www.trjfas.org
ISSN 1303-2712
Turkish Journal of Fisheries and Aquatic Sciences 12: 185-187 (2012) DOI: 10.4194/1303-2712-v12_1_21

SHORT PAPER

Elimination of Pathogenic Bacterium (*Micrococcus sp.*) by the Use of Probiotics

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Received 14 March 2011
Accepted 24 October 2011

Abstract

Probiotics 1 (*Lactobacillus sporogenes*), 2 (*Saccharomyces boulardii*) and 3 (*Nitromonas*, *Rhodococcus*, *Bacilus megaterium*, *Lecheni formis*, *Desulphovibrio sulphuricum*, *Psuedomonas*, *Chromatium*, *Chlorobium*, *Thiobacillus*, *Thioxidants*, *Thiobacilus ferroxidant*, *Methylomonas methyanica*, *Glucon acetobactor*, *Azospirillum*, *Trichoderma*, *Shizophyllum commune* and *Sclertium gluconicum*) were tested against the pathogenic *Micrococcus* sp.^{oxt} *in vitro* as well as *in vivo* for four weeks. *In vitro* experiment revealed that the zone of inhibition of probiotic 1 was higher than probiotic 3 followed by probiotics 2. *In vivo* experiment also revealed that the elimination of pathogenic *Micrococcus* sp.^{oxt} from 1.54 x 10^{11} colony forming units (cfu)/ml to 2.50 x 10^{1} by probiotic 1 was higher than; to 2.00 x 10^{1} cfu/ml by probiotic 3 and; to 3.3 x 10^{1} cfu/ml by probiotics 2. The present investigation confirmed the elimination of pathogenic bacterium, *Micrococcus* sp.^{oxt} in both *in vitro* and *in vivo* experiments.

Keywords: Probiotics, *Lactobacillus sporogenes*, CFU (Colony Forming Units), *Micrococcus* sp., zone of inhibition

Introduction

Probiotic microorganisms may release chemical substances that have a bactericidal or bacteriostatic effects on other microbial populations; they do so by altering interpopulation relationships like competition for chemicals or available energy rich compounds (Zhenming *et al*., 2006). The probiotic microorganisms produce inhibitory substances in the intestine of the host, on its body surface, or in culture medium where organism live and create a barrier against the proliferation of opportunistic pathogens (Balcazar *et al.*, 2006). In aquaculture, non-pathogenic strains of identified bacteria have been successfully used as probiotics to control the diseases in fish (Austin *et al.*, 1995; Gomez-Gil *et al.*, 2002; Chythanya *et al.*, 2002; Capkin and Altinok, 2009). These probiotic bacteria suppress proliferation of pathogenic and opportunistic bacteria in the mucus in intestine as well as ambient environment of the fishes simultaneously (Skjermo and Vadstien, 1999). Consequently the probionts reduce the incidence of diseases. In objective of the present study was to investigate the elimination of pathogenic bacterium (*Micrococcus* sp.) by the use three probiotics.

Materials and Methods

The probiotics 1, 2 and 3 were used to observe *in vitro* and *in vivo* antagonism against pathogenic bacterium, *Micrococcus* sp., Probiotics 1 contained only single bacterium named lactic acid bacteria (*L. sporogenes*); Probiotics 2 contained single fungus yeast, *S. boulardii* while probiotics 3 contained many; soil, pond bottom and water harboring bacteria viz. *Nitromonas*, *Rhodococcus*, *B. megaterium*, *L. formis*, *D. sulphuricum*, *Psuedomonas*, *Chromatium*, *Chlorobium*, *Thioxidants*, *Thiobacillus*, *T. ferroxidant*, *M. methyanica*, *G. acetobactor*, *Azospirillum*, *Trichoderma*, *S. commune* and *S. gluconicum*.

