Effect of Enterococcus avium and Enterococcus casseliflavus supernatant and pellet on Klebsiella pneumoniae biofilm formation

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ABSTRACT

Enterococcus is cocci shaped, gram positive, oxidase and catalase negative, facultatively anaerobic, and does not produce spores. It is one of the principal genera that belong to the group of ‘lactic acid bacteria’ (LAB), which also includes Streptococcus and Lactobacillus. The study aimed to detect Enterococcus spp. bacteria using molecular diagnostics. Twenty-one isolates (21%) from 100 samples belonging to Enterococcus spp. were obtained. Three isolates belonging to (two) Enterococcus avium (9.52%) one of them isolated from Kaymak and the other from yoghurt, the other one was (one) Enterococcus casseliflavus (4.76%) which isolated from Ricotta (Made by buffalo milk). After diagnosing by Vitek-2 system of Klebsiella pneumoniae bacteria and detecting its biofilm formation by ‘Congo Red Agar’ method with antibiotic test, the effect of the Enterococcus avium and Enterococcus casseliflavus (supernatants and pellets) on its biofilm formation was tested. Moreover, when compare; to the control group, the effect was highly significant (p.0.0069).

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1 Introduction

Lactic Acid Bacteria (LAB) are a category of Gram-positive, do not form spores, anaerobic or facultatively aerobic cocci or rods that create lactic acid as one of the primary fermentation products of the metabolism of carbohydrates [1]. Lactic acid bacteria (LAB) are naturally found in milk and dairy products. In addition, [2] shown that lactic acid bacteria could be isolated from soil, lakes, the gastrointestinal tracts of human and animal. Lactic acid bacteria are classified as whichever ‘homofermenters’ or ‘heterofermenters’ according to the end product of glucose fermentation. Examples of ‘homofermenters’ include the bacterium Enterococcus casseliflavus. The fermentation of glucose results in the production of lactic acid, carbon dioxide, acetic acid, and ethanol by the heterofermenters, the evidence suggests that [3, 4]. Enterococci are an important part of the microorganisms in many cheese products and their metabolic activities contribute to the unique sensory properties associated with them [5].

Klebsiella pneumoniae, often known as K. pneumoniae, is one of the primary agents responsible for the development of infectious illnesses such as ‘urinary tract infections’ (UTI), ‘pneumonia’, ‘intra-abdominal infections’, ‘blood stream infections’ (BSI), ‘meningitis’, and ‘pyogenic liver abscesses’ (‘PLA’) [6]. During an infection, Klebsiella pneumoniae is able to live because it possesses a number of virulence factors, most notably fimbriae, antiphagocytic capsule capsular polysaccharide, (CPS), lipopolysaccharides (LPS), membrane transporters, and siderophores [7]. These virulence factors allow K. pneumoniae to evade the immune system and survive. The capacity of K. pneumoniae to build biofilm protects the pathogen from the immunological responses of the host and also protects it from the effects of antibiotics, hence increasing its ability to survive on epithelial tissues and the surfaces of medical devices [8]. Through the synthesis of enzymes such as ‘Extended Spectrum β-Lactamase’ (‘ESBLs’) and Carbapenemase, it has been discovered that K. pneumoniae may acquire resistance to antibiotics more quickly than the majority of bacteria [9,10].

The propensity of Klebsiella pneumoniae to create biofilms, which are communities of bacteria embedded in an extracellular matrix, is another one of the pathogen’s well-known characteristics. This matrix is made up of DNA, lipopeptides, exopolysaccharides, and proteins [11]. The purpose of the research was to separate and identify naturally occurring lactic acid bacteria that were present in milk and dairy products purchased from a grocery store. In the governorate of Erbil using the usual culture technique, ‘16S rRNA’ gene analysis, and the measurement of the inhibitory impact of LAB on the biofilm of the disease-causing bacterium Klebsiella pneumoniae.

2 Materials and methods

2.1 Sampling:
One hundred different samples were collected from some supermarkets in Erbil governorate of certain areas in the governorate included (Erbil, Bahareke and Pirmam) between 15 of September to 20 of November 2021, samples included commercial food, dairy products, and animal rumen contents, such as human milk (Breast Milk), yoghurt, cheese, cream cheese, milk, yoghurt drink, Kaymak, cream and Ricotta from different sources and company, all the rumen contents was taken from different animals (cow, goats, sheep and buffaloes). Physiological, biochemical, and molecular tests have also been used to diagnose lactic acid bacterial species.

