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Characterization and Antimicrobial Potential of *Enterococcus Faecium* Isolates from Raw Bovine Milk and Yoghurt

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Abstract

This study focuses on the isolation of enterococci, a subset of lactic acid bacteria (LAB). The study involves the identification of these isolates through morphological, biochemical, as well as molecular identification using 16S rRNA gene sequencing methods, alongside an exploration of their antimicrobial efficacy. Three isolates were obtained from milk and yoghurt samples, identified as *Enterococcus faecium*. Tolerance of the isolates to bile salts (up to 40%) and mild acidity (pH=4.5), makes them survive in guts and thus applicable as probiotics. The cell-free supernatants (CFSs) derived from these isolates exhibited significant antibacterial activity where inhibition zones reached 9.3-10.33 mm against *Proteus vulgaris*, *Staphylococcus aureus* and *Staphylococcus albus;* while zones recorded against *Pseudomonas aeruginosa*, *Serratia marcescens*, *Klebsiella pneumoniae* and *Escherichia coli*, reached 3-8.3 mm. By contrast, they showed no efficacy against fungi. Notably, the antimicrobial activity of CFSs was maintained at various temperatures, including autoclaving conditions (121 °C). The isolates displayed tolerance across a wide pH range (2.5-9.5), with enhanced activity observed at acidic pH levels compared to basic ones. Heat and pH stability of supernatants encourage their use as bio-preservatives.

Keywords: *Enterococcus faecium*, Biochemical characterization, 16S rRNA, Antimicrobial activity

Introduction

Various genera of cocci lactic acid bacteria, encompassing *Pediococcus*, *Leuconostoc*, *Weissella*, *Lactococcus*, *Enterococcus*, and *Streptococcus* (Whitman *et al.*, 2015), exhibit distinctive characteristics. Lactic acid bacteria (LAB) are Gram-positive, non-motile, catalase-negative, and non-spore-forming microorganisms with the ability to produce lactic acid. This bacterial group holds significant technological relevance, showcasing features such as proteolytic activity, polysaccharide production, and remarkable resistance to freezing and freeze-drying. Additionally, LAB exhibit probiotic properties, including adhesion and colonization in the digestive mucosa, vitamin production, and the synthesis of antimicrobial compounds (Ananou *et al.*, 2007; LeBlanc *et al.*, 2011; Oliveira *et al.*,

2008). Notably, LAB demonstrate the inhibition of various bacteria, such as *Escherichia*, *Staphylococcus*, *Salmonella*, *Shigella*, and *Bacillus*, along with antifungal activity against *Candida* sp. (Adikari *et al.*, 2021; Islam *et al.*, 2020).

The use of probiotic strains in treatments is considered both safe and stable, avoiding an increase in the risk of multi-drug resistance among pathogens (Roghmann & McGrail, 2006). The antagonistic mechanism between LAB and harmful genera relies on the production of metabolites, including organic acids (such as lactic and acetic acid, leading to a pH decrease that is unfavorable to some pathogens and spoilage microorganisms), bacteriocins, hydrogen peroxide, antifungal peptides, and competition for nutrients (Vasiljevic & Shah, 2008; Rahmeh *et al.*, 2019). LAB coatings present a viable alternative to chemical compounds, enhancing the shelf life and safety of fresh-cut fruits, such as pineapple (Lee *et al.*, 2020 ; Tenea *et al.*, 2020 ; Yang & Moon, 2021).

Enterococcus faecium emerges as a potential bio-preservative in dairy and meat products to control *Listeria monocytogenes*, capable of growth at refrigeration temperatures (4 °C) (Lee *et al.*, 2020). Lactic acid bacteria find applications in diverse sectors, including ruminants like cattle, poultry, and beekeeping, contributing to health, growth, reproductive success, and protection against diseases (Yang & Moon, 2021). The supplementation of milk with probiotic lactic acid bacteria, such as *E. faecium*, *L. plantarum*, and *L. acidophilus*, has been linked to increased weight in young calves (Frizzo *et al.*, 2011). In the production of artisanal cheeses, numerous enterococci (e.g., *E. avium*, *E. durans*, *E. faecalis*, *E. faecium*, *E. hirae*, *E. lactis*, among others) play a vital role in imparting unique flavors (Dapkevicius *et al.*, 2021).

