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Isolation and genetic diagnosis of local isolate *Enterococcus faecium* with evaluation of inhibitory against bacterial pathogens

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Abstract

Objective: Isolation the bacteria from food source has a probiotic characterization and evaluate the inhibitory activity against bacterial pathogens. **Methods:** Lactic acid bacteria from various sources are isolated by the MRS-$CaCO_3$Agar medium, the isolates were purified and microscopic, biochemical were tested. The antibacterial activity of cell free supernatant (CFS) against *Bacillus subtilis* , *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli* also testing. The isolate was selected based on high inhibitory activity and genetic diagnosis were performed. **Results**: the highest inhibitory diameter against Gram-positive bacteria was recorded with a diameter of 13 mm to *Staph aureus*. The results showed that the bacterial isolate Gram positive, immobile, negative for catalyze, Esculin hydrolysis, and CO_2 production from glucose, grows at 10- 45˚C, 2- 6.5% Nacl, and at pH 4.5- 6.9. It had the ability to ferment of sucrose, mannitol, arabinose, sorbitol and xylose. The diagnosis of isolation has been genetically confirmed to the species *Enterococcus faecium*. **Conclusion:** The *E. faecium* can be considered as probiotics in a safe manner to adaptive immune, in addition to the possibility of using it as a potential source of natural antibacterial agent in food and pharmaceutical industries.

Keywords: *Enterococcus faecium*, PCR, Antibacterial, pathogens.

Introduction

Probiotics refer to groups of non-pathogenic microorganisms which have many benefits on physiology or human health. Enterococci were introduced as a probiotic that improves the immune system of the host against pathogens. Accordingly, it has been the potential factors for prevention or help in the treatment of human infectious diseases, especially intestinal disorders. Probiotics must meet certain requirements. Therefore, it must be isolated from the hosts for whom it is supposed to be used, in addition to its ability to survive in the digestive tract, characterized by the production of compounds with bacteria static activity [1, 2].

The strain *E. faecium* species is a gram positive bacteria, Enterococcus genus belong to Enterococcaceae family, Lactobacillales order, bacilli class and Firmicutes phylum, which exhibits some probiotic properties and proposed as a probiotic [3]. The most species frequently detected in human feces are *E. faecium* and *E. faecalis*, representing a common presence within the Enterococcus species. They have been used for many years as starter cultures in food fermentation due to their ability to act as protective cultures and control bacterial contamination and thus increase the storage and biopreservation period of food [4, 5]. Escherichia fecal has been considered a probiotic and several investigations have evaluated effects on immune responses in animal and human models. They were categorized into three categories including effects on innate immunity, adaptive immunity, and effects on modulating immune responses during pro-inflammatory diseases [6].

Some enterococcal strains in the dairy industry contribute to texture, flavor, and aroma improvement of fermented milk products. It can hydrolyze proteins to amino acids, peptides and citrates to produce aromatic compounds and with proteolytic and lipolytic features [7, 8]. They are used as starter in different food and dairy products due to ability the lipolysis and proteolysis and metabolism of pyruvate and citrate. Some food-borne can prevent by bacteriocin (enterocin) produce from enterococci that inhibit food pathogens or food poisoners such as *S. aureus*, *Listeria monocytogenes*, *Clostridium* spp., *Bacillus* spp. and *Vibrio cholera.*

Moreover, the bacteria inhibit the growth of spoilage microorganisms by the production of enterocins. Thus, bacteriocins may be used as food preservatives In addition, enterococci compounds such as acetoin, acetaldehyde, diacetyl, or 2,3 -butanediol [9, 10]. The aim of the study is to isolate food bacteria with safe properties and investigate their inhibitory activity against pathogenic bacteria.