2.2 Preparation of Sample
After adding one gram or one milliliter (according to the type of sample, 1 gram of solid sample or 1 milliliter of watery sample) to 4 ml of ‘de Man, Rogosa, and Sharpe’ (MRS) broth (Oxoid, Basingstoke, UK), the mixture was homogenized by using a vortex mixer. This process was repeated for each sample. The inoculated broth samples were then placed in an anaerobic jar and incubated at ‘37 Cº’ for 24–48’ hours without the presence of oxygen. A loop full of the growing culture broth was plated onto de Man, Rogosa, Sharpe agar (MRSA) plates [12].

2.3 Biochemical test of Lactic Acid Bacteria
The cultures were considered as Lactic Acid Bacteria transferred to laboratory and diagnosed by performing some tests on them such as “Gram staining” and microscopic examination. In addition to growing them on MRS agar medium, and for more accuracy in diagnosis, the colony shape was investigated, additionally colonies were characterized by catalase and Oxidase test.

2.4 Molecular Study

1- DNA Extraction:
Genomic DNA was isolated from bacteria colony of 21 different pure culture petri plate by Bacterial DNA Extraction Kit was manufactured by (BETA Bavarian-Germany).

2- Polymerase Chain Reaction (PCR) Amplification ‘16S’ ribosomal RNA’ (‘16S rRNA’):
The PCR amplification of the ‘16S rRNA’ sectional gene was performed in a reaction mixture containing 50 µl; ‘2x Taq’ DNA Polymerase Master Mix (‘AMPLIQON’ A/S ‘Stenhuugervej 22’), ten Picomol (‘pmol’) of the forward primer ‘16S’ (‘AGA GTT TGA TCC TGG CT C AG’), ten pmol of inverse primer 16S (‘AAG GAG GTG ATC CAG C’), DNase free water, and template DNA (‘Table 1’) by Bioresearch ‘PTC-200’ Gradient thermocycler.
The temperature profile contained step one, which is an initial denaturation at ‘ninetty-five’ degrees Celsius for ‘five’ minutes; step 2, which was followed by thirty-five cycles of a denaturation at ninety-five degrees Celsius for thirty-five seconds; a primer annealing at ‘fifty-eight degrees Celsius’ for ‘forty-five’ seconds; an extension at ‘seventy-two degrees Celsius’ for one minute; and step three, which was an additional extension at 72 degrees Celsius for ten minutes.

3. Visualization of DNA fragments

After thirty minutes in the electric field of electrophoresis, an intercalating dye made of ethidium bromide is added to 1.5 percent melted agarose gel in 1X TBA buffer. The position of bands is identified by observing the gel under UV trans-illuminator [13].

2.5 Antibiotic susceptibility testing of Klebsiella pneumoniae:

These tests were carried out by culture on nutrient agar using the Vittek-2 technique on a total of eight different Klebsiella pneumoniae isolates to determine whether or not they were susceptible to any of Twelve commonly prescribed antibiotics. Using antibiotic sensitivity test by Vithek test (‘AST-GN’), Gram Negative Susceptibility card (AST-N222”) manufactured by Biomerieux Inc Company-USA, which included antimicrobial agents as follows: ‘Piperacillin’ (PRL) 100 μg/disk, ‘Piperacillin/tazobactam’ (PTZ) 100/10 μg/disk, ‘Cefazidime’ (CAZ) 30 μg/disk, ‘Cefepime’ (CPM) 10 μg/disk, ‘Aztreonam’ (AZT) 30 μg/disk, ‘Imipenem’ (IPM) 10 μg/disk, ‘Meropenem’ (MEM) 10 μg/disk, ‘Amikacin’ (AMK) 30 μg/disk, ‘Gentamicin’ (GEN) 10 μg/disk, ‘Tobramycin’ (TOB) 10 μg/disk, ‘Ciprofloxacin’ (CIP) 5 μg/disk, ‘Trimethoprim /sulphamethoxazole’ (SXT) 25 μg/disk.

2.6 Detection of Klebsiella pneumoniae biofilm formation:

2.6.1 ‘Congo Red Agar method’

Klebsiella pneumonia biofilm formation was detected by using Congo-Red Agar’ (CRA) media according to [14]. In order to make CRA medium, thirty-seven gram/Liter of brain heart-infusion broth, fifteen gram/Liter of sucrose, 10 g/L. of agar ‘No. 1’- (“Oxoid, UK”), and 8 gram/Liter of Congo-Red indicator were combined. First, the Congo Red stain was made as a concentrated aqueous solution, and then it was autoclaved at 121 °C for fifteen ‘minutes’ in a separate chamber from the other components of the medium. After that, it was mixed into autoclaved brain heart infusion agar with sucrose at a temperature of 55 °C [15]. (CRA) plates were inoculated with test organisms and then incubated aerobically at thirty-seven degrees Celsius for twenty-four hours. The presence of black colonies with a dry and crystalline nature suggested the formation of biofilm [15].

