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GROWTH ABILITY OF BACTERIOCINOGENIC STRAINS IN MILK AND THEIR BACTERIOCIN ACTIVITY AGAINST CHEESE STARTER CULTURES

Nilgün Öncül[1*], Zeliha Yıldırım[2]

[1]Muğla Sıtkı Koçman University, Faculty of Fethiye Health Sciences, Department of Nutrition and Dietetics, Fethiye-Muğla, Turkey

[2]Niğde Ömer Halisdemir University, Faculty of Engineering, Department of Food Engineering, Niğde, Turkey

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ABSTRACT

In this study, the growing ability of *Lactococcus lactis* ssp. *lactis* BZ and *Enterococcus faecalis* KP in different fat content milk at 30°C for 48 hours and the efficiency of their bacteriocins, lactococcin BZ and enterocin KP, against three types of cheese starter (Choozit MA 11LYO 50, Choozit MA 19LYO 50 and DI-Prox M265) in full fat milk were determined. The growth and bacteriocin production abilities of *L. lactis* ssp. *lactis* BZ and *E. faecalis* KP in milk were examined for 48 hours at 30°C. As a result of the study, it was determined that both bacteria grew better in milk with low fat content and produced more bacteriocin. It was found that lactococcin BZ (1600 AU/mL) and enterocin KP (1600 AU/mL) had an inhibitory effect against three different cheese starter cultures analyzed when used separately or in combination.

Keywords: *Lactococcus lactis* ssp. *lactis* BZ, *Enterococcus faecalis* KP, bacteriocin, lactococcin BZ, enterocin KP, milk, cheese starter cultures

BAKTERİYOSİNOJENİK SUŞLARIN SÜTTE GELİŞMELERİ VE BAKTERİYOSİNLERİNİN PEYNİR KÜLTÜRLERİNE KARŞI AKTİVİTELERİ

ÖZ

Bu çalışmada; farklı yağ içeriğine sahip sütlerde bakteriyosin üreticisi olan *Lactococcus lactis* ssp. *lactis* BZ ve *Enterococcus faecalis* KP'nin gelişme ve bakteriyosin üretme yetenekleri ile bakteriyosinleri olan laktokoksin BZ ve enterosin KP'nin tam yağlı sütte peynir üretiminde kullanılan starter kültürlere (Choozit MA 11LYO 50, Choozit MA 19LYO 50 ve DI-Prox M265) karşı etkileri belirlenmiştir. *L. lactis* ssp. *lactis* BZ ve *E. faecalis* KP'nin sütte gelişme ve bakteriyosin üretme yetenekleri 30°C'de 48 saat boyunca incelenmiştir. Çalışma sonucunda, her iki bakterinin düşük yağ içeriğine sahip sütte daha iyi geliştikleri ve daha fazla bakteriyosin ürettikleri tespit edilmiştir. Laktokoksin BZ (1600 AU/mL) ve enterosin KP (1600 AU/mL) hem tek başlarına hem de kombine olarak kullanıldıklarında analiz edilen üç farklı peynir starter kültürlerine karşı inhibitör etkinliğe sahip olduğu bulunmuştur.

Anahtar kelimeler: *Lactococcus lactis* ssp. *lactis* BZ, *Enterococcus faecalis* KP, bakteriyosin, laktokoksin BZ, enterosin KP, süt, peynir starter kültürleri

[*] *Corresponding author* / Yazışmalardan sorumlu yazar
✉: nilgunoncul@hotmail.com; nilgunoncul@mu.edu.tr, ✆:(+90) 252 211 5733 🖷:(+90) 252 211 1352

Nilgün Öncül; ORCID no: 0000-0002-2865-7958
Zeliha Yıldırım; ORCID no: 0000-0002-6155-6921

INTRODUCTION

Lactic acid bacteria (LAB) are Gram-positive, motionless, nonsporulating, acid-tolerant, microaerophilic, either rod- or cocci-shaped bacteria whose main fermentation product from carbohydrates is lactic acid. LAB are attained as "food grade" microorganisms (Beasley, 2004). Microorganisms of genera *Lactococcus, Carnobacterium, Lactobacillus, Leuconostoc, Pediococcus, Tetragenococus, Streptococcus, Weissella, Vagococcus, Enterococcus, Oenococcus,* and *Lactosphaera* are recognized as LAB (Kuipers et al., 1997; O'Sullivan et al., 2002; Galvez et al., 2007). In the fermentation process, LAB produce an array of metabolites which have antagonistic activity against pathogenic and saprophytic microorganism in foods (Chen and Hoover, 2003; de Vuyst and Leroy, 2007). LAB have demonstrated antagonism against other bacteria by competing for nutrients or producing metabolites. These bacteria produce several metabolic products that have antimicrobial effects such: organic acids, diacetyl, acetaldehyde, reuterin, hydrogen peroxide, fatty acids, ethanol, enzymes, and bacteriocins. Especially, there has been great interest in bacteriocins because of their antibacterial activity against foodborne bacteria (Khalid et al., 1999; Soomro et al., 2002).

Bacteriocins produced by LAB are small antimicrobial peptides that are characterized as cationic and hydrophobic or amphiphilic. The genera of *Enterococcus, Lactococcus, Streptococccus, Lactobacillus, Pediococcus, Leuconostoc,* and *Corynebacterium* have been stated in several studies with their ability to produce bacteriocins. Bacteriocins, secreted by the ribosome, are antagonistic to strains closely related to the producer microorganism, food spoilage, and foodborne pathogenic bacteria, but confer immunity to the host strain (Klaenhammer, 1993; Cleveland et al., 2001; Chen and Hoover, 2003; Rodriguez et al., 2003).

