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Antibacterial and Antibiofilm Activity of the Mixed Lactobacilli Against the Most Bacterial Etiology of Otitis Media

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Received: 4 July 2022 **Accepted: 14 August 2022**

DOI: https://dx.doi.org/10.24237/ASJ.01.01.633B

Abstract

The present study aimed to investigate the anti-biofilm activity of Cell Free Supernatant (CFS) and Biosurfactant (BS) of mixed lactobacilli (*L. acidophilus* and *L. plantarum*) against biofilm formation by bacterial pathogens isolated from chronic otitis media. In addition, the antimicrobial potential of ciprofloxacin in combination with CFS of lactobacilli species was evaluated againt the tested pathogens. The automated identification of bacterial isolates was peroformed by VITEK2 compact system their antibiotics susceptibility was evaluated using disc diffusion method. The minimum inhibitory concentrations (MIC) of the mixed lactobacilli CFS and ciprofloxacin were determine against the bacterial isolates using broth micro-dilution assay. Anti-biofilm activities of CFS and bio-surfactant of the mixed lactobacilli were evaluated against the biofilm–associated bacterial isolates. The minimum biofilm inhibitory concentrations ($MBIC_{50}$) were determined. In the current study, one hundred sixty-two ear swab were collected from had otitis media cases with chronic discharging. These patients attended to the ENT department in the medical consultation clinic at Baquba Teaching Hospital and to private doctors' clinics. Ear swabs were taken from both genders during September 2021 to the end of December. The most bacterial species isolated from otitis media samples were *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Ciprofloxacin was an effective antibiotic against the tested pathogenic bacteria. The mixed lactobacilli CFS showed an inhibitory effect at MIC_{90} (25%) against *P. mirabilis* but (50%) against *P. aeruginosa*, *S.aureus*, and *K.*

pneumoniae. The data illustrated that CFS of mixed lactobacilli was synergized ciprofloxacin against otitis media-bacterial isolates. The CFS of mixed lactobacilli exhibited a higher $MBIC_{50}$ against *K. pneumoniae* (50%) compared to other bacterial isolates. In addition, a higher $MBIC_{50}$ of the mixed lactobacilli bio-surfactant was reported against *P. aeruginosa* 50%, compred to the other bacterial species. The authors concluded that tested probiotics cells, the mixed lactobacilli, and their products possessed an effective antimicrobial potential and enhanced ciprofloxacin activity to control the chronic otitis media-associated bacteria and inhibit their persistent biofilm formation.

Keywords: Chronic otitis media; Mixed lactobacilli; Biosurfactant; Antimicrobial; Antibiofilm.

الفعالية المضادة للبكتريا وللغشاء الحيوي للعصيات اللبنية المختلطة ضد أكثر المسببات البكتيرية لالتهاب الأذن الوسطى

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الخلاصة

هدفت الدراسة الحالية إلى معرفة النشاط المضاد للغشاء الحيوي للراشح البكتيري من خلايا طافية (CFS) والمستحلب الحيوي (BS) للعصيات اللبنية المختلطة (*L. acidophilus* و *L. plantarum*) ضد تكوين الأغشية الحيوية بواسطة مسببات الأمراض البكتيرية المعزولة من حالات التهاب الأذن الوسطى المزمن. بالإضافة إلى ذلك ، تم تقييم إمكانية مضادات الميكروبات للسيبروفلوكساسين بالاشتراك مع الراشح البكتيري (CFS) لأنواع العصيات اللبنية على العوامل الممرضة المختبرة. تم تنفيذ التشخيص الآلي للعزلات البكتيرية بواسطة نظام VITEK2 المضغوط و تم تقييم حساسيتها للمضادات الحيوية باستخدام طريقة انتشار القرص. تم تحديد التركيز المثبط الأدنى (MIC) لراشح العصيات اللبنية المختلطة (CFS) والسيبروفلوكساسين ضد العزلات البكتيرية باستخدام اختبار التخفيف الدقيق للمرق. تم تقييم الأنشطة المضادة للغشاء الحيوي للراشح البكتيري (CFS) والمستحلب الحيوي (BS) للعصيات اللبنية المختلطة ضد العزلات البكتيرية المرتبطة بالغشاء الحيوي. تم تحديد الحد الأدنى من تركيزات مثبطة للبيوفيلم ($MBIC_{50}$). أظهرت النتائج أن أكثر الأنواع البكتيرية المعزولة من عينات التهاب الأذن الوسطى هي *Pseudomonas aeruginosa* و *Staphylococcus aureus*. لوحظ أن سيبروفلوكساسين مضاد حيوي فعال ضد البكتيريا المسببة للأمراض المختبرة. أظهر راشح بكتريا العصيات

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اللبنية المختلطة (CFS) تأثيرًا مثبطًا MIC_{90} عند (25%) ضد *P. mirabilis* ولكن (50%) ضد *P. aeruginosa* و *S.aureus* و *K. pneumoniae*. أوضحت البيانات أن الراشح البكتيري من العصيات اللبنية المختلطة كانت متآزرة مع سيبروفلوكساسين ضد العزلات البكتيرية لالتهاب الأذن الوسطى. أظهرالراشح البكتيري (CFS) للعصيات اللبنية المختلطة نسبة $MBIC_{50}$ أعلى ضد (50%) *K.pneumoniae* مقارنة بالعزلات البكتيرية الأخرى. بالإضافة إلى ذلك ، تم التسجيل عن وجود مستوى أعلى من $MBIC_{50}$ من المستحلب الحيوي لبكتريا العصيات اللبنية المختلطة ضد *P. aeruginosa* بنسبة 50% مقارنة بالأنواع البكتيرية الأخرى. استنتج الباحثون أن خلايا البروبايوتيك المختبرة ، العصيات اللبنية المختلطة ، ومنتجاتها تمتلك إمكانيات فعالة لمضادات الميكروبات وتعزز نشاط سيبروفلوكساسين للسيطرة على البكتيريا المرتبطة بالتهاب الأذن الوسطى المزمن وتثبيط تكوين الأغشية الحيوية المستمرة.

