Original Research

BIOTECHINTELLECT, 2025,2 (1) e3 (1-14)
https://jbiotechintel.com/index.php/biotechintel

eISSN: 3115-7920

OPEN ACCESS

Safety Assessment and Anticancer Potential of Fermented Milk Containing *Enterococcus faecium* KMJC93 Isolated from Traditional Sources

Mandana Mahmoudi[1], Morteza Khomeiri[1*], Mohsen Saeidi[2], Homa Davoodi[3], Ali Memarian[2]

1- Department of Food Science and Technology, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran
2- Stem Cell Research Center, Golestan University of Medical Sciences, Gorgan, Iran
3- Cancer Research Center, Golestan University of Medical Sciences, Gorgan, Iran

Article history:
Received 15 November 2024
Revised 17 December 2024
Accepted 30 December 2024
Published online 01 January 2025

Keywords: *Anticancer, Apoptosis, Enterococcus, Fermented milk, Probiotic, Safety*

How to cite this article: Mahmoudi, M., Khomeiri, M., Saeidi, M., Davoodi, H., & Memarian, A. (2025). Safety Assessment and Anticancer Potential of Fermented Milk Containing *Enterococcus faecium* KMJC93 Isolated from Traditional Sources. *BiotechIntellect, 2(1)*, e3 (1-14).
https://doi.org/10.61838/biotechintellect.18

*corresponding author's email:
khomeiri@gau.ac.ir
Tel: 00989111777143,

ABSTRACT

The objective of the present research was to assess the safety and anticancer effect of fermented milk containing *Enterococcus* strains. Initially, six autochthonous *Enterococcus* strains were molecularly identified. To demonstrate their safety *in vitro*, six strains were evaluated for hemolysis and vancomycin sensitivity. Furthermore, they were also studied for their milk fermentation ability. To confirm the safety of fermented milk, its toxic effect on normal mouse fibroblast cell (L929) was examined. Selected fermented milk was evaluated for anticancer activity against human colon cancer cell (HT-29). The findings showed that six *Enterococcus* strains were *E. faecium* KMJC41 (OP764046), *E. faecalis* KMJC54 (OP764047), *E. faecalis* KMJC62 (OP764048), *E. faecium* KMJC71 (OP764049), *E. faecium* KMJC93 (OP764050), *E. faecium* KMCH3 (OP764051). Among them, three strains including *E. faecium* KMJC93, *E. faecium* KMJC41 and *E. faecalis* KMJC62 did not show hemolysis and were sensitive to vancomycin. *E. faecium* KMJC93 and *E. faecium* KMJC41 showed milk fermentation capability. The cytotoxicity of fermented milk with *E. faecium* KMJC93 and *E. faecium* KMJC41 on normal mouse fibroblast cell was 7% and 24%, respectively. Since HT-29 cell viability treated with milk fermented by *E. faecium* KMJC41 was below 90%, hence, fermented milk with *E. faecium* KMJC41 was excluded from the apoptosis test. Fermented milk with *E. faecium* KMJC93 induced 46% apoptosis in HT-29 cells. Therefore, it was concluded that the fermented milk with *E. faecium* KMJC93 was safe and presented promising anticancer properties.

What is "already known":	• Probiotic bacteria generally have lower milk fermentation capability compared to commercial starter cultures. • The safety of probiotic fermented milk is commonly evaluated by assessing its cytotoxicity toward normal mammalian cells, where cell viability above 90% is considered acceptable. • The cytotoxic effects of fermented milk or probiotic metabolites on cancer cells are typically dose- and time-dependent. • Apoptosis is recognized as the most desirable mechanism for inhibiting cancer cell growth without damaging surrounding normal cells. • *Enterococcus* strains are found in dairy products and some exhibit probiotic potential. • Some *Enterococcus* isolates have been studied for antimicrobial or probiotic properties, but their anticancer potential in fermented milk systems remains poorly understood.
What this article adds:	• Safe *Enterococcus faecium* KMJC93 was isolated from dairy sources, showing a non-hemolytic and vancomycin-sensitive profile. • The fermented milk does not sjow cytotoxic effects on normal fibroblast (L929) cells. • Fermented milk with *E. faecium* KMJC93 induced 46% apoptosis in HT-29 colon cancer. • Treated HT-29 cells exhibited significantly higher early apoptosis and lower late apoptosis compared to untreated controls. • No significant necrosis was observed, as the fermented milk supernatant did not affect necrosis in HT-29 cells compared to the control.

1. Introduction

Functional foods have gained widespread acceptance among consumers in various countries due to their associated health benefits. Probiotics, commonly found in dairy-based fermented foods, offer a range of health-promoting properties. Previous researches have explored the health benefits of probiotics and their bioactive components, including anticancer, antimicrobial, antihypertension and antioxidant properties, in various dairy products such as fermented milk [1, 2], synbiotic yogurt [3], kefir [4] and cheese [5]. While, *Lactobacillus* and *Lactococcus* species have been extensively studied for their probiotic potential, certain enterococci strains have also been investigated for their health-promoting effects. For instance, peptides derived from fermented milk with *Enterococcus faecalis* have demonstrated antihypertensive activity [6], and bacteriocin from *E. thailandicus* has exhibited anticancer activity without side effects on normal cells [7]. Moreover, *Enterococcus* strains showed antimicrobial activities against both Gram-negative and Gram-positive pathogenic bacteria [8, 9], as well as cholesterol-lowering activity and bile salt hydrolases capability [10, 11]. Furthermore, in some countries enterococci are utilized as starter cultures in cheese production to enhance flavor during ripening [12].

Despite of the health benefits associated with enterococci in fermented foods, some of strains have been identified as opportunistic pathogens. *E. faecalis* and *E. faecium* are implicated as the two species of enterococci responsible for causing infection [13, 14]. Furthermore, the potential presence of virulence genes or transferable vancomycin resistance genes in certain enterococci strains raises safety concerns, limiting their use in food fermentation [15]. Nevertheless, laboratory safety evaluation methods can distinguish between pathogenic and non-pathogenic enterococci, and the safety of functional foods produced with enterococci can be assessed using normal cell lines.

One of the remarkable functional properties of probiotics is their anticancer effect. Some of the mechanisms used by probiotics to prevent intestinal cancer including altering in the metabolic activity of the intestinal microbiota, the binding and neutralization of carcinogenic compounds in the intestine, the production of short-chain fatty acids, enhancement of the immune response in the host and the inhibition of the proliferation of cancer cell [16]. Induction of apoptosis, or programmed cell death, is a key mechanism in cancer treatment, as resistance to chemotherapy often arises from a lack of apoptosis in cancer cells [17]. Thus, agents capable of inhibiting cancer cell proliferation without harming normal cells hold promise for cancer prevention and treatment [18]. Probiotics represent a potential avenue for discovering anticancer agents with minimal side effects. In this regard, Mahmoudi et al. [1] reported that fermented milk with single culture of *Lactiplantibacillus plantarum* KMJC4, *Lactococcus lactis* KMCM3 and *Lactobacillus helveticus* KMCH1 induced apoptosis in HT-29 cell without side effects on normal mouse fibroblast cell (L929). Additionally, the anticancer effect of fermented goat milk with *Lactiplantibacillus plantarum* and *Lacticaseibacillus paracasei* against HeLa cancer cells was observed [19]. Kefir was introduced as a probiotic product with anticancer effect [20]. But until now, the anticancer effect of fermented milk with enterococci has not been studied.

