Melioration in Anti-staphylococcal Activity of Conventional Antibiotic(s) by Organic Acids Present in the Cell Free Supernatant of *Lactobacillus paraplantarum*

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Received: 18 April 2017 / Accepted: 3 July 2017 / Published online: 11 July 2017
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Abstract In view of emerging drug resistance in pathogens, there is a need to explore alternative strategies to combat infections. Use of probiotics is one such option. In this regard, efficacy of *Lactobacillus plantarum* has been reported against *Staphylococcus aureus*. Here, we propose that cell free supernatant (CFS) of *Lactobacillus paraplantarum* when used in combination with conventional antibiotics viz. ampicillin and oxacillin [to which the methicillin resistant *Staphylococcus aureus* (MRSA) strains were originally resistant] reduce the minimum inhibitory concentrations of these antibiotics, rendering the combination either synergistic or additive against the tested MRSA strain. The anti-staphylococcal activity was observed to be due to organic acids (acetic acid and lactic acid as confirmed by HPLC analysis) present in the CFS, as neutralization of the CFS with an alkali, sodium hydroxide (NaOH), caused the complete abrogation of its activity. The role of H₂O₂ and bacteriocin present in the CFS was also ruled out. The findings of this study suggest that cell free supernatant and ampicillin/oxacillin combination(s) might help in rejuvenating the use of conventional anti-staphylococcal antibiotics for the treatment of multi-drug resistant strains.

Keywords Anti-staphylococcal activity · Cell free supernatant · Conventional antibiotics · *Lactobacillus paraplantarum* · Organic acids · Synergistic/additive

Introduction

Staphylococcus aureus is an opportunistic pathogen causing a variety of clinical manifestations including nosocomial and community acquired infections [1]. A wide array of diseases resulting from *S. aureus* infection range from mild skin infections to life-threatening diseases such as, meningitis, osteomyelitis, endocarditis, toxic shock syndrome, bacteraemia and sepsis [2]. Emerging drug resistant *S. aureus* strains such as methicillin resistant *Staphylococcus aureus* (MRSA), oxacillin resistant *Staphylococcus aureus* (ORSA) and the newly emerging vancomycin-intermediate *Staphylococcus aureus* (VISA) have complicated the current scenario of advocating antibiotic therapy [3–5]. Therefore, there is a need to look for alternative treatment options to combat the emergence of deadly resistant strains [6]. The role of various probiotics in the prevention and treatment of several infections has been indicated [7–11]. In the present study, we have explored the use of CFS of *L. paraplantarum* in conjunction with conventional antibiotics keeping in view the multiprong approach of the agents to tackle the pathogens. To the best of our knowledge, this is the first report on augmentation of anti-staphylococcal activity of conventional antibiotics viz. ampicillin and oxacillin in the presence of cell free extract of *L. paraplantarum*.

The antibiogram of standard *S. aureus* (ATCC 9144) strain using Combi 513 octodisc revealed the sensitivity to all the antibiotics tested. However, the methicillin sensitive strain (MSSA 1) exhibited resistance to ampicillin and was

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intermediately resistant to ceftriaxone and cefuroxime (Table 1), whereas the resistant strain (MRSA 1) was found to be resistant to three antibiotics i.e. ampicillin, ceftriaxone and cefuroxime. The same pattern was observed in other methicillin resistant clinical isolates (Table 1). Resistance of sensitive strain (MSSA) to ampicillin observed in the present study may be due to the production of β -lactamase which might have inactivated ampicillin by acting on β -lactam ring thereby preventing it to inhibit cell wall synthesis. However, antibiogram of MRSA revealed resistance to ampicillin, cefuroxime (2nd generation cephalosporin) and ceftriaxone (3rd generation). These observations are in agreement with the known fact that MRSA strains develop resistance to other antibiotics as well [12]. This may be attributed to the expression of *mecA* gene which may inhibit the binding of antibiotics to penicillin binding proteins (PBPs), thereby, inhibiting the transpeptidation step involved in peptidoglycan synthesis required for bacterial cell wall [13]. Further, it may be speculated that some efflux pumps may be involved in conferring such resistance. However, sensitivity of MRSA to other classes of antibiotics such as co-trimoxazole, gentamicin and ciprofloxacin may be due to the inhibition or interference with folate synthesis, protein synthesis (30S) and DNA gyrase, respectively [14–16].

