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Research Paper

Evaluation of Antimicrobial Activity of Buforin I and Nisin and the Synergistic Effect of Their Combination as a Novel Antimicrobial Preservative

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ABSTRACT

One of the most effective methods for increasing the antimicrobial activity of a substance is to combine it with one or more other antimicrobial agents. The aim of the present study was to evaluate the antimicrobial effect of buforin I and nisin alone and investigate the synergistic action of these compounds against the most important food spoilage microorganisms, including *Bacillus subtilis, Staphylococcus epidermidis, Listeria innocua, Escherichia coli, Salmonella* serovar Enteritidis, *Aspergillus oryzae, Rhodotorula glutinis,* and *Geotrichum candidum*. The results of MIC and MBC or minimum fungicidal concentration examinations showed that buforin I had higher antimicrobial activity than nisin on all microbial strains used in this study ($P \leq 0.5$). *E. coli* was the most resistant to both antimicrobial agents, whereas *L. innocua* and *S. epidermidis* were the most sensitive to nisin and buforin I, respectively. The results of synergistic interaction between buforin I and nisin indicated that the combination of buforin I and nisin on *B. subtilis, S. epidermidis,* and *A. oryzae* showed a synergistic effect, whereas it had no effect on *Salmonella* serovar Enteritidis and *G. candidum*. The combination of buforin I and nisin showed a partial synergistic effect on *L. innocua, E. coli,* and *R. glutinis*. Assessment of viability of the microorganisms under the antimicrobial agents alone and in combination with each other at MICs and fraction inhibitory concentrations indicated that use of these antimicrobial agents in combination enhances antimicrobial activity at lower concentrations of both agents. The present study investigated the antimicrobial properties of buforin I against food spoilage microorganisms for the first time and suggests that its use alone or with nisin may provide a clear horizon for the application of antimicrobial peptides as natural preservatives. Thus, the combination of antimicrobial peptides and traditional antimicrobial food preservatives could be a promising option for the prevention of contamination, spoilage, and infestation of food and beverage products.

HIGHLIGHTS

- Buforin I had higher antimicrobial activity than nisin.
- The combination of nisin and buforin eliminates the limitation of the antimicrobial effect of nisin.
- Buforin I may provide a horizon for application of antimicrobial peptides as natural preservatives.

Key words: Antimicrobial effects; Buforin I; Nisin; Synergistic effects

Despite significant advances in food production and preservation methods, food safety remains a global challenge *(15)*. Although global health levels have improved, the rates of foodborne diseases and poisoning continue to be high, and unsafe food causes 600 million cases of foodborne diseases and 420,000 deaths annually. In addition, 30% of foodborne deaths occur among children younger than 5 years of age *(75)*. In conflict with these rates, the World Health Organization has many recommendations for reducing salt consumption to reduce the incidence of cardiovascular diseases *(74)*. Because salt has antimicrobial properties, its removal from or reduction in food products in which it has a protective role leads to decreased shelf life; hence, other antimicrobial agents may be needed to maintain the safety of foods *(27)*. In addition, increased concern over synthetic preservatives, the prevalence of foodborne pathogens with resistance to classical antibiotics, restrictions or prohibition of the use of some chemical preservatives in some countries, and increased consumer tendency for fresh or minimally processed foods have created many technological challenges in the food industry *(58)*. Therefore, it is necessary to study and investigate new natural antimicrobial compounds with a

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broad spectrum of antimicrobial activity *(62)* or to find ways to increase the effectiveness of the compounds or methods used *(39)*.

