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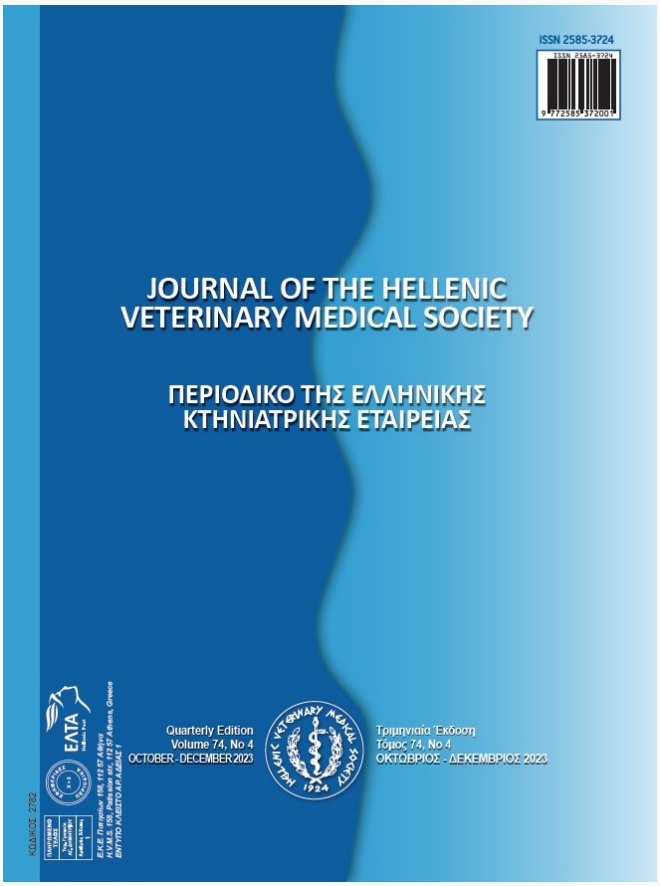

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Research article
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In vitro probiotic properties of enterocin-producing *Enterococcus mundtii* and *Enterococcus faecium* strains isolated from sheep and goat colostrum

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ABSTRACT: The aim of this study was to investigate the *in vitro* probiotic properties of enterocin-producing *E. mundtii* and *E. faecium* strains previously isolated from sheep and goat colostrum. It was determined that only *E. mundtii* HC26.1, HC56.3, HC73.1, HC73.2, HC147.1, and HC166.5 survived after 3 hours at pH 3. Survival rates of *E. mundtii* HC26.1, HC56.3, HC73.1, HC73.2, HC147.1, and HC166.5 strains were calculated as 27.98%, 27.43%, 29.16%, 25.89%, 25.66%, and 29.61%, respectively. However, none of them survived in artificial gastric juice. The survival rates of *Enterococcus* strains in MRS broth containing 0.3%, 0.5%, and 1% bile salt were found to be at least 47.42±0.74%, 41.63±1.41%, and 17.15±0.00% after 24 hours, respectively. It was determined that all of the strains were grown in a medium containing lysozyme and tolerated the presence of phenol. All of the strains showed tauro-deoxylic acid and glycodeoxycholic acid (except for *E. faecium* HC161.1) hydrolase activities. All strains except *E. faecium* HC161.1 showed over 40% hydrophobicity. After 24 hours, the autoaggregation and coaggregation values of the strains were found to be between 34.33-49.17% and 24.78-46.47%, respectively. As a result of the study's findings, it is believed that *Enterococcus* strains might be employed as probiotic cultures if microencapsulation increases their resistance to the harsh conditions of the stomach.

Keywords: Colostrum; *Enterococcus*; enterocin; probiotic properties

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INTRODUCTION

Enterococcus is the third largest lactic acid bacteria genus, which could be isolated in different environments (soil, water, etc.), raw foods (milk, vegetables, meat, and fish), and fermented food products (Ben Braïek and Smaoui, 2019). Enterococci serve as a starter or adjunct culture involved in the production and preservation of fermented meat, dairy, and vegetable products (Krawczyk et al., 2021). Some members of *E. faecalis*, *E. faecium*, and other *Enterococcus* species such as *E. mundtii* are able to synthesize bacteriocins known as enterocins, which are cationic, hydrophobic, and heat-stable antimicrobial peptides and exhibit inhibitory activity against a variety of foodborne pathogens, particularly *Listeria monocytogenes* (Alvarez-Cisneros et al., 2011; Almeida-Santos et al., 2021; Öztürk et al., 2023). The ability of enterococci to synthesize bacteriocins is one of the main reasons for their use as a preservative and probiotic culture. Many studies have been conducted proving the possibilities of using enterococci, which are part of the natural microbiota of the human and animal gastrointestinal tracts, as potential probiotics to treat some bacterial, fungal, and viral human and animal diseases (Ermolenko et al., 2019). The most common and most studied probiotic strain among enterococci is *E. faecium* SF68 (Cernivet® and Cylactin® commercial strain NCIMB 10415). In a double-blind, placebo-controlled clinical trial, *E. faecium* SF68 proved effective in reducing the incidence of antibiotic-induced diarrhea (Wunderlich et al., 1989). *E. faecium* SF68 has also been found to significantly increase immune function in puppies by adding it to dry dog foods as a probiotic supplement (Benyacoub et al., 2003). Probiotic enterococci have been used to treat conditions including diarrhea (Wunderlich et al., 1989) and irritable bowel syndrome (Enck et al., 2008), and they also have health-promoting qualities like immunostimulating (Habermann et al., 2002) and hypocholesterolemic (Guo et al., 2015) effects. *E. faecalis* DSM 16431 is supplied as a medication in Germany under the trade name Symbioflor 1 (SymbioPharm, Herborn, Germany) and is suggested for the treatment of acute and recurrent sinusitis or bronchitis (Krawczyk et al., 2021). In addition, probiotic enterococci have been applied for the exclusion and immunostimulation of gastrointestinal pathogens by generating competitive flora in animals such as poultry, pigs, and cattle (Franz et al., 2011).

In this study, the probiotic properties of bacteriocin-producing *E. mundtii* and *E. faecium* strains, which were previously isolated from sheep and goat colostrum samples obtained from the provinces of Isparta and Antalya in Türkiye, were investigated.

