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Characterization of Antimycobacterial Activity of Bacteriocins Isolated from Fish-Gut Associated Lactic Acid Bacteria

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ABSTRACT

Tuberculosis (TB) is a contagious airborne disease caused by the pathogen, *Mycobacterium tuberculosis* (MTB). The conventional anti-tubercular drugs such as isoniazid and rifampicin have maximum activity and lengthy duration of therapy. The risk of serious adverse events such as hepatotoxicity, discourage both patients and providers. The big challenge here has been to find a new drug effective against TB. Lactic acid bacteria (LAB) are widely distributed in dairy products and fermented foods and also from non-dairy sources. LAB would produce novel antimicrobial peptides (bacteriocins) with unique structural characteristics and applications. The antimycobacterial properties of bacteriocins isolated from lactic acid bacteria have been studied. In this study, we isolated lactic acid bacteria from fish gut and evaluated their bacteriocins for antimycobacterial activity. Different genus of LAB was isolated and characterized viz. *Pediococcus sp., Aerococcus sp, Lactobacillus sp*. from fish gut. All the isolates were screened for their antibacterial and antimycobacterial activity. Based on the activity against *M. smegmatis* MC^2155, *Lactobacillus spp*. (BFO21) was selected and characterized by 16S rRNA gene sequence analysis. Bacteriocin were isolated from *Lactobacillus plantarum* BFO21 and partially purified using chloroform solvent extraction method. About 98% of RLU reduction in terms of inhibition was found with bacteriocins of *L. plantarum* BFO21 against *M. tuberculosis* H37Rv through LRP assay. According to our results, the isolate *Lactobacillus plantarum* BFO21 should be evaluated for further characterization of its bacteriocins to explore their anti-tubercular activity against both replicating drug resistant *M. tuberculosis* and non-replicating *M. tuberculosis* (Latent TB)..

KEY WORDS: TUBERCULOSIS; BACTERIOCINS; FISH GUT; LACTOBACILLUS; ANTI-TUBERCULAR ACTIVITY; LRP ASSAY.

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INTRODUCTION

Tuberculosis (TB) poses serious epidemics around the world caused by the pathogen *Mycobacterium tuberculosis* (Mtb). The increasing rate of HIV-related TB, multi-drug resistant TB (MDR- TB) and Extensively Drug Resistant TB (XDR-TB) also concerned globally. Treatment for tuberculosis requires 6-8 months for new cases and 18-24 months for MDR TB whereas the treatment for XDR-TB is ineffective making the treatment options seriously limited. The big challenge here has been to find new drugs effective against tuberculosis. In this context, antimicrobial peptides such as bacteriocins produced by lactic acid bacteria has been emphasized for their prominent antimycobacterial properties due to cationic characteristics which likely binds to the anionic lipids in membrane (Sivaraj et al., 2018). There are only few studies carried out on bacteriocins focused on their antimicrobial or antitubercular activity but many studies focused on their potential applications as food preservatives (Perez et al., 2018). Bacteriocins exhibit significant potency against multidrug-resistant bacteria and also offer promising lead compound as substitutes or conjugates to current therapeutic compounds (Meade et al., 2020).

The concept of research on bacteriocins applications are expanding from food preservative to human health including novel drug delivery systems, anti-tubercular treatment and anticancer treatment applications (Meade et al., 2020). Lactic Acid Bacteria (LAB) is a group of Gram positive, catalase negative, often non-motile organisms that are grouped in to two distinct phyla, such as Firmicutes and Actinobacteria. Around 15 different genera of LAB have been reported and some of them are dominant genera viz. *Lactobacillus, Streptococcus, Pediococcus, Enterococcus, Aerococcus*, etc. LAB have been isolated from various sources like raw milk, cultured milk products, meat products, fish, grains, green plants, fermenting vegetables, mucosal surface of animals (Lindgren and Dobogosz, 1990). Fish gut is considered as complex ecosystem that contains numerous microorganisms (Wong et al., 2013). The microbiota of fishes helps in antagonistic activity against pathogens and is in various immune responses (Huber et al. 2004). LAB isolated from the gastrointestinal tract (GIT) of fishes had been reported to have the potential as probiotic agents by protecting the aquatic species from various aquatic infections and also by killing the pathogens involved in the spoilage of fish products (Meade et al., 2020).

