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Diversity and antimicrobial activity of Lactic Acid Bacteria from the gut of marine fish *Rastrelliger kanagurta* against fish, shrimp and human pathogens

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Original Article

Abstract
Emergence of antibiotic resistance among aquaculture pathogens has made it necessary to look into environment friendly, effective and sustainable methods such as probiotic and immunostimulants among others.. In the present study, LAB were isolated from the gut of fish species namely *Rastrelliger kanagurta* and analyzed for their antibacterial activity against various fish, shrimp and human pathogens. Different LAB species such as *Lactobacillus plantarum, L. bulgaricus, L. brevis and L. viridiscens* were encountered in the gut of *R. kanagurta.* Several strains showed good activity against fish, shrimp and human pathogens. LAB from the gut of such marine species may be developed as possible probiont for environment friendly health management of fresh water, estuarine and marine species currently exploited in aquaculture.

Keywords: *Rastrelliger kanagurta, lactic acid bacteria, probiotic, antimicrobial activity, pathogen, shrimp, fish.*

Introduction

Lactic Acid Bacteria (LAB) are a group of Gram- positive, catalase- negative, non- spore forming, rod or cocci shaped fastidious organism which have "Generally Recognized as Safe" (GRAS) status. It contains different genera as *Lactobacillus, Lactococcus, Pediococcus, Leuconostoc, and Oenococcus* among others. The LAB is found throughout the gastrointestinal tract including the stomach and the duodenum. The mechanisms of inhibition of pathogenic bacteria by the probiotic strains are generally mediated through the production of Bacteriocin. There are different types of Bacteriocin which are produced by the LAB and they can be classified as follows: Class 1 or Lantibiotics (less than 5KD), Class II or Non Lantibiotics (usually less than 10kDa) and Class III bacteriocin (generally larger than 30kDa). A prominent Lantibiotic which has significant antibacterial activity against Gram positive organisms like *Listeria* sp., *Micrococcus* sp. and also against sporulating bacteria like *Bacillus* is Nisin (Parada *et al*., 2007).

Most of the preliminary reports of isolation of LAB came from diverse sources as grains, dairy and meat products, fermenting vegetables and even from the mucosal surface of animals (Mahantesh *et al*., 2007; Schrezenmer and Verse, 2001) has defined probiotic as viable microbial food supplements which beneficially influence the health of the host. Lilley and Stillwell (1965) described them as substances secreted by one microorganism, which promotes the growth of other.

In the current study, an attempt has been made to study the LAB flora from the gut of *Rastrelliger kanagurta*, a marine fish species. Adults of *R. kanagurta* occur in coastal bays, harbors

and deep lagoons, usually in some turbid plankton-rich waters. It forms schools and feed on phytoplankton (diatoms) and small zooplankton (cladocerans, ostracods, larval polychaetes, etc.). Adult individuals feed on macro plankton such as larval shrimps and fish. They are generally marketed fresh, frozen, canned, dried- salted, and smoked; also made into fish sauce.

Material and methods

Isolation and screening for LAB from fish gut

Fresh samples of *R. kanagurta* were procured from the brackish water fish landing centre at Fort Kochi and from the Broadway market at Ernakulam. The fishes were packed in ice boxes and transferred to the laboratory within 2 hours (Nair & Surendran, 2005). The fishes were surface washed, weighed and then the gut was removed by making a "T" shaped incision on the ventral surface. The weight of the fishes ranged from 78 - 137 g. The guts were then weighed in an electronic balance (Keroy, India). The weight of the gut ranged from 20.54 to 28.6 g. The guts were then surface washed with sterile physiological saline so as to remove any extraneous matter. Depending upon the weight of the gut obtained they were mixed with 80-110 ml of sterile saline and were homogenized in a Masticator (IUL Instruments, Spain) for about 3-5 minutes, till the gut tissues appeared visibly macerated. The supernatant of this homogenized gut tissue was then transferred into 200 ml 1% Peptone broth containing 0.5% Sodium Chloride and was kept for enrichment for 24 hours. Next, 1 ml of the enriched broth were serially diluted to 10-1 and 10-2 dilutions and were pour plated onto De Man Rogossa and Sharpe (MRS) agar (Hi Media, Mumbai) and incubated at 31-32° C for 5-7 days. White colored, well isolated colonies of 2-3 mm diameter with round margin were obtained within the agar, and where as the surface of the agar was dominated by diffuse white colored and sometimes ill defined colonies. The colonies were picked up and transferred into MRS broth (Hi Media, Mumbai) and thereafter transferred into MRS agar slants for storage at room temperature. All further work was carried out from the stored cultures.

