

*Full Length Research Paper*

# ***In vitro* and *in vivo* studies of antibacterial effect of ceftriaxone moxifloxacin combination against methicillin resistant *Staphylococcus aureus* biofilms formed on biomedical implants**

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**Methicillin resistant *Staphylococcus aureus* (MRSA) is the etiologic agent of a wide range of diseases worldwide including the Middle East. Biofilm production is an important virulence attribute in the pathogenesis of device-related MRSA infection. Our aim was to study the bactericidal effect of cephalosporin/fluoroquinolone combinations against MRSA biofilm *in vitro* and *in vivo*. The minimal inhibitory concentrations (MICs) were evaluated by microdilution method. All studied MRSA strains were highly resistant to cephalosporins (MIC<sub>90</sub>, 500 - 1,000 µg/ml). Moxifloxacin showed higher activity than levofloxacin (MIC<sub>90</sub>, 6.25 and 12.5 µg/ml, respectively). The combinations were studied using checkerboard technique. Ceftriaxone/moxifloxacin revealed 50% synergistic effect contrary to ceftriaxone/levofloxacin combination (16.7%). Rate of biofilm inhibition was determined by the time kill assay. When biofilm coated catheter was exposed to ceftriaxone and/or moxifloxacin, the combination showed 3–7.5 log reduction compared to the starting point after 24 h while it was only 1-3 and 2-4 log reduction with ceftriaxone and moxifloxacin, respectively. Levels of inflammatory markers as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) were evaluated by Enzyme-linked immunosorbent assay (ELISA). Co-administration of both antibiotics to bacterial strain challenged rats showed significant reduction in TNF-α and IL-6 levels (P < 0.001).**

**Key words:** MRSA biofilm, ceftriaxone, moxifloxacin, levofloxacin, checkerboard technique, serum inflammatory markers.

## **INTRODUCTION**

Catheter-related infections are among the most common nosocomial infections, accounting for significant morbidity and mortality (Mermel, 2000). The most common

etiologic agent of catheter related infections worldwide including the Middle East - especially in Saudi Arabia - is MRSA (Baddour et al., 2006). For many patients, surgical implantation of bioengineered medical devices can be life saving. However, implant-associated infections are associated with considerable morbidity, repeated surgeries, and prolonged antibiotic therapy (Zimmerli et al., 2004). The key to the pathogenesis of device

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infections is bacterial adherence to the prosthetic surface and formation of a bacterial biofilm. Biofilm associated bacteria are 100 - 1,000 times less susceptible to antibiotics than are planktonic bacteria (Donlan, 2000). Resistance of biofilm bacteria to antibiotics may be due to a variety of factors, including changes in cell wall composition and surface structures or presence of specific resistance genes (Madigan and Martinko, 2006).

The ability of biofilm-embedded *S. aureus* to resist clearance by antimicrobial agents points to the importance of a continuous search for novel agents that are effective against bacteria in this mode of growth or work in synergy with the currently available myriad of antimicrobial agents. Limited established treatment options exist for the treatment of serious, invasive infections caused by multidrug-resistant *S. aureus*. Therefore, a number of alternative approaches to antimicrobial treatment have been proposed (Micek, 2007).

Although vancomycin represents the gold standard for therapy of such invasive infections, reports of increasing *in vitro* resistance to vancomycin, combined with reports of clinical failures (with this and other antistaphylococcal agents), underscore the need for alternative therapies (Moise et al., 2007, 2008). Combinations of older agents to overcome resistance is a possibility now being explored (Huang and Rybak, 2005).

Cephalosporins are bactericidal and have the same mode of action as other beta-lactam antibiotics (such as penicillins) but are less susceptible to penicillinases. Cephalosporins disrupt the synthesis of the peptidoglycan layer of bacterial cell wall. The final transpeptidation step in the synthesis of the peptidoglycan is facilitated by transpeptidases known as penicillin-binding proteins (PBPs). PBPs bind to the D-Ala-D-Ala at the end of mucopeptides (peptidoglycan precursors) to crosslink the peptidoglycan. Beta-lactam antibiotics mimic the D-Ala-D-Ala site, thereby competitively inhibiting PBP crosslinking of peptidoglycan (Senior, 2002). Ceftriaxone, third generation cephalosporin, has a very high plasma protein binding (up to 98%). This high protein binding results in high concentrations in plasma that are frequently related to the anti-infective activity (Kovar et al., 1997).

Recent combinations involving ceftriaxone were tried. Vancoplus is a brand of ceftriaxone and vancomycin produced by Venus-Remedies for MRSA treatment. It is the only remedy after vaccination to treat MRSA like meningitis, pneumonia, typhoid, septicaemia, urinary tract infection, skin and skin infections and staphylococcal endocarditis. The drug restricts the production of toxin by MRSA pathogens and also reduces the treatment time, cost and adverse effects (<http://tahilla.typepad.com/mrsawatch/2011/08/venus-remedies>), while Shrivastava et al. (2009) found that 2:1 of ceftriaxone and sulbactam has the best *in vitro* efficacy against MRSA and their combination has less chances of development of resistance than ceftriaxone alone.

Limited data were found concerning ceftriaxone combinations with quinolones. Moxifloxacin and levofloxacin are fourth-generation synthetic fluoroquinolone antibacterial agents. They are used to treat a number of infections including: respiratory tract infections, cellulitis, anthrax, intraabdominal infections, endocarditis, meningitis, and tuberculosis (Nelson et al., 2007). They are broad-spectrum antibiotics that are active against both Gram-positive and Gram-negative bacteria. They function by inhibiting DNA gyrase, a type II topoisomerase, and topoisomerase IV, enzymes necessary to separate bacterial DNA, thereby inhibiting cell replication (Drlica and Zhao, 1997). Their roles against *S. aureus* have not been properly evaluated although data suggests that they have good *in vitro* as well as *in vivo* activity against MRSA with a low propensity to select for resistance. Furthermore, they might be a cost-effective alternative to vancomycin in MRSA infections (Entenza et al., 2001).