Nisin, the widely used bacteriocin as a food bio-preservative, shows decreased stability and a narrow pH range (5.0 - 7.0), with only slight effects on gram-negative bacteria. This prompts the exploration of new antimicrobial components with a broad spectrum (Héchard & Sahl, 2002). The safety and efficiency of antimicrobial compounds from lactic acid bacteria, have garnered considerable attention in recent research as potential natural alternatives to antibiotics and chemical preservatives in the food industry (Bhakta *et al.*, 2023).

Thus, this study aims to investigate and characterize the antimicrobial components of lactic acid bacteria, with a specific focus on enterococci, to explore their potential as natural alternatives to antibiotics and chemical preservatives in the food industry.

Materials and Methods:

Isolation of Lactic Acid Bacteria (LAB):

A 10^{-1} dilution of selected food samples (bovine milk, yoghurt) was prepared in approximately 10 ml sterile distilled water (SDW). Each diluted sample (0.5 ml) was plated on de Man, Rogosa, and Sharpe (MRS) agar plates (de Man *et al.*, 1960) and incubated under aerobic conditions at 37 °C for 3 days.

Morphological Characterization:

Colonies with white, convex or raised, smooth surfaces and diameters (≤ 2 mm) were selected for purification (on MRS agar) and Gram staining (Grange & Lyne, 2004). Gram-positive cocci isolates were purified through repeated streaking on MRS agar plates, with strains isolated from milk and yoghurt denoted by the letters (M) and (Y), respectively.

Preservation of Isolates:

Short-term storage (for 1 month at 4 °C) involved three methods: agar slant, stab inoculation using semi-solid MRS medium (with 0.3% $CaCl_2$ as pH neutralizer) as described by Björkroth and Holzapfel, 2006), and inoculation of MRS broth with young bacterial culture. For long-term storage, isolates were maintained as glycerol stocks at -20 °C (Spencer & de Spencer, 2008). One isolate were deposited in the Culture Collection Ain Shams University (CCASU) of the World Data Centre for Microorganisms (WDCM) under specific codes (*Enterococcus faecium*, CCASU-2023-62) (https://doi.org/10.12210/ccinfo.1186) (Table 5).

Motility Test:

Stab inoculation in tubes of semi-solid MRS medium was performed as described by MacFaddin (2000), and motility was assessed

after incubation at 37 °C for 48 hrs.

Biochemical Characterization:

Various biochemical tests were conducted in MRS broth (Somasegaran & Hoben, 2012), including gas (CO_2) production (Schillinger & Lücke, 1987), catalase production (Kozaki *et al.*, 1992) , gelatin hydrolysis (Aneja, 2007), starch hydrolysis (Evans *et al.*, 2004), tryptophanase activity (Kovacs, 1928), nitrate reduction (Reddy *et al.*, 2007), citrate utilization (Mithun *et al.*, 2015), hydrolysis of arginine (Samelis *et al.*, 1994), NaCl tolerance (Ni *et al.*, 2015), growth at acidic and alkaline pH (Ni *et al.*, 2015), growth at different temperatures (Samelis *et al.*, 1994), Voges-Proskauer test (Barritt, 1936), production of dextran (slime) from sucrose (Hitchener *et al.*, 1982), production of hydrogen sulfide (H_2S) (Shay & Egan, 1981), methylene blue reduction (Abanoz & Kunduhoglu, 2018), carbon source utilization (Abanoz & Kunduhoglu, 2018), urease test (Steadham, 1979) and bile salts tolerance (Menconi *et al.*, 2014).

Amplification of 16S rRNA Gene:

Genomic DNA was extracted as described by Spencer and de Spencer (2008), and PCR amplification of the 16S rRNA gene was performed using universal primers 27 F (5′-AGAGTTTGATCCTGGCTCAG- 3′) and the reverse primer 1492 R (5′-GGTTACCTTGTTACGACTT- 3′). The PCR product was sequenced, and BLAST analysis was conducted to determine sequence similarities.

Sequence Analysis:

Obtained sequences were edited and analyzed using Lasergene 7.1.0. A phylogenetic tree was constructed to assess evolutionary relationships with sequences from GenBank.

