The Egyptian Journal of Hospital Medicine (January 2023) Vol. 90, Page 321-327

Probiotic Characteristics of *Enterococcus spp* Bacteria Isolated from Different Sources

Yaqeen Dheyaa Faris, Ahmed Khassaf Atya
College of Science, University of Thi-Qar, Thi-Qar, 64001, Iraq, Department of Biology
Corresponding authors: Yaqeen Dheyaa Faris, **E-mail: yageen.far.bio@sci.utq.edu.iq**

ABSTRACT

Background: Probiotics are "live bacteria that, when provided in suitable amounts, impose a health benefit on the host," according to the World Health Organization. **Objective**: This study aims to assess the probiotic properties of *Enterococcus* bacteria and the safety and effectiveness of it as a probiotic agent.

Methods: Lactic acid bacteria, were isolated, using MRS media. Through biochemical tests, and PCR and sequencing methods the isolates appeared to be members of the *Enterococcus species*. So blood agar was used to grow them as a safety test. The presence of bacteriocin was then verified in the bacteria using a partial purification process. Its autoaggregation, hydrophobicity, biofilm formation, and other probiotic characteristics of the bacteria were investigated.

Result: When cultivated on blood agar media, the lactic acid bacteria were not hemolytic. The probiotics' characteristics included substantial capacity for auto aggregation, hydrophobicity, and biofilm formation, The results showed that the *Etetrococcus* employed in this study had bacteriocin, a pectocin variant effective against a variety of dangerous bacterial species.

Conclusion: Although it is not hemolytic when growing on blood agar medium, *Enterococcus spp*. isolated from conventional cheese and waterfowl intestines, in this study are unsatisfactory because of this safety trait. One of the properties of bacteria that makes them advantageous is that they produce bacteriocin and inhibit the growth of gram-negative bacteria.

Keywords: Lactic acid bacteria, Probiotics, *Enterococci*, Bacteriocins, probiotic properties**.**

INTRODUCTION

According to science, probiotics are non-pathogenic bacteria that improve consumer health when consumed regularly and at recommended dosages. Probiotics mostly fall within the category of lactic acid bacteria (LAB). The probiotic strains from the LAB category that are most widely used in food items include *Bifidobacterium*, *Lactobacillus*, *Enterococcus*, and *Lactococcus*[(1)].

The member species of the genus *Enterococci* are widespread and can be discovered in many ecosystems, such as freshwater, soils, sediments, sea water, beach sand, and a variety of plants [(2)]. The typical gastrointestinal (GI) flora of both humans and livestock frequently includes *Enterococci* species [(3)].

Despite being thought of as commensals in humans, *Enterococcus spp*. is an important opportunistic pathogen that can cause bacteremia, biliary and stomach infections, endocarditis, and urinary tract infections in addition to biofilms on catheters and implanted medical devices[(4)]. The bacteriocins (enterocins) produced by the enterococci can increase the shelf life and safety of items by acting as antibacterial peptides[(5)].

The only food preservatives used nowadays are bacteriocins produced by lactic acid bacteria, which offer significant advantages over chemically produced food preservatives and antibiotics. Bacteriocin preparations such as propionicin, acidocin, and nisin, as well as pure and mixed cultures of lactic acid-producing bacteria, are actively used as antibacterial agents against bacteria and pathogens[(6)]

MATERIALS AND METHODS

16S rRNA sequencing for isolate identification samples was plated onto an MRS agar medium and cultivated for 48 hours at 37 °C to isolate the bacteria. DNA marker amplification for 16S rRNA and representative isolates was found utilizing a primer set and 16S rRNA sequencing on the isolates to be identified on the countable plates (30-300 CFU/plate). Amplicons were sequenced after being purified with a PCR purification kit. Using the BLAST algorithm, 16S rRNA sequences from isolates were compared.

Probiotic properties

1-Hemolytic Activity

Through inoculation on blood agar medium containing 5% (w/v) human blood or sheep blood, 10 l of a 24-hour-old culture, the hemolytic activity of fresh cultures isolated from cheese and intestines of ducks was examined. At 37 °C, the plates were incubated for 48 hours. Following this time, they were examined for hemolytic activity by looking for distinct haloes around the colonies.

