World Journal of Dairy & Food Sciences 5 (2): 145-151, 2010
ISSN 1817-308X

Comparison of Antimicrobial Activity of Probiotic Bacterium *Streptococcus phocae* PI80, *Enterococcus faecium* MC13 and *Carnobacterium divergens* Against Fish Pathogen

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Abstract: In this study, the antimicrobial activity of *Streptococcus phocae* PI80, *Enterococcus faecium* MC13 and *Carnobacterium divergens* against Gram positive and Gram negative bacteria using Agar spot-on lawn, Disc diffusion and Agar well diffusion method was examined. The probiotic bacterium *S. phocae* PI80 and *E. faecium* MC13 inhibited almost all indicator organisms tested except *Escherichia coli* CSH57 and *E. coli* SK39. *Vibrio parahaemolyticus*, *V. anguillarum* and *L. monocytogenes* and *E. coli* DH5α were significantly inhibited at probability level ($P<0.05$) by *S. phocae* and *E. faecium*. On the other hand, in agar spot on lawn and disc diffusion, *V. parahaemolyticus*, *V. anguillarum*, *Listeria monocytogenes* and *E. coli* DH5α were the most sensitive indicator strains to *S. phocae* PI80. Moreover, *S. phocae* PI80 significantly inhibited most of the pathogenic strains in agar well diffusion method when compared with *E. faecium* and control *C. divergens*. Also, the maximum antimicrobial or bacteriocin activity (16900 $AUml^{-1}$) was observed in *S. phocae* PI80 and *E. faecium* MC13. These findings encountered that probiotics *S. phocae* PI80 and *E. faecium* MC13 have a broad spectrum antimicrobial effect than *C. divergens*.

Key words: Probiotics · Antimicrobial activity · *Streptococcus phocae* PI80 · *Enterococcus faecium* MC13 · *Carnobacterium divergens*

INTRODUCTION

Probiotic cultures have been associated historically with cultured milk, dairy products. There is substantial evidence for positive effects on human health and general well-being, because of using it as probiotics [1]. Lactic acid bacteria (LAB) are among the most important probiotic microorganisms typically associated with gastrointestinal tract whereas they exercise beneficial effects. Several *in vitro* and *in vivo* experiments on antagonism of different lactobacillus strains against *Helicobacter pylori, Clostridium difficile*, *Vibrio* spp. *Aeromonas salmonicida* and *E. coli* were performed [2, 3]. Currently in both shrimp hatcheries and forming industries, probiotic bacteria were used for controlling pathogenic *Vibrio's*. Attempts were being made in food industries for controlling food spoilage microorganisms like *Listeria monocytogenes* and *Pseudomonas* and other food born pathogen. The probiotic bacteria produce antimicrobial metabolites like lactic acid, diacetyl, hydrogen peroxide and bacteriocin or bacteriocin like compounds [4 - 6]. Lactic acid secreted by this probionts reduces the pH in the fermented medium [7] and hydrogen peroxide which is a non stable thermodynamic compound destroys bacterial enzymatic activity [8]. Use of beneficial bacteria (probiotic) to displace pathogens by competitive process is being used in the shrimp hatchery as a better remedy than administering antibiotics and is now gaining acceptance for control of pathogens in aquaculture [9].

In recent years, there have been many reports on bacteriocin that are produced by probiotic bacteria. However, most reports deal with bacteriocins produced by various *Lactococci, Pediococci, Leuconostoc, Enterococci* and *Lactobacilli* [10, 11]. Bacteriocins are proteinaceous antibacterial compounds that mainly exhibit bactericidal activity against closely related species to the

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producer strain [12]. Satish kumar and Arul [13] reported that the antibacterial proteinaceous culture free supernatant of these probiotic bacterial strains can also inhibit opportunistic pathogens including *P. aeruginosa, B. cereus, S. aureus* and *P. vulgaris*.