Antimicrobial Activity Test:

Isolates were grown in MRS broth, and cell-free supernatants (CFSs) were obtained. The well diffusion method (Sonbol *et al.*, 2020) was employed to test antimicrobial activity against various indicator organisms, for example: *Staphylococcus aureus*, *S. albus*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Klebsiella pneumoniae*, *Escherichia coli*, *Aspergillus niger*, *Aspergillus flavus* and *Candida albicans*.

Thermal and pH Stability of CFS:

CFSs were subjected to different temperatures and autoclaving, as well as varying pH levels. *Staphylococcus albus* was chosen as an indicator to assess supernatant activity (Oliveira *et al.*, 2008 ; Abanoz & Kunduhoglu, 2018).

Data statistical analysis:

Data are presented as mean ± SE by applying the SAS program (version 9.4, 2013). Duncan's test was used to determine the significance of the mean differences. The probability was considered significant at $p < 0.05$.

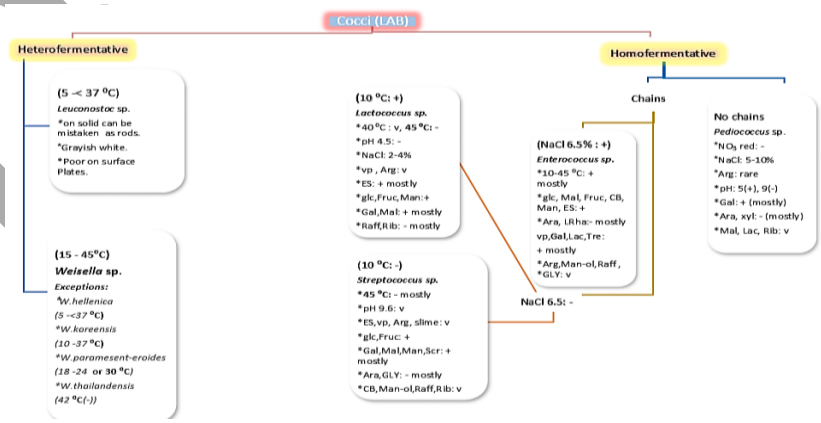

Fig.1: Schematic representation for differentiation between cocci Lactic Acid bacteria (LAB).
*+ = Growth observed, - = No growth, **v** = variable, **Gal**=galactose, **Fruc**=fructose, **Glc**=glucose, **GLY**=glycerol, **Man**=D-mannose, **Ara**=D-arabinose, **Rib**=ribose, **LRha**=L-rhamnose, **Man-ol**= mannitol, **Lac**=lactose, **Mal**=maltose, **CB**=cellobiose, **Raff**= raffinose, **Scr**=sucrose, **ES**=esculin, **Arg**= arginine hydrolysis, **Xyl**=xylose, **Tre**=trehalose, **VP**= acetoin production

Results:

Isolation and Identification of Enterococci:

Three isolates of cocci LAB were successfully obtained, with two originating from milk (4M and 7M) and one from yoghurt (6Y). Colonies exhibited circular, convex morphology, ranging from translucent to opaque, with off-white to pale-white color, smooth surfaces, and entire margins. Under microscopic examination, the strains appeared oval, Gram-positive, and non-spore formers (Table 1).

All isolates exhibited catalase negativity and acid production from glucose without gas formation. Negative results were observed for motility, indole, nitrate reduction, urease, starch and gelatin hydrolysis, and H_2S production. Positive outcomes were recorded for acetoin production, slime formation, methylene blue reduction, milk coagulation, arginine hydrolysis, and growth in bile salts up to 40% (Table 2).

Positive carbon source utilization was observed for lactose, mannose, fructose, galactose, cellobiose, mannitol, ribose, raffinose, glycerol, and maltose. Negative results were noted for citrate and arabinose; while only one isolate utilized rhamnose (7M) (Table 3). Isolates demonstrated growth at temperatures ranging from 5 to 45 °C, with tolerance to salinity up to 6.5 % and pH levels between 4.5 and 9.5. None of the isolates grew at pH 3 (Table 4).

