Tikrit Journal of Pure Science Vol. 28 (3) 2023

TJPS

https://doi.org/10.25130/tjps.v28i3.1420

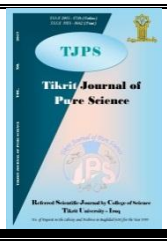
IASJ

المجلات الاكاديمية العراقية

Tikrit Journal of Pure Science

ISSN: 1813 – 1662 (Print) --- E-ISSN: 2415 – 1726 (Online)

Journal Homepage: http://tjps.tu.edu.iq/index.php/j



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

Keywan jebrail Chicho, Sawsan Mohammed Sorchee

Department of Biology, College of Education, Salahaddin University-Erbil, Erbil, Kurdistan Region, Iraq

ARTICLE INFO.

Article history:

-Received: 8 / 7 / 2022

-Received in revised form: 3 / 8 / 2022

-Accepted: 28 / 8 / 2022

-Final Proofreading: 12 / 6 / 2023

-Available online: 25 / 6 / 2023

Keywords: *Enterococcus avium, Enterococcus casseliflavus*, Antibiofim. *Klebsiella pneumoniae*

Corresponding Author:

Name: Keywan jebrail Chicho

E-mail:

Keywan.chichoo@student.su.edu.krd

Sawsan.ahdulla@su.edu.krd

Tel:

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).

تأثير بكتيريا طاف والحبيبات *Enterococcus casseliflavus* و *Enterococcus avium* على تكوين الغشاء الحيوي لبكتريا الرئوية *Klebsiella pneumoniae*

كيوان جبرائيل جيج ، سوسن محمد عبدالله السورجي

كلية التربية ، جامعة صلاح الدين ، اربيل ، كوردستان العراق

الملخص

المكورات المعوية مكورة الشكل ، موجبة الجرام ، أوكسيديز وكتاليز سالب، لاهوائية اختيارية ، ولا تنتج السبورات. إنه أحد الأجناس الرئيسية التي تنتمي إلى مجموعة "بكتيريا حمض اللاكتيك" (LAB)، والتي تشمل أيضًا المكورات العقدية و *Lactobacillus*. هدفت الدراسة إلى الكشف عن *Enterococcus* spp. باستخدام التشخيص الجزيئي احدى وعشرون عزلة (21٪) من مئة عينة تنتمي إلى *Enterococcus* spp حيث تم الحصول على النوع (عزلتين) تنتمي إلى (9.52 *Enterococcus avium*٪ (واحدة منها معزولة من القيمر والأخرى من اللبن ، والنوع الاخر (عزلة واحدة) (4.76 *Enterococcus casseliflavus*٪(معزولة من Ricotta (مصنوع من حليب الجاموس).و بعد التشخيص عن طريق نظام Vitek-2 لبكتيريا *Klebsiella pneumoniae* واكتشاف تكوين الغشاء الحيوي الخاص بها بواسطة طريقة "Congo Red Agar" كونكو ريد اكار مع اختبار المضادات الحيوية، علاوة على ذلك تم اختبار تأثير *Enterococcus avium* و *Enterococcus casseliflavus* (طاف وحبيبات) على تكوين الغشاء الحيوي، وعند المقارنة مع مجموعة السيطرة، كان التأثير معنويا مرتفعا (p.0.0069.

https://doi.org/10.25130/tjps.v28i3.1420

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 Bayern-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 'Stenhuggervej 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 CC'), DNase free water, and template DNA ('Table 1') by Bioresearch 'PTC-200' Gradient thermocycler.

https://doi.org/10.25130/tjps.v28i3.1420

Table 1: 16S rRNA PCR Amplification Reagents

No.	PCR components	Concentration	Volume (µl)
1	Master Mix	2x	25
2	Forward Primer	20 Pmol	3
3	Reverse Primer	20 Pmol	3
4	DNase free Water	-	15
5	Template DNA	50ng/µl	4
Total			50

The temperature profile contained step one, which is an initial denaturation at 'ninety-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 Vitek-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 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, 'Ceftazidime' (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, 'Trimethoprime /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 ºC 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 ºC [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 pneomoniae*

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 OD_{570} 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 OD_{570} values of the remaining isolates. Isolates with an OD_{570} value between 0.065 and 0.130 were categorized as having a low capacity for biofilm formation; those with an OD_{570} value between 0.130 and 0.259 were considered to be capable of moderate biofilm formation; and those with an OD_{570} 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 OD_{570} values, which indicated that they had the highest inhibitory effects.

https://doi.org/10.25130/tjps.v28i3.1420

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

Samples Resource	location	Bacteria Species	Cell Shape	Colony shape	Colony Color
Kaymak	Dugondan	*Enterococcus avium*	Cocci	Smooth	White
Buffalo Ricotta	Erbil	*Enterococcus casseliflavus*	Cocci	Smooth	White

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.

