

Full Length Research Paper

Antibacterial activity of isolated human intestinal microbiota *Lactobacillus* strains against methicillin resistant and susceptible *Staphylococcus aureus*

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Accepted 19 April, 2013

In the present study, lactic acid bacteria were screened for anti-methicillin resistant activity. Isolation of lactic acid bacteria from 100 faecal samples of healthy adults was conducted using de Man-Rogosa Sharpe agar (MRS) and three *Lactobacilli* strains were isolated. The three isolates of human intestinal microbiota were identified on the basis of morphological and biochemical characteristics. The incidence of *Lactobacillus plantarum*, *Lactobacillus acidophilus* and *Lactobacillus casei* were found to be 43.47, 34.78 and 21.73% respectively. The antibacterial activity of these three isolates was determined against five strains of *Staphylococcus aureus*, including three methicillin resistant *Staphylococcus aureus* strains. Maximum inhibitory potential was seen in *L. casei*. Therapeutic efficacy of the *lactobacilli* strains was evaluated to control the re-emerging MRSA infection.

Key words: Antibacterial activity, *Lactobacilli*, methicillin resistant *Staphylococcus aureus* (MRSA).

INTRODUCTION

Healthcare-associated infections (HAI) are those infections that patients acquire while under the healthcare institution. Methicillin-resistant *Staphylococcus aureus* (MRSA) is the most common HAI causing agent and is resistant to certain antibiotics (oxacillin, penicillin and amoxicillin). In 1972, MRSA accounted for 2% percentage of all *S. aureus* HAIs reported to the centres for Disease Control and Prevention (CDC) in the U.S. Today, MRSA accounts for 60% of *S. aureus* infections. MRSA infections occur in wounds of the skin burns and other places where intravenous tubes enter the body. Infection also occurs in eyes, bones, heart and blood. Approximately 23% mortality rate is among patients with MRSA bacteremia (bacterial infections of bloodstream). Disease caused by MRSA can range from skin infections to food borne illness and severe infections such as endocarditis, osteomyelitis and sepsis (Lowly, 1998). The nasal carriage of *S. aureus* is common in 20-50% of the

population (Cespedes et al., 2005). Intestinal carriage appears to be increasing among hospitalized patients and infants (Kluytmans et al., 1997).

Methicillin resistance in *staphylococcus aureus* is mediated by the *mecA* gene, which encodes for the penicillin-binding protein 2A (PBP2A) resulting in reduced affinity for the beta-lactam antibiotics including the penicillinase-resistant penicillin (Khadri and Alzohairy 2010). The emergence of MRSA isolates with reduced susceptibility to glycopeptides has created need for new agents for the treatment of MRSA infections (Griethuysen et al., 1999). Widespread antibiotic usage exerts a selective pressure that acts as a driving force in the development of antibiotic resistance. The effectiveness of currently available antibiotics is decreasing due to the increasing number of resistant strains causing infections. MRSA is one such bacteria and it causes wide range of syndromes, from minor

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skin and soft tissue to life-threatening pneumonia and toxinoses such as toxic shock syndrome (Nawaz et al., 2008).

Lactic acid bacteria (LAB) are commonly defined as gram-positive, non-sporulating, catalase negative, aero tolerant, acid tolerant, nutritionally fastidious, strictly fermentative organisms that produce lactic acid as end product of carbohydrate metabolism (Hartnett et al., 2002). LAB are famous as friendly bacteria for human health. Most of the *Lactobacillus* species are normal and non-pathogenic microorganisms in human and animal intestine and are a vital part of the intestinal microbial ecosystem. Lactic acid bacteria have a number of properties which render them highly suitable for probiotic therapeutics which are of pharmaceutical interest. They play a significant physiological role in the maintenance of the ecological balance because their lactic acid production is responsible for low pH level in the tracts. In addition, they also produce other inhibitory substances such as hydrogen peroxide, bacteriocins and some organic acids. Other mechanisms proposed for their microbial antagonism are competition for nutrition, adhesion inhibition of pathogens to surfaces and stimulation of the immune system (Voravuthikunchai et al., 2006).

