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ORIGINAL ARTICLE

Antimicrobial Activity of Mixed Lactobacilli in Combination with Antibiotics Against Bacterial Pathogens Causing Chronic Suppurative Otitis Media

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ABSTRACT

This study aimed to determine the antimicrobial activity of CFS of the selected lactobacilli, alone and in combination with antibiotics against pathogenic bacteria isolated from patients with chronic otitis media. In addition, we investigated the ability of tested lactobacilli (a mixed of Lactobacillus acidophilus and Lactobacillus plantarum) to co-aggregate with the isolated pathogens.. One hundred sixty-two ear swab were collected from patients had otitis media 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 sexes, male and female starting from September to the end of December, 2021. The isolates were identified based on their morphological features of the colonies and some biochemical tests. The final identification and antibiotics susceptibility of bacterial isolates was confirmed by VITEK2 compact system. The results showed that 113 samples were positive for bacterial culture, while 30 samples were negative growth, mostly identified as fungi. A total of 113 bacterial species isolated from 162 otitis media samples were: Pseudomonas aeruginosa, Staphylococcus aureus, Proteus mirabilis, and Klebsiella pneumonia. The highest predominant bacterial agents were P. aeruginosa 52 (32%), S. aureus 40 (24.7%), followed by P. mirabilis 14 (8.64%), and K. pneumoniae 7 (4.32%). The obtained results showed that the highest rate of infection was identified in male rather than in female. In antibiotic susceptibility testing, 16 types of antibiotics was evaluated against four bacterial species using VITEK2 system. In addition, disk diffusion assay was utilized in order to perform antimicrobial combinations. The susceptibility of bacterial species were various among these antibiotics. It was noticed that ciprofloxacin was the most effective antibiotic against all the isolated bacteria with the lowest MIC in comparison to the other antibiotics. We evaluated the effect of ciprofloxacin alone and in combination with the cell-free supernatants (CFSs) of mixed lactobacilli against the four isolates using disk diffusion assay. Our results showed that the CFS produced by the mixed lactobacilli have a significant inhibitory effect on the growth of bacterial pathogens. Moreover, CFS in combination with ciprofloxacin was more effective against pathogenic isolates than using ciprofloxacin alone. Co-aggregation of the selected mixed lactobacilli strains with the four bacterial isolates were evaluated in order to investigate the competitive activity of the tested lactobacilli with the isolated pathogens.

Keywords: Otitis media**,** Mixed lactobacilli, Cell Free Supernatant, Co-aggregation, Antimicrobial combinations

INTRODUCTION

Ear infections, otitis media and otitis externa, are common worldwide conditions that affect both genders in all age groups; infants, children, and adults (Meherali et al., 2019). Otitis media (OM) is an infection of the middle ear that affects patients, children, and outpatients who use antibiotics randomly (Meherali et al., 2019). OM can be connected with consequences including hearing loss, recurrent acute otitis media, persistence of middle ear effusion, mastoiditis, and chronic otitis media (Protasova et al., 2017).

Otitis media is a polymicrobial infection caused by viruses, bacteria, fungi, or a combination of these microbial agents. In regards to bacterial OM, Streptococcus pneumoniae, Moraxella catarhalis, and Haemophilus influenza are the most commonly caused acute cases (AOM) (Ubukata et al., 2018). Whereas, the most etiological agents of chronic infections include Pseudomonas aeruginosa, Proteus spp., Klebsiella pneumonia, Escherichia coli, as gram-negative species, and Staphylococcus spp., as gram-positive species (Basavaraj and Jyothi, 2015).

Controlling of such infection is a challenging issue. Antibiotics are commonly prescribed by physicians in order to eliminate the bacterial otitis media. However, abuse and/ or overuse of these antibiotics lead to develop and emerge of multi-drug-resistant bacteria which are an important health problem associated with increasing mortality and morbidity rates (Chan et al., 2017).

Therefore, it is important to invetigate an alternative antimicrobial therapies that are inhibit/kill the resistant microorganisms and possess long-term effect, such as probiotics.

Probiotics are defined by the World Health Organization (WHO) as living microorganisms that provide a health benefit to the host when given in sufficient amounts (FAO/WHO, 2002; Hill et al., 2014).