Usually, diseases caused by *S. aureus* can elicit a systemic or topical inflammatory response syndrome. The innate immune response is important to the upregulation of cytokine production (Capparelli et al., 2007). MRSA infection contributes to an increased production of several inflammatory cytokines such as IL-6, IL-4 and interferon (Gu et al., 2011). On the other hand, it has been shown that suppression of the inflammatory immune response prevents the development of chronic biofilm infection due to MRSA (Prabhakara et al., 2011).

For all of that, our study is aimed to; (i) determine the *in vitro* susceptibility of different cephalosporin and fluoroquinolone antibiotics against MRSA biofilm forming strains, (ii) study the effect of some cephalosporin fluoroquinolone combinations against MRSA biofilm forming strains, (iii) evaluate the time-dependent effects of the most potent synergistic combination against selected MRSA strains, (iv) study the *in vivo* effects of single cephalosporin and single fluoroquinolone and their combination against already formed MRSA biofilm implant, and finally (v) determine serum inflammatory cytokines level during inflammatory response elicited by MRSA biofilm, and the effect of different antibiotic regimens on such response.

## MATERIALS AND METHODS

### Isolation and identification of MRSA strains

Thirty six MRSA clinical strains were collected and isolated from several clinical samples (central venous catheters, orthopaedic implantations and urinary catheters) obtained from inpatient departments of different teaching hospitals in Riyadh, Saudi Arabia. The identification of isolates was done according to standard method described by the Clinical Laboratory Standards Institute (CLSI, 2011). Methicillin resistance was confirmed by oxacillin and cefoxitin disk test in accordance with CLSI (2007). One isolate for a patient was considered in order to avoid duplicates. All isolates were stored in brain heart infusion broth containing 16% (w/v)

glycerol at - 80°C until further use.

### Antimicrobial agents and chemicals

Ceftriaxone (CRO) (Hoffmann-La Roche Ltd, Switzerland), cefotaxime (CTX) (Sanofi-Aventis, France), cefepime (CEF) (Bristol Myers Squibb, USA), moxifloxacin (MOX) (Bayer AG, Germany), and levofloxacin (LVX) (Sanofi-Aventis, France) were all purchased commercially from the manufacturers and were supplied as powders of stated potency. All other chemicals as Xyaline and Ketamine used in this study were analytically pure product of Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

### *In vitro* study

#### Determination of MICs

Determination of MICs of used antimicrobial agents was performed in cation-adjusted Müller Hinton broth (MHB) by means of microdilution broth method in accordance to National Committee for Clinical Laboratory Standards (2003a, 2003b). MIC<sub>50</sub> and MIC<sub>90</sub> were calculated for each antibiotic.

#### Evaluation of antibiotic combinations

MICs of antibiotic combinations were determined by broth microdilution technique (Eliopoulos and Moellering, 1991; National Committee for Clinical Laboratory Standards, 2003a, 2003b). For each combination, a two-dimensional checkerboard with twofold dilutions of each drug was used for the study. Growth control wells containing medium were included in each plate. Each test was performed in duplicate. The fractional inhibitory concentration (FIC) was calculated as follows: FIC of drug A = MIC of drug A in combination/MIC of drug A alone, FIC of drug B = MIC of drug B in combination/MIC of drug B alone, FIC of drug C = MIC of drug C in combination/MIC of drug C alone. FIC Index (FIC<sub>i</sub>), calculated as the sum of each FIC, was interpreted as follows: FIC<sub>i</sub> ≤ 0.5 is synergism, 1 > FIC<sub>i</sub> > 0.5 is partial synergism, FIC<sub>i</sub> = 1 is additive, 4 ≥ FIC<sub>i</sub> > 1 is indifferent reaction and FIC<sub>i</sub> > 4.0 is antagonism (Lorenzo et al., 2007).

#### Biofilm formation

Biofilm formation was determined by using a modified microtitre plate test (Stepanović et al., 2000). Briefly, bacteria were grown overnight on Müller Hinton agar plates (Oxoid) and then resuspended in trypticase soy broth (TSB) plus 5% glucose. Dilutions containing approximately 10<sup>8</sup>cfu/ml were made. Aliquots of 100 µl were inoculated in nine parallel wells of a 96-well polystyrene plate. After incubation for 5 days at 37°C, the plates were softly shaken to remove planktonic bacteria. The wells were rinsed with phosphate buffer saline (PBS) and fixed with 150 µl absolute methanol for 10 min. Attached bacterial material was then stained by adding 150 µl crystal violet (1% w/v) for 20 min. The plates were rinsed with tap water and the amount of attached material was measured by solubilisation of the crystal violet dye in 150 µl of 33% glacial acetic acid. The A<sub>570</sub> was measured using an ELISA reader.

#### Time-kill curves

Five, strong biofilm formers, MRSA strains were selected and renamed as (MRSA-I through MRSA-V). The ability of ceftriaxone

and moxifloxacin either as single agent or combined together to inhibit growth of biofilm of the strains developed on polyurethane catheters (triple-lumen intravenous cut into 6 mm discs) was evaluated based on the plotting of time-death curves. Each of the selected strains was grown in TSB media for 18 h, then, several catheter discs were soaked for 5 days in each. Thereafter, the discs were removed, washed twice with PBS and reinoculated into sterile water. Catheters were then divided into four groups as follows: untreated organisms as the control group, treated with ceftriaxone alone group, treated with moxifloxacin alone group and treated with ceftriaxone/moxifloxacin combination group. The concentration of the antimicrobials used was 2XMIC. After 0, 4, 8 and 24 h of incubation at 37°C, each group was subjected to sonication (ultrasonic processor XL, NY, USA). Aliquots of bacterial culture were serially diluted and then plated on to MHA for colony counts. Parallel controls were carried out in antimicrobial-free medium.