Lactobacilli, despite of their origin, have potential to inhibit the growth of pathogens, including problematic antibiotic resistant isolates due to their production of several antimicrobial compounds (Petrova et al., 2009). Verdenelli et al. (2009) reported that two *Lactobacillus* strains had an inhibitory effect on potentially pathogenic microorganisms such as *Escherichia coli*, *Staphylococcus aureus*, *Candida albicans* and *Candida perfringens*. Lactic acid bacteria (LAB) work via different mechanisms to exert an antimicrobial effect, but the cell envelope is generally the target. Studies have revealed that *Lactobacillus reuteri* isolated from healthy vaginal ecosystem (Voravuthikunchai et al., 2006) and *Lactobacillus fermentum* (Nawaz et al., 2008) have significantly inhibited methicillin resistant *S. aureus*. It has also been demonstrated that *L. rhamnosus* has the capacity to displace and kill *S. aureus* adhering to human intestine mucus by 39 to 44% (Vesterlund et al., 2006).

Methicillin resistant bacterial infections provide a tough challenge in the selection of antibiotics. Traditional use of antibiotics is worsening the problem. Although antibiotics are available for the treatment of MRSA infections, because of their numerous adverse effects and development of resistant strains, there is an urgent need to search for alternatives to synthetic antibiotics for treating MRSA infections. Studies were carried out to search for alternatives to synthetic antibiotics against MRSA. LAB demonstrates many properties which make them highly suitable for probiotic therapeutics of pharmaceutical interest. LAB has become an attractive option of modern medical practice. Recently attention has been paid to their health promoting properties. Of particular importance are their probiotic properties and

specially the ability to compete with pathogens.

The present study was carried out to isolate *Lactobacilli* and to observe its efficacy against MRSA, in controlling MRSA infections as well as providing a new strategy to treat re-emerging infections.

MATERIALS AND METHODS

Test pathogen

Five isolates of *S. aureus* were collected from Microbial Culture Collection Bank (MCCB) of the Department of Microbiology and Fermentation Technology. The *S. aureus* strains were MCCB0045, MCCB0046, MCCB0065, MCCB0066 and MCCB0067.

Isolation of lactic acid bacteria

100 human faecal samples were collected and 10³ dilutions of faecal samples were prepared in sterile normal saline, then swab inoculated on de Mann Rogosa Sharpe (MRS) agar plates. The plates were incubated anaerobically using anaerobic jar, at 37°C for 48 h to obtain colonies (De Man et al., 1960).

Identification of *Lactobacillus* species

The bacterial colonies on incubated plates were identified on the basis of cultural, morphological and biochemical characteristics as described in the Bergey's Manual of Determinative Bacteriology (Holt et al., 1984).

Cultural characteristics

The isolates were identified on the basis of different colony characteristics like diameter, consistency, colour, texture, elevation, margin, etc.

Morphological characteristics

The organism was subjected to Gram's staining and observed under 100 x objective for observing the morphological characteristics such as the shape and arrangement (clusters or chains) of cells, and gram-reaction (gram-positive or gram-negative).

Biochemical characteristics

The following biochemical tests were performed for the identification of lactic acid bacteria.

Indole test

The test tubes containing tryptophan broth were inoculated with the test organism and incubated aerobically at 37°C for 24 h. After incubation, 0.5 ml Kovac's reagent was added gently. Red colour formed in the alcohol layer indicated positive reaction.

Methyl red test

Five drops of 0.04% solution of methyl red were added to the

culture in glucose phosphate broth which had been incubated at 30°C for five days. Red colour indicated positive test while yellow colour indicated negative test.

Citrate utilization test

Simmons Citrate medium was prepared, inoculated from a saline suspension of organism to be tested and incubated for 24 h at 37°C. Growth in the medium with a change in colour from green to blue indicated a positive result.