They exert their following effects:(i) re-balancing of gut microbiota, (ii) enhancing of immunological function, (iii) competing with the pathogenic bacteria for nutrients and adhesion sites on epithelial surfaces, and (iv) producing bacteriocins and other inhibitory chemicals, which could be used, to prevent biofilm formation (Hao et al., 2015).

Antimicrobial activity of Lactic acid bacteria and their metabolite (s) alone and in combination with traditional antibiotics were reported in many publications. Antimicrobial combinations have advantages include; lowering the doses of the antimicrobials that used to control infections, in addition to reduce the cost and toxic side effects when antibiotics were administered alone in high concentrations.

In this study, we determined the antimicrobial activity of CFS of the selected lactobacilli, alone and in combination with antibiotics against the isolated pathogens from patients CSOM. In addition, we investigated the ability of tested lactobacilli (a mixed of Lactobacillus acidophilus and Lactobacillus plantarum) to co-aggregate with the these pathogens..

MATERIALS AND METHODS

This study was accompanied during the period from September to the end of December 2021. This study included 162 patients with chronic ear discharge attended to the consulting clinic\ ENT department at Baquba Teaching Hospital and to the private clinics

Samples collection and bacterial growth: A total of 162 clinical samples were collected from patients with ear discharge including males and females of different ages.

The collected specimens were cultured directly and separately on the following prepared solid media: Blood agar, MacConkey agar, Mannitol salt agar, and Chocolate agar using streaking method. Incubation of MacConkey agar, blood agar, and Mannitol Salt agar plates were in aerobic conditions, whereas the

chocolate plates were incubated in a candle jar anaerobically, which can generate about 5% CO2. All plates were incubated at 37°C for 18–24 hrs.

Bacterial identification : The diagnosis of bacterial cells was, initially, based on the phenotypic characteristics (on the culture media), microscopic examination (by staining them with Gram stain) and initial biochemical tests. The identification of bacterial species was confirmed using VITEK 2 system (Levinson, 2014; Procep et al., 2017). The antimicrobial susceptibility test of isolated pathogenic isolates (P. aeruginosa, P. mirabilis, S. aureus, and K. pneumoniae) to antibiotics was carried out by VITEK 2 system using AST-GN (Gram-negative) card and a AST-GP (Gram-positive) card. Commercial probiotic strain, the mixed lactobacilli (L. acidophilus and L. plantarum), was previously procured from Vitalactic B (Germany). It was activated in skimmed milk for 48 hrs at 37◦C under aerobic conditions, and then inoculated in De Mann, Rogosa and Sharpe (MRS) agar (Liofilchem, Italy) followed by incubation aerobically at 37◦C for 24-48 h.

Antibiotic susceptibility test: An antimicrobial susceptibility test of isolated pathogenic bacteria to antibiotics was carried out by VITEK 2 system . In addition, the antibiotics , alone and in combination with CFS of lactobacilli spp. (explained below) was evaluated against the bacterial isolates using the modified Kirby-Bauer method based on the CLSI (2021). The antibiotic discs included; aztereonam (30 µg), meropenem (10 µg), ceftazideme (30 µg), piperacillin (100 µg),ciprofloxacin (5 µg), oxacillin (5 µg), gentamycin (10 µg) and trimethoprim-sulfamethoxazole (1.25/23.75 µg). The tested antibiotics were selected based on the recommendation of WHO (2004) for the treatment of CSOM. Standard bacterial suspensions of the pathogens were prepared equivalent to McFarland standards No. 0.5 and the suspension was spread on the surface of Mueller–Hinton agar media using a sterile cotton swab.

Then, the selected antibiotics discs were picked up using a sterile forceps and applied onto the surface of the Muller Hinton agar previously inoculated with the bacterial isolates. The agar plates were incubated at 37ºC for 24 hrs under aerobic conditions. After incubation, the diameter of inhibition zone around the discs were measured by millimeter (mm) using a metric ruler and the bacterial susceptibility/ resistance to the tested antibiotics and their interpretations were carried out according to the CLSI (2021) guidelines.

Preparation and isolation of Cell-Free Supernatant (CFS) from mixed lactobacilli: The CFS of the selected lactobacilli is prepared according to (Sutyak et al., 2008) with minor modifications. The mixed lactobacilli was inoculated into MRS broth and incubated at 37 ◦C for 48 hrs under aerobic condition. After incubation, the bacterial cells were removed by centrifugation at 6000 rpm at 4°C for 30 min. The CFS was collected and filtered through a sterilized millipore 0.45 µm syringe filter and stored at 4 °C for further use.