Nowadays, bacteriocinogenic strain of LAB can be used in several ways in fermented dairy products from the use of bacteriocin-producing strains directly in food as a starter, co-culture, probiotic, or protection cultures to the use of concentrated bacteriocin preparations as antimicrobials (Cintas et al., 2001; O'Sullivan et al., 2002; Papagianni, 2003). *Lactococcus lactis, Streptococcus thermophilus, Lactobacillus bulgaricus, Lactobacillus acidophilus, Lactobacillus helveticus, Enterococcus faecium, Enterococcus faecalis, Leuconostoc mesenteriodis,* and their subspecies are the bacteriocinogenic strains of LAB which are the most commonly used in fermented milk production processes (Cleveland et al., 2001; Deegan et al., 2006).

Bacteriocinogenic strain of LAB can be added into raw materials as a protective adjunct for inhibiting the growth of pathogenic or spoilage microorganisms and extending shelf life, or they can be applied in processing or to end products. There are basic factors that determine at which stage of protective culture will attend into a food. These factors are the interaction between starter and protective culture, and the functional properties of protective culture. Bacteriocinogenic strains can be used either directly as starter cultures, as adjunct or co-cultures in combination with a starter culture, or as protective cultures (especially in the case of symbiotic against starter culture, and having a positive or a neutral impact on flavor) in pre-treated raw materials. If the strains perform inhibitor activity towards starter culture, it can be incorporated into foods after the fermentation step or added to end products (Deegan et al., 2006; Gálvez et al., 2007). There have been several applications in the use of bacteriocinogenic strains. In general, the following strains are used when producing foods for the industry: (i) *L. lactis (L. lactis* ssp. *lactis, L. lactis* ssp. *lactis* biovar *diasetilactis, L. lactis* ssp. *cremoris*) is as a starter, protective and probiotic culture in cheese, buttermilk, butter, kefir, and koumiss; (ii) *L. helveticus* is as a starter and protective culture in some type of cheese; (iii) *E. faecium* and *E. faecalis* are as an adjunct, protective, and probiotic culture in some type of cheese; (iv) *L. acidophilus, L. bulgaricus,* and *S. thermophilus* are as a starter, protective, and probiotic culture in yogurt and cheese; (v) *Pediococcus acidolactici* and *P. pentasaues* are protective culture in cheese (O'Sullivan et al.,

2002; Soomro et al., 2002; Deegan et al., 2006; de Vuyst and Leroy, 2007; Lopez and Belloso, 2008).

In previous studies, *L. lactis* ssp. *lactis* BZ and *E. faecalis* KP which are bacteriocinogenic strains were isolated from Boza and White cheese. In the same studies, their bacteriocins were also characterized and named as lactococcin BZ and enterocin KP, respectively. The bacteriocins have individually a broad inhibitory spectrum in the medium (Şahingil et al., 2011; Isleroglu et al., 2012). However, the bacteriocin production ability of these bacteriocinogenic bacteria in the food matrix is limited. Therefore, the uses of these bacteriocin producing strains as a protective culture have been exhibited for the dairy industry in this research. So, the objectives of this study were to determine the growth and bacteriocin producing ability of *L. lactis* ssp. *lactis* BZ and *E. faecalis* KP, and to explore their bacteriocins activity against some commercial cheese starter cultures in milk.

MATERIAL AND METHODS

Material

Milk samples and commercial starter cultures

Full fat (3.0%), half fat (1.5%), and low fat (<0.1% fat) UHT milk samples were purchased as an industrial product from local supermarkets. In this study, three types of mesophilic cheese starter cultures were provided from dairy companies. The strain compositions of freeze-dried starter cultures were *L. lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris*. Two of the cheese starters (Choozit MA 11LYO 50 and 19LYO 50) were produced from Danisco (Denmark), and the other starter was obtained from DI-Prox M265 (France). Choozit MA 11LYO 50, Choozit MA 19LYO 50, and DI-Prox M265 cheese starters were named as CS1, CS2, and CS3, respectively.

Bacterial strains and media

L. lactis ssp. *lactis* BZ producing lactococcin BZ and *E. faecalis* KP producing enterocin KP were propagated in De Mann Rogosa and Sharp broth (MRS, Fluka, Germany) at 30°C for 24 hours. *Lactobacillus plantarum* DSM 2601 (9 log CFU/mL) is the most sensitive indicator organism to lactococcin BZ and enterocin KP (Şahingil et al., 2011; Isleroglu et al., 2012). So, it was used as an indicator bacterium for determining bacteriocin activity and was also cultivated in MRS broth at 30°C. All the strains were maintained in a frozen stock at -80°C in MRS broth with 20% glycerol (Merck, Germany) and propagated twice before used in experiments.

Methods

Growth of bacteriocin producer strains and bacteriocin production in milk samples

Twice activated culture of *L. lactis* ssp. *lactis* BZ and *E. faecalis* KP were centrifuged (4000×g at 4°C, 10 min), and supernatants were discarded. The pellets were collected and washed twice with 0.1% sterile peptone water (PW, Merck, Germany) and then pellets were re-suspended with PW to the beginning volume. The beginning value of *L. lactis* ssp. *lactis* BZ and *E. faecalis* KP were 9.66 log CFU/mL and 8.55 log CFU/mL. The bacteriocin producer strains were inoculated separately into full, half, and low milk samples at 1%, and then incubated at 30°C for 48 hours. The samples were taken at certain intervals and tested for pH values, bacteriocin activity (AU/mL), and colony forming unit (log10 CFU/mL) during the incubation. For determining the colony forming unit, decimal dilutions were prepared with PW and viable cell numbers were enumerated by surface plating on MRS agar. The MRS agar plates were incubated at 30°C for 48 hours. The UHT milk samples without bacteriocinogenic strains were used as control.