الكلمات المفتاحية: التهاب الأذن الوسطى المزمن ، العصيات اللبنية المختلطة ، المستحلب الحيوي ، التركيبات المضادة للميكروبات ، النشاط المضاد للغشاء الحيوي.

Introduction

Otitis media (OM) is an infection of the middle ear that affects patients, adult and children, who abuse and overuse of antibiotics [1]. The complications of OM infection may lead to hearing loss, recurrent acute otitis media, persistence of middle ear effusion, mastoiditis, and chronic otitis media [2]. The acute cases of otitis media (AOM) is characterized by the accumulation of fluids in the middle ear and the presence of symptoms of the middle ear infection [3]. The chronic suppurative otitis media (CSOM), known as chronic otitis media, which is a persistent or recurrent otorrhoea is noticed, lasting 2 to 6 weeks due to a tympanic membrane rupture or a ventilation tube. The pathogenic bacteria may play a key role in the acute and/or chronic infection process [4].

Antibiotics, including ciprofloxacin, are commonly prescribed by physicians in order to eliminate the bacterial otitis media. However, antibiotic resistance of otopathogens which mostly related to their capability to polymicrobial biofilms formation was reported [5].

Biofilms defined as bacterial aggregations attached to a surface and to each other and embedded in a self-produced structure composed of proteins (for example, fibrin), polysaccharides (for example, alginate), and extracellular DNA [6]. Biofilm is an important feature that aid pathogens avoiding host immune responses, survive at high levels of antibiotics, and establish

a chronic infection. The bacteria within a biofilm are 1000-fold more resistant to antibiotic treatment than the planktonic state [7]. Therefore, it is important to investigate an alternative antimicrobial therapies that are inhibit/kill the resistant microorganisms and possess long-term effect.

Probiotics are defined according to World Health Organization (WHO) as "living microorganisms that provide a health benefit to the host when given in sufficient amounts" [8]. Probiotics importance are mostly related to re-balancing of gut microbiota, enhancing of immunological function, producing bacteriocins and other inhibitory chemicals, which could be used, to prevent biofilm formation [9,10].

This study comes to evaluate the antimicrobial potential of ciprofloxacin and Cell Free Superatant (CFS) of lactobacilli species, alone and in combination, against the tested pathogens. In addition to assess antibiofilm activity of CFS and BS of the mixed lactobacilli against the isolated pathogens from patients with chronic suppurative otitis media (CSOM).

Materials and METHODS

Collection of samples

This study included one hundred sixty-two ear swab were collected form patients with chronic ear discharge attended to the consulting clinic\ ENT department at Baquba Teaching Hospital and to the private clinics. From 162 otitis media samples, 113 bacterial species (69.76%) were identified as *P. aeruginosa* 52 (32.1%), *S.aureus* 40 (24.7%), *P. mirabilis* 14 (8.64%) and *K. pneumoniae* 7 (4.32%) which were mostly isolated and 19 (11.73%) were macroscopically identified as fungi while 30 (18.51%) showed no growth despite the presence of infection.

Specimens collection, isolation and identification of bacterial isolates

The collected specimens were inoculated on the culture media and incubated under aerobically at 37°C for 18–24 hrs. The bacterial cells were identified, initially, based on their phenotypic characteristics (on the culture media), microscopic examination (by staining them with gram stain) and initial biochemical reactions [11,12]. The identification of bacterial species was

confirmed using VITEK 2 system (BioMérieux, Marcy-l'Étoile, France). The commercial probiotic strain, *L. acidophilus* and *L. plantarum*(Vitalactic B Ltd, Wells Ave, Congers, USA) was activated, and then inoculated in Mann, Rogosa and Sharpe (MRS) agar (Liofilchem, Italy) under aerobic conditions at 37◦C for 24-48 h.

Antibiotic susceptibility test

The antibiotic susceptibility of the tested pathogens and the tested lactobacilli strain to ciprofloxacin was evaluated using Kirby-Bauer method based on the Clinical and Laboratory Standards Institute (CLSI), (2021). The tested antibiotic,ciprofloxacin (5 µg) were selected based on the recommendation of WHO [13] for the treatment of CSOM. The bacterial suspensions were prepared equivalent to McFarland No. 0.5 and spreaded on the surface of Muller Hinton (MH) agar in three directions. Then, ciprofloxacin disc was picked up using a sterile forceps and applied onto the surface of the MH agar inoculated with the tested pathogens. The agar plates were incubated at 37ºC for 24 hrs under aerobic conditions. The diameter of inhibition zone around the discs were measured by millimeter (mm) after incubation and the bacterial susceptibility/ resistance to ciprofloxacin. The results were interpretated based on CLSI guidelines [14].

Preparation of Cell-Free Supernatant of mixed lactobacilli

The lactobacilli CFS is prepared according to [15] with some modifications. The lactobacilli species were inoculated into MRS broth and incubated aerobically at 37 ˚C for 24 hrs.The bacterial cells were centrifuged and removed after incubation at 6000 *rpm* at 4°C for 30 min. The CFS was filtered using a sterilized syringe filter (millipore 0.45 µm) and Kept at 4 °C.