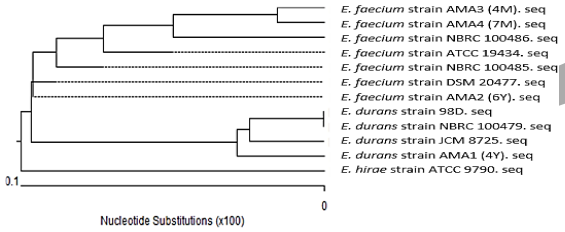

Fig. 2: Phylogenetic tree indicating relationship between our isolates and enterococci strains based on 16S rRNA gene sequence.

Table 1: Colony morphology and microscopic examination of *Enterococcus faecium* isolates.

Isolate	Shape	Transparency	Color	Margin	Surface	Diameter	Elevation	Cell shape
6Y	Circular	Translucent	Off white	Entire	Smooth	0.5 mm	Convex	Oval in chains
4M	Circular	Opaque with translucent margin	Pale white	Entire	Smooth	1.5 mm	Convex	Oval in chains
7M	Circular	Opaque	Off white	Entire	Smooth	1 mm	Convex	Oval in chains

Table 2: Biochemical properties of *Enterococcus faecium* isolates.

Isolate	Acid/gas	Ct	Mot	Ind	NO_3 red	U	St	Gel	H_2S	Vp	Slime	MB red	Milk coag	Arg	Bile 3 %	Bile 40 %
6Y	+/-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+
4M	+/-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+
7M	+/-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+

***** **+** = Growth observed, **-** = No growth**, Ct**=catalase, **Mot**=motility, **Ind**=indole production, **NO_3 red**= nitrate reduction, **U** = urease, **St** = starch hydrolysis, **Gel** = gelatinase, **H_2S**= H_2S production, **Vp**= acetoin production**, MB red**= methylene blue reduction, **milk coag**= coagulation, **Arg**= arginine hydrolysis

Table 3: Carbon sources utilization of *Enterococcus faecium* isolates

Isolate	Lac	Man	Fruc	Gal	CB	Man-ol	Rib	Raff	Mal	GLY	Ara	Cit	LRha
6Y	+	+	+	+	+	+	+	+	+	+	-	-	-
4M	+	+	+	+	+	+	+	+	+	+	-	-	-
7M	+	+	+	+	+	+	+	+	+	+	-	-	+

***** **+** = Growth observed, **-** = No growth, **Lac**=lactose, **Man**=D-mannose, **Fruc**=fructose, **Gal**=galactose, **CB**=cellobiose, **Man-ol=** mannitol, **Rib**=ribose, **Raff**= D-raffinose, **Mal**=maltose, **GLY**=glycerol, **Ara**=D-arabinose, **Cit=** citrate**, LRha**=L-rhamnose

Table 4: Effect of temperature, NaCl and pH on growth of *Enterococcus faecium* isolates

Isolate	Temperature				NaCl						pH				
	5 ºC	37 ºC	40 ºC	45 ºC	3 %	4%	5%	6.5%	8%	9%	3	4.5	7.2	8.7	9.5
6Y	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+
4M	+	+	+	+	+	+	+	w	-	-	-	+	+	+	+
7M	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+

***** **+** = Growth observed, **-** = No growth, **W**= Weak growth.

The amplified 16S rRNA gene, visualized by agarose gel electrophoresis, exhibited a size of approximately 1.5 kbp. Sequences were deposited in GenBank with accession numbers provided (Table 5). The three isolates were identified as *Enterococcus faecium*. A phylogenetic tree illustrating the relationship with other enterococci strains in GenBank is presented in Figure (2).

Antibacterial and Antifungal Activity of Enterococcus sp.*:*

The isolated bacterial strains did not exhibit antagonistic effects against each other but demonstrated significant antimicrobial activity against other bacteria, particularly Gram-positive strains (Fig. 3, Table 6). Inhibition zones were prominent, reaching 9.3 mm or more in the case of *Proteus vulgaris*, *Staphylococcus aureus*, and *Staphylococcus albus*, while the remaining indicators (*Klebsiella pneumoniae*, *Escherichia coli*, and *Pseudomonas aeruginosa*) had zone < 5.3. A similar pattern was observed against *Serratia marcescens* (with 4M displaying a 8.3 mm inhibition zone). However, the tested fungi (*Candida albicans*, *Aspergillus niger*, and *Aspergillus flavus*) resisted the effects of cell-free supernatants from the isolates (Fig. 3, Table 6). Statistical analysis revealed that supernatants of these isolates weren't significantly different against *S. albus*, *P. aeruginosa* and *E. coli*, but had significant variance against *P. vulgaris*, *S. aureus*, *S. marcescens* and *k. pneumoniae*.

