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Inhibition of *Pseudomonas aeruginosa* by antibiotics and probiotics combinations- *In vitro* study

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ABSTRACT

Pseudomonas aeruginosa is the most important agent causing nosocomial infections. It is due to its resistance to common antibiotics and antiseptics that it establishes itself widely in hospitals. Probiotic strains (Lactobacillus rhamnosus, Saccharomyces boulardii, Streptococcus faecalis and Lactobacillus acidophilus) have been found to enhance the antagonistic activity of antibiotics (Azetreonam, Amikacin, Meropenam and Ciprofoxacin) by above mentioned Probiotic strains against P. aeruginosa (MTCC1688 and clinical isolates of human). In this study, probiotic strains were obtained from the commercial probiotic products and their inhibitory activity was seen by using Kirby bauer disc diffusion method. It was observed that in 71.875 % cases, the zone of inhibition of antibiotics was enhanced by probiotic strains. No enhancement was seen in 25 % of cases while reduction of zone was observed in 3.125 % cases. All the Probiotic strains showed maximum enhancement of zone in combination with Azetreonam (7 to 25 mm) followed by Amikacin (0 mm to 8 mm), Meropenam (-5 to 5 mm) and ciprofloxacin (0 to 4 mm). This study indicates that Probiotic strains can be used to overcome the increasing drug resistance of P. aeruginosa.

Key words: Antagonistic effect, Probiotics, Antibiotics, *Pseudomonas aeruginosa*, *Lactobacillus rhamnosus*, *Saccharomyces boulardii*, *Streptococcus faecalis* and *Lactobacillus acidophilus*.

INTRODUCTION

Pseudomonas aeruginosa, is highly prevalent opportunistic pathogen. It is, in spite of its lack of invasiveness, causes severe diseases like urinary tract infections, acute purulent meningitis, otitis media, otitis external, eye infections, wound and burn infections, septicaemia and infantile diarrhoea. The multiple resistance to antibiotics is quite common in *P. aeruginosa*, it is intrinsically resistance to most of the commonly used antibiotics, which is attributable to a concerted action of multidrug efflux pumps with a chromosomally encoded antibiotic resistance genes and the low permeability of bacterial cellular envelopes [1]. So, there is a considerable interest in developing low cost large-scale alternative solutions to prevent or reduce the increasing resistance of *P. aeruginosa*. In this regard, probiotics may close the therapeutic gap. Probiotics are living microbial species that, on administration, has positive effect on the health of individual [2]**.** Probiotics have been proven to be useful in the treatment of several infections and gastrointestinal diseases such as acute diarrhea or pouchitis [3], [4], [5]. Multiple mechanisms have

been proposed to justify the protective and therapeutic role of probiotics including lactose digestion [6], production of antimicrobial agents [7], [8], pathogen exclusion and immunomodulation [9], [10]. Commercially available probiotic preparations including lactic acid bacilli, LAB (*Lactobacillus rhamnosus, Lactobacillus acidophilus* etc.) alone or in combination with *Streptococcus* and *Saccharomyces* species have shown the beneficial effects [11].

Antibiotics are among the most frequently prescribed medication in modern medicines. Antibiotics not only put the severe side effects on health but also destroy the protective good bacteria in the body causing gastrointestinal infections such as antibiotic associated diarrhoea and colitis caused by *Clostridium difficile* and *Clostridium perfrigens* [12]. A combination of antibiotics and probiotics can restore the normal predominance beneficial enteric bacteria on one hand and can inhibit the harmful bacteria on the other hand. There is a exhaustive list of *in vivo* [13], [14], [15], [16] and *in vitro* [17], [18], [19], [20] studies indicating the potential antimicrobial effect of probiotics against the pathogens. This study is an attempt to measure the probiotic potential of enhancing the antimicrobial effect antibiotics. Positive outcomes of this study can reduce the duration and as a result the cost of antibiotic therapy, putting probiotics as a prophylactic and preventive medicine if not an alternative to antibiotic therapy. Present investigation evaluates the potentiation of antimicrobial activity of the Antibiotics, Azetreonam (AT), Amikacin (AK), Meropenam (MRP) and Ciprofloxacin (CIP) by the probiotic strains *Lactobacillus rhamnosus*, *Sachharomyces boulardii*, *Streptococcus faecalis* and *Lactobacillus acidophilus*, *in vitro* against standard and the clinical isolates of *P. aeruginosa.*

MATERIALS AND METHODS

Bacterial isolation and cultivation :

