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APPLICATION OF LACTOCOCCIN BZ AND ENTEROCIN KP AGAINST *LISTERIA MONOCYTOGENES* IN MILK AS BIOPRESERVATION AGENTS

Z. YILDIRIM[a]*, N. ÖNCÜL[b], M. YILDIRIM[a] and Ş. KARABIYIKLI[b]

[a]Department of Food Engineering, Niğde University, 51240 Niğde. Turkey
[b]Department of Food Engineering, Gaziosmanpaşa University, 60250 Taşlıçiftlik, Tokat. Turkey

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This study was performed to evaluate the effect of lactococcin BZ and enterocin KP against *Listeria monocytogenes* ATCC 7644 in skim (0.1%), half (1.5%), and full fat (3.0%) UHT milks. The milk samples were inoculated with *L. monocytogenes* at the level of approximately 2.60, 4.76, and 6.45 log CFU ml^{-1}, and then treated with various concentrations (400, 800, 1600, or 2500 AU ml^{-1}) of lactococcin BZ, enterocin KP, or their combination (1:1). Lactococcin BZ at 400–2500 AU ml^{-1} level displayed strong antilisterial activity, and decreased the viable cell numbers of *L. monocytogenes* to an undetectable level in all types of milk samples during the entire storage periods at 4 °C or 20 °C. Enterocin KP also had a high antilisterial effect, but it decreased as both the fat content of milk and inoculation amount of *L. monocytogenes* increased.

Keywords: bacteriocin, lactococcin BZ, enterocin KP, *Listeria monocytogenes,* milk

Milk and dairy products could be easily contaminated with foodborne pathogens such as *Listeria monocytogenes* under poor hygienic conditions. *L. monocytogenes* is an intracellular, facultative anaerobic, psychrotrophic and opportunistic pathogenic bacterium, which causes listeriosis. Listeriosis is a serious foodborne disease for newborns and infants, pregnant women or immunocompromised people (JEMMI & STEPHAN, 2006). Listeriosis has been associated with raw milk, pasteurized milk, and other dairy products, vegetables, and meat products. Because of its resistance to thermal, drying, and freezing processes, it can be a serious threat especially in dairy products. Outbreaks of human infections associated with consumption of dairy products have occurred with increased frequency during the past decade (JEMMI & STEPHAN, 2006; KIM et al., 2008).

One of the current trends in food preservation is biopreservation. Biopreservation refers to extended storage life and enhanced safety of foods using the natural microflora or their antibacterial products (STILES, 1996). Bacteriocins produced by lactic acid bacteria are one of the most important biopreservatives due to their proteinaceous nature and inhibitory effect against foodborne pathogens and spoilage bacteria (DEEGAN et al., 2006).

In previous studies, we isolated two bacteriocinogenic strains, *Lactococcus lactis* spp. *lactis* BZ and *Enterococcus faecalis* KP, from traditionally produced Boza and White cheese (ŞAHINGIL et al., 2011; ISLEROGLU et al., 2012). Lactococcin BZ produced by *L. lactis* spp. *lactis* BZ has antibacterial activity against either Gram-positive or Gram-negative bacteria, including some strains of *Listeria, Bacillus, Enterobacter, Escherichia*, *Salmonella*, *Yersinia*, *Citrobacter, Lactobacillus, Enterococcus,* and *Leuconostoc* (ŞAHINGIL et al., 2011). Enterocin

* To whom correspondence should be addressed.
Phone: +90 388 2252354; fax: +93 388 2250112; e-mail: zeliha.yildirim@nigde.edu.tr

KP produced by *E. faecalis* KP is active against only Gram-positive bacteria such as some strains of *Listeria, Bacillus, Enterococcus, Lactobacillus, Leuconostoc*, and *Lactococcus* (Isleroglu et al., 2012).

The objective of this study was to investigate the antimicrobial effects of lactococcin BZ and enterocin KP by itself or in combination toward *L. monocytogenes* in skim (0.1%), half fat (1.5%), and full fat (3.0%) milks.

1. Materials and methods

1.1. Materials

Skim (0.1%), half fat (1.5%), and full fat (3.0%) UHT milks were obtained from local supermarkets and they were stored at 4–5 °C in their original aseptic packages until used.

L. lactis spp. *lactis* BZ and *E. faecalis* KP were grown in de Man Rogosa and Sharpe Medium (MRS, Fluka, Germany) and stocked at –80 °C in MRS broth containing 20% glycerol. Brain Heart Infusion (BHI, Merck, Germany) broth was used for culturing *L. monocytogenes* ATCC 7644, and it was kept at –80 °C in BHI broth containing 20% glycerol.

