

Antagonistic Compounds Produced by Consortium of Probiotic Bacteria against Fish Pathogens

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Abstract: In this study, the antimicrobial activity of three solvent extracts of consortium (mixed culture) of commercial available probiotic bacteria against eight fish pathogens using well diffusion and disc diffusion assay method. The probiotic bacteria inhibited almost all the fish pathogens tested. All tested strains were significantly inhibited at probability level ($P < 0.5$). Zone of inhibition was higher in well diffusion method than disc diffusion method because of diffusible rate was higher in well method. These findings encountered that mixed probiotic culture have a broad spectrum antimicrobial effect.

Keywords: Probiotic bacteria, Antimicrobial activity, fish pathogens, solvents

1. Introduction

Probiotic have been also defined as “Mono or mixed culture of live microorganism that applied to animal (or) man, affect beneficially by the host by improving the properties of the indigenous microflora (1). The word “probiotic” was introduced by Parker (2). The concept of micro floral manipulation was first appreciated by Metchnikoff (3) who examined the consumption of yoghurt and found an effect on the longevity of Bulgarian peasants.

A Beneficial effect by application of certain beneficial bacteria in human, pig, cattle and poultry nutrition has been well documented (4 and 5). Several beneficial function have been reported for probiotic bacteria, e.g. vitamin production, production of important digestive enzymes, prevention and treatment of diarrhea, establishment of a healthy flora in premature babies, alleviation of the symptoms of lactose indigestion, stimulation of the immune system, suppression of tumor and cholesterol reduction.

Probiotic also contain bacteria belonging to the genera *Leuconostoc*, *Pediococcus*, *propionibacterium* and *Bacillus*. Yeasts (*Saccharomyces* and *Candida pintolopesii*) and moulds (*Aspergillus niger* and *A.oryzae*) are also used but mainly in animal product (6). Currently available probiotic preparation contains *Lactobacillus delbreuckii subsp. bulgaricus*, *L.acidophilus*, *L. casei*, *L. plantarum*, *L.brevis*, *L.cellobiosus*, *L.lactis* and *L.reuteri*. The first use of streptococci as probiotic was in the form of soured milk and yoghurt. The yoghurt starter *Streptococcus salivarius subsp.thermophilus* is still common probiotic organism. According to Austin *et al.*, (7) the bacteria *vibrio alginolyticus* was found to reduce disease in Atlantic salmon (*Salmo salar*) caused by infection of common pathogen strain (*Aeromonas salmonicida*, *vibrio anguillaram*) because it acting as a probiotic effect. Beneficial bacteria, such as *Lactobacillus* and *B.bifidum*, have the ability to kill off other bacteria by secreting small quantities of antibiotic-like substances, including lactic-acid, acetic acid, benzoic-acid, hydrogen peroxide acidolin, lactocidin and acidophilin (8).

Lactobacilli of human intestinal origin have been shown to exhibit antagonistic activity both Gram positive and Gram negative (8). *Bifidobacteria* are a focus of intensive international research for their essential role in fermented food especially for their ability to produce various antimicrobial compounds promoting probiotic properties (9). Microbial extracts have been and continue to be productive source of new biologically active molecules for drug discovery (10). Microbial secondary metabolites include antibiotics, pigments, toxins, effectors of ecological competition and symbiosis, pheromones, enzymes inhibitors, immunomodulating agents, receptor antagonists and agonists, pesticides, antitumor agents and growth promoters of animals and plants (11).

The purpose of this study was to investigate to enrich the probiotic cultures in laboratory condition for production of secondary metabolites. These metabolites are extracted with three solvents. Then the purified compound was subjected to study the efficiency for antimicrobial activity against fish pathogens.

2. Materials and methods

2.1 Sample collection:

Commercial available probiotic capsule was purchased from pharma company (Becelac PB, Dr.Reddy, Hyderabad). Eight different fish pathogen (*Aeromonas hydrophila*, *Bacillus sp*, *E.coli*, *staphylococcus*, *streptococcus*, *Vibrio.sp*, *Vibrio harvi*, *Pseudomonas*) were isolated from infected fish. The isolated fish pathogens were identified by Bergey’s manual of systematic bacteriology (12).

2.2 Separation of secondary metabolites:

Man Rogasa Sharpe (MRS) broth was prepared and inoculated the probiotic organisms allowed incubated to 24 hours at 37°C. After incubation, centrifuge the culture and take the supernatant was store in container. The supernatant was mixed with different solvents (Ethyl acetate, Toluene, Benzene) separately and poured in to the separation funnel, mix well and allowed to stand for 2 hours for separation. After separation, clear solution was treated with distillation. We get the crude metabolite compound in the form of paste. The compound is mixed with DMSO reagent because the reagent is helpful for stable the metabolic compound.