Probiotic strains *L. rhamnosus* and *S. boulardii* were isolated from commercially available capsule 'Darolac'. For this, half of ampoule was suspended in MRS broth in anaerobic condition at 37^0C for 24 hrs and half was used to inoculate the sabraoud's agar and kept at 37^0C for 24 hrs in aerobic condition. After incubation a loopful MRS broth was dispensed to MRS agar and kept in Mc intosch jar with an anaerobic gas packet for 48 hrs at 37^oC. *S. boulardii* was isolated from sabraoud's plate while *L. rhamnosus* was isolated from MRS plate. *S. faecalis* and *L. acidophillus* were isolated from the commercial product 'Prepro' with the only difference that the *S. faecalis* was subculture on blood agar from the mixed colonies appeared on MRS agar. Pure colonies were obtained by repeated subculturing. All the probiotic strains were confirmed by Gram's staining, cell and colony morphology.

Culture of *P. aerruginosa* MTCC1688 was obtained from Imtech, Chandigarh, India. The clinical isolate of *P. aerruginosa* was obtained from the Department of Microbiology, S. N. Medical College, Agra (India) and confirmed by cultural and biochemical test. Bacterial Stocks were kept in Brain heart infusion agar slant at 4^oC.

Antibiotic resistance :

Antibiotic resistance of probiotic strains was assessed using antibiotic discs (Hi Media, India) by using Disc Diffusion Method [21] according to the National Committee for Clinical Laboratory Standards (NCCLS) guidelines. Mueller hinton agar was swabbed by probiotic suspention of 0.5 Mc farland standards. The antibiotic discs of Amikacin (AK 30ug), Ceftazidime (CAZ 30ug), Meropenem (MRP 10ug), Azithromycin (AZM 15ug), Aztreonam (AT 30ug), Nitrofurantoin (NIT 300ug), Amoxicillin/Clavulanate (AMC 20/10ug), Piperacillin/Tozobactum (PIT 100/10ug), Ciprofloxacin (CIP 5ug), Levofloxacin (LE 5ug) and Chloramphenicol (C 30ug) were placed on MHA surface and kept at 37^0C for 24 hrs.

Antagonistic activity :

The antagonistic activity of antibiotic and probiotic combinations were assessed by modified Disc Diffusion Method according to the NCCLS guidelines. The two MHA (60ml) plates of 120 mm diameter were swabbed by *P. aeruginosa*, MTCC 1688 and *P. aeruginosa*, from clinical sample separately and kept for 3 hrs at 37^0C. Now the readymade antibiotic disc of AT, AK, MRP and CIP were dipped in the 24 hrs old probiotic suspensions and kept for 1 hr at 37^0C to allow the maximum absorption. The MHA plates were seeded with the above antibiotic disc impregnated with probiotic along with plain antibiotic disc taking as positive control. Now the MHA plates were kept at 4^oC for 1 hr to allow the proper diffusion. The two MHA plates were now kept at 37^0C for 24 hrs. Zone of inhibition were measured by using a caliper micrometer against the back of the petri plates [21].

Minimum Inhibitory Concentration (MIC) of probiotic strains :

Three dilutions of turbidity equal of ≠ 1.0, (3×10^8 cfu/ml), 1/10 (3×10^7 cfu/ml) and 1/100 (3×10^6 cfu/ml) were prepared. Now the 20ul of each was transferred to plain sterile disc of 6mm. These impregnated discs now contained approximately 6×10^6 cfu/disc (for Mac Farland standard ≠ 1.0), 6×10^5 cfu/disc (for 1/10 serial suspensions) and 6×10^4 cfu/disc (for 1/100 serial suspension).

A plate of MHA was swabbed with *P. aeruginosa*, MTCC 1688 and clinical isolate of *P. aeruginosa* and kept at 37°C for 3 hrs. Now the probiotic discs were dispensed on MHA surface, taking the sterile water discs as negative control. The plates were kept at 4°C for 1 hr for diffusion and then at 37°C for 24 hrs zones of inhibition were measured**.**

RESULTS AND DISCUSSION

Both the *Lactobacilli* (*L. rhamnosus* and *L. acidophillus*) showed round, small colonies without any pigment and white to cream in colour. Both appeared as gram +ve bacilli. *S. boulardii.* appeared as oval shaped cells under microscope. *S. faecalis* viewed as gram +ve cocci in chains. *P. aeruginosa* produced smooth, large, translucent, low convex, greenish blue colonies with an aromatic odour, on nutrient agar and viewed as gram -ve bacilli. The biochemical kit testing for *P. aeruginosa* gave a negative Indole, methyl red, *Voges proskauer* and positive citrate utilization test (IM ViC, ---+). It utilized glucose but –ve for other carbohydrate utilization tests.