2.7 Determination of Antibiofilm Activity of LAB against Klebsiella pneumoniae

Lactic acid bacteria that were effectively cultivated from samples as well as (Human Breast milk, Yoghurt, Cream Yoghurt, Cheese, Cream Cheese, Milk, Yoghurt, Drink, Kaymak, Cream and Ricotta) were sub-cultured in fifteen ml tubes containing five ml of (MRS) broth and then incubated for sixteen hours at thirty-seven degrees Celsius. After being allowed to incubate, the LAB cultures were centrifuged at a speed of 10,000 rpm for twenty minutes. This allowed for the separation of the cell free supernatants- (CFS) and the pellets. Isolates of K. pneumoniae were re-cultured in Luria Bertani (LB) broth and then allowed to grow at 37 degrees Celsius for eighteen hours. The next day, after determining that the culture had reached confluence using an OD570 McFarland standard solution, one hundred microliters of the culture was dispensed into each well of a ninety-two-well plate. After this step, 100 microliters of LAB CFS or pellet was added, which was then diluted with MRS medium. The (MRS) broth medium used as the negative-control for this experiment. Plates were kept in an incubator at 37 degrees Celsius for a period of 16 hours [16]. The effectiveness of crystal violet in inhibiting biofilm was evaluated [16]. The cultures were thrown away, and the plates were cleaned three times by being completely submerged in water. 125 microliter of a crystal violet solution containing 0.1 percent was added to each well, and then the plates were incubated at room temperature for 15 minutes (RT). To get rid of any remaining stain on the plate, the staining solution was discarded, and it was rinsed three times with sterile water. After incubation for 15 minutes at room temperature, the optical density (OD) of both the supernatant- and the pellet was measured at - (570 nm) using an enzyme-linked immunosorbent assay (ELISA) reader. The resultant biofilms were diluted with 125 microliters at a concentration 30 percent acetic acid in each well. After removing the planktonic cells, the ability to form biofilm was evaluated based on the OD570 values of the remaining isolates. Isolates with an OD570 value between 0.065 and 0.130 were categorized as having a low capacity for biofilm formation; those with an OD570 value between 0.130 and 0.259 were considered to be capable of moderate biofilm formation; and those with an OD570 value more than 0.259 were classified to be highly capable biofilm producers [17]. Because the supernatants and pellets produced by two different LAB isolates had the lowest OD570 values, which indicated that they had the highest inhibitory effects.
2.8 Statistical analysis
A one-way analysis of variance was carried out in order to determine how the CFS and pellet of LAB affected the in vitro biofilm inhibition test and the viability test (optical density). After conducting ANOVAs, we employed Tukey's test to do multiple pairwise comparisons between the different treatment levels. The threshold of significance was established at $P$ value less than 0.0069 for each comparison. GraphPad Prism 8 was used for all of the statistical analyses that were performed.

3 Results

3.1 Isolation, Phenotypic Characterization
Enterococcus species were isolated by growing them on MRSA medium, and the morphology of each colony was analyzed to determine its characteristics as showed in (Table 2) and (Figure 3).

### Table 2: Samples Resource location, Bacteria Species, Cell Shape, Colony shape and Colony Color

<table>
<thead>
<tr>
<th>Samples Resource</th>
<th>location</th>
<th>Bacteria Species</th>
<th>Cell Shape</th>
<th>Colony shape</th>
<th>Colony Color</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kaymak</td>
<td>Dugondan</td>
<td>Enterococcus avium</td>
<td>Coci</td>
<td>Smooth</td>
<td>White</td>
</tr>
<tr>
<td>Buffalo Ricotta</td>
<td>Erbil</td>
<td>Enterococcus casseliflavus</td>
<td>Coci</td>
<td>Smooth</td>
<td>White</td>
</tr>
</tbody>
</table>

According to the findings of Holdeman, Moore, and Cato's study [18], the isolates that have been incubated and developed on selective medium were then put through a gram staining test along with a catalase test. These features were found in the bacteria that were tested. On the surface of the MRS agar, each isolated isolate manifested as a spherical colony that was opaque, creamy, or milky white in color. According to the findings of our research, there were a total of 21 isolates of Enterococcus, all of which were classified as belonging to two distinct species (Table 2 & Figure 1).