Determining pH Values

The pH values were measured by a calibrated pH meter (WTW Inolab pH Level 1, Germany) (AOAC, 1995).

Bacteriocin activity assay

The agar spot technique was used for determining the activity of bacteriocins. The samples were heated at 75°C for 10 min and diluted as two-fold dilutions (1/2, 1/4, 1/8, etc.) before analyses. The samples from each dilution (20 µl) were spotted onto a bottom layer of MRS soft agar (0.8% agar) which inoculated with the indicator strain *L. plantarum*. After pre-diffusion at room temperature (~25ºC) for 30 min, the plates were

incubated at 30ºC for 24 hours. A clear zone (at least 2 mm) was recorded as positive after incubation. The bacteriocin activity was expressed in arbitrary units (AU/mL), the reciprocal of the highest serial 2-fold dilution showing a clear zone of growth inhibition of the indicator strain (Mayr-Harting et al., 1972).

Preparations of bacteriocins

Twice activated culture of *E. faecalis* KP and *L. lactis* ssp. *lactis* BZ were inoculated into MRS broth at 1% and then incubated at 30°C and 25°C for 18 hours, respectively. After the incubation period, the cultures were centrifuged (7000×g at 4°C, 20 min), and the pellet was discarded. The supernatants were collected and sterilized by membrane filter (with 0.45 μm pore diameter). The cell-free supernatants were frozen and dried by lyophilization. Then the bacteriocins were kept at -80°C until used (Moreno et al., 2002).

Determining the activity of bacteriocins against cheese starter cultures

The full fat milk samples were inoculated with cheese starter cultures at 1% and then treated with lactococcin BZ (1600 AU/mL) or enterocin KP (1600 AU/mL) or a combination of 50% lactococcin BZ (800 AU/mL) and 50% enterocin KP (800 AU/mL). The viable cell numbers of starters were counted at certain intervals (0, 1, 3, 5, 9, and 24 hours) on MRS agar at 30°C. Bacteria count performed 30 min after the addition of bacteriocin to the milk samples was accepted day 0 (T=0). The pH values were also determined during the incubation. The UHT milk samples containing only cheese starters at the same level were used as a positive control. The UHT milk samples with or without bacteriocin were used as a negative control.

Statistical analyses

The counts of bacteria were expressed as log CFU/mL. All experiments were carried out three times. The significant difference between the means was established by Tukey tests. All data were analyzed using the general linear models procedure of SAS Institute Inc. (1998) to determine differences between treatment means.

RESULT AND DISCUSSION

The growth and bacteriocin producing of *L. lactis* ssp. *lactis* BZ and *E. feacalis* KP in milks

L. lactis ssp. *lactis* BZ can grow in all types of milk samples and the difference between the milk type is not significant ($P > 0.05$) (Fig.1a). It was observed that *L. lactis* ssp. *lactis* BZ was produced more bacteriocin than the others in low fat milk samples (Fig.1b). Lactococcin BZ was first synthesized in low fat milk after 15 hours of incubations (100 AU/mL) and it was reached the maximum value at the 30th hour of incubation (800 AU/mL). However, the first bacteriocin activity was detected in full (200 AU/mL) and half fat (175 AU/mL) milk samples at the 30th hour of incubation. The bacteriocin activity of lactococcin BZ was lower in full and half fat milk samples than low fat milk. The main reason may be dependent on the hydrophobic interaction between lactococcin BZ and milk fat globules. The bacteriocin activity was decreased at the 48 hours of incubation time in all kinds of milk. The probable reasons are proteolytic degradation, protein aggregation, and bacteriocin adsorption to the cells (de Vuyst et al., 1996; Aasen et al., 2000). The bacteriocin activity of the samples due to the fat milk content is significantly different ($P < 0.01$). The pH values of the milk samples were measured as 6.63, 6.63, and 6.60 at the beginning of the incubation and decreased to 4.49, 4.60, and 4.69 at the end of the period in full, half, and low fat milk samples, respectively. In a previous study, it was indicated that *L. lactis* ssp. *lactis* BZ was produced the bacteriocin after 6 hours of incubation at 32°C in MRS medium and the maximum level (400 AU/mL) was found at the 18th hour. Similarly, it was also mentioned that bacteriocin activity was decreasing by the increasing incubation time (Şahingil et al., 2010). The main reason for the different bacteriocin producing ability of *L. lactis* ssp. *lactis* BZ in medium or milk is having different components of milk with MRS. MRS, is a special medium that provides sources of carbon, nitrogen, and vitamins for lactic acid bacterial growth. The MRS also contains vitamins and amino acids specifically required by Lactobacilli. The bacteriocin producing was started early in MRS

than milk because *L. lactis* have low proteolytic activity and MRS medium provides nitrogen sources for *L. lactis*.