Determining Lactobacillus CFS activity alone and in combination with antibiotics discs by disk diffusion method: The evaluation of antimicrobial activity of mixed lactobacilli CFS alone or in combination with antibiotics against the isolated pathogenic bacteria was done by an modified disc diffusion assay according to CLSI (2016) and Algburi et al., (2020a) with some modifications. Briefly, 3-5 of overnight grown bacterial colonies were used and inoculated in BHI broth to make a bacterial cells suspension. Then, a sterile swab was dipped into the bacterial suspension and streaked on Muller Hinton agar at 3 different planes. In the combination method, three types of discs were used (an antibiotic disc alone, an antibiotic disc saturated with 20 µl of CFS of mixed lactobacilli, a blank disc with mixed lactobacilli CFS alone). With a sterile forceps, the antibiotic disc was picked up and placed on the surface of the MH agar inoculated previously with the bacterial isolates. The agar plates were then incubated at 37°C for 24 hours and the diameter zones of bacterial growth inhibition were measured.

Co-aggregation of mixed lactobacilli with the bacterial isolates: Co-aggregations of mixed lactobacilli with the isolated bacterial species were performed according to method of Collado et al. (2008) with slight modifications. The four bacterial isolates and the selected mixed lactobacilli were separately cultured at 37°C for 24 hrs in BHI and MRS broth, respectively. After that, they were harvested from planktonic growth by centrifugation (4480 rpm, 15 min, 23°C), and washed twice with a sterile phosphate buffer saline (PBS). Bacterial cells precipitates were re-suspended in the same buffer and their optical density (OD_{630}) was adjusted to 0.25. Using 96-well microtiter plate, equal volumes of the Lactobacillus and pathogenic bacteria were mixed and incubated at 37 °C without agitation, their OD_{630} were reported at 0, 2, 4, and 24 hrs of incubation.

The co-aggregation % was calculated using the following equation as described by Collado et al. (2008) :

Co-aggregation (%)= (X-Y)/(X) × 100

Where X denotes the absorbance at time 0 (before incubation) and Y denotes the absorbance at time 2, 4, 24 hrs (after incubation per-time point). Our experiment was done in duplicates.

RESULTS AND DISCUSSION

Bacterial isolation and identification: This study identified four bacterial species which were 52 (32%) Pseudomonas aeruginosa, 40 (24.7%) Staphylococcus aureus, 14 (24.7%) Proteus mirabilis 14 (8.64%), and 7 (4.32%) were Klebsiella pneumoniae, as shown in table (1).

Table 1: Numbers and percentages of bacterial isolates from patients with ear infection

Types of isolates	Number of isolates	%
P. aeruginosa	52	32.1
S. aureus	40	24.7
P.mirabilis	14	8.64
K. pneumoniae	7	4.32
Other microbial growth (Fungi)	19	11.73
No growth	30	18.51
Total	162	100%

The results of our study were in agreement with Kadhim et al. (2018) in Iraq who showed that the highest rate of ear infection was by P. aeruginosa (35%) followed by S. aureus (26%). While, disagreement with Asima and Karthik (2017) who found that S. aureus was the predominant bacterial species isolated from (34.9%), followed by P. aeruginosa (20.9%).

According to the gender, our study demonstrated that the incidence of CSOM in male were more than female as (53.7%) and (46.3%), respectively, table (2).

Table 2: Distribution of ear infection according to the gender

Genders	Number of the positive cases	%
Male	87	53.7
Female	75	46.3
Total	162	100

Our data was in line with the several investigators in Iraq (Agha & Al-Delaimi, 2021), Pakistan (Javed et al., 2020), Ethiopia (Wasihun & Zemene, 2015), and Uganda (Justin et al., 2018). However, the study of Asima & Karthik (2017) and Jik et al. (2015) found the opposite, they reported that the infection percentage in female were more than male.

