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Isolation, identification, and characterization of probiotic bacteria from the intestine of *Etroplus suratensis.*

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ABSTRACT

In the present study, an attempt was made to isolate, identify, and characterize probiotic bacteria from the intestine of native freshwater fish *Etroplus suratensis*. Intestine of three fishes were dissected out, homogenized and serially diluted to 10^{-1}-10^{-5} dilutions. Diluted samples were spread plated and 16 bacterial colonies showing morphological variations were sub-cultured. Out of which, seven bacterial strains which were able to grow in MRS (De Man, Rogosa and Sharpe) broth were evaluated for probiotic properties. Antimicrobial test was done by well diffusion method on the selected bacterial strains against *Bacillus subtilis*, *Bacillus cereus*, and *Staphylococcus aureus*. Four bacterial strains which showed antimicrobial properties were then tested for antibiotic sensitivity on ampicillin, chloramphenicol, erythromycin, tetracycline and vancomycin through disc diffusion method, followed by pH tolerance test at 5,6,7 and 8. The three bacterial strains which showed tolerance to varying pH were subjected to bile salt tolerance at concentrations, 0%, 0.15% and 0.30%. All the three strains were found to be tolerant to different bile salt concentrations and later tested for *in vitro* antioxidant activity using DPPH (2,2-Diphenyl 1-picryl hydrazyl) radical scavenging assay. The bacteria which showed more potent activity in all the experiments was subjected to gram staining and was found to be gram negative. Biochemical tests on the selected bacteria showed negative result for Indole, Methyl red, Voges Proskauer and Oxidase test and proved positive for Citrate test, TSI (Triple Sugar Iron) test, Mannitol motility test and Catalase test. The bacterium was identified as *Enterobacter asburiae* by molecular characterization using BLAST with 98.79% homology. The result of the above study opens the sustainable utilization of *Etroplus suratensis* gut as a probiotic source, which is otherwise considered as a waste product in aquaculture.

Keywords: Gut microbiota, Antimicrobial, Antibiotic susceptibility, Antioxidant, *Enterobacter asburiae.*

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INTRODUCTION

Probiotics are live microbial adjunct having a positive effect on host which helps in modifying host associated microbial community by excluding pathogens, producing substance having beneficial effect and improving immune response (Nagpal *et. al,* 2012). A good probiotic strain must have acid- bile salt tolerance, immunomodulation, antimicrobial activity, and antioxidant properties (Muthukumar and Kandeepan, 2015; Sugita *et. al,* 1996).

Probiotics provide various health benefits including treatment and prevention of diseases such as infectious diarrhoea, inflammatory bowel disease, irritable bowel syndrome, colon cancer, coronary heart disease, bacterial vaginosis (Alvarez-Olmos *et al.*, 2001; Al-Ghazzewi, 2016; An *et al.*, 2011; Grandy *et al.*, 2010; Mego *et al.*, 2005; Nazir *et al.*, 2018; Saavedra *et al.*, 1994). Probiotics are also reported to limit free radicals and reduce oxidative stress (Amaretti *et al.*, 2012; Wang *et. al,* 2011). Certain probiotics seems to improve lactose metabolism and provide microbial stimulation for developing immune system in infants (Fassio *et al.*, 2018; Morya *et al.*, 2017; Sophia Oak, 2019). Probiotics are also used in cattle and poultry feed for increasing productivity, controlling enteric pathogens, increase milk yield and increase quality of eggs produced (Kabir, 2009; Musa *et al.*, 2009).

This study aims at isolating probiotic bacteria from fish intestine which is considered as a waste material and unsuitable for human consumption. Indigenous freshwater Cichilid, *Etroplus suratensis* has been investigated for gut microbial probiotics, as it is a major inland fish, widely cultivated in Kerala. The study aims to find probiotic strains having antibacterial activity, acid and bile salt tolerance, antibiotic susceptibility, and antioxidant properties**.**

MATERIALS AND METHODS

Collection and processing of fish

The fish *Etroplus suratensis,* was collected from Punnamada lake located at Alappuzha district, with the help of fishermen using fishing rod. The fishes were cleaned with water and dissected out the digestive tracts, which was homogenized and serially diluted to 10^{-1} to 10^{-5} dilutions under sterile conditions. Each of the dilutions were spread platted in nutrient agar and incubated for 24 hours. Individual colonies having specific morphological characteristics were sub-cultured (Muthukumar and Kandeepan, 2015), and the colonies which were able to grow in MRS broth were tested for probiotic properties.