All the probiotic strains were highly resistance to Aztreonam and Ceftazidime followed by Amoxicillin/Clavulanate (maximum zone of inhibition, 6-8 mm), Azithromycin (19mm), Nitrofurantoin (20 mm), Piperacillin/Tozobactum (upto 26 mm) Chloramphenical (28), Amikacin (29mm), Ciprofloxacin (30mm), Leavofloxacin and Meropenam (35mm).

Zones of inhibition of all the 4 probiotic strains, *L. rhamnosus*, *S. boulardii*, *S. faecalis* and *L. acidophillus* in combination with the drugs AT^{30}, AK^{30}, $MRP^{10}$and CIP^5 were measured against the both *P. aeruginosa*, MTCC 1688 and clinical isolate of *P. aeruginosa*. These zones were compared with the zone of inhibition of antibiotic drug used as +ve control to see the enhancement of zone by probiotic strain. Maximum enhancement was shown by AT & probiotic combinations (upto 25mm) followed by probiotic combination with the drugs, AK (8 mm), MRP (5 mm) and CIP (4 mm). Reduction in zone was noticed only in MRP and *S. feacalis* combination (Table-1, Figure- 1 and 2).

Table-1 : Antimicrobial activity of antibiotics and antibiotics + Probiotic combination against *P. aeruginosa*

Test Microorganism	***P. aeruginosa* MTCC 1688**												***P. aeruginosa* clinical isolate**											
	Diameter of the zone of Inhibition (in mm)												**Diameter of the zone of Inhibition (in mm)**											
	L. rham.			***S. boul.***			***S. face.***			***L. acido.***			***L. rham.***			***S. boul.***			***S.fae.***			***L. acido.***		
Drugs	A	A+L rham.	E	A	A+ S. boul.	E	A	A+S. face.	E	A	A+ L. acido.	E	A	A+ L. rham.	E	A	A+ S. boul.	E	A	A+ S.fae.	E	A	A+ L. acido.	E
AT	0	25	25	0	25	25	0	25	25	0	10	10	0	17	17	0	16	16	7	16	9	0	16	16
AK	30	32	2	28	36	8	30	30	0	31	31	0	30	31	1	34	36	2	34	34	0	30	32	2
MRP	35	37	2	33	38	5	35	30	-5	35	35	0	31	35	4	37	39	2	35	35	0	30	35	5
CIP	27	30	3	26	38	4	26	27	1	29	29	0	31	31	0	30	31	1	32	32	0	29	31	2

Figure-1: Comparison of zone of inhibition of antibiotics and antibiotics + Probiotic combination against *P. aeruginosa* MTCC 1866

