IJCRT.ORG ISSN : 2320-2882

INTERNATIONAL JOURNAL OF CREATIVE RESEARCH THOUGHTS (IJCRT)

An International Open Access, Peer-reviewed, Refereed Journal

Antimicrobial Activity Of Different Cellular Compounds Of 4 Probiotic Strains MBTU_PB1, MBTU_PB2, MBTU_PB3 And MBTU_PB4 Against Bacterial Fish Pathogens

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1. ABSTRACT

Aquaculture is one of the fastest growing sector in the world, especially in Asia. it has become an important economic activity for many countries. Various kind of diseases are the major problem associated with this sector it reduces the yield considerably. Usually antibiotics are used against diseases. But antibiotic administration has many problems; to overcome these problems probiotic can be used as an alternative.Anatagonestic activity is a major characteristic of probiotics. Probiotics produce an array of antimicrobial compounds , so the location and characterization and effect of these compounds against the fish pathogens are very much beneficial. The compounds may be present either intracellularly or extrecellularly. So the antimicrobial activity determination of all these components is necessary. For that 4 cellular components such as intracellular product(ICP), extracellular product(ECP), heat killed whole cell product(HKWCP), and whole cell product(WCP) can be used, and their antimicrobial activity pattern determination and comparison of this with standard antibiotic will show their activity. Then the highest activity showing fraction then subjected to various analysis to identify which is the component which contributes this effect; for that Dialysis, Tricine SDS –PAGE, TLC analysis etc can be done . After that the effective component should be used for some field trails to find whether thy are effective in invivo conditions or not.

2. INTRODUCTION

Aquaculture is one of the fastest developing growth sector in the world. Especially in Asia. Asia contributes about 90% to the global production. It has become an important economic activity in many countries. Intensive aquaculture system produces substantially higher yield. But this intensification results in stressful environmental changes which can produce various kinds of problems in aquatic organisms, principally diseases of diverse etiology. So the control of disease is become of great importance in aquaculture. The most common way to disease control is the use of antibiotics. But the use of antibiotics as a preventive measure has been questioned by acquisition of genes that results in antibiotics resistance and horizontal gene transfer from fish pathogens to human.

The use of probiotics in aquaculture is based on the antagonistic activities shown by them. Microbes produces an extra-ordinary array of microbial defence systems. These include broad spectrum of classical antibiotics so critical to human health concerns, metabolic by - products like lactic acids produced by lactobacilli, lytic agents such as lysozymes found in many foods, numerous types of proteins exotoxins, and bacteriocins, which are loosely defined as active protein moieties with a bacteriocidal mode of action. Bacterial strains appear to be an excellent candidate for a friendly alternative since bacteriocin would be used as an antibiotic substitute (Riley MA and Wertz JE, 2002), whereas bacteria would be a potential probiotic.

In contrast to the currently used antibiotics, Bacteriocins are often considered more natural because they are thought to have been present in many of the foods eaten since ancient times. The administration of bacteriocin-producing bacteria rather than the bacteriocins themselves might be a more cost-effective approach, but significant progress in developing suitable producer strains will have to be made before such an approach will be feasible.

As with any antimicrobial compounds, the tissue of resistance also has to be considered for bacteriocins. Although the mechanism of action is not known for all bacteriocins, most of the low molecular weight bacteriocins appear to interact with the bacterial membrane. Resistance is therefore usually the result of changes in the membrane of bacteria targeted by a bacteriocin.

Bacteriocins are commonly divided into three or four groups. Nisin was discovered in 1928, and subtilin, a nisin analogue differing by 12 amino acid residuces, was discovered in 1948. Both belong to class I, termed lantibiotics. Class I is being further subdivided into class Ia and class Ib. In general, Class I peptides typically have from 19 to more than 50 amino acids. Class I bacteriocins are characterized by their unusual amino acids, such as lanthionine, methyl- lanthionine, dehydroubtyrine and dehydroalanine. Class Ia bacteriocins, which include nisin, consist of cationic and hydrophobic peptides that form pores in target membranes and have a flexible structure compared to the more rigid class Ib. Class Ib bacteriocins, which are globular peptides, have no net charge or a net negative charge.

Class II contains small heat-stable, non-modified peptides, and can be further subdivided. According to conventional classification, class IIa includes Pediocin like Listeria active peptides with a conserved N-terminal sequence Tyr-Gly-Asn-Gly-Val and two cysteines forming a S-S bridge in the N-terminal half of the peptide. Bacteriocins composed of two different peptides comprise Class IIb. The two-peptide bacteriocins need both peptides to be fully active. The primary amino acid sequences of the peptides are different. Though each is encoded by its own adjacent genes, only one immunity gene is needed. Class IIc was orginally proposed to contain the bacteriocins that are secreated by the general sec-system. Since this proposal, it has been shown that Class IIa bacteriocins can use the secretory system and consequently the sub-class IIe should eradicated.

The large and heat labile bacteriocins makes up the Class III bacteriocins for which there is much less information available. A fourth class consists of bacteriocins that form large complexes with other macromolecules, has been proposed. However, presently, no such bacteriocins have been purified and there is good reason to believe that this type of bacteriocin is an artifical due to the cationic and hydrophobic properties of bacteriocins which result in complexing with other macromolecules in the crude extract. This phenomenon has been shown in the case of plantaricin S. First, it was calimed to be a large complex molecule, but later the activity was purified as a small peptide, and the complex disintergrated while the activity was maintained. The Class I and II bacteriocins since they are the best understood and most likely to be used in food applications due to their target specificity and robustness.

SIDEROPHORES.

Siderophore is another biocontrol agent produced by some microorganisms. Sideropheres are low- molecular weight, ferric iron specific chelating agent that can dissolve and precipitated iron and make it available for microbial Growth (Verschuere et al, 2000) . The significance of sideorphores resides in their capacity to scavenge an essential nutrient from environment and deprive competitors for them. Since pathogens lack siderophore they can't survive in and iron limiting environment. The ecological significant of siderophore in soil as important tool for iron acquisition by microorganisms and plants, and their involvement in suppression of plant root pathogens (Williams, 1993).

Harmless bacteria which can produce siderphores could be used as probiotics to complete with pathogens for iron from solution. The possible effectiveness of siderophore producing probiotics can be illustrated by the study of Gatesoupe (1997), in which the addition of the resistance of turbot larvae challenged with the pathogenic strain Vibrio P. The addition of sidereophore producing Vibrio strain protected the turbot larves slightly more.