Phenotypic characterization

The isolates were subjected to morphological, cultural, physiological and biochemical characterization. Each isolate was initially examined for colony and cell morphologies, cell grouping, presence or absence of spores, Gram staining, catalase production and spore formation (Cappucinno and Sherman, 2004).

The isolates were checked for acid production from the following sugars (1% w/v) (Abegaz, 2007) – D- arabinose, Sucrose, Mannose, Melibiose, Lactose, Aesculin, Cellobiose, Rhamnose, Maltose, Galactose, Sorbitol, Fructose, Mannitol, Raffinose, Amygdalin, Inositol and Ribose in Purple Broth Base containing Peptone (10 g/L), NaCl (5 g/L) and Bromocresol Purple (0.02 g/L). All the carbohydrate broths except glucose were autoclaved at 12 lbs for 15 minutes. Glucose broth was autoclaved at 15 lbs for 15 minutes. The results were assessed with reference to the control after aerobic incubation at 37°C for 5 days (Abegaz, 2007).

Identification of the gut isolates

The isolates were identified by the software PIB-Win (Probabilistic Identification of Bacteria for Windows Version 2) which provides probabilistic identification of unknown bacterial isolates against identification matrices of known strains. The results are matched with the database and a list of most probable organisms are produced based on the Identity Score (I. D. Score). A minimum threshold value of 0.95 is desirable for a good match. The software also provides selection of additional tests to differentiate between possible species, if identification is not achieved (Bryant, 2004).

Antibiogram of LAB

The objective of the current work is to project and develop gut associated LAB as possible environment friendly alternative to antibiotics in treating various pathogens. For this, it is necessary to ensure that the LAB isolates themselves are host friendly and are not pathogens themselves and that they can be contained by commonly used antibiotics. All the isolates were screened for the presence of antibacterial property following the agar gel diffusion method as devised by Bauer *et al.* (1966) by disc and as well as by well method. The antibacterial property of the gut isolates were tested against 12 WHO type strain of human pathogens. For the antibiogram, 50-100 microlitres of the untreated MRS broth cultures were either absorbed into sterile filter paper discs or were poured into the wells in the Muller Hinton agar plates swabbed with a 24 hour broth culture of the test pathogens. Zone of inhibition, if any was noted after 10-12 hours. Sensitivity of a pathogen to the LAB isolates was defined by the absence of visible growth of the test organism around the disc or the well. Zone of inhibition ranging from 0-10 mm was accepted as poor, 11-15 mm as moderate and beyond 15 mm as good result.

Results and discussion

All the isolates obtained from the gut of *R. kanagurta* were found to be Gram- positive. Morphologically, the isolates fell into two broad categories, namely cocci and cocco-bacilli; though the demarcation was not very clear always. Four different types of colonies were isolated. Small creamy white round colonies on the agar; white shady colonies below

the agar with a prominent centre; white pinpoint colonies within the agar and white round disc like colonies always transversely present inside the agar. Different kinds of cell arrangement were noticed. Cocci were found in three different conformations: chains (43.75%), clumped (6.25%) and solitary (31.25%). Cocco- bacilli were found to be clumped (18.75%). Among the chained cocci, again two variations were noticed. The first type consisted of the ones where the cells were arranged more or less in a linear manner, almost resembling the English alphabets of T, L, I, Y, X and U on many occasions. This category consisted of 85.71% of the chained cocci. The other group (14.29%) of chained cocci displayed intense coiling resembling a super helical structure or a mesh of tangled wire. Cocci in clumped conformation were found in diads and on few occasions showing one or two chains. The cocco- bacilli were present in clumps of 2, 4, 5, 6 and sometimes even in bigger clumps. However, diads were the predominant ones. The cocco bacilli group was mostly densely packed and was devoid of any chain formation.