The (Figure 1) represents: Enterococcus spp. Number and percentage of isolated enterococci of milk and various dairy products, and the percentages were varied according to the various milk products.

### Table 3: LAB isolated number and percentage of Enterococcus avium and Enterococcus casseliflavus from various milk and milk products.

<table>
<thead>
<tr>
<th>Genus</th>
<th>Raw Milk</th>
<th>Kaymak</th>
<th>Cheese</th>
<th>Ricotta</th>
<th>Yoghurt</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Enterococcus avium</em></td>
<td>-</td>
<td>1 (4.76%)</td>
<td>-</td>
<td>-</td>
<td>1 (4.76%)</td>
</tr>
<tr>
<td><em>Enterococcus casseliflavus</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1 (4.76%)</td>
<td>-</td>
</tr>
</tbody>
</table>

Fig. 1: isolated number and percentage of Enterococcus Sun. from various milk and milk

In (Table 3) Two species have been diagnosed *Enterococcus avium* (2n) and *Enterococcus casseliflavus* (1n), the number and percentage identified in (Figure 2).

### Table 3: Samples Resource location, Bacteria Species, Cell Shape, Colony shape and Colony Color

<table>
<thead>
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<tr>
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<td>Erbil</td>
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<td>Coci</td>
<td>Smooth</td>
<td>White</td>
</tr>
</tbody>
</table>

Fig. 2: LAB isolated number and percentage of Enterococcus avium and Enterococcus casseliflavus from various milk and milk products.

After the culture diagnosis (Figure 3) there is a difference between the two species of bacteria in shape, color and texture as well as (Figure 4) represents as a result of the dye, it was revealed that it is a Gram-positive bacterium under a light microscope.
When examined under the microscope after being stained with gram stain, they appeared as rods or spheres of a purple color, indicating that they were gram positive bacteria as seen in (Figure 4) [19].

Fig. 3: Colony shape and color of Enterococcus Spp.

Fig. 4: Enterococcus avium and Enterococcus casseliflavus Gram staining

3.2 Biochemical tests for LAB: The biochemical tests for 2 species isolates of lactic acid bacteria showed that 21 (100%) are negative for oxidase and catalase test (Table 4). These results agreed with most of the studies were show that these bacteria have all mentioned properties [20].

Table 4: Biochemical tests for LAB (Oxidase and Catalase test)

<table>
<thead>
<tr>
<th>Biochemical tests</th>
<th>N. of Enterococcus isolates</th>
<th>N and % of (+)</th>
<th>N and % of (-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidase test</td>
<td>21</td>
<td>0 (0%)</td>
<td>21 (100%)</td>
</tr>
<tr>
<td>Catalase test</td>
<td>21</td>
<td>0 (0%)</td>
<td>21 (100%)</td>
</tr>
</tbody>
</table>

3.3 Molecular Detection of LAB

3.3.1 Genomic DNA isolated

Genomic DNA was isolated from 2 different pure culture bacterial species; Each specimens was extracted by PGA Bacterial DNA Extraction Kit was manufactured by BETA Bayern-Germany. The isolated DNA was electrophorized in 1% Agarose gel (Figure 5).

Fig. 5: Genomic DNA isolated from 15 isolates of bacteria

3.3.2 ‘PCR’ amplification of partial ‘16S rRNA’ gene

Ribosomal gene specific primers were designed for the using the sequences of ‘16S –rRNA’ Synthesized by Micro-Gene Company (South Korea) The primers could yield a band ~1200bp. The PCR product was electrophoresed and visualized by 1.5% Agarose gel. - (Figure 6).
Fig. 6: PCR amplification of partial 16S rRNA gene from 3 bacterial colonies which wells include M; Ladder (1500 bp-100 bp) lanes from 1-15 indicate 16S rRNA gene with 1200 bp amplified and C indicate negative control without band.

Table 5: Enterococcus avium and Enterococcus casseliflavus After molecular diagnosis.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Bacterial Identified</th>
<th>Source</th>
<th>Accession Numbers</th>
<th>Query Cover %</th>
<th>Identic Number %</th>
<th>Accession Number of BLAST Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Enterococcus avium</td>
<td>Kaymak</td>
<td>ON651138</td>
<td>100</td>
<td>100</td>
<td>CP024590</td>
</tr>
<tr>
<td>2</td>
<td>Enterococcus casseliflavus</td>
<td>Cheese</td>
<td>ON651440</td>
<td>100</td>
<td>100</td>
<td>MT613602</td>
</tr>
</tbody>
</table>