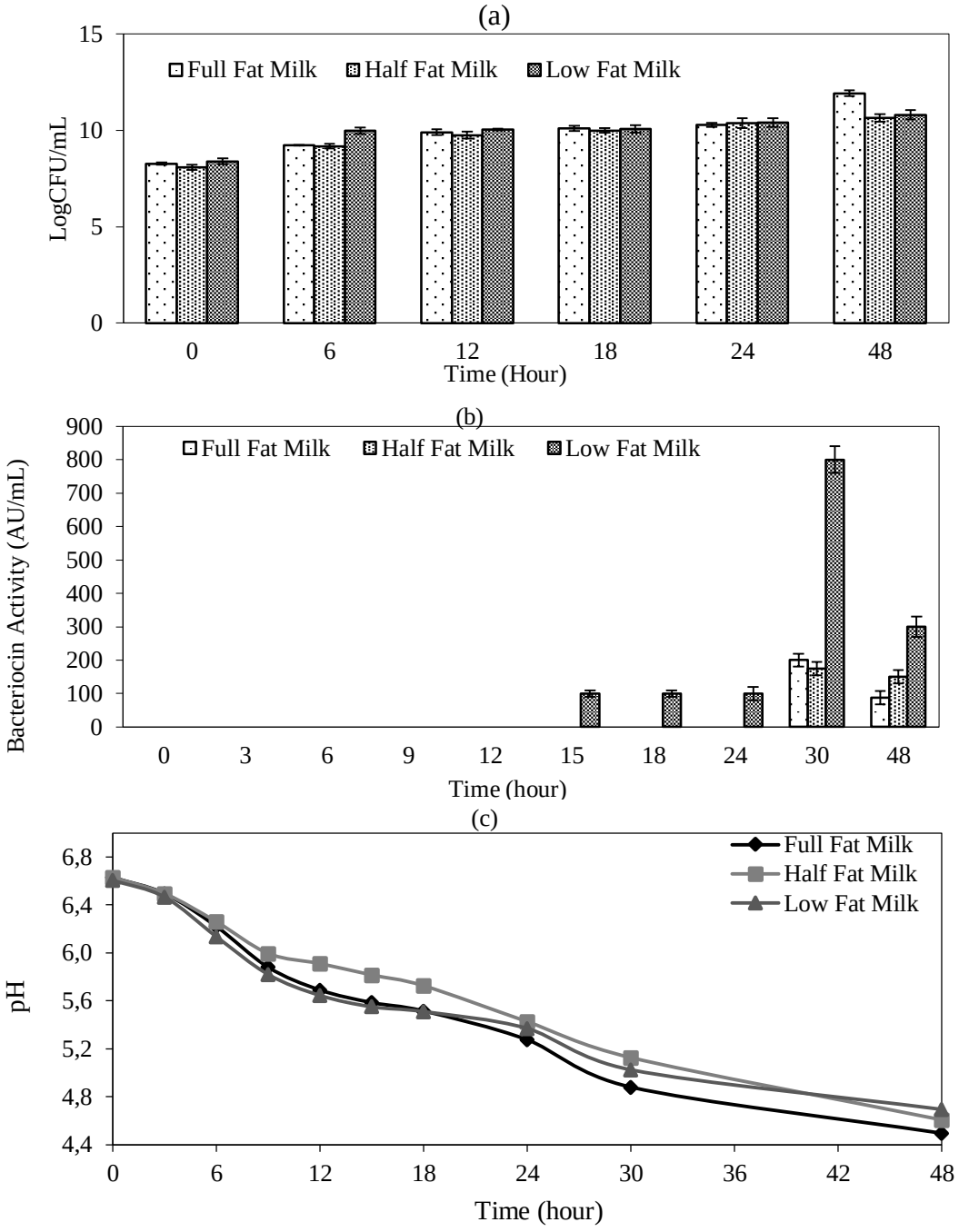
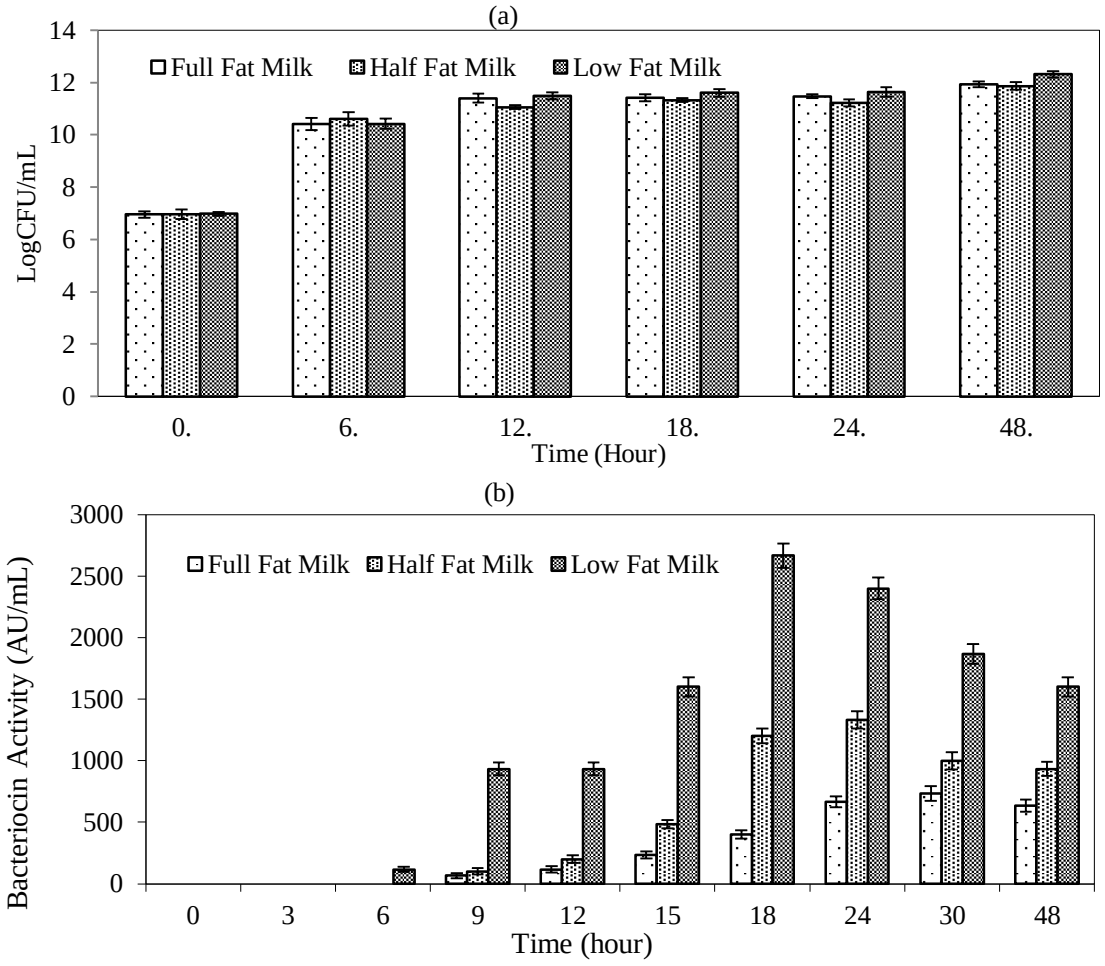