Preparation of Biosurfactant from mixed lactobacilli

Biosurfactant isolation from the selected mixed lactobacilli was applied as previously described by [16] with minor modifications. Briefly, 600 ml of MRS culture broth was inoculated with 10 ml of an overnight culture of lactobacilli species and incubated aerobically for 24 hrs at 37°C. After incubation, cell pellets were removed by centrifugation (10000 *rpm*, 10 min at 10°C), and washed twice in demineralized water, and re-suspended in 100 ml of phosphate

buffer saline. Afterward, this solution was shaken gently for 2 hrs at room temperature to release the cell-bound BS. After 2 hrs, bacterial cells were separated by centrifugation and the supernatant liquid was collected by filtering through a millipore 0.45 µm syringe filter (Difco, USA). The filtered supernatant was kept at 4°C in a sterile tubes until be used. This is considered as a stock solution of BS of lactobacilli strain.

Determination of Minimum Inhibitory Concentration (MIC)

The MIC_{90} determination of the tested mixed lactobacilli CFS was performed according to [17] with minor modifications. Briefly, the CFS (100%) of mixed lactobacilli was prepared as stock solution. A series of two-folds dilution of CFSs of the tested lactobacilli was made in a 96 well of microtiter plate with 100 µl of fresh Brain Heart Infusion (BHI) broth. Then, each well was inoculated separately with 100 µl of previously prepared overnight culture of bacteria (1.5×10^8 CFU/ml), the final volume in each well was 200 µl. The micro plates were incubated at 37°C for 24 h. After the incubation, MIC results were reported using a micro plate reader (Kevin, Germany), measuring the optical density (OD) at 630 nm. The MIC was defined as "the lowest concentration of antimicrobial can inhibit visible growth of microorganism or give a 20% reading less than the reading of positive control" [18].

In regards to antimicrobial combinations between the CFSs of lactobacilli species and ciprofloxacin against the isolated bacterial pathogens, checkerboard assay was performed as described by [19,20] with slight modifications. Briefly, each antimicrobial agent was diluted two-folds into BHI broth into two separate 96-well micro-plates as following; 50 µl from each dilution of Antimicrobial A (CFS of lactobacilli strain) was taken and added horizontally over 50 µl of antimicrobial B (ciprofloxacin). Then, 100 µl of suspension of each bacterial species which were diluted previously with BHI broth to achieve ($1.5x10^8$ CFU/ml) was added to the pre-determined concentration of antimicrobial combinations. The concentrations (15.6, 7.8, 3.9, 1.95, 0.98, 0.49, 0.24 µg/ml) of ciprofloxacin and (50, 25, 12.5 and 6.25 %) of the CFSs of the tested lactobacilli strains which were selected and utilized based on their MICs values. A positive (non-treated bacterial cells) and negative controls (broth only, broth + antimicrobials) were used in duplicate. The MICs of each antimicrobial combinations were determined after 24

hrs incubation. After incubation, the growth of the treated wells were measured using a microplate reader at OD630 to determine the MIC_{90} of the antimicrobials combination. Isobolograms were used to analyze the nature of antimicrobial combinations and identify if they are synergised, antagonised, or have an additive effect against the tested pathogenic bacteria.

Determination of Minimum Biofilm Inhibitory Concentration (MBIC) of CFS and BS of mixed lactobacilli

Biofilm inhibition assay was performed as described by [21], with minor modifications. Briefly, the selected mixed lactobacilli CFS and BS were diluted two-fold with an appropriate volume of fresh BHI broth that supplemented with 1% glucose (BHIG). In each well, the final volume of each CFS/ BS diluted into the BHIG broth was 100 µl. The overnight culture of each bacterial cells was adjusted to a final concentration $1.5x10^8$ CFU/ml using BHIG broth. Then, 100 µl of diluted bacterial cells were separately transferred into the wells containing pre-determined concentrations of lactobacilli CFS /BS. The positive and negative controls were used in duplicates. The micro plates were covered with a lid and incubated at 37◦C under aerobic conditions for 24 hrs. Following incubation, the multichannel pippete was used to remove the non-adherent cells gently from each well without disrupting the biofilm construction and transferred into a new sterile microtiter plates. The turbidity of non-adherent cells was measured at OD630 nm by a microplate reader. Then the wells were gently washed three times with 200 µl of sterile distilled water. The biofilm cells were fixed by heating for 60 min at 60°C by oven. After fixation, 150 µl of 0.1% crystal violet (BDH, England) was added to each treated wells and left for 15-20 min at room temperature. The residue of crystal violet was removed and each well was washed three times with 200 µl of distilled water, air- dried, then 200 µl of 95% ethanol was added into each well to solubilized the dye bound to the adherent cells. The micro plates, then, incubated for 30 min at 4°C. After incubation, 125 µl were transferred from each treated well into a new 96 well microtiter plates. The absorbance readings were made at 630 nm using microplate spectrophotometer system. The percentage of biofilm inhibition was then, calculated compared to the positive control, untreated biofilm.

Statistical Analysis

Software was used to analyze continuous variables, the mean and standard error was calculated. The statistical analysis was performed using the one-way analysis of variance (ANOVA) test. P-value ≤ 0.05 was measured to indicate a statistically significant difference.

Results and Discussion

Bacterial isolation, identification and antibiotic susceptibilty

This four bacterial species which mostly isolated from patients with CSOM were *P. aeruginosa*, *S. aureus*, *P. mirabilis* and *K. pneumoniae*. The bacterial isolates were initially identified based on their macroscopic features and their biochemical tests using manual and automated system.