### *In vivo* study

#### Experimental animals

The animals used for the *in vivo* experiments were 200 g specific-pathogen-free male Wistar albino rats. All animals were obtained from Experimental Animal Care Center, College of Pharmacy, King Saud University (KSU), Riyadh, Saudi Arabia (SA). Rats were housed in stainless steel cages (5 animals/cage). Animals were acclimated with free access to tap water and standard pellet diet (Purina Chow) in a facility with controlled temperature (22–24°C) and humidity (50–60%), on a 12 h light/12 h dark cycle, for at least 1 week before the experiments. The protocol of this study was carried out according to the regulations and recommendation of the Animal Research Ethics Committee of College of Pharmacy, KSU, Saudi Arabia.

#### *In vivo* biofilm model

Two MRSA strains (MRSA-I and MRSA-IV) were selected for further studies on the basis of their biofilm forming ability and showing the highest synergistic effect to the tested drugs. Briefly, as described by Ishida et al. (1998), bacteria were incubated in trypticase soy agar with 5% glucose for 24 h at 37°C and re-suspended in saline adjusted to 0.5McFarland turbidity. Then, 200 µl of this suspension and the catheter pieces of 6 mm diameter were added to 18.8 ml of TSB with 1 ml of 5% glucose and incubated for 5 days at 37°C. After incubation, catheters were washed twice with PBS before being implanted under the skin of rats as described previously (Van Wijngaerden et al., 1999; Massonet et al., 2006). Rats of each group were intraperitoneally anesthetized with xylazine (8 mg/kg body weight) and ketamine (30 mg/kg body weight), the hair on the lower back was shaved and the skin was cleansed with antiseptic solution (10% povidone-iodine solution). A 10 mm incision was made longitudinally and the subcutis was carefully dissected to create subcutaneous tunnels for implantation of catheter fragments. The incision was closed after implantation with surgical sutures, and disinfected with 10% povidone-iodine solution. The animals were returned to individual cages and thoroughly examined daily.

#### Experimental protocol

Rats were randomly allocated into nine groups; control group: comprised of normal control group without bacterial challenge to evaluate the sterility of surgical procedure. Group I (GI); challenged control group with MRSA-I that did not receive any antibiotic. Group

**Table 1.** Comparative *in vitro* activities of different antibiotics against 36 clinical MRSA isolates.

Antibiotics	Antibiotics tested ( $\mu\text{g/ml}$ )				% susceptibility	
	Range	<sup>a</sup> MIC <sub>50</sub>	<sup>b</sup> MIC <sub>90</sub>	<sup>c</sup> Breakpoint		
				S	R	
Cefotaxime	16 - > 2000	250	1000	-	-	*ND
Cefepime	4 - >2000	128	1000	-	-	*ND
Ceftriaxone	8 - >2000	16	500	-	-	*ND
Moxifloxacin	0.09-12.5	0.8	6.25	$\leq 0.5$	$\geq 2$	45%
Levofloxacin	0.39-12.5	12.5	12.5	$\leq 1$	$\geq 4$	12%

<sup>a</sup>The minimal concentration at which 50% of the isolates was inhibited, <sup>b</sup>The minimal concentration at which 90% of the isolates was inhibited, <sup>c</sup>CLSI, 2007, \*ND: not determined.

II (GII): challenged control group with MRSA-IV that did not receive any antibiotic. For each bacterial strain, three challenged groups received the following: group III (GIII): challenged group with MRSA-I received moxifloxacin 100 mg/kg/day orally (Dalhoff, 2001); group IV (GIV): challenged group with MRSA-I received 100 mg/kg/day ceftriaxone orally (Kovar et al., 1997); and group V (GV): challenged group with MRSA-I received oral moxifloxacin plus ceftriaxone at the above concentrations. From group VI to group VIII (GVI-GVIII), animals were challenged with MRSA-IV and received the same treatment as in GIII, GIV, and GV respectively. All antibiotic treatments were started 48 h after implantation and continued for seven days. For catheter recovery, after 24 h from last dose injection, rats were anesthetized, their skin was disinfected, and catheter fragments were removed from under the subcutaneous tissue. Then, rats were sacrificed and the blood was collected. Freshly isolated serum was used for the assessment of inflammatory cytokines (Gul et al., 2010).

#### Infection assessment

The harvested catheters from rats treated with either ceftriaxone, moxifloxacin or their combination were gently washed in PBS solution and placed in tubes containing 5 ml of PBS solution. They were then sonicated to remove the adherent bacteria from the catheters and then placed in sterile melted nutrient agar. Quantification of viable bacteria was done by culturing serial 10 fold dilutions of the bacterial suspension onto nutrient agar plates. All plates were incubated at 37°C for 48 h and evaluated for the presence of the MRSA strain under test. The organisms were quantified by counting the number of cfu/system.

#### Determination of serum inflammatory markers concentrations

The concentration of serum inflammatory cytokines, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-6 (IL-6), were determined using commercially available ELISA assays following the instructions supplied by the manufacturer (DuoSet kits, R and D Systems; Minneapolis, MN, USA). The results are shown as pg/ml.

#### Statistical analysis

Data were expressed as means  $\pm$  S.D. For multi-variable comparisons, one-way ANOVA was conducted, followed by Bonferroni testing using the GRAPHADA INSTAT (ISI Software) computer program. Differences were considered significant at  $P < 0.05$ .

## RESULTS

The present study included 36 bacterial isolates of MRSA. The yields were subjected to several treatment regimens either by single antibiotics or combined therapy in both their planktonic and settling states. They were also evaluated under the effect of these antibiotics *in vivo* and *in vitro*.

