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The synergistic antibacterial effect of bacteriocin produced by *Lactobacillus casei* ATCC 39392 and iron oxide nanoparticles (IONPs) on selected foodborne pathogens

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ABSTRACT

Bacteriocins are antimicrobial proteins, which show antagonistic effects on pathogenic bacteria. The most important problems of bacteriocins are low stability and specific antibacterial activities. Using the antibacterial activity of nanoparticles in combination with bacteriocins is a new strategy against pathogenic bacteria. This study aimed to investigate the combined effects of iron oxide nanoparticles (IONPs) and bacteriocins produced by *Lactobacillus casei* ATCC 39392 against selected foodborne pathogens. Iron oxide nanoparticles (IONPs) were synthesized by hydrothermal method and characterized using a field-emission scanning electron microscope, transmission electron microscope, and dynamic light scattering analysis. Minimum inhibitory concentration and antibacterial activity of bacteriocins produced by *L. casei* ATCC 39392 alone, and in the combination of (IONPs) evaluated against selected foodborne pathogens including; *Listeria monocytogenes* PTCC1294, *Bacillus cereus* PTCC1857, *Staphylococcus aureus* PTCC1917 and, *Escherichia coli* PTCC1276 using checkerboard assay method. The obtained results showed that bacteriocin produced by *L. casei* ATCC 39392 combined with (IONPs) showed a fully synergistic effect on *S. aureus* 1917 and *E. coli* PTCC 1276 with fractional inhibitory concentration index (FIC) values of 0.3 and 0.4, respectively. The FIC indexes for *L. monocytogenes* PTCC1294 and *B. cereus* PTCC1857 were 0.9 (additive) and 1.5 (indifferent), respectively. Therefore, it concluded that using a combination of nanoparticles with bacteriocins has a promising candidate for the prevention of foodborne pathogens in food industries.

1. Introduction

The recent increase of antibiotic resistance cases in pathogenic bacteria has encouraged scientists to reassess alternative therapeutic options (Holmes et al., 2016; Michael et al., 2014). Unfortunately, the rate of antibiotic resistance is worrying since progress in the discovery of novel antibiotics with different modes of action has slowed significantly(Mathur et al., 2017). A better option to overcome these problems is to combine antimicrobial agents such as bacteriocins with other compounds such for instance nanomaterial and nanoparticles. Using nanoparticles that function synergistically with bacteriocins may probably expedite each other's killing effects, thereby possibly reducing the likelihood of resistance development to either the bacteriocin or other antimicrobial agents. Furthermore, combinations of bacteriocins with nanoparticles can decrease the concentration of bacteriocins required for the target pathogen. The most common genera for the production of probiotics are *Lactobacillus*

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spp. and *Lactobacillus casei* is the most commonly utilized probiotic species(Aktas et al., 2016).

Also, *L. casei* is one of the most widely studied lactic acid bacteria in the food industry, including cheese, yogurt, meat, fermented soybean milk. Furthermore, *L. casei* have proven to show beneficial probiotic properties, such as antioxidative activity, tumor inhibitory, reduction of cholesterol, and improvement of gastrointestinal microflora(Ma et al., 2020; Qian et al., 2020).

Different methods have been described for assessing antimicrobial synergy in laboratory conditions including the broth-based checkerboard assay, as well as agar-based screens such as E-tests (bioMérieux) to evaluate synergy(Soltani et al., 2012). Both checkerboard and time-kill synergy methods provide valuable information on the activity of antimicrobial combinations and are particularly useful for evaluating potential novel therapeutic options for highly resistant bacterial pathogens. Synergy measurements by checkerboard analysis used to determine the impact on the potency of antibiotics in comparison to their individual activities. This comparison is then representing as the Fractional Inhibitory Concentration (FIC) index value. In addition, the time-kill test is the most appropriate method for determining the bactericidal or fungicidal effect. It is a strong tool for obtaining information regarding the dynamic interaction between the anti- microbial agent and the microbial strain. The time-kill test reveals a time-dependent or a concentration-dependent antimicrobial effect (Balouiri et al., 2016; Brennan-Krohn & Kirby, 2019). As mentioned, the checkerboard method permits the determination of the fractional inhibitory concentration (FIC) index. Although some researchers disagree about the interpretation of results obtained with checkerboard assays, there appear to be five different effects on which the majority of researchers have reached a consensus. These five effects are (i) full synergy (FIC $\leq$ 0.5) (Mattila-Sandholm et al., 2002), partial synergy (0.5 $\leq$ FIC $\leq$ 0.75), (iii) additive effects (0.75 $\leq$ FIC $\leq$ 1.0), (iv) indifferent effects (1.0 $\leq$ FIC $\leq$ 2.0) and (v) antagonistic effects (FIC $\geq$ 2.0) (Bacon et al., 1991; Orhan et al., 2005).