MATERIAL AND METHODS

Enterococcus strains and culture media

In this study, 11 *E. mundtii* and two *E. faecium* enterocin producer strains isolated from sheep and goat colostrum samples, were used. *Enterococcus* strains were identified at the species level by 16S rDNA sequence analysis. Furthermore, these results were supported by *Enterococcus* genus- and species-specific polymerase chain reactions. The genetic differences between strains were determined by phylogenetic analysis of the 16S rRNA gene sequence and random amplified polymorphic DNA (RAPD-PCR) (Öztürk et al., 2023). All *Enterococcus* strains were grown in de Man Rogosa and Sharpe broth (MRS, Biokar, France) at 37 °C for 18 hours. Stock cultures of *Enterococcus* strains were kept in a 20% (v/v) sterile, glycerol-added MRS broth at -32 °C.

Survival at low pH

After growing Enterococcus cultures in MRS broth at 37 °C for 18 hours, they were centrifuged at 3000 x g for 10 minutes at 4 °C (Sigma 2-16KL, Rotor No. 12148, Germany). The cell debris was washed once in phosphate-buffered saline (PBS, pH 7.2), and then it was resuspended to one-tenth of the volume of the culture. 0.1 mL of the prepared cell suspensions were taken and inoculated into tubes containing 2 mL of PBS adjusted to pH 1, 3, 5, and 7.2 (control). The tubes were incubated at 37 °C, and after 0, 1, 2, 3, and 4 hours, samples were taken to count the live cells on MRS agar (Conway et al., 1987).

Resistance to bile salt

To determine the bile salt resistance, *Enterococcus* strains (8-9 log10 cfu/mL) cultured in MRS broth medium at 37 °C for 18 hours, were added to MRS broth with or without 0.3%, 0.5%, or 1% (v/v) bile salt and incubated at 37 °C. The viability of the cells was determined by colony counting on MRS agar after 0, 2, 4, and 24 hours of incubation. Samples without added bile salt were used as controls (Gilliland and Walker, 1990). The survival percentage of *Enterococcus* cells was calculated using the following formula:

Survival (%) = [test group cfu/mL / control group cfu/mL] x 100

Bile salt deconjugation

Enterococcus strains were grown in Elliker broth (Sigma-Aldrich, 17123) for 18 hours at 37 °C. Then, 10 µL of cell cultures were taken with a micropipette and transferred to Elliker agar containing 0.5% (w/v) of taurocholic acid (TC) (Sigma-Aldrich, T9034), taurodeoxylic acid (TDC) (Sigma-Aldrich, T0565), glycocholic acid (GC) (Sigma-Aldrich, G7132), or sodium salts of glycodeoxycholic acid (GDC) (Sigma-Aldrich, G9910). Elliker agar without added bile salt was used as a control. The Petri dishes were incubated at 37 °C for 72 hours, and the presence of bile salt deconjugation around the colonies at the end of the period was evaluated as a positive result. Cell growth was classified as either growth positive (g), poor growth (zg), or no growth (-) in the presence of bile salt (Yerlikaya and Akbulut, 2020).

Resistance to simulated gastric juice

Enterococcus strains were cultured in 30 mL of MRS broth at 37 °C for 18 hours. Active cultures were centrifuged at 6000 x *g* at 5 °C for 20 minutes, and cell pellets were washed twice with 50 mM K_2HPO_4 (pH 6.5) buffer. Cell pellets were then resuspended in 3 mL of 50 mM K_2HPO_4 buffer. One mL of the washed cell suspension was centrifuged at 12000 x *g* for 5 minutes at 5 °C. The cell pellet was dissolved in 10 mL of simulated gastric juice containing 0.3% (w/v) pepsin and 0.5% (w/v) NaCl at pH 2 and 3, and the tubes were incubated at 37 °C. Viable cell numbers were determined at 0 and after 3 hours of incubation using MRS agar (Vinderola and Reinheimer, 2003).

Hydrophobicity

Enterococcus strains were cultured in MRS broth for 18 hours at 37 °C before being centrifuged at 12000 x *g* for 5 minutes. The supernatant was decanted, and the cell pellets were washed twice with 50 mM K_2HPO_4 (pH 6.5) buffer. The cell pellets were resuspended with the same buffer. The absorbance of the cell suspensions was adjusted to approximately 1.0 at 560 nm using a UV/VIS spectrophotometer (Soif UV-5100, Türkiye) (A_0). Then, 0.6 mL of n-hexadecane (Sigma-Aldrich) was added to 3 mL of cell suspension and mixed for 120 seconds in a vortex. For phase separation to occur, tubes were kept at 37°C. The aqueous phase was carefully taken, and its absorbance at 560 nm was measured. The reduction in absorbance of the aqueous phase was calculated using the following formula as a measure of cell hydrophobicity (%H) (Vinderola and Reinheimer, 2003). A_0 and A are the absorbance values before and after extraction with n-hexadecane, respectively.

$$\text{Hydrophobicity (\%)} = [(A_0 - A) / A_0] \times 100$$

Autoaggregation and coaggregation

The autoaggregation and coaggregation abilities of *Enterococcus* strains were detected according to the method of Jeon et al. (2017). *Enterococcus* cultures were cultured in MRS broth medium at 37 °C for 18 hours, centrifuged at 12000 x *g* for 5 minutes, and cell pellets were washed twice with PBS. Then, the absorbance of cell suspensions was adjusted to 0.3±0.005.

