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Research Article

Antimicrobial Activity of TP4 Recombinant Peptide Alone and in Combination With ZnO NPs on Foodborne Bacteria: Potential as a Milk Preservative

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Bacterial resistance, including resistant foodborne pathogenic bacteria, is developing daily, thus becoming a growing public health concern worldwide. Antimicrobial peptides (AMPs) such as tilapia piscidin 4 (TP4) are a class of small peptides widely present in nature that are promising antimicrobial agents that could potentially be considered food preservatives. This study is aimed at investigating the effect of TP4 alone and in combination with zinc oxide nanoparticles (ZnO NPs) on foodborne bacteria in milk. First, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of TP4 and its combination with ZnO NPs (synergistic effect) were measured against four standard foodborne bacteria using the microdilution and checkerboard methods, respectively. According to the results, TP4 showed a high antibacterial effect against four tested strains. Also, TP4 combination with ZnO NPs showed a relative synergistic effect against three tested strains. Then, a time-kill assay was performed to evaluate the antibacterial effect of TP4 over time on selected bacteria. The results showed that within 24 h, a TP4 concentration of $\geq 1 \times$ MIC prevented the growth of Gram-negative and Gram-positive bacteria. The microbial load of pasteurized and raw milk was also associated with population reduction against the $2 \times$ MIC concentration of TP4. To evaluate the cytotoxicity of TP4, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) analysis was performed on the human embryonic kidney-293 (HEK-293) cell line and the half-maximal inhibitory concentration (IC50) was evaluated as 71.61 μg/mL, indicating the nontoxicity of this peptide. Finally, the results showed that TP4 peptide reduces the microbial load of milk, and due to its safety, it can be used as a food preservative.

Keywords: antimicrobial peptide; dairy products; foodborne bacteria; TP4; zinc oxide nanoparticles

1. Introduction

Consuming food contaminated with bacteria or microbial toxins can lead to foodborne illness or food poisoning [1]. Symptoms such as stomach pain, diarrhea, vomiting, and fever may be observed in patients with food poisoning [2, 3]. Food spoilage caused by microorganisms can be affected by pH, temperature, humidity, and oxygen [4]. Dairy products such as milk support the growth of bacteria, including pathogenic bacteria [5]. *Yersinia enterocolitica* is a Gram-negative bacterium that can grow at refrigerator temperature and be transmitted to humans. Raw and pasteurized milk are considered suitable food sources for its growth. In addition, the antibiotic resistance of *Y. enterocolitica* should be given more serious attention, because this bacterium shows resistance to some antibiotics such as penicillin and oxacillin in dairy products, according to some reports [6]. *Escherichia coli O157:H7* is a Gram-negative bacterium that can be transmitted through water, vegetables, raw milk, dairy products, poultry, domestic cattle, and their meat. It can grow at refrigerated temperatures [7]. *E. coli O157:H7* has become resistant to antibiotics such as amoxicillin and teicoplanin [8], and due to its enterotoxin, which is stable at pasteurization temperatures, resistance to enzymatic degradation, and

wide pH range, it is dangerous [9]. At a temperature of 20°C–40°C, dairy products such as milk are prone to the growth of *Staphylococcus* bacteria [10]. *Bacillus cereus*, which produces cereulide toxin, is one of the most important spoilage microorganisms in dairy environments [11]. Consuming foods containing cereulide toxin causes vomiting within 6 h [12]. Antimicrobial resistance (AMR) is an increasing threat to global health and should be considered the most controversial health issue [13]. Antimicrobial preservatives address food spoilage caused by microorganisms that are either naturally present in food or can be added to food. In general, mineral acids and their salts, such as nitrate, and organic acids, such as benzoate, are considered artificial preservatives, and there is some debate over their use; safe alternatives must be used instead [14]. Sorbic acid and hydrogen peroxide (H_2O_2) are common GRAS (generally recognized as safe)-approved preservatives used in milk. However, they have disadvantages, including the occurrence of resistance in some molds and yeasts and side effects such as nausea, shock, convulsions, and metabolic acidosis [15]. Ideally, food prepared with natural additives rather than chemical ones is preferred for health reasons for consumers [16]. Following the overuse of antimicrobial agents over the years, AMR has developed, and antibiotic drug residues are detected in meat, milk, and egg products [17]. Owing to many advantages such as their variety and efficacy [18], fast killing ability [19], having different mechanisms of action of antimicrobial peptide (AMPs) against the bacteria, and limiting the development of resistance, AMPs are considered promising alternatives to antibiotics [20]. AMPs, such as nisin, poly-lysine, pediocin PA-1, enterocin, AS-48, and CCM4231 enterocin, are some of the newest antibiotic alternatives and food preservatives [21, 22]. Although AMPs are not affected by acids, bases, or even high temperatures, they are irreversibly degraded by proteases [22]. Nisin is an AMP with a low molecular weight of less than 5 kDa that is effective against Gram-positive bacteria [23, 24] and is GRAS-approved for use in food preservation system [21]. However, it has disadvantages such as being unstable at neutral pH, emerging resistance, and not having a significant effect on dairy products [25]. Tilapia piscidin 4 (TP4) is a broad-spectrum AMP that also plays a role in cancer therapy and wound healing. The TP4 peptide also regulates and modulates the immune system and antibiofilm activity [26]. It is a 25-amino acid alpha-helix peptide isolated from the mast cells of *Nile tilapia* (*Oreochromis niloticus*). TP4 has been cloned in *Pichia pastoris GS115*, and its recombinant form was produced at appropriate concentrations, which was economically cost-beneficial [27]. Recombinant AMPs can also be used as food preservatives [28].