On the other hand, CFS of all the four *Lactobacillus* strains (*L. acidophilus*, *L. casei*, *L. plantarum*, *L. paraplantarum*) revealed the anti-staphylococcal activity for the standard, methicillin sensitive and methicillin resistant strains (Table 2). However, CFS of *L. paraplantarum* showed pronounced activity. The anti-staphylococcal activity was observed only with the neat (undiluted) CFS against each resistant clinical isolate. The activity observed was thought to be due to the presence of inhibitory compounds like organic acids, H_2O_2 or bacteriocins of *L. paraplantarum* in the CFS. In order to find out that which component of CFS (organic acids, H_2O_2 or bacteriocin) was responsible for anti-staphylococcal activity, the CFS was given various treatments. For this, the CFS was treated with 1 N NaOH (neutralized to pH 6.5), catalase and proteolytic enzymes (trypsin, pepsin and proteinase K) separately and incubated at 37 °C for 2 h (Fig. 1). Enzyme controls were also run simultaneously. The results indicated that H_2O_2 or any proteinaceous moiety were not responsible for the antimicrobial activity of the CFS as its activity was retained even after treatment with catalase as well as after digestion with the proteolytic enzymes. However, on neutralization with NaOH, the CFS completely lost its activity, indicating the role of acidic moiety present in the CFS. To further strengthen this observation, the CFS was given treatment with 2% diaions for 3 h so that the proteinaceous components (including bacteriocins) can be separated from the CFS. During this treatment, the proteins bind to the diaions,

leaving a fraction of CFS which has no proteins. The protein fraction bound to diaions was also extracted with methanol and the antimicrobial activity of the two fractions so obtained was evaluated. Excitingly, the anti-staphylococcal activity was observed only in the fraction that was free of protein component (removed after binding to diaions). Furthermore, HPLC analysis confirmed the presence of acetic acid and lactic acid in the CFS (Fig. 1). Various organic acids like lactic acid and acetic acid are one of the best characterised antimicrobials produced by lactic acid bacteria. The inhibitory effect of these acids has been reported to be due to diffusion across the cell membrane towards the more alkaline cytosol which interferes with the essential metabolic functions of the cell. The toxic effects of these acids include the reduction of intracellular pH and dissipation of the membrane potential [17, 18].

Keeping these observations in mind, *L. paraplantarum* was selected for further studies and its antibiogram was determined to check its feasibility to be used in combination with the antibiotics. The antibiogram of *L. paraplantarum* showed complete resistance to ampicillin, methicillin as well as oxacillin (Table 1). Resistance of *L. paraplantarum* to these antibiotics may again be either due to production of β -lactamases or some efflux pumps may be involved. Genetic profiling of this organism may throw more light on the expression of the genes present in this organism. Since treatment with proteases did not alter the activity of CFS, the latter can be perceived as a better choice for in vivo use.

On the basis of the above mentioned results, efficacy of both the combinations (ampicillin-CFS and oxacillin-CFS) was evaluated. The MIC of ampicillin against resistant strains was determined and it was found to be in the range of 8–16 $\mu\text{g/ml}$ and that of the CFS was found to be in the range of 0.5–1 AU/ml. MIC of oxacillin was also checked against all the resistant strains (MRSA) and was found to be in the range of 4–8 $\mu\text{g/ml}$ (Table 3). Further, checker-board microtitre test was performed to find out whether CFS of *L. paraplantarum* augments the activity of the selected conventional antibiotics or not. Using ampicillin-CFS and oxacillin-CFS in combination revealed that the concentration of both the agents were reduced significantly as compared to the MICs when the agents were used alone (Table 3). Though, the MIC of ampicillin when used in combination was reduced significantly but the strains still remained resistant to ampicillin as per the CLSI guidelines. Therefore, this indicates that guidelines for cut off points specifically, when the agents are used in combination need to be revisited. However, when the CFS was used in combination with oxacillin, it is worth mentioning that the strains which were resistant to oxacillin earlier, were rendered sensitive as per the CLSI guidelines. The combinations against *S. aureus* strains were found to be either