In the present study, we compared the antimicrobial activity of probiotic bacterium *S. phocae, E. faecium* and *C. divergens,* using well known methods such as disc diffusion, agar spot- on lawn and agar well diffusion.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions: The strains used in this study are listed in the Table 1. *L. monocytogenes* MTCC-657 was procured from Microbial type culture collection, IMTECH, Chandigarh, India. *Vibrio parahaemolyticus* and *Vibrio anguillarum* were obtained from Central Institute of Brackish water Aquaculture (CIBA), Chennai. Cultures were maintained on plates or slants of brain heart infusion agar and in tryptone soy agar. *Streptococcus phocae* PI80 and *Enterococcus faecium* MC13 was isolated from shrimp and fish intestine [14]. *Carnobacterium divergens* collected from NRRL in USA was used as control strain to compare with our isolated probiotic strains.

Table 1: Reference of bacterial strains

Probiotic bacteria	Source/Origin
Streptococcus phocae PI80	*Penaeus indicus* (Shrimp)
Enterococcus faecium MC13	*Mugil cephalus* (Fish)
Carnobacterium divergens	NRRL, USA
Indicator strains	
Vibrio parahaemolyticus	CIBA, Chennai
V. harveyi	Hatchery water
*V. vulnificus*1145	MTCC, Chandigarh
*V. fischeri*1738	"
V. anguillarum	CIBA, Chennai
Aeromonas hydrophila	Diseased fish
*Aeromonas hydrophila*646	MTCC, Chandigarh
*Aeromonas salmonicida*1945	"
Escherichia coli DH5-α	Reference strain
E. coli KL-16	"
E. coli KL-96	"
E. coli CSH-57	"
E. coli SK-39	"
Pseudomonas aeruginosa	Spoilage food
Klebshiela pneumonia 30	Human middle ears
Proteus vulgaris	CIBA, Chennai
Bacillus cereus	Soil
Listeria monocytogenes 657	MTCC, Chandigarh
Lactobacillus plantarum	"
L. acidophilus	"
L. rhamnosus	"

Preparation of Indicator Cells: Fresh cells of indicator strains were prepared as described by Kivanc [15]. Briefly, all cultures were grown in appropriate growth media at 37°C for 16 hr and the cells were separated by centrifugation at 800 X g for 10 min. The supernatant was discarded and remaining pellet was washed twice with 0.85 % saline. Cell concentration of 10^5 CFUml^{-1} was used for this study.

Preparation of Culture Free Supernatant: *S. phocae* PI80, *E. faecium* MC13 and *C. divergens* were grown in Lactobacilli MRS broth at 37°C for 16 hr. After incubation, cell free culture supernatant was separated by centrifugation (800 X g for 10 min at 4°C). The supernatant was adjusted to pH 6.5 by means of 1M NaOH to exclude the antimicrobial effect of organic acid, followed by filtration of the supernatants through a 0.22 μm cellulose acetate membrane filter. Filterate was used directly to study the antagonistic effect.

Agar Spot –On Lawn Method: Culture free supernatant (24 hr) of *S. phocae* PI80, *E. faecium* MC13 and *C. divergens* were spotted on surface of MRS agar plates (2mm diameter) and incubated at 37°C to dry the supernatant. 10 ml of TSA soft-agar containing indicator strain (10^5 CFUml^{-1}) was overlaid on to the MRS agar plates containing the spots of antimicrobial product. Sterile MRS broth served as a control. The antimicrobial activity (mm) was measured after 24 hr [16].

Disc Diffusion Method: In this method, sterile paper discs (6 mm, Himedia) were placed over BHI agar plates seeded with indicator strains. 50 μl of culture free supernatant was added to the sterile paper discs and incubated at 37°C for 24 hr. A sterile paper disc served as control. After incubation, antimicrobial activity (mm) was measured around the paper discs and tabulated [17].

Agar Well Diffusion Method: Agar well diffusion assay described by Lyon and Glatz [18] was used for comparing the antimicrobial activity of probiotic isolates. The wells of 6 mm were made using well borer and bottom of the wells were sealed with a few drops of MRS agar media. 100 μl of culture free supernatant was added to the wells and kept at 4°C. After 2 hr of incubation, the agar base was loosened from edge of the petri dish with spatula and filliped into the petri dish lid. 10 ml of BHI soft agar

containing indicator strains (10^5 CFUml^{-1}) were overlaid on the agar base. After 24 hr of incubation, zone of inhibition was measured and tabulated.