Research on zinc nanoparticles (NPs) synthesized by the green method has shown that they are effective against foodborne bacteria [29]. In this regard, some studies have shown that zinc oxide NPs (ZnO NPs), along with chitosan [30, 31] and silver NPs, are effective against *Staphylococcus aureus* and *E. coli* [32]. Dairy products are among the widely consumed foods with high nutritional value in the human daily diet [33]. Spoilage and pathogenic bacteria can grow in dairy products and cause food spoilage and foodborne illness, respectively. It is therefore very important to find cheap and effective methods to control food spoilage and foodborne diseases. Meanwhile, AMPs are relatively new and effective agents to combat food bacteria [34]. Due to its cost-effective production in *Pichia pastoris* yeast [27], recombinant TP4 peptide has a special potential for use as a food and dairy preservative and its effectiveness on food spoilage and foodborne bacteria.

The present study is aimed at investigating the effect of TP4 peptide on the microbial load of milk and against standard pathogenic foodborne bacteria, to assess its cytotoxicity on human normal cell line and to evaluate the synergistic effect of TP4 and ZnO NPs against the mentioned bacteria. It should be noted that our research is the first study on the potential effect of this peptide in inhibiting the growth of pathogenic bacteria transmitted through food and preventing food spoilage.

2. Materials and Methods

2.1. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). The MIC and the MBC of TP4 recombinant peptide (accession number: MK515149, Mashhad Medical School) and biosynthesized ZnO NPs (Microbial Biotechnology Laboratory at Ferdowsi University of Mashhad) against *B. cereus* (PTCC 1015) and *S. aureus* (PTCC 1784) (Persian Type Culture Collection) as Gram-positive bacteria and *E. coli O157:H7* (PTCC 1860) and *Y. enterocolitica* (PTCC 1785) (Persian Type Culture Collection) as Gram-negative bacteria were evaluated using the microdilution method according to Clinical Laboratory Standards Institute (CLSI) protocols [35]. First, serial dilutions of TP4 recombinant peptide were prepared in PBS (64–1/8 μg/mL), and 100 μL of each was added to each microplate well containing 100 μL 2X Mueller Hinton Broth (MHB) (QUELab, Canada). Data from previous research were used for ZnO NPs (1000–1.953 μg/mL) [36]. The wells of microplates were also inoculated with 20 μL of 5×10^6 CFU/mL bacteria. Culture media with and without bacteria were used as positive and negative controls, respectively. Then, the microplate was placed in a 37°C incubator for 24 h. Then, 50 μL triphenyltetrazolium chloride (TTC) reagent (Merck, Germany) at a concentration of 5 mg/mL was added to each well and incubated for 1 h at 37°C. The microplate wells were checked for color changes. MICs were defined as the lowest concentration of the tested compound at which the reduction of TTC to red formazan was not observed after incubation.

To perform the MBC test, 10 μL of each well with no growth in the MIC test was cultured on Mueller Hinton Agar (MHA) medium and incubated at 37°C for 24 h. The lowest concentration of the peptide that prevented visible bacterial growth was considered MBC.

2.2. Evaluation of the Synergistic Effect. The synergistic effect of the TP4 peptide and ZnO NPs was evaluated using the checkerboard method. First, 50 μL of NB (2X) was added to each microplate well. Next, the TP4 peptide and NP were prepared in separate microtubes at concentrations of 2 to

1/16 MIC. Then, 50 μL of peptide and 100 μL of ZnO NPs were added to each well, which was inoculated with 20 μL of 1/20 concentration 0.5 McFarland bacterium. The bacteria were incubated in a 37°C shaker incubator for 24 h.

