ISSN : 0974 - 7532 Volume 10 Issue 8

Research & Reviews in

BioSciences

Regular Paper

RRBS, 10(8), 2015 [299-304]

Lactobacillus acidophilus la-5 inhibits growth and biofilm formation of methicillin resistant *staphylococcus aureus* isolated from wounds of hospitalized patients

Salahuddin Khan*, Salma Mahmud

Department of Microbiology, Chittagong Medical College Hospital

***IQC Research Consultancy, House 5, Lane 2, Road 1, Block H, Halishahar Housing Estate, Chittagong-4216**

E-mail: salahuddin1187@gmail.com

ABSTRACT

The aim of the present study was to assess the ability of predominantly used probiotic strain *Lactobacillus acidophilus* La-5 to inhibit the growth and biofilm formation by Methicillin Resistant clinical isolates of *S. aureus*. A total of 41 clinical isolates of *S. aureus* were collected from wound samples of patients. Biofilm formation assay was performed using microtiter plate. At 24h incubation, 13 (31.70%, P<0.5) of the clinical isolates were strongly adherent, 25 (60.97%, P<0.5) were moderately adherent and only 3 (7.31%, P<0.5) were weakly adherent. Agar overlay interference test was employed to study the ability of *L. acidophilus* La-5 to inhibit the growth of *S. aureus*. The growth was completely inhibited at higher cell concentrations of *L. acidophilus* (10^7-10^9). The inhibitory effects declined with lowering of cell concentration. The assay to study the inhibition of biofilm formation was conducted using microtiter plate and staining with crystal violet. Only 5 of the 13 strong biofilm producers were able to form biofilm upon introduction of probiotic strain. There were only 6 out of 25 moderate biofilm producers and none of the three weak biofilm formers were able to form biofilm upon co-inoculation with *L. acidophilus* La-5.

KEYWORDS

MRSA;
Probiotic;
Staphylococcus aureus;
Lactobacillus acidophilus.

INTRODUCTION

Staphylococci are the most frequent pathogens of nosocomial infections and infections on indwelling medical devices that involve the formation of biofilm[1]. The biofilm-associated infections caused by *Staphylococcus aureus* are often difficult to treat with antibiotics and require frequent replacements of the indwelling medical devices. They are one of the most prevalent opportunistic pathogens that can be found in damaged host tissues, nasal passages and human skin. Biofilms are complex communities of microorganisms that stick together in extracellular polymeric substances (EPS) and are capable of adhering to any surface. The EPS, which contain the conglomerate of proteins, polysaccharides and extracellular DNA are often referred to as slime. Probiotics are a group of microorganisms that pro-

Regular Paper

mote health conditions. Recent studies have established the health benefits of probiotic treatment that ranges from acute viral gastritis through pediatric post-antibiotic-associated diarrhea, certain pediatric allergic disorders, necrotizing enterocolitis in preterm infants, inflammatory bowel diseases and post-surgical pouchitis [2] (Vuotto et al., 2014). Lactobacilli are a group of probiotic bacteria that administer several health benefits to the host. These bacteria are often included in various commercial dairy products such as milk, cheese and yogurt as well as chewing gums and fruit drinks[3] The ability of common probiotic bacterium *Lactobacillus acidophilus* has been shown to demonstrate antagonistic effect against biofilm formation in previous studies. The present study was conducted to determine the possible effect of *L. acidophilus* on growth and biofilm formation of *S. aureus* isolated from wounds.

EXPERIMENTAL

Isolation of bacterial strains

The 41- biofilm forming clinical isolates of Methicillin Resistant *S. aureus* were obtained from patients with wound infection during 2012-2013, at Chittagong Medical College Hospital. The clinical isolates were grown on blood agar plates supplemented with 5% horse blood for 24h at 37°C. The pure cultures of *S. aureus* were sub-cultured on Tryptone Soya Agar and incubated for another 24h at 37°C. The isolates were identified and confirmed according procedures described previously[4]. The *L. acidophilus* La-5 strain was obtained from laboratory of the Department of Health. The strains were characterized by the API 50 CH system to confirm their identity. The bacteria were initially cultured for 16-20 h on MRS agar (de Man, Rogosa, Sharpe). A distinct colony of each bacterium was then transferred to 4.5 ml MRS broth for further 16-20 h of incubation.