1.2. Methods

1.2.1. Bacteriocin production. L. lactis spp. *lactis* BZ and *E. faecalis* KP cultures were grown in MRS broth at 30 °C and 25 °C for 18 h, respectively (Şahingil et al., 2011; Isleroglu et al., 2012). At the end of incubation, each culture was centrifuged (Beoco, Germany) at 7000×*g* for 20 min, and the supernatant was sterilized by membrane filtration (0.45 µm). Both bacteriocins were partially purified by ammonium sulphate precipitation (50% of saturation) and then organic solvent precipitation (methanol/chloroform mixture, 1:2, v/v) (Moreno et al., 2002). During each step of the bacteriocin preparation, the bacteriocin activity was tested against *L. monocytogenes* by using agar spot method mentioned previously by Isleroglu and co-workers (2012).

1.2.2. Inhibitory effect of bacteriocins toward L. monocytogenes *in milk samples.* Skim, half fat, and full fat milk samples were divided into 100 ml portions under aseptic conditions, inoculated with various doses of *L. monocytogenes* (approximately 10^2, 10^4, and 10^6 CFU ml^{-1}) and then treated with different concentrations (400, 800, 1600, and 2500 AU ml^{-1}) of lactococcin BZ, enterocin KP, or their combination (1:1, total activity 400, 800, 1600, or 2500 AU ml^{-1}). The samples were stored for 25 days at 4 ºC or 20 °C. During storage period, cell numbers of *L. monocytogenes* were determined on PALCAM agar (Sigma, Germany) after incubation at 37 °C for 24–48 h. The milk samples without bacteriocins and *L. monocytogenes* or containing just bacteriocin or *L. monocytogenes* were used as control samples.

1.2.3. Statistical analyses. Three replicates were done for each experiment. All data were analysed using the general linear models procedure of SAS Institute Inc. (1998) in order to determine differences between treatment means. Pairwise comparisons of all treatment means were achieved by using Least Significant Difference (LSD) procedure at $P<0.05$.

2. Results and discussion

2.1. Effect of bacteriocins on the survival of L. monocytogenes *in milk samples stored at 4 °C*

Lactococcin BZ at all treatment levels (400–2500 AU ml^{-1}) decreased *Listeria* counts in all kinds of milk samples containing 2.53, 4.76, and 6.44 log CFU ml^{-1} to an undetectable level during the storage period. Inhibitory effect of enterocin KP was similar to that of lactococcin BZ in all milk samples inoculated with 2.53, 4.76, and 6.44 log CFU ml^{-1} when it was used at the concentrations of 800, 1600, or 2500 AU ml^{-1}. Antilisterial activity of enterocin KP at 400 AU ml^{-1} decreased by increasing the amount of *L. monocytogenes* or the fat content of the milk. Enterocin KP at 400 AU ml^{-1} reduced *Listeria* counts to an undetectable level from day 0 to day 25 of the storage in all milk types inoculated with 2.53 log CFU ml^{-1}. At the same level, enterocin KP decreased *Listeria* counts to an undetectable level at day 3, 12, and 15 in skim, half fat, and full fat milk samples containing 4.76 log CFU ml^{-1}, respectively. However, similar reduction in *Listeria* counts with the same enterocin KP concentration was achieved at day 15 in skim milk inoculated with 6.44 log CFU ml^{-1} (Table 1). Additionally, enterocin KP at the same concentration did not reduce *Listeria* counts to an undetectable level even after 15 days storage in half and full fat milk samples containing 6.44 log CFU ml^{-1}. At the end of 25 days storage, half fat and full fat milk samples still contained 1.55 and 1.76 log CFU ml^{-1} *Listeria*, respectively (Table 1). Unlike enterocin KP, the fat content of the milk samples did not affect the activity of lactococcin BZ, probably because of its low hydrophobicity. These results showed that milk fat is not an important factor for biological activity of lactococcin BZ; however, it is an important factor for enterocin KP. Lactococcin BZ activity being unaffected by fat content could be quite important for food industry, especially for dairy industry. This result indicates that lactococcin BZ could be a good alternative antimicrobial agent to control pathogens in foods containing fat. Similar to enterocin KP, the inhibitory activity of nisin (Jung et al., 1992; Bhatti et al., 2004; Meena et al., 2004; Yoon et al., 2011) and pediocin 5 from *Pediococcus acidilactici* UL5 (Huang et al., 1994) declined by increasing fat content of dairy products. According to those authors, the decrease in bacteriocin activity was probably due to the fact that bacteriocin might be adsorbed to the milk fat globules and become unavailable to destroy bacterial cells.