The results were analyzed using fractional inhibitory concentration (FIC) and sum FIC index analysis following equations, in which *B* represents TP4 recombinant peptide, and *C* represents ZnO NPs.

$$\text{FIC} = \frac{\text{MIC } B \text{ or } C \text{ in combination}}{\text{MIC } B \text{ or } C \text{ alone}} \quad (1)$$

$$\text{Sum FIC}_{BC} = \text{FIC}_B + \text{FIC}_C \quad (2)$$

If the values obtained from Equation (2) are smaller than or equal to 0.5, it indicates synergism, and if it is larger than 0.5 and smaller than one, it indicates a partially synergistic effect. A value equal to one indicates an additive effect, a value between one and four indicates neutrality, and a value greater than or equal to four indicates the antagonistic effect of two antibacterial agents [37].

2.3. Bacterial Time-Kill Assay. To do this, 0, 1/4, 1/2, 1, and 2 × MIC concentrations were prepared from recombinant TP4 peptide in PBS. Then, each MIC concentration was added to a microtube containing an equal volume of 0.5 McFarland bacteria. Next, of these mixtures, at 0, 1/2, 1, 2, 4, 6, 8, and 24 hours, serial dilutions of 10^{-1} to 10^{-6} were prepared in PBS. Finally, 10 μL of each dilution was cultured on MHA (QUELab, Canada) and incubated at 37°C for 18 h according to the method of Yi et al. with some modifications using the following equation [38].

$$\text{CFU} = \log \frac{\text{colony count}}{\text{dilution factor} * \text{volume}} \text{ (CFU/mL)} \quad (3)$$

2.4. The Effect of TP4 Recombinant Peptide on the Microbial Load of Raw and Pasteurized Milk. Raw milk and pasteurized milk (local retailer, Mashhad) was exposed to TP4 peptide to evaluate the reduction rate of existing microorganisms. First, the initial microbial load was obtained by plating 500 μL of original samples, 500 μL of 1/10 dilution, and 500 μL of 1/100 dilution on Plate Count Agar (PCA) (QUELab, Canada) and incubating at 37°C for 18 h. As the resulting colonies were uncountable, concentrations of 0, 1/2, 1, and 2-fold of the maximum MIC value, which was 4 μg/mL, were prepared in PBS. Then, each prepared concentration was separately added to the microtube containing an equal volume of raw or pasteurized milk. Next, at 0, 1/2, 1, 2, 4, 6, and 8 h, serial dilutions of 10^{-1} to 10^{-6} were prepared in PBS, 10 μL of each dilution was inoculated in PCA, and the plates were incubated at 37°C for 18–24 h. Finally, the appropriate plate containing 30–300 bacterial colonies was selected and counted, and the value was used to obtain log CFU/mL using Equation (3).

2.5. Cytotoxicity of TP4 Recombinant Peptide. The cytotoxicity of the TP4 peptide on the human embryonic kidney-293 (HEK-293) normal cell line (Cell Bank of the Biotechnology Research Institute at the Ferdowsi University of Mashhad) was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) test [39]. Two million cells (HEK-293) were added to 4 mL of Dulbecco's modified Eagle's Medium (DMEM) supplemented with FBS 10% and antibiotic penicillin-streptomycin 1% and incubated under 5% CO_2 and 98% humidity at 37°C for 24 h. A total of 5000 cells were seeded in each well containing DMEM and incubated at 37°C under 5% CO_2 for 24 h. Next, 100 μL of different concentrations (30–0.1 μg/mL) of TP4 peptide was added to the fresh culture medium in each well and incubated at 37°C under 5% CO_2 for 48 h. A positive control column containing the cells in DMEM without treatment was considered. Subsequently, 20 μL of MTT at a concentration of 5 mg/mL was added to each well and incubated for another 3 h. In doing so, MTT is metabolized by living cells, and purple formazan crystals are formed. Then, the medium solution containing MTT was removed, and 100 μL of dimethyl sulfoxide (DMSO) was added to the wells to dissolve the crystals. Using a microplate reader (Stat Fax 2100, USA), the absorption of the color corresponding to each well was measured at 570 nm, and the cell viability was calculated through the following equation [40].

$$\text{cell viability (\%)} = \frac{\text{sample absorbance}}{\text{control absorbance}} \times 100 \quad (4)$$

2.6. Statistical Analysis. The data were analyzed using SPSS 26.0 software, and one-way analysis of variance (ANOVA) was utilized to analyze the statistical significance.

All experiments were performed in triplicate.

