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Assessment of bacteriocin producing *Enterococcus faecium* HZ as adjunct culture to improve the aroma formation and antimicrobial activity in white-brined cheese

S. Ozturkoglu-Budak[1*], M. Arkadaş[2], Z. Yıldırım[3] and Y.K. Avşar[4]

[1] Department of Dairy Technology, Faculty of Agriculture, Ankara University, Ankara, Türkiye

[2] Graduate School of Natural and Applied Sciences, Ankara University, Ankara, Türkiye

[3] Department of Food Engineering, Faculty of Engineering, Nigde Ömer Halisdemir University, Nigde, Türkiye

[4] Department of Food Engineering, Faculty of Agriculture, Hatay Mustafa Kemal University, Antakya, Türkiye

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ABSTRACT

Cultures used in dairy products make it possible to obtain standard industrial products. However, they all provide a uniform taste and aroma. Generally, non-starter lactic acid bacteria (NSLAB) isolated from raw-milk or artisanal cheeses offer varied sensory characteristics when integrated in cheese provided that biosafety criteria are met. *Enterococcus faecium* HZ was previously isolated from traditional Turkish cheese and determined to have strong antibacterial activity as well as no gelatinase and hemolysis activities. In this study, this strain was used as adjunct culture in white-brined cheese to improve the physicochemical, textural, and aromatic properties, as well as antimicrobial activity. Cheeses with *E. faecium* HZ had a higher sensory score, which could be due to the aroma-active compounds produced by this strain. The incorporation of *E. faecium* HZ also improved the microbial quality of cheeses and showed an inhibitory effect via a stable enterocin production on indicator microorganisms.

* Corresponding author. E-mail: budak@ankara.edu.tr

KEYWORDS

Enterococcus faecium, cheese, adjunct culture, aroma, antimicrobial

1. INTRODUCTION

Enterococcus spp. are common bacteria found in many fermented foods due to their ability to withstand heat stress and adverse environmental conditions (Özkan et al., 2021). They are mostly found in high numbers in traditional or raw-milk cheeses. They contribute to cheese ripening via biochemical reactions and provide a unique taste and flavor to products (Centeno et al., 2022). In addition, *Enterococcus* spp. have antimicrobial activity enabling them to compete with other microorganisms. The antimicrobial properties are derived from the production of antimicrobial metabolites such as organic acids, hydrogen peroxide, diacetyl, bacteriocins, and exopolysaccharides, which prevent the growth of foodborne pathogens (Khan et al., 2010; Oruc et al., 2021; Kavitake et al., 2023).

In recent years, consumers expect diversity in sensory properties of cheese. However, starter cultures used in cheese industry provide limited and similar sensory properties. This is most likely due to the inability of industrial cultures to produce aroma-active compounds. *Enterococcus* spp. are present naturally in many traditional cheese microbiota, but include both pathogenic and non-pathogenic species. Therefore, there is a need to isolate non-pathogenic *Enterococcus* species and determine their safety and technological properties.

Enterococcus faecium HZ is a native strain previously isolated from traditional Turkish cheese. Antibiotic resistancy, hemolytic and gelatinase activities of this strain were also determined (Yildirim et al., 2014). Enterocin HZ, its bacteriocin, was also characterised in terms of both growth kinetics and antimicrobial activities. It was found to be sensitive to papain and trypsin, biologically active at pH 2.0–9.0, and resistant to heat treatment (90 °C for 30 min). It was synthesised at the highest level both in MRS broth at 32 °C and M17 broth at 37 °C, and also demonstrated strong inhibitory activity against some Gram-positive foodborne bacteria, particularly against *Listeria monocytogenes* in UHT milk.

In this study, non-pathogenic and bacteriocin producer *E. faecium* HZ strain was evaluated as an adjunct culture for use in industrial production of white-brined cheese in terms of its biopreservation and aroma production capacities. Aroma forming capacity of *E. faecium* HZ in white-brined cheese was monitored both by chromatographic and olfactometric techniques during 90 days of storage. The bacteriocin activity of this strain and the stability of formed bacteriocin during cheese storage were evaluated. The contribution of this strain on the microbiological, chemical, and textural properties of cheeses was also determined.