In the past two decades, iron oxide nanoparticles (IONPs) have become a frequently employed material in biomedical research. These IONPs can be used to non-invasively visualize labeled cells by magnetic resonance imaging (MRI) after transplantation, enhance the efficiency of drug or gene delivery, or be used as tools for magnetic hyperthermia cancer treatment (Duguet et al., 2006; Soenen et al., 2009).

In addition, an ideal bactericidal compound should be lethal to bacteria, but safe to human cells. One candidate is iron and its compounds. Iron-oxide nanoparticles (IONPs) are non-toxic materials. The U.S. Food and Drug Administration approved feraheme/ferumoxytol containing superparamagnetic (IONPs) as an iron supplement for treatment of iron deficiency in patients with renal failure is not only non-toxic, but also its byproduct, degraded iron from the cores, and apparently accumulates in natural iron stores in the body. Properly bio-functionalized IONPs have been shown to inhibit the growth of *Staphylococcus aureus* and *Escherichia coli*, prevent biofilm formation by *Pseudomonas aeruginosa* and *Streptococcus mutans* and exhibit bactericidal activity against a range of Gram-negative and Gram-positive bacterial species. As the use of IONPs clearly increasing, the lack of information regarding the interaction of these particles with antimicrobial such as bacteriocins is of great importance. This study aimed to investigate the combined effects of iron oxide nanoparticles (IONPs) and bacteriocin produced by *Lactobacillus casei* against foodborne pathogens including *Listeria monocytogenes* PTCC1294, *Bacillus cereus* PTCC1857, *Staphylococcus aureus* PTCC29213 and, *Escherichia coli* PTCC1276 checkerboard assay method.

2. Materials and Methods

2.1. Bacterial strains, media, and growth conditions

All media and materials used in this study obtained from (Merck Co. Darmstadt, Germany). The strain *Lactobacillus casei* ATCC 39392 was obtained from (Ibresco, Life Science, Karaj, Iran). Foodborne pathogens including *Listeria monocytogenes* PTCC1294 (native strain), *Bacillus cereus* PTCC1857 (native strain), *Staphylococcus aureus* ATCC 29213 and, *Escherichia coli* PTCC1276 (native

strain) obtained from Persian Type Culture Collection (PTCC, Tehran, Iran). For primary culture, a lyophilized vial of *L. casei* ATCC 39392 was streaked on (MRS) agar and incubated at 35°C for 48 h.

2.2. Bacteriocin production and activity determination

For bacteriocin production, the method used by (Thirumurugan et al., 2013) *L. casei* ATCC 39392 was grown in MRS broth (Merck Co. Darmstadt, Germany) at pH 6.8 and maintained aerobically at 35°C for 48 h. Then, bacterial cells were removed from the growth medium by centrifugation (10000X g for 30 min at 4°C) and passed through the Whatman membrane filter (0.2 µm diam. 47 mm). The cell-free supernatant was with adjusted pH 6.0 using 1N NaOH and used as crude bacteriocin. Bacteriocin activity was determined by the agar well diffusion method. The activity of cell-free supernatant was explained in arbitrary units per ml (AU/ml). A unit activity of the bacteriocin was defined as an arbitrary unit (Mathur et al.); 1 AU is a unit area of inhibition zone per unit volume, in this case, mm^2/ml (Usmiati & Marwati, 2009). The bacteriocin activity was calculated using the following formula: Bacteriocin activity (mm^2/ml) =Lz-Ls/V which the parameters are LZ=clear zone area (mm^2), Ls=well area (mm^2), and V=volume of the sample (ml).

2.3. Synthesis of iron oxide nanoparticles

IONPs were prepared by the method used by Lin et al., (Lin et al., 2018) with some modifications via a ligand-assisted method by the introduction of oleic amine (Sigma-Aldrich, USA) and sodium citrate as co-coordinating agents through a hydrothermal reaction. For synthesis, 1 mL of oleic amine (OA) was dissolved in 10 mL of ethylene glycol (Sigma-Aldrich, USA) to form a clear solution; next, 20 mmol of iron (III) chloride hexahydrate ($FeCl_3$·6H2O, Sigma-Aldrich, USA) and 20 mmol of sodium acetate anhydrous (Sigma, Aldrich, USA) were added. Then, the mixture was mixed at 70 °C for 24 h to generate a homogeneous solution. Then, the mixture was placed in a Teflon-lined stainless steel autoclave for hydrothermal reactions at 220 °C for 8 h. After the reaction, the mixture cooled down to room temperature for another 3 h. The magnetic particles were collected by centrifugation at 8,000 rpm for 10 min and, washed three times with ethanol 3. The particles dried under vacuum overnight at room temperature. The morphologies of IONPs were characterized using a field emission scanning microscope (FE-SEM, JEOL-6700, Japan) and transmission electron microscopy (TEM, Zeiss electron microscope, EM10C – 150 kV; Carl Zeiss Meditec AG, Jena, Germany). For transmission electron microscopy, samples were prepared using the sonication method by a sonicator (Misonix S3000) for 30 minutes. The hydrodynamic radius of IONPs was characterized using dynamic light scattering (DLS; Brookhaven Instrument, Holtsville, NY, USA) technique.