To find out the autoaggregation values of *Enterococcus* strains, cell suspensions were kept at 37 °C for 4 and 24 hours. The absorbance of the cell suspensions was measured at 600 nm after 0, 4, and 24 hours. Autoaggregation percentages of *Enterococcus* strains were calculated using the formula below. A_0 is the absorbance value at 0 hour, and A_t is the absorbance value (4^{th} and 24^{th} hours) after incubation.

$$\text{Auto-aggregation (\%)} = (1 - A_t/A_0) \times 100$$

To determine the coaggregation abilities of *Enterococcus* strains, 2 mL of cell suspension was mixed with 2 mL of pathogenic bacterium suspension and incubated at 37 °C for 4 and 24 hours. *L. monocytogenes* ATCC 19115, *S.* Typhimurium ATCC 14028, *S. aureus* ATCC 43300 and *E. coli* ATCC 25922 were used as pathogenic bacteria in coaggregation experiments. Coaggregation percentages of *Enterococcus* strains were calculated using the formula below. A_P refers to the absorbance value of the pathogenic bacteria at 0 hours, A_B refers to the absorbance value of the test bacteria at 0 hours, and A_{mix} refers to the absorbance value of the pathogen and tested bacteria mixture measured after incubation.

$$\text{Coaggregation (\%)}: [((A_P+A_B)/2) - A_{mix}] / (A_P+A_B)/2) \times 100$$

Lysozyme treatment

The effect of lysozyme on the growth of *Enterococcus* strains was tested in MRS broth containing 100 mg/L of lysozyme (Sigma-Aldrich). Overnight cultures of *Enterococcus* strains grown in MRS broth at 37 °C were inoculated at a rate of 2% (v/v) into MRS broth with or without lysozyme and incubated at 37 °C for 24 hours. MRS agar was used to count the cells at 0, 3, and 24 hours after incubation (Brennan et

al., 1986). The percentage increase in cell numbers at the conclution of the 3rd and 24th hours of incubation was calculated using the formula below.

Increase (%) = [(final cfu/mL) - (initial cfu/mL) / (final cfu/mL)] x 100

Phenol treatment

Survival of *Enterococcus* strains in the presence of phenol (Sigma-Aldrich) has been tested according to Teply et al. (1984). Overnight cultures of *Enterococcus* strains grown in MRS broth at 37 °C were inoculated at a rate of 2% (v/v) into MRS broth with or without phenol (4 g/L) and incubated for 24 hours. The cell counts were determined using MRS agar at 0, 3, and 24 hours of incubation. The percentage of inhibition in the cell numbers was calculated using the formula below.

Inhibition (%) = [(initial cfu/mL) - (final cfu/mL) / (initial cfu/mL)] x100

RESULTS

Survival at low pH

The surviving cell numbers of *Enterococcus* strains as a result of low pH survival trials are given in Table 1. It was determined that all of the *Enterococcus* strains decreased to an undetectable level (<1 log10 cfu/mL) by zero hour at pH 1. It was determined that *E. mundtii* HC26.1, HC56.3, HC73.1, HC73.2, HC147.1, and HC166.5 survived after 3 hours of incubation at pH 3. After 3 hours of incubation at pH 3, the survival rates of *E. mundtii* HC26.1, HC56.3, HC73.1, HC73.2, HC147.1, and HC166.5 strains were calculated as 27.98%, 27.43%, 29.16%, 25.89%, 25.66%, and 29.61%, respectively. After 4 hours of incubation at pH 5, it was determined that all of the strains remained viable (Table 1).

Resistance to bile salt

The survival percentage of *Enterococcus* strains at different bile salt concentrations at the end of the incubation period of 2, 4, and 24 hours are given in Table 2. It was determined that all *Enterococcus* strains survived in MRS broth containing 0.3%, 0.5%, and 1% bile salt.

Bile salt deconjugation

The bile salt deconjugation activities of *Enterococcus* strains are given in Table 3. It was determined that *Enterococcus* strains generally showed poor growth on MRS agar containing taurocholic acid and glycocholic acid. On the other hand, it was determined that all of the isolates deconjugated taurodeoxycholic acid. Similarly, all isolates except *E. faecium* HC161.1 deconjugated glycodeoxycolic acid.

Resistance to simulated gastric juice

The tests for resistance to simulated gastric juice revealed that at the end of the three-hour incubation period, the number of *Enterococcus* strains had dropped to an undetectable level (1 log10 cfu/mL).

Hydrophobicity

The hydrophobicity rates (%) of the *Enterococcus* strains are given in Table 4. As a result of hydrophobicity tests, it was determined that all *Enterococcus* strains except *E. faecium* HC161.1 (8.63±1.66%) had high hydrophobicity rates between 47.44±1.52% and 96.31±1.19%. It was determined that *E. mundtii* HC166.8 had the highest hydrophobicity rate with 96.31±1.19%

Autoaggregation and coaggregation

Autoaggregation percentages of *Enterococcus* strains are given in Table 4. At the end of the four-hour incubation period, it was determined that *Enterococcus* strains had autoaggregation rates ranging from 9.62±0.14% to 14.69±0.64%. Among the bacteriocin producing strains, the highest autoaggregation value was detected in the *E. mundtii* HC26.1, HC112.1, and *E. faecium* HC161.1 strains. Autoaggregation rates of isolates at the end of the 24 hour incubation period were found to be between 34.33±0.17% and 49.17±0.27%.

It was determined that the coaggregation values of *Enterococcus* strains increased at the end of the incubation period of 24 hours compared to 4 hours (Table 5). At the end of the 24-hour incubation period, the coaggregation values of *Enterococcus* strains were found to be between 38.04±1.14 - 45.35±0.34 % with *L. monocytogenes* ATCC 19115, 32.85±0.73 - 43.66±1.26 % with *S.* Typhimurium ATCC 14028, 24.78±0.30 - 37.86±0.50 % with *S. aureus* ATCC 43300, and 36.00±1.89 - 46.47±0.96 % with *E. coli* ATCC 25922.

Lysozyme treatment

After lysozyme treatment, it was determined that all *Enterococcus* strains were resistant to lysozyme (Table 4). It was determined that the *E. faecium* HC161.1 strain reached the highest cell count at the

Tabel 1. Cell numbers of bacteriocin-producing *Enterococcus* strains surviving at low pH