Antibacterial activity of isolated bacterial strains

The pathogens, *Bacillus subtilis*, *Bacillus cereus* and *Staphylococcus aureus* were used to test the antibacterial activity of the isolated strain using well diffusion method. The pathogenic bacteria were cultured in TSB (Tryptone Soya Broth) and incubated at 30^{o} C for 24 hours. Thereafter 30µl of bacterial cultures with 10^{3} CFU/ml was spread on TSA (Tryptone Soya Agar) by swab. The isolated bacterial strains grown in the MRS broth were harvested by centrifugation at 8000 rpm and 4^{o} C for 5 min and the supernatants were used for antibacterial test. The supernatants were transferred to the wells using a micropipette and incubated at 30℃ for 24 hours (Allamesh *et al.,* 2012; Balcazar *et al.,* 2008; Muthukumar and Kandeepan, 2015).

Antibiotic susceptibility test

Antibiotic susceptibility test was carried out by disc diffusion method. The nutrient agar medium inoculated with bacterial strains were impregnated with 6 antibiotics discs viz, Ampicillin, Chloramphenicol, Erythromycin, Tetracycline and Vancomycin and is incubated at room temperature for 24 hours. The zone of inhibition formed were measured and recorded (Bauer *et al.,*1996). The experiments were done in triplicate.

pH tolerance test

MRS broth having different pH such as 5, 6, 7 and 8 were prepared using 1% HCl and 1N NaOH and divided in universal bottles and inoculated with 30 µl of bacterial strains and incubated at 30^{o}C. After two hours of incubation optical density was measured by spectrophotometer at 600nm (Allamesh *et al.,* 2012; Balcazar *et al.,* 2008; Muthukumar and Kandeepan, 2015).

Bile salt tolerance test

Acid tolerance of the cultures was studied by incubating the organisms in MRS broth supplements with different concentrations of oxgall bile salt. Oxgall bile salt is the purified, dried form of ox bile. Oxgall bile salt added to MRS broth in three different concentrations 0.0, 0.15 and 0.3% (w/v) and were inoculated by 30 µl of bacterial strains and incubated at 30^{o}C. Optical density was measured using spectrophotometer at 600 nm during 0, 2, 4 and 8 hours of incubation period for assessing the growth rate (Allamesh *et al.,* 2012; Balcazar *et al.,* 2008; Muthukumar and Kandeepan,2015).

DPPH scavenging assay

For testing *in vitro* antioxidant activity of the isolated bacteria, DPPH radical scavenging assay was followed. 1mg of ascorbic acid dissolved in 1ml of ethanol served as standard solution and 1% DPPH solution in 1ml ethanol served as control. The final concentration of the test samples used was 0.125, 0.25 and 0.5µg/ml. The samples including standard and control were incubated at 37°C for 30 minutes and the absorbance of each solution were measured at 540 nm, using UV-spectrophotometer (Alam *et al*., 2012; Hwang *et al.*, 2001). All experiments were done in triplicate. The calculation of percentage inhibition was as per the equation below.

$$\% \text{ inhibition} = \frac{(\text{Absorbance of control} - \text{absorbance of standard/test})}{\text{Absorbance of control}} \times 100$$

Morphological, biochemical, and molecular characterization of probiotic bacteria

Gram staining of the isolated bacteria was conducted by the method suggested by Whitman *et al*., 2009. Biochemical tests such as Indole test, Methyl red test, Voges Proskauer test, Citrate test, Mannitol motility test, Triple sugar iron agar test, Catalase test and Oxidase test (Whitman *et al*., 2009) were conducted. Molecular characterization of the probiotic bacteria was done by DNA isolation, agarose gel electrophoresis, polymerase chain reaction. Sequence similarity search was done using BLAST.

RESULTS AND DISCUSSION

The sample strains were selected based on their ability to grow in MRS broth which were named as K1, K2, K3, K4, K6, K8 and K20 and further tested for probiotic properties. These strains were also tested for antimicrobial property. The antimicrobial activities of tested strains are shown in Figure 1. Among the above sample strains only four strains K2, K4, K6 and K20 showed anti-microbial activities against the tested pathogens. Strains K1, K3 and K8 did not show any inhibition for the 3 pathogens tested. Strains K2, K6 and K20 showed antimicrobial activity against all the three pathogens while the activity of K4 was limited to the pathogen *B.subtilis* (Fig.1). The average value and standard deviations obtained were calculated for accuracy in comparison and screening of the most potent strain.