2.4. Determination of minimum inhibitory concentration

For the determination of minimum inhibitory concentration, the standard broth dilution method for the compounds of interest (IONPs, crude bacteriocin, and selected antibiotics) was used in 70192 Mueller Hinton broth (Sigma-Aldrich) medium, according to CLSI M7-A8 and M100-S28-2018 guidelines. Serial two-fold dilution (25, 12.5, 6.25, 3.125, 1.56, 0.78 and 0.39 µg/ml) of IONPs, the cell-free extract of bacteriocin, and antibiotics (64, 32, 16, 8, 4, 2 and 1µg/ml) with adjusted bacterial concentration (10^8 CFU/ml 0.5 McFarland's standard) were used to determine MIC in MH broth. The control contained only inoculated broth and incubated for 24 h at 37° C. The MIC endpoint is the lowest concentration of antimicrobial components where no visible growth is seen in the tubes. The visual turbidity of the tubes was distinguished, both before and after incubation to approve the MIC value.

2.5. Minimum bactericidal concentration determination

For determination of minimum bactericidal concentration, aliquots of 50 µl from all the tubes, which showed no visible bacterial growth, were cultured on Mueller Hinton agar plates and incubated for 24 h at 37 °C. When 99.9% of the bacterial population is killed at the lowest

concentration of an antimicrobial agent, it is termed as MBC endpoint.

2.6. Evaluating the antimicrobial activity of bacteriocin and IONPs using microtitration assay

For microtitration assay, a temperature-controlled Dynex 96-well plate reader MRX (Vodičkova, Czech Republic) was used to monitor the optical density of the microbial test strains. Wells used to evaluate the effects of the two antimicrobials combinations contained a 50:50 mixture of each bacteriocin and IONPs in stock solutions. The combined and alone (bacteriocin (40 µl), bacteriocin+ nanoparticles (40 µl), and nanoparticles as control (40 µl) were poured into the corresponding wells. All foodborne test strains were diluted in fresh 70192 Mueller Hinton broth to achieve $10^5 – 10^6$ CFU $m1^{-1}$, and 100 µl of each test strain was added to microplate wells. Then the microtitre plate was incubated at 37°C for 24 h with OD_{600} read every half an hour immediately following a 5-s shake cycle. All assays were performed in triplicate.

2.7. Fractional inhibitory concentration determination

Synergy was determined using the fractional inhibitory concentration (FIC) index by using the checkerboard assay method. The index is calculated by utilizing the minimum inhibitory concentrations (MIC) of the antimicrobial compounds alone and the respective MIC when the compounds are combined. The formula is ([A]/MICA) + ([B]/MICB), where MICA and MICB are the MIC of the compounds alone and [A] and [B] are the MIC of the compounds when used together.

2.8. Determining the antibacterial activity using scanning electron microscopy

For determining of antibacterial activity of bacteriocin and IONPs in combination on the selected foodborne pathogens, a field emission scanning microscope (FE-SEM, JEOL-6700, Japan) was used.

2.9. Statistical analysis

Data represented as mean ± SD of three independent experiments. The statistics of experiment results were calculated by one-way analysis of variance and Student's *t*-test. $P<0.05$ was chosen as the significance level for all analyses performed.

3. Results

3.1. Nanoparticle characterization

IONPs were characterized by SEM, TEM, and DLS methods. TEM observation showed that the size of IONPs in the dried state was about 100 nm exhibited a nearly homogeneous shape (Figure 1 A and B). Transmission electron micrographs of IONPs were shown in Figures (1 C and D) with higher and lower resolution. DLS studies demonstrated that the hydrodynamic radius of Fe_2O_3 NPs was 175 nm (Figure 2). This difference between DLS and electron microscopy studies showed the presence of a hydrated shell on the NP surface in the DLS investigations. Thus, it concluded that Fe_2O_3 NPs exhibit pronounced colloidal stability.

3.2. Inhibitory activity of individual bacteriocin

The minimal inhibitory concentrations of the bacteriocin against foodborne pathogens are summarized in Table 1. Minimum inhibitory concentrations of bacteriocin against *L. monocytogenes* PTCC 1294, *Bacillus cereus* PTCC 1857, *E. coli* PTCC1267, and *S. aureus* PTCC1917 were >25, >25, 12.5, and 25 µg/l, respectively. According to the above order, the MIC for IONPs against the selected foodborne pathogens was>25, >25, 12.5, and 6.25, respectively. The MIC of bacteriocin and IONPs in combination were 25, 25, 3.125, and 6.25, respectively.