Carbohydrate fermentation test

10 ml of carbohydrate fermentation broth was transferred in culture tubes along with the inverted Durham's tube and autoclaved at 121°C for 15 to 20 min. The carbohydrate was prepared at 1% concentration in distilled water and autoclaved at 10 lbs/inch². Separately, one loopful of organism from the culture plate was transferred in the tubes containing 10 ml carbohydrate fermentation broth and 1 ml of each sugar solution. A control was taken; tube that is not inoculated (without microorganism the tubes were incubated at 37°C for 24 to 48 h). After incubation, the tubes were examined for acid production and gas production. Change in media colour from purple to yellow indicated the production of acid, which was a positive test. No colour change indicated negative test. Accumulation of gas bubbles in Durham's tube indicated the gas production and gave a positive test.

Catalase test

A loopful of culture was transferred from tube to a clean glass slide. Thereafter, a drop of hydrogen peroxide was added and mixed well. Evolution of bubbles indicated a positive test, whereas no evolution of bubbles indicated a negative test.

Nitrate reduction

Equal amount of α naphthol and sulphanilic acid were added to 24 to 48 h culture in nitrate reduction broth. The test tubes were examined for the appearance of red colour and gas bubbles. The occurrence of red colour and gas bubbles were presumptive for denitrification and indicated a positive test.

Hugh and Liefson's test

2.3 ml of 10% solution of glucose was added to Hugh and Liefson's oxidation and fermentation medium tubes and inoculated in duplicate and incubated aerobically at 37°C from 24 to 48 h. Paraffin was spread over the media in one of the test tube after inoculation and then incubated to check for anaerobic bacteria. Positive tubes showed change in colour of the media green to yellow.

Motility test

The tube containing motility agar medium was stab inoculated. Positive test is indicated by the growth around the stab line that radiated outwards in all directions while no growth around stab line indicated negative test.

Hydrogen sulphide test

Tubes containing sulphide indole motility agar were stab inoculated

and kept for incubation at 37°C for 24 h. Blackening of the culture medium indicated a positive test.

Antibiotic susceptibility pattern of *S. aureus* against selected antibiotics

The test organism was tested for its sensitivity towards the given antibiotics using the Disk Diffusion Method (Bauer et al., 1966). Overnight, broth cultures were spread on the surface of Nutrient Agar Media plates with the help of sterile swabs. The antibiotic discs were placed on the agar surface and plates were incubated aerobically at 37°C for 24 h. Zone of inhibition was measured in 'mm' and the result interpreted on the basis of CLSI standards (Wayne, 2003). Antibiotic discs (Hi Media) which were used in the present study were Methicillin (M, 5 µg), Erythromycin (E, 15 µg), Ciprofloxacin (Cf, 5 µg), Tetracycline (T, 30 µg), Kanamycin (K, 30 µg), Gentamicin (G, 10 µg), Rifampicin (R, 30 µg), Linezolid (L, 30 µg), Vancomycin (Va, 30 µg) and Tobramycin (Tb, 10 µg).

Screening of LAB isolates for anti-MRSA activity

The antibacterial activity was assayed against isolates of *S. aureus* using Agar Well Diffusion method (Schillinger and Lucke, 1989). The five strains of *S. aureus* were incubated in Nutrient Broth at 37°C for 24 h. The broth culture was swab inoculated on Nutrient Agar. For the detection of antibacterial activity, the *Lactobacilli* strains were cultured in MRS broth, incubated anaerobically at 37°C for 36 h. The broth culture of *Lactobacilli* was subjected to centrifugation at 5000 g for 15 min. The supernatant was discarded and the pellet was washed twice with sterile normal saline. The washed pellet was then inoculated in Skim Milk Broth and incubated aerobically at 37°C for 36 h. The inoculated Skim Milk Broth was subjected to centrifugation at 5000 g for 15 min and 20 µl of the supernatant was filled on each 5 mm well on Nutrient Agar plates. Wells filled with sterile Nutrient Broth were included as control. Inhibition zones were measured after incubating the plates aerobically at 37°C for 24 h (Hassan et al., 2011). Inhibition was scored positive if the width of the clear zone around the well was observed.