Strains	pH	Cell number (log_{10} cfu/mL)				
		0. hour	1. hour	2. hours	3. hours	4. hours
E. mundtii HC26.1	1	< 1	< 1	< 1	< 1	< 1
	3	6.47±0.07	3.63±0.06	2.98±0.03	1.81±0.03	< 1
	5	6.69±0.09	6.63±0.13	6.56±0.07	6.42±0.10	5.75±0.18
	7.2 (control)	6.73±0.05	6.69±0.09	6.63±0.06	6.63±0.06	6.56±0.07
E. mundtii HC56.3	1	< 1	< 1	< 1	< 1	< 1
	3	6.49±0.04	3.00±0.13	2.80±0.08	1.78±0.04	< 1
	5	7.10±0.09	6.68±0.14	6.39±0.36	6.26±0.24	6.09±0.13
	7.2 (control)	7.13±0.04	7.12±0.02	7.10±0.004	7.09±0.02	7.05±0.02
E. mundtii HC73.1	1	< 1	< 1	< 1	< 1	< 1
	3	6.55±0.06	3.73±0.05	3.15±.07	1.91±0.06	< 1
	5	6.89±0.19	6.83±0.13	6.26±0.24	6.10±0.17	5.96±0.10
	7.2 (control)	7.14±0.06	7.12±0.04	7.08±0.04	7.00±0.04	6.97±0.03
E. mundtii HC73.2	1	< 1	< 1	< 1	< 1	< 1
	3	6.49±0.10	2.98±0.03	2.67±0.06	1.68±0.03	< 1
	5	6.80±0.08	6.49±0.20	6.36±0.10	6.20±0.17	5.33±0.09
	7.2 (control)	6.86±0.03	6.84±0.06	6.80±0.04	6.73±0.05	6.56±0.07
E. mundtii	1	< 1	< 1	< 1	< 1	< 1
HC112.1	3	6.41±0.06	1.82±0.05	< 1	< 1	< 1
	5	6.82±0.20	6.59±0.11	6.56±0.24	6.53±0.21	6.30±0.04
	7.2 (control)	6.94±0.06	6.84±0.06	6.80±0.04	6.69±0.09	6.63±0.06
E. faecium	1	< 1	< 1	< 1	< 1	< 1
HC121.4	3	6.59±0.05	3.40±0.17	2.02±0.03	< 1	< 1
	5	6.57±0.23	6.59±0.11	6.49±0.20	5.86±0.07	4.01±0.09
	7.2 (control)	6.69±0.09	6.67±0.06	6.63±0.04	6.62±0.15	6.58±0.17
E. mundtii	1	< 1	< 1	< 1	< 1	< 1
HC147.1	3	6.43±0.03	2.88±0.17	2.42±0.10	1.65±0.05	< 1
	5	6.79±0.10	6.62±0.15	6.48±0.00	6.46±0.15	6.03±0.11
	7.2 (control)	6.88±0.03	6.86±0.03	6.80±0.08	6.75±0.08	6.67±0.06
E. mundtii	1	< 1	< 1	< 1	< 1	< 1
HC155.2	3	6.42±0.03	1.98±0.05	< 1	< 1	< 1
	5	7.08±0.07	6.67±0.06	6.50±0.17	6.27±0.03	6.26±0.04
	7.2 (control)	7.06±0.08	7.04±0.04	7.00±0.04	6.97±0.03	6.94±0.06
E. faecium	1	< 1	< 1	< 1	< 1	< 1
HC161.1	3	6.49±0.02	2.06±0.02	< 1	< 1	< 1
	5	6.53±0.21	6.65±0.16	6.42±0.10	6.20±0.05	6.18±0.04
	7.2 (control)	6.76±0.25	6.64±0.19	6.42±0.10	6.40±0.17	6.16±0.28
E. mundtii	1	< 1	< 1	< 1	< 1	< 1
HC165.3	3	6.54±0.08	1.80±0.03	< 1	< 1	< 1
	5	6.93±0.08	6.63±0.13	6.30±0.30	6.08±0.07	5.91±0.19
	7.2 (control)	6.97±0.06	6.92±0.03	6.84±0.06	6.77±0.07	6.75±0.08
E. mundtii	1	< 1	< 1	< 1	< 1	< 1
HC166.3	3	6.48±0.03	2.67±0.05	1.64±0.06	< 1	< 1
	5	6.62±0.15	6.56±0.07	6.57±0.23	6.24±0.06	6.22±0.03
	7.2 (control)	6.74±0.13	6.69±0.09	6.63±0.13	6.59±0.11	6.56±0.07
E. mundtii	1	< 1	< 1	< 1	< 1	< 1
HC166.5	3	6.45±0.03	3.43±0.23	2.78±0.15	1.91±0.02	< 1
	5	6.86±0.28	6.90±0.10	6.49±0.20	6.04±0.04	6.02±0.10
	7.2 (control)	7.04±0.04	7.03±0.02	7.00±0.04	6.97±0.03	6.93±0.08
E. mundtii	1	< 1	< 1	< 1	< 1	< 1
HC166.8	3	6.48±0.01	1.62±0.05	< 1	< 1	< 1
	5	7.00±0.14	6.59±0.26	6.42±0.10	5.94±0.06	3.93±0.06
	7.2 (control)	7.00±0.07	6.98±0.05	6.94±0.03	6.88±0.03	6.82±0.11

Table 2. Survival percentage (%) of bacteriocin-producing *Enterococcus* strains at different bile salt concentrations after 2, 4, and 24 hours incubation period

Strains	0.3% bile salt			0.5% bile salt			1% bile salt		
	2 hours	4 hours	24 hours	2 hours	4 hours	24 hours	2 hours	4 hours	24 hours
E. mundtii HC26.1	89.49±0.75	80.76±0.55	73.88±1.03	76.40±1.00	67.88±1.14	49.23±1.33	59.95±0.72	48.48±0.37	35.66±0.72
E. mundtii HC56.3	89.61±0.56	80.14±1.04	69.09±0.92	78.51±2.35	63.56±1.61	50.25±0.46	66.16±7.99	50.99±0.22	32.40±1.24
E. mundtii HC73.1	86.61±2.94	79.18±1.50	60.96±0.92	73.57±0.89	58.87±2.09	51.08±1.95	60.98±0.93	40.78±0.70	26.94±1.86
E. mundtii HC73.2	89.24±1.71	78.38±0.70	63.62±0.17	62.36±2.12	50.90±3.69	42.52±0.63	59.51±1.81	54.25±4.18	17.15±0.00
E. mundtii HC112.1	89.37±0.37	87.15±0.78	80.12±0.33	69.08±3.59	58.84±1.00	48.96±2.81	70.04±4.20	38.91±1.01	25.25±2.29
E. faecium HC121.4	86.16±2.88	83.53±1.32	75.50±3.22	74.31±3.52	52.73±2.66	48.26±2.53	62.41±3.82	39.84±0.71	35.79±0.78
E. mundtii HC147.1	82.50±1.50	69.06±0.77	47.42±0.74	68.55±1.89	55.48±2.86	41.63±1.41	59.73±1.61	52.19±1.95	40.91±1.17
E. mundtii HC155.2	89.52±0.22	79.71±1.54	64.75±1.95	79.26±1.81	69.36±1.16	44.35±1.57	53.45±1.95	45.51±0.75	32.50±0.83
E. faecium HC161.1	83.73±3.84	76.99±0.23	53.13±1.59	63.78±1.20	55.68±2.44	47.32±1.02	43.37±0.84	40.02±1.57	36.26±2.01
E. mundtii HC165.3	77.77±0.91	68.62±0.85	48.58±0.81	77.00±1.05	66.94±0.19	47.04±0.93	60.12±0.40	35.36±1.44	33.42±0.79
E. mundtii HC166.3	89.14±1.70	78.64±0.12	53.34±2.33	75.44±1.59	71.21±0.50	62.65±0.66	57.13±2.34	50.80±2.10	32.40±0.35
E. mundtii HC166.5	90.24±0.62	78.55±0.23	65.19±1.80	73.32±2.97	67.33±2.07	46.96±0.00	58.89±0.39	51.24±1.53	37.39±0.22
E. mundtii HC166.8	90.73±0.52	80.60±0.45	57.30±1.63	74.70±1.71	64.68±1.42	48.75±1.32	57.92±0.00	52.65±1.60	34.91±0.71