Figure 1. Growth and lactococcin BZ production of *L. lactis* ssp. *lactis* BZ in milk at 30°C. (a) Count of *L. lactis* ssp. *lactis* BZ, (b) activity of lactococcin BZ, and (c) pH of milk containing *L. lactis* ssp. *lactis* BZ

E. feacalis KP can grow in all types of milk samples and the difference between the milk type is not significant ($P > 0.05$) (Fig.2a). *E. feacalis* KP was produced more bacteriocin in low fat milk samples among the others like *L. lactis* ssp. *lactis* BZ. However, *E. feacalis* KP was synthesized bacteriocin earlier than *L. lactis*. Enterocin synthesis was started at the 6th hour in low fat milk and at the 9th hour in half and full fat milk samples (Fig.2b). *E. feacalis* KP was started to synthesize bacteriocin earlier in contrast *L. lactis* BZ in low fat milk because *E. feacalis* KP is required less nutrient for growth and they have better proteolytic activity. The bacteriocin activity of the samples due to the fat milk content is significantly different ($P < 0.01$). The maximum bacteriocin activity was observed at the 18th, 24th, and 30th hour of incubation in low (2600 AU/mL), half (1300 AU/mL), and full fat (700 AU/mL) milk samples, respectively. The bacteriocin activity was lower in half and full fat milk samples because of the hydrophobic interaction of enterocin KP with milk fat globules. The pH values of the milk samples were decreased from 6.65, 6.65, and 6.51 to 4.33, 4.42, and 4.32 during the period in full, half, and low fat milk samples, respectively (Fig.2). Isleroglu et al. (2012) was mentioned that *E. feacalis* KP was synthesized bacteriocin at the 6th hour of incubation and reached the maximum level (1600 AU/mL) at the 18th hour of the period at 32°C in MRS medium. The bacteriocin producing time of *E. feacalis* KP was similar in MRS and low fat milk; however, the activity of enterocin KP was higher in low fat milk samples.