The bacterial isolates were initially identified based on their macroscopic features. Colonies of P. aeruginosa isolates were pale as non-lactose fermentator, large and flat with irregular edge, and grape-like odor when grown on MacConkey agar. On blood agar medium, a positive hemolysis activity (β-hemolysis) around colonies appeared which is due to the production of hemolysin enzyme. These properties were in agreement with that published by Ghazi & Kahya (2021).Isolates of S. aureus on blood agar appeared large circular colonies, slightly raised and yellow to

golden in color, surrounded by a transparent and narrow zone which indicates their hemolysis. On Mannitol Salt agar, colonies appeared yellow due to acid production from mannitol fermentation (Hashim & Atya, 2019).Colonies characteristics of K. Pneumoniae were distinctive pink shiny colored due to lactose fermenter, with a regular and round edge, large in size and mucoid colonies. While on Blood agar, the large mucoid colonies appeared white to gray in color (Sathyavathy & Madhusudhan, 2019).Proteus mirabilis appeared as pale colored, lactose non-fermented, single circular colonies of medium size with smooth edges, and of rotting fish smell when grew on MacConkey agar. On a Blood agar, colonies are of a clear swarming movement, the primary diagnostic characteristics of P. mirabilis (Little et al., 2019).

In regards to biochemical test, the results of the biochemical tests as shown in table (3) were in accordance with the approved diagnostic systems and for all studied bacterial isolates, as presented by (Levinson, 2014; Procop et al., 2017)

Table 3: Biochemical test for identification of bacterial isolates

Bacteria	Catalase	Coagulase	Oxidase	TSI				Urease	Indole	Citrate
				Gas	H2S	Slant	But			
P. aeruginosa	+	-	+	+	-	K	K	-	-	+
S. aureus	+	+	-	-	-	A	A	-	-	-
P. mirabilis	+	-	-	+	+	K	A	+	-	+
K. pneumoniae	+	-	-	+	-	A	A	+	-	+

Antibiotic Susceptibility of bacterial isolates Using VITEK 2 System: The antimicrobial susceptibility test for Gram-negative and Gram-positive bacterial isolates was determined by VITEK 2 system. Bacterial susceptibility was tested towards (16) antimicrobial agents, the minimum inhibitory concentration (MIC) of these antibiotic was identified. The table below explain the sensitivity of Gram-negative bacteria to antibiotic, table (4).

Table 4: Antibiotic sensitivity of the Gram-negative isolates using VITEK2 system, the MIC values were determined.

Bacterial isolates / Antibiotics	P. aeruginosa (MIC- µg/ml)	K. pneumoniae (MIC- µg/ml)	P. mirabilis (MIC- µg/ml)
Ticarcillin	S (16)	R (≥128)	R (≥128)
Ticarcillin/Clavulanic	S (16)	R (≥128)	R (≤8)
Piperacillin	R (≥128)	R (≥128)	R (≥128)
Ceftazidime	R (16)	R (16)	S (≤1)
Piperacillin/Tazobactam	S (8)	S (32)	S (≤4)
Cefepime	S (2)	R (32)	S (≤1)
Aztreonam	R (≥64)	R (≥64)	S (≤1)
Imipenem	S (1)	S (≤0.25)	R (4)
Meropenem	S (1)	S (≤0.25)	S (≤0.25)
Amikacin	S (≤2)	S (≤2)	S (≤2)
Gentamicin	R (≥16)	R (≥16)	R (8)
Tobramycin	R (≥16)	R (≥16)	R (8)
Ciprofloxacin	S (≤0.25)	S (≤0.25)	S (1)
Minocyclin	-	S (≤1)	R (≥16)
Trimethoprim/sulfamethoxazole	-	R (≥320)	R (≥320)
Colistin	S (≤0.5)	-	-

Our data were agree and disagree with several studies. Hussain & Hasan (2016) showed that P. aeruginosa was highly resistance to piperacillin but sensitive to other tested antibiotics. Al-Taai & Jasim (2017) reported that the bacterial isolate was more resistant to ceftazidime. However, our data were disagree with some studies such as Pungcharoenkijkul et al. (2021) and Asghar and Ahmed (2018) who reported no ceftazidime resistance.

our datum about K. pneumoniae was in agreement with the findings of Abbas & Jarallah (2016), while disagree with the results of Mohsen et al. (2016). Bacterial resistance to piperacillin, aztreonam, and ceftazidime in our study, was close to the data of a local study done by Al–Zubaidi & Al–Taai (2020), and Al-obadi (2014), While it disagree with the results of the study conducted by Hosseinzadeh et al. (2018) in Iran.