2.3 Antibacterial activity assay of bacterial probiotic metabolites

The antibacterial activity of bacterial secondary metabolites extracted with different solvent was tested by agar diffusion assay and disc diffusion assay method. The plates were incubated at 37°C for 24h during which activity was evidenced by the presence of a zone of incubation surrounding the well and disc. Each test was repeated three times and the antibacterial activity was expressed as the mean of diameter of the incubation zone (mm) produced by the secondary metabolite when compared to control.

3. Results and Discussion

In this study the role of enriched probiotic bacteria such as *Streptococcus faecalis* (30 millions), *Clostridium butricum* (2 millions), *Bacillus mesentericus* (1 million) and *Lactobacillus sporogens* (50 millions) from commercial probiotic capsule (Becelac PB,) are potential competitors of the pathogenic bacteria was investigated. The use of probiotics in aquaculture is a new potential and natural prevention mechanism against fish disease caused by pathogenic bacteria.

The enriched probiotic bacteria exhibited a marked antagonistic activity against all the bacterial pathogens (Table 1 and 2). The Toluene extract (25 µl, 50 µl and 75 µl) obtained from the probiotic mixture showed high significant antimicrobial activity against selected gram positive and Gram negative fish pathogens in disc diffusion method, *Aeromonas hydrophilus* (19mm), *Bacillus* sp (23mm), *E.coli* (24mm), *Pseudomonas aerogenosa* (20mm), *Streptococcus* sp (22mm), *Vibrio harveyi* (20mm) *Staphylococcus* sp (23mm) and *Vibrio* sp (20mm) when compared with the standard streptomycin (22mm) for 25µg/disc (Table 1). The antibacterial activity exhibited by the solvent extract was equivalent to that of the activity of streptomycin. The ability of consortium probiotic bacteria to suppress pathogen in vitro conditions suggests that it is a promising probiotic bacteria. Among the three solvent used in these study, ethyl acetate extract have given less antibacterial activity (Table 1 and fig 1). Nestle yogurt probiotic were bactericidal for *S.aureus* and *P.aeruginosa*, but were inhibitory for *S.typhi*. Neslac probiotic s killed the test organisms *E.coli* and *S.typhi*. They were only inhibitory for *S.aureus* and *C.albicans*. Results of the study showed the antimicrobial activity of the probiotic enriched from the commercial probiotic capsule. This may be due to the production of acetic acid and lactic acid that lower the pH of the medium, and stimulate the production of hydrogen peroxide and bacteriocins (13).

The solvent extracted bacterial supernatant of mixed culture of *Streptococcus faecalis*, *Clostridium butricum*, *Bacillus mesentericus* and *Lactobacillus sporogens* exhibited greater inhibitory activity against eight bacterial fish pathogens in well diffusion method (Table 2 and fig 2). The highest inhibitory zone was observed in the ethyl extract of probiotic metabolites around the well against pathogen *Streptococcus* sp was 11-13 mm in diameter. In toluene extract, the highest zone inhibition was 18-30 mm against *Vibrio harveyi*. The highest zone of inhibition of benzene extracts was 18-29 mm

against *Pseudomonas aerogenosa*. In our finding, inhibitory activity has vary from solvent to solvent, pathogens to pathogens and concentrations (25µl, 50µl and 75µl). The *B.subtilis* UTM 126 inhibited growth of *V.alginolyticus*, *V.para haemolyticus* and *V.harveyi*. The inhibitory mechanism is production of volatile and non volatile compounds. *Bacillus* produces polypeptide antibiotics such as bacitracin, gramicidin and polymyxin, which are active against a wide range of gram positive and gram negative bacteria (14).

Balcazar and Rojas Luna, (15) reported that the fish food supplementation with probiotic for shrimp culture, the reduction of shrimp mortality was found from *V. harveyi* infection. Vaseeharan and Ramasamy,(2003) found that growth of pathogenic *V.harveyi* in tiger shrimp was controlled by the probiotic effect of *B.subtilis* BT23 in vitro and in vivo. Kanatani et al(16) has stated that a bacteriocin from *L.acidophilus* TK9201 had inhibitory effect on closely related lactic acid bacteria and food born pathogens including *L.monocytogenes*. Itoh et al., (17) indicated that *L.gasseri* LA39 was one of the most active bacteriocins for use against enteric pathogens. *L.plantarum* strains give an inhibition diameter of 20mm for *S.aureus*, 11mm for *Bacillus* sp and 10mm for *E.coli* (18). Generally the medium composition values recorded for production of metabolites by measuring the diameter of the high inhibition zone, show that the Gram positive bacteria than Gram negative bacteria. Todorov and Dicks (19) observed that a high level of bacteriocin was produced when the cells were grown in the presence of K_2HPO_4 . The antimicrobial activity (mm) was measured and the data represented as mean \pm SE (including disc diameter), all the pathogens were inhibited significantly at probability level ($P < 0.05$).

