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Effect of genetic diversity, Antimicrobial activity, and Safety Evaluation Between probiotic isolates From Groups of *Enterococcus faecium* Isolates

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ABSTRACT

Marine probiotic sh6 and sh7 have been isolated from the Mediterranean and the Red Sea beaches of Egypt, respectivily. Were identified as *"Enterococcus faecium* AUMC B-450 and AUMC B-451 "by molecular identification These novel strains were compared to (*LAB*) as a reference for probiotic properties. The two strains were analysed for hemolytic activity the results were non hemolytic blood. The bacteria were discovered to be resistant to the gastrointestinal tract's severe environment (acidity pH3 and bile salt 0.5%). Susceptibility of *E. faecium* AUMC B-450 and AUMC B-451 strains to some different commercially available antibiotic discs: (tetracycline, penicillin, streptomycin, erythromycin, Nalidixic acid, Ciprofloxacin, chloramphenicol, and gentamicin) have been tested the method of disc diffusion using antibiotics discs. The result demonstrated that *E. faecium* AUMC B-450 is sensitive to:(penicillin-streptomycin-erythromycin-Nalidixic acid-Ciprofloxacin-and chloramphenicol) but Insensitive to tetracycline - penicillin – streptomycin - erythromycin- Nalidixic.acid-Ciprofloxacin-gentamicin -and chloramphenicol) but *E. faecium* AUMC B-451 sensitive to(tetracycline-penicillin- streptomycin- erythromycin-Nalidixic acid-Ciprofloxacin and chloramphenicol)but Insensitive to (Nalidixic acid, Ciprofloxacin, chloramphenicol). These two strains *E. faecium* AUMC B-450 and AUMC B-451 exhibited wide antimicrobial efficacy against the most common Gram-positive , Gram-negative and fungi pathogenic. The degree of its safety the strains was measured by a toxicity test, and it was found that the two strains *E. faecium* AUMC B-450 and AUMC B-451 have a high degree of safety. According to the findings, *E. faecium* AUMC B-450 and AUMC B-451 appears to be suitable for application as a probiotic in the food and feed industries in the future.

KEYWORDS: *Enterococcus faecium*, probiotics, pathogen inhibition, Cytotoxicity.

1. INTRODUCTION

Enterococcus are common bacteria found in both human's and animals' gastrointestinal tracts. These bacteria have been applied in the food industry as probiotic microorganisms when they were Known in nature (Franz, C. M., *et al*., 2003) Additionally, as a culture starter for high-salt and low-pH cheese manufacturing (Wessels *et al*., 1991). Fermented (fruits-vegetables) contain them such as sausages and olives and they help to give classic Mediterranean cheeses their characteristic flavor. Because they produce bacteriocins, certain strains There have been suggested for long food shelf life (enterocytes), and Contain antimicrobial peptides that can stop hazardous and decaying germs from growing (Khan, H., *et al* ., 2010 , Franz, C. M., *et al* ., 2011). Some enterococci strains produce many bacteriocins at the same time, giving them an edge over other bacteria in terms of Controlling niches and colonization (Hanchi, H., *et al*., 2018). recurrent chronic sinusitis-Irritable bowel syndrome-and bronchitis is currently treated using *Enterococcus* strains known as Cylactins (Hoffmann-La..Roche, Basel, Switzerland), Fargo 688s (Quest International_Naarden_Netherlands"), ECO FLOOR "(Walthers Health Care, DenHaag, Netherlands") or (Symbioflor 1 SymbioPharm, Her) (Franz *et al*., 2003; Foulquié Moreno *et al*., 2006).

The aim of this research was to isolate probiotic bacteria from various marine sources.and tested the hemolitic activity and the safety was determined using a "cytotoxicity assay , for two selective isolated , after they have been tested positive for gastric pH resistance tolerance against 0,3% bile concentration and antibacterial efficacy against the most common pathogenic, and Measuring their sensitivity to some commercially available antibiotics and molecular identification

techniques were used to identify it. Phylogenetic tree formed from the 16S RNA sequence data of the bacterial isolated in the this study for their application in a variety of foods and feed

