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Isolation, Identification and Growth Characteristics of Enterococcus faecalis Strains isolated from Tulum cheese

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Abstract:

The current study aimed to characterize the growth characteristics of three Enterococcus (E.) faecalis isolates that were newly isolated from Tulum cheeses. The stains were investigated for their growth properties in different temperatures, alcohol, NaCl and H2O2 concentrations. All Enterococcus faecalis stains had similar tolerance to hydrogen peroxide, resistant to frozen storage conditions, and very well growth under 45 ℃ but the growth rate was declined under 4 ℃. Among these three E.faecalis stains, isolate 388 showed survivability at 12% alcohol concentration while all strains were able to tolerate up to 6% alcohol concentration. Also, all strains showed tolerance to 3 and 5% NaCl concentrations except for 388 maintained their viability at 6.5% NaCl concentration. In conclusion, three strains fulfilled sufficient criteria to be a potential candidate for further in vivo investigations as a starter culture of Tulum cheese or other dairy products.

Keywords: *Enterococcus faecalis*, growth properties

INTRODUCTION

In the food and dairy industry, enterococci are among the lactic acid bacteria that are important because of their proteolytic and lipolytic activities, citrate metabolism, probiotic properties and bacteriocin production (Schirru et al. 2012). Enterococci are Gram-positive cocci widely known for their role in increasing the shelf life, developing flavour compounds in food products, treating gastroenteritis in humans and animals, and improving the intestinal microbial balance. These bacteria are used in many probiotic formulations because of their known health benefits (Khalkhali and Mojgani 2017). In addition to their commensal characteristics, these microorganisms colonize the gastrointestinal tract of humans and animals and are also found in many different food sources, such as meats, milk and cheese (Aspri et al. 2017).

These bacteria can grow and survive under harsh environmental conditions, like those found in various soils, surface water, raw plants and animal products as well as being able to survive extreme environments, such as 6.5 NaCl, pH of 9.6, high heat (Banwo et al. 2013). Due to their ability to survive and compete in the gastrointestinal tract, the last few decades witnessed an increased interest in dealing with the use of enterococci with desirable technological and metabolic properties as starter cultures, co-cultures or probiotics (Pieniz et al. 2014).

Tulum cheese is a traditional Turkish cheese which has a wihte or creamy color, high amount of fat and a crumbly and semihard texture. The flavor is buttery and pungent. This cheese manufactured from raw ewe's milk and ripened for 150 day in goat's skin bags (tulums) or plastic containers (Hayaloglu et al. 2007). Overall, the main goal of this study is to determine probiotic properties, starter potentials and critical virulence factors of three Enterococcus faecalis isolated from traditional Turkish tulum cheeses manufactured in different regions.

The main goal of this study is to determine the growth properties and starters of three Enterococcus faecalis isolated and identified from traditional Turkish Tulum cheeses manufactured in different regions. In addition, these strains were considered safe to use as starter culture or co-culture depending on their result in another study.

MATERIAL AND METHODS

Culture conditions

All *Enterococcus* strains were grown in M17 broth (Merck) at 30 °C for 24 h. Indicator strains *Escherichia coli* ATCC 25922, *Listeria monocytogenes* ATCC 7644, Salmonella Typhimurium ATCC 14028, *Staphylococcus aureus* ATCC 25923, and *Bacillus cereus* ATCC 14579 were used for antimicrobial and co-aggregation analyses, and were supplied from the Provincial Control Laboratory in Konya, Turkey. All indicator strains were incubated at 37 °C for 24 h under aerobic conditions. Purified isolates and indicator strains were stored at -80 °C by adding 20% sterile glycerol (v/v). The cultures were activated before use.

Isolation and molecular identification of Enterococcus strains

Enterococcus strains were isolated using the spread-plate and streaking methods on M17 medium (Merck) (Randazzo *et al.*, 2004). For this purpose, 10 g of a Tulum cheese sample were diluted in a 90 mL sodium citrate solution (2%) (Merck) and homogenized with a Stomacher (HG400V, Mayo International, Italy). Serial dilutions were made and plated on M17 medium. The plates were incubated at 30 °C for 48 h under microaerophilic conditions. After incubation, cultures were gathered randomly from plates. The cultures were then streaked over an agar surface of M17 medium and incubated again under the same growth conditions. This process was repeated twice, and then pure cultures were collected. The cultures were checked regularly for purity using a microscope. The pure cultures were transferred to the M17 broth to maintain their purity and stored under the above mentioned conditions.