In regards to resistance of P. mirabilis to piperacillin, our results were close to the findings of Kadhim (2017). Regarding to imipenem and tobramycin, our results were closed to Kadhim et al. (2014), whereas, mismatching with the result of Pal et al. (2014). Resistance to trimethoprim– sulfamethoxazole was in agreement with Al-Bassam & Al- Kazaz (2013) in their local study.

The results for S. aureus, study of Foster et al. (2017) reported that S. aureus isolates were resistant to a group of penicillins, including Benzyl-penicillin due to its ability to produce beta-lactamase enzyme, which breaks down the bonds of beta-lactam and thus destroys the activity of the antibiotic and this was in agreement with the results of our current study, table (5).

Table 5: Antibiotic sensitivity of S .aureus using VITEK2 system, the MIC values were determined.

Antibiotics	Sensitive (S), Resistant (R) (MIC- µg/ml)
Benzyl penicillin	R (≥0.5)
Oxacillin	R (≥4)
Gentamicin	S (≤0.5)
Ciprofloxacin	S (≤0.5)
Moxifloxacin	S (≤0.25)
Erythromycin	S (≤0.25)
Clindamycin	S (≤0.25)
Linezolid	S (2)
Teicoplanin	S (≤0.5)
Vancomycin	S (1)
Tetracycline	S (≤1)
Tigecycline	S (≤0.12)
Fusidic Acid	S (≤0.5)
Rifampicin	S (≤0.5)
Trimethoprim/Sulfamethoxazole	S (≤10)

Also**,** the sensitivity of bacterial isolates was investigated to seven antibiotics using Kirby-Bauer method. This methods was used to determine the sensitivity or resistance of the tested isolates (pathogenic and probiotic) to antibiotics alone, and later in combination with probiotics metabolites as shown in table (6), (7)

Table 6: Susceptibility of Gram-negative bacteria to antibiotics

Bacterial spp.	Antibiotics						
	ATM	MEM	CAZ	PRL	SXT	CIP	CN
P. aeruginosa	S	S	R	R	R	S	S
P. mirabilis	S	S	S	R	R	S	R
K. pneumoniae	R	S	R	R	R	S	R

(ATM): Aztereonam, (MEM): Meropenem, (CAZ): Ceftazidime, (PRL): Piperacillin, (SXT): Trimethoprim-sulfamethoxazole, (CIP): Ciprofloxacin, (CN): Gentamicin.

Table 7: Susceptibility of Gram-positive bacteria to antibiotics

Bacterial spp.	Antibiotics					
	OX	CN	CAZ	PRL	SXT	CIP
S. aureus	R	S	_	_	S	S

*(OX):Oxacillin, (CN): Gentamicin, (CAZ): Ceftazidime, (PRL): Piperacillin, (SXT): Trimethoprim-sulfamethoxazole, (CIP): Ciprofloxacin.

Table 8: Antibiotic sensitivity of the tested Lactobacillus strains

Lactobacillus strains	Antibiotic disc					
	PRL	MEM	CAZ	CIP	SXT	ATM
Mixed lactobacilli	31	20	Zero	30	zero	Zero

Susceptibility of Lactobacillus strain to antibiotics : The antibiotics susceptibility pattern of the Lactobacillus strain was examined using the Kirby-Bauer diffusion method on MRS agar. Six antibiotics were tested as shown in table (8).

(PRL): Piperacillin , (MEM): Meropenem, (CAZ): Ceftazidime, (CIP): Ciprofloxacin, (SXT): Trimethoprim-sulfamethoxazole, (ATM):Aztereonam.

Results in table (8), showed high levels of resistance by the tested lactobacillus strain against almost tested antibiotics while were susceptible to piperacillin and ciprofloxacin antibiotics. In this study, ciprofloxacin was selected based on World Health Organization WHO (2004) which showed that ciprofloxacin was more effective antibiotic used to control chronic suppurative otitis media. In this study, ciprofloxacin was combined with the CFS of mixed lactobacilli against the isolated organisms.