2. MATERIALS AND METHODS

2.1. Marine probiotic bacteria sampling and isolation

A Total of 5 samples of sea water were collected from different Mediterranean Sea_Egyptian coasts and the Red Sea. To activate the probiotic bacteria, a tenth of a milliliter of each seawater sample was placed in 90 milliliters of MRS broth medium "pH 7" and incubated at 37 °C for 24 hours. Then they were diluted and Suspended sequentially, at 0.9% Man-Rogosa-Sharpe (MRS) broth take 1 ml of each dilution and place it in a Petri dish that has been sterilized, Pour all of the dishes into the MRS agar medium(Toffin, L., *et al* ., 2005) described a 48-hour anaerobic incubation at 37°C to isolate probiotic bacteria. Serial dilutions of 10^{-1} to 10^{-10} were used to select and purify isolated single colonies, which were subsequently At 37°C, the bacteria were grown overnight in MRS broth. It's kept in 30 percent (w/v) glycerol at -20°C (Abdel-Rahman, M. A , *et al* ., 2011).

2.2. Molecular identification of bacteria isolates:

Bacterial isolates were grown in ten milliliters of nutritional broth medium in sterile tubes for testing (Zimbro, M. J., *et al.*, 2015). Before being transported to the Research Centre for "Molecular Biology". Cultures were cultured at 28°C for 48 hours, for DNA extraction at Assiut University Intron Biotechnology Company, Korea, which provided the pathogen spin (DNA-RNA) kit for extraction. The "DNA samples" that were extracted were transferred to (SolGent Company) in Daejeon-South Korea,,for gene sequencing and "polymerase chain reaction" PCR..The '27F'(5'-AGAGTTTGATCC TGGCTCAG-3') and '1492 R' (5'_GGTTACCTTGTTACGACTT_3') -primers that are universal were used to do the PCR. By electrophoresis on a 1 percent agarose gel, (PCR) products that have been filtered 'amplicons' were validated Using nucleotides volume markers"100 base pairs".Dideoxynucleotides [dd NTPs] are added to the reaction mixture, and purified amplicons were 27F and 1492R primers it was used to sequence the genes in both 'sense and antisense'orientations (White, T. J., *et al.*, 1990). The Search Tool for Basic Local Alignment on the (National Center) for NCBI: Biotechnology Information website (BLAST) was previously used to further identify the sequences.[MegAlign-DNA Star] software version (5.05) was used to: perform sequence phylogenetic analysis.

2.3. Hemolytic Activity

The major safety assessment involved determining the hemolytic activity of isolated isolates. All samples were incubated at (37°C) for (24 hours) under anaerobic conditions. The presence: of a ring of {grass green} around the 'colonies' was interpreted as α-hemolytic activity. The formation of A 'clear zone' was created 'around' the colonies was thought to indicate β_hemolytic activity: which means complete hemolysis. 'no hemolysis'or 'γ_hemolytic' activity was defined as colonies that did not alter as in comparison to :(MRS) agar plate (Pieniz *et al.*, 2014).

2.4. Cytotoxicity test

by the method described (Skehan P, *et al.*, 1990) and (Allam RM, *et al.*, 2018).

2.4.1. Cell culture

Cells were grown in (DMEM media) supplemented with 100 mg/mL streptomycin, 100 units/mL penicillin , and 10% heat, inactivated foetal, bovine , serum in a humidified and {5% (v/v)} CO_2 environment, at 37 °C.

2.4.2. Cytotoxicity assay

The (SRB-assay) was used to determine cell viability. Aliquots of (100 L) cell suspension{5x103}were incubated in complete medium for (24 hours) in"96-well"plates. For treatment, the 'cells':second aliquot of (100L) media containing medicines in different dosages was used. Cells were correct by having to replace the medium using (150 mL)of {10% TCA}and incubated for"1 hour", at a temperature of 4 °C after 3 days of treatment exposure. when was removing the (TCA) solution, the cells were then washed five times by drops of distilled water. (70 µL)SRB liquid{0.4 % w/v}was applied in aliquots and incubated at room temperature for (10 minutes)in the night time. Plates were started cleaning 3 times using (1% acetic acid) and dried overnight in the air. After 150 L of TRIS"10 mM"was added to remove the protein bound (SRB) dye. the absorbance was read at(540 nm) using a{BMG LABTECH® ,FLUOstar Omega}microplate reader {Ortenberg-German}.