Materials and methods

Samples

The Samples were collected from Basra local markets, cheese Locally named braids (Dhafaer), pickled cucumbers, and dates, as they were brought in sterile plastic containers and activated on the MRS broth (Hi-media company), serial dilutions prepared by homogenates the samples in sterile peptone water (1 g/L) and plated on the MRS-agar with 1% w/v Cycloheximide (USA. Sigma Chemicals, St Louis) to inhibit the growth the yeast, and 1% (w/v) calcium carbonate(CaCO3) which has been added, clear zone is an indication of lactic acid production for [11].

Microscopic test

The isolates tested on Gram staining, and motion examination by hanging drop technique [12].

Biochemical Test

These tests were conducted according to [13], the strains typed based on hydrolysis of esculin, catalase test, and growth in the brain heart infusion broth containing 2- 7NaCl. Carbohydrate fermentation test carried out using glucose-free MRS medium and addition of sucrose, mannitol, arabinose, sorbitol and xylose singly and with red-chlorophenol reagent. And temperature tolerance is checked by inoculating isolates into tubes of MRS and incubated at 5, 10, 37 and 45 ◦C for 3-5 days. Also, tested its growth on different hydrogen bases 3, 4.5, 6.9 and 7. Also, tested for the production of gas by using Durham tube [14].

Pathogenic bacterial cultures

Four types of pathogenic bacterial cultures *B. subtilis*, *S. aureus*, *P. aeruginosa*, and *E.coli* obtained from the Laboratories of Biology Sciences/College of Science/University of Basra. Activated on nutrient broth (Sigma Company) at 37 °C for 24 hours [15].

Inhibition activity determination

The pathogenic bacteria *B. subtilis*, *S. aureus*, *P. aeruginosa*, and *E.coli* were inoculated on Mueller-Hinton agar media using a sterile glass rod and then dried. The drilling was done with a sterile 6 mm diameter metal drill and contained 50 µl of *E. faecium* CFS (after 6000 rpm centrifugation cell separation for 15 minutes) and plated and incubated at of 37°C for 24-48 hours and the inhibition diameter was measured with the ruler according to the method outlined by [16].

Genetic Identification

Isolation and detection of DNA

The Cell pellets were suspended in lysis buffer 180 ml (20 mg /ml lysozyme, 1.2%, Triton X -100, 20 mM Tris- HCl, 2 mM EDTA) , incubated for 30 min at 37◦C, and then proteinase K (20 ml) was added to each reaction, followed to manufacturer's instructions (QIAamp DNA Mini Kit, QIAGEN) to chromosomal DNA isolated. The DNA integrity was confirmed using a 0.8% agarose gel. Strain identification confirmation was carried out by multiplex PCR assay as discussed above by [17].

Genetic diagnosis

The polymerized chain reaction technique (PCR) of enzyme 16Sr DNA has been used in genetic diagnosis using the Primers of (16Sr DNA) according to [18]. The electrophoresis method of amplification samples was carried out by adding 7 µL DNA ladder to the first well of (500-10000) bp. After the migration process, transfer the gel to the UV unit to detect the beams. They were then made ready to read series of nitrogenous bases by the company Geneaid Biotech Ltd. in preparation to send them to the Korean company BIONEER. Local isolates were genetically diagnosed using the Blast software of the National Center for Information Service (NCBI).

Statistical analysis

The experiment was carry out in a factorial experiment under Complete Randomize Design (CRD) with 3 replicates, the statistical package SPPS (2023). The mean differences were compared using the least significant difference (L.S.D) test at the probability level ≤ 0.01 [19].

Results and discussion

Morphological characterization

It was observed that the isolates were circumscribed into a clear zone on MRSA-CaCO3 convex peak, smooth edges, and creamy white. Diameter of 0.9-1.2 µm, length 3-8 µm.

Microscopic test

Microscopic observation findings revealed t hat all positive Gram stain isolates, immobility, Most of the isolates were cocci shape in coccid in pairs and the remainder are rods, form short chain, this confirms its membership in lactic acid bacteria [20].

The test result in table (1) showed that all the isolates were catalase test negative since they were unable to hydrolyze hydrogen peroxide, and gas production from glucose negative test, which shows they are a homogeneous fermentation LAB, Negative to esculin test, Variation of isolates is observed in carbohydrate fermentation.