Antibiotic assay

All the clinical isolates of *S. aureus* were subjected antibiogram assay using disk diffusion method to determine the sensitivity and resistance pattern of the isolated strains to amikacin (30 μg), clindamycin (2 μg), gentamicin (10 μg), ciprofloxacin (5 μg), trimethoprim-sulfamethoxazole (1.25 / 23.75 μg), erythromycin (15 μg), tetracycline (30 μg) using disk diffusion method according to rec-ommendation of Clinical and Laboratory Standards Insti-tute[5].

Assay of biofilm production

To analyze the ability of the clinical isolates to produce biofilm, an overnight culture of each was grown in Tryptic Soy Broth supplemented with 1% glucose at 37°C for 18-20h. The suspension was adjusted with TSB to 0.5 on the McFarland standard to measure the optical density (OD) at 630nm absorbance in spectrophotometer corresponding to approximately 10^6 cells. A 96-wells polystyrene microtiter plate was transferred with 250 μl suspension of clinical isolates that were grown in TSB. Each of the 41 clinical isolates thus had a corresponding well transferred with the suspension of the same dilution. The blank wells were filled with broth with no organisms. Plates were made in duplicate and allowed to incubate for 24h at 37°C. At 24h, the wells were washed and aspirated with physiological saline. The wells were vigorously shaken to remove non-adherent bacteria. The remaining bacteria were fixed using 96% ethanol and then were allowed to dry. The dried wells were then subjected to staining using Crystal violet. Excess stains were rinsed off. The OD of the each well was measured at a wavelength of 540 nm using ELISA reader[6].

Inhibition of biofilm formation

An overnight culture of *S. aureus* was prepared in TSB supplemented with 1% sucrose. The *L. acidophilus* La-5 strain was also grown in MRS broth. The OD of the both suspensions was measured as described above. Both of the suspensions were mixed (1:1) and studied for biofilm formation as described earlier in another study[6].

Agar overlay interference test

A tenfold serial dilution of *L. acidophilus* La-5 culture was also prepared. The OD at 630nm was measured at 10^1 dilution. Undiluted suspension and cell suspensions corresponding to approximately 10^9, 10^7, 10^5, and 10^3 CFU/ml were used in the inhibition experiments. The interference test was performed

according to a similar previous study[3]. Briefly, one ml from each suspension was grown anaerobically in MRS agar for 24h at 37^0C and a second layer of Vogel-Johnson agar (VJA) base was laid on top of grown lactobacilli. The plates were allowed to set at room temperature for 3h and broth cultures grown in TSB were diluted in the same medium followed by measurement of OD at 500nm wavelength adjusted to 0.2. The plates were subsequently incubated for 24h at 37^0C in an anaerobic chamber. The results of the agar overlay tests were categorized as follows according to Simark-Mattsson et al[7].

Score 0 = complete inhibition (no visible colonies), Score 1 = slight inhibition (at least one visible colony but definitely smaller amounts then in the control plate), and Score 2 = no inhibition (the same growth as on the control plate).

pH – measurements

The pH values of the surfaces of MRS agar plates and VJA plates were measured before and after the final incubation of each lactobacilli strain but without *S. aureus* to determine acid production using pH-meter.

Statistical method

The data were processed with the SPSS software (version 17.0, Chicago Ill, USA) and subjected to chi-square tests. A p-value < 0.05 was considered as statistically significant.

RESULTS

Bacterial strains

The bacterial strains in test were subjected to thorough screening for confirmed identification. Both the *S. aureus* isolates and the *L. acidophilus* La-5 were subjected to characterization using the API 50 CH system. All of the isolates were identified and confirmed without difficulty.

Antibiotic assay

All of the clinical isolates were resistant against a wide range of antibiotics. They were mostly resistant to β-lactam antibiotics. The resistance pattern is summarized in TABLE 2. They were also resistant to fluoroquinolones.

Biofilm formation

The clinical isolates of *S. aureus* showed varying degree of biofilm formation. All of the isolates tested were capable of producing biofilm. At 24h incubation, 13 (31.70%) of the clinical isolates were strongly adherent while 25 (60.97%) others were moderately adherent and remaining three (7.31%) showed very weak adherence according to OD values. Based on biofilm forming capacity, MRSA strains were classified as TABLE 2.