This research aims to evaluate the *in vitro* safety of autochthonous enterococci isolated from traditional fermented dairy products through hemolysis and vancomycin sensitivity tests. Milk fermentation capability by safe strains was also studied. Subsequently, the safety of fermented milk containing selected enterococci strains was assessed using normal mouse fibroblast cell (L929). Additionally, this study investigates, for the first time, the anticancer properties of fermented milk containing enterococci strains.

2. Materials and Methods

2.1. Isolation and initial phenotypic identification of enterococci

A volume of 10 ml of Chal (Iranian traditional fermented camel milk) sample was transferred to 90 ml of sterile saline solution (0.85%) under sterile conditions and mixed. 10 g of Jug cheese samples were homogenized with 90 ml of sterile 2% trisodium citrate solution for 5 min by stomacher (Seward Laboratory, London, UK). 100 µl of diluted and uniformed samples were spread on plates containing de Man, Rogosa, Sharpe (MRS; Merck, Darmstadt, Hesse, Germany) agar. The plates were then incubated for 48 h at 37 °C under anaerobic conditions created by Gas Pack (Anaerocult A, Merck) in anaerobic jar. Next, the colonies grown on each plate were studied by microscopic observation, Gram staining and catalase activity [21, 22]. The characteristics of the samples collected in this research are shown in Table 1.

2.2. Molecular identification and phylogenetic assessment

2.2.1. DNA extraction and Standard PCR

Genomic DNA extraction from the pure culture was done based on the protocol of Yekta Tajhiz Azma kit (Tehran, Iran). The 27F and 1492R universal primers were used for the amplification of 16S rRNA gene. PCR was performed according to the protocol provided by Mahmoudi et al. [22] for optimized amounts of reaction components and thermocycler temperature program.

2.2.2. Sequencing and phylogenetic analysis

PCR products were sequenced by Macrogen Company in South Korea. Then, in order to identify the isolates, the resulting sequences were blasted with the sequences in the NCBI (National Center of Biotechnology Information) database with using the BLAST (Basic Local Alignment Search Tool) program (http://www.ncbi.nlm.nih.gov/BLAST). Sequences that showed an identity percentage above 97% were identified as the same species. Then the sequences were submitted to the NCBI and an accession number was assigned to each sequence.

Molecular phylogenetic analysis was done to compare the genetic relationship of identified strains in the present study with strains of the same species from other foods. For this purpose, fourteen nucleotide sequences (16S rRNA) of other strains were selected from the NCBI. *Acetobacter syzygii* KMCH8 (ON514266) isolated from Chal was used as outgroup. All the sequences were aligned by KLUSTALW in (Molecular Evolutionary Genetics Analysis) MEGA v.7.0 software. Evolutionary analysis was conducted using the maximum likelihood method based on the Kimura 2-parameter model. Bootstrap (1000 replications) method was applied to determine the evolutionary distances on each node.

2.3. Safety assays

2.3.1. Hemolysis

To assess the safety and non-pathogenicity of isolates, hemolysis test was done. Following anaerobic growth facilitated by Gas Pack in Jar (Merck, Darmstadt, Hesse, Germany) at 37 °C, each isolate in the end of logarithmic phase was cultured linearly on blood agar medium containing 5% v/v of sheep blood and incubated under anaerobic conditions at 37 °C for 48 h. Subsequently, the blood agar plates were examined for β-hemolytic activity (clear halo around bacteria), α-hemolytic (green halo around bacteria) and γ-hemolytic activity (no halo around bacteria [23].

2.3.2. Susceptibility of isolates against vancomycin antibiotic

A volume of 50 µl of the culture of the isolates (end the log phase) were spread onto MRS agar plates, and an antibiotic disc containing 30 µg of vancomycin (Padtan Teb Laboratory Instruments, Tehran, Iran) was placed on the surface of these plates.

Table 1. The characteristics of the samples collected in this research

No.	Source of isolation	Ripening time	Sample collection location
1	Jug cheese made from ewe's milk	6 months	Iran: Boukan
2	Jug cheese made from cow's and ewe's milk, 70:30	6 months	Iran: Boukan
3	Jug cheese made from cow's and ewe's milk, 70:30	3 months	Iran: Boukan
4	Jug cheese made from ewe's milk	3 months	Iran: Boukan
5	Jug cheese made from cow's milk, contains some vegetables	3 months	Iran: Salmas
6	Iranian traditional fermented camel milk (Chal)	A week	Iran: Turkmen Sahra

The plates were then incubated under anaerobic conditions at 37 °C. After 24 h, the diameter of the no-growth halo around the disks was measured (in mm). According to [24], the results were reported as resistant, sensitive and moderate sensitivity.

2.4. Preparation of fermented milk supernatant (FMS)

After centrifugation (Model k2042, centurion scientific, Stoughton, UK) of 10 ml of the bacterial culture in MRS broth (end of the log phase) at 4500 × g for 10 min, the resulting cell pellet was washed with sterile 0.85% saline (Merck) solution following the same centrifugation method. Bacterial suspension was then prepared by adding 10 ml of the saline solution to cell pellet (10^8 cfu/ml).

12% w/v reconstituted skim milk (RSM) (skim milk obtained from Golestan Pegah Company, Gorgan, Iran) was sterilized at 115 °C for 15 min and was then cooled to 37 °C to inoculate bacterial culture. Subsequently, 2% (v/v) of the suspension of each bacterium (10^8 cfu/ml) was inoculated into sterile milk and the mixture was incubated at 37 °C until the pH of milk dropped to 4.6 [1]. For preparation of acidified milk as a control, lactic acid was added to sterile 12% w/v RSM until dropping pH to 4.6 [25]. Fermented and acidified milk samples were centrifuged at 11200 × g for

10 min at 4 °C (Model Combi 514R, Hanil Science Industrial, Gimpo-si, South Korea). The supernatants were filtered to clarify from suspended particles by a Glass Fiber filter (Merck) with a pore-size of 2.0 µm and then sterilized by a PES syringe filter with a pore-size of 0.22 µm (Jet Bio-Filtration, Guangzhou, China). Subsequently, the supernatant was dried using a freeze dryer (Model FDB-5503, Operon, Gimpo-si, South Korea) and stored at -20 °C [26]. The concentration of the supernatant dissolved in RPMI 1640 was adjusted to 60 mg/mL.

2.5. Culture of cancer and normal cells

HT-29 (human colon cancer) and L929 (normal mouse fibroblast) cells were obtained from Pasteur Institute of Iran, Tehran, Iran. The cells were grown in an RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS) (Thermo Fisher Scientific) and 1% streptomycin and penicillin solution (Thermo Fisher Scientific), subsequently incubated at 37 °C in 5% carbon dioxide and 95% humidity (INCO153, Memmert, Büchenbach, Baden, Germany) [27].