2.5 Probiotic Properties

2.5.1. Acid and Bile Salts Tolerance

Acid 'tolerance test and bile salt test by the method described (Tulini, F. L., *et al.*, 2013) and (Birkin Elmer, Massachusetts, USA). It's been placed to use with a few changes. The MRS was

adjusted to {pH 3.0} to mimic the 'stomach acid' of the gastrointestinal (gi) tract (Azat, R., *et al.*, 2016). "LAB isolates" were inoculated into (MRS) broth at pH 3 and incubated at (37°C) for 0 , 120 and 240 minutes. Growth was observed using a spectrophotometer was used to measure the optical density of the cultures at (620nm).

By growing isolates from cultures in MRS broth modified with different measures of bile salt , LAB isolates bile tolerance was evaluated at {0.1, 0.2, 0.3, 0.4, 0.5 and 1%}bile salt All samples were incubated for {0 , 120 and 240 min} at 37°C, As previously mentioned, growth was observed.

2.5.2. Antimicrobial Activity

The agar well diffusion method was used to test the ability of the selected marine isolates to inhibit several harmful bacterial strains. (NavinChandran, M., *et al.*, 2014). These tests were performed in triplicate. 20 ml of Dextrose Agar and Miller Agar together with the pathogenic strains (*E.coli - Salmonella typhi – Shigella dysenteriae - Listeria monocytogenes - Staphylococcus aureus - Bacillus cerues - Candida albicans - Aspergillus niger*) were poured into sterile Petri dishes. The plates were kept at 4 °C for 30 min to solidify the agar. Agar was cut with a sterile 6 mm Kursk drill. 100 µl of the bacterial suspension (probiotic isolates) was added. Each well, to assay antimicrobial activity, was incubated at 37 °C for 24 h, and areas of inhibition were measured with calipers in millimeters.

2.5.3.Antibiotic sensitivity test

Probiotic isoltes cultures were analysed for resistance to antibiotics by using disk diffusion and antibiotic discs. One milliliter of actively growing cultures was mixed with (ten milliliters) of MRS agar before being poured in to the Petri dish. After solidification. the antibiotic discs were put on a solidified surface of the agar and the samples were left at (4°C) for (30 minutes) to enable antibiotic diffusion before even being anaerobically incubated at (37°C) for (48 hours)The disc diffusion method has been used to Measure antibiotic resistance that use antibiotic discs of [streptomycin, gentamycin, chloramphenicol, ciprofloxacin, and tetracycline] (Felten, A., *et al.*, 1999). Calipers were used to measure the inhibitory zone in millimeters (del Mar Lleo, M., *et al.*, 1998)

2.6. Statistical analysis

In order to determine whether there is a statistically significant difference among the obtained results variance analyses were carried out using "SPSS" (SPSS Inc., Chicago, IL) software package. by means of independent one-way ANOVA tests. The multiple comparison of each test was assessed by least significant difference test ($P < 0.05$).

3. RESULTS AND DISCUSSION

3.1. Isolates and Identification

five marine samples collected from different Egyptian coasts. the three samples from 'Red Sea' and the two samples from the Mediterranean] were used for isolating marine probiotic, by MRS broth medium at (37 °C for 7 days) under anaerobic conditions. Two isolates of marine probiotics were isolated from Mediterranean and Red Sea sh6 and sh7 repectivily. The colonies were described under microscope , the result was diplo cocci , gram posstive stain and not forming spores .

3.2. Molecular Identification

As identified by the phylogenetic tree in (Fig 1), based on the [16S RNA-sequence] of the two-isolates sh6 and sh7 bacterial samples in the current study, the result of isolate sh6 and sh7 are *Enterococcus faecium* AUMC B- AUMC-B-450, and *Enterococcus faecium* AUMC B- AUMC-B-451 respectivily.