3.3. Synergistic activities of bacteriocin and iron oxide nanoparticles (IONPs)

The effect of combined bacteriocin and (IONPs) on the growth of foodborne pathogens is shown in Table 2. Using bacteriocin and (IONPs) showed fully synergy against *S. aureus* PTCC1917 (FIC index = 0.3) and *E. coli* PTCC1267 (FIC index = 0.4). For *L. monocytogenes* PTCC 1294 (FIC index=0.9) and *B. cereus* PTCC1857 (FIC index=1.5) additive and indifferent effects were obtained, respectively.

3.4. Evaluation of bacterial morphology upon treatment of bacteriocin and IONPs by field emission scanning electron microscopy

The treatment effects of the combination of bacteriocin and IONPs on the morphology of the selected foodborne bacteria were shown in field emission scanning electron micrographs (figures 3 and 4). As shown in Figures 3A and B, the combination effect of IONPs and bacteriocin did not show a significant effect on cell morphology and cellular integrity of *L. monocytogenes* PTCC1294.

The same situation was observed for *B. cereus* PTCC1857 as shown in figure 3C and 3D. However, there was significant loss of membrane integrity and damage when *E. coli* PTCC1267 and *S. aureus* PTCC1917 were treated with IONPs and bacteriocin in combinations. *E. coli* cells were swollen with the size reduction, pores are formed on the cell membrane as shown in figure 4 B in comparison to untreated cells (figure 4A). Also, *S. aureus* ATCC 29213 cells formed clumps and lost membrane integrity in response to IONPs and bacteriocin synergism (figure 4C and D).

