid at pH 3.0, for 3 h. Conversely, *L. plantarum* strains isolated from fermented olives have been reported to survive for a period of 2–6 h at pH 2.0 and pH 3.0 [23]. *Subtilisins* is classified as a proteolytic enzyme which is ubiquitous in occurrence, and are essential for metabolic activities such as cell growth and differentiation. The studies of [24,25] reported that *subtilisin* derived from *B. licheniformis* enhanced the growth and viability of *Lactobacillus reuteri* and *Lactobacillus acidophilus*. *Bacillus subtilis* was reported to have proteolytic enzymes (alkaline serine protease, metalloprotease, and esterase). They are produced during the sporulation phase, possessing high esterolytic but low proteolytic activity [26] and thus play a significant role stimulating the biological process. The HPLC profiles of the crude isolate evidenced transient elution peak of *subtilisin*-propeptide complex and rationalizes the formation of active enzyme. The distinct peaks obtained in the study where in accordance with [27,28].

The *in vivo* studies were designed to assess the acute toxicity of the extracted protein from the soil isolates on zebra fish models which are very sensitive to the external changes in environment caused by different toxicants. The induced concentration of the crude extract has an influence on the AAD and showed significant difference. Studies have showed that antibiotic treatments cause oxidative stress, which also related to the impairment of normal immune functions [29].

This is a novel effort to challenge subtilisin and its protease derivate to combat against AAD. The histopathological examinations also substantiated the disturbed intestine of the animals when exposed to profound dosage of antibiotics. This can impair the mucous layers and interrupt the cellular formation and hamper the cellular development in this tissue, causing a disturbance in its physiology evidenced by histological changes. The muscular layer is also disturbed that can affect the of nutrient absorption and often precede necrosis [30]. The treated samples elucidated the restoration capacity

which depicts the morphology of villi with distinct globular cells and enterocytes. The Molecular docking studies has changed the perspective of *subtilisin* as a lead molecule and remedy towards human ailment rather than being a target to be inhibited. This study serves to be unique in facilitating the significance of *subtilisin* and its derived serine protease [31].

CONCLUSION

The use of antibiotics for anti-infection and growth promotion has caused a menace leading to several adverse effects. The aquatic organisms are affected immensely due to this massive toxicity which can disrupt the ecological system .Probiotic organism accelerates gut health and stimulates the growth of gut microbes by inhibiting the pathogens. This work enumerates the identification of soil resourced organism *Aneurinibacillus migulanus* strain BJ-44 and its probiotic proficiency. Conversely, the effect of extracted crude protease extract on antibiotic induced zebra fish disclosed optimistic weight gain with decreased mortality rate. The molecular docking study embellished *subtilisin* as an inhibitor against *Clostridium difficile* Toxin A. This study enhances the probiotic stimulating efficacy of protease *subtilisin* and institutes it as a therapeutic lead against AAD. Yet the study requires implementing purification and downing streaming process of the extracted crude protein which would disseminate its potential better.

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