2-Biofilm Production through Micro titer Plate Method (MTP)

On a sterile 96-well microtiter plate, biofilm development was carried out. Two ml of brain heart infusion broth (BHIB) and a colony of each isolate were added to tubes, and the tubes were then incubated at 37°C for 24 hours. 200 l of each broth culture was put into microtiter plates and incubated at

Received: 22/08/2022
Accepted: 23/10/2022

37°C for 24 hours after being diluted at a ratio of 1:20 with fresh BHIB. Microtiter plates were emptied, thoroughly cleaned with distilled water three times, and then inverted to blot when the Shaker incubator's incubation process was finished. The next step was to add 200 l of 1% crystal violet to each well and incubate for 15 minutes. Following incubation, the microplates underwent a third round of distilled water rinsing. Following the addition of 200 l of ethanol: acetone combination (80: 20 w/v), each well's OD was measured after being read at 450 nm using an ELISA reader. The negative control was sterile BHIB devoid of bacteria. By mathematically averaging the OD of the wells containing sterile BHIB and adding a standard deviation of +2, the cut-off value was established. Samples having an optical density (OD) greater than the cut-off value were regarded as positive, while those with an OD less than the cut-off were regarded as negative[7].

3-Autoaggregation

In either an MRS agar or broth medium, bacteria were cultivated for 18 hours at 37 °C. To obtain cells with a viable count of 108 CFU/ml, cells were collected by centrifugation (5,000 g, 15 min), twice washed in phosphate saline (PBS) buffer (pH = 7.0), and then resuspended in either their original culture supernatant or in PBS. After vortexing for 10 seconds to combine the cell suspensions (4 ml), autoaggregation was assessed after 5 hours of incubation at room temperature. The absorbance (A) was measured at 600 nm after 0.1 ml of the upper suspension was transferred to a tube containing 3.9 ml of PBS per hour. For mula, the following formula was used to get the autoaggregation percentage: (A0−At) /A0× 100= % autoaggregation Where A0 is the absorbance at time t=0 and At is the absorbance at time t=1, 2, 3, 4 or 5 h. Three independent reruns of this test were conducted. Assays for auto aggregation were carried out following [8].

4-Hydrophobicity

Changes to measurements of microbial adherence to solvents (MATS)[9] with modifications(10). In a nutshell, bacteria were separated from their environment in the stationary phase by centrifugation (5,000 g, 15 min), twice-washing, and resuspension at a concentration of about 108 CFU/ml in phosphate buffer (pH = 7.0). The absorbance of the cell suspension was measured at 600 nm (A0). One milliliter of xylene and three milliliters of cell suspension were mixed (Fluka, Germany). The two-phase system was mixed for two minutes by vortexing after preincubating for ten minutes at room temperature. The hydrous phase was relocated and its absorbance at 600 nm (At) was measured after two hours of room-temperature incubation. The percentage of bacterial adhesion to dissolvent was calculated using the formula below. This experiment was run three times. Hydrophobia (A0At)/A0 100=%

Bacteriocin extraction from *Enterococcus spp*

After the cells were removed, the bacteriocin was isolated from the cell-free supernatant liquid, according to [11]. The following information was provided on lactobacillus spp. bacteriocin purification. A 50 ml of de Man, Rogosa, and Sharpe (MRS) broth with 2 ml of Enterococcus spp. culture added was incubated at 37 °C for 14 hours under anaerobic conditions. Then 100 ml of MRS broth with 1% of an Enterococcus spp. culture added was incubated for 24 hours under anaerobic conditions at 37 °C. 15 minutes of centrifugation at 6000 rpm and 4 °C. In a rotating vacuum evaporator, cell-free supernatant was condensed to a volume of 100 ml. To get 70% saturation, ammonium sulfate was added at 4 °C while being stirred. centrifuged for 20 minutes at 4 C and 1000 rpm. In three days, the precipitate was gathered and resuspended.

Ethical approval: The project was approved by The Local Ethics Committee at Thi-Qar University following University Order No. (5698) on 02/19/2022.

RESULTS

Sequencing Analysis of (*Enterococcus)* isolates

The nucleotide sequences of the HBB gene for the studied region of the 16S rRNA gene in our current study were recorded at the US NCBI and Japanese DDBJ genebank sites.