Antimicrobial peptides are produced as an important part of the immune system in all aspects of life, and antimicrobial activity of cationic antimicrobial peptides has been extensively studied *(1, 4, 9, 12, 31)*. The major benefit of using antimicrobial peptides as new natural food preservatives is that they preserve the food without changing its quality and they are not harmful *(68)*. They are short amino acid sequences (less than 50) with a positive charge (in general +2 to +9 because of basic amino acids, such as lysine and arginine) *(21, 51)* and contain more than 30% of hydrophobic amino acids. Nisin is the only antimicrobial peptide that is widely used in the preservation of food commercially *(61)*. Nisin, because it is nontoxic, flavorless, heat stable, and tolerant of low pH, is the most commonly used bacteriocin *(41, 63)*. Nisin is a polycyclic antibacterial peptide comprising 34-amino-acid residues, with an overall positive charge and amphipathic properties *(41)*. It exerts its antimicrobial activity through impaired membrane function and a permeability period. Buforin I is a 39-amino-acid cationic antimicrobial peptide that was first isolated from the stomach tissue of the Asian toad *Bufo bufo gargarizans* and shows strong antimicrobial activities against a range of microorganisms, including gram-positive bacteria (*Bacillus subtilis, Staphylococcus aureus, Streptococcus mutans,* and *Streptococcus pneumoniae*), gram-negative bacteria (*Escherichia coli, Salmonella* Typhimurium, *Pseudomonas putida,* and *Serratia* species), and fungi (*Candida albicans, Cryptococcus neoformans,* and *Saccharomyces cerevisiae*) *(54)*.

Buforin I belongs to the great buforins family, whose members share the N-terminal region of histone H2A as a common trait. This region specifies the protein's DNA binding activity, and this family consists of histone-derived peptides *(16)*. Histone-derived peptides do not have a role in replication; however, these extracellular histone derivatives have a substantial antimicrobial property *(54)*. Although the mode of action of the buforin family is still unknown, reports indicate that they kill a microorganism by translocating into the cell and binding to nucleic acids and that they cross lipid bilayers via the transient formation of a peptide-lipid supramolecular complex pore *(16)*. Thus, the purpose of this research was to assess the impact of nisin and buforin I and their combination on food spoilage microorganisms, including gram-negative and gram-positive bacterial and fungal isolates.

B. cereus is a psychrotroph and sporogenic microorganism that proved able to grow in products at cold-storage temperatures and even cause alimentary diseases *(29)*. *Listeria monocytogenes* is the only species of the genus *Listeria* that has been involved in known foodborne outbreaks of listeriosis *(30)*. Because of the difficulty and risks of detection of *L. monocytogenes, Listeria innocua,* which appears to be present with *L. monocytogenes,* is often used as an indication for the presence of this bacteria *(19)*. *B. subtilis, S. epidermidis,* and *L. innocua* were studied as the most important representatives of gram-positive bacteria in this study. Gram-negative bacteria not only are associated with foodborne illness and poisoning but also are important factors in food spoilage *(45, 58)*. In this study, *E. coli* and *Salmonella* serovar Enteritidis were used as representatives of gram-negative bacteria. The combination of buforin I and nisin was evaluated against the growth of spoilage fungal strains, including *Aspergillus oryzae, Rhodotorula glutinis,* and *Geotrichum candidum,* which are known to be responsible for the production of allergenic and toxic compounds *(33)*.

MATERIALS AND METHODS

Microorganisms, media, and antimicrobial agents. *B. subtilis* (PTCC 1023), *Staphylococcus epidermidis* (PTCC 1114), *L. innocua* (ATCC 33090), *E. coli* (ATCC 25922), *Salmonella* serovar Enteritidis (PTCC 1735), *A. oryzae* (PTCC 5164), *R. glutinis* (PTCC 5257), and *G. candidum* (ATCC 34614) were procured from the microbial collection of the Department of Food Science and Technology, Faculty of Agriculture, Ferdowsi University of Mashhad. Bacterial strains were cultured 24 h in Mueller-Hinton broth (Sigma-Aldrich, Hamburg, Germany) at 37°C and fungal strains were cultured 48 h in potato dextrose broth (Sigma-Aldrich) at 25°C, before the antimicrobial tests were performed. A 0.5 McFarland standard was used to prepare the microbial suspension, which was equivalent to 10^6 to 10^8 CFU/mL of microorganism *(3)*.