2.6. Cytotoxicity effects of fermented milk supernatant (FMS) on cancer and normal cells

A volume of 200 µL of RPMI 1640 containing 5×10^3 HT-29 and L929 cells were seeded in each well of a 96-well microplate and incubated at 37 °C for 24 h. The cells were then treated with fermented and acidified milk supernatants (FMS and AMS) at concentrations of 1, 4, 8, and 12 mg/mL for 48 and 72 h of incubation. After the mentioned time, the supernatant was removed from the wells and 100 µL of medium and subsequently 20 µL of MTT (M5655, Sigma-Aldrich,

St. Louis, MO, USA) at a concentration of 5 mg in 1 mL of phosphate buffer solution (PBS) were replaced in each well. Then, the cells were again incubated for 4 h at 37 °C in dark condition. After incubation, 100 µL of Dimethyl sulfoxide (Merck) replaced the medium containing the MTT in each well. The cells were then gently shaken in an incubator (Model DHO-101, FIRSTEK, Piscataway, NJ, USA) for 20 min. The absorbance of wells containing formazan was measured at 570 nm using a microplate reader (Model ELX800, Biotech, Hayward, CA, USA) [27]. The viability of HT-29 cell was calculated using the Eq. 1:

The cell viability (%) = (Absorbance of treated well) / (Absorbance of control well) ×100 Eq. 1

2.7. Determination of HT-29 cell apoptosis

HT-29 cell treated with FMS was evaluated for apoptosis by FITC Annexin V Apoptosis Detection Kit with PI (640914, BioLegend, San Diego, CA, USA). Each well of a 12-well plate was seeded with 1.2×10^5 cells and incubated for 24 h at 37 °C in 5% CO_2. The cells were then treated with 12 mg/ml of FMS for 72 h. After incubation, the cells were then detached by 0.25% trypsin-EDTA (Thermo Fisher Scientific). After washing the cells with PBS, the kit solutions were added to each tube based on the optimized values at; 50 µL of binding buffer, 2 µL of FITC Annexin V and 3 µL of propidium iodide (PI) solution. The tubes were kept at room temperature in dark condition for 20 min. Each tube was then supplemented with 200 µL of binding buffer [1]. Data analysis was done by the BD Accuri C6 Flow Cytometry (BD Biosciences, Franklin Lakes, NJ, USA).

2.8. Statistical analysis

A completely randomized design (CRD) was used in this research. The results were analyzed by SAS version 9.1.3 software (SAS Institute Inc., Cary, NC, USA) [28]. To compare significant differences among means, Duncan's test was applied at the 5% probability level.

3. Results and Discussion

3.1. Molecular identification

Following phenotypic preliminary assessments on isolates obtained from six different sources; cocci-shaped, Gram-positive and catalase-negative isolates were selected for molecular identification at the species level. Comparing the resulting sequences with the NCBI database using BLASTn software, *Enterococcus* species with an identity above 97% were identified. The results of molecular identification of the isolates and their accession numbers in NCBI are shown in Table 2. Based on the findings, four strains of *E. faecium* and two strains of *E. faecalis* were identified from the traditional fermented dairy products.

Table 2. Sequencing results of cocci-shaped bacteria isolated from traditional fermented dairy products.

Source of isolation	Bacteria identified at the species level	Strain	GenBank accession numbers
Jug cheese (1)	*Enterococcus faecium*	KMJC41	OP764046
Jug cheese (2)	*Enterococcus faecalis*	KMJC54	OP764047
Jug cheese (3)	*Enterococcus faecalis*	KMJC62	OP764048
Jug cheese (4)	*Enterococcus faecium*	KMJC71	OP764049
Jug cheese (5)	*Enterococcus faecium*	KMJC93	OP764050
Chal (6)	*Enterococcus faecium*	KMCH3	OP764051

Similarly, three species of *Enterococcus* including; *E. faecalis*, *E. durans* and *E. faecium* were dominant in raw and fermented camel milk and raw-milk cheeses [29, 30]. Strains of *E. faecalis* and *E. faecium* isolated from Iranian and Turkish white cheeses was also identified in previous research [31].

The phylogenetic relationship of six autochthonous *E. faecium* and *E. faecalis* (OP764046-OP764051)

strains in the present research with fourteen autochthonous strains isolated from foods with different origins was shown on a phylogenetic tree. The evolutionary history inferred showed that two *Enterococcus* species were grouped in two divergent clusters. Cluster 1 and 2 included *E. faecium* and *E. faecalis* strains, respectively. Cluster 1 contained four sub-clusters: sub-cluster 1 consisted of *E. faecium* KMJC93 and *E. faecium* KMCH3 (OP764050 and OP764051) with the genetic similarity rate of 64%. Sub-cluster 2 consisted of *E. faecium* KMJC71 (OP764049), which had a genetic similarity rate of 61% with latter strains. Sub-cluster 3 consisted of *E. faecium* KMJC41 (OP764046), which had a genetic similarity rate of 83% with *E. faecium* KMJC71 (OP764049). In sub-cluster 4, *E. faecium* strains including; TW40-1, CCEF21, CCEF-20 and DU-1 were grouped with a genetic similarity rate of 52% with *E. faecium* KMJC41. In cluster 2, *E. faecalis* KMJC54 and *E. faecalis* KMJC62 (OP764047 and OP764048) were placed in a sub-cluster with a Bootstrap value of 83%. The genetic similarity rate of *E. faecalis* KMJC54 and *E. faecalis* KMJC62 strains with other *E. faecalis* strains grouped in cluster 2 was 76%.

3.2. Enterococci safety

Enterococcus strains isolated from traditional fermented dairy products showed different safety results (Table 3). According to the findings obtained from the experiment, except for *E. faecalis* KMJC54, the rest of the strains showed sensitivity to vancomycin. *E. faecalis* KMJC62, *E. faecium* KMJC41 and *E. faecium* KMJC93 were not able to hemolysis of red blood cells, as a result, they were identified as safe *Enterococcus* strains in this study. While *E. faecalis* KMJC54, *E. faecium* KMJC71 and *E. faecium* KMCH3 caused incomplete decomposition of red blood cells, hence, they were excluded from further experiments due to the signs of pathogenicity and lack of safety.

Table 3. The results of the hemolysis and antibiotic susceptibility of autochthonous *Enterococcus* strains.

Strains	Hemolysis	Vancomycin
Enterococcus faecium KMJC41	γ	20 (S)*
Enterococcus faecalis KMJC54	α	0 (R)
Enterococcus faecalis KMJC62	γ	23 (S)
Enterococcus faecium KMJC71	α	18 (S)
Enterococcus faecium KMJC93	γ	17 (S)
Enterococcus faecium KMCH3	α	16 (MS)

γ (no hydrolysis of blood), α (incomplete hydrolysis of blood). Diameter of no-growth halo around vancomycin disc is reported in mm. * (S) = Sensitive, (MS) = Moderate sensitivity, (R) = Resistant. Vancomycin: S ≥ 17 mm, MS: 15-16 mm, R ≤ 14 mm.