The sensitivity of bacterial isolates was investigated using Kirby-Bauer method. This methods was used to determine the sensitivity or resistance of the tested isolates (pathogenic and probiotic) to ciprofloxacin. All the tested bacteria (100%) were sensitive to ciprofloxacin.

Minimum Inhibitory Concentration (MIC)

Determination of MIC was performed using broth micro dilution method. The MIC values were determined by selecting the lowest concentration in the well of microtiter plate at which no growth is observed. A series of different concentrations were prepared; from (500-0.95) µg/ml for ciprofloxacin and from (50-6.25) % for CFSs of mixed lactobacilli. The MIC_{90} values of ciprofloxacin were found in the range of (0.95-1.9) µg/ml, while those of CFS were in the range of 25-50%. Figure (1) showed that *P. aeruginosa* displayed a significant inhibition 92.1% when 1.9 µg/ml of ciprofloxacin was used, as a MIC_{90}. Whereas, MIC_{90} values were 0.95 µg/ml for *P. mirabilis*, *K. pneumoniae*, and *S. aureus* causing growth inhibition 90.3%, 93.3%, and 94.9% respectively. The results showed different significant in bacterial growth inhibition when ciprofloxacin was used in all concentrations (31.3, 15.6, 7.8, 3.9, 1.9, and 0.95) compared to zero concentration p-value < 0.001.

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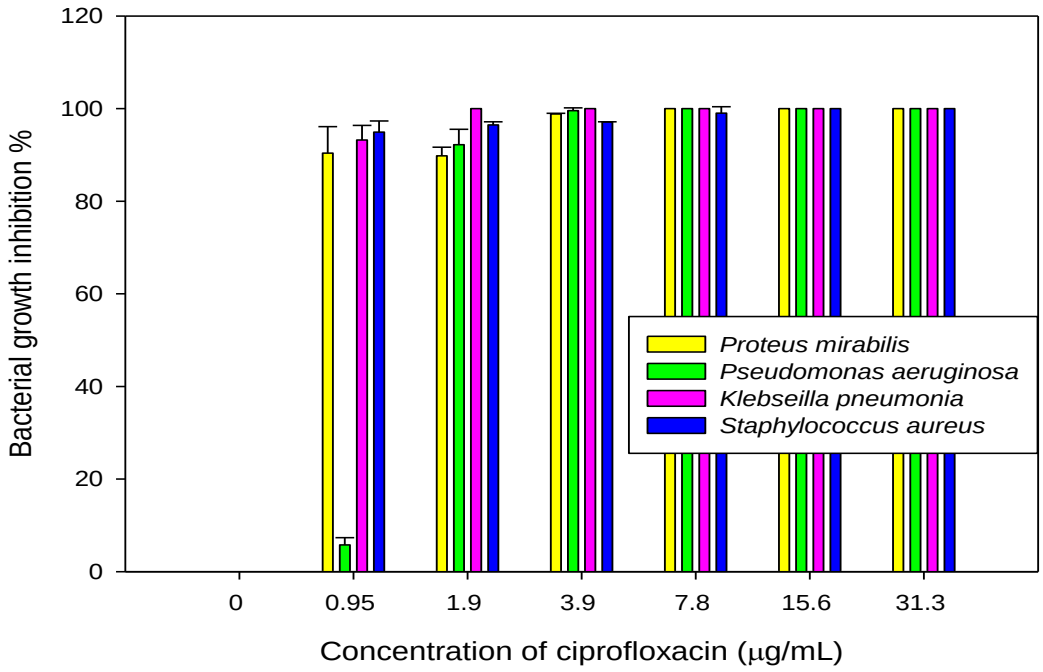

Figure 1: Antibacterial activity of ciprofloxacin against bacterial isolates

Regarding the CFS of mixed lactobacilli, it was found to be 25% against *P. mirabilis* which inhibited 91.4%, while was 50% against *k. pneumoniae*, *P. aeruginosa*, and *S. aureus* caused growth inhibition 93.3%, 90.5%, and 90.5%, respectively, figure (2).

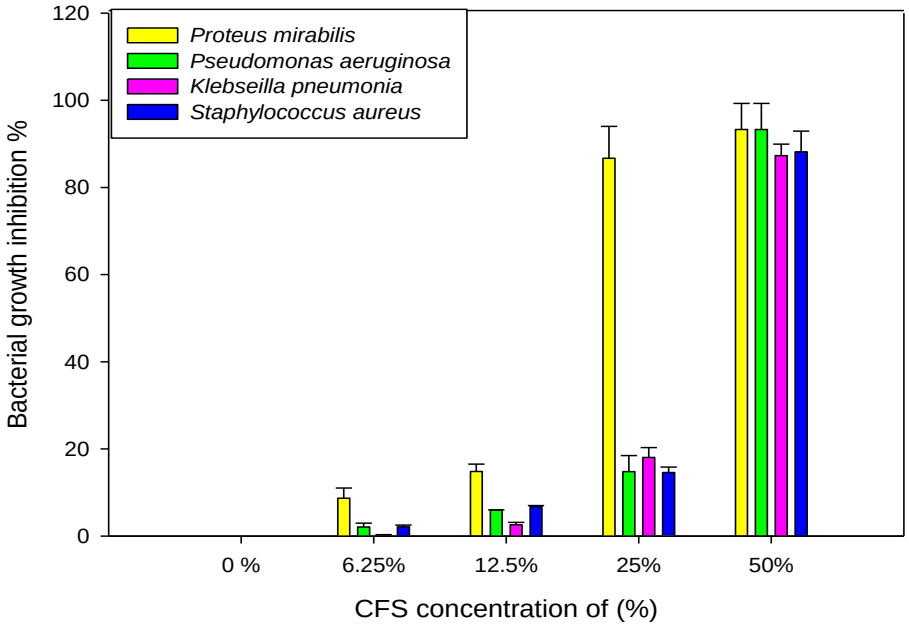

Figure 2: Antibacterial activity of mixed lactobacilli CFS against bacterial isolates