The amino acid sequence of buforin I was determined by the NCBI database (https://www.ncbi.nlm.nih.gov/) with the accession no. P55897 and then synthesized by Mimotopes (Mulgrave, Australia). The purity of buforin I was 96%. Next, 1 mg of buforin I was dissolved in a 1-mL water and dimethyl sulfoxide solution (80:20, v/v) and filter sterilized (0.22 μm) to prepare a 1-mg mL^{-1} stock solution *(64)*. Then, 1 g of nisin (2.5% from *Lactococcus lactis,* CAS 1414-45-5; Sigma-Aldrich) was dissolved in 25 mL of 0.05% acetic acid solution and centrifuged at 4,000 × *g* for 20 min. The supernatant was then filter sterilized to prepare a 1-mg/mL stock solution *(63)*.

MIC. MICs were obtained using the micro broth dilution method. Serial dilutions (1, 2, 4, 6, 8, 10, 12, 14, and 16 μg/mL) of buforin I and serial dilutions (1, 2, 4, 8, 16, 32, 64, 128, 256, and 512 μg/mL) of nisin in Mueller-Hinton broth for bacterial strains and in potato dextrose broth for fungal strains were prepared. Then, 10 μL of microbial suspensions with an optical density at 630 nm (OD_{630}), equal to 0.08 to 0.13, were added to 90 μL of each dilution in the 96-microwell plates. The microwell plates were incubated at 37°C for 24 h for bacterial strains and 25°C for 48 h for fungal strains *(8)*. To determine the MICs, the absorbance was measured at 630 nm by ELISA reader (ELx808; BioTek, Tokyo, Japan). Because increase in turbidity is a sign of growth of microorganisms, MICs were determined as the lowest concentrations that prevented visible growth. Growth medium without inoculum was used for negative control *(37)*.

MBC or MFC. From each well, 100 μL in which growth was not seen was pour-plate cultured in Mueller-Hinton agar for bacterial strains and surface-plate cultured on potato dextrose agar for fungal strains (Sigma-Aldrich). The plates were incubated at 37°C for 24 h for bacterial strains or 25°C for 48 h for fungal strains, and the lowest dilution that yielded complete inhibition of growth was taken as the MBC or minimum fungicidal concentration (MFC) *(70)*. Thus, MBC or MFC was the lowest concentration of antimicrobial agents that prevented visible growth on the subculture plate *(34)*.

Synergistic interaction between buforin I and nisin by checkerboard assay. The synergistic interaction between nisin and buforin I was assessed using the checkerboard method *(28, 44, 53)*. Thus, seven twofold serial dilutions (from 2MIC to MIC/32) of nisin and buforin I, in accordance with obtained MIC in the previous section for each microorganism, were prepared. An equal amount (25 μL) of each dilution was poured into 96-well microplates to obtain a fixed amount of both antimicrobial peptides so that each row (and column) contained a fixed amount of the first agent and increasing amounts of the second one. A total of 50 μL of fresh bacteria and fungi suspension (10^8 CFU/mL) was added to each well and cultured at 37°C for 24 h for bacterial strains and 25°C for 48 h for fungal strains.

The fraction inhibitory concentration index (FIC_I) was calculated using the following formula:

$$FIC_I = \frac{MIC_{A/B}}{MIC_A} + \frac{MIC_{B/A}}{MIC_B}$$

where MIC_A is the MIC of compound A, MIC_B is the MIC of compound B, and $MIC_{A/B}$ is the MIC of compound A in combination with compound B. Total synergism ($FIC_I \leq 0.5$), partial synergism ($0.5 < FIC_I \leq 0.75$), indifference ($0.75 < FIC_I \leq 2$), or antagonism ($FIC_I > 2$) between the two compounds was reduced using FIC_I *(46)*.

Survival curve. The effect of nisin and buforin I was evaluated separately and in combination on the growth of microbial strains through the construction of a survival curve *(48)*. The final concentration of suspension of the strain (adjusted to 10^6 to 10^8 CFU/mL) was added to the wells of 96-well microplates, and 50 μL of the antimicrobial agent (at MICs or at fraction inhibitory concentrations [FICs]) was added to each well. The bacterial strains were cultured at 37°C for 30 h, and fungal strains were cultured at 25°C for 50 h. After incubating for 0, 6, 12, 18, 24, and 30 h for bacterial strains and 0, 10, 20, 30, 40, and 50 h for fungal strains, a 50-μL liquid from each dilution was spread on the surface of the agar plates and incubated at 37°C for 24 h or 25°C for 48 h for bacterial and fungal strains, respectively. Then, the number of CFU per milliliter was counted. The control group was 50 μL of the microbial suspensions without antimicrobial agents. Thereafter, survival curves were constructed by plotting the log number of CFU per milliliter against time (h).