Cytolysin/hemolysin is a protein secreted from virulent enterococci. Hence, strains of *Enterococcus* with hemolytic activity are related to pathogenicity in animal models and increased infectious diseases in human [32]. Based on previous studies, strains of *E. faecium* FL31 and *E. faecium* HY07 isolated from fermented foods are non-hemolytic [23, 33], which they are agreement to γ-hemolytic *Enterococcus* strains in the present study. Despite α-hemolysis, β-hemolysis was not observed on blood agar medium for the tested strains, while some strains of β-hemolytic *E. faecalis* isolated from milk, butter and cheese have been reported (Jeeja, 2019). The virulence genes expression could be related to the source of isolation [32] and subsequently strain.

Similar to our observations in the present research, *E. faecium* strains isolated from other fermented foods were sensitive to vancomycin [23, 34]. In contrast, 16 strains of *Enterococcus* studied including *Enterococcus* sp., *E. faecium* and *E. durans* isolated from water-buffalo mozzarella cheese presented resistance to vancomycin [35]. It could be related to strain and subsequently difference in presence or lack of vancomycin-transmissible genes.

Enterococci have intrinsic resistance to several antibiotics such as cephalosporins, aminoglycosides, *β*-lactams and lincosamides [15]. The sensitivity of

enterococci to vancomycin guarantees their safety. Vancomycin is one of the most important antibiotics studied to screen pathogenic enterococci from non-pathogenic ones [34]. In enterococci resistant to vancomycin, the antibiotic resistance gene is often transmitting via transposon, integrative conjugative element and plasmid [36]. Due to the possibility of transferring the resistance gene from the food chain to human, it is considered a threat to health. Since vancomycin is the last antibiotic prescribed for patients with nosocomial infections resulting from multiple antibiotic-resistant enterococci, the sensitivity of enterococci to vancomycin is important [37], that in the present study screening of autochthonous enterococci sensitive to vancomycin was done.

3.3. Milk fermentation capability by Enterococcus strains

The final pH of fermented dairy products is 4.6 [38]. *E. faecium* KMJC41 and KMJC93 were able to ferment lactose to lactic acid and reduce the pH of milk to 4.6 during 72 h and 64 h of incubation at 37 °C, respectively. In contrast, *E. faecalis* KMJC62 decreased pH of milk to 4.9 during 72 h of incubation. Since this strain was not able to decrease pH of milk to 4.6 during 72 h of incubation, thus it excluded from further experiments. Similarly, weak to moderate acidifying activity of enterococci in milk has been confirmed in previous research [39, 40]. Overall, commercial or autochthonous probiotic LAB strains have less acidifying ability compared to commercial starters [1]. Therefore, prolonged fermentation time was expected to produce more acid values by the tested strains, and this time depend on the *Enterococcus* species and strain due to their different ability to acidify milk. For instance, [41] stated that *E. faecalis* SLT13 reduced the pH of milk to 4.29 during 18 h of incubation at 37 °C. While, *E. faecium* strains isolated from Turkish Tulum cheese displayed faster acidification ability than *E. faecalis* strains [42].

3.4. Cytotoxicity of fermented milk supernatants (FMSs) on HT-29 cell

The cytotoxic effect of FMSs obtained from *E. faecium* KMJC41 and *E. faecium* KMJC93 on HT-29 cell was studied at concentrations of 1, 4, 8, and 12 mg/ml during 48 and 72 h of incubation (Figure 1). The cytotoxicity effect of FMSs on HT-29 cells was based on concentration and time. As the concentration of FMS and incubation time increased, a significant difference was revealed in the viability of HT-29 cell treated with FMSs of *E. faecium* KMJC41 and *E. faecium* KMJC93 compared to control samples 1 and 2 (Figure 2). After 48 h of incubation, the viability of HT-29 cell treated with FMSs of *E. faecium* KMJC41 and *E. faecium* KMJC93 reduced to 58 and 58.5%, respectively, while after 72 h of incubation, the cell viability decreased to 50 and 46.5%, respectively. After 48 and 72 h of incubation, no significant difference was revealed between the cytotoxicity of FMSs of *E. faecium* KMJC41 and *E. faecium* KMJC93 on HT-29 cells. Based on our findings, the greatest effect of cytotoxicity was determined at a concentration of 12 mg/mL for 72 h of incubation. Thus, the mentioned concentration and time were used for the next experiments.

Since skim milk was used in the preparation of fermented milk, thus regardless of the anticancer effects of short chain fatty acids [43, 44] and fat-soluble vitamins such as vitamin D [45], the cytotoxicity effect of fermented milk could be related to postbiotics such as bioactive peptides released in fermented milk. Kordesedehi et al. [46] reported the high ability of *E. faecium* isolated from Iranian camel milk to hydrolyze α_{s1}-casein. They also noted that proteolysis increased with increasing incubation time. The proteolytic activity of *E. faecium* strains in fermented milk has been proven in previous research [47]. As reported in previous research works, sequences of colon cancer inhibiting anticancer peptides were identified from synbiotic yogurt and fermented milk with *Lactobacillus helveticus* [3, 48]. So it is expected that

bioactive peptides released during milk fermentation by enterococci were responsible for the anticancer effect of fermented milk.

However, enterocin secreted from *E. thailandicus* and *E. faecium* showed anticancer properties *in vitro* [7, 49], but until now, not only the anticancer effect of fermented milk with enterococci has not been investigated, but also peptidomics has not been performed to identify anticancer peptides released in fermented milk with enterococci, so more research is needed to elucidate this issue. In general, bioactive peptides have specificity, low molecular weight, high penetration and a flexible conformation that enables them to interact with cell surface receptors, which leads to their functional effects [50, 51]. Anticancer peptides were usually composed of hydrophobic and positively charged amino acid sequences. These peptides interact with the negatively charged membrane of cancer cells, which results in the destruction of the cell membrane, penetration into it, and toxicity in the cancer cell [52]. Apart from targeting the cell membrane, some functions of anticancer peptides include induction of apoptosis, effect on essential cellular proteins and immune cells [53].

.