Broad range of various lactic acid bacteria species found in the GIT of various fish species which include both fresh water and marine water species (Merrifield et al., 2014). It was believed that LAB obtained from the GIT of fishes has the potential to develop as ideal probiotic agents (Gomez-Sala et al., 2015). LAB is considered as "Generally Recognized as Safe (GRAS)" microorganisms, which produce various compounds during lactic acid fermentation including organic acids, diacetyl hydrogen peroxide, bacteriocins, etc. Bacteriocins produced by LAB are considered as small peptides (<10 kDa) that have greater antibacterial activity by means of their unique characteristics such as cationic in nature, heat-stable, amphiphilic and adsorption to the gram-positive cell surfaces. LAB and their metabolic products usages are generally considered as safe (Zacharof and Lovitt, 2012). LAB-bacteriocins are emerging as a novel alternative to antibiotics and known to exert either bacteriostatic or bactericidal activity toward sensitive organisms. Bacteriocins of LAB target specific species and do not affect other population within the same ecosystem (Vieco-Saiz et al., 2019).

Bacteriocins are secreted in the logarithmic growth phase of bacteria with increasing bacterial numbers and optimal culture conditions promoting increasing peptide secretion (Ge et al., 2019; Anbarasu et al., 2020). Bacteriocins are extracellularly released peptides and they are mainly categorized into class I, class II, class III and class IV based on the host producer, molecular weight and amino acid sequence (Meade et al., 2020). The electrostatic interaction between positive charge of bacteriocins and the negative charge of bacterial cell membranes plays a significant role in the initial interaction thereby facilitating the binding of the molecules to the membranes of target cells (Perez et al., 2015). LAB isolated from GI of estuarine fish and freshwater fishes have showed significant antimicrobial activity against various aquaculture pathogens (Sahoo et al., 2015; Hagi et al.,2004; Ghosh et al., 2014).

The antimycobacterial properties of bacteriocins isolated from lactic acid bacteria have been studied by few researchers globally. Lantibiotics are class I bacteriocins that certainly possess sufficient potential for treating tuberculosis in future. Bacteriocins from lactic acid bacteria have showed greater antimycobacterial activity than equal concentrations of rifampicin in vitro model (Carroll and Jim O'Mahony, 2011; Sosunov et al., 2007). Pérez et al., (2018) demonstrated synergistic actions of enterocin AS-48 (bacteriocins produced by LAB) with ethambutol against *Mycobacterium tuberculosis* and revealed its potential role in tuberculosis treatment. In this context, the present study was aimed to isolate lactic acid bacteria from fish gut and to evaluate their bacteriocins for anti-tubercular activity against *M. tuberculosis* using luciferase reporter phage (LRP) assay.

MATERIAL AND METHODS

The fishes such as *Rastrelliger kanagurta*, *Tilapia, Centropristis striata* and *Teuthida* were aseptically dissected to collect intestinal contents, weighed and homogenized. They were each transferred aseptically to 10 ml of saline, shaken well and about 1 ml aliquots were transferred to 10ml of MRS broth each and incubated at 30˚C for 18 hours. After incubation, ten-fold serial dilution of samples was carried out and plated onto MRS agar containing bromocresol (0.04mg/ml) and incubated at 30˚C for 24 hours. Colonies showing yellow zones

were selected and sub-cultured on to MRS agar plate. Isolates that were catalase negative and gram positive further got identified at genus level according to method described by Nikita and Hemangi (2012) and Kalschne et al., (2015).

To test the antimicrobial and antimycobacterial activities, one ml of overnight grown LAB culture was transferred to 100 ml of MRS broth (Himedia, India) and incubated at 30˚C in shaker (100rpm). After 18 hours of incubation, cell free supernatant (CFS) was collected by centrifugation at 5000rpm for 10mins (4˚C). Then pH of CFS was adjusted to 6.5 and treated with catalase (1mg/ml) for testing their inhibitory activity against non-mycobacterial strains such as *S. aureus, B. cereus, E. coli* and *K. pnemuoniae* and mycobacterial strains, *M. smegamtis* mc2155 by agar well diffusion assay (Anbarasu et al., 2019). Briefly, about 5 mm diameter wells were cut on nutrient agar medium containing non-mycobacterial cultures and middlebrook 7H9 agar plate containing *M. smegamtis* mc2155. About 100µl of treated CFS from all isolates were added into each well. Nutrient agar plates were incubated at 30˚C for 18 hours and middlebrook 7H9 agar plates were incubated for 48 hours at 37˚C. Zones of inhibition were measured in mm.