ENZYMES

Probiotics produces various enzymes such as amylase, protease, cellulase and lipases as well as necessary growth factors. The probiotics produces extracellular amylase and protease is improved host carbohydrate and protein digestion, while Degradation of chitin, starch, protein, cellulose and lipids is largely due to the production of extra cellular enzymes; proteases, lipases, chitinases and cellulases by bacteria associated with aquacultures species. Another important enzyme is lysozyme. Lysozymes (muramidases) are a family of enzymes with antimicrobial activity characterized by the ability to damage the cell wall of bacteria. The enzyme acts by catalyzing the hydrolysis of 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl-D- glucosamine residues in peptidoglycans and between the N-acetyl-D-glucosamine residues in chitodextrins. Several workers have reported that bacteria from isolated from fish can digest chitin, strach, protein, cellulose and lipids, Vitamins, carotenoids , short -chain fatty acids and lipids produced by probiotics that also contribute to the improve host nutrition. These are produced as secondary metabolites by probiotic bacteria. Carotenoids, which act as antioxidants and polyunsaturated fatty acids contribute significantly to the total fatty acids contribute significantly to the total fatty acid content of host fish. Improve host digestive processes by producing extra cellular enzymes.

ROLE OF ANTIBIOTICS IN AQUACULTURE

The discovery and development of antibiotics in the twentieth century, the perceived value of these traditional therapies diminished (Bengmark 2001; Meier and Steuerwald 2005). Today, with the efficacy of antibiotics waning and a dramatic resurgence are reconsidering the possible role of probiotics as an alternative to supplement existing antibiotics-dominated therapies (Saavedra 2001; Senok et al. 2005). Over the past 15 years, there has been an increase in research on probiotic bacteria and a rapidly growing commercial interest in the use of probiotic bacteria in food, medicine, and a supplements (Morelli 2002; Scarpellini et al. 2008).

The purpose of this work is to found out the antibiogram of selected aquatic probiotic strains and antagonistic activity of different cellular components against selected fish pathogens like *Alcaligens, Acinetobacter, Flavobacteriam, Vibrio cholerae, Aeromans hydrophila.* The antimicrobial activity is may be due to the presence of bacteriocin, bacteriocin like substances or by some other antimicrobial peptides. Since these are ribosomally synthesized peptides, they may be present either intracellular environment or in the medium, where these found (extracellular). This peptide fractions sometimes found as the part of cell wall too. So to find out the location of these antimicrobial compounds at maximum concentration are also very significant.

3. MATERIALS AND METHODS:

MICROORGANISMS

Microorganisms (Probiotics) used in this study; MBTU_PB1, MBTU_PB2, MBTU_PB3& MBTU_PB4 were collected from the Microbial Biotechnology Lab of School of Biosciences M G University Kottayam. The indicator pathogenic organisms; *Alclingenes* 1424, *Aeromonas hydrophila* 1739, *Acinetobacter* 1271, and *Flavobacterium* 2495 were collected from the Microbial Cultural Collection Centre (MTCC) Chandigarh.

PREPARATION OF BACTERIAL STRAINS

Four types of antimicrobial metabolites, e.g. heat killed whole cell product (HKWCP), whole cell product (WCP), intra cellular product (ICP) and extra cellular product (ECP) were prepared from (MBTU__PBI, MBTU__PB2, MBTU__PB3 and MBTU__PB4 (Das et.al, 2006). Briefly pure cultures of all the four strains were grown separately in sterile conditions in 250 ml of brain heart infusion broth (Himedia, India) at 37^0C for 24hr. Pure cultures of each selected strain were divided into five equal volumes of 50 ml and each 50 ml was taken for preparation of HKWCP, WCP, ECP and ICP separately. The optical density (OD) of 24 h old cultures was taken for each bacterium and simultaneous plating was carried out in tripulate and the colony forming unit/ ml was calculated. The OD 546 of all four strains was adjusted to 0.5 which corresponded to 1.96 x 106 cfu/ml, 1.6x106 cfu/ml 1.78 x 106 cfu/ml and 2.17 x 106 cfu/ml for MBTU__PB2, MBTU___PB3 and MBTU__PB4, respectively.

PREPARATION OF CELLULAR COMPONENTS

(Basanta Kumar Das et al (2006); Aquaculture 2531(19-24)

Preparation of Whole cell product (WCP)

All the isolates (MBTU_PB1, MBTU_PB2, MBTU_PB3 & MBTU_PB4)grown separately in Brain Heart Infusion broth were centrifuged at 12,000rpm for 10 minute at 4^0C. The bacterial pellets were washed twice in Phosphate Buffer Saline (pH 7.2) and after that the pellets were resuspended in Phosphate Buffer Saline. This was used as Whole cell product.

Preparation of Extracelluar products (ECP)

The supernatant obtained after centrifugation of the 24 hour old culture of bacteria in BHI broth were filter sterilized. They were dialysed in benzoylated dialysed bag of against Phosphate Buffer Saline (pH-7.2). After 48 hours, the dialysed portion is concentrated with 20% Poly Ethylene Glycol (PEG 6000).

Preparation of Heat killed whole cell product (HKWCP)

All the bacterial isolates, after 24 hour incubation were centrifuged at 12,000rpm for 10 minutes in 40C. The bacterial pellets obtained after centrifugation were washed twice and resuspended in Phosphate Buffer Saline (pH 7.2). And this were heat killed by placing it in a boiling water bath at 80^0C for 2 hours. This pellets were used for antagonistic study.

Preparation of Intracellular product (ICP)

All the bacteria grown seperately in Brain Heart Infusion broth; after 24hours were centrifuged at 12,000rpm for 10 minute in 4^0C. The bacterial pellets were washed twice and resuspended in Phosphate Buffer Saline (pH-7.2), to 4% of the initial volume. The cell pellets were then sonicated at 50Hz for 5 minutes, and after that these sonicated sample was filtered through a micropore filter with pore size 0.45µm. Finally stored at 4^0C.