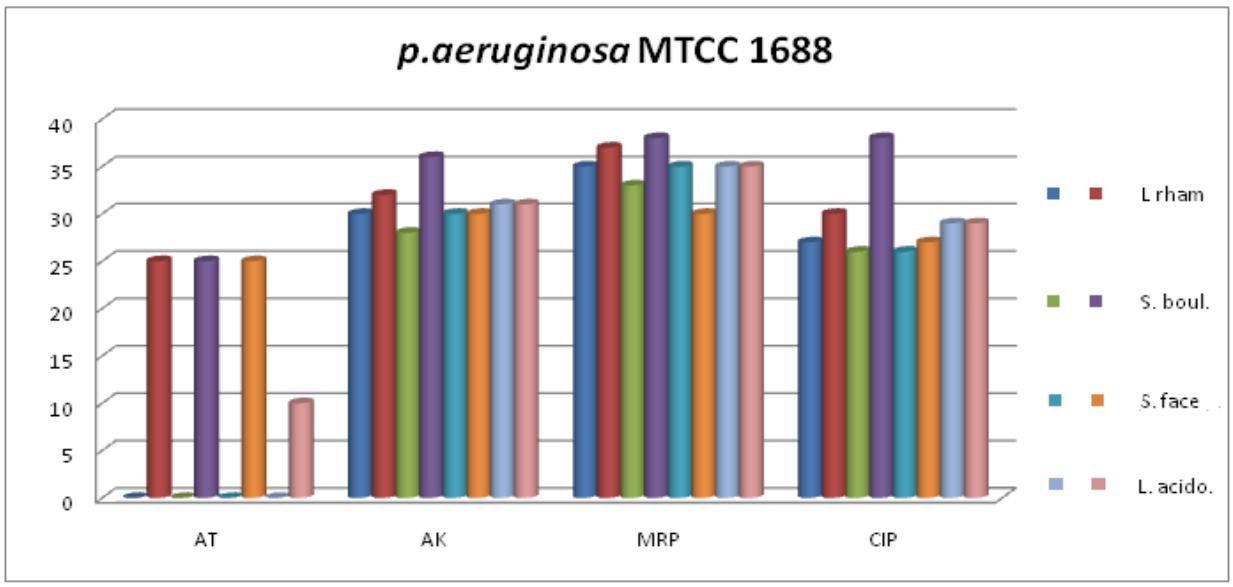
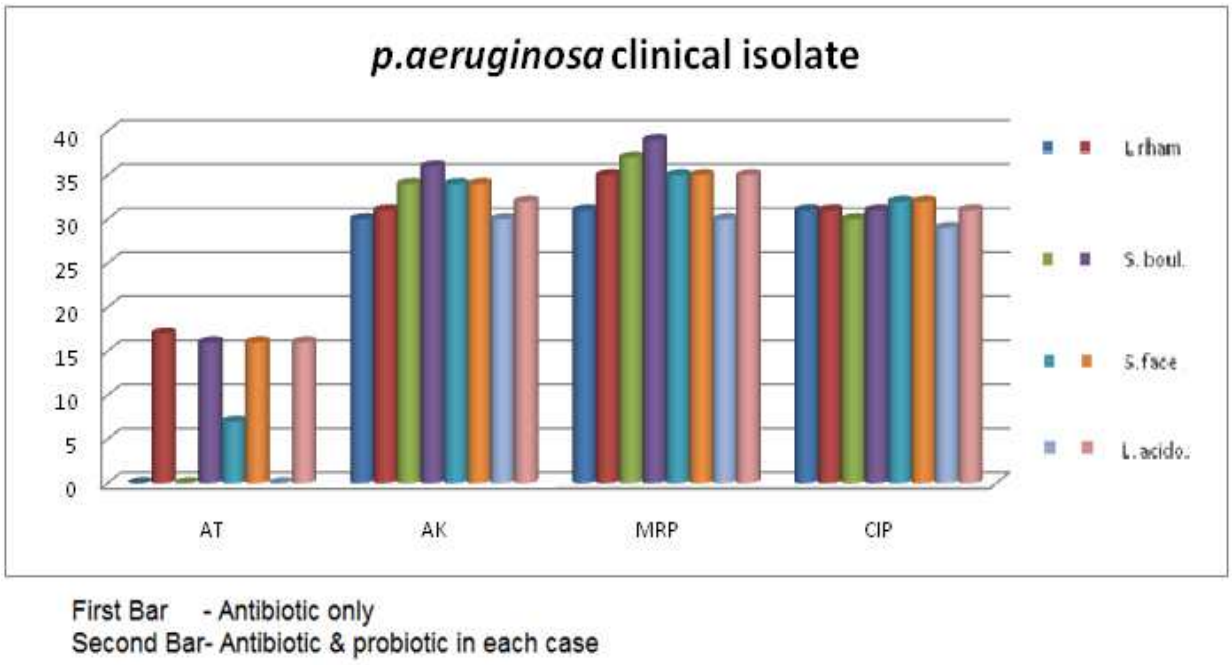


Figure-2 : Comparison of zone of inhibition of antibiotics and antibiotics + Probiotic combination against *P. aeruginosa*



The Minimum Inhibitory Concentration (MIC) of *L. rhamnosus*, *S.boulardii*, *S. faecalis* and *L. acidophillus* was assessed against both *P. aeruginosa*, MTCC 1688 and clinical isolate of *P. aeruginosa* keeping sterile water disc as -ve control and the drug AK^{30} as +ve control. Maximum Inhibitory activity was shown by the Mac farland standard

#1.0(3x108 cfu/ml) followed by 3x107 cfu/ml and 3x106 cfu/ml by both the MTCC (17 mm, 7 mm and 6 mm respectively) and clinical isolates (10 mm, 6 mm and 0 mm respectively).

CONCLUSION

Out of total 32 *in vitro* tests 71.875 % showed enhancement of zone diameter but no enhancement was seen 25 % cases while reduction in zone size was recorded in 3.125 % tests. No reduced zone was seen against the clinical isolate giving rise to 75 % cases with enhanced zone diameter recording 25 % cases with unaffected zone diameter. The intention of this study was to find out the role of probiotics in overcoming the drug resistance *P. aeruginosa.* Almost all the probiotics strains showed maximum enhancement of zone in combination with those drugs for which they have zero susceptibility. In 23 cases probiotic strains were found to reduce the drug resistance of *P. aeruginosa* to greater or smaller extent. Apart from this probiocs also protect the individuals from the harmful effects of the antibiotics, so there seems to be no harm in having these combinations. Definitely there is a room for further *in vitro* and *in vivo* studies in support of above study.

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