Generally, the low MICs values of CFS of tested *lactobacilli* strains against the isolated pathogens reflect its potent inhibitory effect. Diverse studies have highlighted the antimicrobial activity of CFS against several bacterial pathogens. [22] Observed in their study that *Lactobacillus* strains can exhibit inhibitory activity against *P. aeruginosa* and *S. aureus*. Another study done by [23,24] showed that CFS of *L. acidophilus* had an inhibition growth effect on *S. aureus*.

The mechanisms of antimicrobial activity of CSF *lactobacillus* strains include: (i) competitive exclusion of bacteria to adhere and competing for nutrients and adhesion receptors, (ii) co-aggregation, the assembly of microbial communities into distinct, interlinked structures, (iii) an intense production of antimicrobial compounds such as lactic acid which in turn lowers the pH in the reaction environment, hydrogen peroxide (H_2O_2), biosurfactants, and bacteriocins like substances, ultimately inhibiting the growth of bacteria [25]. Bacteriocins are small antimicrobial peptides that have lethal or inhibitory effects against other types of bacteria. Their

adsorption to specialized receptors on the surface of bacteria, causing vital and phenotypic metabolic changes, killing those bacteria [26,27]

The Synergistic effect of mixed lactobacilli CFS in combination with ciprofloxacin against the bacterial isolates

The checkerboard assay was use to evaluate the nature of antimicrobial combinations of the ciprofloxacin and CFS of mixed lactobacilli against the four bacterial isolates, as described by [28]. The laboratory results of the current study were obtained after 24 hrs of incubation using an microplate reader at OD630 nm. Isobolograms was used to analyze the combination of CFS of tested *Lactobacillus* with ciprofloxacin against the bacterial isolates.

In the present study, when ciprofloxacin was combined with the CFS of mixed lactobacilli against *P. aeruginosa*, a synergistic activity was reported, the MICs was 0.98 µg/ml of ciprofloxacin when mixed with 6.25%, 12.5% of CFS mixed lactobacilli was used, see figure (3). The MIC of ciprofloxacin was 1.9 µg/ml and of CFS was 50%. When it was used alone.

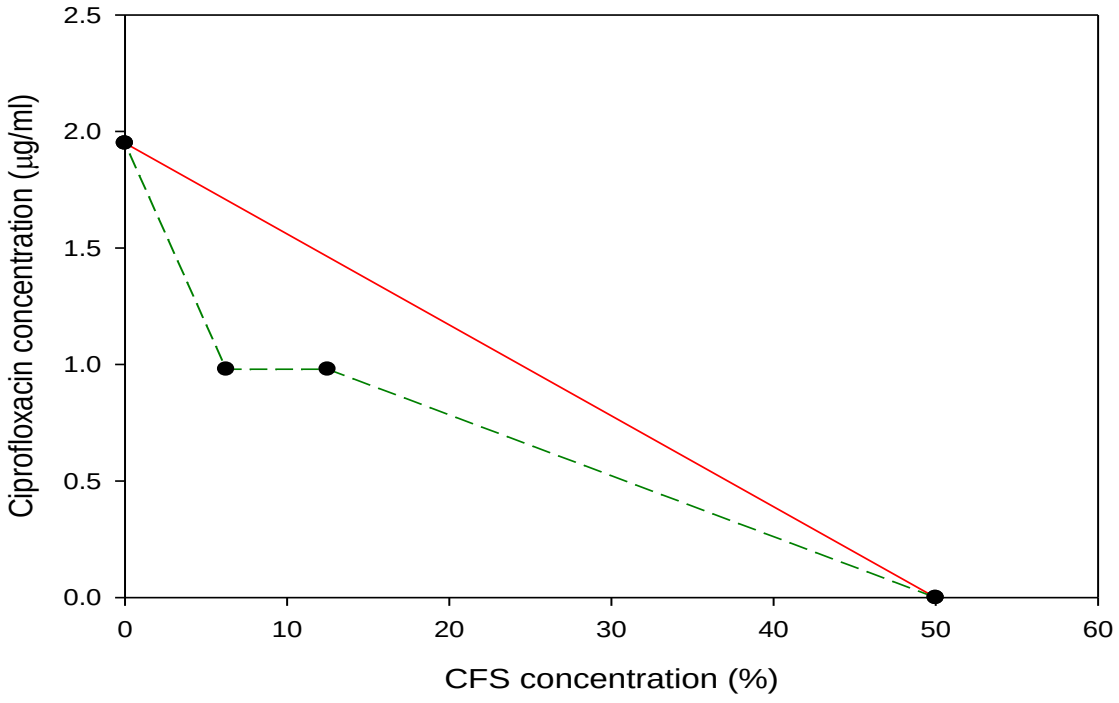

Figure 3: Isobolograms of ciprofloxacin combined with mixed lactobacilli CFS against *P. aeruginosa*.