Our results suggest that the growth and production of secondary metabolites of mixed culture of *S.faecalis*, *Cl.butyricum*, *B.mesentericus* and *L.sporogens* can take into account their interactions with pathogens. Future challenge experiments in secondary metabolites could provide valuable insight into its potential probiotic effect in situation directly relevant to aquaculture conditions.

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Table 1: Antibacterial activity of bacterial Probiotic metabolites extracts against fish pathogens (Disc diffusion method)

Fish pathogens	Zone of Clearance (mm)								
	Ethyl acetate extract			Toluene extract			Benzene extract		
	25µl	50µl	75µl	25µl	50µl	75µl	25µl	50µl	75 µl
Control for Dc (5%-DMSO)	-	-	-	-	-	-	-	-	-
Control for Tc/Bc	-	-	-	-	-	-	-	-	-
<i>Aeromonas hydrophilus</i>	9	11	12	14	16	19	13	18	18
<i>Bacillus sp</i>	7	12	12	18	19	23	14	17	16
<i>E.coli</i>	6	7	8	18	19	24	14	17	18
<i>Pseudomonas aerogenesa</i>	6	9	11	13	19	20	14	17	18
<i>Streptococcus sp</i>	11	12	13	19	19	22	13	16	17
<i>Vibrio harveyi</i>	9	8	10	14	16	20	15	17	19
<i>Staphylococcus sp</i>	8	8	10	19	20	23	16	20	20
<i>Vibrio sp</i>	6	8	10	16	16	20	13	16	17

SD of zone inhibition was calculated from three replicates of each pathogen

Signification; **P<0.05**

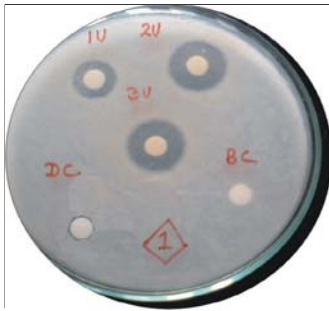
Table 2: Antibacterial activity of bacterial Probiotic metabolites extracts against fish pathogens (well diffusion method)

Fish pathogens	Zone of Clearance (mm)								
	Ethyl acetate			Toluene extract			Benzene		
	25 µl	50 µl	75 µl	25 µl	50 µl	75 µl	25 µl	50 µl	75µl
Control for Dc (5%-DMSO)	-	-	-	-	-	-	-	-	-
Control for Tc/Bc	-	-	-	-	-	-	-	-	-
<i>Aeromonas hydrophilus</i>	9	11	12	16	16	20	19	23	26
<i>Bacillus sp</i>	7	12	12	17	22	25	16	20	23
<i>E.coli</i>	6	7	8	21	23	25	20	22	28
<i>Pseudomonas aerogenesa</i>	6	9	11	12	18	22	18	26	29
<i>Streptococcus sp</i>	12	11	13	21	26	27	21	25	28
<i>Vibrio harveyi</i>	9	8	10	18	27	30	20	27	26
<i>Staphylococcus sp</i>	9	8	10	20	20	22	19	22	25
<i>Vibrio sp</i>	6	8	10	22	24	25	22	23	29

Fig-1 Benzene mixer

Dics diffusion method

Aermonas hydrophilus



Bacillus.ssp



E.coli



Pseudomonas aerogenesa



Streptococcus



Vibrio.harviyi



Staphylococcus.ssp



Vibriio.ssp



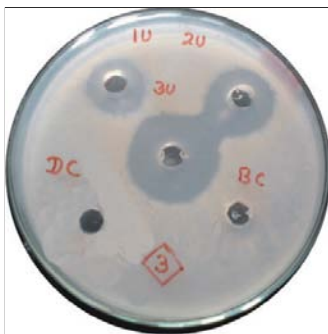
Fig-2 Benzene mixer

Well diffusion method

Aeromonas hydrophilus



E.coli



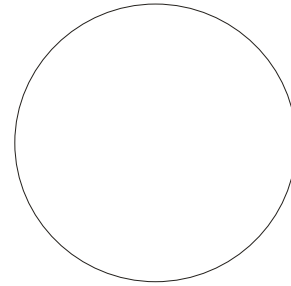
Streptococcus



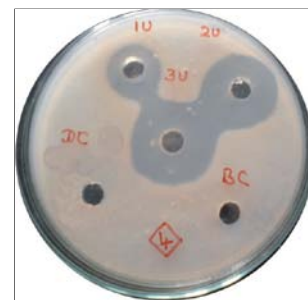
Staphylococcus.spp



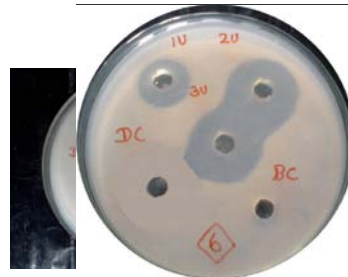
Bacillus.ssp



Pseudomonas aerogenesa



Vibrio.harviyi



Vibrio.ssp