Table 3. Bile salt deconjugation activities of bacteriocin-producing *Enterococcus* strains

Strains	Bile salt deconjugation*				
	Control	TC	TDC	GC	GDC
E. mundtii HC26.1	g	pg	+	pg	+
E. mundtii HC56.3	g	pg	+	pg	+
E. mundtii HC73.1	g	pg	+	pg	+
E. mundtii HC73.2	g	pg	+	pg	+
E. mundtii HC112.1	g	pg	+	pg	+
E. faecium HC121.4	g	pg	+	pg	p+
E. mundtii HC147.1	g	pg	+	pg	+
E. mundtii HC155.2	g	pg	+	pg	+
E. faecium HC161.1	g	g	p+	g	-
E. mundtii HC165.3	g	pg	+	pg	+
E. mundtii HC166.3	g	pg	+	pg	+
E. mundtii HC166.5	g	pg	+	pg	+
E. mundtii HC166.8	g	pg	+	pg	p+

* TC: taurocholic acid, GC: glycocholic acid, GDC: glycodeoxycholic acid, TDC: taurodeoxycholic acid, g: growth, pg: poor growth, +: deconjugation positive, p+: poor deconjugation, -: no growth

Table 4. Hydrophobicity and autoagregation properties, and inhibition percentage (%) against lysozyme and phenol of bacteriocin-producing *Enterococcus* strains

Strains	Hydrophobicity (%)	Autoaggregation (%)		Lysozyme (%)		Phenol (%)	
		4 hours	24 hours	3 hours	24 hours	3 hours	24 hours
E. mundtii HC26.1	77.00±2.87	14.57±0.81	45.16±0.48	17.36±1.02	23.09±0.17	2.06±0.35	4.58±0.05
E. mundtii HC56.3	51.50±0.51	10.18±0.54	42.95±0.41	15.15±0.81	23.42±0.72	0.50±0.21	8.91±1.36
E. mundtii HC73.1	94.29±2.57	9.70±0.64	42.18±0.61	15.91±0.51	21.47±0.82	1.59±0.49	13.44±0.84
E. mundtii HC73.2	91.49±2.56	9.62±0.14	41.87±0.87	18.31±1.26	24.41±0.24	4.42±0.17	9.93±0.77
E. mundtii HC112.1	50.15±1.73	14.69±0.64	49.17±0.27	16.41±0.83	22.57±0.13	0.55±0.14	18.83±1.29
E. faecium HC121.4	47.44±1.52	10.33±0.46	34.33±0.17	14.17±1.38	20.94±1.89	3.21±0.31	-0.60±0.21
E. mundtii HC147.1	82.26±2.03	11.29±0.43	39.72±0.62	18.39±1.12	25.36±1.40	1.13±0.55	10.62±0.95
E. mundtii HC155.2	89.71±0.52	11.84±0.11	41.01±0.48	17.71±0.61	23.63±0.81	3.76±0.56	20.27±1.92
E. faecium HC161.1	8.63±1.66	14.00±0.45	44.22±0.25	20.57±0.98	24.81±0.59	4.52±0.60	16.07±0.22
E. mundtii HC165.3	79.19±1.08	10.74±0.53	37.47±0.64	17.65±0.29	23.57±0.13	1.44±0.19	8.50±1.34
E. mundtii HC166.3	94.35±0.11	11.05±0.40	41.99±0.55	17.91±0.69	22.17±0.85	2.11±0.62	15.47±0.39
E. mundtii HC166.5	94.49±1.13	10.07±0.11	40.48±0.64	13.81±0.59	19.91±0.36	1.47±0.46	10.39±1.41
E. mundtii HC166.8	96.31±1.19	9.91±0.61	40.76±0.36	14.04±0.79	18.74±0.70	4.24±0.64	17.73±1.03

Table 5. Coaggregation percentages (%) of bacteriocin-producing *Enterococcus* strains with pathogenic bacteria