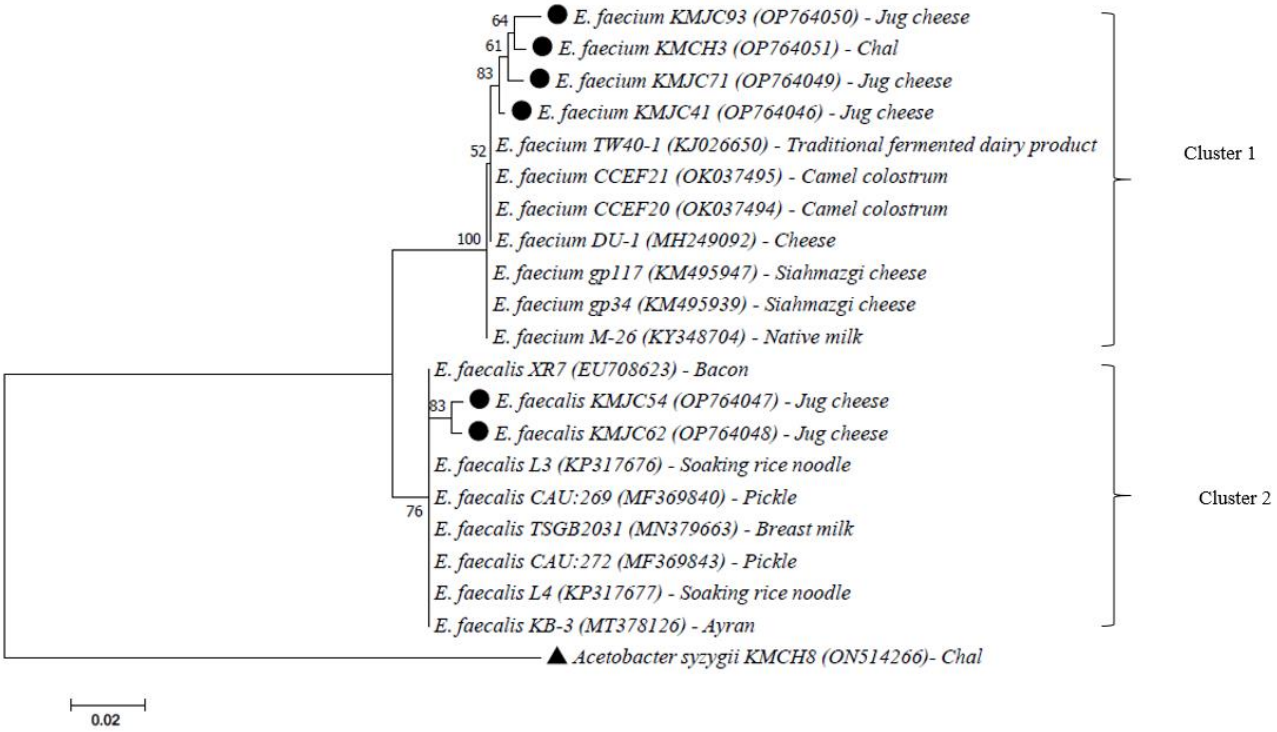

Figure 1. The phylogenetic analysis of six strains of autochthonous *E. faecium* and *E. faecalis* in the present study with fourteen other strains isolated from foods with different origins is presented on the tree. The tree was created using the MEGA7 software by Maximum Likelihood method based on the Kimura 2-parameter model. The confidence level in each node on the tree was evaluated by 1000 Bootstrap replications. The six autochthonous strains were indicated by a black circle (●). *Acetobacter syzygii* KMCH8 was used as outgroup and indicated by a black triangle (▲). The scale bar displays 2% nucleotide substitution per site on the respective branch. This analysis was carried out by 21 nucleotide sequences.

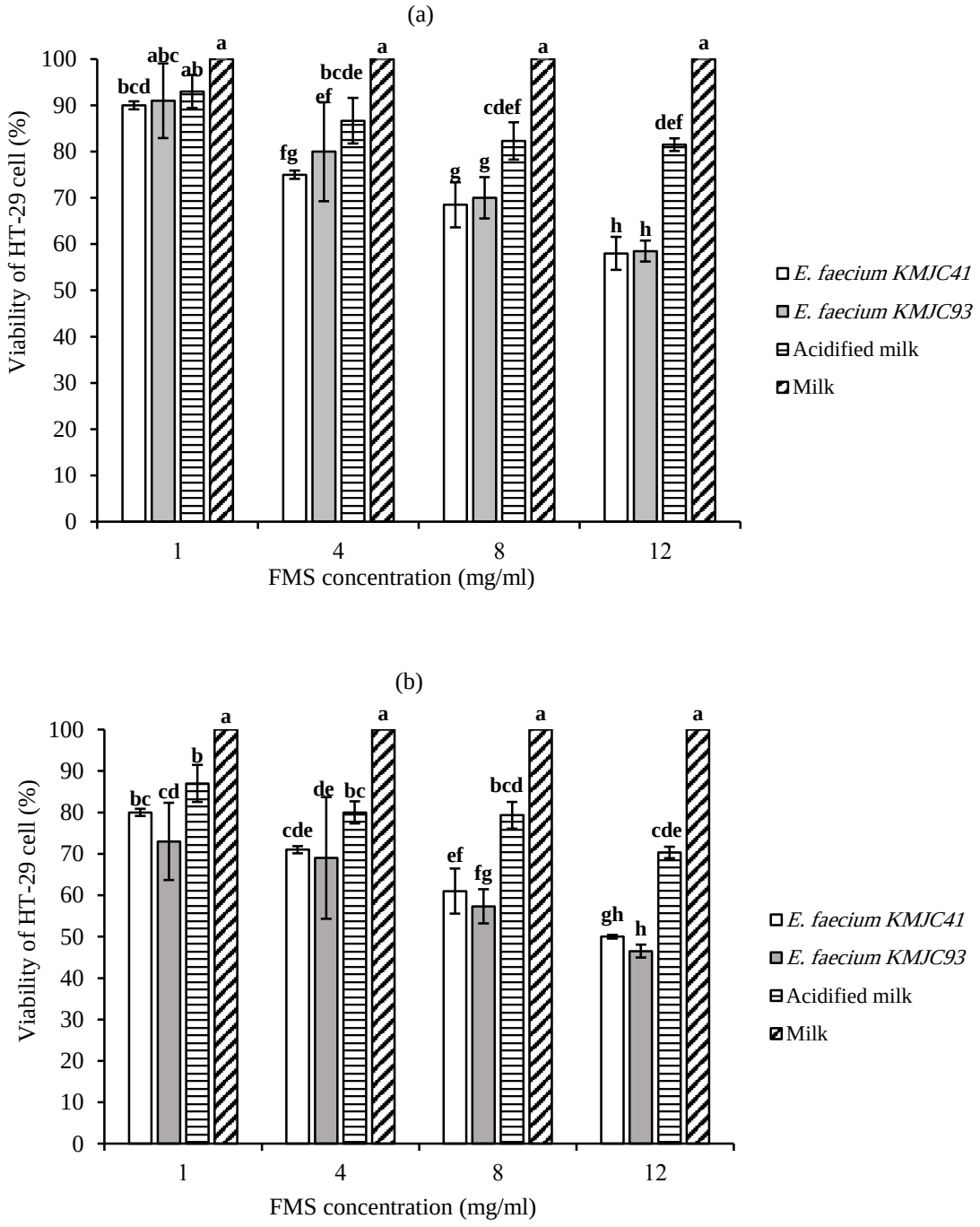

Figure 2. Viability of HT-29 cell treated with FMS (fermented milk supernatant) of *E. faecium* KMJC41 and *E. faecium* KMJC93 at 1,4,8, and 12 mg/ml concentrations for 48 h (a) and 72 h (b) of incubation. Non-fermented sterile reconstituted skim milk was employed as control 1 and acidified milk supernatant was employed as control 2. Different lowercase letters indicate significant differences ($P<0.05$) among means of treatments tested in triplicate.