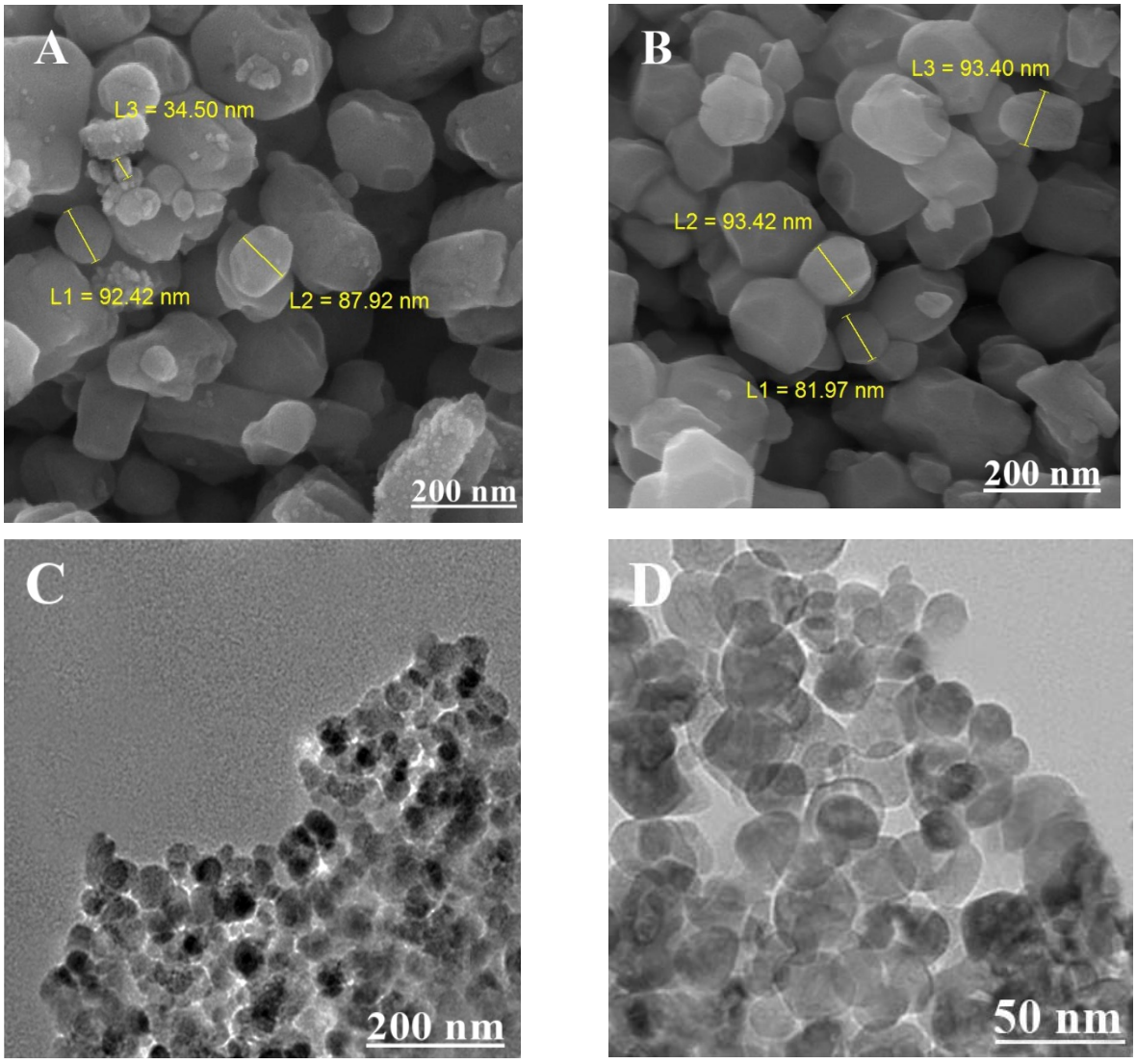

Figure 1. Electron micrographs of IONPs.(A, B) Field emission scanning microscope images with nanoparticle sizes and(C, D) Transmission electron microscopes with higher and lower resolutions.

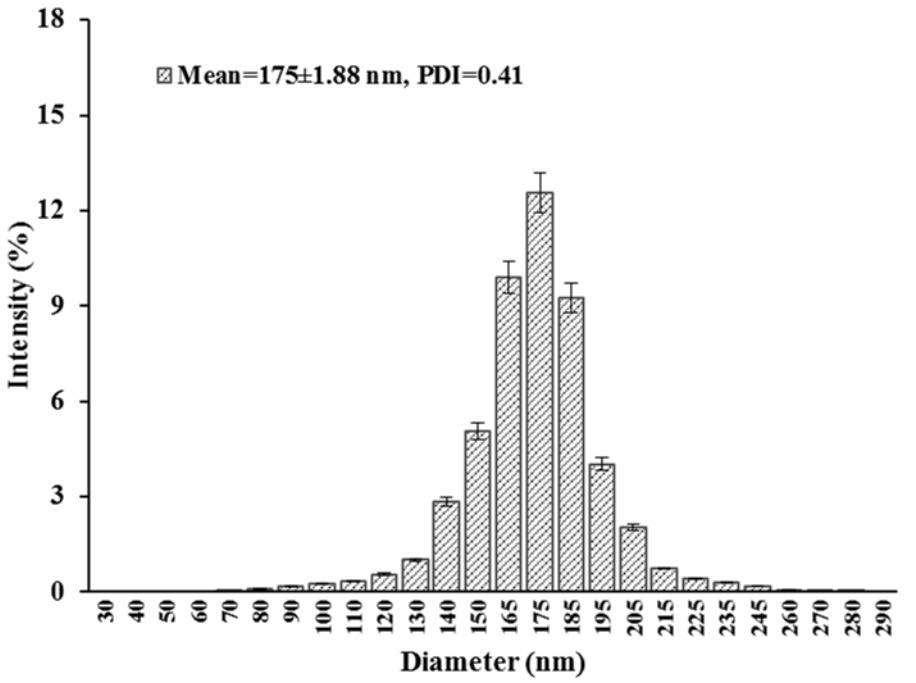

Figure 2. Dynamic light scattering (DLS) of iron oxide nanoparticles (IONPs)

Table 1. Minimum inhibitory concentration (MIC) of IONPs, Bacteriocin, combination of bacteriocin+IONPs and reference antibiotics against selected foodborne pathogens ($\mu g/mL^{-1}$)

	IONPs	Bacteriocin	IONPs+Bacteriocin	Ampicillin	Gentamycin	Chloramphenicol
L.monocytogenes PTCC 1294	>25	>25	25	2	2	aNT
S. aureus ATCC 29213	12.5	25	6.25	aNT	4	8
B. cereus PTCC1857	>25	>25	25	32	16	8
E. coli PTCC1276	6.25	12.5	3.125	8	4	8

a NT, not tested

Table 2. Fractional Inhibitory Concentration (FIC) Index of IONPs and *L.casei* 39392 Bacteriocin against selected foodborne pathogens

Combinations of antibacterial compounds	*L. monocytogenes* FIC interpretation[a]	*S. aureus* FIC interpretation[a]	*B. cereus* FIC interpretation[a]	*E. coli* FIC interpretation[a]
IONPs and Bacteriocin	0.9 Additive	0.3 Synergy	1.5 Indifferent	0.4 Synergy

[a] full synergy (FIC ≤ 0.5), additive effects (0.75 ≤ FIC ≤ 1.0) and indifferent effects (1.0 ≤ FIC ≤ 2.0).