***Enterococcus* strains**	**Pathogenic bacteria**	**Coaggregation (%)**	
		4th hour	**24th hour**
E. mundtii HC26.1	*L. monocytogenes* ATCC 19115	5.56±0.16	38.04±1.14
	S. Typhimurium ATCC 14028	14.29±0.71	35.30±0.79
	S. aureus ATCC 43300	8.38±0.54	30.21±0.81
	E. coli ATCC 25922	13.90±1.49	42.49±1.83
E. mundtii HC56.3	*L. monocytogenes* ATCC 19115	8.95±0.35	41.88±1.15
	S. Typhimurium ATCC 14028	12.47±0.90	41.28±0.93
	S. aureus ATCC 43300	7.14±0.53	34.57±0.48
	E. coli ATCC 25922	11.21±0.20	45.07±0.19
E. mundtii HC73.1	*L. monocytogenes* ATCC 19115	12.79±0.47	41.49±1.15
	S. Typhimurium ATCC 14028	12.27±0.97	32.85±0.73
	S. aureus ATCC 43300	5.56±0.20	24.78±0.30
	E. coli ATCC 25922	8.97±0.45	40.50±0.08
E. mundtii HC73.2	*L. monocytogenes* ATCC 19115	10.83±0.76	41.04±0.62
	S. Typhimurium ATCC 14028	12.93±0.44	37.90±0.71
	S. aureus ATCC 43300	8.19±0.28	32.92±0.14
	E. coli ATCC 25922	12.20±0.70	44.57±0.61
E. mundtii HC112.1	*L. monocytogenes* ATCC 19115	13.52±0.94	43.90±0.92
	S. Typhimurium ATCC 14028	10.55±0.77	43.66±1.26
	S. aureus ATCC 43300	11.04±0.40	37.86±0.50
	E. coli ATCC 25922	13.94±0.46	46.21±0.44
E. faecium HC121.4	*L. monocytogenes* ATCC 19115	9.69±0.68	39.02±0.57
	S. Typhimurium ATCC 14028	9.92±0.27	41.85±0.27
	S. aureus ATCC 43300	7.81±0.24	34.20±1.23
	E. coli ATCC 25922	12.78±0.29	44.00±0.28
E. mundtii HC147.1	*L. monocytogenes* ATCC 19115	13.63±0.41	43.13±0.94
	S. Typhimurium ATCC 14028	12.80±0.32	38.96±0.43
	S. aureus ATCC 43300	8.83±0.40	36.31±0.46
	E. coli ATCC 25922	13.56±0.75	42.45±0.99
E. mundtii HC155.2	*L. monocytogenes* ATCC 19115	14.52±0.69	45.35±0.34
	S. Typhimurium ATCC 14028	12.80±0.33	38.61±0.96
	S. aureus ATCC 43300	9.95±0.24	33.22±0.64
	E. coli ATCC 25922	13.86±0.78	43.02±0.79
E. faecium HC161.1	*L. monocytogenes* ATCC 19115	12.78±0.31	45.33±0.27
	S. Typhimurium ATCC 14028	11.21±1.00	42.55±0.61
	S. aureus ATCC 43300	5.81±0.72	33.30±1.02
	E. coli ATCC 25922	12.97±0.56	46.47±0.96
E. mundtii HC165.3	*L. monocytogenes* ATCC 19115	11.86±0.78	42.97±0.76
	S. Typhimurium ATCC 14028	10.07±0.38	42.28±0.57
	S. aureus ATCC 43300	5.68±0.78	33.07±0.46
	E. coli ATCC 25922	12.58±0.62	45.00±0.22
E. mundtii HC166.3	*L. monocytogenes* ATCC 19115	12.36±0.47	42.50±0.45
	S. Typhimurium ATCC 14028	12.16±0.86	41.59±1.26
	S. aureus ATCC 43300	9.61±0.33	34.41±0.27
	E. coli ATCC 25922	10.81±0.58	38.66±0.94
E. mundtii HC166.5	*L. monocytogenes* ATCC 19115	11.82±0.78	41.32±0.58
	S. Typhimurium ATCC 14028	11.89±2.18	40.88±0.84
	S. aureus ATCC 43300	10.88±0.73	34.95±0.38
	E. coli ATCC 25922	7.99±2.10	36.00±1.89
E. mundtii HC166.8	*L. monocytogenes* ATCC 19115	12.43±0.38	42.40±0.46
	S. Typhimurium ATCC 14028	12.51±0.96	38.99±0.90
	S. aureus ATCC 43300	10.13±0.57	35.09±0.62
	E. coli ATCC 25922	11.22±0.47	44.05±0.55

end of the 3rd hour of incubation in MRS broth containing lysozyme, and *E. mundtii* HC147.1 at the end of the 24th hour. At the end of the three-hour incubation period in the medium containing lysozyme, it was determined that the cell number of *Enterococcus* isolates increased between 13.81±0.59 and 20.57±0.98%. At the end of the 24th hour of incubation, it was determined that the percentage increase in the number of cells varied between 18.74±0.70 and 25.36±1.40.

Phenol treatment

All *Enterococcus* strains were viable in the presence of 0.4% phenol at the end of both the three-hour and 24-hour incubation periods. At the end of the three-hour incubation period, the most resistant strain to phenol was determined as *E. mundtii* HC56.3 with an inhibition rate of 0.50±0.21%, and the most susceptible strain was *E. faecium* HC161.1 with an inhibition rate of 4.52±0.60%. The *E. mundtii* HC155.2 strain had the highest inhibition rate (20.271.92%) after 24 hours of incubation. On the other hand, it was determined that there was an increase in the number of *E. faecium* HC121.4 cells at the end of the 24 hour incubation period (Table 4).

DISCUSSION

Probiotic bacteria must have exclusion mechanisms or be resistant to harsh conditions imposed by the digestive system, such as bile salt and gastric juice pH, in order to reach the gut actively and provide the expected benefits to host health (Zommiti et al., 2018). The pH of the human stomach varies between 1 (during fasting) and 4.5 (after a meal), and the digestion of food can take up to 3 hours (Maragkoudakis et al., 2006). Previous studies have shown that *Enterococcus* strains can be sensitive to low pH depending on the species and strain (Nami et al., 2019; Yerlikaya and Akbulut, 2020; Özkan et al., 2021). Although acid tolerance is desired in the pH range of 2-4 for probiotic cultures, a tolerance to pH 3 for 3 hours is considered sufficient for successful passage of the culture through the stomach (Guo et al., 2015; Tinrat et al., 2018; Shi et al., 2020). *E. mundtii* HC26.1, HC56.3, HC73.1, HC73.2, HC147.1, and HC166.5 strains, whose survival rates were determined to be between 25.66% and 29.61% after 3 hours at pH 3 as a result of low pH survival trials, have the potential to be used as probiotic cultures. On the other hand, microencapsulation can be applied today in order to increase the resistance of LAB to the harsh environmental conditions created by the stomach and to ensure that it can enter the intestinal system in large quantities (Martín et al., 2015).

Bile salt tolerance has been identified as an important factor for intestinal survival and proliferation of LAB (Gilliland and Walker, 1990). Enterococci can grow in the presence of 40% (w/v) bile salts (Fisher and Philips, 2009). It was determined that all bacteriocin-producing *Enterococcus* strains survived after 2, 4, and 24 hours of incubation in MRS broth containing 0.3%, 0.5%, and 1% bile salt. Similar to our findings, Zommiti et al. (2018) reported that all five strains of bacteriocin producer *E. faecium* survived in MRS broth containing 0.3% bile salt. Nami et al. (2019) reported that after 4 hours in M17 broth with 0.3% bile salt, the survival rates of *Enterococcus* strains isolated from dairy products manufactured without a starter culture ranged from 9.1% to 79.8%. Lauková et al. (2020) reported that the bacteriocin-producing *E. mundtii* EM ML2/2 strain found in raw goat milk was resistant to both 3% and 5% bile salts. Özkan et al. (2021) investigated the bile salt tolerance of nine *Enterococcus* strains isolated from Turkish Tulum cheese at different concentrations (0.06-1%) and reported that every isolate survived. The researchers determined that the survival rate of the strains at the end of the 24 hour incubation period at 1% bile salt concentration was between 70.85% and 87.47%.

Bile salt deconjugation occurs through the bile salt hydrolase activity produced by bacteria. The bile salt hydrolase activity of probiotic bacteria in the gastrointestinal tract is generally associated with cholesterol-lowering effects (Kumar et al., 2012). Bile salt deconjugation is a desirable feature in potential probiotic bacteria (Nascimento et al., 2019). This study discovered that bile salt hydrolase activities for TDC and GDC were present in bacteriocin-producing *Enterococcus* strains, which is advantageous for their application as probiotic cultures. Contrary to our findings, Amaral et al. (2017) found that the potential probiotic strain *E.durans* SJRP29 could not hydrolyze both TC and TDC. Similar to our results, Yerlikaya and Akbulut (2020) reported that 25 *E. faecium* and 5 *E. durans* isolated from raw milk and conventional dairy products deconjugate only TDC. Researchers determined that 14 *E. faecium* and 2 *E. durans* deconjugated TDC, 5 *E. faecium* showed weak deconjugation, and 9 isolates developed but did not show deconjugation properties. They also reported that en-

terococci isolates thrive in environments containing TC and GC but cannot deconjugate these bile salts. They found that 13 of the isolates did not grow, 14 of the isolates grew slowly, and 3 of the *E. faecium* isolates grew in GDC containing medium. Pradhan and Tamang (2021) reported that 6 of 26 *Enterococcus* isolates deconjugated TDC. On the other hand, researchers reported that 10 of 26 isolates could also deconjugate TC, contrary to our results.

The ability of a bacterium to function as a potential probiotic and to demonstrate the anticipated positive effects on health is directly related to its capacity to endure the transition from the upper digestive system to the intestine, and this property is a significant prerequisite for probiotic bacteria (Zommiti et al., 2018). It was found that enterocin-producing *Enterococcus* strains could not survive in simulated gastric juice. On the contrary to our results, Nami et al. (2019), Özkan et al. (2021) and Hajikhani et al. (2021) indicate that *Enterococcus* strains isolated from dairy products survive in artificial gastric juice. The inability of bacteriocin producer *Enterococcus* strains to survive in artificial gastric juice is a disadvantage to their use as probiotic cultures. On the other hand, the fact that bacteriocin-producing *Enterococcus* strains are resistant to mouth (lysozyme) and lower digestive tract conditions (bile salt and phenol) is an advantage in terms of being able to be used as probiotic cultures. It is thought that these strains can be microencapsulated to pass through the harsh conditions of the stomach and reach the intestine in large quantities. Today, microencapsulation is frequently preferred in order for probiotic cultures to settle in the host digestive system and gain resistance against negative factors that may prevent their survival (Martín et al., 2015; Pradeep Prasanna and Charalampopoulos, 2018; Arepally and Goswami, 2019; Singh et al., 2019).

It is stated that the hydrophobicity values of bacteria that have the potential to be used as probiotics should be above 40% (Son et al., 2018). As a result of hydrophobicity tests, it was determined that all enterocin producer *Enterococcus* strains, except *E. mundtii* HC161.1, had a hydrophobicity value of over 40%. Previous studies, showed that the hydrophobicity properties of *Enterococcus* strains isolated from different sources differed in species or strain (Bhardwaj et al., 2011; Favaro et al., 2014; dos Santos et al., 2015; Nami et al., 2019; Yerlikaya and Akbulut, 2020; de Castro Santos Melo et al., 2021; Özkan et al., 2021). Contrary to our findings, Bhardwaj et al. (2011) reported that the hydrophobicity values for n-hexadecane of bacteriocin producer *E. faecium* strains ranged from 2.9% to 9.9%. Researchers reported that the hydrophobicity values for xylene for the same strains were between 65.5% and 91%. Favaro et al. (2014) reported that the hydrophobicity of bacteriocin producer *E. faecium* ST209GB, ST278GB, ST315GB, and ST711GB strains isolated from homemade white cheese was 9.16%, 9.85, 7.92, 10.23%, respectively. In the same way, dos Santos et al. (2015) determined that the hydrophobicity of *E. faecium* EM485 and EM925 strains isolated from Brazilian Coalho cheese were 8.18% and 11.33%, respectively. Nami et al. (2019) reported that the hydrophobicity values of *Enterococcus* strains isolated from dairy products produced without the use of starter culture were between 23.3±1.6% and 58.6±2.3%. However, many investigations have found that isolates of the genus *Enterococcus* have very high hydrophobicity values (Amaral et al., 2017; Yerlikaya and Akbulut, 2020; Özkan et al., 2021; de Castro Santos Melo et al., 2021), as confirmed in this study. The hydrophobic nature of the outer surface of microorganisms plays a role in the attachment of bacteria to the host tissue. This feature can provide an important competitive advantage for the colonization of bacteria in the gastrointestinal tract (Vinderola and Reinheimer, 2003; de Melo Pereira et al., 2018). High hydrophobicity properties increase the ability of probiotics to adhere to epithelial cells and thus support host health (Nami et al., 2019). Adhesion to the intestinal mucosa is considered one of the most important criteria in the selection of probiotic bacteria (Yerlikaya and Akbulut, 2020). Therefore, the high hydrophobicity properties of bacteriocin producer *Enterococcus* strains are an important advantage in terms of their potential to be used as probiotics.

It was determined that the autoaggregation values of *Enterococcus* strains were between 9.70% and 14.69% at the end of 4 hours, and between 34.33% and 49.17% at the end of 24 hours. According to Del Re et al. (2000), strains with an autoaggregation value of less than 10% are defined as non-aggregating strains. Contrary to our results, Abushelaibi et al. (2017) reported that the autoaggregation values of potential probiotic LAB isolated from camel milk were between 1.5% and 10.2% at the end of 3 hours and between 2.7% and 38.8% at the end of 24 hours. On the other hand, Özkan et al. (2021) reported that the autoaggregation values of *E. faecium* strains isolated from Tulum cheese were between 15.87% and

33.05% at the end of the 3rd hour of incubation and between 9.7% and 49.09% at the end of the 24th hour, which is similar to our findings. In addition, there are also studies reporting the presence of *Enterococcus* strains with higher autoaggregation values than those determined in this study (Ben Braïek et al., 2018; Zommiti et al., 2018; Nami et al., 2019; Rajput and Dubey, 2020). Zommiti et al. (2018) reported that autoaggregation is strain-specific and may differ within the same taxonomic groups. Cell aggregation is one of the most important phenotypic features that can be used in the selection of a potential probiotic strain (Collado et al., 2007). Bacteria with autoaggregative potential prevent the colonization of pathogenic bacteria by forming a barrier to the intestinal mucosa via autoaggregation (Prince et al., 2012). After 24 hours of incubation, the autoaggregation values obtained in this study show that all enterocin-producing *Enterococcus* strains have the potential to be used as probiotic cultures.

Coaggregation ability is an important feature of probiotic bacteria and is defined as the bacterial accumulation of different species (Campana et al., 2017). When pathogens are present, coaggregation of LAB forms a protective barrier that keeps pathogens from taking up residence in the human intestine (Vidhyasagar and Jeevaratnam, 2013). Collado et al. (2008) and Nami et al. (2019) found that the ability of LAB to coaggregate depends on the strain (probiotic or pathogen) and the length of time it is incubated. Tareb et al. (2013) also reported that the ability of LAB isolates to aggregate with pathogens can be attributed to proteinaceous components present on the cell surface and interactions between carbohydrates and lectins. The coaggregation values of bacteriocin-producing *Enterococcus* strains after 4 hours of incubation were found to be similar to the values obtained by Abushelaibi et al. (2017) and Nami et al. (2019). Abushelaibi et al. (2017) reported that the coaggregation percentages of nine LAB isolates with *E. coli* O157:H7, *S.* Typhimurium, *L. monocytogenes*, and *S. aureus* were found to be between 3.0-16.2, 5.0-19.0, 3.4-17.7, and 4.0-16.9, respectively. Nami et al. (2019) reported that the coaggregation percentages of seven *Enterococcus* strains with antibacterial activity were between 2.2-12.7% with *S. aureus*, 2.8-19.9% with *E. coli*, and 2.5-18.7% with *L. monocytogenes*. Grujovic et al. (2021) reported that the coaggregation values of *Enterococcus* strains isolated from traditional Serbian cheese were between 0 and 23.32% with *E. coli* after 2 hours of incubation. The researchers indicated that *E. faecium* KGPMF14, *E. faecalis* KGPMF48, and KGPMF49 strains did not coaggregate with *E. coli*, but *E. faecalis* KGPMF47, *E. hirae* KGPMF9, and *E. durans* KGPMF10 strains coaggregated at 10.53±1.56%, 15.13±0.44%, and 23.32±0.36%, respectively. Contrary to our results, Bhagwat et al. (2019) found that among 13 *Enterococcus* isolates, especially *E. dispar* S27A (74.137±1.2), *E. canintestini* SB3 (73.37±1.34), and *E. canintestini* S18A (72.49±0.72), had very high coaggregation percentages with *E. coli* after 4 hours of incubation.

At the end of the 3-hour and 24-hour incubation periods, all strains of bacteriocin-producing *Enterococcus* demonstrated a cellular increase in the MRS broth containing lysozyme. Due to the high concentration of lysozyme in saliva, the mouth is the first obstacle to be overcome for probiotic bacteria (Samedi and Charles, 2019). Therefore, the high lysozyme resistance capacity of bacteriocin producer *Enterococcus* strains is an advantage for the strains to survive in saliva. In previous studies, it has been reported that species belonging to *Enterococcus* (Kıvanç et al., 2016) and *Lactobacillus* (Turchi et al., 2013; Rajoka et al., 2018) are resistant to lysozyme at a concentration of 100 mg/L, as confirmed in this study.

Some aromatic amino acids that come out as a result of digesting foods can be deaminated by bacteria and cause phenol formation in the intestines (Aswathy et al., 2008; Özden Tuncer and Tuncer, 2014; Panda et al., 2017). For this reason, it is accepted that probiotic bacteria that are resistant to 0.4% phenol *in vitro* can survive in the intestine (Sarkar et al., 2020). In this study, we determined that bacteriocin-producing *Enterococcus* strains generally tolerated phenol at high levels, which is an advantage in terms of their use as probiotic cultures. Similar to our results, Rajput and Dubey (2020) found that the *E. hirae* G24 strain could survive at a high rate after both 4 and 24 hours of incubation in the presence of 0.4% phenol. On the other hand, Sarkar et al. (2020) found that two *E. faecium* strains that produce an antibacterial substance were only moderately resistant to 0.4% phenol.

CONCLUSION

In this study, the *in vitro* probiotic properties of enterocin-producing *E. mundtii* and *E. faecium* strains were tested. The inability of *Enterococcus* strains to survive in simulated gastric juice is a disadvantage for their use as probiotic cultures. On the other hand, the fact that *Enterococcus* strains are resistant to oral

(lysozyme) and lower digestive tract conditions (bile salt and phenol) and have good hydrophobicity, autoaggregation, and coaggregation values is advantageous in terms of being used as a probiotic culture. For this reason, it is thought that the enterocin-producing strains have the potential to be used as probiotic cultures by increasing their resistance against the harsh environmental conditions created by the stomach through microencapsulation. Further, studies are required for the safety evaluation of the strains.

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CONFLICT OF INTEREST

The authors have declared no conflicts of interest for this article.

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