Several studies have revealed the benefits of administration of probiotics in inhibiting pathogenic microorganisms and controlling opportunistic infections (Rossoni *et al.*, 2017). Studies have shown that probiotics with antimicrobial property can control pathogens or completely kill pathogens. A similar study conducted by Lopes *et al* (2018) reported the effectiveness of a mixture of oral probiotics in decreasing disease caused by *S.aureus*. In a

similar study *Lactobacillus plantarum* was used as a probiotic against *Bacillus cereus* (Zhang *et al*., 2016). The mechanism of action of different probiotic strains may vary, some may produce inhibitory substances such as bacteriocins and hydrogen peroxide and some participate in immune response modulation by phagocytic activity or by altering the release of pro and anti-inflammatory cytokines (Dong *et al*., 2012; Kanmani *et al.*, 2013). The findings in this study suggest that the strains K2, K6 and K20 having a potential probiotic activity.

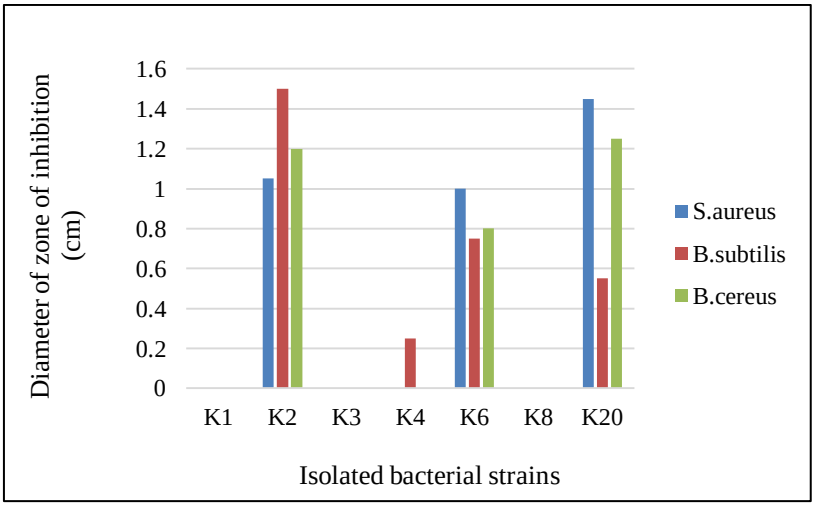

Figure 1: Antimicrobial activity of selected strains against *Bacillus subtilis, Bacillus cereus* and *Staphylococcus aureus*.

Antibiotic susceptibility test for bacterial strains K2, K3, K6 and K20 are shown in Table 1**.** The strains K3, K6 and K20 were susceptible to the antibiotics tested, forming zones of inhibition at different rates. Strain K2 showed inhibition for all other antibiotics except chloramphenicol (Table 1). Strains K6 and K20 showed good zone of inhibition against 5 antibiotics, while K2 and K3 has shown mild zones of inhibition.

Antibiotic susceptibility is important for a probiotic to thrive in an environment where antibiotics are present and still impart its beneficial effects. In similar studies it is discussed that probiotic should be susceptible to common antibiotics and probiotics are considered normal microbiota for certain host microbial environment that may prevent diseased conditions and may improve general health outcome (Franz *et al.*, 2011; Rosander *et al.*, 2008; Munoz-Atienza *et al.*, 2013). However, a risk of pathogenic microbes acquiring antibiotic resistance genes from probiotic microbes exists and many studies are done regarding this matter (Drago *et al.*, 2011; Mater *et al.*, 2008).