Assay of Antimicrobial Activity: The antimicrobial activity of culture free supernatant of probiotics *S. phocae* PI80, *E. faecium* MC13 and *C. divergens* were determined by agar well diffusion method. To the wells, 100 µl of twofold serially diluted supernatant was added and incubated at 4°C. After 2 hr, the agar base was loosened from edge of the petri dish with spatula and the agar medium was flipped into the petri dish lid whish was covered with BHI soft agar containing indicator strains at the concentration of 10^5 CFU ml^{-1}. After 24 hr of incubation, zone of inhibition was measured. Arbitrary units (AUml^{-1})for bacteriocin was calculated as $a^b \times 100$, whereas "a" represents the dilution factors and "b" the last dilution that produces an inhibition zone of at 2 mm in diameter. Activity is expressed per ml multiplication with 100. One Arbitrary unit (AU) of antimicrobial or bacteriocin activity was defined as the reciprocal of the highest twofold dilution that showing a clear zone of growth inhibition [19].

Statistical Analysis: Data were presented as mean ± S.E. The zone of inhibition was analyzed using the one way ANOVA to compare the difference in values among the pathogenic bacterium using the statistical package (SPSS).

RESULTS AND DISCUSSION

Three probiotic strains (*Streptococcus phocae* PI80, *Enterococcus faecium* MC13 and *Carnobacterium divergens)* were tested against Gram positive and Gram negative pathogenic strains (Table 2). More than eighteen G (+) and G (-) pathogenic strains was inhibited by probiotic strains *S. phocae, E. faecium* and *C. divergens*. However, these probiotic strains failed to show the inhibitory activity against *E. coli* CSH57 and *E. coli* SK39. *V. anguillarum* (19.3±0.8), *L. monocytogenes* (15.0±0.8) and *V. parahaemolyticus* (15.0±1.7) were significantly inhibited at probability level ($P<0.05$) by *S. phocae* in agar spot on lawn method (Table 3). Moreover, *E. faecium* also showed significant antimicrobial activity against *L. monocytogenes* (16.3±1.4) and *V. parahaemolyticus* (15.3±0.8) which was higher than the inhibitory

Table 2: Growth medium and incubation temperature of indicator strains and inhibitory spectrum of the cell free supernatant of probiotic bacterium *S. phocae* PI80, *E. faecium* MC13 and *C. divergens*

Indicator strains	Medium	Temperature(°C)	Antimicrobial activity
Vibrio parahaemolyticus	TSA	37	+
V. harveyi	Sea water agar	37	+
*V. vulnificus*1145	TSA	37	+
*V. fischeri*1738	TSA	37	+
V. anguillarum	TSA	37	+
Aeromonas hydrophila	TSA	37	+
*Aeromonas hydrophila*646	TSA	37	+
*Aeromonas salmonicida*1945	TSA	37	+
Escherichia coli DH5-α	BHI	37	+
E. coli KL-16	BHI	37	+
E. coli KL-96	BHI	37	+
E. coli CSH-57	BHI	37	-
E. coli SK-39	BHI	37	-
Pseudomonas aeruginosa	BHI	37	+
Klebshiela pneumonia 30	BHI	37	+
Proteus vulgaris	BHI	37	+
Bacillus cereus	BHI	37	+
Listeria monocytogenes-657	BHI	37	+
Lactobacillus plantarum	BHI	37	+
L. acidophilus	BHI	37	+
L. rhamnosus	BHI	37	+

(+) antimicrobial activity present (-) antimicrobial activity absent

Table 3: Antimicrobial activity of *S. phocae* PI80, *E. faecium* MC13 and *C. divergens* against indicator strains by Agar spot-on lawn method.