Statistical analysis. To confirm the results, the experiments were repeated three times. Results of the study were analyzed by Minitab version 18.0, and differences among the means were determined by one-way analysis of variance for significance at $P < 0.05$.

RESULTS

MICs and MBC/MFCs. MICs and MBCs of antimicrobial agents were evaluated and reported in Table 1. Buforin I and nisin showed different antimicrobial effect against the tested strains. The range of obtained MICs for nisin and buforin I was 32 to 512 and 4 to 16 μg/mL, respectively.

In general, gram-negative bacteria were more resistant to both nisin and buforin I than were gram-positive bacteria. In addition, the antimicrobial effect of buforin I on the microbial strains was higher than that of nisin. The results of MIC and MBC/MFC tests show that *E. coli* was the most resistant strain to both antimicrobial agents, whereas *L. innocua* and *S. epidermidis* were the most sensitive strains to nisin and buforin I, respectively. The results also indicated that nisin showed a bactericidal or fungicidal effect on *L. innocua* and *G. candidum* at 256 μg/mL and on *B. subtilis, S. epidermidis, Salmonella* serovar Enteritidis, and *R. glutinis* at 512 μg/mL. Buforin I showed a fungicidal effect on all fungal strains used in this study and a bactericidal effect on *L. innocua* and *S. epidermidis* at 16 and 10 μg/mL, respectively.

TABLE 1. *MICs and MBCs/MFCs of nisin and buforin I against some food spoilage bacterial and fungal strains*[a]

Microorganism	Nisin (μg/mL)		Buforin I (μg/mL)	
	MIC	MBC/MFC	MIC	MBC/MFC
Listeria innocua	64	256	10	16
Bacillus subtilis	256	512	14	>16
Staphylococcus epidermidis	256	512	4	10
Escherichia coli	512	>512	16	>16
Salmonella serovar Enteritidis	128	512	8	>16
Aspergillus oryzae	256	>512	8	16
Rhodotorula glutinis	512	512	8	16
Geotrichum candidum	128	256	10	16

[a] MFC, minimum fungicidal concentration.

Synergistic interaction between buforin I and nisin by checkerboard assay. The results of synergistic interaction between buforin I and nisin are shown in Table 2. Results indicated that the combination of buforin I and nisin on *B. subtilis, S. epidermidis,* and *A. oryzae* showed synergistic effect, whereas it had no effect on *Salmonella* serovar Enteritidis and *G. candidum*. The combination of buforin I and nisin showed a partial synergistic effect on other microorganisms, including *L. innocua, E. coli,* and *R. glutinis*. As shown in Table 2, smaller amounts of both antimicrobial agents are used to inhibit the growth of microorganisms when viewing the synergistic or the partial synergistic effect.

Survival curve. The survival curve shows the effect of nisin, buforin I, and their combination against the growth of the microbial strains used in this study (Fig. 1). The survival and activity curves showed overlap and confirm the results of each other. For all microbial strains except *Salmonella* serovar Enteritidis and *G. candidum,* the curves represent FIC in a synergism or partial synergism state and placed lower than the curves of buforin I and nisin alone, indicating a higher bacteriostatic or fungistatic effect of the combined use of both agents. For all microbial strains of buforin I, antimicrobial activity was higher than that of nisin.