The molecular identification of the two strains was recorded in the genebank, definition *Enterococcus faecium* strain AUMC B-450 (16S) ribosomal RNA gene.Partial sequence ACCESSION ON614152 and definition *Enterococcus faecium* strain AUMC B451 Partial sequence of the [16S ribosomal] RNA geneACCESSION'ON619889

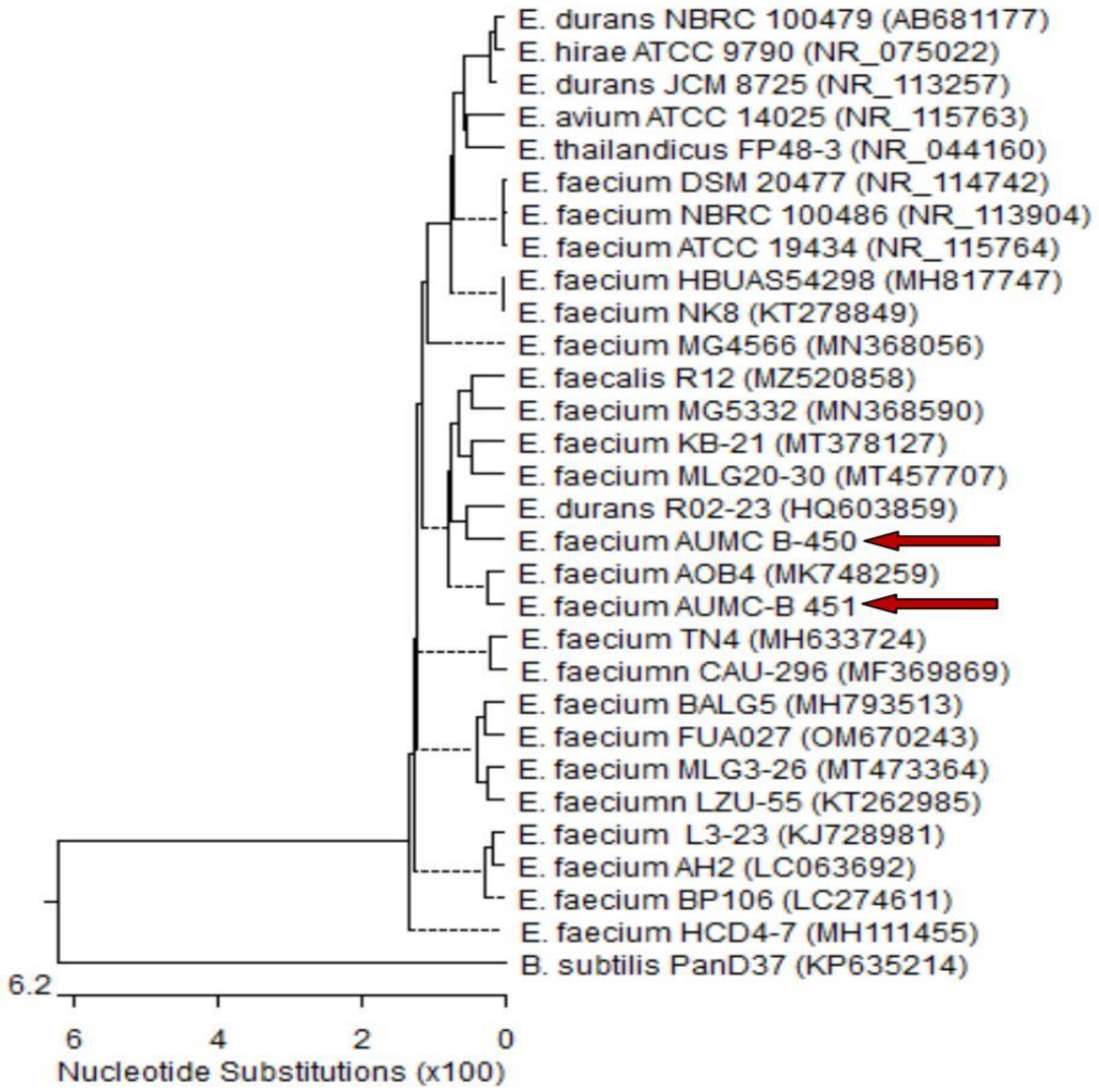

Fig 1. Phylogenetic tree formed from the [16S sequence] data of the two bacterial strains in the this study [*Enterococcus faecium* AUMC B-450 and AUMC-B-451, arrowed] aligned with types of bacteria from the "GenBank" that are closely connected. *Bacillus subtilis* is an out group type of bacteria in the tree.

Notes: a- E.= *Enterococcus*, B.= *Bacillus*.

b- Both strains of *E. faecium* showed 99.79% -99.86% identification and (99.9% - 100.0%) coverage with multiple strains of *E. faecium* including: the type strain ATCC19434 (NR 115764).