Table 1 Antibiogram of different strains of *S. aureus* using methicillin and combi 513 octodisc

	ATCC 9144	MSSA S1	MRSA S1	MRSA R1	MRSA R2	MRSA R3	MRSA R4	MRSA R5	MRSA R6	MRSA R7	MRSA R8	MRSA R9	MRSA R10	<i>L. paraplantarum</i>
COT	S	S	S	S	S	I	S	I	S	S	I	S	S	I
GEN	S	S	S	S	S	S	S	S	S	S	S	S	S	S
CTR	S	I	R	I	I	R	I	I	I	I	I	I	S	S
CIP	S	S	S	S	S	S	S	I	S	S	S	S	I	I
CXM	S	I	R	S	S	S	S	I	R	S	S	S	S	R
AMP	S	R	R	R	R	R	R	R	R	R	R	R	R	R
AK	I	S	S	S	S	I	S	S	I	S	S	S	S	S
CEP	S	S	S	S	S	S	S	S	S	S	S	S	S	S
MET	S	S	R	R	R	R	R	R	R	R	R	R	R	R

Cut-off values of different antibiotics according to CLSI guidelines: COT (25 µg): S ≥ 16, I 11–15, R ≤ 10; GEN (10 µg): S ≥ 15, I 13–14, R < 12; CTR (30 µg): S ≥ 21, I 14–20, R ≤ 13; CIP (5 µg): S ≥ 21, I 16–20, R ≤ 15; CXM (30 µg): S ≥ 18, I 15–17, R ≤ 14; AMP (10 µg): S ≥ 29, R ≤ 28; AK (30 µg): S ≥ 17, I 15–16, R ≤ 14; CEP (30 µg): S ≥ 18, I 15–17, R ≤ 14; MET (5 µg): S ≥ 14, I 10–13, R ≤ 9

COT co-trimoxazole, GEN gentamicin, CTR ceftriaxone, CIP ciprofloxacin, CXM cefuroxime, AMP ampicillin, AK amikacin, CEP cephalothin, MET methicillin, S sensitive, I intermediate, R resistant

Table 2 Zones of inhibition of CFS (from different strains of *Lactobacillus*) against different strains of *S. aureus*

S. no	Strain	<i>L. casei</i>	<i>L. acidophilus</i>	<i>L. plantarum</i>	<i>L. paraplantarum</i>
1.	<i>S. aureus</i> ATCC 9144	++	++	+++	+++
2.	MSSA S1	+	+	++	+++
3.	MRSA R1	+++	+	++	+++
4.	MRSA R2	+	+	++	++
5.	MRSA R3	+	++	++	++
6.	MRSA R4	+	++	++	+++
7.	MRSA R5	++	+++	+	+++
8.	MRSA R6	+++	++	+++	+++
9.	MRSA R7	++	++	++	+++
10.	MRSA R8	++	+	++	+++
11.	MRSA R9	++	++	++	++
12.	MRSA R10	++	++	++	+++

Range of zones of inhibition (diameter): +: 10–13 mm; ++: 14–18 mm; +++: 19–22 mm

Treatment of CFS	Concentration	Zone of Inhibition (mm)
Untreated CFS (Control)	-	14±0.2
NaOH	1 N	--
Catalase	1 mg/ml	13±0.1
Pepsin	1 mg/ml	14±0.2
Trypsin	1 mg/ml	13±0.3
Proteinase K	1 mg/ml	12±0.1
Lipase	1 mg/ml	14±0.2
Diaion HP-20	2%	14±0.2
Diaion protein fraction (extracted with methanol)	2%	--