2. MATERIALS AND METHODS

2.1. Strains

The bacteriocin producer *E. faecium* HZ and *Lactobacillus plantarum* DSM 2601 used in the bacteriocin activity test were obtained from the culture collection of the Niğde Ömer

Halisdemir University. Both bacteria were propagated in de Man Rogosa Sharpe (MRS) broth (Fluka, Darmstadt, Germany) at 32 °C for 18 h. *E. faecium* HZ was subjected to a second activation in reconstituted milk (10%, w/w) before use in cheese milk (1%, v/v).

2.2. Cheese production

Pasteurised milk (72 °C, 15 s) was supplied by the Dairy Plant of Ankara University (Ankara, Turkey). Commercial freeze-dried cultures of *Lactococcus lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis* used in this study were obtained from Chr. Hansen (Horsholm, Denmark), and liquid rennet enzyme was supplied from Mysecoren (Maysa, İstanbul).

Pasteurised milk cooled to 32 °C was divided into two equal parts, and $CaCl_2$ (0.2 g L^{-1}) and commercial starter culture (1%) was added into cheese milk, respectively. Control cheese (C-cheese) was produced by using commercial starter culture, and experimental cheese (E-cheese) was produced with the use of *E. faecium* HZ together with the commercial culture. After a 30 min incubation period, liquid rennet (180 IMCU/mL) was added and it was left left for a 90 min coagulation period. The whey removal was performed, and the obtained curds were divided into blocks of 7 cm^3 and put in brine (14% NaCl) for 14–16 h at room temperature. Cheeses were vacuum-packed (with a composition of Polyethylen/Orevac/Polyamide (44/15/20 µm)) and stored at 4 °C for 90 days.

2.3. Determination of volatile compounds

The headspace solid phase microextraction (HS-SPME) technique was used for the extraction of volatile compounds on gas chromatograph mass spectrometer (GC-MS) (7890A/5975C Agilent Technologies, Santa Clara, CA) equipped with DB-WAX column (30 m × 0.32 mm × 0.25 µm). The method of Lee et al. (2003) was performed. The identification of volatile compounds was carried out by using the libraries of Wiley 7, NIST0.5, and Flavor on Agilent software. Relative amounts of each volatile compound were calculated according to the internal standard used.

2.4. Identification of aroma-active compounds

Aroma-active compounds were extracted and determined according to Deibler et al. (2004). GC-MS equipped with an olfactometry apparatus was used for his purpose. A polar column (DB-WAX column, 30 m × 0.32 mm × 0.25 µm) was used for sniffing. Three panellists with a training of 60 h sniffed cheese volatiles recording the retention time and describing the odours. The contribution of each aroma active compound to the overall aroma was determined by AEDA analysis. Dilutions were done at the injection port using split mode. For this purpose, samples extracted with SPME were injected at different splitting modes based on $\log_5$ (1:1, 1:5, 1:25, 1:125, and 1:625 corresponding to 1, 2, 3, 4, and 5th dilution). The highest dilution where an odour was detected was reported as the flavour dilution factor (FDF) (Grosch, 1993). Kovats' indices were calculated for each compound via using an alkane series (C8–C20). Reference aroma compounds (Sigma-Aldrich, St. Louis, MO, USA) were also used for comparison during identification.

2.5. Determination of bacteriocin activity in cheese

The bacteriocin activity test in cheese was carried out according to Munoz et al. (2004) by the agar spot method. *Lb. plantarum* DSM 2601, which is the bacterium most sensitive to enterocin HZ, was used as indicator to determine the bacteriocin activity.

2.6. Microbiological analyses

10 g of cheese was homogenised with 90 mL Ringer solution in a stomacher (IUL 707/470 Instruments, Spain), and 10-fold serial dilutions were prepared. Total aerobic bacteria (TAMB), lactic acid bacteria (LAB), total coliform, *Enterococcus* spp., *Lactococcus* spp., *Staphylococcus aureus* and yeast-mold analyses were performed according to the media and incubation conditions given by Harrigan and McCance (1998).

2.7. Instrumental texture analyses

Double-cycling compression test was performed using a TA-XT2 Texture Analyzer (Stable Micro Systems, Godalming, Surrey, UK) to determine the textural properties (Avsar, 2010). 35 mm back extrusion probe (A/BE-d35, Stable Micro Systems) and a 5 kg load cell were used during analyses. The probe was moved at a speed of 5 mm s^{-1} from the cheese surface.