3.3. Hemolytic Activity

Bacterial strains of *Enterococcus* non hemolytic blood .

3.4. Cytotoxicity

Cytotoxicity is significant when the viability of the {IPEC-1} cell layer is strongly toxic at about 50%, medium at 60-80%, low at 80-99%, and negative at 100% The two strains showed low toxicity at [100 µg/mL] and non-toxic at (10 µg/mL). Therefore, it is highly safe when used at a concentration of [10 µg/mL], as shown in the Table (1) and as shown test cell viability in the Photo(1).

Table1. Percent of the cytotoxicity of *Enterococcus Faecium* AUMC B-450 (6) and AUMC-B-451(7) strains.

	Viability %		
	Compound	**Strain No. sh6**	**Strain No. sh7**
Conc.	10 ug/ml	103.482	101.589
	Standard deviation(SD)	0.5909	0.84797
	100ug/ml	91.452	93.2767
	Standard deviation(SD	1.9061	2.3048

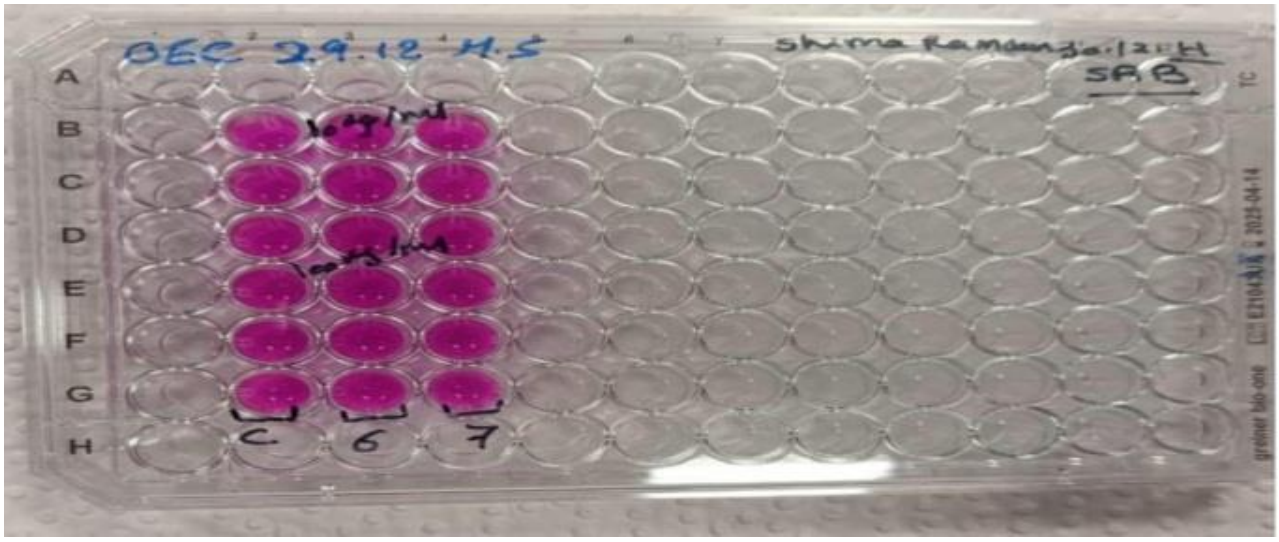

Photo. 1. Test of the cytotoxicity for *Enterococcus faecium* AUMC B-450 (sh6) and AUMC-B-451(sh7).

3.5 Probiotic Properties

3.5.1 Acid and Bile Salts Tolerance

The pH tolerance results illustrated by Figure (2) clearly shows that the highest biomass production strain of *Enterococcus faecium* was (sh7) and the lowest was (sh6) with 0.72 g/mL and 0.33g/mL,respectively , after 4 hours. Our findings are consistent with these other scientists[Yi-Chih, C., (2012) who found that *E. faecium* are resistant at pH 3.