Biochemical test

Table (1). Biochemical test for isolates

Isolate no.	Catalase test	CO_2 production	Esculin	Sucrose	Mannitol	Arabinose	Sorbitol	Xylose
B1	-	-	-	+	-	+	+	+
B5	-	-	-	+	+	+	+	+
C13	-	-	-	+	-	+	+	+

Table (2) showed the ability of bacterial isolates to grow in a different temperature, pH, and NaCl. As the isolates differed in their ability to grow in terms of turbidity in the MRS-broth. The isolation (B5) was able to grow at 10-45°C, 6.5 NaCl, and show high tolerant growth at pH 9.6.

Table (2). Isolates growth at temperature, pH, NaCl

Isolate no.	NaCl %			pH			Temperature °C			
	2	4	6.5	3	4.5	9.6	10°	15°	37°	45°
B1	+	+	+	-	+	-	-	+	+	-
B5	+	+	+	-	+	+	+	+	+	+
C13	-	+	+	-	+	-	-	-	+	-

Antibacterial activity

Figure (2) indicates inhibitory activity of isolated bacterial supernatant towards pathogenic bacteria, as its activity was observed to be more against Gram-positive bacteria. B5 isolation did much better compared to $p \leq 0.01$ the other isolations because it recorded the greatest inhibitory diameter on the *S. aureus* and *B. subtilis* that measured (13, 12) mm respectively. The effect of isolates on the negative bacteria of Gram negative bacteria was less, with a range of 6-10 mm. This variation can be due to certain chemical structures and physiology of microorganisms, e.g., some bacteria may need high energy to transfer the inhibitor to cell membrane this depends on the oxidation and phosphorylation system of the bacteria and availability of the correct amounts of oxygen, can also be due to intrinsic differences in the structure of the able cell membrane protein on binding to the inhibitor,

which is the basis which leads to variation in the sensitivity of different bacteria to the inhibitory filtrate containing bacteriocin. The antibacterial activity can be due to the resistance of certain bacteria to bacteriocin [21,22]. While [23] found that the *E. faecium* inhibit *P. aeruginosa* bacteria with a diameter of 23 mm.

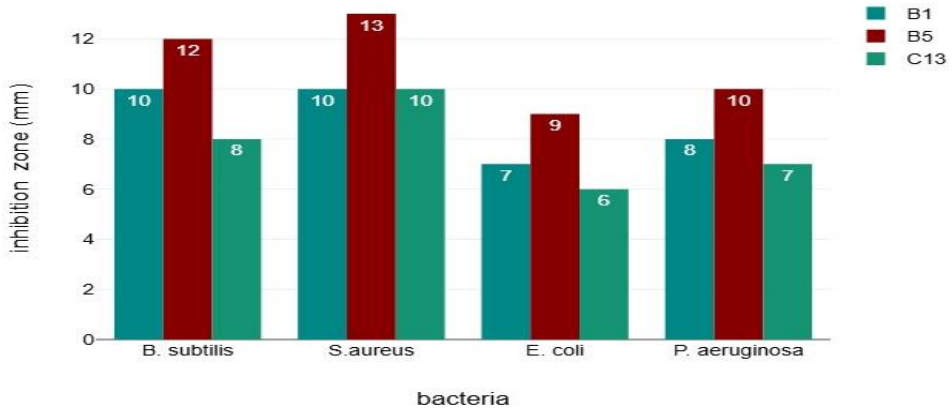

Figure 2. Effect of isolated bacterial supernatant on pathological bacteria

Genetic Identification

DNA extraction and gel electrophoresis

The Bacteria were diagnosed on species basis by molecular diagnosis as shown in figure (2),DNA bands of bacterial isolates were observed in agarose gel, the Universal Primer, the 16S rDNA gene have been shown in the 1500 base pair (bp).