When ciprofloxacin was combined with the CFS of *Lactobacillus* mix against *S. aureus*, a synergistic activity was identified when CFS of mixed lactobacilli was combined with ciprofloxacin. The MICs of ciprofloxacin were 0.49 µg/ml when added to 3.13% and 12.5% of CFS of mixed lactobacilli, (figure 4). The MIC of ciprofloxacin was 0.95 µg/ml and of CFS was 50% When it was used alone.

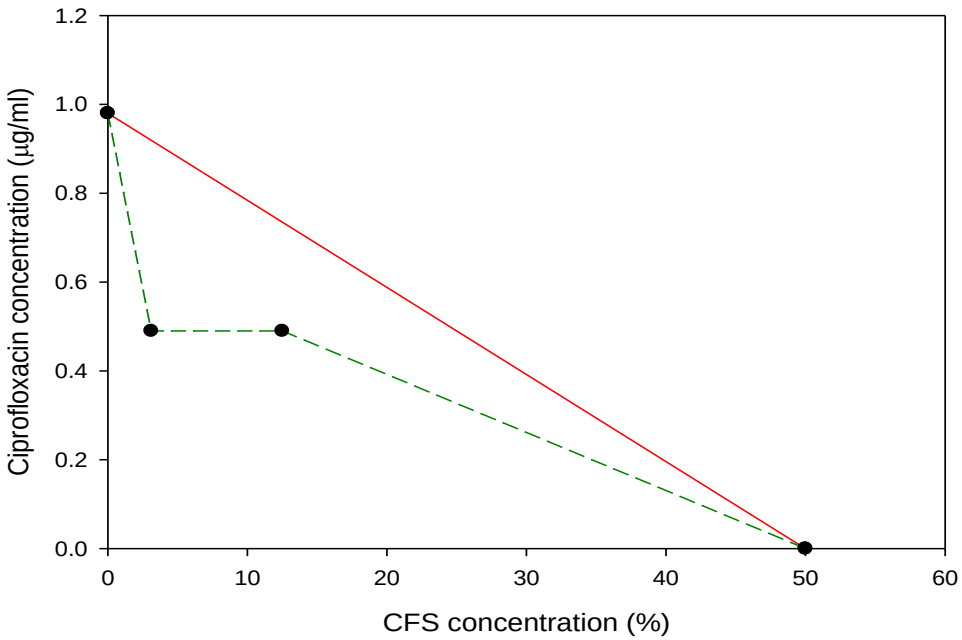

Figure 4: Isobolograms of ciprofloxacin combined with mixed lactobacilli CFS against *S. aureus.*

When ciprofloxacin was mixed with CFS of mixed lactobacilli, a synergistic activity was observed against *P. mirabilis*, the MICs of combinations were 0.49 µg/ml for ciprofloxacin when combined with 6.25%, 12.5% of CFS mixed lactobacilli. The MIC of ciprofloxacin was 0.95 µg/ml and of CFS was 25% When it was used alone (figure 5).

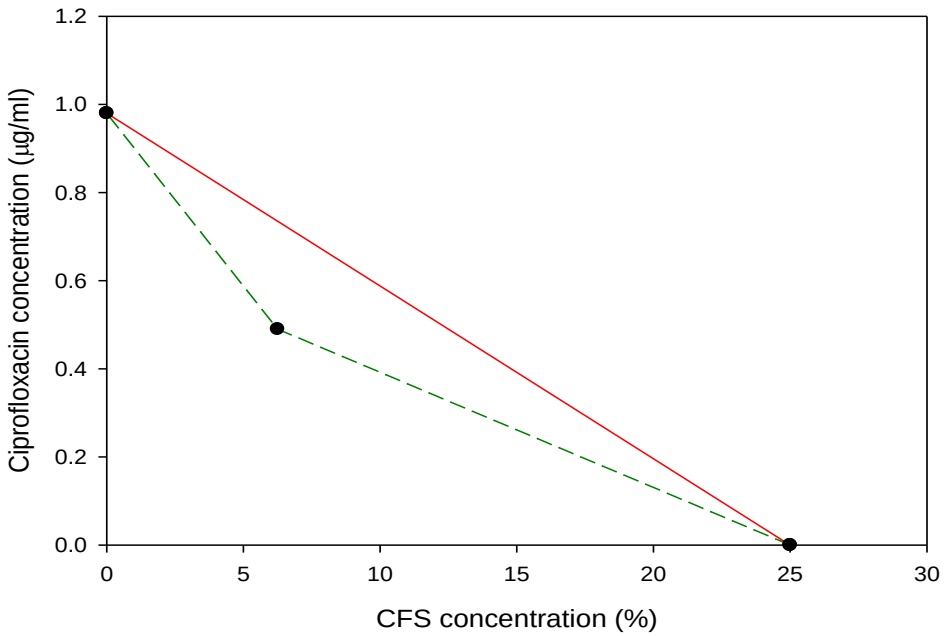

Figure 5: Isobolograms of ciprofloxacin combined with mixed lactobacilli CFS against *P. mirabilis*

A synergistic activity also was reported when ciprofloxacin was combined with CFS of *Lactobacillus* mix against *K. pneumoniae*. The MICs of ciprofloxacin were 0.49 µg/ml when mixed 3.13% and 6.25% of CFS of mixed lactobacilli (figure 6). The MIC of ciprofloxacin was 0.95 µg/ml and of CFS was 50% When it was used alone.