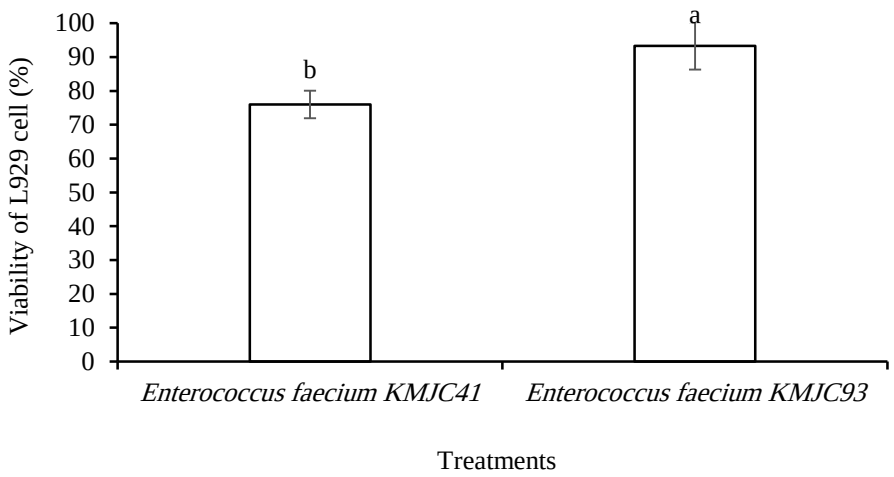

Figure 3. Viability of L929 cell treated with FMS (fermented milk supernatant) of *E. faecium* KMJC41 and *E. faecium* KMJC93 at a 12 mg/ml concentration for 72 h of incubation. The results were presented as mean ± standard deviation of triplicates. Different lowercase letters indicate significant (P<0.05) differences among means in the Duncan test.

This result showed that the treated HT-29 cells underwent more early apoptosis and less late apoptosis than the control (untreated) cell (P<0.05). Moreover, the rate of early apoptosis was significantly higher than late apoptosis in treated cells (P<0.05). Additionally, FMS of *E. faecium* KMJC93 was not effective on cell necrosis compared to control (P>0.05). The results of this research proved that FMS of *E. faecium* KMJC93 inhibited the proliferation of HT-29 cell by inducing apoptosis. The induction of apoptosis in HT-29 cell could be related to the proteolytic activity of *E. faecium* KMJC93 and the release of anticancer peptides. Bioactive compounds including polysaccharides and peptides found in kefir inhibited the proliferation of tumor cells by inducing apoptosis [56]. Apoptosis has an important effect on the removal of cells by phagocytes and prevent adverse immunity [57]. One of the advantages of late apoptosis less than early apoptosis, is that when clearing dead cells, phagocytosis of early apoptotic cells is not associated with inflammation, while the removal of late apoptotic cells is inflammatory and enhances immunity-autoimmunity [58]. Interestingly, the positive effect of FMS of *E. faecium* KMJC93 on the late apoptosis of the treated cell compared to the control cell was revealed. So that it increased the population of early apoptotic cells compared to late apoptosis in treated cells.

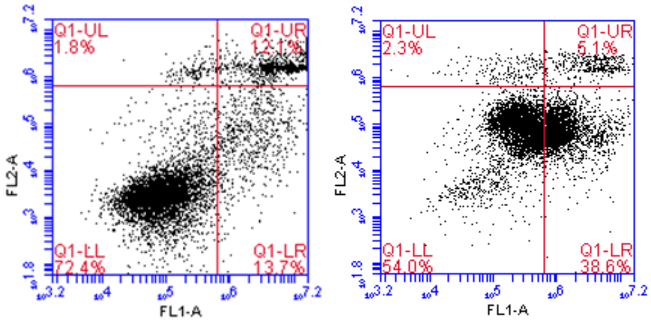

Figure 4. Flow cytometry analysis of HT-29 cell treated with FMS of *E. faecium* KMJC93 at a 12mg/ml concentration for 72 h of incubation. (a) Control, untreated HT-29 cell and (b) HT-29 cell treated with FMS. Annexin V and PI distinguish cells into four panels: bottom left panel for viable cells (Annexin V−/PI−), bottom right panel for cells in early apoptosis (Annexin V+/PI−), top right panel for cells in late apoptosis (Annexin V+/PI+), and top left panel for necrotic cells (Annexin V−/PI+).

Table 4. Flow cytometry results of HT-29 cell treated with of FMS* of *E. faecium* KMJC93 compared to control.

Samples	Early apoptosis (%)	Late apoptosis (%)	Necrosis (%)
Control (untreated cell)	$14^{b} \pm 0.58$	$11^{a} \pm 1.00$	$1.70^{a} \pm 0.30$
FMS of *E. faecium* KMJC93	$40.50^{a} \pm 1.73$	$5.50^{b} \pm 0.58$	$2.00^{a} \pm 0.30$

*FMS: Fermented Milk Supernatant. HT-29 cell was treated with FMS at a concentration of 12mg/ml for 72 h of incubation. The results were presented as mean ± standard deviation of triplicates. Different lowercase letters indicate significant differences ($P<0.05$) among means in the Duncan test.

4. Conclusion

In the present study, three strains (*E. faecium* KMJC41, *E. faecium* KMJC93 and *E. faecalis* KMJC62) were identified as safe strains due to their sensitivity to vancomycin and lack of hemolytic activity *in vitro*. *E. faecium* KMJC41 and *E. faecium* KMJC93 exhibited the milk fermentation capability. Fermented milk with *E. faecium* KMJC93 demonstrated a safety profile of over 90% on normal mouse fibroblast cell (L929). The result revealed that fermented milk with *E. faecium* KMJC93 included a total apoptosis rate of 46% in HT-29 cancer cells, indicating cytotoxicity. *E. faecium* KMJC93 holds promise as a probiotic to produce functional dairy products. Further research involving *in vivo* studies is recommended to better assess the safety and anticancer effects of this novel product.

5. Declarations

5.1. Acknowledgments

The authors gratefully acknowledge the support provided by the Vice President for Research and Technology at the Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran and Golestan University of Medical sciences, Gorgan, Iran.

5.2. Authors' Contributions

Mandana Mahmoudi: Conceptualization, Data curation, Methodology, Investigation, Formal analysis, Writing – original draft and Writing – review & editing; Morteza Khomeiri: Supervision, Conceptualization, Data curation, Methodology, Project administration, Writing – review & editing; Mohsen Saeidi: Supervision, Conceptualization, Data curation, Methodology, Project administration, Writing – review & editing; Homa Davoodi: Data curation, Methodology, Investigation, Writing – review & editing; Ali Memarian: Data curation, Methodology, Formal analysis, Writing – review & editing.

5.3. Declaration of Interest

The authors of this article declared no conflict of interest.