Since all the isolates displayed varying degree of antimicrobial property against different classes of pathogens, all of them were chosen for biochemical characterization. Most of the isolates were found to be able to utilize 16 varied sugar sources. All the isolates were found to utilize Ribose (Table 1). The authors have not gone into nor do they wish to attach any special significance to the ability of the isolates to utilize Ribose, within the scope of the current work. Since Ribose was the only sugar which all the isolates were able to utilize,

Table 1. Prevalence of various lactobacilli in the gut of *Rastrelliger kanagurta*

Lactobacillus species	Percentage occurrence
L. plantarum	29%
L. viridiscens	12%
L. bulgaricus	12%
L. brevis	12%
Unknown	35%

a mention to that effect has only been made. The isolates were tested against 3 fish, 5 shrimp (Table 2) and 12 human pathogens (WHO Type Strains) and 14 multi drug resistant *Enterobacter cloacae* (Table 3). In this context, it is appropriate to note that, the gut flora of the salt water fishes are generally dominated by *Vibrio and Pseudomonas* (Onarheim *et al.*, 1994) whereas that of the fresh water fishes are dominated by the members of the genera like *Aeromonas* and *Plesiomonas* and obligate anaerobic bacteria of the genera *Bacteroides, Fusubacterium and Eubacterium* (Sakata, 1990). Nagvenkar *et al.* (2006) reported the presence of 8 species of bacteria from the gut of *Mugil* which included *Pseudomonas* sp., *V. parahemolyticus*, *Vibrio* sp., *Enterobacter* sp., *Streptococcus* sp., *Proteus vulgaris, Bacillus subtilis and Serratia mercesens;* also majority of the isolates from the gut were reported to be Gram-negative organisms.

However, in the current study, all the isolates were found to be Gram-positive ones. *Lactobacillus* dominated the flora among the Gram-positive and catalase-negative isolates. There is no mention of *Lactobacillus* being even isolated from gut, gills or from the external surface of another widely consumed fish-grey mullets (*Mugil cephalus*) in the study of Nagvenkar *et al.* (2006) even though it does mention the absence of *Azotobacter* from the gut of *M. cephalus*. Ringo and Vadstein (1998) had reported that, LAB even though are present in the gut of human (as *Bifidobacterium*) and swine and birds (as *Lactobacillus*) and as *Enterococcus* in carnivores - are generally subdominant in the gut of the fishes and if at all, are mostly represented by the genus *Carnobacterium* (Gildberg and Mikkelsen, 1998).

Isolate 246 showed good antimicrobial activity against the fish pathogens, while isolates 229, 232 and 234 displayed commendable activity against the shrimp pathogens (Table 2). Of the gut isolates screened, 45% were found to be effective against 50% or more of the WHO pathogens, the highest degree of efficacy observed being 92%. Forty percent

Table 2. Antibacterial activity of Lactic Acid Bacilli from the gut of *R. kanagurta* against fish and shrimp pathogens

Fish/ shrimp pathogen	Diameter of zone of inhibition (in mm) by Lactic acid Bacilli					
	L 229	L 232	L 234	L 246	L 247	L 249
Aeromonas hydrophila	13	10	9	13	0	0
Vibrio harveyi	0	0	0	12	0	0
V. metschnikovii	0	11	11	0	0	0
V. corallilyticus	11	16	11	0	10	15
V. fluvialis	11	12	9	0	0	14
V. hollisae	10	10	0	0	0	0
V. mimicus	13	13	16	0	0	0

Table 3. Antibacterial activity of Lactic Acid Bacilli from gut of *R. kanagurta* against human pathogens