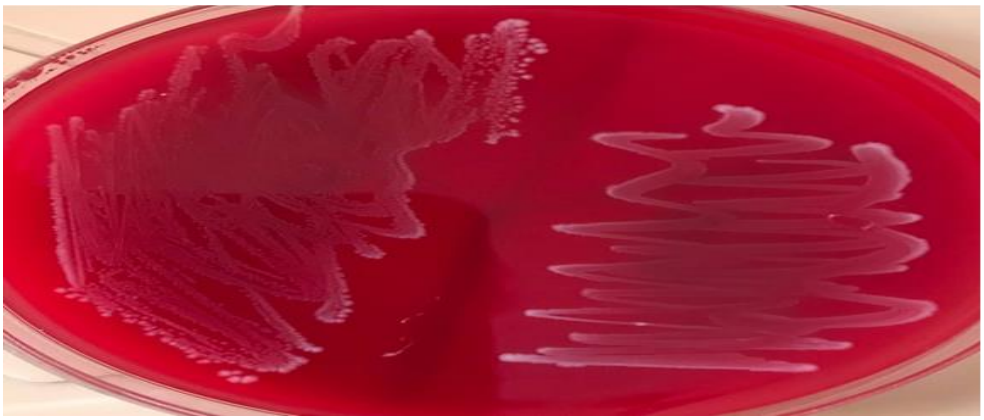
Table (1): The nucleotide sequences of the HBB gene for the studied region of the 16S rRNA gene

Gene	No.	Isolated Accession Number	Species	Identity with NCBI Accession Number
(16S ribosomal RNA)	19	LC723777	*E. durans*	The isolates are 100% identical to each other.
	26	LC723778	*E. faecium*	100% Identical with MT634243.1.China &KJ026650.1. UEA
	34	LC723779	*E. faecium*	100% Identical with MT634243.1.China &KJ026650.1. UAE
	43	LC723780	*E. durans*	99.88% Identical with MT545097.China
	50	LC723782	*E. durans*	100% Identical with MF582908.China & MF582835. China
	45	LC723781	*E. lactis*	100% Identical with MT544960/ China
	54	LC723783	*E. faecium*	99.77% Identical with MT545055/ China

Results of probiotic properties

(LAB) hemolytic Activity

Hemolytic activity is considered a safety factor when choosing probiotic strains. Blood agar plates were used in this study to test the hemolytic activities of chosen LAB strains. No hemolytic effects (γ-hemolysis) were found given as shown in **Figure** (1)



Figure(1): Hemolytic activity of LAB isolates.

Biofilm formation by(LAB) isolates

Results showed that all the strains in this study were biofilm producers in the brain heart infusion broth. The formation of the biofilm was strain-dependent. All of the strains examined were vigorous producers as determined by the OD. The most significant increases over 1 were seen for i26(2.36), i34(2.04), c19(1.08), c43(1.35), c45(1.34), c50(1.75) and c54(1.04) as shown in **Figure (2)**.