Fig. 1 Effect of NaOH, catalase, various proteolytic enzymes and diaion treatment on the inhibitory activity of CFS produced by *L. paraplantarum* against *S. aureus*. The results are expressed as mean $\pm$ SD of three replicate experiments. **a** HPLC showing

chromatogram for mix standard solution of organic acids 1 lactic acid, 2 acetic acid, 3 propionic acid, 4 butyric acid **b** showing the elution time for organic acids (1 lactic acid, 2 acetic acid) present in the test sample of CFS produced by *L. paraplantarum*

Table 3 MIC values and FIC index of antibiotics and CFS alone and in combination against different *S. aureus* strains as determined by broth dilution technique and checkerboard method

Sr. no.	Isolates	MIC of ampicillin (µg/ml)		CFS (AU/ml)		FIC index	MIC of oxacillin (µg/ml)		CFS (AU/ml)		FIC index
		Alone	Combination	Alone	Combination		Alone	Combination	Alone	Combination	
1.	Standard <i>S. aureus</i> 9144	1	0.25	0.5 (neat/2)	0.125 (neat/8)	0.5	1	0.5	0.5 (neat/2)	0.0625 (neat/16)	0.625
2.	MSSA S1	8	8	0.5 (neat/2)	0.25 (neat/4)	1.5	2	0.25	0.5 (neat/2)	0.0625 (neat/16)	0.25
3.	MRSA R1	16	4	1 (neat)	0.25 (neat/4)	0.5	4	1	1 (neat)	0.125 (neat/8)	0.375
4.	MRSA R2	16	4	0.5 (neat/2)	0.25 (neat/4)	0.75	8	2	0.5 (neat/2)	0.125 (neat/8)	0.50
5.	MRSA R3	8	2	1 (neat)	0.25 (neat/4)	0.5	4	1	1 (neat)	0.25 (neat/4)	0.50
6.	MRSA R4	8	2	0.5 (neat/2)	0.25 (neat/4)	1	4	2	0.5 (neat/2)	0.125 (neat/8)	0.75
7.	MRSA R5	8	2	1 (neat)	0.125 (neat/8)	0.375	8	1	1 (neat)	0.125 (neat/8)	0.250
8.	MRSA R6	16	4	1 (neat)	0.125 (neat/8)	0.375	8	2	1 (neat)	0.125 (neat/8)	0.375
9.	MRSA R7	8	4	0.5 (neat/2)	0.125 (neat/4)	0.75	4	1	0.5 (neat/2)	0.5 (neat/2)	1.25
10.	MRSA R8	16	2	1 (neat)	0.5 (neat/2)	0.625	4	1	1 (neat)	0.125 (neat/8)	0.375
11.	MRSA R9	16	2	1 (neat)	0.125 (neat/8)	0.250	8	2	1 (neat)	0.25 (neat/4)	0.5
12.	MRSA R10	8	2	0.5 (neat/2)	0.125 (neat/8)	0.5	4	1.0	0.5 (neat/2)	0.125 (neat/8)	0.5

Synergistic ≤ 0.5 , additive or indifferent >0.5 and ≤ 2.0 , antagonistic >2.0

synergistic or additive indicating that the combinations do augment the efficacy of conventional antibiotics. The important finding of this study may be helpful in making the future strategies for rejuvenating the antibiotics in presence of probiotics or their CFS due to the multipronged mechanism to target the pathogen in addition to the health benefits provided due to the immuno-modulatory properties of probiotics or their CFS. Taking into account all the observations, the study revealed that CFS and ampicillin/oxacillin combination might prove as an effective option. However, in future more studies need to be carried out by including larger sample size from different geographical areas due to clinical variability of the strains and by validating the in vivo efficacy in MRSA/VISA mediated systemic or local infections.

Acknowledgements We are thankful to the Indian Council of Medical Research (ICMR) for providing Short-Term Studentship

(STS) to Lavanya Rishi for carrying out this work during the medical course. The authors are also grateful to Central Sophisticated Instrument Cell, PGIMER Chandigarh for providing HPLC facility.

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