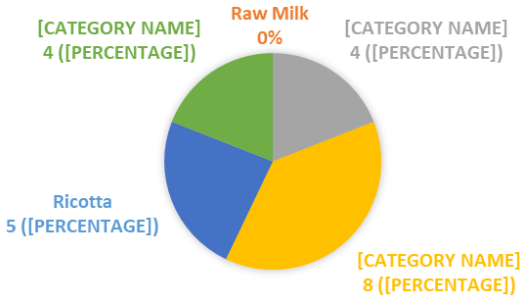


Fig. 1: isolated number and percentage of *Enterococcus* Spp. from various milk and milk

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

Genus	Raw Milk	Kaymak	Cheese	Ricotta	Yoghurt
Enterococcus avium	-	**1 (4.76%)**	-	-	**1 (4.76%)**
Enterococcus casseliflavus	-	-	-	**1 (4.76%)**	-

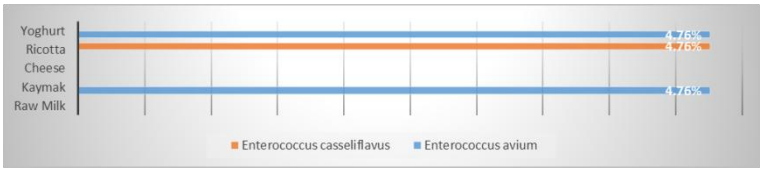
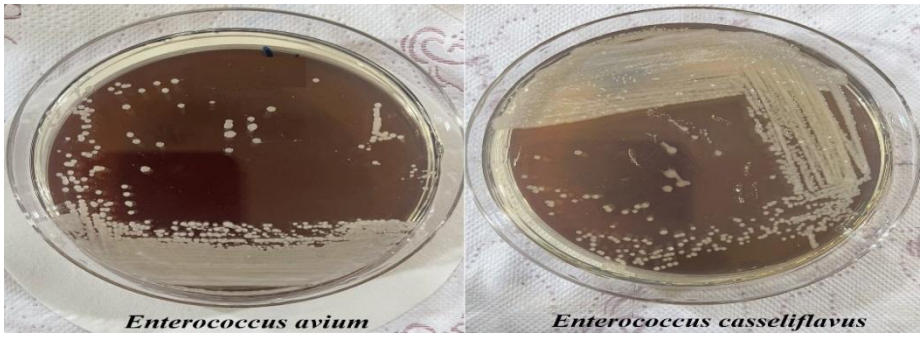


Fig. 2: LAB isolated number and percentage of *Enterococcus avium* and *Enterococcus casseliflavus* from various milk and milk products.

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.



https://doi.org/10.25130/tjps.v28i3.1420

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

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].

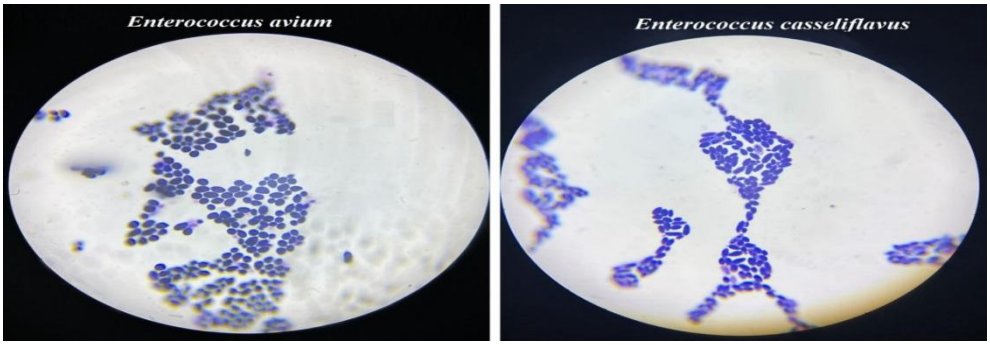


Fig. 4: *Enterococcus avium* and *Enterococcus casseliflavus* Gram staining

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)

Biochemical tests	N. of *Enterococcus* isolates	N and % of (+)	N and % of (-)
Oxidase test	21	0 (0%)	21 (100%)
Catalase test	21	0 (0%)	21 (100%)

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).