Human pathogens	Diameter of zone of inhibition (in mm) by Lactic Acid Bacilli					
	L 229	L 232	L 234	L 246	L 247	L 249
Salmonella enteritidis	11	0	12	12	15	13
S. brandenberg	0	0	0	0	17	21
S. muenster	10	0	10	0	0	16
S. bredney	10	0	0	0	0	25
S. sandiego	0	0	0	15	12	15
S. worthigton	0	0	0	0	15	13
S. albany	11	0	11	9	11	14
S. oranienberg	9	0	0	11	11	12
S. thompson	0	0	0	10	0	15
S. javiana	12	0	12	0	12	12
S. meleagridis	0	0	0	0	0	17
Campylobacter jejuni	12	0	12	9	15	13
Vibrio cholerae	11	13	7	0	0	15
Enterobacter cloaccae	14	15	19	13	0	20

of the gut isolates were found to be effective against 30-49% of the same pathogens, while 15% of the isolates did not show any antagonistic property. Among the pathogens, *Salmonella enterica* serovar *Albany* and *Campylobacter jejuni* showed sensitivity to 85% of the isolates which was highest. *S. meleagridis* was the most resistant organism, showing sensitivity to only 5% of the isolates. Campylobacter is one of the most common agents of food poisoning and is one of the most common agents of human gastroenteritis in the world. *C. jejuni* is commonly associated with poultry, and it naturally colonises the digestive tract of many bird species. It is also common in cattle, as commensal of the gastrointestinal tract in these animals, but occasionally causing campylobacteriosis in calves. It has also been isolated from wombat and kangaroo feces, being a cause of bushwalkers' diarrhea. Contaminated drinking water and unpasteurized milk provide an efficient means for distribution while contaminated food is a major source of isolated infections, with incorrectly prepared meat and poultry as the primary source of the bacteria. Hence, the reported sensitivity of *S. albany* and *C. jejuni* to the isolated gut associated bacteria assumes significance. Of the four selected isolates further screened for their ability to inhibit multi- drug resistant strains of *Enterobacter cloacae* (Table 3) two isolates (246 and 249) inhibited 88.88% of the test pathogens, while a third one (247) inhibited 38.88% of the test pathogens. *Enterobacter* infections are known to cause bacteremia, lower respiratory tract infections, skin and soft-tissue infections, urinary tract infections (UTIs), endocarditis, intra-abdominal infections, septic arthritis, osteomyelitis, CNS infections, and ophthalmic infections. Hence the report of sensitivity of multi drug resistant *Enterobacter* to the gut isolates is encouraging.The fourth isolate (248) displayed a very poor affectivity against only 5% of the test organisms. The highest zone of inhibition that was observed was 17mm which is accepted as a commendable result as per laid down standards.

In the current scenario, where large scale production and breeding facilities in aquaculture have come into vogue, not discounting the cut throat competition, the stakes are high for the fish farmers. This leads to an increase in the indiscriminate and uninformed usage of commercially available antibiotics, which ultimately leads to the emergence of antibiotic resistance among the fish pathogens. In the European Union and Switzerland, around 1600 tons of antibiotics have been used for growth promotion purpose in 1997, which constitute 30% of the total antibiotic usage in that year (SCAN, 2003). These amounts of antibiotics have exerted a very strong selection pressure towards resistance amongst bacteria, which have adapted to the situation, by a horizontal and promiscus flow of resistance genes (SCAN, 2003). Flow of resistance genes among the bacterial population can take place either by chromosomal mutation or by acquisitions of plasmids- the later being a rapid process (Lewin, 1992). Apart from these processes, transfer of antibiotic resistance can also take place by viral transduction or even by direct transformation from DNA absorbed to the particles in water or sediment particles. Cases that warrant a mention here are transference of multi-drug resistance in Ecuador and transference of Florophenicol and Chloramphenicol resistance from *Photobacterium*

damsela - a fish pathogen to *S. typhimurium* DT 104 (Angulo, 2000). In the prevailing scenario, thus, usages of probiotic have assumed considerable significance by the virtue of it inherently being sustainable, environment friendly and organic in nature.