Statistical analysis

The data was analyzed using Chi-square test, Z-test and two way classification of ANOVA.

RESULTS AND DISCUSSION

Isolation and identification of *Lactobacilli* from human faecal samples

In the present investigation of the hundred human faecal samples collected from healthy adults, 23% were positive for *Lactobacilli*. On the basis of the biochemical characteristics of the 23 *Lactobacilli* isolates, it was observed that the three different *Lactobacilli* species isolated were *L. plantarum*, *L. acidophilus* and *L. casei*. Out of the 23 isolates, ten isolates were *L. plantarum*, eight were *L. acidophilus* and five were *L. casei* (Tables 1 and 2). On analyzing statistically, the data was non-significant at 0.05% probability. Study conducted by Dhewa and Goyal (2009) described incidence of LAB

Table 1. Incidence and distribution of *Lactobacilli* species in human faecal samples.

Total number of sample	Total number of <i>Lactobacilli</i> isolates (%)	Number of <i>Lactobacillus plantarum</i> (%)	Number of <i>Lactobacillus acidophilus</i> (%)	Number of <i>Lactobacillus casei</i> (%)
100	23	10 (43.47)	8 (34.78)	5 (21.73)

$\chi^2=5.99$ (tabulated value), $\chi^2=24.32$ (calculated value), N.S=non-significant.

Table 2. Cultural, morphological and biochemical identification of *Lactobacilli* Isolates.

Characteristic		Organism		
		<i>Lactobacillus plantarum</i>	<i>Lactobacillus acidophilus</i>	<i>Lactobacillus casei</i>
Cultural characteristics	Colour of colony	Cream	White	Off white
	Shape of colony	Small, spherical	Small, spherical	Small, spherical
	Elevation	Convex	Convex	Convex
	Margin	Entire edges	Entire edges	Entire edges
Morphological characteristics	Gram's reaction	Gram positive	Gram positive	Gram positive
	Arrangement of cells	Long slender rods in chains	Rods in pairs and chains	Small rods in chains
	Spore formation	Non -sporing	Non -sporing	Non -sporing
	Indole test	Negative	Negative	Negative
Biochemical characteristics	Methyl red test	Positive	Positive	Positive
	Citrate test	Negative	Negative	Negative
	catalase test	Negative	Negative	Negative
	Motility test	Negative	Negative	Negative
	Nitrate reduction test	Positive	Positive	Positive
	H ₂ S production test	Negative	Negative	Negative
Carbohydrate fermentation	D-Glucose	A ⁺ G ⁻	A ⁺ G ⁻	A ⁺ G ⁻
	D- Galactose	A ⁺ G ⁻	A ⁺ G ⁻	A ⁺ G ⁻
	Sucrose	A ⁺ G ⁻	A ⁺ G ⁻	A ⁺ G ⁻
	Maltose	A ⁺ G ⁻	A ⁺ G ⁻	A ⁺ G ⁻
	Esculin	A ⁺ G ⁻	A ⁺ G ⁻	W A ⁺ G ⁻
	Fructose	A ⁺ G ⁻	A ⁺ G ⁻	A ⁺ G ⁻
	D-Mannitol	A ⁺ G ⁻	A ⁻ G ⁻	A ⁺ G ⁻
	Arabinose	W A ⁺ G ⁻	A ⁻ G ⁻	A ⁺ G ⁻

A⁺, Acid positive; A⁻, acid negative; G⁺, gas positive; G⁻, gas negative; WA⁺, weakly positive.