3.4 Klebsiella pneumonia identification by Vitek-2
In this study, a total of 8 different clinical specimens were collected from some hospitals in Erbil city. Including Sputum (3), blood (1), Urine (2), wound (1) and Nasal Swab (1) samples. They were diagnosed with a Vitek-2 system.
These tests were done by culture Klebsiella pneumoniae on the nutrient agar using Vitek-2 system. Using antibiotic sensitivity test by Vitek test (AST-GN) Gram Negative Susceptibility card (AST-N222’) manufactured by Biomerieux Inc Company-USA, which included antimicrobial agents as follows: ‘Piperacillin’ (PRL) 100 μg/disk, ‘Piperacillin/tazobactam’ (PTZ) 100/10 μg/disk, ‘Cefazidime’ (CAZ) 30 μg/disk, ‘Cefepime’ (CM) 10 μg/disk, ‘Aztreonam’ (AZT) 30 μg/disk, ‘Imipenem’ (IPM) 10 μg/disk, ‘Meropenem’ (MEM) 10 μg/disk, ‘Amikacin’ (AMK) 30 μg/disk, ‘Gentamicin’ (GEN) 10 μg/disk, ‘Tobramycin’ (TOB) 10 μg/disk, ‘Ciprofloxacin’ (CIP) 5 μg/disk, ‘Trimethoprim /sulphamethoxazole’ (SXT) 25 μg/disk. The (Table 6) illustrates that all isolates vary in their response to the use of antimicrobial agents and the highest sensitivity percentage was to Amikacin (87.5%), ‘Imipenem’ (75%), Meropenem (75%) and Gentamicin (62.5%) respectively, and less sensitivity recorded for Piperacillin, Cefazidime, Cefepime, and Aztreonam (12.5%), Trimethoprim/sulphamethoxazole (25%).

Table 6: Antibiotic susceptibility test for Klebsiella pneumoniae

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>Disk content μg/disk</th>
<th>Number of Sensitive %</th>
<th>Number of Resistant %</th>
<th>Number of Intermediate %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Piperacillin</td>
<td>100</td>
<td>1(12.5%)</td>
<td>7(87.5%)</td>
<td>0</td>
</tr>
<tr>
<td>Piperacillin/tazobactam</td>
<td>100/10</td>
<td>4(50%)</td>
<td>3(37.5%)</td>
<td>1(12.5%)</td>
</tr>
<tr>
<td>Cefazidime</td>
<td>30</td>
<td>1(12.5%)</td>
<td>7(87.5%)</td>
<td>0</td>
</tr>
<tr>
<td>Cefepime</td>
<td>10</td>
<td>1(12.5%)</td>
<td>7(87.5%)</td>
<td>0</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>30</td>
<td>1(12.5%)</td>
<td>7(87.5%)</td>
<td>0</td>
</tr>
<tr>
<td>Imipenem</td>
<td>10</td>
<td>6(75%)</td>
<td>1(12.5%)</td>
<td>1(12.5%)</td>
</tr>
<tr>
<td>Meropenem</td>
<td>10</td>
<td>6(75%)</td>
<td>2(25%)</td>
<td>0</td>
</tr>
<tr>
<td>Amikacin</td>
<td>30</td>
<td>7(87.5%)</td>
<td>1(12.5%)</td>
<td>0</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>10</td>
<td>5(62.5%)</td>
<td>2(25%)</td>
<td>1(12.5%)</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>10</td>
<td>3(37.5%)</td>
<td>2(25%)</td>
<td>3(37.5%)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>5</td>
<td>4(50%)</td>
<td>4(50%)</td>
<td>0</td>
</tr>
<tr>
<td>Trimethoprim/ sulphamethoxazole</td>
<td>25</td>
<td>2(25%)</td>
<td>6(75%)</td>
<td>0</td>
</tr>
</tbody>
</table>

(S) sensitive (R) resistance (I) Intermediate
3.5 Klebsiella pneumonia biofilm formation assay

In this study, all eight isolates of Klebsiella pneumoniae were biofilm positive on Congo red agar as in (Figure 7) and (Table 7).

The biofilm-positive bacteria resulted in the formation of black colonies, whereas the biofilm-negative strains resulted in the formation of pink colonies.