There have been various school of thoughts as to how exactly a LAB is beneficial to its host. Numerous studies in the past few decades have pointed out that there are different methods by which the probiotic could beneficially influence the host such as competitive exclusion of pathogens (Garriques and Arevalo, 1995; Moriarty, 1997) source of nutrients and aid in digestion by the production of enzymes (Sakata, 1990; Priuer *et al*., 1990) and increase direct uptake of dissolved organic matter mediated by the bacteria (Garriques and Arevalo, 1995; Moriarty, 1997). Other possible beneficial roles might be in up- regulating the immune system of the host (Rengpipat *et al*., 2000; Iriantano and Austin, 2002). Lactic acid bacteria are known to help cellulolytic bacteria to become predominant microbial flora in the rumen and increase the degradation of cellulose (Kalmokoff and Teather, 1997). Lactic acid bacteria are also known to prevent acidosis caused by *Streptococcus bovis* when a cattle consumes grain based diet, thus helping to maintain rumen homeostasis (Morovsky *et al*., 1998). In 1969, after a joint committee of WHO/FAO, approved the usage of Nisin as an antimicrobial substance in food, 50 countries accepted the usage of Nisin for preservative purpose. Nisin has been widely used to prevent the spoilage of canned food products (Tagg *et al*., 1976). Various workers in this area have more often isolated LAB like *Carnobacterium* from rotifers (Gatesoupe, 1994), intestines of Atlantic salmon (Robertson *et al*., 2000) or from the digestive tract of Rainbow trout (Irianto and Austin, 2002) and have used them on turbot larvae, Atlantic cod fry or on Rainbow trout. Nikoskelainen *et al.* (2001) have also reported the use of *L. rhamnosus* ATCC 53103 on Rainbow trout. Also most of the probiotic employed in aquaculture of crustaceans and molluscans come from digestive tract of chicken (Phianphak *et al*., 1999), or from micro algal cultures (Riquelme *et al*., 2000). Even Villamil *et al.* (2003) who have employed *L. casei, L. brevis, L. helveticus, Leuconostoc mesenteroids* and *Pediococcus* on *Artemia* nauplii have used strains from Culture collection centers. Thus, we can see that, cases of indigenous gut LAB flora of fish being used to treat aquaculture pathogens are few and far between.

In the present study, it has been observed that the gut LAB flora have been proved to be effective against various serovars of *Salmonella*, a potent human pathogen and other fish and shrimp pathogens to varying degree. What attracts our attention here is, that these strains of *Lactobacillus* have been effective against Gram- negative organisms, whereas they are generally known to be effective against Gram-positive organisms, because of their cell wall composition. Analysis of the carbohydrate profile of the isolates by PIB-Win (Version 2) indicated that, *Lactobacillus plantarum* consisted of 29.41% while *L. viridiscens* and *L. brevis* each accounted for 11.76% of the isolates. Isolates, for which the I.D. value fell below 0.95, were categorized as others. This group consisted of 47.07% of the isolates and many of them displayed commendable antimicrobial property. Even in the case of such isolates, where in the threshold value of 0.95 was reached, variance with the standard result (provided in the matrix) was observed.

Also, majority of the LAB isolates found to possess good antibacterial activity have been identified as *L. plantarum* which underscore their prospect as a potent bioagent for many diseases. The presence of strains of bacteria which can tolerate a wide range of salinity, as in a marine fish like *R. kanagurta*, assumes importance since they can be harvested to fight aquaculture pathogens from both brackish and saline water fishes. Recent works as that of Nagvenkar *et al.* (2006) on euryhaline species of *M. cephalus* does not mention the presence of LAB in the gut of the grey mullets. Also, earlier reports on LAB from the gut of marine fishes have been that of *Carnobacterium* sp. from the intestinal tract of Atlantic salmon (Gildberg and Mikkelsen, 1998; Robertson *et al*., 2000). Yongjin *et al.* (2007) reportedly mixed LAB cultures in the sausage prepared from silver carp to inhibit the growth of spoilage microbes and the accumulation of histamine, cadaverine, putrescine, tryptamine and tyramine. Again, LAB such as *Pediococcus pentasaceous* and *P. acidilactici* were used to prevent the growth of spoilage bacteria in mackerel fish chunks (Kannappan and Manja, 2004). LAB coating also depleted salts and nitrites present in the fish (Chang *et al*., 2004).

Reports on LAB from the gut of a marine fish as *R. kanagurta* - a fish widely consumed in India, could not be found by the authors of the current study. The presence of antagonistic properties against strains of human pathogen *Salmonella* provide encouragement for further studies in this field. Further screenings of the isolates against a wider range of pathogens as well as 16S rRNA identification of the isolates are underway.

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