LAB isolate (BFO21) showing activity against *M. smegmatis* mc2155 was identified by 16S rRNA gene sequencing. Briefly, genomic DNA was isolated using a modified phenol-chloroform protocol. PCR was performed using the following primer pair-forward primer 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and reverse primer 1492R (5'-TAC GGT TAC CTT GTT ACG ACT T-3') at the following conditions: initial denaturation (97°C for 5 min followed by 35 cycles at 94°C for 2 min), annealing (51°C for 1 min) extension (72°C for 2 min) and final extension (72 °C for 5 min). PCR products were purified and subjected to sanger sequencing for 16s rRNA gene sequence analysis. The homology of the obtained sequence was analyzed using NCBI BLAST.

Table 1a. Isolation and characterization of lactic acid bacteria from fish gut

S. No	Reference ID	Source (Fish)	Morphology	Gas Production	Growth @10˚C	Growth @45˚C	Growth @pH 4.4	Growth @pH 9.6	Growth @ 6.5% NaCl	Growth @ 18% NaCl
1	BFO21	*Tilapia*	Gram positive bacilli	–	–	+	+	+	+	+
2	BFO22	*Tilapia*	Gram positive bacilli	–	+	+	+	+	+	+
3	BFO27	*Rastrelliger kanagurta*	Gram positive cocci (tetrads)	–	–	–	–	+	+	–
4	BFO28	*Rastrelliger kanagurta*	Gram positive cocci (tetrads)	–	+	+	–	+	–	–
5	BFO29	*Centropristis striata*	Gram positive cocci (tetrads)	–	+	+	+	+	+	–
6	BFO30	*Teuthida*	Gram positive cocci (tetrads)	–	+	+	+	+	–	–

Table 1b. LAB identified at genus level by biochemical methods

S. No	Reference ID	Genus Identified
1	BFO21	*Lactobacillus sp.*
2	BFO22	*Lactobacillus sp.*
3	BFO27	*Streptococcus sp.*
4	BFO28	*Aerococcus sp.*
5	BFO29	*Pediococcus sp.*
6	BFO30	*Pediococcus sp.*

Table 2. Inhibitory activity of cell free supernatant by agar well diffusion assay

S. No	Reference ID	Zone of Inhibition (mm)				
		S. aureus	*B. cereus*	*E. coli*	*K. pneumoniae*	*M. smegmatis*
1	BFO21	-	11	-	-	12
2	BFO22	-	10	-	-	-
3	BFO27	-	11	-	-	-
4	BFO28	-	-	-	-	-
5	BFO29	-	8	-	-	-
6	BFO30	-	10	-	-	-

Lactobacillus spp. (BFO21) was subjected to partial purification of bacteriocin using solvent extraction method. Briefly, overnight grown LAB isolate was inoculated to 500ml of MRS broth and incubated at 30°C for 18 hours in shaker, 100 rpm. CFS were collected by centrifugation at 5000 rpm for 15minutes at 4°C, added with equal amount of chloroform and kept in magnetic stirrer for 20minutes at 4°C. Resulting white precipitate was collected by centrifugation at 5000rpm for 30 minutes, lyophilized and stored in PBS buffer

(pH 6.0) at -20°C. The precipitated bacteriocin was filtered through 0.45micron syringe driven filter and subjected to antimycobacterial screening.

Table 3. Anti-tubercular activity of partially purified bacteriocin by LRP assay

S. No	Reference ID	Percentage of Reduction in RLU	Result
1	Rifampicin	91.93	Inhibition
2	BFO21*	98.00	Inhibition

*Bacteriocins produced by *L. plantarum*.