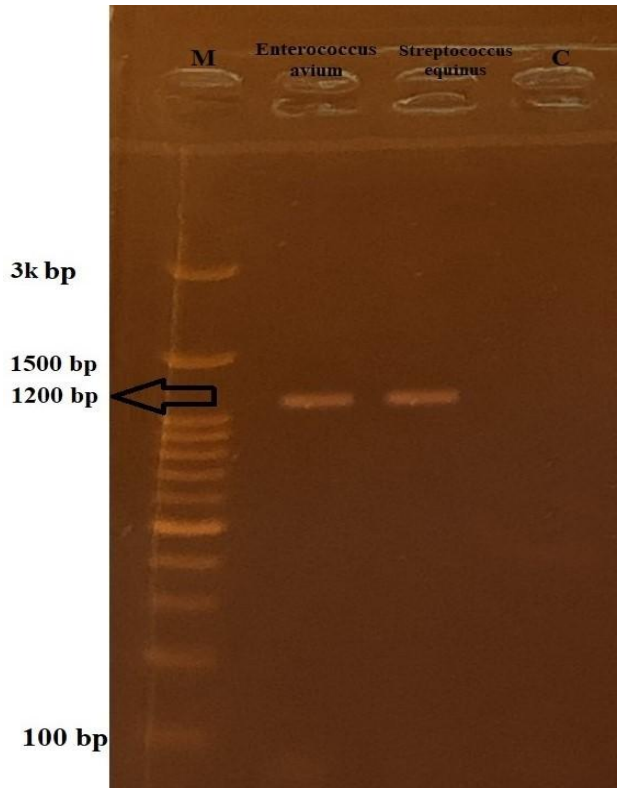


Fig. 5: Genomic DNA isolated from 15 isolates of bacteria

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).

https://doi.org/10.25130/tjps.v28i3.1420

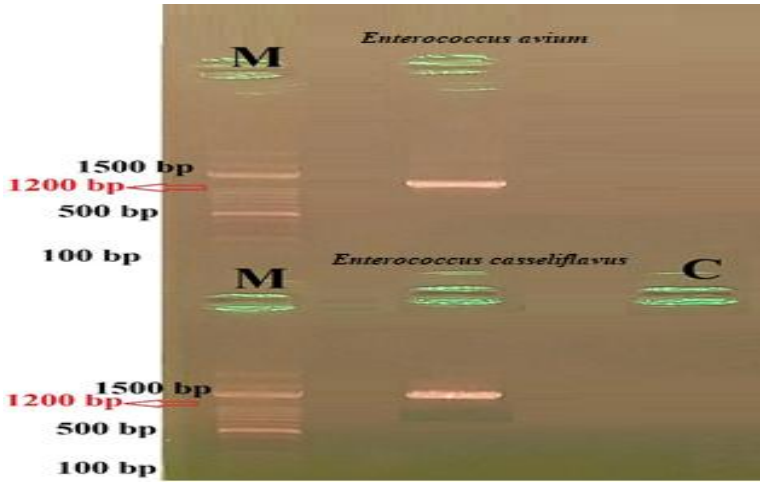


Fig. 6: PCR amplification of partial 16S rRNA gene from 3 bacterial colonies which wells include M; Ladder (1500 bp-100 bp) lanes from 1-15 indicate 16S rRNA gene with 1200 bp amplified and C indicate negative control without band.

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

Samples	Bacterial Identified	Source	Accession Numbers	Query Cover %	Identic Number %	Accession Number of BLAST Identification
1	*Enterococcus avium*	Kaymak	ON651138	100	100	CP024590
						MT613602
2	*Enterococcus casseliflavus*	Cheese	ON651440	100	100	KX953853
						MN213357

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, 'Ceftazidime' (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, 'Trimethoprime /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, Ceftazidime, Cefepime, and Aztreonam (12.5%), Trimethoprime/ sulphamethoxazole (25%).