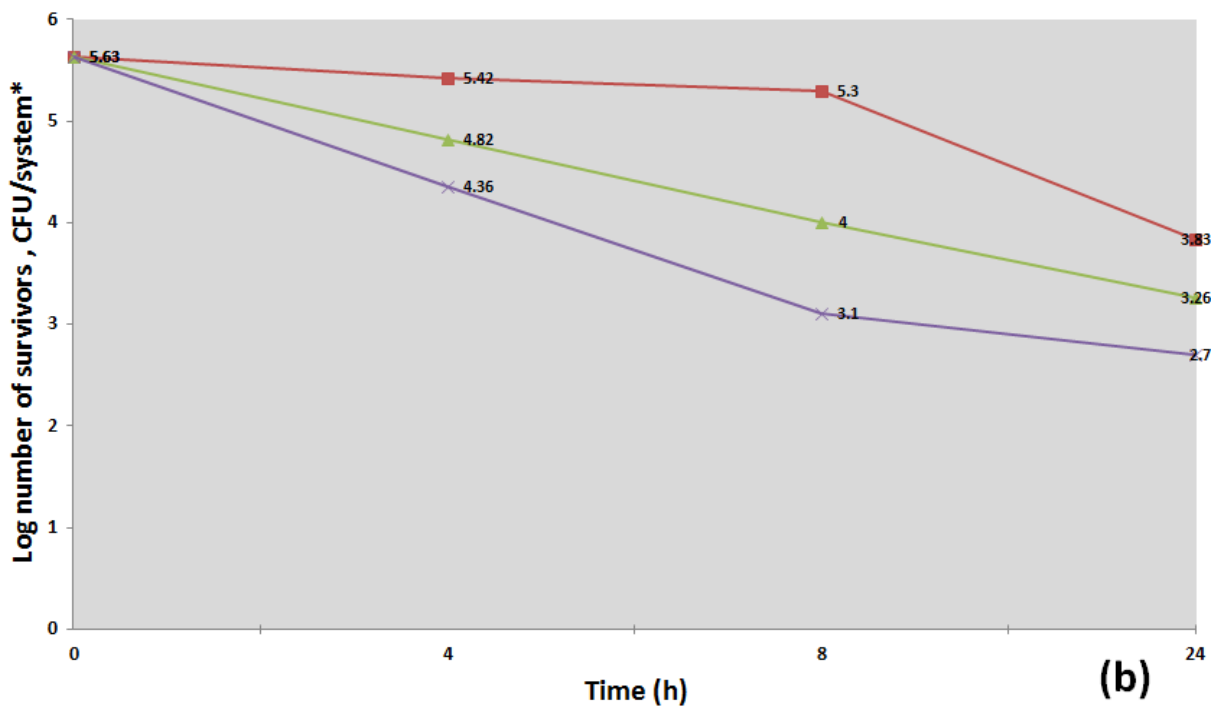
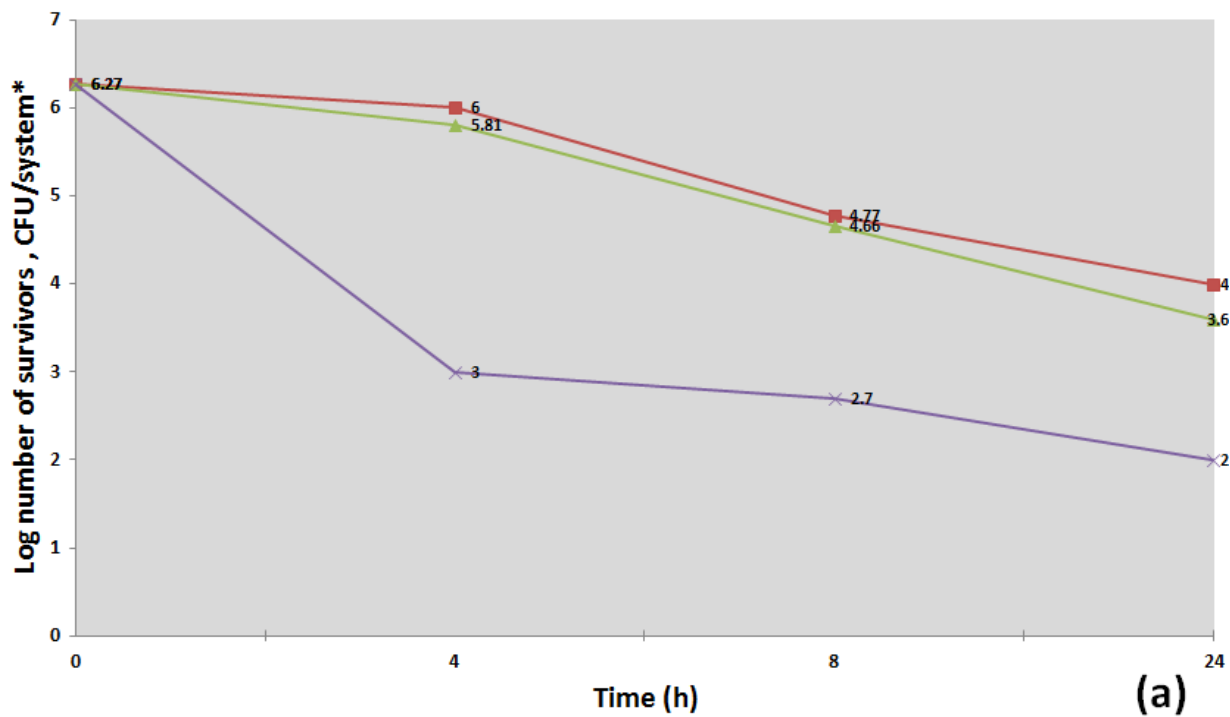
The *in vitro* comparative study of the activities of different antibiotics used against 36 studied clinical MRSA isolates is shown in Table 1. According to the CLSI guidelines, all tested MRSA strains were highly resistant to cephalosporins. The MIC<sub>90</sub> for cefotaxime and cefepime was 1000  $\mu\text{g/ml}$  while their MIC<sub>50</sub> was 250 and 128 respectively. Ceftriaxone was more active than the other cephalosporins tested (MIC<sub>50/90</sub> was 16/500  $\mu\text{g/ml}$ ). Against the 36 MRSA strains tested, moxifloxacin demonstrated good activity, with MICs for all strains in the range of 0.09-12.5  $\mu\text{g/ml}$ . Moreover, it showed higher activity against the tested strains than levofloxacin demonstrating MIC<sub>50</sub> of 0.8 vs. 12.5  $\mu\text{g/ml}$  and MIC<sub>90</sub> of 6.25 vs. 12.5  $\mu\text{g/ml}$ . The percentage of MRSA fully resistant to moxifloxacin was 55% while it was 88% in the case of levofloxacin.