In vitro antagonism tests of three probiotics against (*Micrococcus* sp.) were carried out by using agar well diffusion method (Gram *et al*., 2004), which is based on poisoning of culture medium with pathogenic bacteria at 1.64×10^{10} colony forming units (cfu)/ml and then allows the concerned probiotic to grow on medium in the bored well. To observe the antagonism of three probiotics against pathogenic bacterium; zone of inhibition were measured (in millimeters) by agar well diffusion method (Table 1). The following procedure was followed step by step:

Table 1. Colony Forming Units of pathogenic bacterium, *Micrococcus* sp.^{oxt} for *in vitro* and *in vivo* antagonism with probiotics

Treatments		CFU per ml
T1	Control	200µl of PBS
T2	Control + *Micrococcus* sp.^{oxt}	1.64×10^{10}
T3	Control + probiotic 1	1.64×10^{10}
T4	Control + probiotic 2	1.64×10^{10}
T5	Control + probiotic 3	1.64×10^{10}

i) Pathogenic bacteria (*Micrococcus* sp.) of known cfu (colony forming units) was poured into melted nutrient agar (NA; beef extract 3 g, peptone, 5 g, Sodium chloride 5 g, agar 15 g for one liter volume at pH 7.0±0.2; autoclaved at 15 atm pressure, for 20 minutes and then kept in deep freezer at 4°C till use), at 60-62 °C; and mixed well by shaking then poured into petri plates, and allowed to solidify in laminar flow with ultraviolet light remained off for 15-20 minutes.

ii) Three well were bored in solidified NA containing pathogenic bacterium (*Micrococcus* sp.) by the well borer and every time the borer pipe was sterilized on the flame.

iii) Then 5-10 µl melted water agar (15 g Agar + 1 liter distilled water) was added at the bottom of the each well with micropipette; to prevent the seepage of the probiotic bacterial suspension to the bottom of petri plates.

iv) Then the probiotics (50 µl) were added to each well and plates were incubated in B.O.D. at 35-37 °C for 18-24 h.

v) The zone of inhibition was measured by using simple scale and recorded.

Antibiotic oxytetracycline resistant *Micrococcus* sp.^{oxt} was taken as pathogenic microorganism for inoculation in Indian magur catfish (*Clarius batrachus* L.). The treatments were given to the magur catfishes in triplicates of every treatment (Table 1).

One catfish from each replicate of every treatment was sacrificed then tissues from liver, kidney and gills were taken and macerated at weekly interval and, viable counts of *Micrococcus* sp.^{oxt} were worked out by serial dilution method by growing supernatant on NA containing oxytetracycline at 50 µg/ml. The obtained results were analyzed statistically using completely randomized design (CRD) to evaluate differences among different treatments means at 0.05 significant levels following Snedecor *et al*. (1989).

Results

In vitro antagonism

Inhibition zone of probiotic against *Micrococcus* sp. was found to be different in each treatment. Probiotic 1 showed bigger inhibition zone as compared to probiotic 2 and probiotic 3 against each bacterium. From these results, it is concluded that probiotic 1 was better than probiotic 3 and probiotic 3 was better than the probiotic 2, in gushing out the pathogenic bacteria-*Micrococcus* sp. from diseased catfishes (Table 2, Figure 1).

Effect of Probiotics on the Viable Count of *Micrococcus* sp.^{oxt} Injected into Indian Magur (*C. batrachus* L.) Under *in vivo* Induced Pathogenicity

The results of viable counts of pathogenic bacterium *Micrococcus* sp.^{oxt} under different treatments over a period of five weeks were presented in Table 3. In control, the catfishes were injected with *Micrococcus* sp.^{oxt} showed progressive increase in the number of viable counts from 1.68×10^{10} in first week to 4.7×10^{12} cells/ml until the catfishes died after five weeks. The viable counts of *Micrococcus* sp. became so high in fifth week that the catfishes could not

Table 2. Inhibition zones (in millimeters) of three probiotics against pathogenic bacteria *Micrococcus* sp.^{oxt}

Probiotics	Inhibition zones against *Micrococcus* sp.^{oxt} (mm)
Probiotic 1	23.00 ±0 .58
Probiotic 2	20.00 ± 0.00
Probiotic 3	22.00 ± 0.58
C.D. Value	1.66