Table 6: Antibiotic susceptibility test for *Klebsiella pneumoniae*

Antimicrobial agent	Disk content µg/disk	Number of Sensitive %	Number of Resistant %	Number of Intermediate %
Piperacillin	100	1(12.5%)	7(87.5%)	0
Piperacillin/tazobactam	100/10	4(50%)	3(37.5%)	1(12.5%)
Ceftazidime	30	1(12.5%)	7(87.5%)	0
Cefepime	10	1(12.5%)	7(87.5%)	0
Aztreonam	30	1(12.5%)	7(87.5%)	0
Imipenem	10	6(75%)	1(12.5%)	1(12.5%)
Meropenem	10	6(75%)	2(25%)	0
Amikacin	30	7(87.5%)	1(12.5%)	0
Gentamicin	10	5(62.5)	2(25%)	1(12.5%)
Tobramycin	10	3(37.5%)	2(25%)	3(37.5%)
Ciprofloxacin	5	4(50%)	4(50%)	0
Trimethoprime/ sulphamethoxazole	25	2(25%)	6(75%)	0

(S) sensitive (R) resistance (I) Intermediate

https://doi.org/10.25130/tjps.v28i3.1420

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.

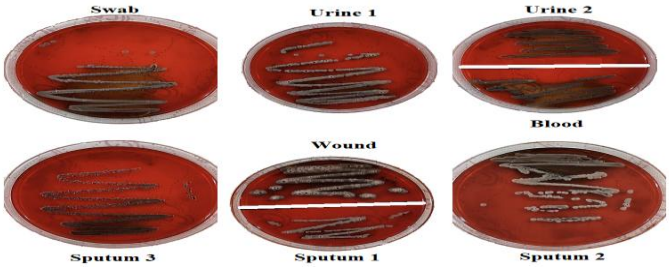


Fig. 7: biofilm formation by congo-red agar)

Table 7: biofilm formation on congo-red agar

Isolated Bacteria	Source	Positive
Klebsiella pneumoniae	Swab	+
	Wound	
	Sputum 1	
	Urine 1	
	Sputum 2	
	Sputum 3	
	Blood	
	Urine 2	

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* shown an increased capacity to produce a strong biofilm. The optical density measurements of *Klebsiella pneumonia* 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

Name of Probiotic Bacteria	Mean and biofilm Optical Density measurement		*p*-value
	Supernatant	Pellet	
Enterococcus avium	**0.056**	**0.060**	**0.0069****
Enterococcus casseliflavus	**0.050**	**0.057**	
Control (mean)	**0.377**		

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.

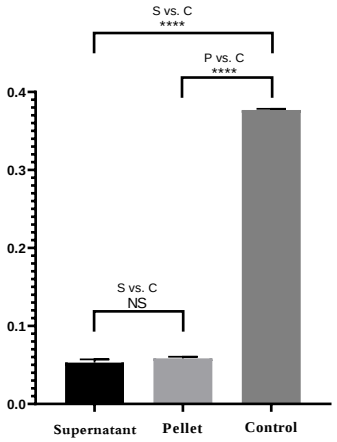


Fig. 8: Pellet and supernatant of *Enterococcus avium* and *Enterococcus casseliflavus* effect on biofilm (p. 0.0069).

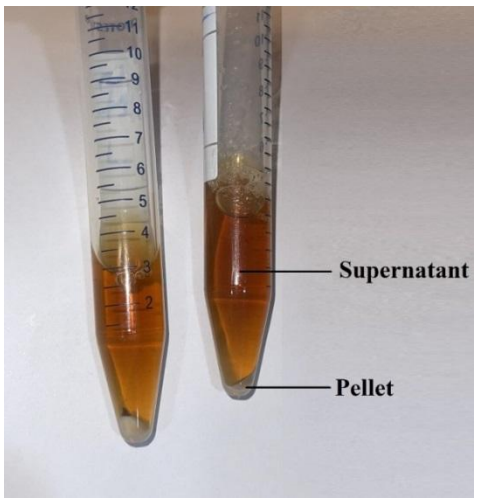


Fig. 9: Pellet and supernatant of *Enterococcus avium* and *Enterococcus casseliflavus*

https://doi.org/10.25130/tjps.v28i3.1420

4 Discussion

4.1 Isolation, Phenotypic Characterization

Enterococcus species were isolated by utilizing de Man, Rogosa and Sharpe agar (MRSA) 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 Coccoid 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%), this 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 *Entrococcus* 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 agarose 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 also been 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 Piperacillin, Ceftazidime, Cefepime, and Aztreonam (12.5%), Trimethoprime/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

https://doi.org/10.25130/tjps.v28i3.1420

to ampicillin (81.5%), tetracycline (74.1%) and trimoxazole (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 pneumonia*

In this study, all eight isolates of *Klebsiella pneumonia* 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. –typhii'*.