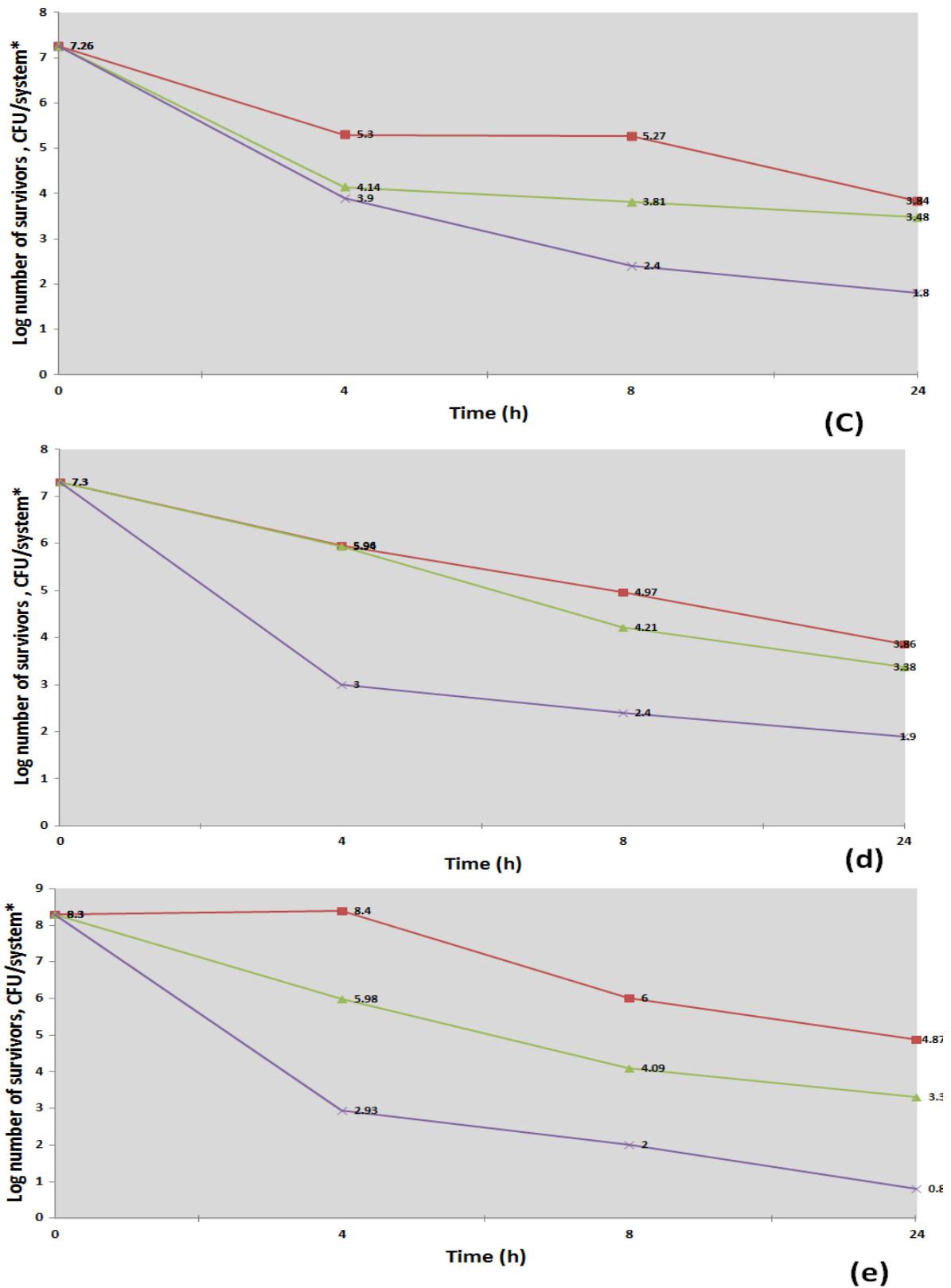
The determined FIC<sub>i</sub> values are summarized in Table 2. In checkerboard studies, FIC<sub>i</sub> values were calculated. Fifty percentage of strains (18/36 strains) showed synergistic reaction among ceftriaxone/moxifloxacin combination (FIC range was 0.1-0.4, that is, FIC  $\leq 0.5$ ). While, only 16.7% of studied strains achieved synergistic reaction with ceftriaxone/levofloxacin combination at FIC range of 0.04-0.15. Partial synergism and additive reaction together were achieved among 11.2 and 11.1% of the studied strains with ceftriaxone/moxifloxacin and ceftriaxone/levofloxacin combinations, respectively. The antagonistic reaction was more apparent with ceftriaxone/levofloxacin combination than with ceftriaxone/moxifloxacin combination (72.2% vs. 22.2%, respectively).