3. Results and Discussion

3.1. MIC and MBC. According to the results, the TP4 peptide showed a high antibacterial effect with the lowest MIC value, which was recorded for *E. coli O157:H7* and some activities against other bacteria at low concentrations (Table 1), which is in line with research conducted by Neshani et al. [27]. As shown in Table 1, TP4 peptide has a high antibacterial effect on Gram-positive as well as Gram-negative bacteria that makes it a valuable potential antibacterial agent. The ratio between MBC and MIC values for *E. coli O157:H7* was eight (> 4), indicating its bacteriostatic activity. However, this value for the rest of the bacterial strains was lower than four, showing its bactericidal effect [41, 42]. It should be noted that no research has been conducted on the effect of TP4 on foodborne bacteria alone or in combination with ZnO NPs. The current study was the first study assessing the effect of TP4 peptide on food bacteria. In the study performed by Amiri et al., the antimicrobial effects of LA-5 and BB-12 peptides with bacterial origins on some foodborne bacteria were investigated. According to their results, Gram-negative bacteria were more resistant when exposed to these peptides. It should be noted that the lowest MIC value obtained was 8 μg/mL [43], which was higher than the highest MIC value of the TP4 peptide in our study, which was 4 μg/mL showing the high value of the antibacterial effect of TP4 peptide. Additionally, in a study performed by Ma et al., 32 μg/mL recombinant OVTp12 peptide derived from egg ovotransferrin inhibited

Table 1: MIC and MBC results of TP4 peptide against foodborne bacteria.

	MIC (μg/mL)	MBC (μg/mL)
B. cereus	4	4
E. coli O157:H7	2	16
S. aureus	4	8
Y. enterocolitica	4	4

the growth of standard bacterial strains such as *E. coli* and *S. aureus*, which were the most sensitive strains to the OVTp12 peptide [44]. Compared to OVTp12, the TP4 peptide inhibits *E. coli* and *S. aureus* at a much lower concentration, showing the valuable antibacterial effect of the TP4 peptide. On the other hand, Yang et al. investigated the food preservation and antimicrobial properties of the LCH4 peptide, a *Larimichthys crocea* fish peptide, and accordingly, the MIC of the peptide against *S. aureus* and *Vibrio parahaemolyticus* was shown to be 12.5 μg/mL and 25 μg/mL, respectively. They concluded that the LCH4 AMP can be used as a food preservative [45]. Compared to LCH4, the TP4 peptide showed lower MIC values against selected bacteria, indicating its higher potential as a food preservative. In another study conducted by Habibi Najafi et al., it was shown that the Lasioglossin *III* recombinant peptide has significant antibacterial activity against standard foodborne bacterial strains such as *S. aureus*, *Salmonella typhimurium*, *Listeria monocytogenes*, *E. coli*, and *Enterococcus faecalis*. The MIC and MBC for *S. aureus* were 3.851 μg/mL and 7.703 μg/mL, respectively [46], which are in line with our results. The (CBD) 2-DrsB1 recombinant peptide was studied by Varasteh Shams, Nazarian-Firouzabadi, and Ismaili, and its antibacterial effect on standard pathogenic bacterial strains, including *E. coli*, *Pseudomonas aeruginosa*, *S. aureus*, *E. faecalis*, and *Bacillus subtilis*, was evaluated. Gram-positive bacteria were more sensitive to the peptide [47], which is inconsistent with our results.

The TP4 peptide acts by bacterial membrane penetration, disrupting the membrane potential and bacterial lysis, as a result [27, 48, 49]. Considering the above information, the TP4 recombinant peptide has a high potential for use as a foodborne antibacterial agent.

Additionally, comparing the ZnO NP results [36] with the TP4 results indicated higher antibacterial effects of the TP4 peptide than NPs.

ZnO NPs and their results obtained from previous research were used to determine FIC. According to the results, the MIC of ZnO NPs against foodborne bacteria showed that *S. aureus* and *B. cereus* were the most sensitive and resistant to ZnO NPs, respectively. The MBC results showed that, except for *B. cereus*, the NPs had no bactericidal effect on other bacterial strains [36].

3.2. Determination of FIC. The checkerboard method was used to measure the synergistic effects of the TP4 peptide and ZnO NPs against the bacterial strains (Table 2). Accordingly, the combination of TP4 peptide and ZnO NPs did not show any effect on *E. coli*. The FICI for the rest of the bacteria was $0.5 <$ and ≤ 0.75, indicating a partial synergistic effect in which the MIC of the peptide and NPs was reduced by half and 1/16-fold, respectively. It has been shown that NPs have many advantages when used in combination with AMPs such as lower frequency AMP administration, maximization in biological activity, cost reduction, controlled AMP release, and the AMP protection against environmental conditions [50]. The combination of NPs and AMPs can be considered a new and effective treatment method against drug-resistant bacteria [51]. A study performed by Narayana et al. showed a significant synergistic effect against *Helicobacter pylori* using TP4 peptide along with commonly used antibiotics such as amoxicillin and metronidazole, in which the MIC was decreased by 1/4 and 1/2-fold for amoxicillin and metronidazole, respectively [48]. Additionally, using ZnO NPs in combination with ciprofloxacin has resulted in even greater efficacy against resistant bacteria [52]. It should be noted that the synergistic effect of ZnO NPs and TP4 peptide has not been investigated thus far. Finally, our results showed that the combination of TP4 and ZnO NPs has a partially synergistic effect against foodborne bacteria.