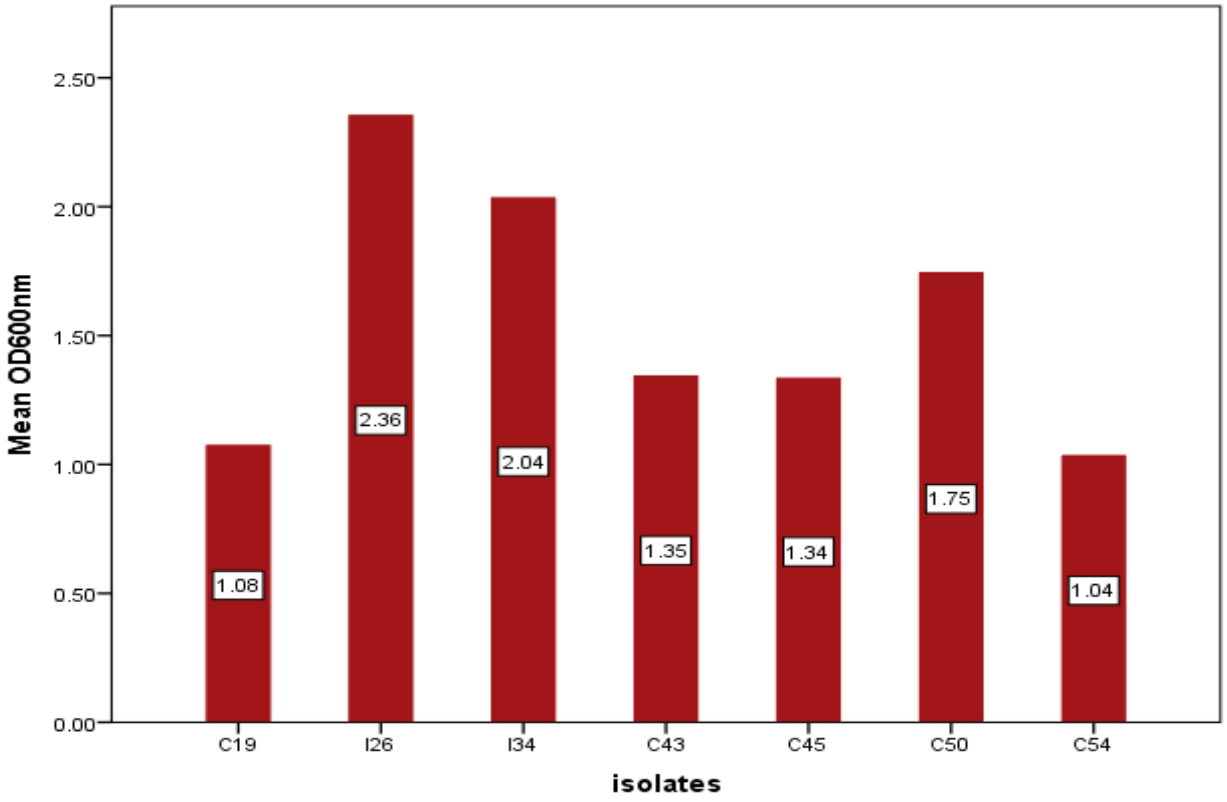


Figure (2): Biofilm formation of (LAB) isolates**.**

Auto aggregation of *Enterococcus* isolates

Seven strains of *Lactobacillus* showed auto aggregation ranging from 65.5% to 75.9% after four hours of room temperature incubation and a rate ranging from 64.5% to 79.8% after five hours of room temperature incubation **Table 2**.

Table (2): Auto aggregation for ***Enterococcus*** isolated after 4 and 5 hours of incubation

Isolates number	Isolates	First reading	After 4h		After 5h	
			Reading	%	Reading	%
I26	***E. faecium***	67	17	74.6	15	77.6
I34	***E. faecium***	39	12	69.7	11	71.7
C19	***E. durans***	62	27	65.5	18	64.5
C43	***E. durans***	59	22	62.7	19	67.7
C45	***E. lactis***	65	18	72.3	16	75.3
C50	***E. durans***	104	25	75.9	21	79.8
C54	***E. faecium***	88	27	69.3	18	79.5
CalX^2= 20.363 TabX^2= 2103 DF= 12 p. value 0.061						

Hydrophobicity for *Enterococcus* isolates

Seven strains of *Lactobacillus* showed hydrophobicity ranging from 54% to 71% after incubation at room temperature **Table 3**.

Table (3): Hydrophobicity of ***Enterococcus*** isolates

Isolates	Isolates name	First reading	Reading after 3H	percentage
C19	***Enterococcus durans***	1145	510	56%
I26	***Enterococcus faecium***	1178	544	54%
I34	***Enterococcus faecium***	1155	331	71%
C43	***Enterococcus durans***	1107	435	61%
C45	***Enterococcus lactis***	1165	518	56%
C50	***Enterococcus durans***	1197	517	57%
C54	***Enterococcus faecium***	1126	353	69%
		CalX^2= 4.585 TabX^2= 12.59 DF= 6 p. value 0.598		

Semi-purified bacteriocin and its activity against some gram-negative bacteria

In this experiment, bacteriocin was extracted from four probiotic isolates then a sensitivity test for these isolates against some gram-negative pathogenic bacteria was performed as shown in **Table 4 and Figure 3.**

Table(4): The antimicrobial activity of bacteriocin against some gram-negative bacteria