Table 7: biofilm formation on congo-red agar

<table>
<thead>
<tr>
<th>Isolated Bacteria</th>
<th>Source</th>
<th>Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Klebsiella pneumonia</td>
<td>Swab</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Wound</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sputum 1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Urine 1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sputum 2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sputum 3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Blood</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Urine 2</td>
<td></td>
</tr>
</tbody>
</table>

3.6 Determination of Antibiofilm Activity of Enterococcus casseliflavus and Enterococcus avium against Klebsiella pneumonia:

Both of these isolates were effectively extracted from their respective whole-sample sources. Both the supernatants (CFS) and the pellets (Figure 9) of the isolates were put through a series of tests to determine whether or not they had the potential to prevent the formation of K. pneumoniae biofilms.

The absorbance following crystal violet staining was used to test whether or not the isolated exhibited anti-biofilm activity. K. pneumoniae that had been cultivated in the absence of Enterococcus casseliflavus and Enterococcus avium showed an increased capacity to produce a strong biofilm. The optical density measurements of Klebsiella pneumoniae was 0.377 before mixed with LAB pellet and supernatant (Table 8).

Table 8: Effect of Enterococcus casseliflavus and Enterococcus avium supernatant and pellet on Klebsiella pneumonia biofilm formation

<table>
<thead>
<tr>
<th>Name of Probiotic Bacteria</th>
<th>Mean and biofilm Optical Density measurement</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Supernatant</td>
<td>Pellet</td>
</tr>
<tr>
<td>Enterococcus avium</td>
<td>0.056</td>
<td>0.060</td>
</tr>
<tr>
<td>Enterococcus casseliflavus</td>
<td>0.050</td>
<td>0.057</td>
</tr>
<tr>
<td>Control (mean)</td>
<td>0.377</td>
<td></td>
</tr>
</tbody>
</table>

On the other hand, (Figure 8) represents the significant effect of two species of bacteria (supernatant and pellet) on the formation of a biofilm of Klebsiella pneumoniae bacteria.

Fig. 7: Biofilm formation by congo-red agar

Fig. 8: Pellet and supernatant of Enterococcus avium and Enterococcus casseliflavus effect on biofilm (p. 0.0069).
4 Discussion

4.1 Isolation, Phenotypic Characterization

Enterococcus species were isolated by utilizing de Man, Rogosa and Sharpe agar (MRS) medium, and the morphology of each colony was analyzed to determine its characteristics. According to the results of the microscopic examination, it was determined that all of the colonies had a round shape, a convex surface, a dimension of 1-2 millimeters, and a color that was either cream or milky white as in (Figure 3). Our result agreed by [18]. Based on the current study, according to the findings of our investigation according and molecular diagnosis after culture, a total of 21 (21%) Enterococcus spp. isolates and categorized into 2 different spp., (2) Enterococcus avium (9.52%) one from Kaymak and the other one from yoghurt and (1) Enterococcus casseliflavus (4.76%) from Ricotta made by (Buffalo milk) (Table 3 & Figure 2).

In the [21] study, the percentage found for Enterococcus spp. was only 6 (18.75%) from the 32 samples analyzed. This is a concerning result for this controversial genus among lactic acid bacteria because of the pathogenicity of various species to humans [22], and their growth in thermally treated milk and refrigerated milk [23].

According to the [24] A total of fifty food samples were taken from various sources (twenty of which were dairy items) and collected in Baghdad. All of the E. faecium and E. avium that were recovered from the twenty samples of dairy products, E. faecium was the most prevalent species that was recovered from the samples (62.5 percent).

Depending on current study as showed in (Figure 4) Enterococcus avium and Enterococcus casseliflavus, they suggested that gram-positive bacteria were present due to their appearance under the microscope, which was of a purple color and spherical. Compatible with result of [25] agreed with that Enterococcus casseliflavus are Coccolid cells usually in pairs or short chains motile. Colonies that form on the surface of (MRS) agar are round, smooth, and entire creamy or milky white as showed in (Figure 3), this was the agreement of [12], Enterococcus avium ovoid cells elongated in the direction of the chain, mostly in pairs or short chains, Nonmotile.

In study of [26] the LAB isolates were: Enterococcus spp. had 2 (11.1%), these isolates were obtained from fermented milk and Yoghurt from various locations in Alnajaf. These results are not close to the results of our research, where the number of diagnosed isolates was 21 as in (Figure 1), distributed in different percentages on milk products such as Kaymak, Cheese, Ricotta and Yoghurt, 19.04, 38.09, 23.80 and 19.04 respectively.

4.2 Biochemical tests for LAB

The biochemical tests for Enterococcus species isolates of lactic acid bacteria showed that 21 isolates (100%) are negative for oxidase and catalase test, (Table 4). These results agreed with most of the studies were show that these bacteria have all mentioned properties [12&20].