2.8. Sensory evaluation

The sensory properties of cheeses during 90 days of storage were evaluated by 7 trained panellists from the Department of Dairy Technology, Ankara University, according to Clark and Costello (2009). Sensory attributes were evaluated based on appearance, texture, and flavour. All sensory attributes were graded on a ten-point hedonic scale.

2.9. Statistical analysis

Statistical analyses were performed using the Minitab package program (version Minitab ®16.1.1, Minitab Inc., State College, PA, USA). One-way analysis of variance (ANOVA) was conducted to determine statistical differences ($P < 0.05$) among storage days (1, 30, 60, and 90) and cheese types (control and experimental). Tukey's Multiple Range Test was also carried out to determine statistically significant differences.

3. RESULTS AND DISCUSSION

3.1. Volatile compound profiling

The determined volatile compounds and their average relative amounts in C and E-cheeses are given in 8 different categories in Table S1 (in Supplement available at the server of Publisher).

Alcohols were determined at highest in both cheeses. Different alcohol groups such as primary, secondary, branched, phenolic, sulphur-containing, and unsaturated were determined. Among them, phenethyl alcohol and 3-methylbutan-1-ol were found in the highest amounts. These two alcohols were more abundant in C-cheese compared to E-cheese, even though the amounts fluctuated in both cheeses. This situation can be interpreted as the enzymatic system of *E. faecium* used either prevents the formation of this molecule or changed it to another metabolite via a different pathway.

The second most determined group in quantity of both cheeses is alkanes. It can be argued that the presence of high amounts of alkanes may result from the hydrolytic effect of the starter and adjunct cultures used and the feeding of the dairy animal.

The group that the most significant difference was observed between C and E-cheese was acids. Pentanoic, hexanoic, octanoic, nonanoic, decanoic, and dodecanoic acids were detected only in E-cheese. The presence of high and different types of free fatty acids in E-cheese can be attributed to the lipolytic activity of *E. faecium* HZ. Centeno et al. (2022) reported acids are mainly formed during lipolysis of triglycerides in milk via the microbial activities. This could be the reason of higher quantities obtained in E-cheeses.

No significant difference was observed in terms of the variety and quantity of aldehydes in both cheeses. 3-methylbutyl aldehyde and phenylacetaldehyde were the most common aldehydes found.

As to ketones, the amount of 3-hydroxy-2-butanone (acetoin) was detected approximately four times higher in E-cheese than in C-cheese. This compound is known to form by LAB through citrate metabolism (Curioni and Bosset, 2002). Therefore, in this study *E. faecium* HZ was thought to be responsible for the formation of this compound.

The relative amounts of esters in cheeses appeared to fluctuate. Methyl and ethyl esters were found in high variety in both types, although no significant differences in terms of their quantities were found.

3.2. Aroma active compounds and their contribution to the overall aroma

Retention index (RI) and aroma extraction dilution analysis (AEDA) results of aroma-active compounds identified during the storage period in C and E-cheeses are given in Tables 1 and 2, respectively. According to aroma active compounds determined by olfactometry, ester and acid groups were found more in E-cheese compared to C-cheese. Methyl hexanoate was identified by fruity, floral, and pineapple aromas, hexanoic acid was identified by acidic, goat cheese aroma, and 2-phenyl acetaldehyde was characterised as honey, sweet, and floral aromas were the main aroma active compounds specific to E-cheese.

3.3. Bacteriocin activity in cheeses

E. faecium HZ maintained its activity during storage in the cheese samples. The filtrate obtained from E-cheese showed an inhibitory effect against *L. plantarum* (Fig. 1). Inhibitory activity tests have shown that *E. faecium* HZ maintains its activity in cheese during the storage process. This result indicates that both early and late contamination of the cheese can be prevented with stable *in situ* enterocin production. Yildirim et al. (2014) stated that *E. faecium* HZ can develop and produce enterocin in MRS medium and milk. Similarly, Aspri (2017) reported that bacteriocin-producing *E. faecium* isolated from donkeys could be used as a bio preservative agent against *L. monocytogenes* in fresh whey cheese. Pingitore et al. (2012) stated that bacteriocinogenic *E. faecium* ST88Ch could be used as starter cultures and have a potential benefit on the biocontrol of *L. monocytogenes* in cheese.