To screen for anti-tubercular activity of partially purified bacteriocin against *M. tuberculosis* H37Rv, LRP assay was used as mentioned (Anbarasu et al., 2019). Briefly, 400µl of middelbrook 7H9 broth was added to two cryo vials (Control), 400µl of middlebrook 7H9 broth containing rifampicin at 2µg/ml concentration (drug control). The test cryo vial was added with 350µl of middlebrook 7H9 broth and 50µl of partially purified bacteriocin (10mg/ml). About 100µl of *M. tuberculosis* H37Rv suspension (Mcfarland Unit 2) was added to all the vials and incubated at 37˚C. After 72 hours of incubation, 40µl of 0.1M $CaCl_2$ and 50µl of mycobacteriophage (phAE202) were added to all the vials and incubated for 4 hours at 37˚C. Then 100µl of the cell-phage mixture from each vial was added with 100µl of D-Luciferin and Relative Light Units (RLU) was measured using Luminometer (model: Lumat^3 LB 9508, make: Berthold). The inhibitory activity against *M. tuberculosis* was calculated based on the formula: Percentage RLU reduction = Control RLU– Test RLU / Control RLU × 100. The test RLU reduction by 50% or more when compared to control RLU was considered as positive for anti-tubercular activity.

RESULTS AND DISCUSSION

In the present study, *Pediococcus sp.* (2) was isolated from *Centropristis striata* and *Teuthida fish* intestinal tract. *Lactobacillus sp.* (2) were isolated from *Tilapia*. *Aerococcus sp.* and *Streptococcus spp.* were isolated from the gut of *Rastrelliger kanagurta* (Table 1a; Table 1b). The antibacterial and antimycobacterial activity of treated CFS samples are summarized in Table 2. CFS of five LAB isolates viz. BFO21, BFO22, BFO27, BFO29, BFO30 showed activity against *B. cereus* alone whereas BFO28 isolate have not shown inhibitory activity against any of the tested organisms. Plantaricin LPL-1 produced by *L. plantarum* have showed significant antibacterial activity against *S. aureus, L. monocytogenes, B. pumilus, B. amyloliquefaciens, E. faecalis* (Wang et al., 2018). In our study, BFO21 (*Lactobacillus sp.*) alone showed inhibition against *M. smegmatis* mc2155 by agar well diffusion assay indicating its antimycobacterial potential. Comparative 16S rRNA gene sequence analysis confirmed that *Lactobacillus spp.* BFO21 displayed 99.86% homology with *Lactobacillus plantarum* (GenBank accession number: MN367969). Silva et al., (2014) revealed that *L. plantarum* is one of the most important and versatile species and has many applications in various industries including pharmaceutical industries (Bravo et al., 2019).

The isolated *Lactobacillus plantarum* BFO21 was chosen for further purification of bacteriocin. Chloroform solvent extraction method was applied to purify the bacteriocin and the resultant lyophilized bacteriocin was screened against *M. tuberculosis* H37Rv using LRP assay. Bacteriocin of *Lactobacillus plantarum* BFO21 has shown reduction in RLU values by 98.00% at concentration of 35.53µg/ml. The result was compared with known anti-TB drug rifampicin at 2µg/ml that showed 91.93% of RLU reduction (Table 3). Previous studies have found that lactobacilli isolated from badger feces, wild boar feces or fermented milk products exhibited antimycobacterial activity against BCG and *M. bovis* (Mariam, 2009; Macuamule et al., 2016; Stedman et al., 2018; Bravo et al., 2019). To the best of our knowledge, this is the first study that showed the partially purified bacteriocin from *Lactobacillus* of *Tilapia* fish intestinal tract showing anti-tubercular properties (Bravo et al., 2019).

CONCLUSION

The burden of tuberculosis was reported high in India and treatment requires 6-8 months for new cases and 18-24 months for MDR TB. Lactic acid bacteria (LAB) and their bacteriocins received significant attention in the past decade due to its Generally Recognized as Safe (GRAS) status. Hits with improved cell penetration and with activity against *M. tuberculosis* should be prioritized. Bacteriocins of LAB certainly possess sufficient potential to merit hope for future therapies for treating intracellular infections. In this study, the *Lactobacillus plantarum* BFO21 obtained from fish gut has shown to produce antibacterial and antimycobacterial bacteriocins. The in vitro LRP assay of partially purified bacteriocins have shown significant anti-tubercular activity against *M. tuberculosis* H37Rv. Further purification and characterization of bacteriocins should be done to explore the possibility to develop the same as lead anti-tubercular compounds.

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Compliance with Ethical Standards: There are no laboratory animals and human subjects involved. The lactic acid bacteria were isolated from the intestinal tract of fishes obtained from fish slaughter-house.

Conflict of interest: All the authors declare that they have no conflict of interest.

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