	Antimicrobial activity (mm) diameter for 24hrs		
Indicator strains	*S. phocae* PI80	*E. faecium* MC13	*C. divergens*
V. parahaemolyticus	15.0±1.7*	15.3±0.8*	12.0±1.1
V. harveyi	14.0±1.1	13.0±0.5	12.3±0.8
V. vulnificus 1145	14.0±1.1	16.0±1.1	14.0±0.5
*V. fischeri*1738	16.3±0.8	16.3±0.8	14.0±0.5
V. anguillarum	19.3±0.8*	16.3±1.4	14.0±0.5
A. hydrophila	14.3±0.8	14.0±0.5	13.0±1.1
E. coli DH5-α	16.0±1.1	15.0±1.1	14.0±1.1
E. coli KL-16	16.3±1.4	15.6±1.7	14.3±0.3
E. coli KL-96	13.6±0.8	13.0±0.5	13.0±1.1
P. aeruginosa	15.0±1.1	13.6±0.8	14.3±0.8
Bacillus cereus	13.3±0.8	14.0±1.1	14.0±1.1
L. monocytogenes 657	15.6±0.8*	16.3±1.4*	12.6±0.8

*P<0.05 significant

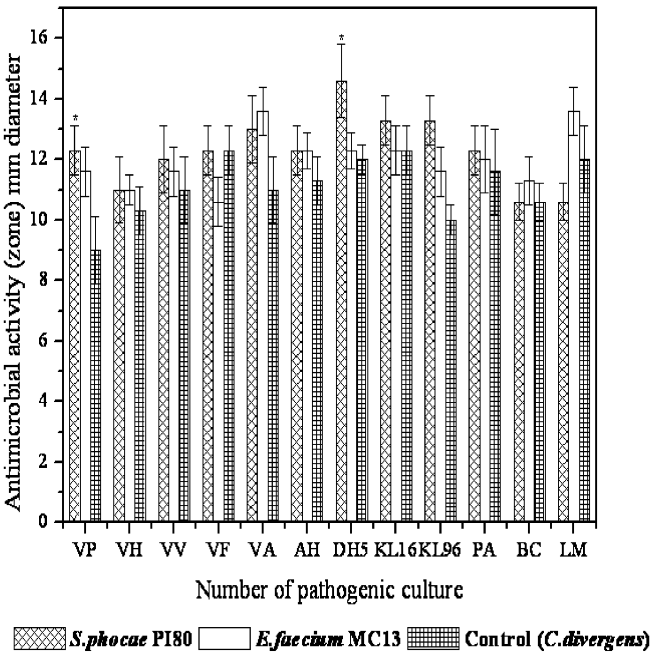

Fig. 1: Antimicrobial activity of *S. phocae* PI80, *E. faecium* MC13 and *C. divergens* against indicator strains by Disc diffusion method

activity produced by *S. phocae* and control bacterium *C. divergens.* Kabuki *et al*. [20], Kayalvizhi and Gunasekaran [21] reported the absence of inhibition in *L. monocytogenes* by *S. thermophilus* SBT1277 and *B. licheniformis* MKU3. Also, poor inhibition was observed for *L. monocytogenes* (7mm and 9mm) by *Lactobacillus plantarum* F1 and *L. brevis* OG1 [22]. However, our probiotic culture *S. phocae* and *E. faecium* exhibit well anti listerial effect by producing their own antimicrobial compound. *L. monocytogenes* widely distributed in the environment is capable of exponential growth at low temperatures [23] which cause listeriosis outbreaks associated with many food products.

S. phocae PI80 showed significant antimicrobial activity against *E. coli* DH5-α (14.6±1.2) and *V. parahaemolyticus* (12.3±0.8) as compared with control bacterium *C. divergens* in disc diffusion assay whereas *E. faecium* MC13 did not exhibit significant antimicrobial activity against pathogen (Fig. 1). These results clearly indicated that the zone of inhibitory activity depends on

Table 4: Antimicrobial activity of *S. phocae* PI80, *E. faecium* MC13 and *C. divergens* against indicator strains by Agar well diffusion method.