NO.	Pathogenic bacteria	C43	C45	I26	I34
		Enterococcus durans	***Enterococcus lactis***	***Enterococcus faecium***	***Enterococcus faecium***
1	*E. coli 1*	11mm	12mm	0	0
2	*E. coli 2*	0	12mm	0	0
3	*E. coli 3*	11mm	12mm	14mm	13mm
4	*E. coli 4*	0	0	11mm	0
5	*E. coli 5*	11mm	12mm	0	11mm
6	*E. coli 6*	14mm	0	0	12mm
7	*E. coli 7*	15mm	16mm	11mm	14mm
8	*PS.1*	13mm	14mm	12mm	14mm
9	*PS.3*	0	12mm	11mm	11mm
10	*PS.4*	11mm	15mm	0	13mm
11	*Proteus 1*	13mm	16mm	11mm	15mm
12	*Proteus 2*	0	12mm	0	0
13	*Klebseilla 1*	11mm	13mm	12mm	11mm
14	*Klebseilla 2*	16mm	16mm	0	17mm
15	*Serratia*	12mm	16	13mm	14mm

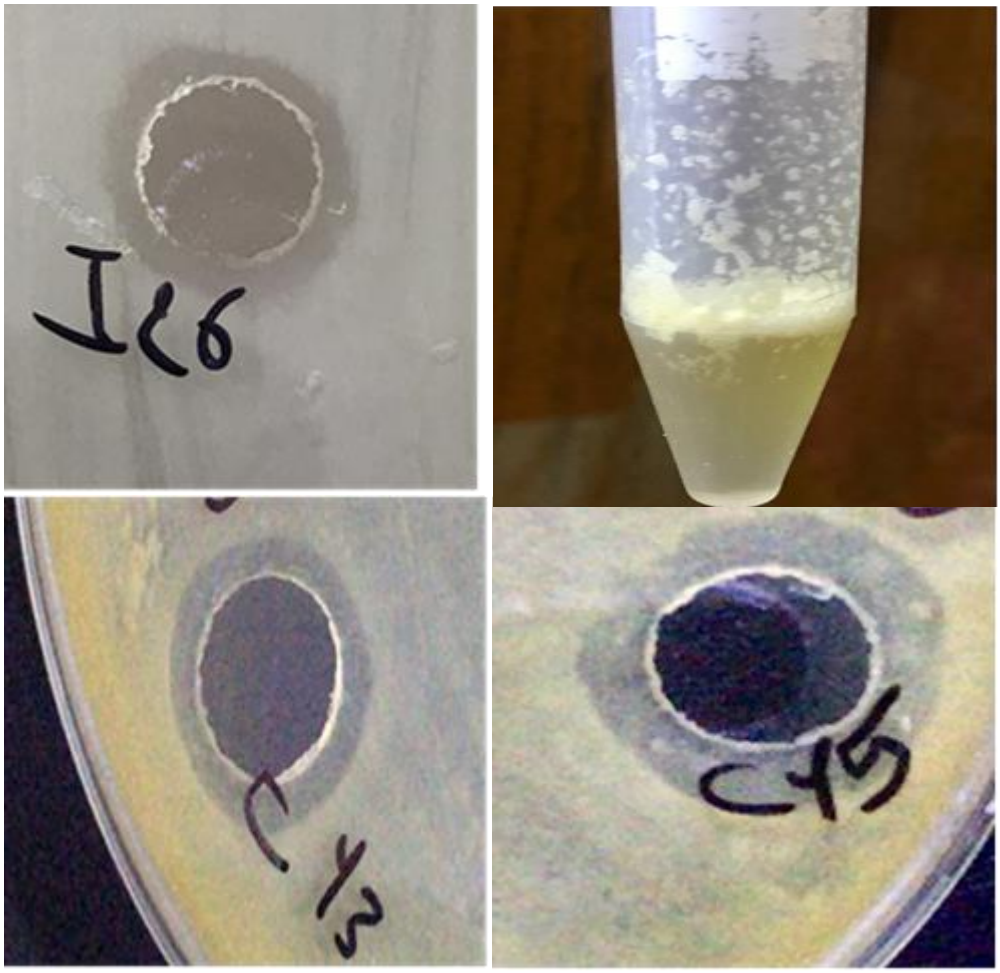


Figure (3): The effect of bacteriocin on the pathogenic bacteria.

DISCUSSION

Sequencing Analysis of (*Enterococcus)* isolates

Currently, PCR techniques should focus on virulence genes, housekeeping genes, or 16S rRNA sequences to detect enterococci [12,13,14]. The nucleotide sequences of the HBB gene for the studied region of the 16S rRNA gene in our current study were recorded at the US NCBI and Japanese DDBJ genebank sites The similarity of nucleotides was 100% with *Enterococcus* spp.