PROTEIN ESTIMATION OF DIFFERENT CELLULAR COMPONENTS (Bradford method, 1976)

The protein estimation of all the protein fraction of the four isolates (MBTU__PBI, MBTU__PB2, MBTU__PB3 and MBTU__PB4) was determined according to Bradford Method (1976). Protein concentration ranging from 10-100µg were prepared by pipetting out 0.2 , 0.4, 0.6, 0.8 and 1.0ml of the working standard solution into a series of test tubes containing required quantity of 0.1M NaOH to make up the volume to 1.0 ml. In each tube, 5.0 ml of Bradford dye solution was added to

make the final volume to 6.0 ml .The contents were mixed well and allowed to stand for five minutes but no longer than 30 minutes . A reagent blank was maintained essentially as above adding working standard solution. Absorbance was measured at 595 nm against the reagent blank. A standard graph was drawn by plotting the concentration of protein along the X-axis and absorbance along the y-axis. From the standard graph, the amounts of protein in the samples were calculated. For biocontrol study, the protein concentration of different cellular components of all the strains were standardized to 20µg/ml of protein.

BIOCONTROL STUDY

Overnight growth cultures of 5 indicator pathogens; *Aeromonas hydrophila, Acenetobacter, Vibrio, Alkaligens, Flavens, Alkaligens* were seperately lawn on Tryptone Soya Agar (TSA, Himedia-India) by lawn culture method. Then 6mm diameter wells were made in each plate and the different cellular components containing 20µgm of protein were introduced into the wells, sterile Phosphate Buffer saline (PBS) in one well was the control. Incubated the plates at 33^0C for 24 hours.

UV-VIS ANALYSIS

The lyophilized samples of Extracellular product of all the 4 selected strains MBTUPBI, MBTUPB2, MBTUPB3 and MBTUPB4 were scanned under UV light (UV-VIS Spectrophotometer- HITACHI, U-1990) within a range of 220-360 nm. All the lyophilized samples were diluted with double distilled water in order to reduce the concentrations.

MOLECULAR WEIGHT DETERMINATIONS

Tricine sds-page (oxford Laboratory Technology 2007)

To estimate the molecular mass of each of the partially purified, PEG concentrated cell free supernatant of the extracellular sample, the pretreated samples from the four selected bacterial strains, obtained as described above the (preparation of extracellular product) were subjected to Tricine-SDS –polyacrylyamide protein of below 35 kDa. Initiate polymerization by adding 40ul of TEMED (pure liquid) to 12.0% separating gel. Transfer to the gel cassettes; bring to approximately 1.5 cm from the top of gel blank (which is reserved for the stacking gel) and gently overlay with a small volume (ca. 0.5 ml) of 0.1% (w/v) SDS. Polymerize for 30 min. Remove layer of SDS after the gel has polymerized Initiate polymerization by adding 20 Ul of TEMED (pure liquid) to 4.0% stacking gel. Transfer to the gel cassettes and insert comb. Clamp along the top with binder clips to insure well formed well. Polymerize the 30 min. The samples were prepared by mixing an equivalent volume of sample with an equivalent volume NOVEX 2X Tricine SDS sample buffer. The samples are then heated for 5 min in a boiling water bath, centrifuged for one minute. Prior to loading the gel, fill the upper buffer chamber with cathode buffer and the lower chamber with 250 ml of anode buffer. Electrophoresis is done at constant (5 Ma) until the samples have migrated through the stack, while 30 Ma is used for the remainder of the run until the tracking dye has migrated out from the lower slot . Staining is accomplished by soaking the gel in a staining solution on a top shaker for 30 minutes. The gel is then destained by soaking in a destaining solution on a table top shaker. Periodically replace with fresh solution until desired destaining is achived. After destaining , the gel is prepared for drying by soaking it for five minutes in gel drying solution. Next, soak a single piece of cellophane in the same gel dry solution for 30 seconds. Lay down the cellophane first, and the gel on top of this. Next soak the second piece of cellophane in gel drying solution for 30 seconds, and then overlay this on the gel to make a sandwich, squeeze out any air bubbles and then clamp on upper frame. Dry at room temperature.

ANTIBIOGRAM DETERMINATION (Kirby-Bauer method)

Antibiotic sensitivity test is used to detect one of the main characters of the probiotics, i.e., the resistance of the bacterial strains against common antibiotics. Antibiogram of isolated strains and indicator strains were prepared using selected antibiotics discs (Gentamicin (10 mcg/disc) Chloramphenicol (10 mcg/disc) Streptomycin (25 mcg/disc), Tetracycline (10 mcg/disc) , Vancomycin (10 mcg/disc) Carbencillin (100 mcg/disc) Kanamycin (10 mcg/disc) Methicillin (5 mcg/disc) , Ciprofloxacin (30 mcg/disc) , Erythromycin (10 mcg/disc) Amikacin (30 mcg/ disc) Amikacin (30 mcg /disc) , Ampicillin (10 mcg/disc) Penicillin G (10 mcg/disc) which were placed on Muller –Hinton agar (HIMedia, India) Plates previously swabbed with the selected bacterial strains pathogens. Plates were incubated at 37^0 C for 24 hours. Resistant bacterial strains around the antibiotic

discs. Clear zones around the discs with respect to the standard MIC of each antibiotics, the sensitivity of the bacterial strains against the antibiotics was evaluated.

4. RESULT

PROTEIN ESTIMATION OF DIFFERENT CELLULAR COMPONENTS

The protein content of ICP, WCP, HKWCP & ECP of MBTU_PB1, MBTU_PB2, MBTU_PB3 and MBTU_PB4 are given in the table 2. Among the different cellular components ICP of all the strains contain highest protein concentration. And the lowest protein concentration was observed in ECP of all the strains.