Table 5: Isolates' identification (using Bergey's Manual of Systematics of Archaea and Bacteria; and 16S rRNA similarity).

Isolate	Homology	Accession Number	Identity	Expected species	Accession Number	Deposition Code
6Y	*Enterococcus faecium* strain DSM 20477	NR_114742.1	100 %	*Enterococcus faecium* strain AMA2	OP648140	*Enterococcus faecium*, CCASU-2023-62
4M	*Enterococcus faecium* strain NBRC100485	NR_113903.1	99.82 %	*Enterococcus faecium* strain AMA3	OP648141	Not deposited
7M	*Enterococcus faecium* strain ATCC 19434	NR_115764.1	99.85 %	*Enterococcus faecium* strain AMA4	OP648142	Not deposited

Table 6: Antagonistic test of *Enterococcus faecium* against bacteria and fungi.

Indicators / Isolate	Gram Positive Bacteria			Gram Negative Bacteria				Fungi		
	P. vulgaris	***S. aureus***	***S. albus***	***P. aeruginosa***	***S. marcescens***	***k. pneumoniae***	***E. coli***	***A. niger***	***A. flavus***	***C. albicans***
6Y	9.30^{a}	10.33^{a}	9.60^{a}	3.00^{b}	4.60^{b}	3.30^{ab}	3.00^{b}	-	-	-
4M	10.03^{a}	10.36^{a}	9.30^{a}	3.60^{ab}	8.30^{a}	4.30^{ab}	3.00^{b}	-	-	-
7M	10.03^{a}	10.06^{b}	9.60^{a}	4.60^{a}	3.00^{bc}	5.30^{a}	4.30^{a}	-	-	-
SE	0.50	0.72	0.344	0.37	0.57	0.60	0.37			
p-value	<.0001	0.0290	0.9826	0.4442	0.0003	0.0372	0.0539			

*Diameter of inhibition zone measured in (mm).

Thermal and pH Stability of CFS:

Figures 4, 5 & 6 illustrate the impact of temperatures and pH on the cell-free supernatant (CFS) of our cultures. Supernatants remained active at temperatures ranging from 50 ºC to 100 ºC, and even after autoclaving (121 ºC for 15 min); they produced an inhibition zone of approximately 10 mm. Though heating didn't cause notable decrease of inhibition zone, statistical analysis showed that inhibitory effects of supernatants significantly decreased by increasing temperature with *p*-value = 0.0004 (6Y), 0.0002(4M) and 0.0085(7M). The final pH of supernatants for *Enterococcus* cultures stabilized at 4.5 after 3 days of incubation. Following pH adjustment to different values, supernatants retained activity within a pH range of 2.5-9.5. Notably, the cultures exhibited higher activity at acidic pH levels (2.5, 3.5, and 5.5), with inhibition zones reaching about 10 mm, compared to alkaline pH levels (8.5 and 9.5), where the diameter of the inhibition zone was nearly 5 mm (Fig. 6). These observations were confirmed by statistical analysis of data, as increasing pH level of supernatants significantly decreased their activity (p-value=<.0001).

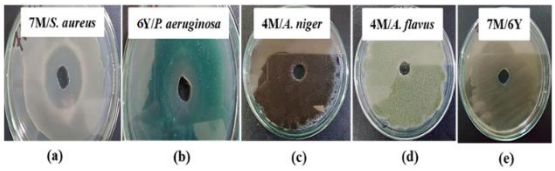

Fig. 3: Antagonistic effect of *E. faecium*

*The indicator organisms are**:**
(a) *S. aureus* **(b)** *P. aeruginosa* **(c)** *A. niger*
(d) *A. flavus* **(e)** *Enterococcus* isolates used in this study.

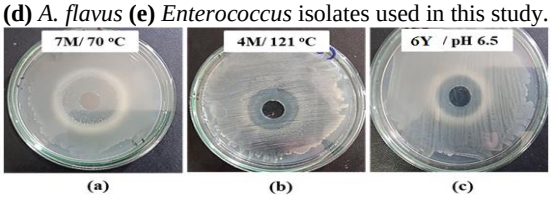

Fig. 4: Effect of heat and pH on activity of Cell-Free Supernatant (CFS) of *E. faecium*

(a) At 70 ºC**, (b)** at 121 ºC**, (c)** After exposure to pH 6.5 for 1 h.