The antilisterial activity of lactococcin BZ or enterocin KP is higher than those of the bacteriocins studied previously such as cerein A8 and nisin. The addition of 160 AU ml^{-1} cerein 8A produced by *B. cereus* to UHT milk resulted in a decrease of 3 log cycles in the cell counts of *L. monocytogenes* within the 14-day period at 4 °C (Bizani et al., 2008). Nisin (50 IU ml^{-1}) caused 7, 3, and 1 log reduction in the counts of *L. monocytogenes* Scott A in skim milk (0% fat), milk with 4% fat, and milk containing 12.9% fat, respectively (Jung et al., 1992). Most of the studies declared that cell counts of *L. monocytogenes* in milk challenged with different concentrations of nisin (Bhatti et al., 2004; Kim et al., 2008; Yoon et al., 2011) or pediocin 5 (Huang et al., 1994) decreased to an undetectable level at the beginning of the incubation, but then increased during the incubation period.

The cell count of *L. monocytogenes* in the milk samples decreased abruptly as soon as lactococcin BZ and enterocin KP in combination (1:1, total activity 400, 800, 1600, or 2500 AU ml^{-1}) was added. Cell numbers decreased to undetectable level in skim, half fat, and full fat milk samples containing 2.53, 4.76, and 6.44 log CFU ml^{-1} *Listeria* cells. It was observed that when enterocin KP was used with lactococcin BZ (total bacteriocin activity 400 AU ml^{-1}), the antilisterial effect increased in full fat and half fat milk containing 6.44 log unit *L. monocytogenes* compared to 400 AU ml^{-1} of enterocin KP used alone (Table 1).

Table 1. Inhibitory effect of bacteriocins against *L. monocytogenes* at the level of 10^6 CFU ml^{-1} in all types of milk at 4 or 20 °C

Milk	samples	Storage (day)																
		4 °C								20 °C								
		0	1	3	6	9	12	15	25	0	1	2	4	6	9	12	15	25
Full fat	C	6.46‡	6.93	7.22	7.36	8.14	8.37	8.15	8.70	6.46	8.50	8.61	8.93	8.93	8.81	8.87	8.82	8.70
	L400–2500†	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	E400	3.8	3.51	3.24	3.01	2.83	2.54	2.23	1.76	3.84	3.68	3.46	3.23	3.05	2.84	2.55	2.22	2.02
	E800–2500	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	EL400	1.62	1.35	1.1	N	N	N	N	N	1.65	1.46	1.15	N	N	N	N	N	N
	EL800–2500	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Half fat	C	6.46	6.93	7.22	7.36	8.14	8.37	8.15	8.70	6.46	8.50	8.61	8.93	8.93	8.81	8.87	8.82	8.70
	L400–2500	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	E400	3.42	2.82	2.59	2.31	2.09	1.96	1.71	1.55	3.45	3.12	2.81	2.64	2.32	2.12	1.94	1.75	1.6
	E800–2500	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	EL400	N	N	N	N	N	N	N	N	1.32	1.07	N	N	N	N	N	N	N
	EL800–2500	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Skim	C	6.46	6.93	7.22	7.36	8.14	8.37	8.15	8.70	6.46	8.50	8.61	8.93	8.93	8.81	8.87	8.82	8.70
	L400–2500	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	E400	2.8	2.42	2.15	1.89	1.58	1.26	N	<1	2.83	2.55	2.23	2.08	1.8	1.52	1.13	N	N
	E800–2500	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	EL400–2500	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N

C: control, inoculated but not treated with bacteriocins; †: AU ml^{-1}; ‡: log CFU ml^{-1}; N: log CFU ml^{-1}<1; L: lactococcin BZ; E: enterocin KP; EL: enterocin KP+lactococcin BZ
N: below detection limit (1 CFU ml^{-1})

The initial counts of *L. monocytogenes* in the control samples increased from 2.53, 4.76, and 6.44 log CFU ml^{-1} to 7.73, 7.63, and 8.57 log CFU ml^{-1} by the end of the storage period. Also, lactococcin BZ and enterocin KP maintained their activities in all milk samples throughout storage period.