from human faecal sample. Human intestinal mucous isolated from faeces of healthy Egyptian infants of 36 months of age were used as a substratum for the adhesion of probiotic *Lactobacilli* strains (Khalil et al., 2007). *Lactobacillus acidophilus* was the most frequently recovered species from infant faecal samples. This finding indicates that this species survived better in the gastrointestinal tract than the other strains (Khalil et al., 2007). Another study showed comparable results in which *Lactobacilli* were isolated from 50 faecal samples of 3 to 30-day old infants. The isolated *Lactobacillus* strains were further identified as *L. acidophilus*, *Lactobacillus brevis*, *L. casei*, *L. plantarum*, *Lactobacillus fermentum*, *Lactobacillus reuteri* and *L. rhamnosus* (Ozgun and Vural, 2011). *Lactobacillus paracasei*,

Lactobacillus gasseri, *Lactobacillus delbrueckii*, and *Lactobacillus plantarum* were isolated from faeces of healthy adults on MRS agar (Delgado et al., 2007).

Antibiotic susceptibility pattern of *S. aureus*

As a result of the antibiotic susceptibility test for the five strains of *S. aureus*, MCCB0065 was most resistant towards the ten antibiotics used, whereas MCCB0045 was most susceptible. Among the ten antibiotics, Tobramycin (Tb) possessed least inhibitory potential whereas Erythromycin (E) had maximum inhibitory effect against the five strains of *S. aureus*. On the basis of resistance against Methicillin, three out of five *S. aureus*

Table 3. Antibiotic susceptibility profile of *Staphylococcus aureus* strains.

<i>S. aureus</i> strain	Antibiotic									
	E (15 µg)	Cf (5 µg)	T (30 µg)	K (30 µg)	G (10 µg)	R (30 µg)	L (30 µg)	Va (30 µg)	Tb (10 µg)	M (5 µg)
MCCB0045	+++	+++	++	++	+++	++	+++	+++	-	-
MCCB0046	++	++	-	++	++	++	+++	++	++	-
MCCB0065	++	++	-	++	++	+	++	++	-	-
MCCB0066	++	++	-	++	++	+++	-	++	++	++
MCCB0067	++	++	++	++	-	+++	++	++	++	+

+++ , Sensitive; ++, Intermediate; -, Resistant. E, Erythromycin; R, Rifampicin; Cf, Ciprofloxacin; G, Gentamicin; T, Tetracycline; L, Linezolid; K, Kanamycin; Va, Vancomycin; Tb, Tobramycin; M, Methicillin.

strains (MCCB0045, MCCB0046, and MCCB0065) were MRSA and the rest two strains (MCCB0066, MCCB0067) were MSSA. *S. aureus* strain MCCB0065 was multi-drug resistant as it was resistant to Tobramycin, Tetracycline and Methicillin (Table 3).

In a previous study, the antibiotic susceptibility of the MSSA and MRSA isolates was tested. The study conducted to test the antibiotic susceptibility pattern of *Staphylococcus aureus* strains showed that both MRSA and MSSA were susceptible towards Gentamicin, Ciprofloxacin and Tetracycline (Sivakumari and Shanthy, 2009). This was comparable with the reports of other workers (Nwankwoet al., 2010; Khadri and Alzohairy, 2010).

Antibacterial activity of *Lactobacilli* species against MRSA

It was observed from the result of the antibacterial activity test of the three isolated strains of *lactobacilli* that *L. casei* showed maximum inhibition against MCCB0045, MCCB0065, MCCB0067 but showed moderate inhibition against MCCB0066 and minimum inhibition was seen against MCCB0046 (Figure 1). In *L. plantarum*, maximum inhibition was observed against MCCB0045, MCCB0067 whereas moderate inhibition was seen against MCCB0046, MCCB0065 and minimum inhibition was seen against MCCB0066. In *L. acidophilus*, maximum inhibition was observed against MCCB0067 whereas moderate inhibition was scored against MCCB0045, MCCB0065, MCCB0066 and minimum inhibition was scored against MCCB0046. On the basis of the above observation, it was observed that *L. casei* had maximum inhibitory potential, showing maximum inhibition against three strains of *S. aureus* whereas *L. acidophilus* showed minimum inhibitory potential, as it showed maximum inhibition against one strain of *S. aureus*. On considering the resistance potential of the five pathogen strains of *S. aureus* towards the three isolated *lactobacilli* strains, it was observed that MCCB0067 was most resistant whereas MCCB0046 was most susceptible towards the

three *Lactobacilli* strains. The range of the width of inhibition zones remained between 6.5 to 15.5 mm (Table 4).