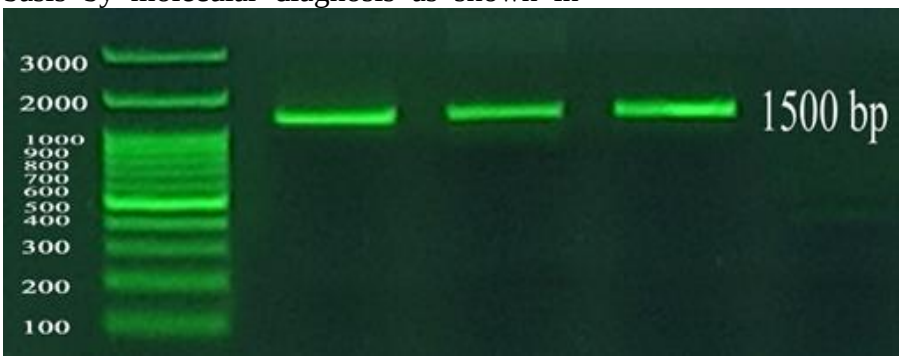

Fig.2 Agarose gel electrophoresis for 16srRNA Gene 1500 bp

Nitrogen Bases Sequencing

The isolate B5 was characterized at the strain level based on reading a nitrogen base sequence as disclosed in Table (3) and when queried in Gene bank had a 99.89% similarity with the reference strain Enterococcus faecium strain OQ940305 as disclosed in table 4.

Table 3. Nitrogen Bases sequencing for isolate B5

multiple sequence alignment between \ PV299116 (our isolate & reference copies: OQ940305 & MW090290) design by CLUSTAL Omega toll V(1.2.4) website:
https://www.ebi.ac.uk/jdispatcher/msa/clustalo

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OQ940305.E.faecium      ACGGGTGAGTAACACGTGGGTAACCTGCCCATCAGAAGGGGATAACACTTGGAAACAGGT  60
MW090290.E.faecium      ACGGGTGAGTAACACGTGGGTAACCTGCCCATCAGAAGGGGATAACACTTGGAAACAGGT  60
PV299116.E.faecium      ACGGGTGAGTAACACGTGGGTAACCTGCCCATCAGAAGGGGATAACACTTGGAAACAGGT  60
                        ************************************************************

OQ940305.E.faecium      GCTAATACCGTATAACAATCGAAACCGCATGGTTTTGATTTGAAAGGCGCTTTCGGGTGT  120
MW090290.E.faecium      GCTAATACCGTATAACAATCGAAACCGCATGGTTTTGATTTGAAAGGCGCTTTCGGGTGT  120
PV299116.E.faecium      GCTAATACCGTATAACAATCGAAACCGCATGGTTTTGATTTGAAAGGCGCTTTCGGGTGT  120
                        ************************************************************

OQ940305.E.faecium      CGCTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCC  180
MW090290.E.faecium      CGCTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCC  180
PV299116.E.faecium      CGCTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCC  180
                        ************************************************************

OQ940305.E.faecium      ACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCAAA  240
MW090290.E.faecium      ACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCAAA  240
PV299116.E.faecium      ACGATGCATAGCCCACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCAAA  240
                        ************ ************************************************

OQ940305.E.faecium      CTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCAAC  300
MW090290.E.faecium      CTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCAAC  300
PV299116.E.faecium      CTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCAAC  300
                        ************************************************************

OQ940305.E.faecium      GCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGAGAAGAACAAGGA  360
MW090290.E.faecium      GCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGAGAAGAACAAGGA  360
PV299116.E.faecium      GCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGAGAAGAACAAGGA  360
                        ************************************************************

OQ940305.E.faecium      TGAGAGTAACTGTTCATCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTACGTGC  420
MW090290.E.faecium      TGAGAGTAACTGTTCATCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTACGTGC  420
PV299116.E.faecium      TGAGAGTAACTGTTCATCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTACGTGC  420
                        ************************************************************