Isolated strains were identified by 16S rRNA sequencing. In this context, F365 (5'-ACWCCTACGGGWGGCWGC-3') and R1064 (5'-AYCTCACGRCACGAGCTGAC-3') universal primers, designed from an invariant region in the 16S rRNA sequences for LAB, were used in PCR amplification and were obtained from the Sentegen Biotech Co., Turkey. The PCR amplification was performed in a final 30 µl reaction volume using a BioRAD thermal cycler (T100™, Foster City, California, USA). The PCR conditions for the amplification procedure were as follows: initial denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 30 s, primer annealing at 58 °C for 30 s and extension at 72 °C for 45 s and one cycle of final extension at 72 °C for 10 min. Presence of PCR products and their purity were verified by agarose gel electrophoresis. The PCR products were sent to Sentegen Biotech (Turkey) for sequencing. *Enterococcus* strains were identified by comparing the sequence results with the DNA sequence database present at the National Centre for

Biotechnology Information (NCBI) using the BLAST algorithm (http://ncbi.nlm.nih.gov/BLAST).

DETERMINATION OF GROWTH PROPERTIES OF ENTEROCOCCUS FAECALIS STRAINS

Temperature tolerance

Representative colonies growing on M17 agar plates, and incubated at different temperatures 4, 15 and 45 ℃ (Tassou *et al.*, 2002).

Alcohol tolerance

According to G-Alegria *et al.* (2004) Three *E. faecalis* strains were included in the study of ethanol tolerance, for which M17 broth supplemented with ethanol to final concentrations of 3, 6, 12 and 15% (v/v) was used.

NaCl tolerance

The viabilities of *E. faecalis* strains under different NaCl concentrations (3, 5, 6.5, 8 and 9%) were determined in M17 broth according to the method of Karasu *et al.* (2010).

H_2O_2 tolerance

The method of Li *et al.* (2012) was used with some modifications. The overnight cultures of the *E. faecalis* strains were inoculated at 1% (v/v) into M17 broth containing 0.4, 0.7 or 1.0 mM hydrogen peroxide (Sigma–Aldrich Chemicals, Steinheim, Germany), and incubated at 37°C for 8 h. The cell growth was measured spectrophotometrically (Cary 300 UV–Vis, Varian Inc., Palo Alto, CA) at 600 nm. Results were given as optical density (OD).

Freezing tolerance

The method proposed by Karasu *et al*, (2010) to determine the viability levels of *E. faecalis* strains against freezing was followed with minor modifications. Cells were centrifuged after incubation in M17 medium at 30°C. Pellets were then resuspended in 0.5 mL sterilized skim milk and frozen at −70°C. The viability rates of *Enterococcus* strains after freezing was calculated as a percentage of the initial count of each strain.

Statistical analysis

All data were given as the mean and standard deviation of two observations. The results were subjected to one-way ANOVA in Minitab software version 17 (State College, USA) to determine significant differences between means of bacterial cultures and treatments. The means of results were compared with the Tukey test with a confidence interval set at 95%.

RESULTS AND DISCUSSION

Growth characteristics E. faecalis isolates

The growth characteristics of LAB under stress conditions such as temperature, alcohol, NaCl, hydrogen peroxide, and freezing are accepted as an important criterion for the selection of starter cultures to be used in fermented foods (Ertekin & Çon, 2014). Growth properties of *E. faecalis* strains are given in some Tables. All isolates grew very well at 15, 30, and 45 ℃; but they showed weak growth at 4 ℃ (Table 1). Likewise, Akoğlu (2020) has observed that *E. durans*, *E. faecalis*, *E. faecium,* and *E. lactis* isolated from local cheeses in Turkey showed better viability at 20 and 37 ℃ as compared to 4 ℃. LAB may require to survive ethanol stress throughout the fermentation process because ethanol is one of the key products of fermentation (Shin *et*