On considering the three MRSA strains, MCCB0045 was most susceptible towards the three isolated *Lactobacilli* strains. MCCB0065 showed moderate susceptibility whereas MCCB0046 was least susceptible. On analyzing statistically, the data was significant due to *Lactobacilli* spp, at 5% probability and non-significant due to *S. aureus* strains at 5% probability. Studies have shown that *L. acidophilus* has the capacity to produce numerous metabolites that kill pathogenic bacteria (Oh et al., 2000). LAB were screened previously for anti-MRSA activity and *L. fermentum* was able to inhibit MRSA strains (Nawaz et al., 2008). Bactericidal proteins with antagonistic activities are produced by some strains of *L. acidophilus*. Among *lactobacilli*, strains belonging to species of the *L. acidophilus* and *L. casei* are frequently used as probiotic agents (Klaenhammer and Kullen, 1999). In a previous study, the isolated *Lactobacillus* strains exhibited the highest zone of inhibition (15 mm) against *S. aureus* (Arokiyarnary and Sivakumar, 2011).

Conclusion

Out of a total of 100 faecal samples of healthy adults studied, 23% were positive for *Lactobacilli*. The 23 *Lactobacilli* strains isolated from 100 human faecal samples in which 43.47% were *L. plantarum*, 34.78% were *L. acidophilus* and 21.73% were *L. casei*. Statistically, the differences were non-significant. *S. aureus* strains MCCB0045, MCCB0046, MCCB0065 were MRSA whereas MCCB0066, MCCB0067 were MSSA and strain MCCB0065 was multi-drug resistant. *L. plantarum*, *L. acidophilus*, and *L. casei* demonstrated antimicrobial potential to inhibit *S. aureus* including MRSA strains. *L. casei* showed maximum inhibitory potential. On analysing statistically the differences were significant. It is recommended that they may be considered beneficial, as compared to the regular antibiotics. Lactic acid bacteria will be ideal in producing