4.3 Molecular Detection of LAB

4.3.1 Genomic DNA isolated

Genomic DNA was isolated from 21 different pure culture bacterial species; Each specimens was extracted by PGA Bacterial DNA Extraction Kit was manufactured by BETA Bayern-Germany. The isolated DNA was electrophorized in 1% Agarose gel (Figure 5).

4.3.2 PCR amplification of partial 16S rRNA gene

Ribosomal gene specific primers were designed for the using the sequences of 16S rRNA synthesized by Micro-Gene Company (South Korea) the primers could yield a band ~1200bp. The PCR product was electrophoresed and visualized by 1.5% Agarose gel. (Figure 6).

The general bacterial gene 16S rRNA was confirmed by PCR detection of isolated bacteria, as shown in (Figures 5&6) and (Table 5). The results suggest that presences of the bacteria may be detected by identifying the genes 16s rRNA in a variety of different sources. PCR products for 16s rRNA-based primers produced bands on agarase gel that corresponded to a ~1200 base pair product when compared to the molecular ladder. Additionally, PCR products for 16S rRNA-based primers produced bands on agarose gel that corresponded to a ~1200 base pair product.

In the current investigation, the PCR technique was used to identify the isolates, and a total of 21 different Enterococcus species were identified, we identified (2) Enterococcus avium (9.52%), one from Kaymak and other from yoghurt, and (1) 4.76% Enterococcus casseliflavus from Ricotta of 21 samples (Table 3).

In the study of [27] used polymerase chain reaction to identification of Enterococcus spp., There were a total of one hundred and ten samples isolated from - cheese from a selected border region of ‘Slovakia’ with ‘Hungary’. [27] discovered three bacterial species that were present: ‘E. faecium’ (12), ‘E. faecalis’ (28), and ‘E. durans’ (12).

In addition to ‘E. faecium’ (52.6 percent), ‘E. durans’ (17.7 percent), ‘E. hirae’ (16.4 percent), and ‘E. faecalis’ (12.8 percent), it has been also reported that these types of bacteria are often found in Serbian cheeses [28].

4.4 ‘Klebsiella pneumonia’ identification by Vitek-2 And ‘Antibiotic Resistance’

All of the isolates had different responses to the use of antimicrobial drugs as in (Table 6), and it shows that amikacin (87.5%) had the greatest percentage of sensitivity out of all of them, Imipenem (75%), Meropenem (75%) and Gentamicin (62.5%) respectively, and less sensitivity recorded for Piperaclillin, Ceftazidime, Cefepime, and Aztreonam (12.5%), Trimethoprim/sulphamethoxazole (25%).

The results of our study were similar with Harith et al., [29] dedicate that the highest resistance percentages of the K. pneumoniae isolates were found
to ampicillin (81.5%), tetracycline (74.1%) and trimoxoazole (74.1%). Moreover, 59.3%, 51.9%, 48.1%, 40.7%, 40.7%, 33.3% and 22.2% of the *K. pneumoniae* isolates were resistant to nitrofurantoin, cefotaxime, Tobramycin, nalidixic acid, ciprofloxacin, ceftriaxon and Gentamicin, respectively.

The majority of the *K. pneumoniae* that were identified in the research conducted by [30] exhibited resistance to a broad spectrum of antibiotics. *K. pneumoniae* showed only a good sensitivity to meropenem (98.60 percent), amikacin (95.80 percent), and piperacillin-tazobactam among biofilm producer isolates (90.00 percent). In contrast, *K. pneumoniae* from non-biofilm producing isolates shown excellent sensitivity to meropenem, levofloxacin, amikacin, piperacillin-tazobactam, and ciprofloxacin, with corresponding levels of sensitivity of 100 percent, 95.83 percent, 91.67 percent, 87.50 percent, and 86.67 percent.

### 4.5 Biofilm formation assay and Determination of Antibiofilm Activity of *Enterococcus casseliflavus* and *Enterococcus avium* against *Klebsiella pneumoniae*