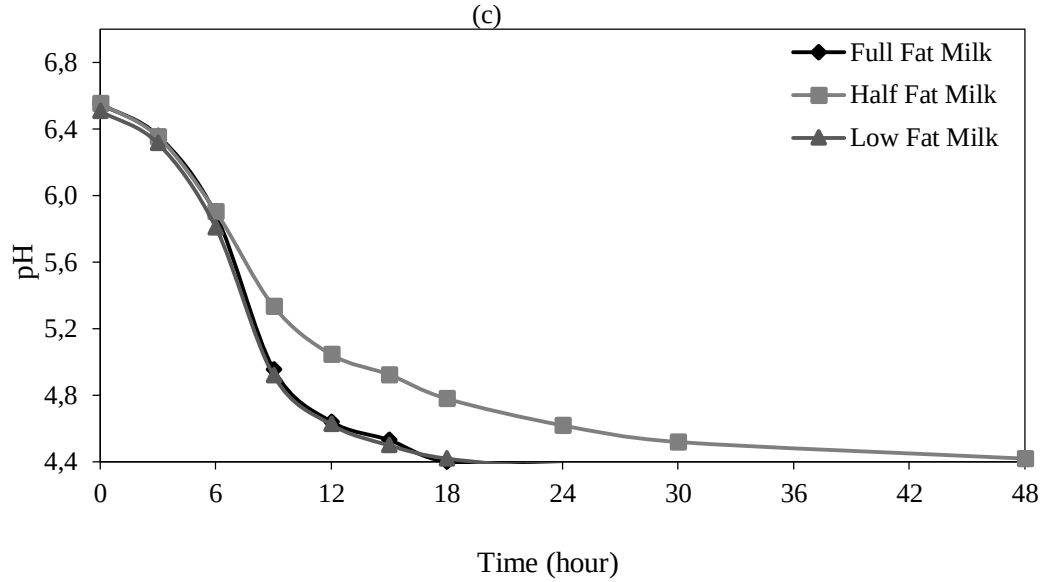


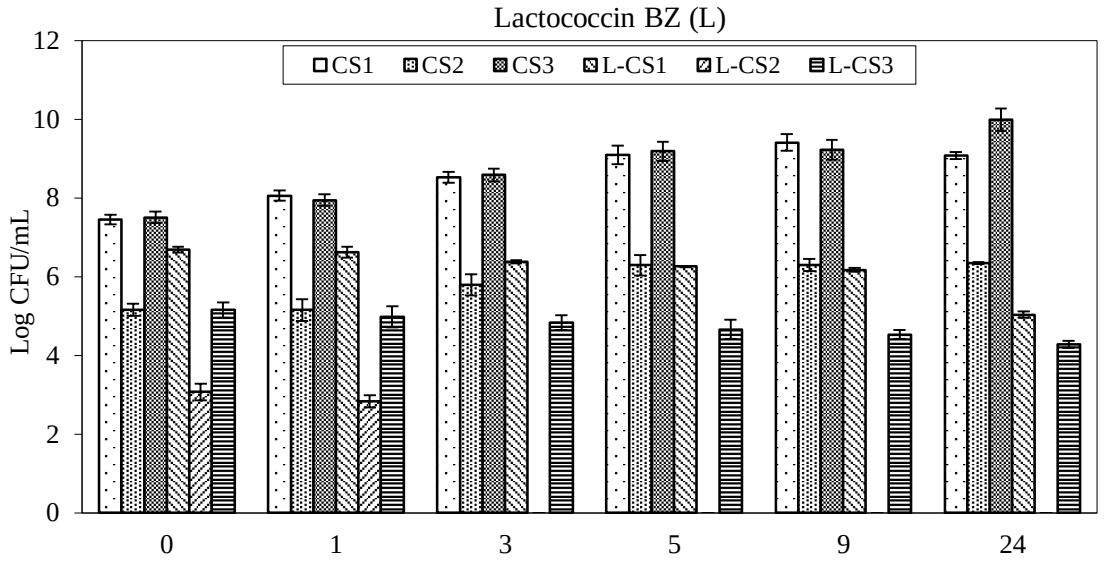
Figure 2. Growth and enterocin KP production of *E. faecalis* KP in milk at 30°C.
(a) Count of *E. faecalis* KP, (b) activity of enterocin KP, and (c) pH of milk containing *E. faecalis* KP

Foulquie´ Moreno et al. (2003) also found that *E. faecium* RZS C5 was produced bacteriocin in low fat milk (40 hours later) than MRS medium (2 hours) at 37°C. The bacteriocin producing ability of *L. lactis* ssp. *lactis* BZ in low fat milk was seen to be higher when compared with *E. faecium* RZS C5. Penna et al. (2005) were indicated that *L. lactis* subsp. *lactis* ATCC 11454 was produced more nisin and reached the maximum level (3.6 g/L) when low fat milk was added to MRS and M17 medium at the rate of 25%. Benmouna et al. (2018) achieved similar results. While no antimicrobial activity was detected in MRS medium, a clear inhibitory activity was observed after adding milk (25%) and yeast extract (%2.5) in MRS medium for three LAB strains. Avila et al. (2020) was tested a nisinogenic *L. lactic* INIA 415 as a starter in cheese that was produced with cow milk. The viable count of starter was 9.6, 9.7, and 9.6 log CFU/g for the 1st, 14th, and 21st days of the ripening time, respectively. The inhibition zone was also recorded to 26.6, 21.8, and 16.5 mm for analysis intervals. In another study, a nisin and lacticin 481 producing lactic acid bacteria was used as a starter culture to make cheese from ovine milk. The cheeses were kept at 12°C for 120 days for ripening. It was stated that LAB counts of the cheeses were similar at the beginning and the end of the ripening. The bacteriocin activity was decreased to 19.2 mm after 120 days (Garde et al., 2011).

The activity of Lactococcin BZ and Enterocin KP against cheese starter cultures

The activity of bacteriocins on cheese starters was analyzed at 30°C for 24 hours. Lactococcin BZ and enterocin KP individually or together had an inhibitory effect against all tested starters (P <0.01) (Fig.3). The counts of CS1 and CS3 cheese starters were decreased by lactococcin BZ at 1st and after 24 hours with counts 0.84 and 2.43 log CFU/mL for CS1, 2.52, and 3.22 log CFU/mL for CS3. The counts of CS2 cheese starter were detected below 1 log unit after 3 hours of application. Enterocin KP treated to starters resulted in reductions of CS1, CS2, and CS3 with count 0.34, 1.14, and 2.38 log CFU/mL after 1 hour, respectively. The effect of enterocin KP was lowered the counts of CS1, CS2, and CS3 to 1.23, 0.08, and 3.10 log CFU/mL after 24 hours of treatment. Synergistic antimicrobial activity was observed between lactococcin BZ and enterocin KP on the cheese starter cultures. The results are in concordance with total inhibition of CS1 and CS2 starters after 3 hours of application. The losses of the viable cell number of CS3 were 2.92