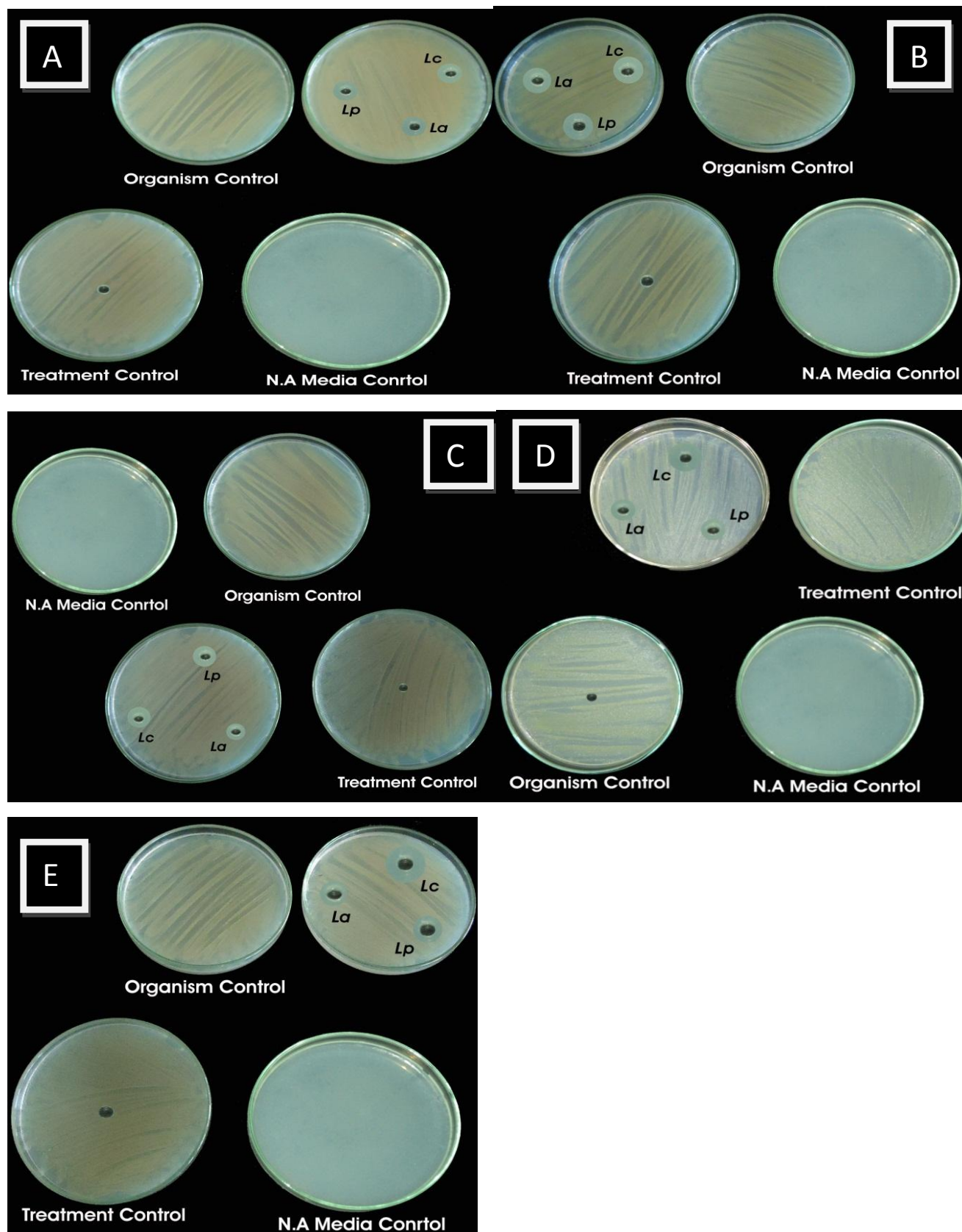


Figure 1. Antibacterial activity of *Lactobacilli* strains against test pathogen *Staphylococcus aureus*. A, *Staphylococcus aureus* MCCB0045; B, *S.aureus* MCCB0046; C, *Staphylococcus aureus* MCCB0065; D, *S. aureus* MCCB0066; E, *S. aureus* MCCB0067; La, *L. actobacillus acidophilus*; Lc, *L. actobacillus casei*; Lp, *Lactobacillus plantarum*.

Table 4. Antibacterial activity of *Lactobacilli* strains against test pathogen *Staphylococcus aureus*.

S/N	<i>Lactobacilli</i>	Zone of inhibition (mm)				
		MCCB0045	MCCB0046	MCCB0065	MCCB0066	MCCB0067
1	<i>Lactobacillus plantarum</i>	15.00	10.50	11.00	6.00	15.50
2	<i>Lactobacillus acidophilus</i>	11.50	7.00	12.00	11.50	14.50
3	<i>Lactobacillus casei</i>	15.50	6.00	13.00	8.00	14.00

Due to *Lactobacilli*: F (Cal) =5.98; F (Tab) =3.84; C.D at 5%=3.044; Result=S (significant). Due to *Staphylococcus aureus*: F (Cal) =0.03; F (Tab) =.4.46; C.D at 5%=3.044.

antimicrobial compounds effective against MRSA. The *Lactobacilli* strains isolated in the present study demonstrated anti-methicillin resistant *Staphylococcus aureus* activity. The isolated strains of *L. plantarum*, *L. acidophilus*, and *L. casei* can also be used as probiotic (after the positive reports of *in vivo* experimentations). This strategy should be applied for the control of re-emerging MRSA infections.

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