5.4. Ethical Considerations

This research was approved by the Ethics Committee of the Research Deputy of Golestan University of Medical Sciences (Ethics Code: IR.GOUMS.REC.1394.261) and Gorgan University of Agricultural Sciences and Natural Resources (Approval No.: 4.1225). The experiments were conducted using human cell lines and followed all institutional and national guidelines for the ethical use of biological materials. The cell lines used in this study were obtained from a certified and reputable source.

5.5. Transparency of Data

Data are available from the corresponding author upon request (email: khomeiri@gau.ac.ir).

5.6. Funding

This research was carried out independently with personal funding and without the financial support of any governmental or private institution or organization.

5.7. Using Artificial Intelligent chatbots

No AI chatbot has been used in this study.

6. References

1. Mahmoudi M, Khomeiri M, Saeidi M, Davoodi H, Memarian A. Anticancer potential of fermented milk with autochthonous lactic acid bacteria. J Appl Microbiol. 2023;134(1):1-9.

2. Loghman S, Moayedi A, Mahmoudi M, Khomeiri M, Gómez-Mascaraque LG, Garavand F. Single and co-cultures of proteolytic lactic acid bacteria in the manufacture of fermented milk with high ACE inhibitory and antioxidant activities. Fermentation. 2022;8(9).

3. Sah BNP, Vasiljevic T, McKechnie S, Donkor ON. Antioxidant peptides isolated from synbiotic yoghurt exhibit antiproliferative activities against HT-29 colon cancer cells. Int Dairy J. 2016;63:99-106.

4. Jalali F, Sharifi M, Salehi R. Kefir induces apoptosis and inhibits cell proliferation in human acute erythroleukemia. Med Oncol. 2016;33(1).

5. Dal Bello B, Cocolin L, Zeppa G, Field D, Cotter PD, Hill C. Technological characterization of bacteriocin producing Lactococcus lactis strains employed to control Listeria monocytogenes in Cottage cheese. Int J Food Microbiol. 2012;153(1-2):58-65.

6. Miguel M, Recio I, Ramos M, Delgado MA, Aleixandre MA. Antihypertensive effect of peptides obtained from Enterococcus faecalis-fermented milk in rats. J Dairy Sci. 2006;89(9):3352-9.

7. Al-Madboly LA, El-Deeb NM, Kabbash A, Nael MA, Kenawy AM, Ragab AE. Purification, characterization, identification, and anticancer activity of a circular bacteriocin from Enterococcus thailandicus. Front Bioeng Biotechnol. 2020;8.

8. García-Cano I, Serrano-Maldonado CE, Olvera-García M, Delgado-Arciniega E, Peña-Montes C, Mendoza-Hernández G, et al. Antibacterial activity produced by Enterococcus spp. isolated from an artisanal Mexican dairy product, Cotija cheese. LWT - Food Sci Technol. 2014;59(1):26-34.

9. García-Vela S, Ben Said L, Soltani S, Guerbaa R, Fernández-Fernández R, Ben Yahia H, et al. Targeting enterococci with antimicrobial activity against Clostridium perfringens from poultry. Antibiotics. 2023;12(2).

10. Singhal N, Maurya AK, Mohanty S, Kumar M, Virdi JS. Evaluation of bile salt hydrolases, cholesterol-lowering capabilities, and probiotic potential of Enterococcus faecium isolated from rhizosphere. Front Microbiol. 2019;10.

11. Yang L, Xie X, Li Y, Wu L, Fan C, Liang T, et al. Evaluation of the cholesterol-lowering mechanism of Enterococcus faecium strain 132 and Lactobacillus paracasei strain 201 in hypercholesterolemia rats. Nutrients. 2021;13(6).

12. Foulquié Moreno MR, Sarantinopoulos P, Tsakalidou E, De Vuyst L. The role and application of enterococci in food and health. Int J Food Microbiol. 2006;106(1):1-24.

13. Veljović K, Terzić-Vidojević A, Tolinački M, Mihajlović S, Vukotić G, Golić N, et al. Molecular characterization of natural dairy isolates of Enterococcus faecalis and evaluation of their antimicrobial potential. In: Mack HL, editor. Enterococcus faecalis: Molecular characteristics, role in nosocomial infections and antimicrobial effects. New York: Nova Science Publishers; 2014. p. 123-35.

14. Jeeja V. Characterization of food and clinical isolates of enterococci. Acta Sci Microbiol. 2019;2(9):56-61.

15. Mariam SH. A sampling survey of enterococci within pasteurized, fermented dairy products and their virulence and antibiotic resistance properties. PLoS One. 2021;16(7).

16. Rafter J. Probiotics and colon cancer. Best Pract Res Clin Gastroenterol. 2003;17(5):849-59.

17. Lekshmi A, Varadarajan SN, Lupitha SS, Indira D, Mathew KA, Chandrasekharan Nair A, et al. A quantitative

real-time approach for discriminating apoptosis and necrosis. Cell Death Discov. 2017;3.

18. Mego M, Holec V, Drgona L, Hainova K, Ciernikova S, Zajac V. Probiotic bacteria in cancer patients undergoing chemotherapy and radiation therapy. Complement Ther Med. 2013;21(6):712-23.

19. Nandhini B, Palaniswamy M. Anticancer effect of goat milk fermented by Lactobacillus plantarum and Lactobacillus paracasei. Int J Pharm Pharm Sci. 2013;5(3):898-901.

20. Fatahi A, Soleimani N, Afrough P. Anticancer activity of kefir on glioblastoma cancer cell as a new treatment. Int J Food Sci. 2021;2021.

21. Mahmoudi M, Khomeiri M, Saeidi M, Kashaninejad M, Davoodi H. Study of potential probiotic properties of lactic acid bacteria isolated from raw and traditional fermented camel milk. J Agric Sci Technol. 2019;21:1161-72.

22. Mahmoudi M, Khomeiri M, Saeidi M, Davoodi H. Lactobacillus species from Iranian Jug cheese: Identification and selection of probiotic based on safety and functional properties. Appl Food Biotechnol. 2021;8(1):47-56.

23. Chakchouk-Mtibaa A, Sellem I, Kamoun Y, Smaoui S, Karray-Rebai I, Mellouli L. Safety aspect of Enterococcus faecium FL31 strain and antibacterial mechanism of its hydroxylated bacteriocin BacFL31 against Listeria monocytogenes. Biomed Res Int. 2018;2018.

24. Clinical, Laboratory Standards I. Performance standards for antimicrobial susceptibility testing. CLSI, 2018.

25. Tulini FL, Hymery N, Choiset Y, Chobert JM, Haertlé T, De Martinis ECP, et al. Milk fermented with the probiotic candidate Lactobacillus paracasei FT700 induces differentiation of monocytes toward macrophages in vitro. J Funct Foods. 2015;15:533-40.

26. Sharma S. Antivirulence activities of bioactive peptides produced by Lactobacillus helveticus and Lactobacillus acidophilus against Salmonella enterica serovar Typhimurium. Guelph: University of Guelph; 2014.