log in the first hour of incubation and over the 24 hours incubation period the decreases were reached 3.67 log unit. The pH values of the UHT milk samples containing only cheese starters were changed due to the metabolic activity of cultures (Fig.4). Besides, the UHT milk samples with bacteriocin showed no significant change in pH values during incubation ($P > 0.05$). In a previous study, lactococcin BZ and enterocin KP were tested against three types of yogurt starter cultures at 42°C for 24 hours. It was established that lactococcin BZ had an inhibitory effect against all kinds of starters whereas enterocin KP was effective in only two of them (Öncül et al., 2015). A bacteriocin, piscocolin 126, was tested against cheese starter strains including *L. lactis* subsp. *cremoris* (6), *L. lactis* subsp. *lactis* (6), *L. lactis* subsp. *diacetylactis* (4), *Leuconostoc* (4), and *S. thermophilus* (6). It was stated that the tested cultures were resistant against the piscocolin 126. The starters could grow in cheese milk and so, the acidity was developed (Wan et al., 1997). In another study, a bacteriocin produced from *L. bulgaricus* BB18 was analyzed on 11 yogurt starter cultures and these starters were also resistant to the tested bacteriocin (Simova et al., 2008). Eissa et al. (2018) were evaluated the bacteriocins from *L. acidophilus* and *L. rhamnosus* on yogurt starter culture which contain *L. bulgaricus* and *S. thermophilus* with 3% inulin. Both starters were resistant to the bacteriocins and were counted during the whole storage period (up to 44 days, at 4°C). The uses of bacteriocin producing strains like *L. lactis* ssp. *lactis*, *L. helveticus, E. faecium, E. faecalis, P. acidolactisi, P. pentasaues* as a starter or protective culture, and the application of their bacteriocins as an antimicrobial agent were caused to inhibition of pathogenic microorganisms in cheese during processing. Also, it has been demonstrated that both fermentation and maturation time was shortened when the bacteriocins were combined with a sensitive cheese starter culture, as well as cheese processing was become safer (Wan et al., 1997; Lauková and Czikková, 2001; Rodriguez et al., 2005; Deegan et al., 2006; Lopez and Belloso, 2008; Lacrois, 2008; Bizani et al., 2008). Like other bacteriocins, lactococcin BZ and enterocin KP have potential usage with resistant or sensitive cheese starter cultures.



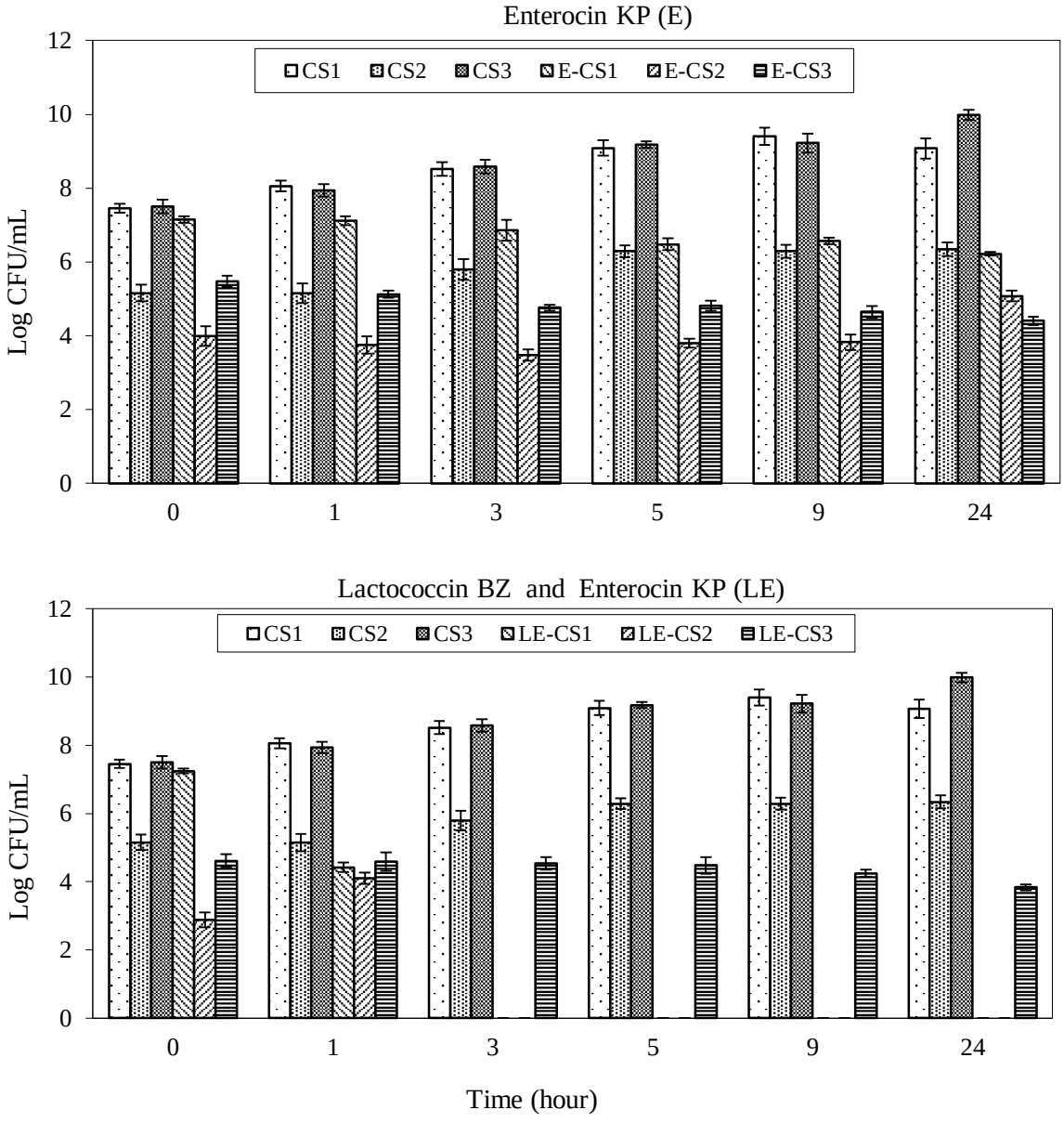


Figure 3. Activity of lactococcin BZ (L) and enterocin KP (E) alone or in combination (LE) against cheese starter cultures