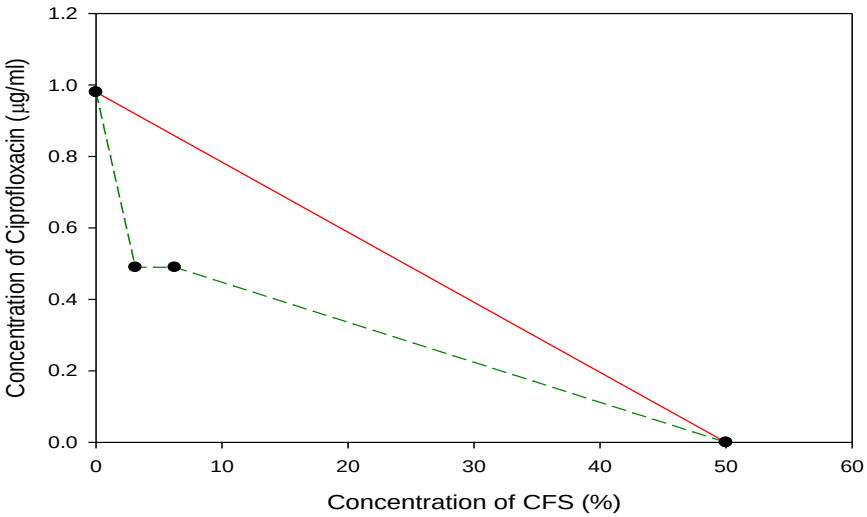

Figure 6: Isobolograms of ciprofloxacin combined with mixed lactobacilli CFS against *K. pneumoniae*

Our results were corresponded with several studies that focused in CFS combination with antibiotics. A study done by [29,30] noticed a synergistic interactions between CFS of *Lactobacillus* spp. and ciprofloxacin against *P. aeruginosa* growth. Similarly, [31] showed in their study that there was a synergistic effect between antibiotics and probiotics. The synergistic reaction of *Lactobacillus* and antibiotics enhancing their antibacterial properties and restores its ability to destroy bacteria that have acquired resistance to it [32].

In the present study, the combination of ciprofloxacin with CFS of the tested *Lactobacillus* showed a synergistic activity when tested against bacterial isolates. Furthermore, in the most cases, the efficacy of a combination of probiotics and antibiotics was found to be greater than that of antibiotics alone [33]. Synergism has the following benefits: (i) expanding of antimicrobial spectrum; (ii) reducing the required dose of the conventional antibiotics; (iii) neutralizing toxicity of high concentration of antibiotic; and (iv) prevention of the emergence bacterial resistance [34].

Minimum Biofilm Inhibitory Concentrations (MBIC)

The CFS and BS *Lactobacillus* spp. were analyzed for their antibiofilm activity against the four pathogenic organisms. $MBIC_{50}$ was determined using the broth micro-dilution method as described by [35]. The anti-biofilm effect of CFS of mixed lactobacilli on *P. aeruginosa*, showed there was a significant reduction in biofilm formation at 12.5%, removing 64.2%, while BS removed 64.3% of biofilm at higher $MBIC_{50}$, (25%). as in figure (7). On the other hand, in regards to *P. aeruginosa* planktonic growth as shown in figure (7) we noticed a significant planktonic growth when 25% and and less of both BS and CFS of mixed lactobacilli were used.

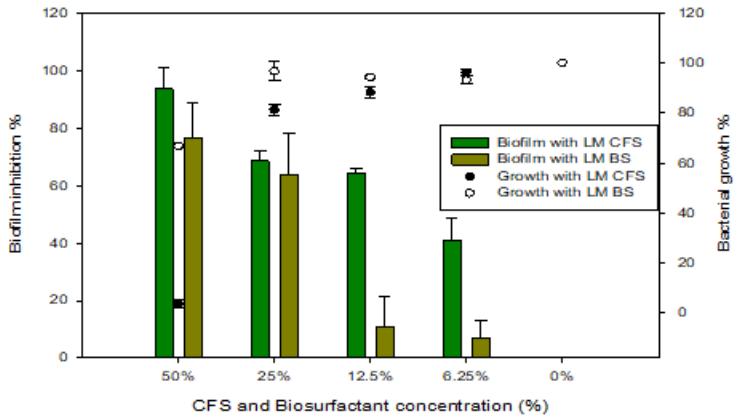

Figure 7: Effect of CFS and BS of mixed lactobacilli on *P. aeruginosa* biofilm and planktonic growth. Results expressed as mean MBIC50±SD (µg/ml) to three independent experiments

The $MBIC_{50}$ of CFS mixed lactobacilli against *S. aureus* was at 6.25% which inhibited 73.7% of biofilm formation but when 50% of BS was used, caused biofilm removal (78.2%), figure (8). Regarding to *S. aureus* growth. In figure (8), a significant growth was observed, compared with control, ($P<0.001$) when 12.5%, 25% and 50% of BS and CFS mixed lactobacilli was used respectively.

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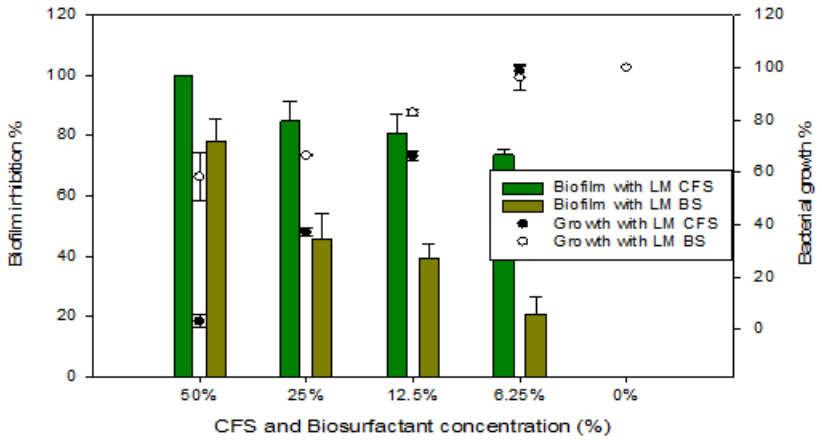