OQ940305.E.faecium      CAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGA  480
MW090290.E.faecium      CAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGA  480
PV299116.E.faecium      CAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGA  480
                        ************************************************************

OQ940305.E.faecium      GCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGGAGGGTCATTG  540
MW090290.E.faecium      GCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGGAGGGTCATTG  540
PV299116.E.faecium      GCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGGAGGGTCATTG  540
                        ************************************************************

OQ940305.E.faecium      GAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATG  600
MW090290.E.faecium      GAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATG  600
PV299116.E.faecium      GAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATG  600
                        ************************************************************

OQ940305.E.faecium      CGTAGATATATGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAACTGACGCTG  660
MW090290.E.faecium      CGTAGATATATGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAACTGACGCTG  660
PV299116.E.faecium      CGTAGATATATGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAACTGACGCTG  660
                        ************************************************************

OQ940305.E.faecium      AGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACG  720
MW090290.E.faecium      AGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACG  720
PV299116.E.faecium      AGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACG  720
                        ************************************************************

OQ940305.E.faecium      ATGAGTGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAAC    769
MW090290.E.faecium      ATGAGTGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAAC    769
PV299116.E.faecium      ATGAGTGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAAC    769
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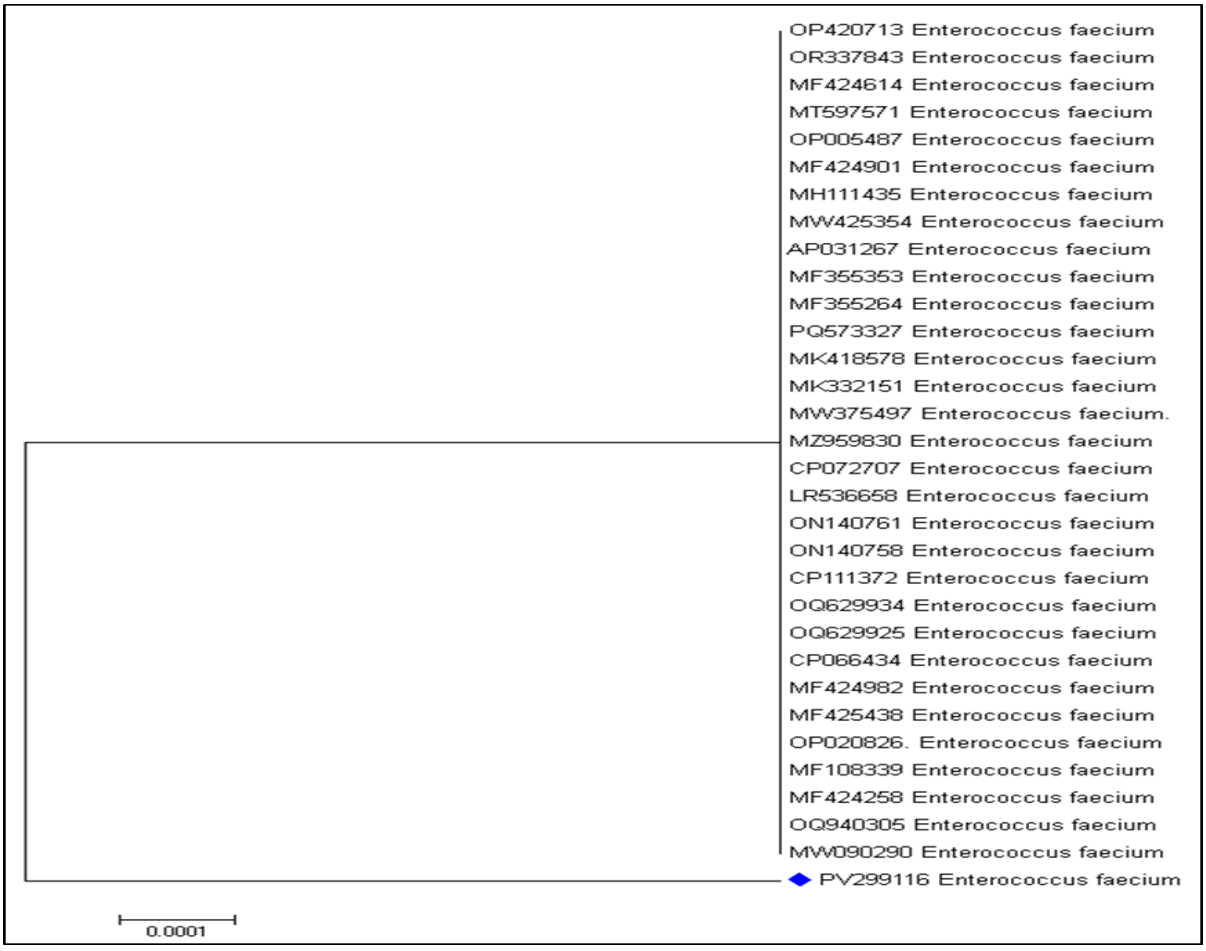
Table 4. NCBI similarity with reference *E. faecium*