27. Liu CT, Chu FJ, Chou CC, Yu RC. Antiproliferative and anticytotoxic effects of cell fractions and exopolysaccharides from Lactobacillus casei 01. Mutat Res Genet Toxicol Environ Mutagen. 2011;721(3):157-62.

28. Institute SAS. SAS 9.1.3 Output Delivery System: User's Guide. 2006.

29. Akhmetsadykova SH, Baubekova A, Konuspayeva G, Akhmetsadykov N, Faye B, Loiseau G. Lactic acid bacteria biodiversity in raw and fermented camel milk. Afr J Food Sci Technol. 2015;6(3):84-8.

30. Terzić-Vidojević A, Veljović K, Tolinački M, Živković M, Lukić J, Lozo J, et al. Diversity of non-starter lactic acid bacteria in autochthonous dairy products from Western Balkan Countries—Technological and probiotic properties. Food Res Int. 2020;136.

31. Oruc O, Cetin O, Darilmaz DO, Yüsekdag ZN. Determination of the biosafety of potential probiotic Enterococcus faecalis and Enterococcus faecium strains isolated from traditional white cheeses. LWT - Food Sci Technol. 2021;148.

32. Araújo TF, Ferreira C. The genus Enterococcus as probiotic: Safety concerns. Braz Arch Biol Technol. 2013;56(3):457-66.

33. Zhou H, Hu Y, Jiang L, Zhou H, Ma J, Liu C. Antilisterial activity of bacteriocin HY07 from Enterococcus faecium HY07 isolated from Chinese sausages. Food Biotechnol. 2015;29(1):51-68.

34. Zommiti M, Cambronel M, Maillot O, Barreau M, Sebei K, Feuilloley M, et al. Evaluation of probiotic properties and safety of Enterococcus faecium isolated from artisanal Tunisian meat "dried Ossban". Front Microbiol. 2018;9.

35. Nascimento LCS, Casarotti SN, Todorov SD, Penna ALB. Probiotic potential and safety of enterococci strains. Ann Microbiol. 2019;69:241-52.

36. Partridge SR, Kwong SM, Firth N, Jensen SO. Mobile genetic elements associated with antimicrobial resistance. Clin Microbiol Rev. 2018;31(4).

37. Terzić-Vidojević A, Veljović K, Popović N, Tolinački M, Golić N. Enterococci from raw-milk cheeses: Current knowledge on safety, technological, and probiotic concerns. Foods. 2021;10(11).

38. Maragkoudakis PA, Zoumpopoulou G, Miaris C, Kalantzopoulos G, Pot B, Tsakalidou E. Probiotic potential of Lactobacillus strains isolated from dairy products. Int Dairy J. 2006;16(3):189-99.

39. Giraffa G. Functionality of enterococci in dairy products. Int J Food Microbiol. 2003;88(2-3):215-22.

40. Mahmoudi M, Khosrowshahi Asl A, Zomorodi S. The influence of probiotic bacteria on the properties of Iranian white cheese. Int J Dairy Technol. 2012;65(4):561-7.

41. Ziadi M, M'Hir S, Dubois-Dauphin R, Chambellon E, Yvon M, Thonart P, et al. Analysis of volatile compounds, amino acid catabolism and some technological properties of Enterococcus faecalis strain SLT13 isolated from artisanal Tunisian fermented milk. Br Microbiol Res J. 2016;14(4):1-17.

42. Tuncer Y. Some technological properties of phenotypically identified enterococci strains isolated from Turkish Tulum cheese. Afr J Biotechnol. 2009;8(24):7008-16.

43. Cousin FJ, Jouan-Lanhouet S, Dimanche-Boitrel MT, Corcos L, Jan G. Milk fermented by Propionibacterium freudenreichii induces apoptosis of HGT-1 human gastric cancer cells. PLoS One. 2012;7(3).

44. Mirzaei R, Afaghi A, Babakhani S, Sohrabi MR, Hosseini-Fard SR, Babolhavaeji K, et al. Role of microbiota-derived short-chain fatty acids in cancer development and prevention. Biomed Pharmacother. 2021;139.

45. Skrajnowska D, Bobrowska-Korczak B. Potential molecular mechanisms of the anti-cancer activity of vitamin D. Anticancer Res. 2019;39(6):3353-63.

46. Kordesedehi R, Taheri-Kafrani A, Rabbani-Khorasgani M, Kazemi R, Mutangadura D, Haertle T. Modification of IgE binding to αS1-casein by proteolytic activity of Enterococcus faecium isolated from Iranian camel milk samples. J Biotechnol. 2018;276-277:10-4.

47. Dos Santos KMO, Vieira ADS, Salles HO, Oliveira JS, Rocha CRC, Borges MF, et al. Safety, beneficial and technological properties of Enterococcus faecium isolated from Brazilian cheeses. Braz J Microbiol. 2015;46(1):237-49.

48. Elfahri K. Anticarcinogenic peptides released from milk proteins by Lactobacillus strains. Melbourne: Victoria University; 2018.

49. Sharma P, Kaur S, Singh Chadha B, Kaur R, Kaur M, Kaur S. Anticancer and antimicrobial potential of enterocin 12a from Enterococcus faecium. BMC Microbiol. 2021;21.

50. Martínez-Sánchez SM, Gabaldón-Hernández JA, Montoro-García S. Unravelling the molecular mechanisms associated with the role of food-derived bioactive peptides in promoting cardiovascular health. J Funct Foods. 2020;64.

51. Ghaly G, Tallima H, Dabbish E, Badr ElDin N, Abd El-Rahman MK, Ibrahim MAA, et al. Anti-cancer peptides: Status and future prospects. Molecules. 2023;28(3).

52. Dai Y, Cai X, Shi W, Bi X, Su X, Pan M, et al. Pro-apoptotic cationic host defense peptides rich in lysine or arginine to reverse drug resistance by disrupting tumor cell membrane. Amino Acids. 2017;49(9):1601-10.

53. Wu D, Gao Y, Qi Y, Chen L, Ma Y, Li Y. Peptide-based cancer therapy: Opportunity and challenge. Cancer Lett. 2014;351(1):13-22.

54. Chang JH, Shim YY, Cha SK, Chee KM. Probiotic characteristics of lactic acid bacteria isolated from kimchi. J Appl Microbiol. 2010;109(1):220-30.

55. Moradi M, Kousheh SA, Almasi H, Alizadeh A, Guimarães JT, Yılmaz N, et al. Postbiotics produced by lactic acid bacteria: The next frontier in food safety. Compr Rev Food Sci Food Saf. 2020;19(6):3390-415.

56. Sharifi M, Moridnia A, Mortazavi D, Salehi M, Bagheri M, Sheikhi A. Kefir: A powerful probiotics with anticancer properties. Med Oncol. 2017;34(11).

57. Singh V, Khurana A, Navik U, Allawadhi P, Bharani KK, Weiskirchen R. Apoptosis and pharmacological therapies for targeting thereof for cancer therapeutics. Science. 2022;4(2).

58. Poon IKH, Hulett MD, Parish CR. Molecular mechanisms of late apoptotic/necrotic cell clearance. Cell Death Differ. 2010;17:381-97.