Figure 8: Effect of CFS and BS of mixed lactobacilli on *S. aureus* biofilm and planktonic growth. Results expressed as mean MBIC50±SD (µg/ml) to three independent experiments

Results in figure (9) for *P. mirabilis* showed that $MBIC_{50}$-CFS of mixed *lactobacilli* was 12.5% which removed 49.6% of the biofilm formation while BS 12.5% caused a significant reduction by 54.2% in biofilm formation. In regard to planktonic growth of *P. mirabilis*, a significant differences ($P<0.001$) was noticed when treated with 50% of CFS and BS of mixed lactobacilli, figure (9).

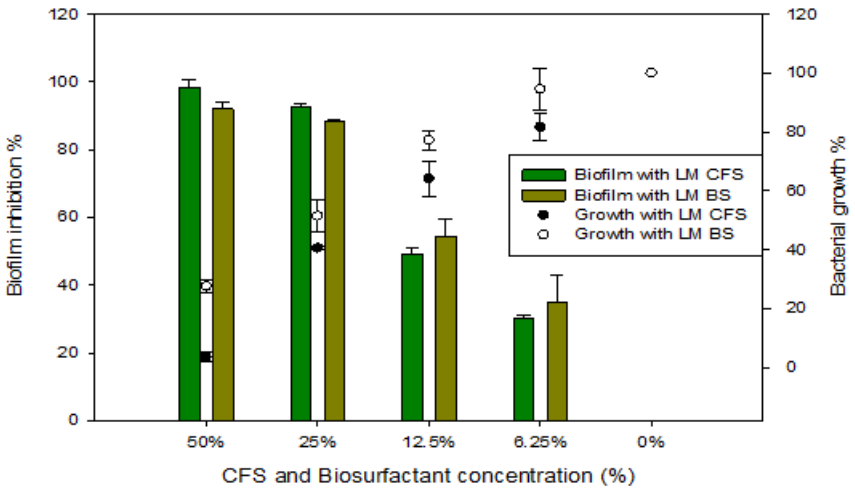

Figure 9: Effect of CFS and BS of mixed lactobacilli on *P. mirabilis* biofilm and planktonic growth. Results expressed as mean MBIC50±SD (µg/ml) to three independent experiments.

Figure (10) illustrated the $MBIC_{50}$ of mixed lactobacilli CFS for *K. pneumoniae*, A 50% of CFS mixed Lactobacilli causes complete inhibition (99.7%) of biofilm. Whereas, BS at 50% prevented 47.1% of the biofilm formation. Regarding to planktonic growth percentages, there was a significance differences when 50% of CFS and BS mixed lactobacilli was used in compared with the control ($P<0.001$), figure (10).

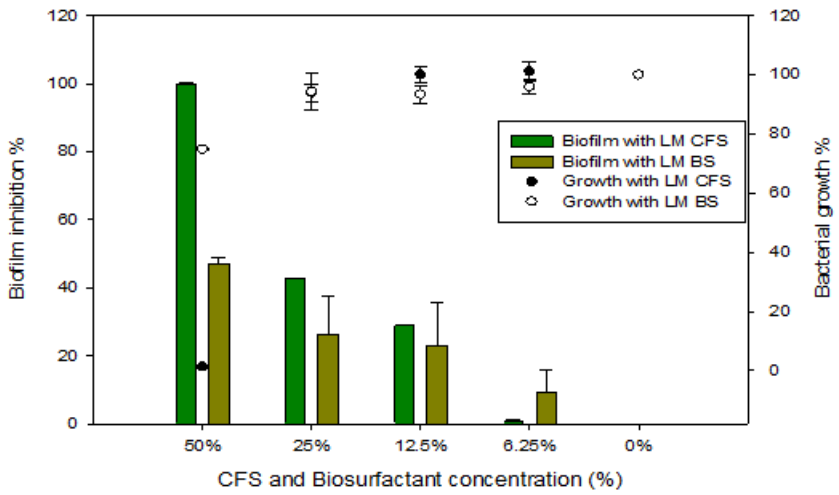

Figure 10: Effect of CFS and BS of mixed lactobacilli on *K. pneumoniae* biofilm and planktonic growth. Results expressed as mean MBIC50±SD (µg/ml) to three independent experiments.

Probiotics such as Lactic acid bacteria (LAB), especially, lactobacilli, have been found to prevent or dispersed of pathogenic biofilms formation by attacking the bacterial membrane leading to a rough and wrinkled membrane that may lead, eventually, to the inhibition of biofilm formation [36,37]. This activity is belong to ability of lactobacilli to interfere with harmful bacteria through competition for nutrients, co-aggregation and production of the antimicrobials; bacteriocin, hydrogen peroxide, and organic acids [38,39].

Conclusions

The common bacteria isolates from chronic suppurative otitis media was *P. aeruginosa*, as Gram-negative and *S. aureus*, as Gram-positive. Mixed lactobacilli CFS possess a significant antibacterial potential against and, when combined, enhace the antibiotic activity against the pathogenic isolates. Furthermore, the best inhibitory effect on biofilm formation but not

planktonic growth inhibition was seen when mixed lactobacilli CFS was used in comparison to BS. Mixed probiotic cells are good candidates could be ever used as effective and safe alternative antimicrobial to control the biofilm-associated CSOM.

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