No.	Sample ID	Percentage of Identity %	Sequence size	Accession Number (our study)	Scientific Name Reference copy (NCBI Data)
1	Z2	99.89%	769 bp	PV299116	Enterococcus faecium (OQ940305)

Table 5. Phylogenic tree for Enterococcus faecium bacteria (PV299116 (our isolate) & reference copies: OQ940305 & MW090290) design by omega V. 11



Conclusion

The strain *E. faecium* can use as probiotics, treatment against the pathogens factors in animals and human. Some strains of them can be used in vivo with a safe manner and adaptive immune. Besides the possible application as a source of natural antibacterial agent in food and drug industries.

Knowledge

I would like to gratitude Food Science Department, College of Agriculture, Basra University for supporting this research work.

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عزل والتشخيص الجيني للبكتريا المعزولة محلياً *Enterococcus faecium* وتقييم تأثيرها التثبيطي ضد البكتريا المرضية

الخلاصة

الهدف: عزل بكتيريا حامض اللاكتيك لها صفات بروبيوتك من مصادر الغذاء وتقييم نشاطها المثبط ضد مسببات البكتريا المرضية. **مواد وطرق العمل:** تم عزل بكتيريا حامض اللاكتيك من مصادر مختلفة بواسطة وسط MRS-CaCO3Agar ، وتنقية العزلات للحصول على مستعمرات نقية مفردة واجراء فحص مجهري وفحوصات بايوكيميائية، اضافة الى الفعالية التثبيطية للراشح الخالي من الخلايا ضد *Pseudomonas aeruginosa* و *Staphylococcus aureus* و *Bacillus subtilis* و *Escherichia coli*. **النتائج:** تم تشخيص العزلة جينياً بناءاً على اعلى قطر تثبيطي ضد البكتريا المرضية. تم تسجيل أعلى قطر تثبيطي ضد البكتيريا موجبة لصبغة جرام بقطر 13 ملم للمكورات العنقودية الذهبية. كما أظهرت النتائج أن العزلة البكتيرية موجبة لصبغة جرام ، غير متحركة ، وعدم مقدرتها على التحلل المائي للإسكولين ، وإنتاج ثاني أكسيد الكربون من الجلوكوز ، تنمو عند 10-45 درجة مئوية ، 2-6.5٪ كلوريد الصوديوم ، وعند اس هيدروجيني 4.5-6.9. كما استطاعت العزلة تخمير السكروز والمانيتول والأرابينوز والسوربيتول والزيلوز. تم تأكيد تشخيص العزلة وراثيا واثبتت عائديتها الى النوع *Enterococcus faecium*. **الاستنتاج:** يعد النوع *Enterococcus faecium* بالإضافة بروبيوتيك آمنة للمناعة التكيفية ، إلى إمكانية استخدامها كمصدر محتمل للمثبط الطبيعي المضاد للبكتيريا في الصناعات الغذائية والصيدلانية.