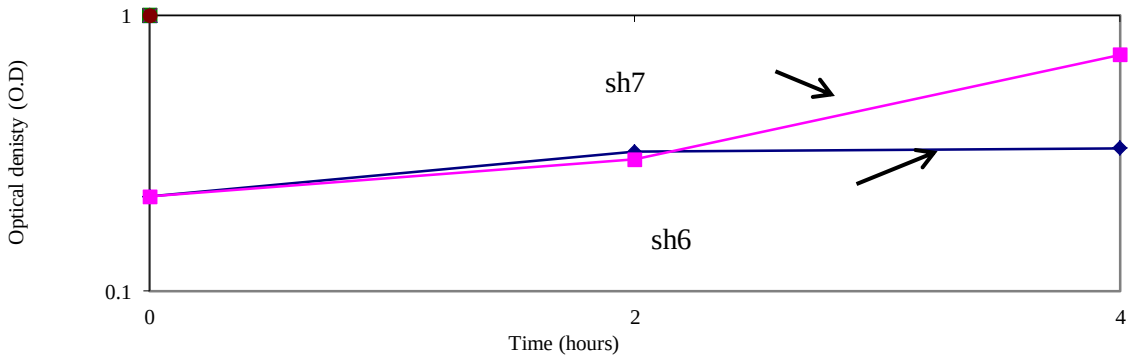

Fig 2. Growth curves of *Enterococcus faecium* strains No (sh6 &sh7) in (pH 3.0) during 0,2 and4 hours at 37 ºC using static culture .

Data presented in Figure (3) showed that, using 0.3% bile salt concentration in medium led to increase in the biomass production by *Enterococcus faecium* strains. The highest biomass production was observed on strain no. (sh6) and the lowest was (sh7) with 0.083 g/mL and 0.078g/mL, respectively, after 4hours.

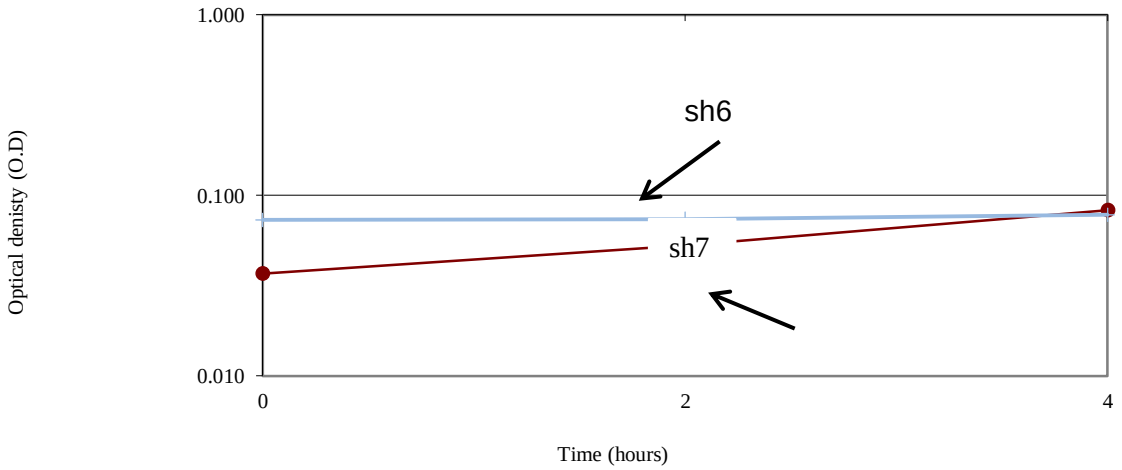

Fig 3. Growth curves of *Enterococcus faecium* strains No. (sh6 &sh7) at (0.3 %) bile salt concentration during 0,2 and 4 hours at 37 ºC using static culture.

3.5.2Antimicrobial Activity

The *Enterococcus* strains (sh6 &sh7) were screened for The antimicrobial activities. Data presented in Table (2) shows that, the highest zone inhibition with *Shigella dysenteriae* was 19 mm but the lowest zone inhibition with *E.coil* and *Bacillus cereus* was 11 mm by sh6 strain. In other hand the highest zone inhibition with *Salmonella typhi* was 21 mm but the lowest zone inhibition with *Aspergillus niger* was 11 mm by strain sh7. All strain of *Enterococcus faecium* no. (sh6 &sh7) gave non inhibition with *Candida albicans*.

Our findings are consistent with these other scientists Yerlikaya, O. (2019) who found that *E. faecium* are antimicrobial with *(Listeria monocytogenes, Escherichia coli, Staphylococcus aureus*).