The synergistic effect of ciprofloxacin in combination with CFS's Lactobacillus; modified disc diffusion method: The modified disc diffusion method (modified Kirby-Bauer) was performed to determine the nature of antimicrobial reactions in the paper discs, by measuring the diameter of the bacterial inhibition zones.

Table (9) referred to the means of diameters of inhibition zones when ciprofloxacin was combined with CFS. The zones of bacterial growth inhibition in case of antimicrobial combinations were higher than that of using ciprofloxacin alone. It was observed that ciprofloxacin alone, produced an inhibition zone (27 mm) around P. aeruginosa and K. pneumoniae , 35 mm around P. mirabilis and 31 mm around S. aureus. When CFS of mixed Lactobacilli was combined with ciprofloxacin, the diameters of growth inhibition zones for P. aeruginosa, P. mirabilis, K. pneumoniae, and S.aureus were 30, 38, 31, and 33 mm respectively.

Table 9: Effect of combination of CFS with ciprofloxacin against pathogenic bacterial otitis media using disk diffusion assay

Bacterial isolates	Diameters of inhibition zone (mm)		
	Antibiotic alone		Antibiotic & CFS of L.M
P. aeruginosa	CIP	27	30
P. mirabilis	CIP	35	38
K. pneumoniae	CIP	27	31
S. aureus	CIP	31	33

* L.M: Lactobacillus mix *CIP: Ciprofloxacin * CFS: Cell-Free-Supernatant

The results in the present study were in agreement with similar study conducted by Aminnezhad et al. (2016) who showed a significant increase in the diameter of growth inhibition zones of P. aeruginosa by CFS of L. plantarum when combined with antibiotic**.** Furthermore, another study of Isayenko et al. (2020) showed an increase in the diameter of growth inhibition of A. baumannii and S. aureus when the metabolic complexes of Lactobacilli and Saccharomycetes were combined with antibiotics.

Co-aggregation of Lactobacillus strain with isolated bacteria: The co-aggregation assays were evaluated based on the turbidometric methods at 2, 4, and 24 hrs. The highest co-aggregation percentages were identified after 24 hrs incubation. The highest aggregation percentages were reported when mixed lactobacilli was incubated with P. aeruginosa (66.4%). Whereas, the co-aggregation percentages of P. mirabilis, K. pneumoniae, and S. aureus with mixed lactobacilli were as the following: 50.3%, 54.8%, and 61.8%, respectively (table 10).

The results of the current study also showed that the tested mixed lactobacilli was able to co-aggregate with the isolated pathogenic bacteria and the percentages of interaction gradually increased with time and it was the highest after 24 hrs of incubation. These data were matching the data of Hojjati & Falah (2020) who reported a strong co-aggregation (76%) between L. brevis gp104 and S. aureus after 24 hrs of incubation (Al-Dulaimi et al., 2021).

Table 1: The co-aggregation percentages between the bacterial isolates and mixed lactobacilli

Bacterial isolates	Co-aggregation%		
	Time		
	2hrs	4hrs	24hrs
Mixed lactobacilli+ P. aeruginosa	10.3	26.9	66.4
Mixed lactobacilli + P. mirabilis	7.9	23.4	50.3
Mixed lactobacilli + K. pneumoniae	8.01	23.4	54.8
Mixed lactobacilli + S. aureus	8.4	21.1	61.8

The co-aggregation of LAB with the bacterial isolates is important for (i) adherence to the mucosal surfaces of the host, (ii) enhancing the production of inhibitory substances directly to pathogens, (iii) formation of a barrier that prevents colonization by pathogens (Abdulla et al., 2014). Also it is a mechanism that promotes exclusion/competition behavior (Santos et al., 2016).

CONCLUSIONS

According to the results of the present study, we found that Pseudomonas aeruginosa was the common bacteria isolated from ear swabs of chronic suppurative otitis media. The antibiotic susceptibility test revealed that the most active antibiotic against the isolated bacterial otitis media was ciprofloxacin. The mixed lactobacilli possess a significant antibacterial activity against the pathogenic isolate. In addition, higher percentages of co-aggregation was seen between mixed lactobacilli and the bacterial isolates, especially at 24 hrs of incubation. We concluded that probiotic and their metabolites could be utilized, alone or in combination with conventional antibiotics, as the effective and safe strategy for controlling CSOM-associated bacterial infection.

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