Table 1: Antibiotic susceptibility test of selected strains against selected antibiotics

SAMPLE	CHLORAMPHENICOL	TETRACYCLINE	ERYTHROMYCIN	VANCOMYCIN	AMPICILLIN
K2	_	+	+	+	+
K3	+	+	+	+	+
K6	+	+	+	+	+
K20	+	+	+	+	+

Among the tested bacterial strains, all the three bacterial strains, K2, K6 and K20 were tolerant to pH ranging from 5 to 8 at varying levels (Fig. 2). The tolerance level of these strains came between 0.4 and 0.7. The bacterial strains were found to grow maximum at a pH of 7 and the bacterial strains were able to grow and survive in decreased pH of 5 and 6. Probiotics must survive in acidic gastric environment to reach small intestine and colonize inside the host. Hence acid tolerance is accepted as one of the desirable properties of a potential probiotic strain (Corcoran *et al.*, 2005). Muthukumar and Kandeepan (2015) observed similar growth pattern at pH 7 and 8 by strains, *Bacillus spp, Lactobacillus spp* and *Macrococcus spp*. The highest activity and growth were attained at pH 7. In a normal intestinal condition, tolerance to pH less than 7 can be considered as required pH tolerance of bacterial strains (Ghannoum *et al.*, 2019).

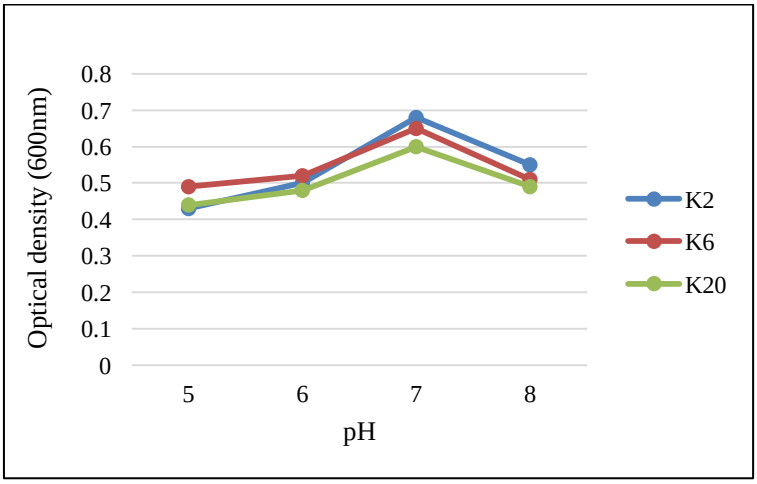

Figure 2: pH tolerance of the bacterial strains to varying pH

The bile salt tolerance test for strains, K2, K6 and K20 are shown in Fig 3. Measurement of optical density of all the three stains K2, K6 and K20 showed significant tolerance against varying concentration of bile salt of 0%, 0.15% and 0.30%. Tolerance to bile salt is a desirable property for probiotic strain as the strain must pass through the adverse conditions

in stomach. The tolerance level of these strains came between 0.08 and 0.7. The tested bacterial strains, K2, K6 and K20 were able to survive in a higher concentration of 0.3% bile salt. Similar pattern of bacterial growth was observed by Muthukumar and Kandeepan (2015) where *Bacillus spp*, *Lactobacillus spp* and *Macrococcus spp*, could survive in 0.3% bile concentration. Sahadeva *et al.* (2011) observed that, *Lactobacillus acidophilus, Lactobacillus casei, Streptococcus thermophilus* and *Bifidobacterium* were tolerant to 0.3% of bile salt in which *Lactobacillus casei* was the most tolerant. Another study done on *Lactobacillus* also showed the tolerance of bacteria at 0.3% of bile salt (Hassanzadazar *et al.*, 2012). The survival of bacteria in high concentration of bile salt also depends on the duration of exposure to bile salt, those strains that tolerate higher concentration for longer time is more potent probiotic which can grow and impart the benefits (Succi *et al.*,2005)