The time-dependent effects of ceftriaxone/moxifloxacin combination (at 2XMIC for each) against established biofilms of the selected five different MRSA isolates were

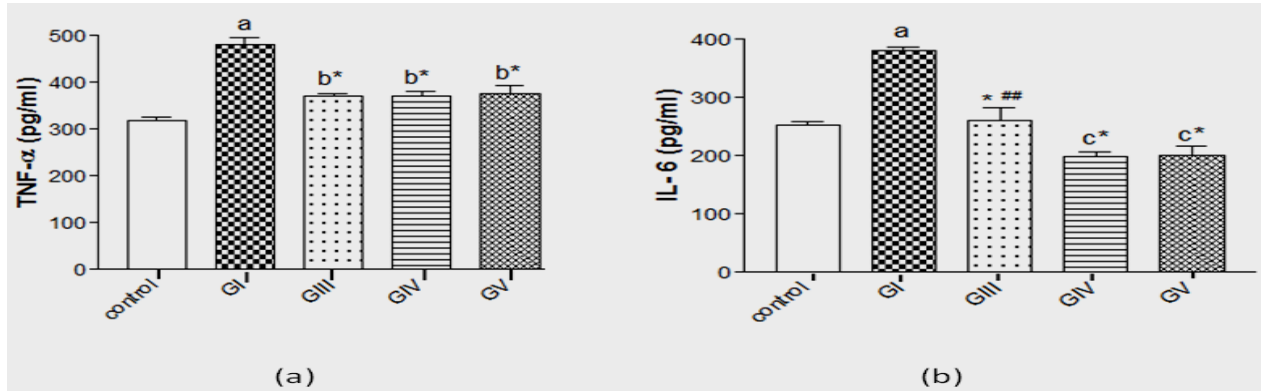
**Table 2.** Fractional inhibitory concentration (FIC) indices of ceftriaxone/fluoroquinolone combinations against 36 MRSA isolates.

Combined antibiotics tested	FIC indices				
	Synergistic reaction	Partial synergism	Additive reaction	Indifferent reaction	Antagonistic reaction
	(FIC ≤ 0.5)	(0.5 < FIC < 1)	(FIC = 1)	(1 < FIC ≤ 4)	(FIC > 4)
Ceftriaxone/moxifloxacin	50 (0.1 - 0.4)	5.6 (0.6)	5.6 (1)	16.6 (1.4 - 1.7)	22.2 (9 - 74)
Ceftriaxone/levofloxacin	16.7 (0.04 - 0.15)	0 (-)	11.1 (1)	0 (-)	72.2 (4.25 - 16.5)

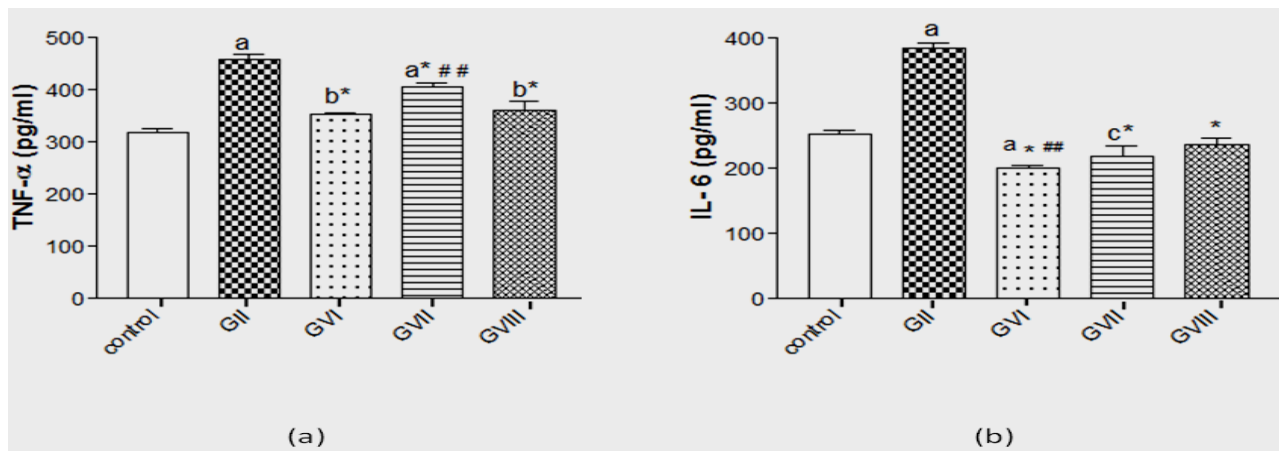




**Figure 1.** The time-dependent effects of ceftriaxone/moxifloxacin combination against established biofilms of five selected MRSA isolates. —■— Ceftriaxone, —▲— Moxifloxacin, —×— Ceftriaxone /Moxifloxacin Combination, \*cfu of the MRSA adhered on the catheter in the 5 ml sonicate. (a) MRSA I, (b) MRSA II, (c) MRSA III, (d) MRSA IV and (e) MRSA V.



**Figure 2.** Effect of moxifloxacin and/or ceftriaxone on serum TNF- $\alpha$  (a) and IL-6 (b) in MRSA-I challenged rats. The values are expressed as mean  $\pm$  S.D. <sup>a</sup>P < 0.001, <sup>b</sup>P < 0.01, <sup>c</sup>P < 0.05 compared to normal control group, \*P < 0.001 compared to MRSA-I challenged group, ##P < 0.01 compared to combination treated group, respectively, using ANOVA followed by Bonferroni as a post-ANOVA test.



**Figure 3.** Effect of moxifloxacin and/or ceftriaxone on serum TNF- $\alpha$  (a) and IL-6 (b) in MRSA IV challenged rats. The values are expressed as mean  $\pm$  S.D. <sup>a</sup>P < 0.001, <sup>b</sup>P < 0.01, <sup>c</sup>P < 0.05 compared to normal control group, \*P < 0.001 compared to MRSA-IV challenged group, ##P < 0.01 compared to combination treated group, respectively, using ANOVA followed by Bonferroni as a post-ANOVA test.