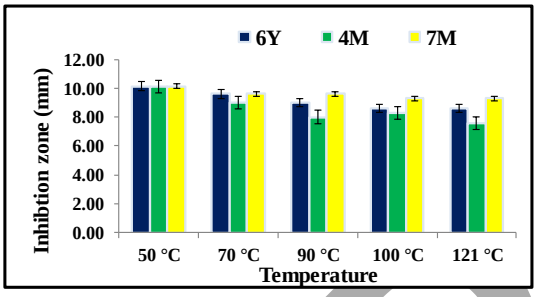

Fig. 5: Thermal stability for Cell-Free Supernatant (CFS) of *E. faecium*

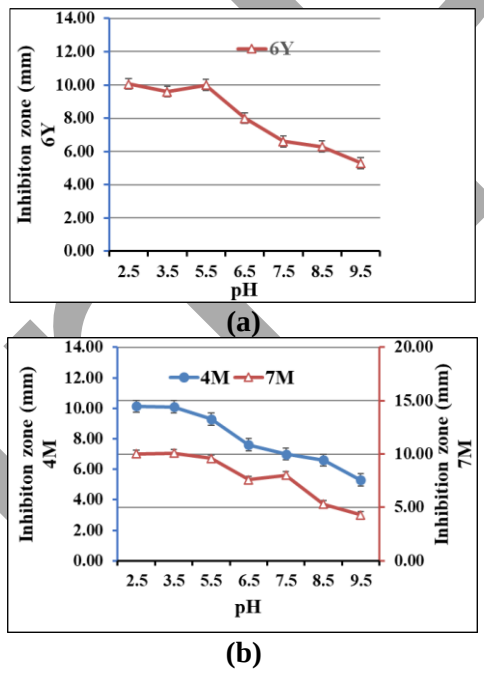

Fig. 6: pH stability for Cell-Free Supernatant (CFS) of *E. faecium*
*(a): Yoghurt isolate = 6Y, (b): Milk isolates = 4M & 7M

Discussion:

Isolation and Identification of Enterococci:

Lactic acid bacteria (LAB) are a well-defined group of Gram-positive, non-spore-forming, and catalase-negative bacteria known for their ability to produce acids from glucose. The identification of enterococci, a subset of LAB, was crucial in our study. Our isolates displayed characteristics consistent with *Enterococcus*, such as Gram-positive cocci arranged in chains, catalase negativity, and growth under homofermentative conditions (Abanoz & Kunduhoglu, 2018; Andrighetto *et al.*, 2001). The absence of gas production ruled out membership in heterofermentative genera like *Leuconostoc* or *Weisella*.

Sequencing the 16S rRNA gene validated the identification of all isolates as *Enterococcus*. A schematic representation based on Bergey's Manual of Systematics of Archaea and Bacteria provided an insightful summary of the features distinguishing various genera of cocci LAB (Fig. 1). Within the genus *Enterococcus*, our isolates were identified at the species level, as *E. faecium*. The biochemical characteristics, such as growth conditions, Voges–Proskauer and arginine dehydrolase tests, helped differentiate *E. faecium* strains. The versatility of *E. faecium* was evident in its isolation from diverse sources, including milk, clinical materials, food, and the environment (Morandi *et al.*, 2012 ; Yerlikaya & Akbulut, 2020). Other *Enterococcus* species, such as *E. lactis*, *E. durans*, and *E. hirae*, were also discussed, highlighting the variations in their acidification abilities for different substrates. The genomic identification through 16S rRNA sequencing aligned with the biochemical characterization. The deposition of isolates in GenBank further enhances the reliability of our findings. The phylogenetic tree illustrated the relationship between our isolates and other members of the *Enterococcus* genus (Fig. 2).