DISCUSSION

The antibacterial activity of various cationic peptides on foodborne and food spoilage microorganisms like *B. subtilis, Bacillus cereus, S. aureus, L. monocytogenes,*

TABLE 2. *FIC_I and FIC of nisin in combination with buforin I against some food spoilage bacterial and fungal strains*[a]

Microorganism	FIC_I	Nisin + buforin I FIC (μg/mL)	Synergistic interaction
Listeria innocua	0.75	64+3.5	Partial synergism
Bacillus subtilis	0.5	128+4	Synergism
Staphylococcus epidermidis	0.5	128+1	Synergism
Escherichia coli	0.75	256+3.5	Partial synergism
Salmonella serovar Enteritidis	1.25	32+8	No effect
Aspergillus oryzae	0.375	32+2	Synergism
Rhodotorula glutinis	0.625	256+1	Partial synergism
Geotrichum candidum	2	128+10	No effect

[a] FIC, fraction inhibitory concentration; FIC_I, fraction inhibitory concentration index.

Salmonella Typhimurium, *Shigella sonnei, E. coli* O157:H7, and *P. aeruginosa* have been investigated in many studies *(13, 20, 22, 61)*. Nisin is one of the most attractive cationic peptides in the field of food microbiology *(2),* and its antimicrobial effect on many gram-positive foodborne and food spoilage microorganisms, including *S. aureus (11), L. innocua (65),* and *L. monocytogenes* and *B. subtilis (44)* with MICs equal to 7.8, 1, 250, and 125 μg/mL, respectively, has been investigated. Nisin's inhibitory effect on the outgrowth of spores of *Bacillus* species and *Clostridium* species has been recorded *(26)*. Ashari et al. *(5)* reported MICs of nisin for *E. coli* FNCC 0091, *Pseudomonas fluorescens* FNCC 0070, and *Aspergillus niger* FNCC 6080, were 500, 500, and 250 IU, respectively. Yosef et al. *(77)* reported that nisin can inhibit growth of *Aspergillus parasiticus* and accumulation of aflatoxin B_1 and G_1 over 3 days; with continued incubating for 10 days, the growth inhibitory effect of nisin was decreased, whereas its inhibitory effect on toxin production was still observed. Similar results were reported by Gourama and Bullerman *(36)* when they investigated inhibition of growth and aflatoxin production of *Aspergillus flavus* subsp. *parasiticus* by *Lactobacillus* species. Not only did growing *Lactobacillus* cells inhibit germination of mold spores, but even culture supernatant broth from the mixture of strains inhibited mold growth. These authors reported that *Lactobacillus* species prevented mold growth because of low pH and a microbial competition effect; however, the inhibition of aflatoxins in this study probably resulted from a low-molecular-weight bacterial metabolite or metabolites *(36)*. Numerous studies have shown that these low-molecular-weight bacterial metabolites are bacteriocin, with a molecular weight of about 3.4 *(7),* and nisin, which is the main metabolite produced by *Lactobacillus* spp. *(24, 32)*. Le Lay et al. *(43)* recorded that nisin reduced *C. albicans* proliferation and that nisin inhibited *C. albicans* growth beginning at 500 μg/mL.

Many studies have confirmed and reported that nisin binds to lipid II (the peptidoglycan precursor), just like other lantibiotics, and leads to pore formation and inhibition of cell wall biosynthesis *(25, 63, 71–73)*. Gram-negative bacteria, because of their lipopolysaccharides in the outer membrane, show less sensitivity or resistance to nisin *(63)*. Reports of the impact of the nisin effect on gram-negative bacteria because of their outer membrane and fungal strains because of their rigid cell wall (a complex structure consisting of glucan cross-linked with chitin and cell wall protein) *(26)* show variable results that range from ineffective *(44)* to meaningful *(50)* effects.