Table 1: The standard curve preparation by Braford method using BSA standard

S.No	Volume of Standard BSA (mL)	Concentration of BSA (mg)	Volume of 0.1M NaOH (mL)	Bradford reagent (mL)		Optical density at 595 nm
Blank	0.0	0.0	1.0	5.0	**Kept at room temperature for 5 minutes**	0.0
1	0.2	20.0	0.8			0.179
2	0.4	40.0	0.6			0.275
3	0.6	60.0	0.4			0.393
4	0.8	80.0	0.2			0.463
5	1.0	100.0	0.0			0.522

Concentration of BSA standard = 5 mg/ml

Table3: protein concentration of different cellular components of MBTU_PB1, MBTU_PB2, MBTU_PB3 and MBTU_PB4

Cellular component / Bacterial isolate	Concentration of WCP (µg/ml)	Concentration of ECP (µg/ml)	Concentration of HKWCP (µg/ml)	Concentration of ICP (µg/ml)
MBTU_PB1	18	16	22	28
MBTU_PB2	36	20	40	42
MBTU_PB3	20	18	20	45
MBTU_PB4	20	18	21	45

BIOCONTROL STUDY

Results obtained from the WCP, HKWCP, ECP and ICP of the four selected strains against the five indicator pathogens are shown in fig no: 1, 2, 3 & 4. The WCP of all the four isolates showed no inhibitory activity against all the investigated indicator strains, as compared to PBS control (data not shown). It was revealed that among the different cellular components the ECP of all the four selected strains was found to be highly effective and produced a zone of inhibition ranging from 18 to 35 mm against all the five indicator strains. The next highest recorded inhibition zone was obtained by HKWCP of the four selected strains against *Alcaligenes* (26 mm ± 0.7 mm, 22 mm ± 0.6 mm and 26 mm ± 0.3 mm for MBTU_PB1, MBTU_PB2 and

MBTU_PB3, respectively) while MBTU_PB4 showed the zone of inhibition for HKWCP (24 mm ± 0.3 mm) with *Flavobacterium*. This result clearly indicate that out of the three cellular products (HKWCP, ECP & ICP), ICP of the stains showed the lowest inhibitory activity against all indicator pathogens.

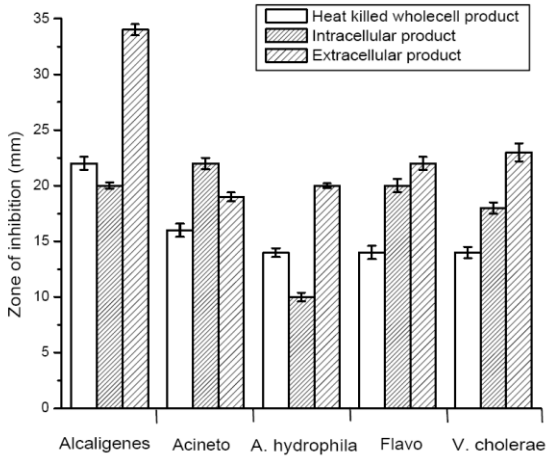

Fig1a: MBTU_PB1

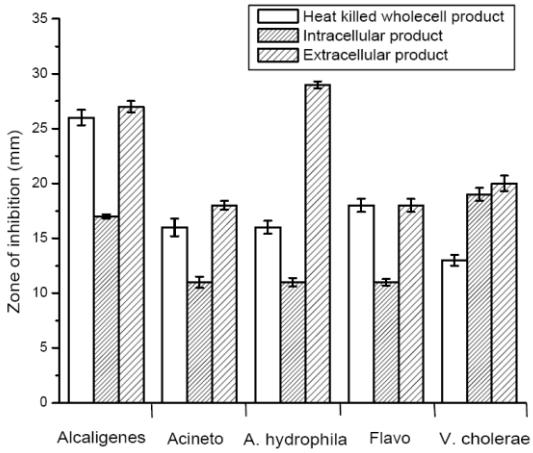

Fig1b: MBTU_PB2

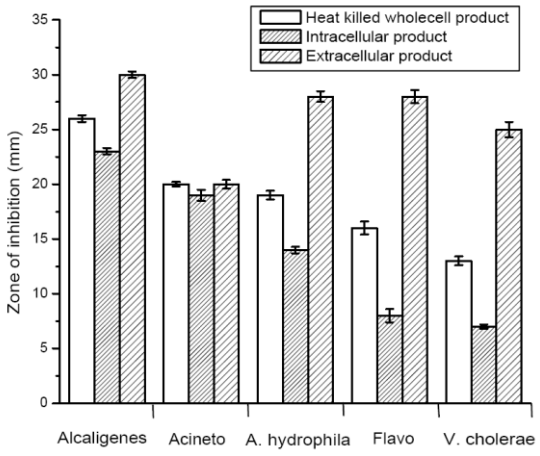

Fig1c: MBTU_PB3

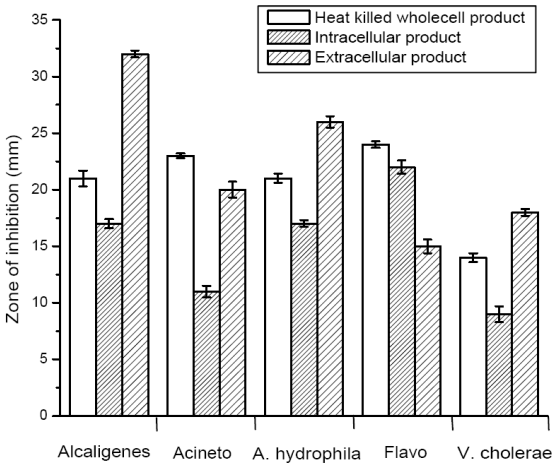

Fig1d: MBTU_PB4

Figure 1: Fig 1a, 1b, 1c and 1d showed zones of inhibition of four crude fractions of MBTU_PB1, MBTU_PB2, MBTU_PB3, and MBTU_PB4 against five indicator pathogens respectively. Values are represented as mean ± standard error.

Figure 2a

Figure 2b

Figure 2: Figure 2a and 2b, Zone of inhibition shown by ECP of all the strains against *Alcaligenes* and *Falvobacterium* respectively.