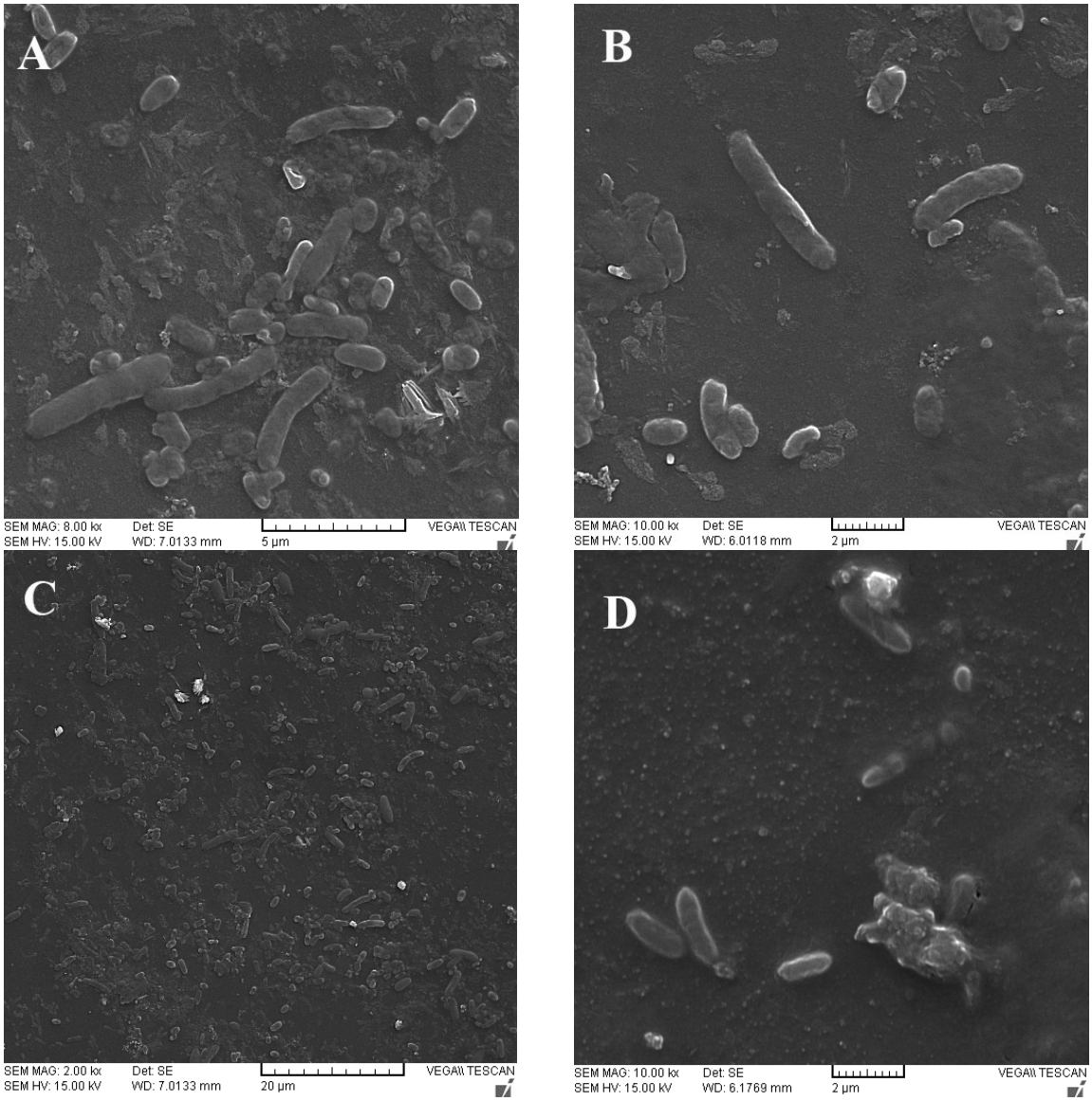

Figure 3. Field emission scanning microscope images of the combination of IONPs and bacteriocin on *Listeria monocytogenes* PTCC1294 (A, normal cells and B, treated cells) and *Bacillus cereus* PTCC1857 (C, normal cells and D, treated cells).