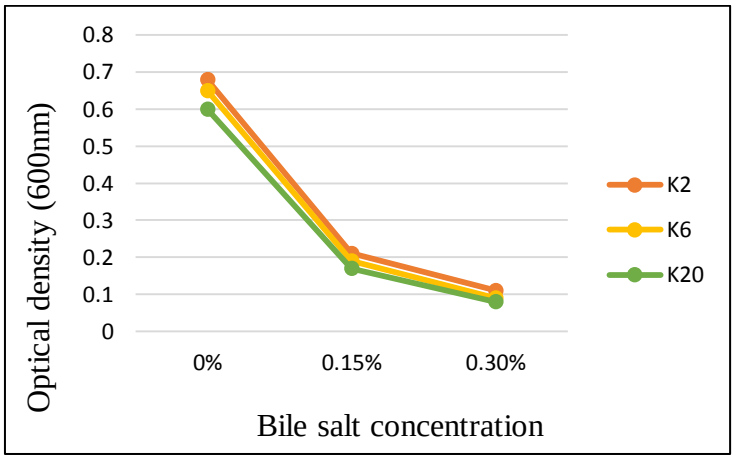

Fig 3: Bile salt tolerance test for selected bacterial strains

The bacterial strains, tolerant to the bile salt conditions were analysed for *in-vitro* antioxidant activity using DPPH free radicals. DPPH free radical method is an antioxidant assay based on electron transfer (Garcia *et al.*, 2012). The values of K2, K6 and K20 were comparable to the value of the standard, ascorbic acid. K2 showed comparatively good antioxidant activity than others and K6 showed the least activity (Fig. 4). Studies have shown that most of the probiotic strains show antioxidant activity and can reduce damages caused by oxidation (Pieniz *et al.*, 2014; Wang *et al.*, 2017). The increased free radical scavenging property shows the increased antioxidant value which can reduce oxidative damage and has high potency as probiotics (Mishra *et al.*, 2015). On a similar study Kim.*et al*. (2020) showed probiotic strains of *Lactobacilli* and *Bifidobacteria*, in mitigating oxidative stress related symptoms. In the present study K2 showed a comparatively increased rate of inhibition of free radical and can be was considered a potent probiotic strain.

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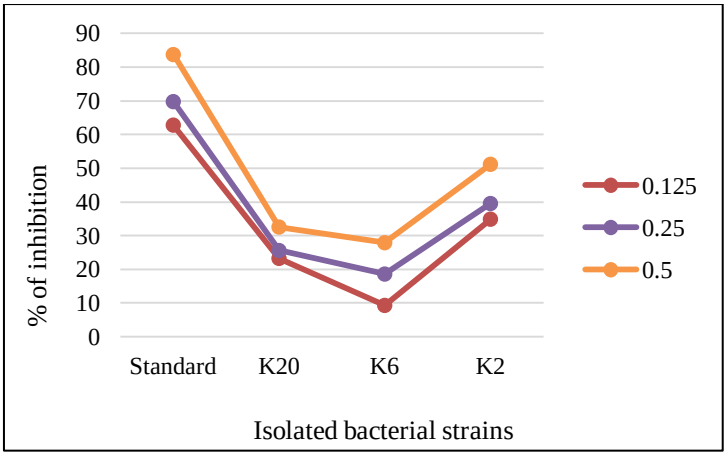

Fig 4: Rate of inhibition of DPPH radicals by selected bacterial strains

The results of gram staining of sample K2, showed that the bacteria is Gram negative (Fig.5).

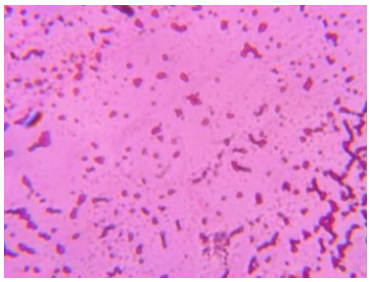

Fig 5: Gram staining image of selected strain K2

Biochemical tests of sample K2 showed negative results in Indole test, Methyl red test, Voges Proskauer test and Oxidase test. Positive results were obtained in Citrate test, TSI test; Mannitol Motility test and Catalase test (Table 2).

Table 2: Biochemical tests of the selected bacterial strains

TESTS	RESULTS
Gram staining	Gram negative, rod shaped
Indole test	Negative (-)
Methyl red test	Negative (-)
Voges Proskauer test	Negative (-)
Citrate test	Positive (+)
TSI test	Positive (+); Glucose, lactose fermenting
Mannitol Motility test	Positive (+); Motile and fermenting
Catalase test	Positive (+)
Oxidase test	Negative (-)

From biochemical and molecular test and BLAST sequence similarity test, it was observed the isolated bacterial strain to be *Enterobacter asburiae* with 98.79% homology in BLAST summary.

The present work confirms the probiotic potential of *E. asburiae*. As an extended study *E. asburiae* can be incorporated in fish food or in animal feeds as a promising probiotic. Further studies must be done on this strain to know its harmful effects if any when consumed as a probiotic. The application of *E. asburiae* as a commercial probiotic supplement for humans or other animals, require further study and trials.

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