3.4. Microbial counts

Microbial counts of C and E-cheeses are given in Table 3. TAMB content of C-cheese was determined to increase throughout the storage period. However, a decrease was determined in E-cheese on day 60. This decrease is estimated to be due to the inhibitory effect of *E. faecium* HZ. In terms of *Lactococcus* spp., a significant difference was determined between C and E-cheeses at the beginning ($P < 0.05$), but they both increased their numbers until the 30th

Table 1. Retention index (RI) and aroma extraction dilution analysis (AEDA) results of aroma-active compounds identified in C-cheese during storage

					C-cheese			
					Log_5 FDF[4]			
Compound	CAS#	Odour description[1]	RI[2]	RI_{REF}[3]	Day 1	Day 30	Day 60	Day 90
Butan-2,3-dione	431-03-8	buttery	980	980	–	–	1	1
Ethyl butanoate	105-54-4	pineapple	1,044	1,043	3	3	3	4
Pentane-2,3-dione	600-14-6	buttery	1,059	1,055	–	3	1	4
Heptane-2-one	110-43-0	fruity, banana-like	1,186	1,187	–	–	1	1
3-methylbutane-1-ol	123-51-3	malt, whisky, chemical	1,219	1,220	–	1	4	5
Ethyl hexanoate	123-66-0	floral	1,224	1,224	–	1	1	1
Pentane-1-ol	71-41-0	chemical	1,234	1,255	–	1	1	1
Acetoin	513-86-0	buttery, acidic	1,298	1,297	–	–	1	1
Acetic acid	64-19-7	vinegar	1,444	1,450	3	4	4	5
Methional	3268-49-3	meaty, smoky, potato	1,460	1,458	2	4	4	5
Butanoic acid	107-92-6	butyric, buttery, waxy	1,620	1,619	3	3	4	5
3-methylbutanoic acid	503-74-2	dirty, feet		1,665	3	4	4	5

[1]: Odour description at the sniffing port during gas chromatography-olfactometry.
[2]: Retention indices calculated from gas chromatography-olfactometry results.
[3]: Retention indices of reference compounds calculated from gas chromatography-olfactometry results.
[4]: Odour intensities as Flavor Dilution Factor on DB-Wax column.

day of storage. After day 30, a decrease in the number of *Lactococcus* has been determined in E-cheeses. This result could be due to the growth ability of *E. faecium* HZ in M17 and MRS mediums (Yildirim et al., 2014). As to LAB counts, there was no significant difference between C and E-cheeses. Enterococcus was only detected in E-cheese during the storage showing an increasing trend. The total coliform content of C-cheese was determined higher than of E-cheese. The initial coliform counts decreased approximately 1–2 $\log_{10}$ CFU mL^{-1} in both cheeses during storage but the decrease ratio in E-cheese was higher. This low number could be due to enterocin formation by *E. faecium* HZ.

Similar to our results, many enterococcal strains that produce enterocins are reported to be effective in controlling contamination of foodborne pathogens in cheeses without adversely affecting the acid-generating activity of the starter culture and the organoleptic properties of the final product (Khan et al., 2010; Pingitore et al., 2012; Aspri et al., 2017).

3.5. Instrumental texture profile

Figure 2 shows the changes in the texture profile of C and E-cheeses during storage. The hardness, gumminess, and chewiness of C-cheese first increased and then decreased during storage, while the E-cheese showed an opposite trend. This situation can be interpreted as a

Table 2. Retention index (RI) and aroma extraction dilution analysis (AEDA) results of aroma-active compounds identified in E-cheese during storage

Compound	CAS#	Odour description[1]	RI[2]	RI_{REF}[3]	E-Cheese Log_5 FDF[4]			
					Day 1	Day 30	Day 60	Day 90
Butane-2,3-dione	431-03-8	buttery	980	980	1	1	1	1
Ethyl butanoate	105-54-4	pineapple	1,044	1,043	1	3	3	3
Pentane-2,3-dione	600-14-6	buttery	1,059	1,055	1	1	1	1
Methyl hexanoate	106-70-7	fruity, fresh, pineapple	1,191	1,202	1	1	1	1
3-methylbutane-1-ol	123-51-3	malt, whisky, chemical	1,219	1,220	1	1	3	4
Ethyl hexanoate	123-66-0	floral	1,224	1,224	–	1	1	1
3-methylbutyl butanoate	106-27-4	fruity, green banana	1,259	1,266	1	–	1	–
Acetoin	513-86-0	buttery, acidic	1,305	1,297	3	1	2	2
Acetic acid	64-19-7	vinegar	1,444	1,450	2	4	4	5
Methional	3268-49-3	meaty, smoky, potato	1,460	1,458	2	4	4	5
Butanoic acid	107-92-6	butyric, buttery, waxy	1,620	1,619	3	3	4	5
2-phenylacetaldehyde	122-78-1	honey, sweety, floral		1,636	–	1	1	1
3-methylbutanoic acid	503-74-2	dirty, feet		1,665	3	4	3	4
Hexanoic acid	142-62-1	acidic, goat cheese	1,841	1,832	1	1	1	1