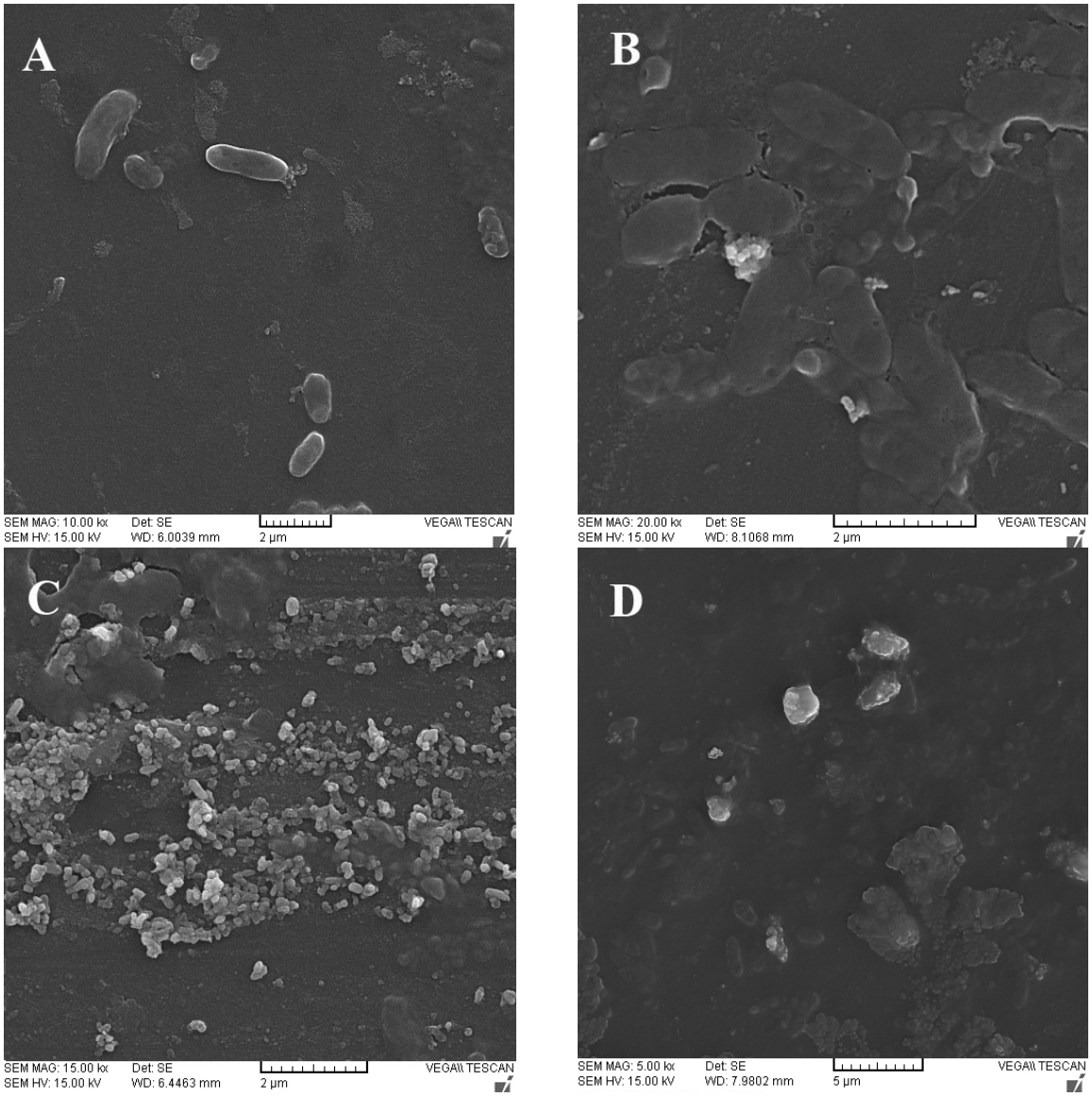

Figure 4. Field emission scanning microscope images of the combination of IONPs and bacteriocin on *Escherichia coli* PTCC1276 (A, normal cells and B, treated cells) and *Staphylococcus aureus* ATCC 29213 (C, normal cells and D, treated cells).

4. Discussion

In the food industry, disease-causing and spoilage organisms have tremendous implications in morbidity/mortality, as well as financial implications. Several bacterial pathogens exist in food systems, both in planktonic and biofilm forms. There is an emphasis on trying to replace chemically derived antimicrobials in food with more natural antimicrobials such as bacteriocins and plant-derived essential oils. In addition, the increase in the extent of global food distribution, in conjunction with more frequent travel has elicited an increase in the dissemination of food-borne diseases, and solutions are required to combat this development (Hussain and Dawson, 2013). While the use of chemical preservatives, as well as heat treatment, have proven to be successful in the past in limiting food-borne pathogens as part of the obstacle effect, such treatments can have an impact on the organoleptic properties of food. In addition, increasing pressure from consumers for the production of safe food, which is minimally processed, has ignited an interest in the

development of effective natural antimicrobials or antimicrobial combinations to control food-borne pathogens(Hussain & Dawson, 2013; Mathur et al., 2017).

Infectious bacteria are fast-developing antibiotic resistance and thus pose serious obstacles to treatment. The use of bacteriocins as anti-infective drugs offers several advantages over current antibiotic treatments. The combination of bacteriocins with nanoparticles could become a particularly effective approach to overcoming the resistance problem. In addition, nanoparticles have great potential as nanomedicine for the treatment of different bacterial diseases. The ultra-small size of nanoparticles may help their diffusion through pore channels of the bacterial cell wall to apply their antibacterial action via destruction of the cell envelope and intracellular damage(Ansari et al., 2017).