3.3. Effects of TP4 Recombinant Peptide on the Growth and Survival of Foodborne Bacteria. Time-kill assays of selected foodborne bacteria treated with a specific concentration of TP4 peptide were performed.

According to Figure 1, *B. cereus* showed more than 99% reduction ($\log \geq 2$) in bacterial population in 24 h at 1 × MIC. Finally, after only 4 h and at a concentration of 2 × MIC, a significant 99.9999% ($\log \geq 6$) reduction in bacterial population (zero bacteria) was observed, indicating bacteriostatic and bactericidal effects of the TP4 peptide on *B. cereus* at different concentrations [53]. Our results are in line with the findings obtained by Yi et al., who concluded a decrease of more than 99.9% in the bacterial population [54].

Unlike *B. cereus*, at 24 h, *Y. enterocolitica* showed a decrease of more than 90% ($\log \geq 1$) at a concentration of 1/2 × MIC of the TP4 peptide, but a reduction of less than 99.9% ($\log \leq 3$) at the concentration of 1 MIC. At a concentration of 2 MIC, there was a significant decrease in the number of bacterial strains by more than 99.9999999% ($\log \geq 9$), showing its bactericidal effect at ≥ 2 MIC at 24 h. At 24 h, the TP4 peptide also showed a bactericidal effect on *B. cereus* at a 2 × MIC concentration. Even though the MIC and MBC of the TP4 peptide against both *B. cereus* and *Y. enterocolitica* were 4 μg/mL, at 2 × MIC concentrations and 4 h, the TP4 peptide showed a higher bactericidal effect on *B. cereus* than on *Y. enterocolitica*, showing the different effects of the peptide on Gram-negative and Gram-positive bacteria. This can be due to the special hydrophilic structure of the Gram-negative plasma membrane, which allows only small hydrophilic molecules to pass into the cell cytosol and delays the entry of antibacterial compounds [55].

One-way ANOVA was used for all comparisons, and various data showed significant differences at the $p < 0.05$ level.

The time-kill curve of *E. coli O157:H7* treated with zp37 AMP showed a significant reduction of eight log units at

Table 2: The results of the combined effect of TP4 peptide and ZnO NPs using the checkerboard method.

Bacterial strains	Antibacterial agent	MIC alone (μg/mL)	MIC in combination (μg/mL)	FIC	Sum FIC	Result
B. cereus	TP4 peptide	4	2	0.5	0.562	Partial synergy
	ZnO NPs	500	31.25	0.062		
S. aureus	TP4 peptide	4	2	0.5	0.562	Partial synergy
	ZnO NPs	31.25	1.952	0.062		
E. coli O157:H7	TP4 peptide	2	4	2	2.062	Neutral
	ZnO NPs	62.5	3.905	0.062		
Y. enterocolitica	TP4 peptide	4	2	0.5	0.562	Partial synergy
	ZnO NPs	62.5	3.905	0.062		