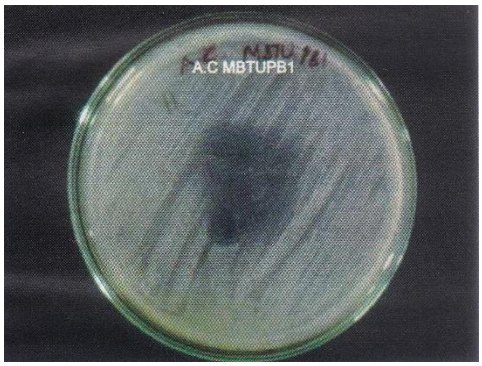

Figure 3a

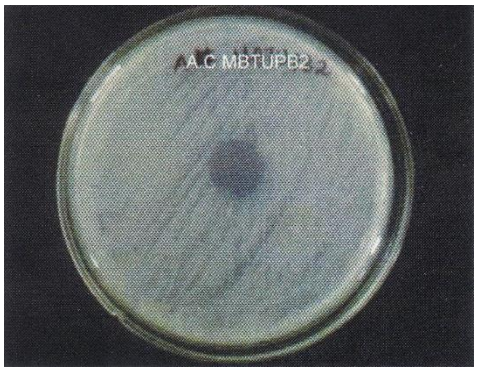

Figure 3b

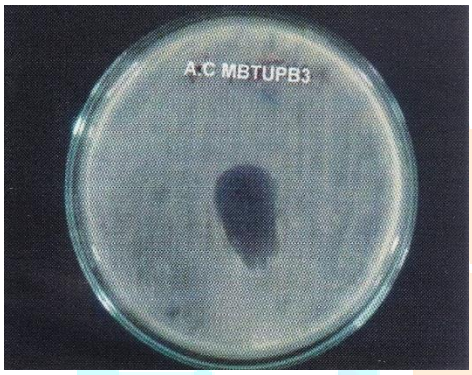

Figure 3c

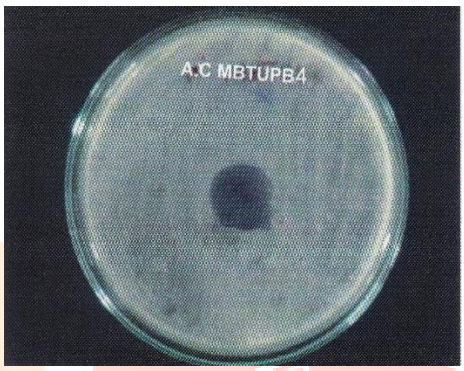

Figure 3d

Figure 3: Figure 3a, 3b, 3c and 3d are zone of inhibition shown by ECP of all the strains against *Acinetobacter*.

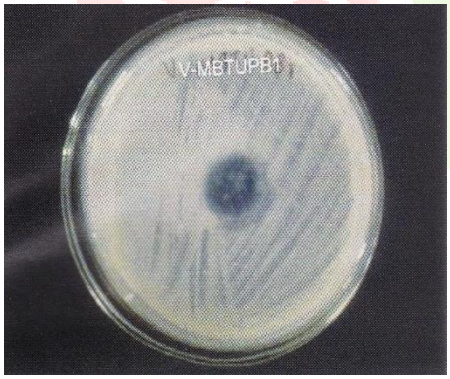

Figure 4a

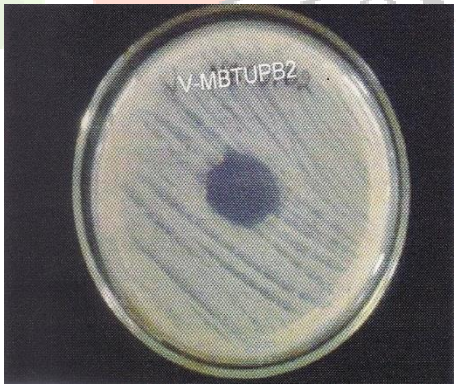

Figure 4b

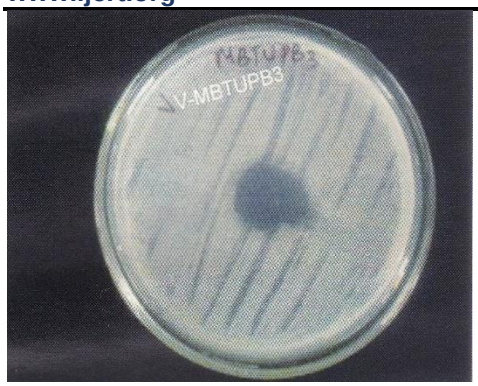

Figure 4c

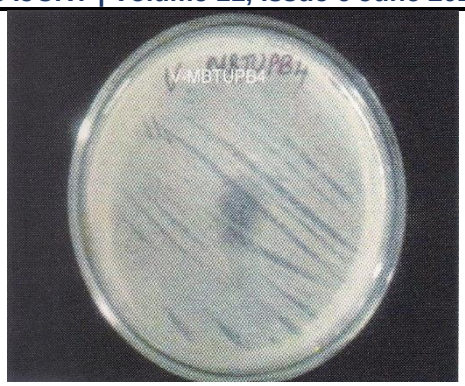

Figure 4d

Figure 4: Figure 4a, 4b, 4c and 4d are zone of inhibition shown by ECP of all the strains against *Vibrio Cholerae.*

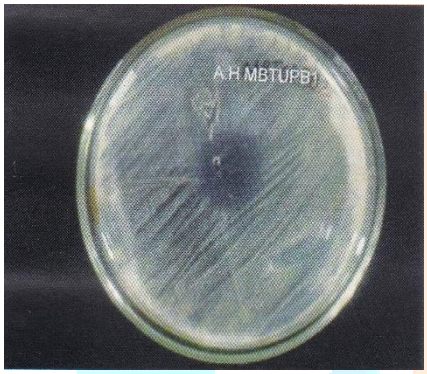

Figure 5a

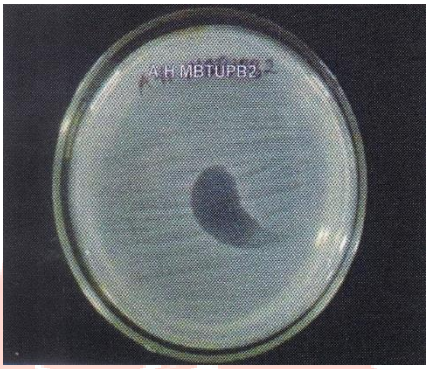

Figure 5b

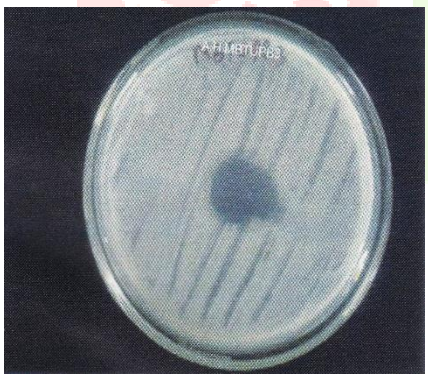

Figure 5c

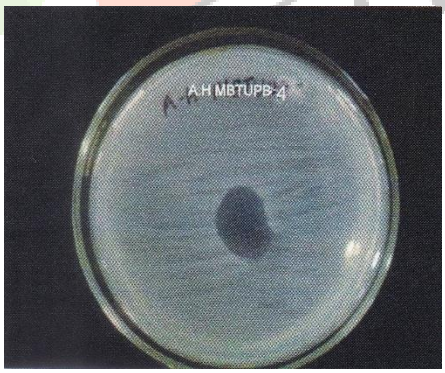

Figure 5d

Figure 5: Figure 5a, 5b, 5c and 5d are zone of inhibition shown by ECP of all the strains against *Aeromonas hydrophila.*

UV-VIS ANALYSIS

The ECP of all the 4 selected strains displayed characteristic protein peak in the UV-absorption spectrum between the ranges of 220-360. This range of spectrum is the characteristic of peptide bonds (Barja *et.al.*, 1989, Lee 1999).