Table 2. Antagonistic activity of *Enterococcus faecium* strains No. (sh6 &sh7) against pathogenic microorganisms.

	Diameter inhibition zone (mm)							
***Enterococcus* strains**	***E.coli***	***Salmonella typhi***	***Bacillus cereus***	***Staphylococcus aureus***	***Shigella dysenteriae***	***Listeria monocytogenes***	***Candida albicans***	***Aspergillus niger***
sh6	11	16	11	16	19	14	0	12
sh7	13	21	12	14	18	12	0	11

3.5.3Antibiotic sensitivity test

Antibiotic activity against *Enterococcus faecium* strains (sh6 &sh7) the strains were sensitive to some antibiotics and insensitive to others, The most sensitive to *Enterococcus faecium* strains (sh6 &sh7) was penicillin which gave the highest zone of inhibition was 29&35 mm with sh6 & sh7 strains, respectively. But the resistance to *Enterococcus faecium* strains sh6 were Tetracycline (30 mg), Nalidixic acid (60 mg), Ciprofloxacin (25 mg) and Gentamicin (10 mg) . In other hand the resistances to *Enterococcus faecium* strains sh7 were Nalidixic acid (60 mg), Ciprofloxacin (25 mg) and Gentamicin (10 mg) which don't give any zone of inhibition. as shown in Table 3.

Table 3. The results of the *Enterococcus faecium* (sh6&sh7) strains sensitive test results for eight commercially distributed antibiotics.

Enterococcus strains	(Diamrter inhibition zone (mm))							
	Tetracycline (30 mg)	Penicillin (10 mg)	Streptomycin (25 mg)	Erythromycin (15 mg)	NALIDIXICACID (60 mg)	CIPROFLOXACIN (25 mg)	Chloramphenicol (30 mg)	Gentamicin (10 mg)
sh6	0 (R)	29 (S)	19(S)	16(S)	0 (R)	0 (R)	20	0(R)
sh7	22 (S)	35 (S)	16 (S)	15 (S)	0 (R)	0 (R)	19	0 (R)

* (R) refers to - Resistant , ** (S) refers to Susceptible

3.5.4.Statistical analysis for isolate *Enterococcus faecium* AUMC B-450 And *Enterococcus faecium* AUMC B-451 at pH3 and bile salt.

The probiotic properties of *Enterococcus faecium* AUMC B-450 were performed. and *Enterococcus faecium* AUMC B-451 by acid and bile tolerance tests. According to the results obtained (Figs 2 and 3).

Change in LAB density at pH 3.0During the different incubation periods at 2 and 4 hours compared to the cell density at the beginning of inocubation at (0 hours) Results of one-way ANOVA shown in Table (4,5).

The data in Table (4) showed that there were significant differences at the level of significance ($p<0.000$) between the three experiments Where the computed value of F is about (100.651).

The data in Table (5) showed that there were significant differences at the level of significance ($p<0.000$) between the three experiments Where the computed value of F is about (225.262).

Table 4. Results of one-way ANOVA for sh 6 strain at pH3

	Enterococcus faecium AUMC B-450				
	Sum of Squares	**Df**	**Mean Square**	**F**	**Sig.**
Between Groups	0.029	2	0.014	100.651	.000
Within Groups	0.002	12	.000		
Total	0.031	14			

Table 5. Results of one-way ANOVA for sh 7 strain at pH3

	Enterococcus faecium AUMC B-451				
	Sum of Squares	**df**	**Mean Square**	**F**	**Sig.**
Between Groups	0.578	2	0.289	225.262	.000
Within Groups	0.015	12	0.001		
Total	0.594	14			

The change in LAB density after adding bile salts to the MRS broth at a concentration of 0.3% w/v during different incubation periods at growth after 2 and 4 hours compared to the cell density at the beginning of inoculation (0 hours). The one-way ANOVA results are shown in Table (6,7).

The data in Table (6) showed that there were significant differences at the level of significance ($p<0.000$) between the three experiments Where the computed value of F is about (925.759).

The data in Table (7) showed that there were significant differences at the level of significance ($p<0.003$) between the three experiments Where the computed value of F is about (9.634).