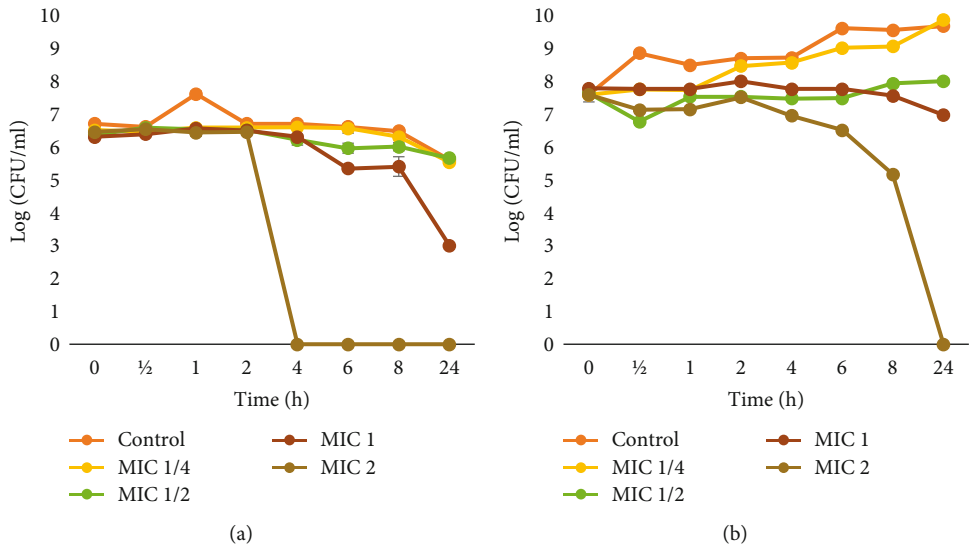

Figure 1: Time-kill curve (TKC) of (a) *B. cereus* and (b) *Y. enterocolitica* in the presence of TP4 peptide. Values are means ± SD. $p < 0.05$.

8 × MIC concentration (128 μM) and 8 h of incubation. The 4 × MIC concentration of peptide after 4 h only resulted in the inhibition of the growth of *E. coli O157:H7* [54]. In another study conducted by Yang et al., the effect of the TGH1 peptide against *V. parahaemolyticus* was evaluated, and the MIC of the peptide against this bacterium was 12.5 μg/mL. Regarding the time-kill curve, it was determined that the 1 × MIC concentration of TGH1 peptide after 5 h of incubation showed a bactericidal effect of a five-log unit reduction for *V. parahaemolyticus*, while for Gram-positive bacteria, *S. aureus* did not show the same effect [56]. Additionally, Yi et al. evaluated the antimicrobial properties of the zp80 peptide against *L. monocytogenes*. They found that the 8 × MIC (16 μM) concentration of the peptide shows a bactericidal effect, and its 16 × MIC within 2 h decreases the population of *L. monocytogenes* by more than eight log units (zero bacteria obtained) [38]. Similarly, Seong Ryul et al. evaluated the MIC of the PAJE AMP against some bacteria, such as *E. coli* and *S. aureus*, at 1 and 4 μM, respectively. The time-kill assays showed that *E. coli* was inhibited at 16 μg/mL and 1 h by 99.9%, and *S. aureus* was inhibited at 16–32 μg/mL and 1 h by 50% [57]. In another study carried out by Liu et al., the MIC of NZX peptide against *Staphylococcus hyicus* was 4 μg/mL, and the time-kill assay showed that the growth of bacteria was temporarily prevented at the 1 × MIC concentration of peptide [58]. Yang et al. showed that the LCWAP peptide inhibited *S. aureus* growth at an MIC of 15.6 μg/mL, and the time-kill assay also indicated a reduction of more than six log units at 1 × MIC and 2 × MIC after 5 h and 4 h of incubation, respectively [59]. Studies have shown that depending on the type of AMP used, the bacteriostatic effect of the peptide on Gram-negative and Gram-positive bacteria directly depends on the peptide concentration and incubation time, in which a low peptide concentration compensates for a high incubation time and vice versa.

3.4. Bacterial Load of Raw and Pasteurized Milk in the Presence and Absence of TP4 Peptide. The effect of the TP4 peptide on the microbial load of raw and pasteurized milk was assessed by the colony count method. The results showed that the number of bacteria in untreated raw and pasteurized milk ranged from 5 to 6 log CFU/mL and 3 to 4 log CFU/mL, respectively, at zero time (Figure 2). On the other hand, when milk was treated with 2 × MIC TP4 peptide, the number of bacteria decreased significantly by more than 99.999% (log ≥ 5) at 4 h for pasteurized milk and by