In the study of [17] investigated the inhibitory impact of *L. fementum* 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

https://doi.org/10.25130/tjps.v28i3.1420

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

[1] Hayek S. A, Ibrahim S. A, Current Limitations and Challenges with Lactic Acid Bacteria: A Review. Food and Nutrition Sciences, 2013:4, 73-87.

[2] Chen Y.S, Yangida F and Shinahara T, Isolation andidentification of lactic acid bacteria, from soil usingenrichment procedure. Lett. Applied Microbiol., 2005:40: 195-200.

[3] Sharma R, Sanodiya B. S, Bagrodia D, Pandey M, Sharma A and Bisen P. S, "Efficacy and Potential of Lactic Acid Bacteria Modulating Human Health," International Journal of Pharma and Bio Sciences, 2012:Vol. 3, No. 4, pp. 935-948.

[4] Steele J, Broadbent J and Kok J, Perspective on the Contribution of Lactic Acid Bacteria to Cheese Flavor Development," Current Opinion inBiotechnology, 2013:Vol. 24, No. 2, pp. 135-141

[5] Maria de Lurdes Enes Dapkevicius, Bruna Sgardioli , Sandra P. A. Câmara ,Patrícia Poeta and Francisco Xavier Malcata 2021.Current Trends of *Enterococci* in Dairy Products:A Comprehensive Review of Their Multiple Roles . Foods 2021, 10, 821. https://doi.org/10.3390/foods10040821

[6] Raras, T.Y.M., Firdausy, A.F., Kinanti, I.R. and Noorhamdani, N., 2019. Anti-Biofilm Activity of Lactic Acid Bacteria Isolated from Kefir Against Multidrug-Resistant *Klebsiella pneumoniae*, J Pure Appl Microbiol., 2019; 13 (2): 983-992. doi: 10.22207/JPAM. 13.2. 35 The Author (s) 2019. Open Access. This article is distributed under the terms of the Creative Commons Attribution, 4.

[7] Clegg, S. and Murphy, C.N., 2017. Epidemiology and virulence of *Klebsiella pneumoniae*. Urinary Tract Infections: Molecular Pathogenesis and Clinical Management, pp.435-457.

[8] Chung, P.Y., 2016. The emerging problems of *Klebsiella pneumoniae* infections: carbapenem resistance and biofilm formation. FEMS microbiology letters, 363(20), p.fnw219.

[9] Miller, W.R., Munita, J.M. and Arias, C.A., 2014. Mechanisms of antibiotic resistance in *enterococci*. Expert review of anti-infective therapy, 12(10), pp.1221-1236.

[10] Bengoechea, J.A. and Sa Pessoa, J., 2019. *Klebsiella pneumoniae* infection biology: living to counteract host defences. FEMS microbiology reviews, 43(2), pp.123-144.

[11] Donlan, R.M., 2002. Biofilms: microbial life on surfaces. Emerging infectious diseases, 8(9), p.881.

[12] Jose, N.M., Bunt, C.R. and Hussain, M.A., 2015. Comparison of microbiological and probiotic characteristics of *lactobacilli* isolates from dairy food products and animal rumen contents. Microorganisms, 3(2), pp.198-212.

[13] Hamasalih, R., Abdulrahman, Z. J. A. E. & Research, E. 2020. Antibiofilm potency of ginger (Zingiber Officinale) and quercetin against *Staphylococcus* aureus isolated from urinary tract catheterized patients. Applied Ecology and Environmental Research, 18, 219-236.

[14] Freeman, D.J., Falkiner, F.R. and Keane, C.T., 1989. New method for detecting slime production by coagulase negative *staphylococci*. Journal of clinical pathology, 42(8), pp.872-874.

[15] Reid, G., 1999. Biofilms in infectious disease and on medical devices. International journal of antimicrobial agents, 11(3-4), pp.223-226.

[16] O'Toole, G.A., 2011. Microtiter dish biofilm formation assay. JoVE (Journal of Visualized Experiments), (47), p.e2437.

[17] Maldonado, N.C., Silva de Ruiz, C., Cecilia, M. and Nader-Macias, M.E., 2007. A simple technique to detect *Klebsiella* biofilm-forming-strains. Inhibitory potential of *Lactobacillus* fermentum CRL 1058 whole cells and products. Communicating current research and educational topics and Trends in Applied Microbiology, pp.52-59.

[18] Holdeman, L.V., Moore, W.E.C. and Cato, E.P., 1977. Anaerobe laboratory manual.