Table 6. Results of one-way ANOVA for sh6 strain at bile salt 0.3 % w/v

	***Enterococcus faecium* AUMC B-450**				
	Sum of Squares	**df**	**Mean Square**	**F**	**Sig.**
Between Groups	0.005	2	0.003	925.759	.000
Within Groups	0	12	.000		
Total	0.005	14			

Table 7. Results of one-way ANOVA for sh 7 strain at bile salt 0.3 % w/v

	***Enterococcus faecium* AUMC B-451**				
	Sum of Squares	**df**	**Mean Square**	**F**	**Sig.**
Between Groups	0	2	.000	9.634	0.003
Within Groups	0	12	.000		
Total	0	14			

4. CONCLUSION

Probiotics are a strain-specific property and the effect of a strain may vary with the genetic diversity of each strain. Then This paper focused heavily on determining the extra added useful properties of the probiotic *Enterococcus faecium*. As previously reported ,along with different probiotic characteristics between *Enterococcus faecium* AUMC B-450 and AUMC-B-451strains this study has been further evaluated and proven successfully the ability of the two strains to resist low acidity and bile salts is resistant to pathogen inhibition and has a high degree of safety based on toxicity test results.
All of these properties make each strain a beneficial probiotic. Which can also be used in vivo in the future

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الملخص العربي

تأثير التنوع الجيني ، نشاط مضادات الميكروبات وتقييم السلامة بين عزلات البروبيوتيك من *Enterococcus faecium*

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[٢]قسم الميكروبيولوجيا الزراعيه – كليه الزراعه – جامعه بني سويف

[٣] قسم الكيمياء الحيويه الزراعيه – كليه زراعه – جامعه بني سويف

تم عزل عزلتين من بكتريا بروبابيوتيك بحريه sh6 و sh7 من السواحل المصرية للبحر الأبيض المتوسط والبحر الأحمر على التوالى. تم تحديدها على أنها *Enterococcus faecium* من خلال التعريف الجزيئي ، بعد ان تم تقييم هذه العزلات الجديدة لخصائص الكائنات الحية المجهرية البروبيوتك مقارنة بـ (LAB) كمرجع. تم تحليل السلالات اولا من حيث تحليلها للدم وكانت نتائج اختبارات السلالات المختاره غير محللين للدم . تم اختبار البكتيريا لمقاومة بيئة الجهاز الهضمي من حيث(الحموضة والاملاح الصفراويه) حيث استطاعت البكتريا تحمل التركيز المنخفض من الحموضه (٣) و تركيز ٠,٥% من املاح الصفراء .تم قياس حساسية سلالات AUMC B-450 and AUMC B-451 *Enterococcus faecium* لاقراص مختلفة من المضادات الحيوية المتاحة تجارياً وهم التتراسيكلين والبنسلين والستربتومايسين والإريثروميسين وحمض الناليديكسيك وسيبروفلوكساسين والكلورامفينيكول والجنتاميسين. تم اختباره بطريقة انتشار القرص باستخدام أقراص المضادات الحيوية. أظهرت النتائج أن *E. faecium* AUMC B-450 حساسة للبنسلين والستربتومايسين والإريثروميسين وحمض الناليديكسيك وسيبروفلوكساسين والكلورامفينيكول ولكنها غير حساسة للتتراسيكلين والبنسلين والستربتومايسين والإريثروميسين وحمض الناليديكسينيك والكلورامبروفلوكساسين وكانت *E. faecium* AUMC B-451 حساسة للتتراسيكلين والبنسلين والستربتومايسين والإريثروميسين وحمض الناليديكسيك وسيبروفلوكساسين والكلورامفينيكول ولكنه غير حساسه لحمض الناليديكسيك وسيبروفلوكساسين والكلورامفينيكول. أظهرت ال AUMC B-450 و *E. faecium* AUMC B-451 نشاطًا واسعًا مضادًا للميكروبات المسببه للامراض الشائعه (إيجابية الجرام وسالبة الجرام). تم قياس درجة سلامتهما عن طريق اختبار السمية الخلويه ، ووجد أن السلالاتين *E. faecium* AUMC B-450 and AUMC B-451لديهما درجة عالية من الامان، وفقًا لهذه النتائج تظهر أن بكتيريا *E. faecium* AUMC B-450 and AUMC B-451آمنة للاستخدام في المستقبل كبروبيوتيك في صناعات الأغذية والأعلاف.