[1]: Odour description at the sniffing port during gas chromatography-olfactometry.
[2]: Retention indices calculated from gas chromatography-olfactometry results.
[3]: Retention indices of reference compounds calculated from gas chromatography-olfactometry results.
[4]: Odour intensities as Flavor Dilution Factor on DB-Wax column.

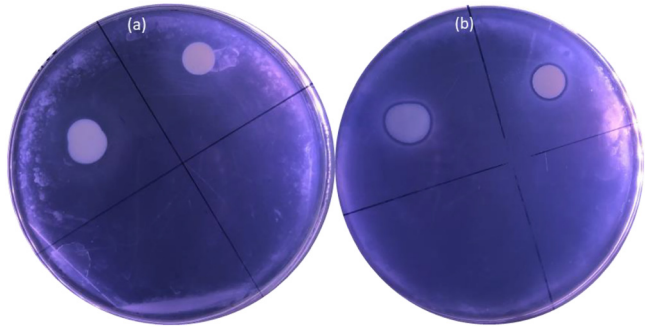

Fig. 1. Inhibitory activities of cheese filtrates against *L. plantarum:* (a) C-cheese (control); (b) E-cheese (experimental)

Table 3. Microbiological quality of cheeses ($\log_{10}$ CFU g^{-1})

	C-cheese				E-cheese			
	Day 1	Day 30	Day 60	Day 90	Day 1	Day 30	Day 60	Day 90
TAMB	6.68 ± 0.44a^{A}	7.66 ± 0.29b^{B}	8.05 ± 0.56c^{C}	8.42 ± 0.18c^{E}	6.25 ± 0.05a^{B}	7.41 ± 0.21ab^{B}	6.93 ± 0.07b^{D}	6.73 ± 0.09b^{AD}
LAB	7.59 ± 0.13a^{A}	8.01 ± 0.09b^{B}	8.37 ± 0.22bc^{BC}	8.51 ± 0.14c^{C}	7.63 ± 0.07a^{A}	8.07 ± 0.07b^{B}	8.47 ± 0.13c^{C}	8.67 ± 0.06c^{C}
Lactococcus spp.	6.06 ± 0.06a^{A}	7.52 ± 0.09b^{CE}	8.35 ± 0.18c^{D}	8.49 ± 0.08c^{D}	7.08 ± 0.15a^{B}	8.46 ± 0.12b^{D}	8.13 ± 0.06b^{D}	7.53 ± 0.10d^{E}
Enterococcus spp.	1>	1>	1>	1>	1>	1>	1>	1>
Total coliform	2.43 ± 0.07a	2.16 ± 0.14a	1.70 ± 0.16b	1.16 ± 0.13c	1.5 × 10^{1}a^{BCD}	0.64 × 10^{1}a^{BD}	<0.30c^{E}	<0.30c^{E}
Staphylococcus spp.	1>	1>	1>	1>	1>	1>	1>	1>
Yeast-mould	1>	1.96 ± 0.04a^{A}	2.30 ± 0.09a^{A}	2.71 ± 0.08b^{B}	1>	1.99 ± 0.07a^{A}	2.25 ± 0.07a^{A}	2.60 ± 0.12b^{B}

TAMB: Total aerobic mesophilic bacteria; LAB: lactic acid bacteria. C-cheese: control cheese; E-cheese: experimental cheese. Different lowercase letters within the same row indicate significant differences during storage ($P < 0.05$). Differences in samples with detected interactions are shown with capital letters ($P < 0.05$).