[19] Bergey, D.H., 1994. Bergey's manual of determinative bacteriology. Lippincott Williams & Wilkins.

[20] Girma, A. and Aemiro, A., 2021. Antibacterial activity of lactic acid bacteria isolated from fermented Ethiopian traditional dairy products against food spoilage and pathogenic bacterial strains. Journal of Food Quality, 2021.

[21] de Siqueira, I.N., Cavalcante, A.A.C., de Sousa, D.L.C., de Aquino, V.V.F., de Oliveira, L.B.S., da Silva, J.G., Santos, C.D.S.A.B., Mota, R.A., de Oliveira Filho, A.A. and de Melo, M.A., 2022. Isolamento e perfil de resistência antimicrobiana de *Enterococcus* spp em linhas de processamento de leite de cabra. Research, Society and Development, 11(9).

[22] Cruz, A.G.D., Zacarchenco, P.B., Oliveira, C.A.F.D. and Corassin, C.H., 2019. Microbiologia, higiene e controle de qualidade no processamento de leites e derivados.

[23] Giraffa, G., 2002. *Enterococci* from foods. FEMS microbiology reviews, 26(2), pp.163-171.

[24] Atiyah, A.S. and Alkhafaji, M.H., 2020. Isolation of *Enterococcus* Species from Food Sources and Its Antibacterial Activity against *Staphylococcus* Aureus. Iraqi Journal of Science, pp.3164-3171.

[25] Collins, M.D., Jones, D., Farrow, J.A.E., Kilpper-Balz, R. and Schleifer, K.H., 1984. *Enterococcus* avium nom. rev., comb. nov.; *E. casseliflavus* nom. rev., comb. nov.; *E. durans* nom. rev., comb. nov.; *E. gallinarum* comb. nov.; and *E. malodoratus* sp. nov. International Journal of

https://doi.org/10.25130/tjps.v28i3.1420

Systematic and Evolutionary Microbiology, 34(2), pp.220-223.

[26] Aldujaili, N.H., 2014 Molecular detection of bacteriocin producing Lactic acid bacteria from fermented milk in Alnajaf . Journal of Kerbala University , 12 (2),56-61

[27] Výrostková, J., Regecová, I., Dudriková, E., Marcincák, S., Vargová, M., Kovácová, M. and Mal'ová, J.(2021). Antimicrobial Resistance of Enterococcus sp. Isolated from Sheep and Goat Cheeses . Foods .10, 1844 .

[28] Bulajić, S., Tambur, Z., Opacić, D., Miljković-Selimović, B., Doder, R. and Cenić-Milošević, D., 2015. Characterization of antibiotic resistance phenotypes and resistance genes in *Enterococcus* spp. isolated from cheeses. Archives of Biological Sciences, 67(1), pp.139-146.

[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. pnumoniae* 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.

[30] Nirwati, H., Sinanjung, K., Fahrunissa, F., Wijaya, F., Napitupulu, S., Hati, V.P., Hakim, M.S., Meliala, A., Aman, A.T. and Nuryastuti, T., 2019, December. Biofilm formation and antibiotic resistance of *Klebsiella pneumoniae* isolated from clinical samples in a tertiary care hospital, Klaten, Indonesia. In BMC proceedings (Vol. 13, No. 11, pp. 1-8). BioMed Central.

[31] Ansari, M.A., Khan, H.M., Khan, A.A., Cameotra, S.S. and Pal, R., 2014. Antibiofilm efficacy of silver nanoparticles against biofilm of extended spectrum β-lactamase isolates of Escherichia coli and *Klebsiella pneumoniae*. Applied Nanoscience, 4(7), pp.859-868.

[32] Vieco-Saiz, N., Belguesmia, Y., Raspoet, R., Auclair, E., Gancel, F., Kempf, I. and Drider, D., 2019. Benefits and inputs from lactic acid bacteria and their bacteriocins as alternatives to antibiotic growth promoters during food-animal production. Frontiers in microbiology, 10, p.57.

[33] Prabhurajeshwar, C. and Chandrakanth, K., 2019. Evaluation of antimicrobial properties and their substances against pathogenic bacteria in-vitro by probiotic *Lactobacilli* strains isolated from commercial yoghurt. Clinical Nutrition Experimental, 23, pp.97-115.

[34] Taye, Y., Degu, T., Fesseha, H. and Mathewos, M., 2021. Isolation and Identification of Lactic Acid Bacteria from Cow Milk and Milk Products. The Scientific World Journal, Volume 2021.