2.2. Effect of bacteriocins on the survival of L. monocytogenes *in milk samples stored at 20 °C*

The results of challenge tests at 20 °C were similar to those at 4 °C. Lactococcin BZ at concentrations of 400–2500 AU ml^{-1} showed very strong antilisterial activity and decreased the numbers of *L. monocytogenes* to an undetectable level in skim, half fat, and full fat milk samples containing 2.66, 4.77, and 6.45 log CFU ml^{-1} *Listeria*, and no cell growth was observed during storage period (25 days). In all treated groups, enterocin KP at 800–2500 AU ml^{-1} resulted in a drastic decline in the initial cell numbers immediately after addition and no cell growth of *L. monocytogenes* was observed over the entire storage period. Enterocin KP at 400 AU ml^{-1} level reduced cell counts of *L. monocytogenes* to uncountable level in all kinds of milk samples containing *L. monocytogenes* at the level of 2.66 log unit. The same concentration (400 AU ml^{-1}) of enterocin KP in skim, half fat, and full fat milk containing *L. monocytogenes* at the level of 4.77 log units reduced *Listeria* counts to an undetectable level on the2nd, 9th, and 15th day of storage, respectively. However, enterocin KP at the level of 400 AU ml^{-1} in skim, half fat, and full fat milk containing *L. monocytogenes* at the level of 6.45 log unit reduced the colony counts of *Listeria* by 3.91, 3.34, and 2.78 log units on 1st day of storage, respectively. In these samples, cell numbers of *Listeria* decreased during the full incubation period (25 days) (Table 1). The effect of decreased activity of enterocin KP in milk sample with high *Listeria* cell counts and high milk fat content could be prevented by increasing the amount of enterocin KP added.

Combination of enterocin KP with lactococcin BZ at the ratio of 1:1 (total bacteriocin activity 400–2500 AU ml^{-1}) led to a decrease in *Listeria* counts to undetectable level in all challenged skim, half fat, and full fat milk samples containing 2.66 and 4.77 log CFU ml^{-1} at day 0. In full fat and half fat milk samples containing *Listeria* of 6.45 log CFU ml^{-1}, the edition of mixture of enterocin KP and lactococcin BZ at the level of total bacteriocin activity 800–2500 AU ml^{-1} decreased *Listeria* cell numbers to the undetectable level. In the same samples, mixture of both bacteriocins used at 400 AU ml^{-1}, the numbers of *L. monocytogenes* declined to 1.32 and 1.65 log units on the1st day of storage, and to undetectable level on the after 2nd and 4th days, respectively (Table 1). It was observed that enterocin KP and lactococcin BZ maintained their biological activities in all milk samples.

Wan and co-workers (1997) reported that piscicolin at a concentration as low as 512 AU ml^{-1} effectively inhibited the growth of *L. monocytogenes* (10^2 CFU ml^{-1}) for the duration of the experiment (20 days) in whole milk. At higher challenge levels of *L. monocytogenes* (10^4 and 10^6 CFU ml^{-1}), piscicolin (2048 AU ml^{-1}) caused a reduction in the viable count of *L. monocytogenes* by more than 4 log CFU ml^{-1} after addition. However, they observed that *L. monocytogenes* started to re-grow within 24 h. Also, piscicolin 126 at higher challenge levels (4096 and 8192 AU ml^{-1}) did not bring about complete inhibition of *L. monocytogenes* further than day 1.

Storage temperature (4 °C or 20 °C) did not affect significantly antilisterial activity of lactococcin BZ, enterocin KP, or the combination of the two ($P>0.05$). At both storage temperatures, lactococcin BZ and enterocin KP showed strong and very quick antilisterial activity. However, some researchers found that the antibacterial activities of bacteriocins

were more pronounced at 5–8 °C than at room temperature, and also high storage temperature and long storage time cause a loss of bacteriocin activity (Delves-Broughton et al., 1996).

In control samples containing only *L. monocytogenes*, the cell number increased from 2.66, 4.77, and 6.45 log CFU ml^{-1} to 8.76, 8.72, and 8.75 log CFU ml^{-1}, respectively, by the end of the storage period.

3. Conclusions

Lactococcin BZ and enterocin KP by itself or their combination have strong antilisterial activity toward *L. monocytogenes* for all inoculum doses tested (2.53–2.66, 4.77–4.76, and 6.44–6.45 log CFU ml^{-1}) in all kinds of milk samples (skim, half fat, and full fat milk) at two storage temperatures (4 °C and 20 °C). Also, their effects are stable until the end of the tested storage periods. However, the low treatment dose of enterocin KP was found less effective than lactococcin BZ under the same conditions. It was also detected that by increasing treatment dose, the test microorganism could successfully be inhibited. Biological activity of lactococcin BZ was not adversely affected by milk fat content, whereas the activity of enterocin KP decreased with the increasing milk fat content. This could be overcome by increasing the concentration of enterocin KP or applying enterocin KP together with lactococcin BZ. The experimental application of lactococcin BZ, enterocin KP, and their combination separately into full fat, half fat, and skim milk to inhibit *L. monocytogenes* resulted in a bactericidal and strong antilisterial effect towards this foodborne pathogen. These results indicate that both bacteriocins may be successful alternatives as biopreservative agents in extending hygienic safety of foods, especially dairy foods.

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