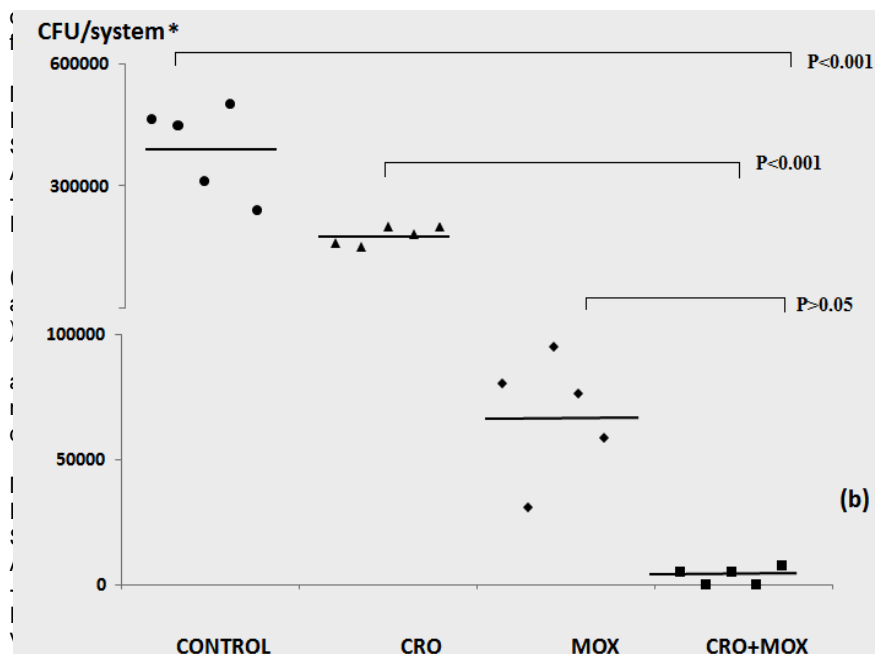
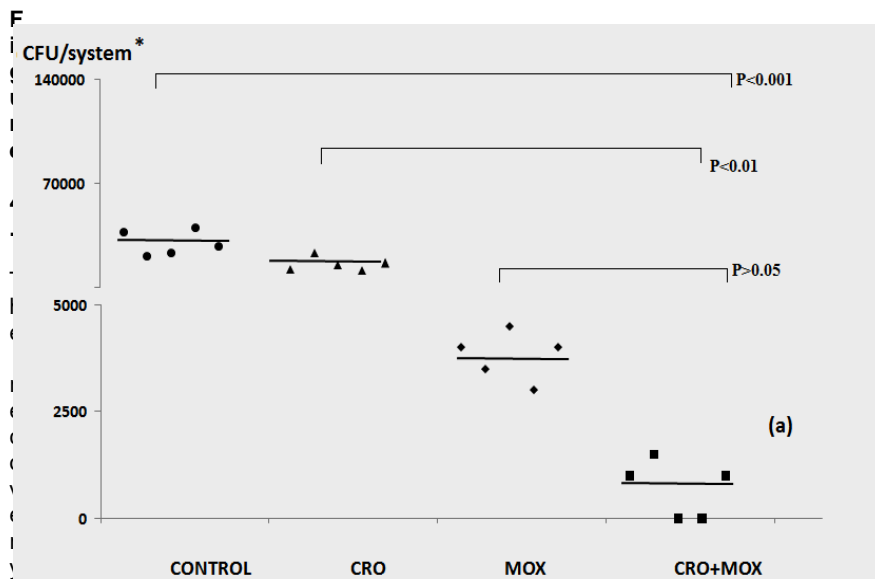
shown in Figure 1. Ceftriaxone/moxifloxacin combination showed 3 – 7.5 log reduction compared to the starting point after 24 h of incubation among the five isolates tested. The most potent reduction in bacterial growth count was observed with MRSA-I, MRSA-IV and MRSA-V isolates where 3 log reductions were achieved at 4 h of incubation (Figure 1a, 1d and 1e). By comparing the antibacterial effects of ceftriaxone and moxifloxacin alone against their combination, the results showed that, the combination was more potent than ceftriaxone alone or moxifloxacin alone among MRSA-I, MRSA-IV and MRSA-V strains (bacterial count reduction was more than 3 logs at 4 h). This result was not obtained with the other tested strains (MRSA-II and MRSA-III) where there was only 1 log reduction difference between each single drug and their combination after 4 h and 2 log reduction differences

at the end of the experiment (24 h).

Since the synergistic effect of the combination was very clear in MRSA I and MRSA IV strains and since their FIC values were the lowest (0.1 and 0.13 respectively) (data not shown) compared to the other strains selected in this experiment they were chosen for the *in-vivo* study of this work.

In the present study, we examined the clinical presentation of the rats in the *in vivo* model. None of the animals died or had clinical evidence of adverse effects anorexia, vomiting, diarrhoea or changes in behaviour.

To examine the effect of the drugs on the inflammatory process in the MRSA-infected rats, serum concentrations of the inflammatory cytokines TNF- $\alpha$  and IL-6 were evaluated. Figures 2 and 3 show serum level of TNF- $\alpha$  and IL-6 in MRSA-I and MRSA-IV challenged rats,



(b) strains following subcutaneous challenge of polyurethane catheter with studied bacteria. \* The number of cfu/system (5 ml catheter sonicate) from the investigated catheters were visualised as scatter dot blot with the horizontal line denotes the mean value.

respectively. Serum level of inflammatory markers was significantly increased in both bacterial strains challenged groups as compared with normal control group ( $P < 0.001$ ). Treatment with either antibiotics or their combination significantly decreased serum TNF- $\alpha$  and IL-6 level compared with MRSA-challenged groups ( $P < 0.05$ ). However, co-administration of moxifloxacin and ceftriaxone to either of the two bacterial strain challenged groups showed significant reduction in serum inflammatory markers level as compared to either of the

two antibiotics-treated rats ( $P < 0.01$ ). These results indicated that both moxifloxacin and ceftriaxone exhibit an immunomodulatory action, independent from their bactericidal activity.

Figures 4a and b shows the recovery of MRSA strain following subcutaneous challenge of polyurethane catheter with approximately  $1 \times 10^8$  cfu/ml bacteria. The number of cfu/system was determined by surface viable count technique. Regarding to recovery of MRSA-I strain (Figure 4a), There was significant differences between



ceftriaxone/moxifloxacin treatment and ceftriaxone alone and control group ( $P < 0.01$  and  $P < 0.001$ , respectively). While, there was no significant difference between combination therapy and moxifloxacin alone ( $P > 0.05$ ). Similar results and statistical analysis were observed against MRSA-IV strain (Figure 4b).