In this study, all eight isolates of *Klebsiella pneumoniae* were biofilm positive on Congo red agar as in (Figure 7) and (Table 7). Infectious bacteria such as *Klebsiella pneumoniae* are rapidly developing strains that are resistant to multiple drugs, a phenomenon known as multidrug resistance (MDR). These bacteria routinely present a significant risk to patients as a result of an increased mortality rate brought on by diminished therapeutic efficacy. Through the synthesis of enzymes such as Extended Spectrum β-Lactamase (ESBLs) and Carbapenemase, it has been discovered that *K. pneumoniae* may acquire resistance to antibiotics more quickly than the majority of bacteria [9,10]. In addition to this, it is famous for its capacity to build biofilms, which are communities of bacteria that are encased in an extracellular matrix. This matrix is made up of DNA, lipopeptides, exopolysaccharides, and proteins [11]. On this basis, the effect of lactic acid bacteria on multi drug resistance *Klebsiella pneumoniae* isolated from sputum was tested, and the results were high when compared with Control represented in the (Table 8) and (Figure 8). In the study of [31] demonstrates that out of forty isolates of *E. coli* and six of *Klebsiella* spp. examined for biofilm formation using Congo red agar (CRA) technique, twenty-six (65 percent) isolates of *E. coli* formed black colonies, while four (66.67 percent) isolates of *Klebsiella* spp. produced black colonies. However, only fourteen (35 percent) of the *E. coli* isolates and 3 (50 percent) of the *Klebsiella* spp. isolates had colonies that were black in color and had a dry crystalline structure. Both of these characteristics are suggestive of the development of biofilm. It was similar to our results. Both the supernatants (CFS) and the pellets of the isolates were put through a series of tests to determine whether or not they had the potential to prevent the formation of *K. pneumoniae* biofilms. The absorbance following crystal violet staining was used to test whether or not the isolated exhibited anti-biofilm activity. *K. pneumoniae* that had been cultivated in the absence of *Enterococcus casseliflavus* and *Enterococcus avium* shown a significant increase in its capacity to generate a strong biofilm. The optical density measurements of *Klebsiella pneumonia* was 0.377 before mixed with LAB pellet and supernatant where the result was 0.5-0.6 after treatment with bacteria lactic acid, the effect of the *Enterococcus avium* and *Enterococcus casseliflavus* (supernatants and pellets) to the control group, was highly ‘significant’ (p. value 0.0069), (Table 8 & Figure 8). Our results were similar and close to the results of the researcher [6], who verified result revealed that addition of cell free supernatant (CFS) as well as pellet of forty isolates LAB on to *K. pneumoniae* culture might decrease their culture absorbance.

Lactic Acid Bacteria exhibit inhibitory effect against *K. pneumoniae*, the acidity of the medium rises as probiotic bacteria multiply, which kills pathogenic bacteria since these bacteria were predisposed to be killed by acidic environments due to their inherent sensitivity to them. [32]. This observation may be associated with the production of inhibitory substances by ‘probiotics’ such as ‘hydrogen peroxide’, ‘lactic acid’, ‘diacetyl’, ‘carbon dioxide’, and ‘bacteriocin’ that have an ‘antimicrobial’ effect. In a study conducted by [33], the researchers found that the *Lactobacillus* strains derived from yoghurt demonstrated antagonistic activities against *E. coli*, *S. aureus*, *K. pneumonia*, *E. faecalis*, *Pseudomonas- aerogenosa*, *Shigella* spp., and *S. typhi*.

In the study of [17] investigated the inhibitory impact of *L. fermentum* acid supernatant on the production of biofilm in addition to its influence on the growth of biofilm. In associative cultures, the strain that was utilized was able to restrict the growth of *Klebsiella pneumonia*, because it produced large quantities of lactic acid and hydrogen peroxide.

### Conclusion

This study was conducted with the intention of providing a clearer characterization of the anti-biofilm activities that are associated with [microbial] crosstalk. According to the findings of this study, the strains of *Enterococcus casseliflavus* and *Enterococcus avium* that were isolated have the ability to prevent the formation of biofilms and also inhibit the growth of multidrug-resistant *Klebsiella pneumoniae*. Both the supernatants (CFS) and the pellets of the isolates were preventing the formation of *K. pneumoniae* biofilms. *K. pneumoniae* in the absence of *Enterococcus casseliflavus* and *Enterococcus avium* generate a strong biofilm. The optical density measurements of *Klebsiella pneumonia* was 0.377 before mixed with LAB pellet.
and supernatant where the result was 0.05-0.06 after treatment with lactic acid bacteria. Our results revealed that addition of ‘cell free supernatant’ (CFS) as well as ‘pellet’ of Enterococcus casseliflavus and Enterococcus avium on to ‘K. pneumoniae’ culture might decline their ability to biofilm formation.

References
[29] Harith et al., (2012) dedicate that Three hundred and twelve specimens of urine, wounds and sputum were collected from patients attending five hospitals in Baghdad, all K. pneumoniae isolates assayed for the production of biofilm. Twenty-two (39.29%) isolates were high producers while 34 (60.71%) isolates were good producers and none of the testes isolates were poor producers.