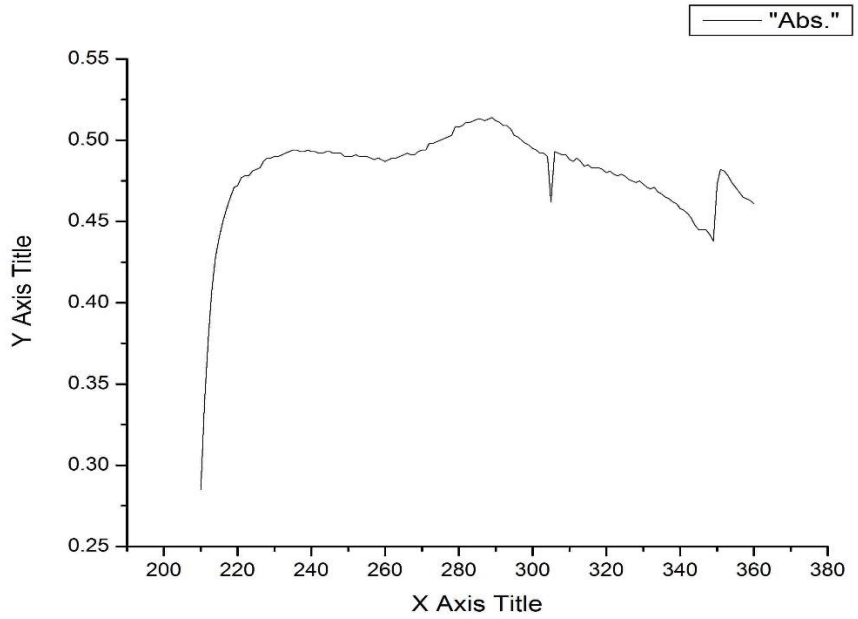

Figure 6: The ECP of strain MBTU_PB1 displayed characteristic protein peak in the UV-absorption spectrum between the ranges of 220-360nm.

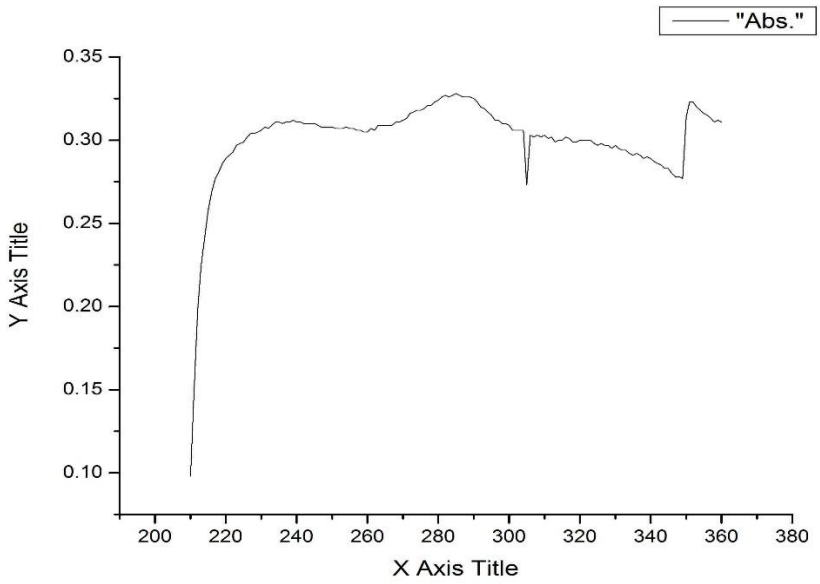

Figure 7: The ECP of strain MBTU_PB2 displayed characteristic protein peak in the UV-absorption spectrum between the ranges of 220-360nm.

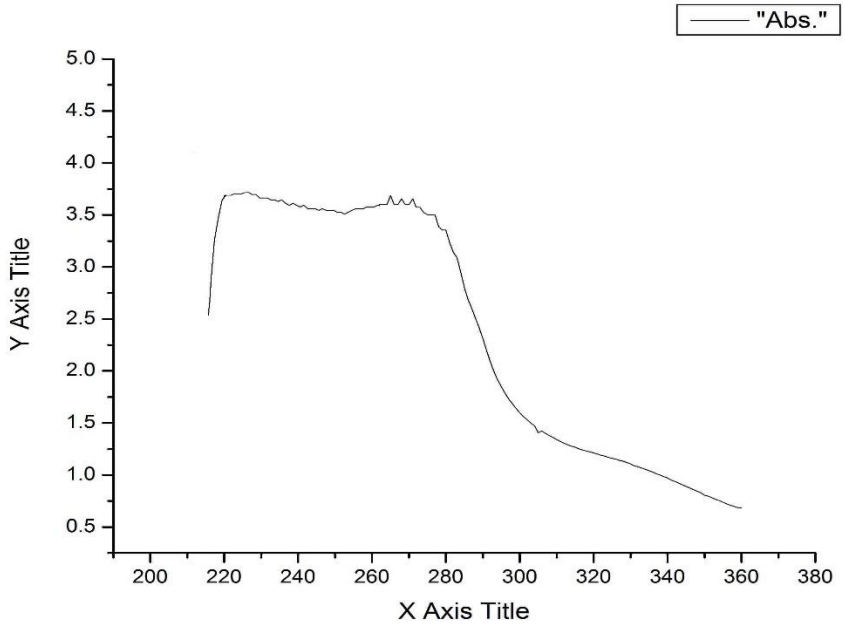

Figure 8: The ECP of strain MBTU_PB3 displayed characteristic protein peak in the UV-absorption spectrum between the ranges of 220-360nm.