## DISCUSSION

Infections involving biomedical devices have significant clinical and economic impact. If bacteria are able to adhere successfully, they will undergo biofilm formation, which alters their properties and renders them resistant to commonly used antibiotics (Garcia et al., 2010). *S. aureus* is the commonest isolate in clinical practice. Over the years, it has acquired resistance to almost all the available antimicrobials and emergence of multi-drug resistant MRSA has been especially troublesome. MRSA now accounts for a major proportion of *S. aureus* infections worldwide (Tenover et al., 2001; CDC, 2002).

In the current investigation, we tested different cephalosporin (cefotaxime, cefepime and ceftriaxone) and different fluoroquinolone (moxifloxacin and levofloxacin) as a single antimicrobial agent. Then we tried novel cephalosporin/fluoroquinolone combination in the form of ceftriaxone/moxifloxacin or ceftriaxone/levofloxacin combination against MRSA biofilm on a medical grade polyurethane catheter. The results revealed that the most active cephalosporin was ceftriaxone while, the isolates were completely resistant to cefotaxime and cefepime. The most potent fluoroquinolone was moxifloxacin followed by levofloxacin as was cited in the study done by Lister which revealed that, moxifloxacin was 4-8 fold more potent than levofloxacin against MRSA, with MICs of 0.03–1 mg/ml for moxifloxacin and 0.25–8 mg/ml for levofloxacin (Lister, 2001). The ceftriaxone/moxifloxacin combination was more synergistic than ceftriaxone/levofloxacin combination (about 56% versus 17%, respectively).

Study results consistently demonstrate the suppression of bacterial growth in biofilm, but no sole agent completely eradicates bacterial colonization. The time-dependent effect of ceftriaxone/moxifloxacin combination showed 3 log reduction within 4 h among MRSA-I, MRSA-IV and MRSA-V and within 24 h among MRSA-II and MRSA-III. By comparing their single antibacterial effects against their combination, the results showed that the combination was more potent than single agent among MRSA-I, MRSA-IV and MRSA-V strains than with the other tested strains (MRSA-II and MRSA-III).

Our *in vivo* model used a direct method of MRSA colonization on the catheter and the explanted grafts were sonicated to remove the adherent bacteria. Therefore, we were able to obtain quantitative cultures of the bacteria included on the catheter material. However this experimental study has several limitations since the

animal model in our study is not directly comparable with graft implantation into the body.

Although moxifloxacin alone demonstrated sufficient activity against the bacteria on polyurethane, it did not completely sterilize the catheter surface *in vivo* after five days of treatment giving a mean cfu/system of  $3.5 \times 10^3$  and  $6 \times 10^4$  for MRSA I and MRSA VI respectively. Combined therapy of ceftriaxone/moxifloxacin resulted in a good therapeutic effect, not only against biofilm growth of bacteria but also on the inflammatory reactions which were suppressed. Since the complete eradication of MRSA-I and MRSA-IV strains was not achieved with ceftriaxone alone, the synergistic effect of ceftriaxone/moxifloxacin combination in our animal model was very interesting. The mean number of bacterial recovery from catheters treated with ceftriaxone alone was  $1.5 \times 10^4$  and  $1.8 \times 10^5$  while it was  $1 \times 10^3$  and  $3.5 \times 10^3$  cfu/system for ceftriaxone/moxifloxacin against MRSA I and MRSA VI, respectively. The mechanism of the synergy is unknown at present; however, the anti-inflammatory effects of both drugs may explain, in part, their effect on biofilms. As it has been proven recently that suppression of the inflammatory immune response prevents the development of chronic biofilm infection due to MRSA (Prabhakara et al., 2011), our results support this idea, as cytokine release such as TNF- $\alpha$  and IL-6 in rat serum was significantly decreased in rats treated with ceftriaxone and/or moxifloxacin, which suggested an immunomodulatory activity of these agents.

Several studies had demonstrated the ability of certain quinolones to confer protective anti-inflammatory effects (Dalhoff and Shalit, 2003). Moxifloxacin, as one of quinolone antibiotics, conferred a protective anti-inflammatory effect in a murine model of *Candida* pneumonia in immunosuppressed animals, resulting in a marked decrease in bronchopneumonia and enhanced survival (Shalit et al., 2002). This protective efficacy was associated with significant reduction in IL-8 and TNF- $\alpha$  in lung homogenates as well as significant inhibition in nuclear factor kappa B (NFkB) nuclear translocation into alveolar macrophages and epithelial cells (Blau et al., 2002). Further *in vitro* studies showed anti-inflammatory effects of moxifloxacin in lipopolysaccharide and cytokine-stimulated human monocytic cells (Weiss et al., 2004). On the other hand, the impact of ceftriaxone on the immune system has been demonstrated in numerous studies. Where, ceftriaxone modulated cytokines and chemokines release and prevented septicemia and death in pneumococcal pneumonia (Wang et al., 2000). Additionally, Grandgirard et al. (2010) had demonstrated that ceftriaxone attenuated cerebrospinal fluid inflammation in experimental pneumococcal meningitis through modulation of several cytokines secretion. All of these data coincidence with our results in which moxifloxacin and/or ceftriaxone significantly decreased serum TNF- $\alpha$  and IL-6 level in MRSA-challenged rats.

Although the biofilms generated in the human body

may be different from those in the rat biofilm model, the use of ceftriaxone, moxifloxacin and other adequate antibiotics in combination may be effective for such biofilm-forming organisms in the clinical setting.

## Conclusion

To sum up, the current study revealed that traditionally used antibiotics such as ceftriaxone and moxifloxacin in combination could be the effective solution against bacterial biofilm caused by MRSA infection rather than searching for new antibiotics for empirical treatment to guard against biofilm formation. Moreover, both tested antibiotics revealed an immunomodulatory effects that could be subjected for further investigation and evaluation.

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