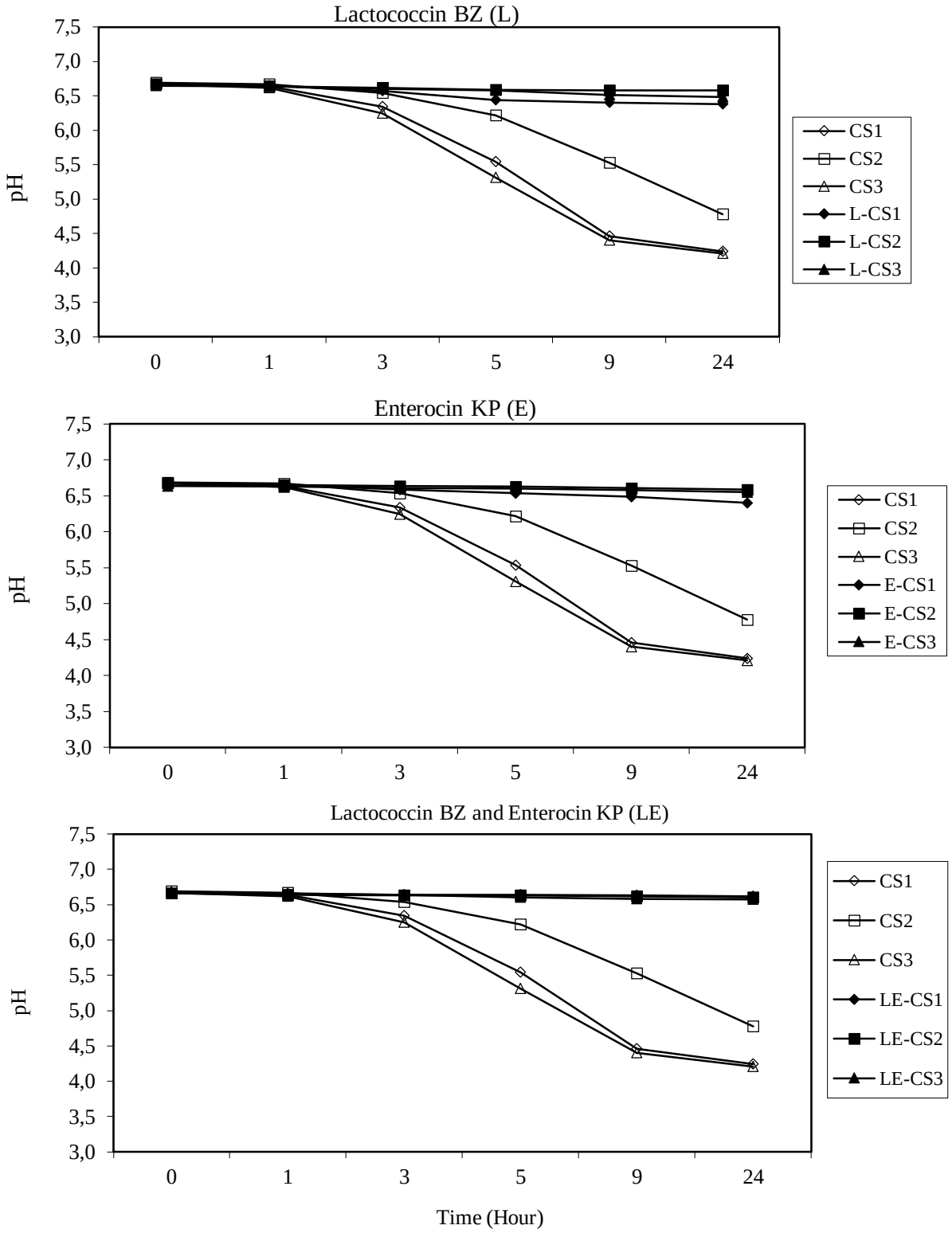


Figure 4. pH values of milk containing cheese culture and treated with lactococcin BZ (L) and enterocin KP (E) alone or in combination (LE)

As a conclusion, it was found that *L. lactis* ssp. *lactis* BZ and *E. feacalis* KP can grow in full, half, and low fat milk. These strains synthesized bacteriocin in all types of milk samples and both of them produced more bacteriocin in low fat milk among the others. So, these bacteriocin producing strains seem to be new biopreservative cultures for the dairy industry. The bacteriocin activity towards three cheese starters was also observed in full fat milk at 1600 AU/mL. Lactococcin BZ produced by *L. lactis* ssp. *lactis* BZ and enterocin KP produced by *E. feacalis* KP individually or together had an inhibitory effect against all tested starter culture. This gained an important property to the bacteriocins that shortened the fermentation process due to the sensitive starter cultures. At the same time, food safety will also be increased by using bacteriocins cause of their antimicrobial properties against pathogenic microorganisms.

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STATEMENT OF CONFLICT OF INTEREST
The authors declare that there are no conflicts of interest among the authors and/or publication of this article.

AUTHORS' CONTRIBUTIONS
NÖ and ZY designed the research. NÖ carried out the analysis. NÖ and ZY made a statistical analysis and wrote the paper. The authors read and approved the final version of the article.

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