Inhibition of biofilm formation

After 24h incubation and following the washing and staining with PBS and CV, 5 of the 13 strongly adherent isolates were able to form biofilm upon co-inoculation with *L. acidophilus*. The finding of the moderately adherent isolates were even more impressive as only 6 out of the 25 moderately adherent isolates were able to form biofilm after incubation with probiotic strain. None of the three weak biofilm formers were able to biofilm after probiotic treatment.

Growth inhibition assay

All the isolates in question were tested against *L. acidophilus* La-5 to assess its ability to deter the growth of *S. aureus* using agar overlay interference test. The result of the inhibition assay is summarized in TABLE 3. At concentrations of *L. acidophilus* La-5 higher than 10^7, all the *S. aureus* strains were unable to grow. However, inhibition got weaker with lowering of concentration. There were weak inhibitions at concentrations ranging from 10^3 to 10^6. The *L. acidophilus* strain showed no inhibitory activity

TABLE 1: Antibiotic resistance pattern of clinical isolates of *S. aureus*

Antibiotic and dosage	Resistance (%)
Amikacin (30 μg)	92.8
Clindamycin (2 μg)	71.3
Gentamicin (10 μg)	76.5
Ciprofloxacin (5 μg)	63.5
Trimethoprim-sulfamethoxazole (1.25 / 23.75 μg),	70.25
Erythromycin (15 μg)	81.6
Tetracycline (30 μg)	85.4

Regular Paper

TABLE 2 : Biofilm forming capacity of the clinical isolates based on OD[8]

OD Reading	Comment
$OD^a \leq OD_C^b$	Non-biofilm producer (Non adherent)
$OD_C < OD \leq 2OD_C$	Weak biofilm producer (Weakly adherent)
$2OD_C < OD \leq 4OD_C$	Moderate biofilm producer (Moderately adherent)
$4OD_C < OD$	Strong biofilm producer (Moderately adherent)

a **-OD= Optical Density of wells with biofilm, *b*- OD_C= Optical Density of blank wells**

TABLE 3 : The result of growth inhibition assay

Concentration of *L. acidophilus* La-5 (cfu/mL)	Inhibition Score		
	Highly adherent *S. aureus* strains	Moderately adherent *S. aureus* strains	Weakly adherent *S. aureus* strains
10^9	0^a	0	0
10^8	0	0	0
10^7	0	0	0
10^6	1^b	0	0
10^5	2^c	0	0
10^4	2	1	1
10^3	2	2	1
10^2	2	2	2
10^1	2	2	2

***a*. 0 = Complete inhibition, *b*. 1= Slight inhibition, and *c*. 2 = no inhibition**

at concentration below 10^2.

DISCUSSION

Experimental studies suggest that staphylococci are one of the major groups of bacteria that form biofilm[9]. The prevalence of methicillin resistant *S. aureus* in clinical isolates of urinary tract infections, nasal carriers and medical devices in several other studies[10][11][12][13]. This study also showed the ability of MRSA isolated from wounds to form biofilm. It is believed that biofilm formation is a survival mechanism of bacterial species in medical setting where the usage of antibiotic is high. The ability of *S. aureus* to form biofilm is also considered a key virulence factor for the pathogen to colonize implanted medical devices and damaged host tissues[14]. The antimicrobial resistance among the bacteria growing in biofilm is 500 to 5000 times higher than their planktonic counterparts[10]. The antibiotic assay revealed that biofilm producing *S. aureus* isolates are insensitive to a wide range of antibiotics including fluoroquinolones which was believed hitherto effective against biofilm producers. This finding is in agreement with Rezaei et al.[10] who reported the high resistance of biofilm forming *S. aureus* against ciprofloxacin. As most of the MRSA isolates tested showed ability to adhere to the surface upon 24h incubation, it demonstrates their possession of protein adhesions family known as MSCRAMM (Microbial Surface Component Recognizing Adhesive Matrix Molecules). The ability of 31.70 % isolates to show strong adherence could be described as the inability of *S. aureus* to produce adhesive molecules isolated from wound compared to isolates of medical devices. Kawamura et al.[13] reported a similar phenomenon where the prevalence of strong biofilm formers in the device group (43.5%) was significantly higher than that in the nondevice group (12.7%) and the colonization group (20 %). However, another experiment suggests a three times higher biofilm production on human fibronectin covered surfaces than those produced on inert polystyrene surfaces[12]. The novel concept of probiotic treatment needs further exploration. *Lactobacillus acidphilus* is one of the major probiotic bacteria that are widely used as a constituent of probiotic for their ability to tolerate acids and bile