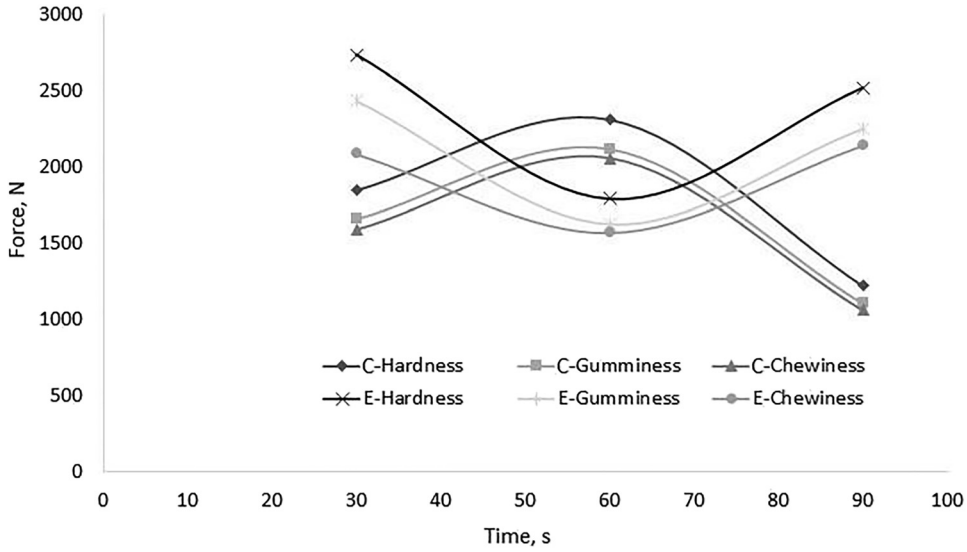

Fig. 2. Textural properties of C-cheese (control) and E-cheese (experimental)

result of salt intake caused by the curd contracts and turning into a harder structure due to the high initial acidity of E-cheese, and then the structure loosens with the dispersion of the salt in the body and re-hardening with serum leakage.

3.6. Sensory evaluation

The sensory properties (appearance, texture, flavour) determined during the storage of C and E-cheeses are given in Fig. 3. The greatest changes have been determined in texture and flavour. When the texture began to soften gradually, it was scored lower than the initial values as the storage progressed. However, flavour scores showed an increasing trend depending on the number and type of active compounds during the ripening. The flavour scores increased significantly more in E-cheese from the second month of storage than in C-cheese. This could be due

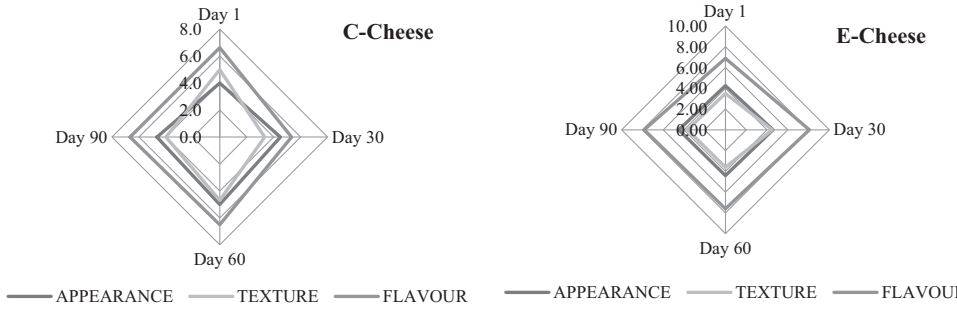

Fig. 3. Sensory characteristics of C-cheese (control) and E-cheese (experimental)

to the aroma-active compounds formed by *E. faecium* HZ. In previous studies, *E. faecium* was reported to cause a preferred aroma via citrate and glucose metabolism (Vaningelgem et al., 2006).

4. CONCLUSIONS

The number of volatile compounds formed in E-cheese was determined higher than in C-cheese. Alcohols, aldehydes, and acids were mainly detected in E-cheese, which can be the reason of having favourable sensory properties that could be due to the aroma-active compounds produced by *E. faecium* HZ. Furthermore, E-cheese showed higher hardness, chewiness, and gumminess than C-cheese during storage. TAMB and coliform counts were determined lower and LAB counts were determined higher in E-cheese, which could be from the bacteriocin activity of *E. faecium* HZ. As a result, *E. faecium* HZ could be an adjunct culture for industrial cheese production to increase shelf-life, to obtain varied sensory properties, and to provide higher quality characteristics.

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SUPPLEMENTARY MATERIALS

Supplementary data to this article can be found online at https://doi.org/10.1556/066.2023.00086.

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