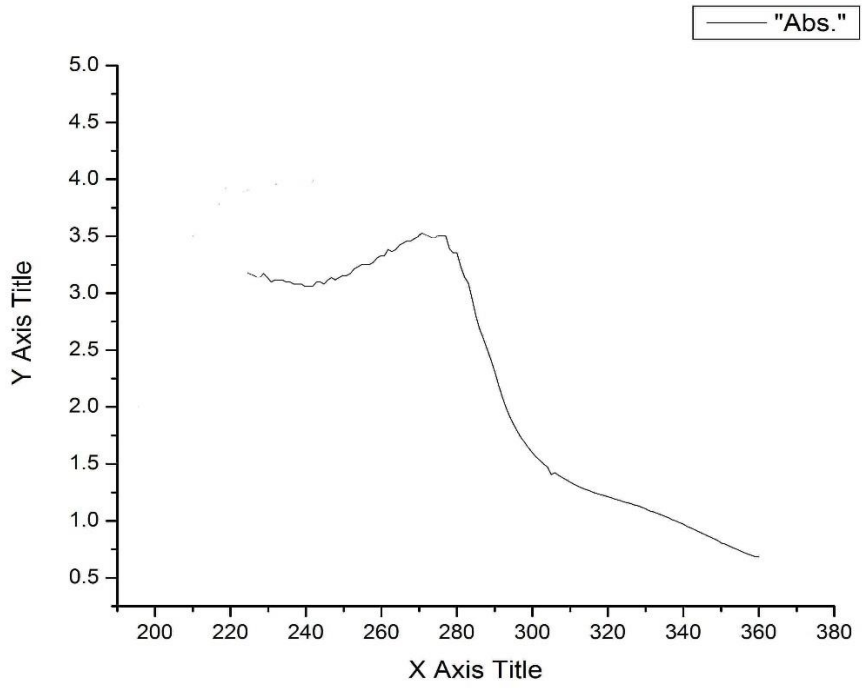

Figure 9: The ECP of strain MBTU_PB4 displayed characteristic protein peak in the UV-absorption spectrum between the ranges of 220-360nm.

MOLECULAR DETERMINATION

The molecular mass of partially purified antimicrobial compound in the ECP all the strain were estimated in the range of approximately 2kDa to 20.4KDa according to Tricine SDS-PAGE(fig:3). MBTU_PB1 shown a band nearly to 8.1kDa of

marker band. MBTU_PB2 showed a band near to 2.5kDa of marker band, MBTU_PB3 and MBTU_PB4 showed bands corresponding to marker band having molecular weight of 20.4kDa.

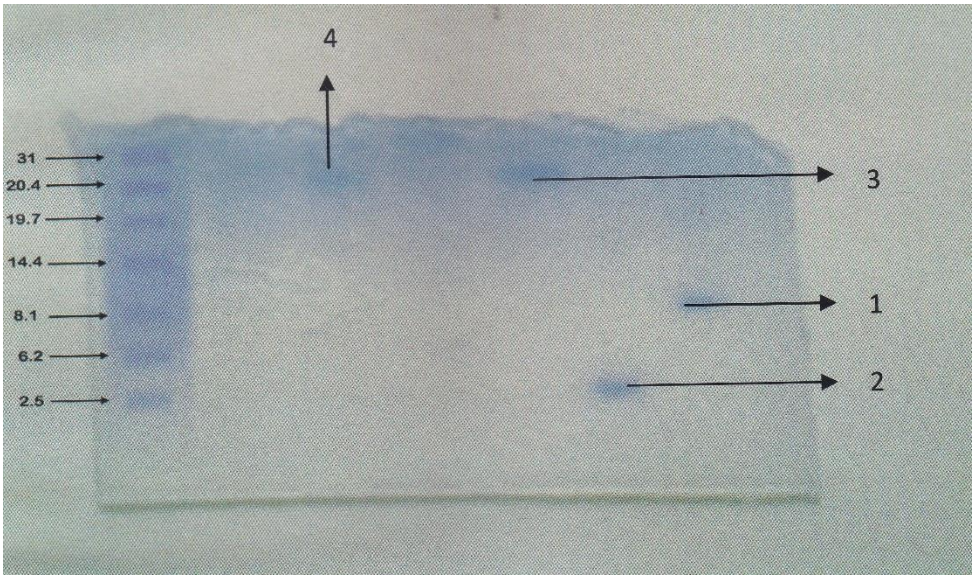

Figure 10: Tricine SDS gel.

ANTIBIOTIC SENSITIVITY PATTERN OF BOTH SELECTED STRAINS AND PATHOGENS

The results obtained after the antibiotic sensitivity test on Muller-Hinton agar plates showed that isolates MBTU_PB2 and MBTU_PB3 were sensitive to all the thirteen investigated antibiotics. MBTU_PB1 was sensitive to all the tested antibiotics except *Tetracycline, Vancomycin, Methicillin, Erythromycin* and *Penicillin*, while MBTU_PB4 was sensitive to all the antibiotics except *Methicillin* and *Penicillin***.** However, in the case of the indicator strains *V. cholerae* and *Flavobacterium* were sensitive to all the thirteen investigated antibiotics, except *Penicillin* (all of the indicator strains were resistant to *Penicillin*). Further, *A. hydrophila* was resistant against *Vancomycin, Methicillin, Erythromycin* and *Ampicillin*, while *Acinetobacter* was resistant against *Vancomycin, Carbencillin, Methicillin* and *Ampicillin*. *Alcaligenes* was resistant against all the antibiotics except *Vancomycin, Methicillin* and *Erythromycin*.