salts. We selected this particular lactobacillus strain (*L. acidophilus* La-5) as it is prevalent in dairy products, fruit drinks, drops, gruels, chewing gums and tablets on the market. Agar overlay method was chosen to study the inhibitory effects as it was successfully used by previous investigators to study the inhibitory effects of lactobacilli strains on mutan streptococci and *Candida albicans*. The reason they used this technique is its ability to test the inhibitory effect on multiple strains on a single plate[3]. The study clearly shows the ability *of L. acidophilus* La-5 to inhibit the growth of *S. aureus* at higher concentrations. But the inhibition lowered with decreasing concentration. This finding could be explained by another similar finding where Hasslof et al.[3] used eight lactobacilli strains to study their inhibitory effects against mutan streptococci and *C. albicans*. The study showed *L. acidophilus* La5 had a statistically significantly weaker inhibition capacity in comparison with the other probiotic strains ($p < 0.05$). They attributed this inability of *L. acidophilus* to produce strong acids after incubation. They assumed that the reaction could be better or even worse *in vivo or* other experimental designs. However, their study was unable to determine the specific cause of this weakness. Another study suggests that the growth of lactic acid bacteria is optimum at slightly higher pH (6.0-6.5) and temperature (30^0C) but the production of bacteriocin was increased with lowering of pH (5.0-5.5) and temperature (25^0C). The study also shows an increased activity of bacteriocins with a slight increase in the final biomass[15]. The simultaneous growth of probiotic strain and clinical isolates of MRSA revealed the inability of most MRSA isolates to form biofilm. This finding is in agreement with Tahmourespour and Kermanshahi[6] who also reported the inhibition biofilm formation of mutan streptococci upon co-inoculation with *L. acidophilus*. Another study demonstrated the impact of bacteriocins on clinical isolates of MRSA. This study reports the varying ability of bacteriocins to inhibit biofilm formation[16]. Walencka et al.[17] reported the ability of *L. acidophilus* derived surfactants to inhibit the deposition rate and biofilm development (and also its maturation) of *S. aureus* and *S. epidermis* without affecting cell growth. It is possible that since lactobacilli strains possess a repertoire of bactericidal agents with varying effects, the accrued impact of these substances might have caused the inhibition of biofilm formation by *S. aureus* at higher *L. acidophilus* concentration.

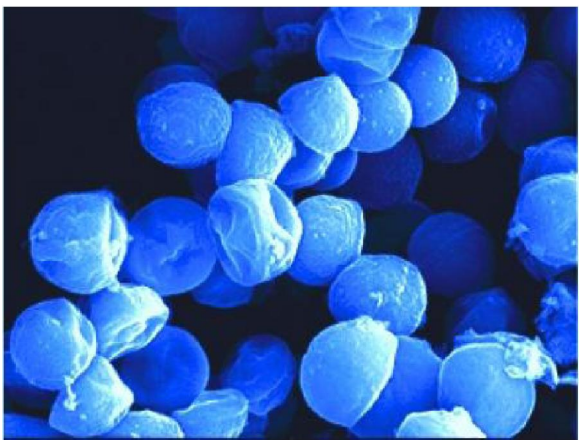

Figure 1

CONCLUSION

The study suggests that *L. acidophilus* La-5 strain has a definite impact on the growth and biofilm forming ability of clinical isolates of Methicillin Resistant S. aureus. However, the researchers could still be interested on how this strain inhibits growth and biofilm formation and the components that are directly responsible for down regulation of biofilm forming accessories.

REFERENCES

[1] M.Otto; Staphylococcal Biofilms, Curr.Top Microbiol Immunol., **322**, 207–228 **(2008)**.

[2] C.Vuotto, F.Longo, G.Donelli; Probiotics to counteract biofilm-associated infections: promising and conflicting data, International Journal of Oral Science, **6**, 189–194 **(2014)**.