al., 2019). While all strains were able to tolerate up to 6% alcohol concentration, only isolate 388 showed survivability at 12% alcohol concentration. Alcohol concentration of 15% inhibited the growth of all isolates (Table 2). These results are in agreement with those reported by G-Alegria *et al.* (2004). In the manufacturing of fermented products, such as cheese, pickle, and sausage, the survival of starter cultures at high NaCl concentrations is high in importance (Perin *et al.*, 2017). In our study, *E. faecalis* strains showed tolerance to 3 and 5% NaCl concentration while all isolates except for 388 maintained their viability at 6.5% NaCl concentration (Table 3). Notwithstanding that, there were no growth at the 8 and 9% NaCl concentrations. Similar results were obtained by Shin *et al.* (2019) who showed that the bacterial growth decreased with the increasing salt concentrations. Resistance to hydrogen peroxide is another feature that is emphasized for probiotic microorganisms and in current study, hydrogen peroxide had no effect on the viability of the *E. faecalis* strains. All three isolates showed tolerance to 0.4, 0.7 and 1.0 mM H_2O_2 concentrations very well (Table 4). In the study of Li *et al.* (2012), *L. plantarum* strains showed similar results at 0.4 and 0.7 mM hydrogen peroxide concentrations but as for 1mM concentration, our strains displayed better growth. Lactic acid starters must be preserved for research and industrial purposes, and freezing is one of the preservation methods of cultures. Therefore, freezing resistance is a crucial feature of LAB for use as starter cultures or probiotics (Béal *et al.*, 2001). As shown in Table 1, after freezing process the viability rates were determined to be between 96.69 and 99.30%. Accordingly, these strains seem to be resistant to freezing and frozen storage conditions (Table 5).

CONCLUSIONS

In conclusion, the results of the current study indicated that three E. faecalis isolates are safe strains with the potential and technological properties. All E. faecalis isolates showed higher resistance to 45°C than 4°C and 15°C. In addition, isolate 28 exhibited tolerances to 6.5% NaCl concentrate, while 388 isolate showed resistance toward 12% alcohol concentrate. Overall, further tests will be needed to investigate its behaviours in food products and in vivo conditions.

Author contributions

Nusiba Alsiddig Badawi: Conceptualization, data curation, formal analysis, investigation, methodology, Writing - review & editing, visualization, roles/writing - original draft; Writing - review & editing; Nihat Akın: Project administration, resources, supervision, data curation.

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Table (1)
Temperature resistant for *Enterococcus faecalis* strain isolates from Tulum Cheeses

Isolate	Temperature (ºC)			
	4	15	30	45
28	+	+++	+++	+++
367	+	++	+++	+++
388	+	+++	+++	+++

(-): no growing ; (+) week; (++): medium; (+++): strong.

Table (2)
Alcohol concenteration Resistant Enterococcus faecalis strain isolates from Tulum Cheeses

Isolate	Alcohol Concentration (%)				
	0	3	6	12	15
28	+++	+++	++	-	-
367	+++	+++	++	-	-
388	+++	+++	++	+	-

(-): no growing ; (+) week; (++): medium; (+++): strong.

Table (3)
Nacl concenteration Resistant Enterococcus faecalis strain isolates from Tulum Cheeses

Isolate	NaCl Concentration (%)					
	0	3	5	6.5	8	9
28	+++	+++	+++	+++	-	-
367	+++	+++	+	+	-	-
388	+++	+++	++	-	-	-

(-): no growing ; (+) week; (++): medium; (+++): strong.

Table (4)
H_2O_2 Concenteration Resistant Enterococcus faecalis strain isolates from Tulum Cheeses

Isolate	H_2O_2 Concentration (mM)			
	0	0.4	0.7	1.0
28	+++	+++	+++	+++
367	+++	+++	+++	+++
388	+++	+++	+++	+++

(-): no growing ; (+) week; (++): medium; (+++): strong.

Table (5)
Freezing Resistant Enterococcus faecalis strain isolates from Tulum Cheeses

Isolate	Freezing resistant (log10 CFU/mL)	
	Kontrol	Freezing
28	9.68 ± 0.15^{a}	9.36 ± 0.06^{abc}
367	8.96 ± 0.05^{ab}	8.88 ± 0.04^{g}
388	9.28 ± 0.04^{ab}	9.21 ± 0.00^{bcde}

The values given are the arithmetic mean of 2 replications and represent the arithmetic mean ± standard deviation.
Significant differences were determined by Tukey test at $p < 0.05$.
Statistical differences in the same column are expressed with lowercase letters.