	Antimicrobial activity (mm) diameter for 24hrs		
Indicator strains	*S. phocae* PI80	*E. faecium* MC13	*C. divergens*
V. parahaemolyticus	16.0±0.8	16.6±1.2	13.3±0.8
V. harveyi	15.1±1.1*	14.3±0.6	11.0±1.1
V. vulnificus 1145	16.6±1.2	17.0±1.1*	12.3±0.8
*V. fischeri*1738	17.3±0.8*	17.3±0.8*	12.6±0.6
V. anguillarum	17.0±1.0*	16.0±0.5	12.3±1.4
A. hydrophila	16.3±0.8	15.3±0.8	12.6±1.4
E. coli DH5-α	17.0±1.0*	17.0±1.1*	12.4±1.1
E. coli KL-16	17.3±0.8*	15.0±1.1	13.3±0.8
E. coli KL-96	15.6±0.6	14.6±1.2	12.6±1.4
P. aeruginosa	15.6±0.6*	15.3±0.8*	11.4±0.5
Bacillus cereus	13.6±1.2	14.3±0.8	14.3±0.8
L. monocytogenes 657	17.1±1.0*	17.0±1.1*	12.3±0.8

**P<0.05 significant*

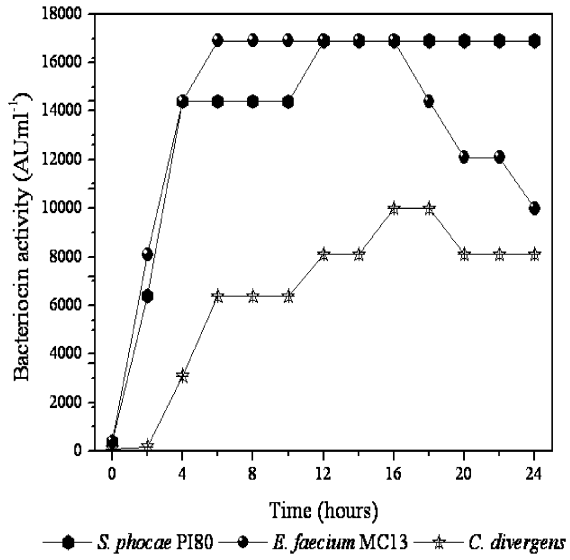

Fig. 2: Effect of probionts on bacteriocin activity (AU ml^{-1}) against indicator strain (*L. monocytogenes* 657) at 37°C for 0-24hrs in agar well diffusion method

the type of method and indicator strains. Most of the indictor strains were significantly inhibited at probability level (P<0.05) by *S. phocae* PI80 in agar well diffusion method. Among the indicator strains, *V. fischeri* (17.3±0.8), *E. coli* KL-16 (17.3±0.8), *L. monocytogenes* (17.1±1.0) *V. anguillarum* (17.0±1.0) and *E. coli* DH5α (17.0±1.0) were highly inhibited by *S. phocae* when it's compared with control bacterium. Also, low inhibitory value was pronounced with *V. harveyi* and *P. aeruginosa* (Table 4). *V. harveyi* is one of the most shrimp pathogen and it has been produced high mortalities in shrimp by causing vibriosis [24]. *P. aeruginosa* spoil food at low temperatures as a result of its lipolytic and proteolytic activity [25]. Control of *P. aeruginosa* by bacteriocin activity of *L. casei* and *L. plantarum* has been reported by Kaya [26]. Also *L. monocytogenes* was prevented by certain bacteriocins produced by lactic acid bacteria [27]. Moreover, *E. faecium* MC13 also exhibited good antimicrobial activity against *V. vulnificus*, *V. fischeri, E. coli* DH5α, *P. aeruginosa* and *L. monocytogenes*. Also, -*E. faecium* showed inhibitory against *B. cereus* equivalent to control bacterium. Kayalvizhi and Gunasekaran [21] observed very low zone of inhibition produced by *B. licheniformis* MKU3 in *E. coli* DH5α (5mm), *B. cereus* (13mm). Also *L. brevis* OG1 exhibited less zone of inhibition (8mm) in *B. cereus* [22]. Kabuki *et al*. [20] suggested that the anti microbial inhibitory compound may be a bacteriocin.