Bacteria / Antibiotic (conc.)	MBTU_PB1	MBTU_PB2	MBTU_PB3	MBTU_PB4	*Vibrio*	*Flavo*	*A.hydrophila*	*Alacligens*	*Acineto*
Gentamicin(10 mcg/disc)	S	S	S	S	S	S	S	R	S
Chloramphenicol (10 mcg/disc)	S	S	S	S	S	S	S	R	S
Streptomycin (25 mcg/disc)	S	S	S	S	S	S	S	R	S
Tetracycline (10 mcg/disc)	R	S	S	S	S	S	S	R	S
Vancomycin (10 mcg/disc)	R	S	S	S	S	S	R	S	R
Carbencillin (100 mcg/disc)	S	S	S	S	S	S	S	R	R
Kanamycin (10 mcg/disc)	S	S	S	S	S	S	S	R	S
Methicillin (5 mcg/disc)	R	S	S	R	S	S	R	S	R
Ciprofloxacin (30 mcg/disc)	S	S	S	S	S	S	S	R	S
Erythromycin (10 mcg/disc)	R	S	S	S	S	S	R	S	S
Amikacin (30 mcg/disc)	S	S	S	S	S	S	S	R	S
Ampicillin (10 mcg/disc)	S	S	S	S	S	S	R	R	R
Penicillin G (10 mcg/disc)	R	S	S	R	R	R	R	R	R

S = sensitive; R = resistant

5. DISCUSSION

Antimicrobial substances produced by different bacteria seem to play an important role in bacterial antagonism in aquatic ecosystem (Depazo et.al., 1998). Normal flora has a well defined role in the immune status of an organism eg, more than 10% of the bacterial isolates of intestinal tract of the fish exhibited antagonistic action (Sugita et.al.,1996).

In the present study we search for the effect of various cellular components of 4 probiotic strains isolated from Guppy, against 5 fish pathogens. From the protein estimation of different cellular components, it was found out that more protein content was observed in the ICP samples (Sonicated sample), and least protein in the ECP (Supernatant) samples of all 4 strains. It may be due to the fact that ECP constitute only extracellularly secreted product and ICP is the total protein content of the organism.

The result of biocontrol study showed that WCP of all the 4 selected strains have no inhibitory activity against the indicatro pathogens (data not shown). This indicated that the bacteria can produce the inhibitory compounds in a specific environmental conditions, such as amount and purity of anti metabolite, culture media, indicator strain & its cellular concentration (Gatesoupe, 1999, de vuyst et.al., 1994) . In this study highest inhibitory activity was showed by ECP samples of all the 4 strains against all the 5 indicator pathogens. Other cellular components i.e, HKWCP & ICP of all the tested strains were also effective as revealed by the zone inhibition to all the tested indicator strains. The order of inhibitions of the 3 cellular components i.e., ECP,HKWCP & ICP is ECP> HKWCP> ICP. This result clearly indicated that the antimicrobial metabolite of all the tested 4 strains produced extracellularly and also present intracellularly that is why the ICP and HKWCP samples showed inhibitory activity. Compare to HKWCP, ICP sample showed low inhibitory activity, this may be due to the presence proteases or any other protein inhibitor (Parente and Riccardi, 1999). Another interesting result obeserved was comparison of HKWCP with ECP, the result revealed that there is only minor variation in the inhibition zone. This clearly indicate that the antimicrobial compound present in the cell membrane of the strains, during heat treatment leaked out from the cell membrane to periphery and this result further indicate that the ant metabolite produced by all the strains was, resistant to temperature to 60^0 C for 1 hour. There are several reports that the cellular components that have the ability to inhibit the growth of fish pathogens in in vitro conditions (Balcazar et. al., 2007, Basanta Kumar Das et. al., 2006).

Hence these e bacterial strains can be considered a very promising alternative to the use of chemotherapeutic agents used in quaculture,particularly against the tested indicated pathogens. Further assays are necessary to characterize the antipathogenic metabolites secreted by the selected 4 strains.

UV-VIS analysis of the ECP of all the 4 strains cleared that the presence of a low molecular weight protein or peptide, because all the samples showed the characteristic absorbance spectra within the range of 220-360nm ((Barja et.al., 1989, Lee, 1999) . The molecular weight determination of ECP samples using. Tricine SDS-PAGE confers the presence low molecular weight protein or peptide .The electrophoretic band shown by all the samples (ECP) was lie within the range of 2.5k Da to 20.4 kDa of marker bands. So from the above all the results we can reach the conclusion that the antimicrobial metabolite present in the ECP of all the 4 strains have potential activity & further, molecular weight determination revealed that all of them were low molecular weight protein or peptide . So we can assume that they may be bacteriocin or bacteriocin like inhibitory compounds (Kleanhammer,1993).

There were many reports of antimicrobial activity of normal flora (Lee et.al., 2001) .Novel probiotic organism should not be resistant for any of the antibiotics (parker et.al., 2008). In the present study the strains MBTU_PB2 and MBTU__PB3 were sensitive to all the tested antibiotics. However, MBTU___PBI was sensitive to all the tested antibiotics except Tetracycline, Methicillin, Erythromycin,Pencillin G and Vancomycin. While MBTU__PB4 was sensitive to all the antibiotics except methicillin and pencillin G. The antibiogram of all the strains showed that all of them are sensitive to most of antibiotics. This indicated the wild type nature of the strains & these strains may not be harbor a plasmid. This observation is very promosing because there is no chance of resistant gene transfer among the organisms; which is a desirable probiotic characteristic.

6. SUMMARY AND CONCLUSION

The use of probiotic is an important management tool, but its efficiency depends on understanding the nature of competition between species or strains, Antagonism to indicator strains was the first step in screening probiotics.

In the present study, antagonistic activity of different cellular components of 4 selected probiotic strains except WCP, all the other components i.e, HKWCP, ECP & ICP were effective against the indicator strains. Even though their degree of inhibition is varies with respect to pathogens. This result revealed that the inhibitory compound located intracellularly and produces extracellularly. Out of the 3 cellular components ECP of all the strains shows maximum inhibition toward all the pathogens & the result also shows that all the antimicrobial compounds are thermo tolerant (active up to 60^0 for 1 hour). The UV-VIS analysis revealed the proteinaceous nature of the anti-metabolites. Molecular weight analysis confers the low molecular weight of proteins or peptides present in the ECP samples. The antibiogram study revealed that 2 strains MBTU_ PB2 & MBTU_PB3 were sensitive to all the antibiotics used, and also other 2 strains were sensitive to majority of the antibiotics. This shows the wild nature of the selected 4 strains. This is a desirable probiotic characteristic.

In the present study we concluded the presence of antimicrobial compound both intracellularly and extracellularly in all the 4 selected strains. With respect to UV-VIS analysis & molecular weight determination of ECP was showed that it is a low molecular weight protein or peptide. So we can assume that it may be a bacteriocin or bacteriocin like substance. It has to further characterized. Present study is very promising to develop these strains in aquaculture system as a substitute for antibiotics after *in vivo* study.

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