The MICs of isolated buforin I from the stomach tissue of *B. bufo gargarizans,* an Asian toad, on the growth of *B. subtilis, E. coli, S. aureus, Salmonella* Typhimurium, *C. albicans,* and *S. cerevisiae* were recorded at 4, 8, 4, 4, 4, and 4 μg/mL, respectively *(54, 55)*. These results demonstrate that buforin I and nisin exhibited different effects against different microorganisms, reflecting potential differences in the inoculum level, experimental temperature, physiological condition of the microorganism *(44),* and culture media and methods *(53)* used for evaluating the antimicrobial effect in different studies. In addition, the origin of the antimicrobial agents that can be extracted from nature or made synthetically must be considered *(6)*. Although consumption of nisin at concentrations of less than 83.25 mg/kg has not shown obvious effects on human health, 1 g of food in the United States and other countries may contain 250 mg/kg nisin, and it can be found at concentrations up to 300 mg/kg in mouthwashes; many studies recorded that 300 to 400 mg/kg nisin has a contraceptive effect in humans *(41, 47, 76)*. Although the U.S. Food and Drug Administration recommended a maximum of 250 μg/mL nisin in finished product in 2017, the European Food Safety Authority reevaluated the toxic potential of nisin and approved an acceptable daily intake of up to 1 mg of nisin per kg of body weight per day for use in certain food products. Therefore, the combination of nisin with another cationic antimicrobial peptide, because of their broad-spectrum activity (gram-positive and gram-negative bacteria, fungi, and viruses) *(9, 14),* low level of induced resistance *(66),* and improvement in nutrient digestibility and modulation of gut microbiota *(61),* can increase its effect on food spoilage or pathogenic microorganisms. Churklam et al. *(17)* recorded the synergistic interaction with FIC_I values ranging from 0.375 to 0.5 for a carvacrol and nisin combination against *L. monocytogenes* 10403S and three food isolates. In addition, they examined the survival of *L. monocytogenes* 10403S under the synergistic effect of carvacrol and nisin during storage of sliced bologna sausages at 4°C and reported that the presence of carvacrol combined with nisin resulted in significant growth rate reductions compared with those of controls *(17)*. Ashari et al. *(5)* recorded that the combination of nisin and essential oil had a synergistic effect against *B. cereus, A. niger,* and *Salmonella* Typhimurium. Liu et al. *(44)* recorded that the combination of ϵ-polylysine and nisin against *E. coli, B. subtilis,* and *S. aureus* had a synergistic effect and against *Micrococcus luteus* and *Hansenula anomala* had a partial synergic effect. Their results showed that the combination of ϵ-polylysine and nisin did not have an effect on *A. niger (44)*. In addition to the mentioned examples, many studies have been conducted to investigate the synergistic effect of different compounds to enhance the antimicrobial effect of nisin *(23, 42, 48, 60)*. What is common among all reports is that the low effect of nisin on