In the present study, the most sensitive indicator organisms to the probiotic strains *S. phocae* PI80 and *E. faecium* MC13 was found to be *L. monocytogenes* 657, *V. anguillarum* and *E. coli* DH5-α. *L. monocytogenes* was found to be more sensitive as well as highly susceptible to bacteriocin produced by *S. phocae* PI80. The maximum antimicrobial or bacteriocin activity (16900 $AUml^{-1}$) was observed in *S. phocae* PI80 with in 12 hr of incubation. The activity remained higher even after 24 hr of incubation. However, in *E. faecium* MC13, maximum antimicrobial activity (16900 $AUml^{-1}$) was observed after 6 hr of incubation. Nevertheless, it lost its activity after 18 hr of incubation. In contrary to the above two strains, maximum bacteriocin activity (10000 $Auml^{-1}$) was

produced by *C. divergens* in 16 hr of incubation period (Fig. 2). Kabuki *et al.* [20] reported that the *S. thermophilus* SBT1277 produced no bacteriocin activity when *L. monocytogenes* was used as indicator strain. Maximum bacteriocin activity (12,800 $AUml^{-1}$) was observed by *S. thermophilus* SBT1277 in *L. helveticus* SBT10511, SBT2171 and SBT1270. However, low level of bacteriocin activity (50 $Auml^{-1}$) was observed in *B. cereus* IFO13494 when used as indicator strain [20]. These results supported that our probiotics *S. phocae* and *E. faecium* exhibited inhibition against *L. monocytogenes* and *V. parahaemolyticus*. Many lactic acid bacteria have been used as probiotics to control bacterial pathogen in fish, shrimp hatchery and food industry. *S. thermophilus*, *L. delbrueckii* sub sp. *bulgaricus* and *L. helveticus* are important dairy starter cultures used for the manufacture of cooked cheese, mozzarella cheese and yogurt [28, 20]. *L. plantarum*, *L. rhamnosus*, *L. lactis*, *B. licheniformis*, *B. subtilis* and *E. faecium* were used as probiotic to restrain the bacterial pathogen in fish and shrimp hatchery [29 - 31]. All the above said studies support the usage of probiotic bacterium in food preservation and shrimp hatchery as they exhibit anti microbial property which was confirmed through different methods.

In conclusion, the probiotic isolates *S. phocae* and *E. faecium* were effectively inhibited most of the fish, shrimp and food spoilage pathogens especially *V. parahaemolyticus* and *L. monocytogenes*. Moreover, the probiotic strains were able to produce higher amount bacteriocin activity when compared with control bacterium. So we concluded that the probiotic bacteria *S. phocae* and *E. faecium* are a better bacterial remedy in aquaculture system and food industry. Also, it is strongly recommended to use probiotic to restrain the aquaculture and food born pathogens.

All pathogenic bacterium were tested against the probiotic *S. phocae* PI80, *E. faecium* MC13 and *C. divergens*. The antimicrobial activity (mm) was measured and the data represented as mean ± S.E. (including spot diameter). *V. parahaemolyticus*, *V. anguillarum* and *L. monocytogenes* were inhibited significantly at probability level ($P<0.05$) by *S. phocae* PI80, *E. faecium* MC13 than control bacterium *C. divergens*.

All pathogenic bacterium were tested against the probiotic *S. phocae* PI80, *E. faecium* MC13 and *C. divergens*. The antimicrobial activity (mm) was measured and the data represented as mean ± S. E. (including wells diameter). Most of the bacterial strains were inhibited significantly by *S. phocae* PI80 than *E. faecium* MC13 and *C. divergens*.

All pathogenic bacterium were tested against the probiotic *S. phocae* PI80, *E. faecium* MC13 and *C. divergens*. The antimicrobial activity (mm) was measured and the data represented as mean ± S.E. (including disc diameter). *V. parahaemolyticus* and *E. coli* DH5α were inhibited significantly at probability level ($*P<0.05$) by *S. phocae* PI80 than control bacterium *C. divergens*.

ACKNOWLEDGMENTS

This study was supported by the Department of Biotechnology, New Delhi.

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