Original Article

Journal of Food Safety and Hygiene

Journal homepage: https://jfsh.tums.ac.ir

Comparative evaluation of antioxidant and antimicrobial properties of bioactive peptides from enzymatically hydrolyzed quail slaughterhouse by-products encapsulated in different nanovesicle systems

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ARTICLE INFO

Article history:
Received 24.04.2026
Received in revised form 21.06.2026
Accepted 28.06.2026

Keywords:
Bioactive peptides;
Quail slaughterhouse by-products;
Nanoliposomes;
Chitosan-coated nanoliposomes;
Nanoniosomes;
Antioxidant activity;
Antimicrobial activity;
Foodborne bacterial pathogens

ABSTRACT

Bioactive peptides derived from protein-rich quail slaughterhouse by-products have potential antioxidant and antibacterial activities; however, their instability may limit their practical applications. This study compared uncoated nanoliposomes, chitosan-coated nanoliposomes, and nanoniosomes as delivery systems for enzymatically produced quail by-product peptides. The peptides were obtained using an alkaline protease from *Bacillus subtilis* under the selected hydrolysis conditions of pH 8.0, 37 °C, an enzyme-to-substrate ratio of 1.5%, and 8 h of hydrolysis. The encapsulation efficiency, peptide release under simulated gastric and intestinal conditions, antioxidant activity based on 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and ferric reducing antioxidant power (FRAP) assays, as well as antibacterial activity based on minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), were evaluated. All nanovesicle assays were performed in triplicate and analyzed using nonparametric statistical tests at $p<0.05$. Encapsulation efficiency significantly differed among uncoated nanoliposomes (68.65 ± 0.19%), chitosan-coated nanoliposomes (73.70 ± 0.22%), and nanoniosomes (70.51 ± 0.25%; overall $p=0.02$), with a significant difference observed only between uncoated and chitosan-coated nanoliposomes (adjusted $p=0.007$). Chitosan-coated nanoliposomes exhibited lower peptide release than uncoated nanoliposomes under both simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) conditions and showed more favorable mean antioxidant activity values, while their differences from nanoniosomes were not statistically significant. Furthermore, peptide-loaded chitosan-coated nanoliposomes and nanoniosomes exhibited the lowest MIC and MBC values, depending on the bacterial species. Overall, chitosan coating enhanced the encapsulation and controlled-release performance of nanoliposomes compared with uncoated nanoliposomes, while both chitosan-coated nanoliposomes and nanoniosomes demonstrated promising potential as peptide-delivery systems with antioxidant and antibacterial functionality *in vitro*.

Citation: Ebrahimi N, Golestan L, Anvar AA, Ataee M. **Comparative evaluation of antioxidant and antimicrobial properties of bioactive peptides from enzymatically hydrolyzed quail slaughterhouse by-products encapsulated in different nanovesicle systems.** J Food Safe & Hyg 2026; 12 (2): 128-142 .https://doi.org/10.18502/jfsh.v12i2.23053

1. Introduction

Nutrition and health are closely related. Bioactive food components have been increasingly studied for their Potential roles in reducing risk factors associated with cardiovascular disease, diabetes, oxidative stress, and cancer. Bioactive peptides (BPs) are short amino-acid sequences released during protein hydrolysis and may

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have antioxidant, antimicrobial, antihypertensive, anti-inflammatory, and hypocholesterolemic activities (1-3). BPs sources include protein-rich plant, dairy, seafood, and animal materials. Quail slaughterhouse by-products, including heads, viscera, and feathers, represent an underused protein-rich waste stream. Enzymatic hydrolysis can convert these materials into peptides with antioxidant and antimicrobial properties, supporting both waste reduction and production of value-added food ingredients (4-7).

Despite their great functional potential, the application of BPs is limited due to their sensitivity to degradation during food processing, storage and gastrointestinal digestion, which can reduce their biological activity and bioavailability. Nanovesicular delivery systems like phospholipid-based nanoliposomes and nanoniosomes based on nonionic surfactants are promising tools for protection of BPs, improving their stability and providing controlled release (1, 8, 9).

Further, coating of nanoliposomes with a biocompatible cationic polysaccharide, chitosan can enhance vesicle stability, retention of peptide and modulation of release under gastrointestinal conditions (9, 10). Studies conducted previously have shown the potential of these delivery systems for the preservation of bioactive compounds. Both liposomes and niosomes were reported by Rezvani et al. to effectively encapsulate the bioactive tripeptide isoleucine-proline-proline to incorporate into functional beverages (9). Reyhani Poul and Yeganeh reported that the chitosan-coated nanoliposomes loaded with peptides derived from prawn waste had higher encapsulation efficiency, slower release of peptides under simulated gastrointestinal conditions and higher antioxidant activity compared to the uncoated nanoliposomes (10). Furthermore, nanoliposomes loaded with myrtle extract showed more potent antioxidant and antimicrobial activities against *Staphylococcus aureus*, *Escherichia coli* and *Salmonella enteritidis* than the corresponding nanoniosomes, but the nanoniosomes demonstrated better physical stability (11). These results support the comparative evaluation of chitosan-coated nanoliposomes and nanoniosomes as delivery systems for antioxidant and antimicrobial peptides from by-products of quail slaughterhouses. Quail abattoir by-products were chosen as an underutilised protein-rich waste stream that can be transformed into value-added BPs through enzymatic hydrolysis (5, 7). Thus, this study generated peptides from the by-products of quails and compared uncoated nanoliposomes, chitosan coated nanoliposomes and nanoniosomes for encapsulation efficiency, simulated gastrointestinal release, antioxidant activity and antibacterial activity.

Previous studies have shown that liposomes and niosomes can be effective carriers for BPs (12), that chitosan coating improves the encapsulation efficiency, gastrointestinal stability, and antioxidant activity of shrimp-processing waste-derived peptides (7), and that the type of nanovesicle has an effect on the antioxidant and antimicrobial activities of encapsulated bioactive compounds (11). However, comparative evidence on BPs from quail abattoir by-products is still scarce. Thus, the present study aimed to obtain BPs through enzymatic hydrolysis of by-products from the quail abattoir and compare antioxidant and antimicrobial properties of peptides encapsulated in chitosan-coated nanoliposomes and nanoniosomes (12). To characterise

the performance of these delivery systems, encapsulation efficiency and peptide release under simulated gastrointestinal conditions were also evaluated. This approach can be used for the sustainable utilisation of by-products from poltry processing and the development of functional peptide delivery systems.

2. Materials and Methods

2.1. Materials and reagents

Quail slaughterhouse by-products (heads, viscera, and feathers) were obtained from a local processing plant and transported to the laboratory on ice. Commercial protease was obtained from Sigma-Aldrich (USA), and the 3-kDa ultrafiltration membrane was obtained from EMD Millipore. Sunflower oil, soy lecithin, cholesterol, Span 60, