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Probiotic Potential of Dairy Western Balkan Countries *Enterococcus faecium* strains

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Abstract

One of the major genera of the lactic acid bacteria family, *Enterococcus* sp., has a controversial status, reflected in the fact that enterococci are utilized as starter cultures and probiotics, in addition to being known to cause nosocomial infections. The qualified presumption of the safety list and the widely acknowledged safe status for *Enterococcus* species are absent. Rich sources of *Enterococcus faecium* species with possible probiotic characteristics can be found in artisanal dairy products, typically made from raw milk. To further understand the probiotic potential and health-promoting effects, this study looked at the presence of virulence factors and adhesion properties of *En. faecium* isolated from artisanal dairy products from Western Balkan countries.

Key words: enterococci, virulence factors, probiotics, extracellular matrix, adhesion, survival

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Introduction

Enterococci, a group of lactic acid bacteria (LAB), are highly debated due to their controversial nature (1). They are facultative anaerobes highly tolerant to diverse environmental conditions such as extreme temperatures, pH, and salt concentrations. This tolerance contributes to their colonization of diverse host niches and persistence in the environment (2, 3). *Enterococcus faecium* is one of the most common types of bacteria that cause infections in humans and is ranked third among multidrug-resistant nosocomial pathogens that cause bacteremia (4). To persist in nosocomial infections, enterococci exhibit a variety of virulence factors, such as gelatinase activity (GelE), enterococcal surface protein (Esp), aggregating substances (Agg), hyaluronidase (Hyl), and cytolysin (Cyl, -hemolysin) (5, 6). Biofilms are intricate communities of microorganisms that are widespread in the natural environment. Biofilm-forming enterococci are a significant cause of various infections, exhibiting increased virulence and antimicrobial resistance (7). Conversely, enterococci are also used as probiotics and starter cultures for various types of cheese. Probiotic enterococci are live microorganisms identified at the strain level that, when given in an appropriate amount, have a beneficial effect on the host's health (8). The first probiotic *En. faecium* SF68® strain is frequently used in veterinary applications to prevent and treat diarrhea in cats and dogs, as well as for treating human digestive system diseases (9, 10). Enterococci are frequently found in Mediterranean-style cheese curds that contribute to the taste and flavor development during cheese ripening, most likely through proteolysis, lipolysis, and citrate breakdown (11). Additionally, enterococci are capable of producing bacteriocins that are effective against pathogenic and spoilage-causing microorganisms in food, as well as suitable probiotic qualities, which are compelling grounds for their use in the fermentation of food (12, 13). Despite their controversial reputation, awareness of enterococci's probiotic potential has recently increased. Because they can survive in harsh digestive conditions, stick to intestinal epithelial cells, and actively keep pathogens out, which are all important qualities of probiotics, they have gained a lot of attention (14). Probiotic enterococci express cell-surface adhesins, facilitating adhesion to host tissue components like mucin, fibronectin, collagen, laminin, or fibrinogen (15, 16). Conversely, pathogenic bacteria also employ specific adhesiveness to collagenous proteins, a crucial factor in early-phase infections and pathogen virulence (17). This interaction enables pathogens to interact with extracellular matrix proteins, ensuring colonization and tissue infection (18).

This study aimed to investigate the probiotic potential of artisanal dairy strains of *En. faecium* isolated from milk and cheese from various locations in the Western Balkans, including survival in simulated GIT conditions, adhesion to the components of the extracellular matrix (ECM) and human intestinal cell line, and the ability to counteract the harmful effects of pathogens.

Material and Methods

Media, Bacterial strains, and Growth factors

Ten dairy isolates of the enterococci species *En. faecium* that had previously been identified were used (Table I). M17 broth (Merck, GmbH, Darmstadt, Germany) supplemented with glucose (0.5% w/v) (GM17) was used to grow enterococci, *Listeria monocytogenes* ATCC19111, and *Lactococcus lactis* subsp. *lactis* at 37 °C and 30 °C, respectively. *Escherichia coli* ATCC25922 and *Salmonella* Enteritidis 654/7E were grown at 37 °C in Luria-Bertani broth (LB), which contained 0.5% NaCl, 0.5% yeast extract (Torlak, Belgrade, Serbia), and 1% tryptone (Torlak). Each broth was mixed with agar (1.7% w/v, Torlak) to create corresponding agar plates.

Table I List of Enterococcus faecium strains used in this study and their origin

Tabela I Spisak sojeva Enterococcus faecium i njihovo poreklo korišćenih u ovoj studiji

Strain	Origin	Region
BGPAS1-3	Cheese	Bosnia and Herzegovina, surrounding Pale mountain city
BGPAS1-4	Cheese	Bosnia and Herzegovina, surrounding Pale mountain city
BGPAS1-10	Cheese	Bosnia and Herzegovina, surrounding Pale mountain city
BGPAS1-20	Cheese	Bosnia and Herzegovina, surrounding Pale mountain city
BGPAS1-58	Cheese	Bosnia and Herzegovina, surrounding Pale mountain city
BGPAS1-71	Cheese	Bosnia and Herzegovina, surrounding Pale mountain city
BGZLM1-5	Milk	Serbia, Zlatar mountain
BGGO9-28	Cheese	Serbia, Golija mountain
BGGO11-27	Cheese	Serbia, Golija mountain
BGGO11-29	Cheese	Serbia, Golija mountain

PCR Detection of Virulence Determinants

According to Parish, the total DNA extracted from ten *En. faecium* species was used in PCR tests to determine whether virulence-related genes were present or absent (19). Table II lists the target gene primer sequences, anticipated amplicon sizes, and annealing temperatures.

Antimicrobial Activity Assay

The deferred antagonism approach was employed for evaluating antimicrobial substances synthesized by enterococci using different indicator strains (14). Table III contains a list of the indicator strains utilized in this test.

Table II List of primers used in this study

Tabela II Spisak prajmera korišćenih u ovoj studiji

Genes	Primers	Product size	T°C[a]	Reference
Virulence factors				
gelE	5′-CGGAAGGCGTTACTGTTGAT-3′ 5′-GAGCCATGGTTTCTGGTTGT-3′	957 bp	46°C	(14)
sprE	5′- TTGAGCTCCGTTCCTGCCGAAAGTCATTC-3′ 5′-TTGGTACCGATTGGGGAACCAGATTGACC-3′	591 bp	58°C	(36)
ace	5′-AAAGTAGAATTAGATCCACAC-3′ 5′-TCTATCACATTCGGTTGCG-3′	320 bp	56°C	(37)
hylN	5′-ACAGAAGAGCTGCAGGAAATG-3′ 5′-GACTGACGTCCAAGTTTCCAA-3′	276 bp	56°C	(38)
agg	5′-AAGAAAAAGAAGTAGACCAAC-3′ 5′-AAACGGCAAGACAAGTAAATA-3′	1553 bp	54°C	(39)
cylA	5′-TGGATGATAGTGATAGGAAGT-3′ 5′-TCTACAGTAAATCTTTCGTCA-3′	517 bp	58°C	(39)
esp	5′-TTGCTAATGCTAGTCCCAGACC-3′ 5′-GCGTCAACACTTGCATTGCCGAA-3′	933 bp	58°C	(39)
efaA^{fs}	5′-GACAGACCCTCACGAATA-3′ 5′-ATGTCATCATGCTGTAGTA-3′	705 bp	56°C	(39)
efaA^{fm}	5′-AACAGATCCGCATGAATA-3′ 5′-CATTTCATCATCTGATAGTA-3′	735 bp	56°C	(39)

Note: [a] - annealing temperature for a given primer pair

Table III The list of indicator strains used in this study

Tabela III Spisak indikatorskih sojeva korišćenih u ovoj studiji

Bacterial strains	Source
Lactococcus lactis subsp. *lactis* BGMN1-596	Laboratory collection
Enterococcus faecalis BG221	Laboratory collection
Listeria monocytogenes ATCC19111	ATCC [a]
Escherichia coli ATCC25922	ATCC
Salmonella Enteritidis 654/7E	Scientific Veterinary Institute ‘Novi Sad’, Serbia

Note: [a] ATCC-American Type Culture Collection

MTT Assay

A microculture tetrazolium [MTT, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] test was used to assess the cytotoxicity of enterococci on the HT29-MTX cell line, which was kindly provided by Dr. T. Lesuffleur (INSERM UMR S938, Paris, France; (20)). After 24 hours of seeding (40–60% confluency), filtered supernatants (using 0.22 µm Nalgene syringe filter units, Sarstedt, Nümbrecht, Germany) and UV-irradiated non-viable bacterial cells were added to the eukaryotic cells in various concentrations (ratios 1:1 and 10:1 bacteria: eukaryotic cell). Live cells exposed to MTT produced formazan crystals that were dissolved in 10% sodium dodecylsulfate (SDS) in 0.01% HCl. After that, the wells were incubated overnight at 37 °C. Plate Reader Infinite 200 Pro was used to measure the adsorption of the dissolved formazan crystals at 570 nm. The percentage of optical density (metabolic activity) relative to the control (cultures of untreated cells), which was utilized to express the results, was calculated as follows: Metabolic activity (%) = 100 divided by (OD of treated cells minus the OD of untreated cells).

Survival in Simulated Gastrointestinal Tract

Survival in a chemically imitated gastrointestinal tract was tested in vitro, as previously described (22). The viable cells recovered after each chemically simulated GIT stage about the initial counts were used to calculate survival. The results were expressed as log colony forming units (CFU) per mL.

Assay for Extracellular Matrix Adhesion

Mucin, collagen, and fibronectin adhesion experiments were done in 96-well polystyrene microtiter plates (Sarsted, Newton, USA) using Valeriano and colleagues' (23) method with slight modifications. At 4 °C for 24 hours, the wells of microtiter plates were coated with 200 L of 100 g/mL porcine stomach mucin-type II (Sigma, Germany), collagen type I (Sigma), and human fibronectin (Serva, Heidelberg, Germany). Wells were rinsed twice with 200 µL PBS before being incubated for 2 hours at 4 °C with 100 µL (20 mg/mL) bovine serum albumin (BSA) (Sigma, Germany). To eliminate detached BSA, the wells were washed twice with 200 µL of PBS. A bacterial culture containing approximately 10^8 CFU/mL (100 µL) was washed, suspended in PBS (pH 7.0), and applied to the wells. Plates were incubated for 2 hours before being rinsed twice with 200 µL of PBS to wash out unattached bacteria. To isolate the adhering bacteria, another 200 µL of 0.5% (v/v) Triton X-100 (Sigma) was added at 37 °C. Plating on GM17 determined the viable cell count expressed as CFU/mL in all cases. The percentage adhesion was obtained by multiplying the viable counts adhered to the mucin, collagen, and fibronectin by the initial count (%) = (CFU/mL recovered bacteria/CFU/mL initial bacteria) ×100.

HT29-MTX Cell Line Adhesion

According to Živković et al. (24), 13 ± 1 day-old cellular monolayers were used for adhesion to HT29-MTX cell line studies. Cellular monolayers were thoroughly cleaned before bacterial suspensions were introduced at a 10:1 (bacteria: eukaryotic cell) ratio. Adhesion tests were performed for 1 hour at 37 °C and 5% CO_2. To calculate the percentage of adhesion we used the formula: (%) = (CFU/mL adhering bacteria/CFU/mL presented bacteria) x 100.

Assay for Pathogen Exclusion

Salmonella Enteritidis 654/7E and *E. coli* ATCC25922 were examined for their capacity to attach to the intestinal epithelium in the presence and absence of enterococci (24). Bacterial suspensions (1 x 10^8 CFU/mL) containing *E. coli*, or a combination of *E. coli* and enterococci (ratio 1:1) and 1 x 10^8 CFU/mL *Salmonella* Enteritidis, or a combination of *Salmonella* Enteritidis and enterococci (ratio 1:1) were added to the HT29-MTX monolayers at a 10:1 (bacteria:eukaryotic cell) ratio and incubated for 1 hour at 37 °C with 5% CO_2. The adhesion percentage was calculated as follows: bacteria attached to HT29-MTX monolayers at 100 CFU/mL / total CFU/mL bacteria introduced (corrected for dilution). Viable cell count expressed as CFU/mL measured by plating on LA was used to determine the count of bacteria. To test the ability of the enterococci to inhibit *E. coli* and *Salmonella* Enteritidis adhesion to HT29-MTX monolayers, the data were compared to that obtained with *E. coli* and *Salmonella* Enteritidis alone (i.e., 100% adhesion).

Statistical Analysis

Each of the experiments was carried out in duplicate and independently performed at least twice. All separate experiments' data are presented as mean values with a standard deviation. For multiple group comparisons, a one-way ANOVA with Tukey's post hoc test was utilized. $p < 0.05$ was considered statistically significant. GraphPad Prism 9 software (California, San Diego, USA) was used to perform statistical analysis and create graphics.

Results and Discussion

Probiotic usage is widely acknowledged as a viable strategy for enhancing or stabilizing the digestive system. The typical microbial community of the human GIT includes enterococci. They can be found naturally in many food products, as well as frequently being linked to various types of traditional fermentations or purposefully added as starting cultures (13). Ten strains of *En. faecium* used in this study were isolated from different dairy products from the Western Balkans (Table I). Previous research has demonstrated that autochthonous dairy products from the Western Balkan counties can be used as a source of novel enterococci probiotic strains (25). Our previous results based on the safety assessment analysis showed that ten strains of *En. faecium* showed sensitivity to nine relevant clinical antibiotics according to the Clinical and Laboratory Standards

Institute (CLSI) standards, and they did not express gelatinase and hemolytic activity (14). The ten strains employed in this research were examined for the presence or absence of the genes associated with virulence encoding aggregation factor (*agg*), collagen adhesin (*ace*), cytolysin (*cylA*), enterococcal surface protein (*esp*), cell wall adhesins ($efaA^{fs}$ and $efaA^{fm}$), gelatinase (*gelE*), hyaluronidase (*hyl*), and serine protease (*sprE*) (Table IV). Six strains (60%) and eight strains (80%), respectively, tested positive for the *esp* gene and the *agg* gene. The $efaA^{fm}$ gene was found to be present in five strains (50%) and the $efaA^{fs}$ gene in three strains (30%). The *ace* gene was not found to be present. It is noteworthy that neither the *cylA* gene, which codes for CylA serine protease, nor the *hyl* gene, which codes for hyaluronidase, a degradative enzyme linked to tissue damage (26), were found. CylA serine protease is involved in processing and activating cytolysin, also known as hemolysin, a bacterial toxin with beta-hemolytic properties in humans. Additionally, neither the *gelE* gene encoding gelatinase nor the *sprE* encoding serine protease were detected in any of the strains. Both gelatinase and serine protease play a part in the pathogenesis of enterococci, supplying the bacteria with nutrients by destroying host tissue, but they also play a part in the development of biofilms (27). Additional crucial probiotic characteristics include persistence in the intestine, competitive exclusion of pathogens, and adhesion to intestinal epithelial cells (IEC), which are essential to colonizing the intestinal mucosa (28, 29). Considering these results, we hypothesize that the presence of genes coding for adhesins, but not for pathogenesis-associated enzymes, enables these strains to adhere to the host surfaces, which can exert health-promoting effects.

Table IV Presence of the virulence genes and genes for biofilm formation

Tabela IV Prisustvo gena virulencije i gena za formiranje biofilma

Strains	Enzymes				Adhesins				
	gelE	*sprE*	*Hyl*	*clyA*	*agg*	*esp*	*ace*	$efaA^{fs}$	$efaA^{fm}$
BGPAS1-3					■				■
BGPAS1-4					■				
BGPAS1-10					■	■			
BGPAS1-20					■	■			■
BGPAS1-58						■			
BGPAS1-71					■	■		■	
BGZLM1-5					■			■	■
BGGO9-28								■	■
BGGO11-27					■	■			
BGGO11-29					■	■			

Note: The shaded areas reflect the presence of the respective gene.

To achieve these effects, probiotic enterococci must survive the unfavorable conditions of the GIT. To examine the survival of enterococci in GIT conditions, ten strains of *En. faecium* were exposed to conditions simulating the GIT (Figure 1). All of the bacteria survived well in highly acidic stomach conditions (from initial 8.79 to 8.23 log CFU/mL), demonstrating the isolates' adaptability to such conditions. After prolonged exposure to lower bile concentrations (0.3%) (8.37–7.40 CFU/mL) and pancreatic enzymes (7.83–7.00 log CFU/mL), the survival rate was either maintained or significantly lowered. This is because earlier research has shown that the GIT is resistant and can survive challenging circumstances like those seen there (14).

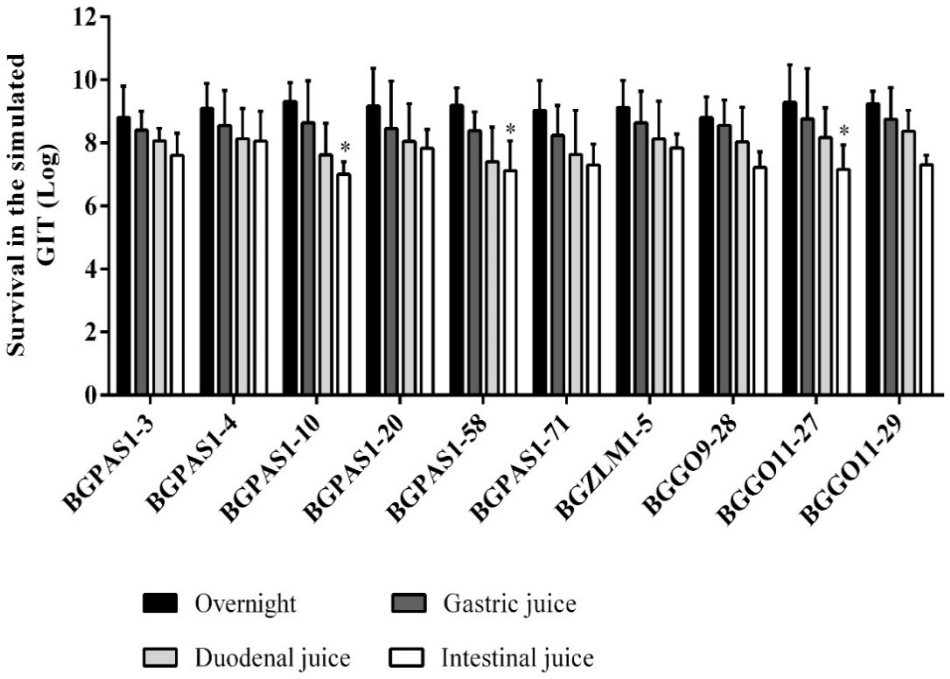

Figure 1. Survival of the enterococci in *in vitro* simulated gastrointestinal conditions. The statistical differences between treatments are annotated with asterisks (*$p < 0.05$).

Slika 1. Preživljavanje enterokoka u *in vitro* simuliranim gastrointestinalnim uslovima. Statističke razlike između tretmana su označene zvezdicama (*$p < 0,05$).

The integrity of the intestinal epithelial barrier is essential for the intestinal epithelium's delicate sensitivity to modulations by commensal and pathogenic microorganisms (30). In addition to the absence of pathogenesis-associated genes, excluding strains with cytotoxicity effects is a very important step. To investigate any potential negative effects, ten *En. faecium* strains were each exposed to the supernatants of overnight bacterial cultures (soluble bacterial products) and UV-irradiated non-viable bacterial cells (surface bacterial cell molecules). The findings of the MTT test showed that none of the

investigated bacteria, their soluble products, or surface chemicals had any significant effects on the metabolic activity of HT29-MTX cells (Figure 2). The data demonstrate the safety of all tested strains because none of the dairy isolates investigated have a cytotoxic effect on the intestinal epithelial barrier. In addition to analyzing the presence of adhesion genes, we further investigated the potential of these strains to adhere to the main components of the ECM, such as mucin (Figure 3A), collagen (Figure 3B), and structural glycoprotein fibronectin (Figure 3C), to assess its probiotic potential (31, 32). Each strain that was evaluated showed a strong affinity for certain ECM elements. Our findings showed that 10 enterococci dairy isolates had a strong ability to bind to mucin, with an average value of 72.9% + 2.77, to collagen, with an average value of 75.9% ± 1.83, and to fibronectin, with an average value of 74% ± 1.74. Interestingly, most strains showed the ability to bind collagen and fibronectin with greater affinity than pathogenic species *Salmonella* Enteritidis 654/7E and *E. coli* ATCC25922, while only BGZLM1-5 and BGGO9-28 bound to mucin were stronger than pathogenic bacteria with significant affinity. To estimate the percentage of attachment of the prospective probiotic strains, the ability of tested enterococci to adhere to the epithelial intestinal cell line HT29-MTX was also assessed. According to the study findings, the tested strains had strong adhesion capabilities, adhering to the HT29-MTX cell line at a rate of 89.7% ± 1.2 (Figure 4). These results are by the properties of enterococcal strains selected in other studies to adhere to components of the ECM and intestinal epithelial cells (14, 33, 34).

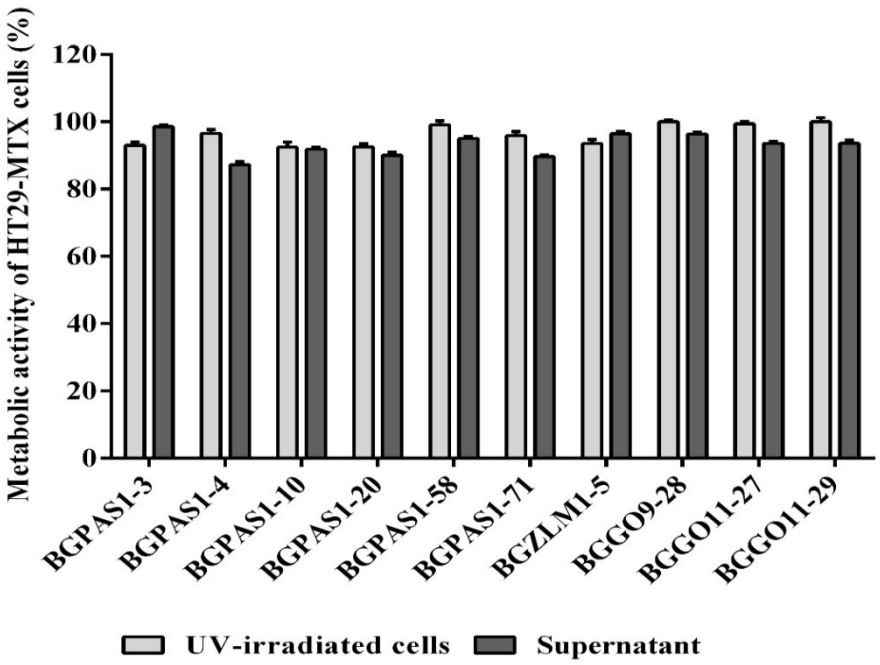

Figure 2. The cytotoxicity of enterococci on HT29-MTX cell line
Slika 2. Citotoksičnost enterokoka na ćelijskoj liniji HT29-MTX

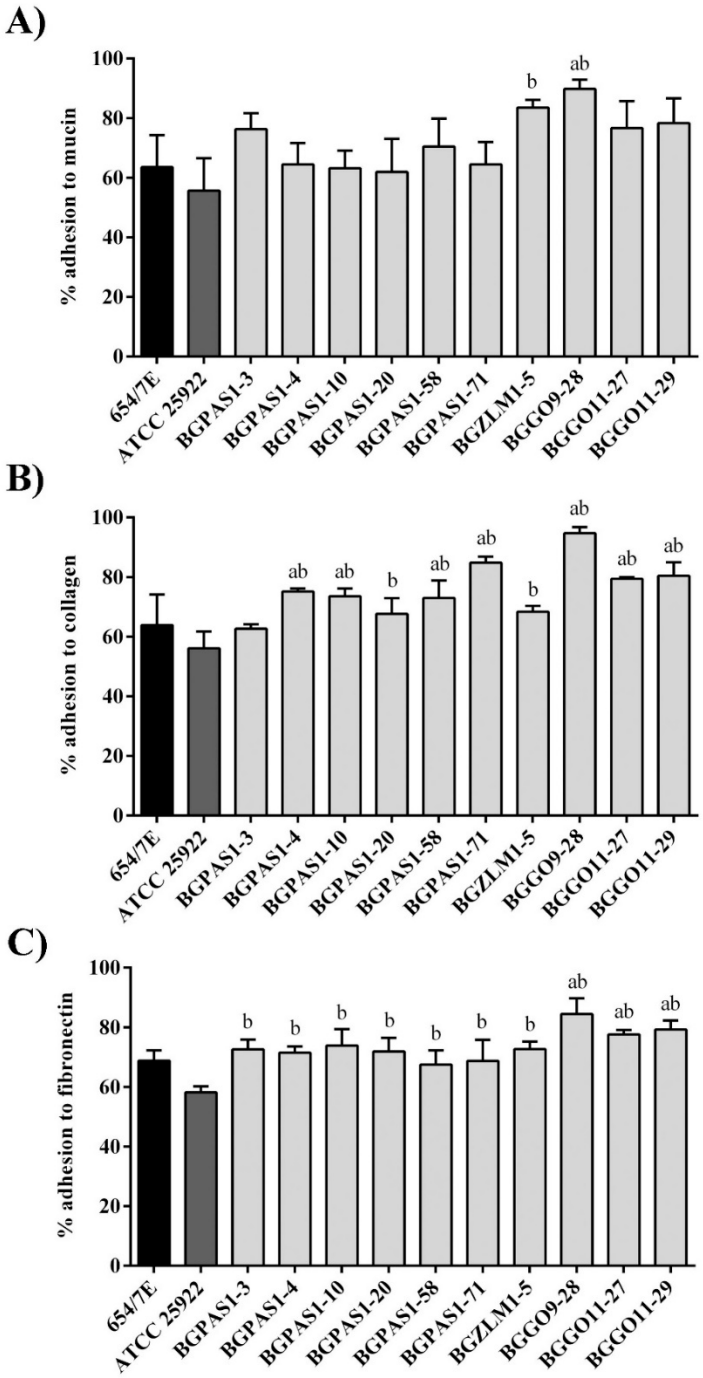

Figure 3. **Adhesion of the enterococci strains to mucin (A), collagen (B), and fibronectin (C). Statistical differences ($p < 0.05$) associated with *Salmonella* Enteritidis 654/7E are marked with the letter a, and the association with *E. coli* ATCC25922 with the letter b.**

Slika 3. **Adhezija sojeva enterokoka za mucin (A), kolagen (B) i fibronektin (C). Statističke razlike ($p < 0,05$) povezane sa *Salmonella* Enteritidis 654/7E označene su slovom a, a povezanost sa *E. coli* ATCC25922 slovom b.**

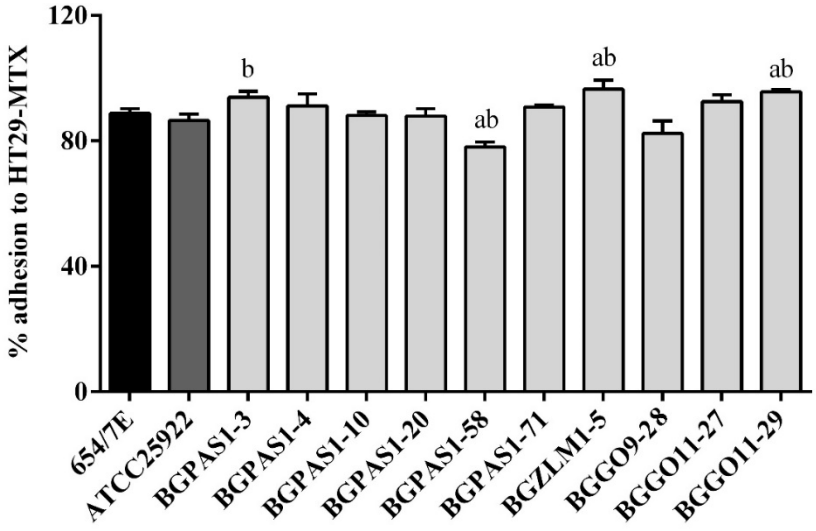

Figure 4. Adhesion of the enterococci strains to the human intestinal epithelial cell line HT29-MTX. Statistical differences ($p < 0.05$) associated with *Salmonella* Enteritidis 654/7E are marked with the letter a, and the association with *E. coli* ATCC25922 with the letter b.

Slika 4. Adhezija sojeva enterokoka za ćelijsku liniju humanog crevnog epitela HT29-MTX. Statističke razlike ($p < 0,05$) povezane sa *Salmonella* Enteritidis 654/7E označene su slovom a, a povezanost sa *E. coli* ATCC25922 slovom b.

One of the first and most common health-promoting properties of probiotic strains is their ability to counteract the harmful effects of pathogens (35). One of the ways they achieve this is through antimicrobial activity, involving the production of bacteriocins, as well as processes like colonization competition and pathogen exclusion (3). We thus showed that only two strains (BGPAS1-3 and BGZLM1-5) have antimicrobial activity (Table V) against specific pathogens; however, none of the tested strains showed activity against *Salmonella* Enteritidis 654/7E and *E. coli* ATCC25922, so we analyzed other potential anti-pathogenic mechanisms. It is proposed that certain cell surface components, such as S-layer macromolecules or auto-aggregation factors, could be essential (3, 11). These components may contribute to the probiotic strains' ability to compete with pathogens for colonization and maintain a healthy microbial balance within the host. The study findings showed that the presence of the two enterococci strains under test decreased the adhesion of *E. coli* ATCC25922 and *Salmonella* Enteritidis 654/7E to HT29-MTX (Figure 5). The adhesion of *Salmonella* Enteritidis 654/7E in the presence of *En. faecium* BGPAS1-3 during co-incubation assay is 94.6% compared to control, whereas the adhesion of *E. coli* ATCC25922 in the presence of *En. faecium* BGPAS1-4 is 96.1% compared to the control.

Table V Antimicrobial activity of *Enterococcus faecium*

Tabela V Antimikrobna aktivnost *Enterococcus faecium*

	BGMN1-596	BG221	ATCC19111	ATCC25922	654/7E
BGPAS1-3					
BGPAS1-4					
BGPAS1-10					
BGPAS1-20					
BGPAS1-58					
BGPAS1-71					
BGZLM1-5					
BGGO9-28					
BGGO11-27					
BGGO11-29					

Note: The shaded areas reflect the presence of antimicrobial activity.

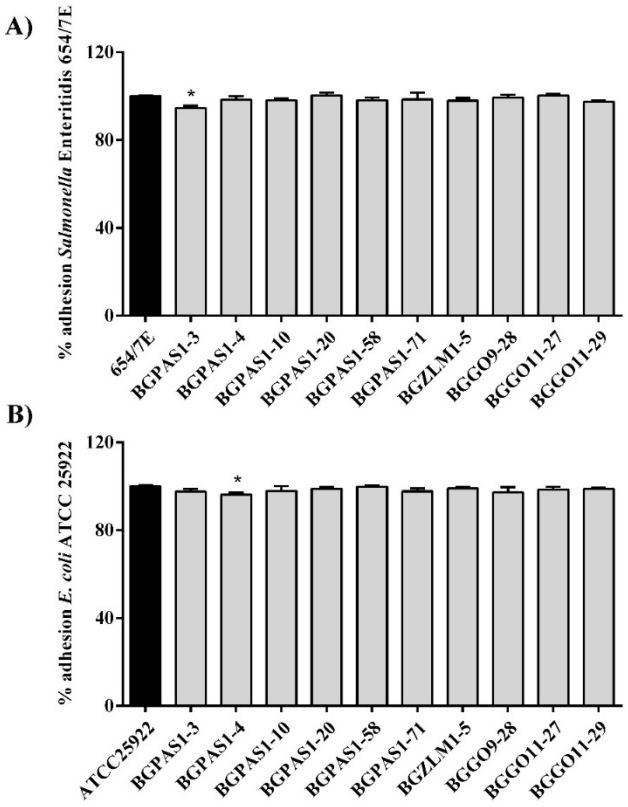

Figure 5. Association of *Salmonella* Enteritidis 654/7E to HT29-MTX cells in the presence of enterococci (A) and association of *E. coli* ATCC25922 to HT29-MTX cells in the presence of enterococci (B). The statistical differences concerning the control strains are annotated with asterisks (*$p < 0.05$).

Slika 5. Adhezija *Salmonella* Enteritidis 654/7E za HT29-MTX ćelijsku liniju u prisustvu enterokoka (A) i adhezija *E. coli* ATCC25922 za HT29-MTX ćelijsku liniju u prisustvu enterokoka (B). Statističke razlike u odnosu na netretirane kontrole su označene zvezdicama (*$p < 0,05$).

Conclusion

As far as we know, this is the first study that combines data on virulence genes and probiotic features of dairy *En. faecium* isolates from the Western Balkan, which expands our understanding of virulence factors implicated in dairy enterococci's probiotic properties. While virulence genes may be sporadically present in enterococci, they could potentially serve as advantageous features, aiding in their successful colonization of the gut.

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Conflict of interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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Probiotički potencijal sojeva *Enterococcus faecium* izolovanih iz mlečnih proizvoda sa područja Zapadnog Balkana

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Kratak sadržaj

Rod *Enterococcus* je jedan od glavnih rodova koji pripada bakterijama mlečne kiseline i ima kontroverzni status, koji se ogleda u činjenici da su enterokoke prepoznate kao uzročnici bolničkih infekcija, dok se istovremeno koriste i kao probiotici i kao starter kulture. Pripadnici vrste *Enterococcus* nemaju opštepriznat bezbedni status, niti su uvršteni na liste bezbednih sojeva Evropske agencije za bezbednost hrane. Autohtoni mlečni proizvodi, posebno oni proizvedeni od sirovog mleka, predstavljaju bogate izvore vrsta *Enterococcus faecium* sa potencijalnim probiotičkim svojstvima. U ovoj studiji je istraživano prisustvo faktora virulencije i sposobnost adhezije vrsta *En. faecium* izolovanih iz mlečnih, autohtonih proizvoda sa područja Zapadnog Balkana sa ciljem boljeg razumevanja njihovog probiotičkog potencijala, kao i efekata koji doprinose unapređenju zdravlja korisnika.

Ključne reči: enterokoke, faktori virulencije, probiotici, ekstracelularni matriks, adhezija, preživljavanje