[3] P.Hasslöf, M.Hedberg, S.Twetman and C.Stecksén-Blicks; Growth inhibition of oral mutans streptococci and candida by commercial probiotic lactobacilli - an in vitro study.BMC Oral Health, **10(18)**, **(2010).**

[4] S.Khan, M.A.Chowdhury, M.A.Hakim; Antibiotic resistance pattern of methicillin resistant staphylococcus aureus isolated from clinical specimens, IOSR Journal of Pharmacy and Biological Sciences (IOSR-

Regular Paper

JPBS), **4(6)**, 37-40 **(2013)**.

[5] Performance Standards for Antimicrobial Susceptibility Testing; Twenty-First Informational Supplement, **31(1)**, **(2011)**.

[6] A.Tahmourespour, R.K.Kermanshah; The effect of a probiotic strain (Lactobacillus acidophilus) on the plaque formation of oral Streptococci.Bosn J Basic Med Sci., **11(1)**, 37-40 **(2011)**.

[7] C.Simark-Mattsson, C.G.Emilson, E.G.Håkansson, C.Jacobsson, K.Roos, S.Holm; Lactobacillus-mediated interference of mutans streptococci in caries-free vs.caries-active subjects, Eur.J.Oral.Sci., **115,** 308-314 **(2007)**.

[8] S.Stepanovic, D.Vukovic, I.Dakic, B.Savic; A modified microtiter plate test for quantification of staphylococcal biofilm formation, J.Microbiol.Meth., **40(2)**, 175-179 **(2000)**.

[9] Y.Yarets, L.Rubanov, I.Novikova, N.Shevchenko; The Biofilm-forming capacity of staphylococcus aureus from chronic wounds can be useful for determining Wound-Bed Preparation methods, EWMA JOURNAL, **13(1)**, 7-13 **(2013)**.

[10] M.Rezaei, R.Moniri, S.G.A.Mousavi, M.J.Shiade; Prevalence of biofilm formation among methicillin resistance staphylococ-cus aureus isolated from nasal carriers, Jundishapur Journal of Microbiology, **6(6)**, e9601 **(2013)**.

[11] E.Ando, K.Monden,R.Mitsuhata, R.Kariyama, H.Kumon; Biofilm formation among methicillin-resistant Staphylococcus aureus isolates from patients with urinary tract infection, Acta Med.Okayama, **58(4)**, 207-214 **(2004)**.

[12] F.A.Ferreira, R.R.Souza, R.R.Bonelli, M.A.Américo,S.E.L.Fracalanzza, A.M.S.Figueiredo; Comparison of in vitro and in vivo systems to study ica-independent Staphylococcus aureus biofilms, J.Microbiol.Methods, **88(3)**, 393–398 **(2012)**.

[13] H.Kawamura, J.Nishi, N.Imuta, K.Tokuda, H.Miyanohara, T.Hashiguchi, M.Zenmyo, T.Yamamoto, K.Ijiri, Y.Kawano, S.Komiya; Quantitative analysis of biofilm formation of methicillin-resistant Staphylococcus aureus (MRSA) strains from patients with orthopaedic device-related infections, FEMS Immunol.Med.Microbiol., **63(1)**, 10-15 **(2011)**.

[14] H.McCarthy, J.K.Rudkin, N.S.Black, L.Gallagher, E.O'Neill, J.P.O'Gara; Methicillin resistance and the biofilm phenotype in Staphylococcus aureus, Front Cell Infect Microbiol., **5(1)**, 1-9 **(2015)**.

[15] M.Mataragas, J.Metaxopoulos, M.Galiotou, E.H.Drosinos; Influence of pH and temperature on growth and bacteriocin production by Leuconostoc mesenteroides L124 and Lactobacillus curvatus L442.Meat Sci., **64(3)**, 265-71 **(2003)**.

[16] K.Okuda, T.Zendo, S.Sugimoto, T.Iwase, A.Tajima, S.Yamada, K.Sonomoto, Y.Mizunoe; Effects of bacteriocins on methicillin-resistant Staphylococcus aureus biofilm.Antimicrob Agents Chemother., **57(11)**, 5572-9 **(2013)**.

[17] E.Walencka, S.Rózalska, B.Sadowska, B.Rózalska; The influence of Lactobacillus acidophilus-derived surfactants on staphylococcal adhesion and biofilm formation.Folia Microbiol (Praha), **53(1)**, 61-66 **(2008)**.