According to the results obtained in this study, the combination of bacteriocin and IONPs could reduce minimum inhibition concentrations against selected foodborne pathogens. The full synergistic effect was observed on *E. coli* PTCC 1267 and *S. aureus* ATCC 20231with FIC 0.4 and 0.3, respectively. According to the obtained field emission scanning electron micrographs, it concluded that *L. casei* bacteriocin interacts with the components of the bacterial cell membrane and facilitate penetration of IONPs to cell envelope leading to damage, followed by the death of the bacterial cells. *E. coli* cells became swollen and deformed, which indicates a typical stress response. Pores are formed on the cell surface of treated *E. coli* cells shown in figure 4B.

The damage was more severe for *S. aureus* cells, destructions in the cell envelope and clumping shown in figure 4D.

Sharma et al. (2012) studied the interaction of bacteriocin-capped silver nanoparticles with some food pathogens including *E. coli*, *Bacillus cereus*, *K. pneumoniae*, *L. monocytogenes*, *Micrococcus luteus*, *Shigella flexneri*, and *S. aureus*. The MIC values suggest that Entrobactin-Capped SNPs showed approximately 2- to 16-fold more inhibitory effects compared to the same concentration of C-SNPs against a range of bacterial pathogens tested. There was a significant improvement in the degree of inhibition in the case of *B. cereus* and *L. monocytogenes*. On the other hand, *K. pneumoniae* was inhibited more effectively by C-SNPs compared to antibacterial peptide enterocin-capped SNPs.

The inhibitory activity bacteriocin produced by *L. casei* ATCC 39392 in combined with IONPs against the representative Gram-negative and positive strains in this study is in agreement with data published by Sharma et al. (2012).

Armijo et al. (2020) investigated the antibacterial activity of iron oxide, iron nitride, and tobramycin conjugated nanoparticles against *Pseudomonas aeruginosa* biofilms(Armijo et al., 2020). They studied the antibacterial effects of iron oxide (nominally magnetite) nanoparticles (NPs) alone as well as alginate-capped iron oxide NPs, and NP-tobramycin conjugates on PAO1 *Pseudomonas aeruginosa*. Their results showed that iron-oxide NPs coated with alginate, as well as alginate-coated magnetite–tobramycin conjugates inhibit *P. aeruginosa* growth and biofilm formation in established colonies. In comparison to this study, we assume that IONPs are very useful nanoparticles using in combination with other antimicrobial compounds to combat pathogenic bacteria (Armijo et al., 2020).

Hanchi et al. (2017) evaluated the efficacy of durancin 61A alone or in combination with nisin, pediocin PA-1, reuterin, microcin J25, vancomycin, or tetracycline as an inhibitor of resistant clinical pathogens. The results showed that durancin in combination with reuterin were effective inhibitors of *Clostridium difficile*, vancomycin-resistant *Enterococcus faecium*, and methicillin-resistant *Staphylococcus aureus*. Fractional inhibitory concentration indexes of durancin and reuterin in combination against *C. difficile* and *S. aureus* ATCC700699 were 0.2 and 0.3, respectively. In our study, the FIC index for *S. aureus* ATCC 20231 was 0.3, comparable to the result obtained by (Hanchi et al., 2017).

In the present work, we demonstrated that bacteriocin produced by *L. casei* ATCC 39392 in combination with iron oxide nanoparticles (IONPs) have significant effects on some foodborne pathogens such as *S. aureus* and *E. coli*. The antimicrobial activity against *L. monocytogenes* PTCC1294, *B. cereus* PTCC 1857 was very significant. So, combined with the other nanoparticles recommended. While numerous bacteriocins have been characterized primarily to facilitate their applications as food additives, their clinical potential as therapeutic

materials remain underexplored. Although there are, studies conducted regarding to promising synergistic interactions between bacteriocins and other stressors such as nanoparticles, it must be emphasized that, since there are a large number of bacteriocin-antimicrobial combinations that have yet to be investigated. In addition, understanding of the mechanism of synergistic interactions of antimicrobial combinations has delayed the progress of alternative therapeutic options of bacteriocin-antimicrobial combinations against target strains, particularly in clinical trials. In clinical trials, it should be noted that many studies have shown that inorganic nanoparticles such as gold and silica with small sizes (<100 nm) can increase tumor cell penetration compared to the larger sizes. For example, Kumar et al. (2014) utilized 5.2 nm gold NPs stabilized with targeting peptides to enhance cellular uptake like gold and iron oxide NPs can be considered as a pharmaceutical candidate in biomedicine and therapy.

Conclusion

According to the results obtained in this study, a combination of bacteriocin produced by *L. casei* ATCC 39392 and IONPs nanoparticles showed antibacterial activity against some selected foodborne pathogens. Therefore, clarification of the mode of action of these synergistic interactions using a combination of genomic, transcriptomic, and proteomic tools is likely to accelerate the processes of these antimicrobial combinations in clinical and food industries.

Conflicts of interest

There are no conflicts of interest.

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Refereces

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