However, prior research revealed that a significant degree of sequence similarity (>99%) was found in the 16S rRNA of the E. faecium group species, *E. durans, E. faecium, E. hirae, E. lactis,* and *E. mundtii*. and lacked enough variation to discriminate between species[15]. For accurate *Enterococcus* species identification, a PCR approach targeting particular DNA markers discovered through comparative genomics was recently developed[16]. These genes offer greater precision than the 16S rRNA gene, although further sequence analysis is needed to identify enterococci following PCR amplification. Furthermore, since the closely related *E. faecium* species, *E. lactis* and *E. mundtii*, lack target genes, several researchers have recently focused on identifying markers for *E. faecalis* and *E. faecium*, as a result, identified distinctive DNA markers that accurately differentiate six Enterococcus species.

Probiotic properties of *Enterococcus spp*.

The characteristics that FAO/WHO suggest[17] GI tract survival, bile tolerance, bile salt deconjugation, aggregation, and the capacity to generate antimicrobial agents against the possible pathogenic bacteria are the factors that go into choosing a strain as a suitable probiotic microbe [18]. In the current study, we addressed some of the characteristics that make bacteria a potential probiotic. By developing the selected strains on blood Agar, we proved in his current study that all the selected strains are not hemolytic, and this is in agreement with the study[19]. As the four on the blood agar plate, some bacteria did not exhibit any hemolytic activity, which indicates their safety and use as a potential probiotic.

Results showed that all the strains in this study were biofilm producers in the brain heart infusion broth. According to the OD, the generation of biofilms was strain-dependent, and all of the strains examined were potent producers. The most significant increases over 1 were seen for i26(2.36), i34(2.04), c19(1.08), c43(1.35), c45(1.34), c50(1.75)

and c54(1.04) Generally, all *Enterococcus* isolates selected in the current study are considered to be strong biofilm components. But, *Enterococcus* isolated from the intestines of waterfowl had higher biofilm formation than the isolates isolated from traditional cheese. This is in agreement with a study[20] where it was shown that some research found that the ability to create biofilms was greater in clinical and oral isolates than in food isolates[21].

In this current study, seven strains of *Enterococcus* showed auto aggregation ranging from 64.5% to 79.8 after five hours of room-temperature incubation. where the ratios of *Enterococcus durans* bacteria ranged from 64.5% to 79.8%, and this is consistent with the results of the study[22]. Also, as explained in his study, at temperatures of 37°C, *Enterococcus durans* SJRP29 displayed a high value of aggregation (63.04%), which is advantageous because aggregation is a crucial component for biofilm development. For LAB bacteria isolated from salmon showed low values of auto-aggregation that varied from 7.2% for E. ET35 *faecium* to 12.1% for E ET05 *faecium*. This differs from what we have achieved, as the rates of auto-aggression for our study for *Enterococcus faecium* ranged between 71.7% and 79.5%, and these percentages are very high compared to a study[23].

In this current study, seven strains of *Enterococcus(LAB)* showed hydrophobicity ranging from 54% to 71% after incubation at room temperature. Also, our study achieved rates of hatred of water less than the percentages achieved by [24] where they recorded in their study that the isolates' cell surface hydrophobicity percentages varied from 3.7 to 70.2 and 2.2 to 76.4, respectively. With toluene and xylene, isolates F15 (70.22.0%) and F12 (76.42.1%) showed the highest hydrophobicity percentages.

In the current study, we investigated that the four isolates of *Enterococcus* with three species (*Enterococcus durans*, *Enterococcus faecium*, and *Enterococcus lactis*), on which the purification experiment was conducted, were bacteriocin-producing. After doing a sensitivity test for gram-negative pathogenic bacteria with extracted bacteriocin, Most of the gram-negative pathogenic bacteria were sensitive to the extracted bacteriocin, although bacteriocin was extracted from the gram-positive *Enterococcus* bacteria.

Our study's findings are somewhat comparable to those of other study [25].**Conclusion**

Because they are not hemolytic when grown on blood agar medium, *Enterococcus* spp. isolated from conventional cheese and waterfowl intestines in this investigation are unsatisfactory. The ability of bacteria to produce bacteriocin and stop the growth of gram-negative pathogenic bacteria is one of their helpful traits, The percentages of biofilms created by *Enterococcus* isolated from duck intestines are higher than those formed by isolates recovered from conventional cheese, investigation of bacteriocin's impact on gram-negative bacteria and its purification from *Enterococcus* species.

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