Antibacterial and Antifungal Activity of Enterococcus sp.:

Our isolates demonstrated significant antimicrobial activity, particularly against Gram-positive bacteria like *Proteus vulgaris*, *Staphylococcus aureus*, and *Staphylococcus albus*. This finding aligns with previous research indicating the antimicrobial potential

of LAB against various pathogens, showcasing their role as probiotics and bio-preservatives (Abanoz & Kunduhoglu, 2018; Abesinghe *et al.*, 2020; Gaaloul *et al.*, 2015). Similarly, bacteriocins from *E. faecium* strain exhibited antimicrobial activity against *L. monocytogenes*, *S. aureus* and *Bacillus cereus* (Aspri *et al.*, 2017).

Enterococcus faecium LCW 44 also exhibited antibacterial activity against *Clostridium*, *Listeria*, *Staphylococcus*, and *Lactobacillus* but not against Gram-negative bacteria (Vimont *et al.*, 2017). *E. faecalis* KT11 showed antimicrobial activity against Gram-negative indicator bacteria, namely, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Serratia marcescens* and *Enterobacter aerogenes*, with inhibition zones ranging from 14 to 18 mm (Abanoz & Kunduhoglu, 2018).

Bacteriocins from LAB have been broadly used as biopreservatives (e.g: Nisin), to control pathogenic bacteria in food products including cheese (Khelissa *et al.*, 2021). Several strains of *Enterococcus* are applied as starter cultures (Moreno *et al.*, 2006), and some are used as probiotics (Holzapfel *et al.*, 2018). The mode of action often involves the production of bacteriocins, proteinaceous substances with broad-spectrum antibacterial properties. The stability of these bacteriocins in a wide range of pH and temperature conditions, as observed in our isolates, adds to their appeal for potential applications in food preservation.

While our isolates did not exhibit antagonistic effects against fungi, this aligns with existing literature (Roy *et al.*, 2009; Roy *et al.*, 1996), emphasizing the selectivity of LAB's antifungal activity. Understanding the mechanisms behind these interactions, including the leakage of DNA and proteins from microbial cells contributes to the broader understanding of LAB's antimicrobial activity. The cell-free supernatant (CFS) of the cultures showed important inhibition zones against *Candida pelliculosa* (18.2–24.85 mm) (Abouloifa *et al.*, 2020).This antifungal activity was noticed against *Candida krusei* and *Candida tropicalis* (Oliveira *et al.*, 2008). Nevertheless, none of our isolates gave antagonistic effect against *Aspergillus* or *Candida*. This is in accordance with results of (Qiao *et al.*, 2020), who mentioned that the enterocin TJUQ1 did not have inhibitory ability against fungi such as *Moniliella pollinis* BH010, *Saccharomyces cerevisiae*, *Botrytis cinereal*, *Fusarium oxysporum* and *Fusarium graminearum* while it could inhibit only the growth of *Zygosaccharomyces rouxii* (Qiao *et al.*, 2020; Liu *et al.*, 2011).

Thermal and pH Stability of CFS:

The cell-free supernatants (CFS) from our isolates demonstrated remarkable stability across a wide pH range (2.5-9.5) and even after autoclaving. This robust stability is consistent with previous studies on LAB-derived bacteriocins, supporting their potential application as natural preservatives (Lü *et al.*, 2014). The ability of our isolates to maintain antimicrobial activity under extreme conditions enhances their appeal for various industrial applications. Enterocins from *E. faecium* (Kumar *et al.*, 2010), *E. hirae* (Gupta *et al.*, 2016) and *E. faecalis* (Khalkhali & Mojgani, 2017) were reported to be stable for 10– 20 min at 121°C.

Bacteriocin KT11 was stable at pH (2 - 11) for 24 h and showed antimicrobial activity against the indicator *S. aureus* ATCC 25923 strain. Maximum bacteriocin activity was recorded at pH 2–5 that is similar to our findings (Abanoz & Kunduhoglu, 2018). It has also been reported that bacteriocins of *E. faecium* (Kumar *et al.*, 2010), and *E. hirae* (Gupta *et al.*, 2016) were stable in the acidic pH levels. The anti-listerial activity of a bacteriocin from *E. durans* was completely retained in the pH range of 2–8 (Du *et al.*, 2017). Bacteriocins stable over a wide pH range have a significant advantage and a potential use as bio-preservatives in food products and fermented foods (Franz *et al.*, 1996).