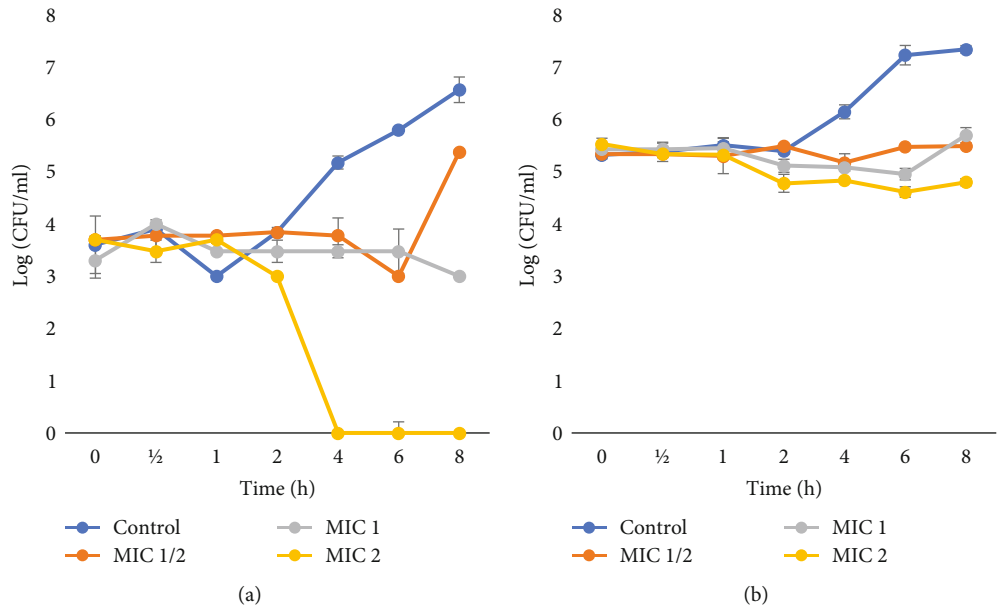

FIGURE 2: Microbial load diagram of (a) pasteurized and (b) raw milk in the presence of TP4 peptide. Values are means ± SD. $p < 0.05$.

99% (log ≥ 2) for raw milk at 6 h. The untreated microbial load of raw and pasteurized milk increased after 8 h of incubation, as expected. Moreover, the number of microbial loads remained almost constant when treated with 1/2 and 1 × MIC of TP4 peptide, with bacteria numbers lower than the initial number. An ANOVA of the obtained results in Figure 2 indicated that the antibacterial activity of each concentration of TP4 was significantly ($p < 0.05$) different from the other concentrations.

In a study by Yang et al., the LCWAP peptide was effective at 2 × MIC (31.2 μg/mL) against *S. aureus* in milk after a 1-day incubation time [59]. In a study carried out by Meng et al., it was shown that bacteriocin NX371 kept the milk pathogens 3.5–4.0 log lower than untreated milk during a week [60]. In another study performed by Stern Bauer and Hayouka, it was shown that the LK 20-mer random peptide mixture and FK 20-mer mixture inhibited the growth of some bacteria in bovine milk, such as *B. cereus*, with MICs of 3.5–50 μg/mL and 12–200 μg/mL, respectively [61].

Our results showed that the TP4 peptide can effectively inhibit microbial growth at 2 × MIC (8 μg/mL) in pasteurized milk during storage, showing its bactericidal effect. Since no research has been conducted on the antimicrobial effects of TP4, it can be used as a suitable option for preserving perishable food such as milk.

Various types of preservation methods can be used in milk systems. For instance, azidiol, the combined chloramphenicol with sodium azide, is a poisonous and non-eco-friendly substance used in some countries worldwide [15, 62]. In addition, using lactoperoxidase in milk collection centers to preserve raw milk cannot replace pasteurization. Instead, adding chemical preservatives such as H_2O_2 has its disadvantages [15, 63]. Although conventional methods applied in milk microbial preservation, such as ultrahigh temperature (UHT) and pasteurization, can make milk safe to consume, such methods cause variation in terms of nutritive value and physicochemical characteristics [64, 65]. Although the extensive use of these ways may not be simply substituted by the other methods, novel technologies can be considered as complementary solutions for pasteurization as a broad definition in the microbe reduction process [66, 67]. While AMPs are expensive [68], the TP4 peptide owing to its cost-effective production may be a suitable candidate for food preservation [27]. In addition, cooling is not readily available in milk collection centers due to the lack of infrastructure. Therefore, it is necessary to provide safe ways to store dairy products [60]. On the other hand, the secondary structure of the TP4 peptide does not change over a wide range of temperature conditions ranging from −20 to +65°C for almost a month [69]. Due to the conditions of transportation and storage systems, the specified expiration date is sometimes inaccurate, leading to spoilage and food poisoning [70]. Among the nonthermal methods, an available functional strategy for milk preservation is ultraviolet radiation, which faces various difficulties, such as its lack of sporicidal ability and low penetration rate [71]. High-pressure processing (HPP) is another nonthermal technology that probably denatures the proteins in milk and reduces the size of its casein micelles. Ultrasound has also been proposed for food preservation, which has disadvantages such as the production of free radicals and, as a result, the destruction of amino and fatty acids in food [72]. However, a combination of nonthermal methods such as HPP and various AMPs such as nisin can inactivate a wide range of microorganisms and even their spores in milk and other types of food [73].