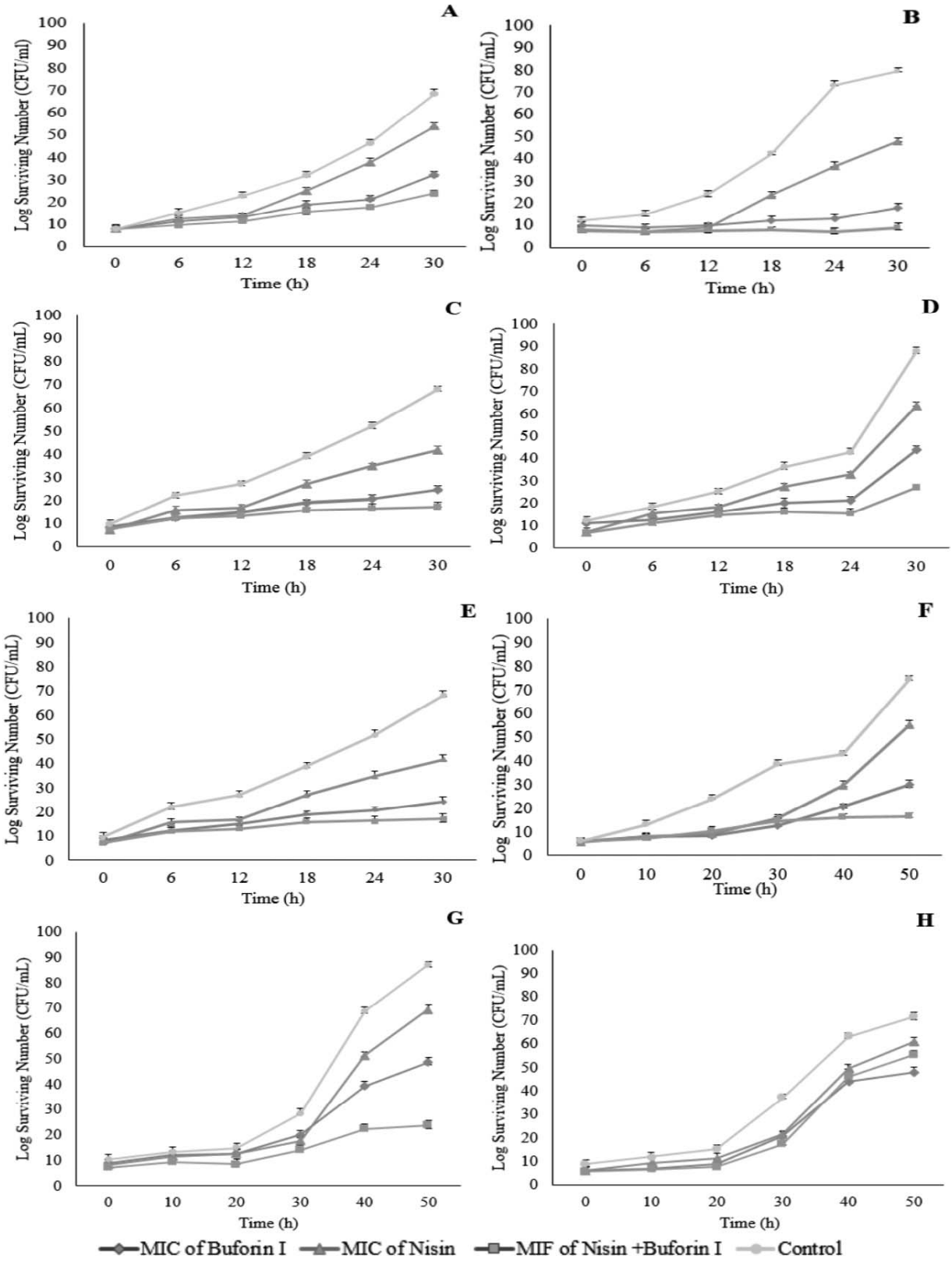

FIGURE 1. *Survival curves of the antimicrobials agents. The microbial suspensions were treated with the antimicrobial agents at MICs of nisin and buforin I separately and FIC$_s$ in combination with nisin and buforin I. The microbial suspensions without antimicrobial agents was used as a control group. (A)* Listeria innocua, *(B)* Bacillus subtilis, *(C)* Staphylococcus epidermidis, *(D)* Escherichia coli, *(E)* Salmonella *serovar Enteritidis, (F)* Aspergillus oryzae, *(G)* Rhodotorula glutinis, *and (H)* Geotrichum candidum*. Vertical error bars represent standard deviation of tree replications.*

some microorganisms, especially gram-negative bacteria or fungal strains, results from nisin restriction on the passage of the outer membrane or rigid membrane, respectively *(50, 52)*. Reports indicate that if a factor can facilitate the passage of nisin through these layers, its inhibitory effect on such microorganisms will also be visible *(10, 59, 69)*.

Although buforin I was first identified in the stomach of Asian toads by Park et al. *(54),* Kim et al. *(40)* showed that histone H2A was the precursor of buforin I, and the H2A nonacetylated histone was converted to buforin I after the secretion from stomach cells and exposure to pepsin. They also investigated the presence of buforin I in humans, cows, and pigs secretions *(40)*. Other studies, like Minn et al. *(49),* recorded that peptides derived from pepsinogen action in the stomach on some proteins, such as buforin I, have a strong antimicrobial activity and are found in most vertebrates, including humans *(49, 67)*. A combination with a similar amino acid sequence was found on sheep's lungs *(49)*. The broad-spectrum activity of buforin I, its high activity, its MIC at low concentrations, and the absence of cell cytotoxicity and a hemolytic effect demonstrate the potential of this compound for use as a food preservative. The results also indicated that the use of buforin I and nisin in combination enhances the antimicrobial activity (the bacteriostatic or fungistatic effect) at lower concentrations of both agents. Although there are many studies on the safe use of cationic peptides at MICs *(18, 35, 37, 56),* they need to be legally approved by a regulatory body.

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