In conclusion, our study unveils three *Enterococcus faecium* strains with promising features, including tolerance to bile salts and acidity, potent antibacterial activity and exceptional thermal stability of cell free supernatants (CFSs). Exopolysaccharides noticed on medium with high concentration of sugar, are applied in fermented milks to improve their texture and manufacture of low-fat cheeses (mozzarella). The application of both traditional biochemical tests and molecular tools, such as 16S rRNA sequencing, ensured accurate identification. These *Enterococcus* strains hold potential as natural preservatives, contributing to the quest for safer and more

sustainable alternatives to chemical preservatives in the food industry.

List of abbreviations

(LAB): Lactic acid bacteria
(MRS): de Man, Rogosa and Sharpe culture medium
(CFSs): Cell free supernatants
(SDW): Sterile distilled water
(GRAS): Generally Recognized as Safe
(EPS): Exopolysaccharides

Declarations

Ethics approval and consent to participate:

This article does not contain any studies with human participants or animals performed by any of the authors.

Availability of data and material:

The datasets utilized and/or examined in the present study can be obtained by contacting the corresponding author. Additionally, the genetic sequence of the strains analyzed has been submitted to the GenBank nucleotide sequence database at the National Library of Medicine, National Center for Biotechnology Information (NCBI). The assigned accession numbers for the sequences are OP648140, OP648141 and OP648142; and are available at the following URLs (respectively).
https://www.ncbi.nlm.nih.gov/nuccore/OP648140.1?report=GenBank
https://www.ncbi.nlm.nih.gov/nuccore/OP648141.2?report=GenBank
https://www.ncbi.nlm.nih.gov/nuccore/OP648142.1?report=GenBank

Author contributions:

HHA: Supervision, Conceptualization, Software, Investigation, Methodology, Writing – original draft, Writing – review & editing, **MAS:** Supervision, Writing – original draft, Resources, Writing – review & editing, **MMI:** Supervision, Conceptualization, Data curation, Resources, Writing – original draft, Writing – review & editing, **AMAA**: Investigation, Methodology, Writing – original draft, Writing – review & editing. All authors read and approved the manuscript.

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الملخص العربي

عنوان البحث: توصيف ودراسة النشاط المضاد للميكروبات لعزلات *Enterococcus Faecium* المعزولة من حليب البقر الخام والزبادي

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تركز هذه الدراسة على عزل enterococci، وهي مجموعة فرعية من بكتيريا حمض اللاكتيك (LAB). تتضمن الدراسة تعريف هذه العزلات من خلال التعريف المورفولوجي والتجارب الحيوية والجزيئية باستخدام طرق تسلسل جينات١٦S rRNA، إلى جانب استكشاف فعاليتها المضادة للميكروبات. تم الحصول على ثلاث عزلات من عينات الحليب والزبادي، تم تحديدها باسم Enterococcus faecium . إن تحمل هذه العزلات لأملاح الصفراء (حتى ٤٠٪) والحموضة المعتدلة (درجة الحموضة = ٤,٥)، يجعلها تبقى على قيد الحياة في الأمعاء وبالتالي قابلة للتطبيق كبروبيوتيك. أظهرت المستخلصات الخالية من الخلايا (CFSs) المشتقة من هذه العزلات نشاطًا مضادًا للبكتيريا بشكل كبير حيث وصلت مناطق التثبيط إلى (٩,٣-١٠,٣٣ مم) ضد Proteus vulgaris و Staphylococcus aureus و Staphylococcus albus. بينما تراوحت مناطق تثبيط النمو ضد Pseudomonas aeruginosaو Serratia marcescens, Klebsiella pneumoniae وEscherichia coli، بين (٣ - ٨,٣ مم). في المقابل لم تُظهر هذه المناطق أي فعالية ضد الفطريات. والجدير بالذكر أن النشاط المضاد للميكروبات لـ CFSs ظل ثابتًا في درجات حرارة مختلفة، بما في ذلك ظروف التعقيم بالبخار (١٢١ درجة مئوية). وأظهرت العزلات تحملًا واسعًا لدرجات الأس الهيدروجيني (2.5-9.5) (pH)، مع ملاحظة زيادة في النشاط عند مستويات الأس الهيدروجيني الحمضية مقارنةً بالمستويات القاعدية. ويحفز استقرار درجة الحموضة والحرارة للمستخلصات على استخدامها كمواد حافظة حيوية.