Biopreservation means adding bioactive molecules such as peptides into food products such as raw milk to extend their shelf life [74]. AMPs are possibly suitable alternatives as food preservatives, owing to their safety, preservation of nutritional quality, great stability, and rapid microbial elimination, particularly against multidrug-resistant bacteria

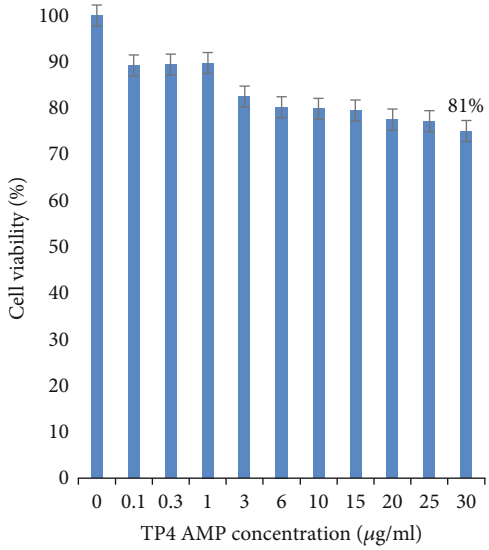

FIGURE 3: MTT analysis of the HEK-293 cell line after 48 h against different concentrations of TP4 recombinant peptide. Values are means ± SE.

[75, 76]. It is more difficult for bacteria to develop resistance to AMPs than conventional antibiotics, and few resistance mechanisms to them have been described. Indeed, the development of bacterial resistance to AMPs cannot be ruled out if the microorganism is repeatedly exposed to them. Thus, proper use of AMPs is essential not only in the treatment of bacterial diseases but also in other diseases to prevent toxicity and limit the development of resistance [77, 78]. Furthermore, using AMPs to preserve foods is not a new approach, and it returns to more than 20 years ago [79]. For instance, in 1996 when nisin was approved, for the first time, the health department in Brazil approved the commercial use of AMP/bacteriocin to preserve cheese [80].

3.5. Cytotoxicity of TP4 Recombinant Peptide. In the present study, the TP4 peptide was evaluated for its possible use as a food preservative; therefore, it is necessary to evaluate its cytotoxicity. The cytotoxicity of TP4 on the normal HEK-293 cell line was assessed by the MTT method in a concentration series of 0.1–30 μg/mL with approximately 81% cell viability at 30 μg/mL (Figure 3), and the half-maximal inhibitory concentration (IC50) was 71.61 μg/mL showing its safety. The IC50 is considered safe when it is between 20 and 100 μg/mL [81].

In a study by Yang et al., the cytotoxicity of the LCWAP peptide was evaluated by the MTT method in a normal human liver cell line (LO2). It was concluded that the peptide at even 16 × MIC (249.6 μg/mL) has no cytotoxicity and, therefore, can be used as a food preservative [59]. In the study performed by Faya et al., the cytotoxic effect of AMP-2 and AMP-3 against the HEK-293 cell line at different concentrations was assessed by the MTT method. They reported that the peptides were nontoxic and suggested the use of these peptides in biomedical applications [82].

Finally, the TP4 AMP, owing to its lack of cytotoxic effects on the HEK-293 cell line and high bacteriostatic/bactericidal activity at very low concentrations (MIC = 4 μg/mL), can be suggested for use in the food industry as a food preservative.

4. Conclusion

Foodborne bacteria cause food poisoning and food spoilage to different degrees. Accordingly, due to their high perishability, dairy products are preserved using food preservatives. Meanwhile, for greater safety, natural preservatives such as AMPs are preferred. In the current study, the antibacterial effects of TP4 peptide alone and along with ZnO NPs were evaluated against four foodborne bacterial strains. The results indicated the high antibacterial effects of the TP4 peptide with 2 and 4 μg/mL for *E. coli* and the rest of bacteria, respectively, and the partial synergistic effect of the peptide along with NPs against most of the mentioned strains ($0.5 < FICI < 0.75$). The number of Gram-negative and Gram-positive bacteria was reduced at low concentrations of TP4 (≥1 × MIC), as determined by the time-kill assay at a short time for Gram-positive bacteria and a relatively long time for Gram-negative bacteria. The microbial load of raw and pasteurized milk was significantly reduced after exposure to 2 × MIC of TP4 peptide in which the number of bacteria decreased significantly by more than 99.999% ($\log \geq 5$) at 4 h for pasteurized milk and by 99% ($\log \geq 2$) for raw milk at 6 h. In addition, ZnO NPs in combination with TP4 are likely to increase food preservation efficiency. The TP4 peptide also showed low cytotoxicity on normal human cells with approximately 81% cell viability at 30 μg/mL, making it a suitable candidate for use in food as a preservative due to its great antibacterial properties at low concentrations. However, it is necessary to investigate the mechanism of action of TP4, conduct additional in vivo studies, and perform more specialized research and studies regarding the effect of this peptide on a wide variety of food bacteria.

Data Availability Statement

All data are available upon request.

Conflicts of Interest

The authors declare no conflicts of interest.

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