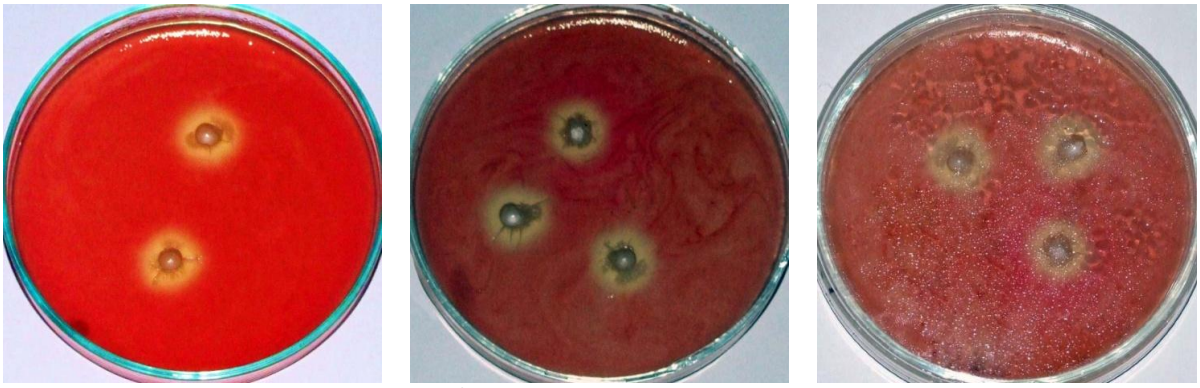

Figure 1. Probiotics 1, 2 and 3 showing zone of inhibition against *Micrococcus* sp.

Table 3. CFU of *Micrococcus* sp.^{oxt} under *in vivo* induced pathogenicity over a period of five weeks

Treatment	Viable counts of *Micrococcus* sp.^{oxt} bacterium in different weeks					
	0	1	2	3	4	5
Micrococcus sp.	1.6×10^{10}	7.0×10^{10}	1.8×10^{11}	7.3×10^{12}	4.7×10^{12}	4.8×10^{12}
Micrococcus sp.+ probiotic 1	1.6×10^{10}	7.3×10^{4}	9.3×10^{3}	6.8×10^{1}	2.8×10^{1}	2.5×10^{1}
Micrococcus sp.+ probiotic 2	1.6×10^{10}	8.6×10^{5}	6.8×10^{4}	5.8×10^{2}	1.9×10^{1}	2.0×10^{1}
Micrococcus sp.+ probiotic 3	1.6×10^{10}	2.6×10^{6}	7.6×10^{4}	7.3×10^{3}	3.5×10^{1}	3.3×10^{1}

tolerate this and subsequently fish died. But the catfishes inoculated with *Micrococcus* sp. and along with the three probiotics showed progressive decline in the viable counts of *Micrococcus* sp. The viable counts of *Micrococcus* sp.^{oxt} along with probiotic 1 in the treatment declined from 1.6×10^{10} cells/ml in first week to 2.50×10^{1} in fifth week; those of *Micrococcus* sp. + probiotic 2 in the treatment declined from 1.6×10^{11} cells/ml in first week to 2.0×10^{1} cells/ml in fourth week while in *Micrococcus* sp. + probiotic 3 treatment viable counts declined from 1.6×10^{10} cells/ml in first week to 3.3×10^{1} bacterial cells/ml in fifth week. *Micrococcus* sp. was eliminated by the all three probiotics 1, 2 and 3 successfully (Table 3).

Discussions

In the present investigation the viable counts or CFU of pathogenic bacterium *Micrococcus* sp.^{oxt} were high in the inoculated catfishes. However, these counts (or cfu) decreased in number in probiotic along with *Micrococcus* sp.^{oxt} treated catfishes. The numbers of viable counts decreased more in probiotic 1 as compared to probiotic 3 followed by probiotic 2 over a period of five weeks. Similar results were observed by Zhou *et al.* (2010), studied the inhibition ability of probiotic, *Lactococcus lactis* RQ516, against *A. hydrophila, in vitro* with 14.77 ± 1.17 mm zones of inhibition and; immunostimulator and growth promoter, *in vivo* in tilapia, *Oreochromis niloticus*. Although, their study was on different fish with different probiotics and pathogenic bacterium, but pattern of inhibition in both in *vitro* as well as *in vivo* was found same. Nimrat and Vuthiphandchai, (2011) also observed similar results in marine shrimp, where they used 12 commercial probiotic products against shrimp pathogenic bacterium *Vibrio harveyi*. In conclusion, pathogenic bacterium, *Micrococcus* sp. can be inhibited by the use of three above mentioned probiotics.

Acknowledgments

We are thankful to Dr. V. P. Sabhlok (Professor and Head, Zoology and Aquaculture) for providing necessary facilities. Help rendered by Mr. Balraj Sehrawat (Fishery Extension Officer, Haryana) during the field surveys and fish sample collection is gratefully acknowledged.

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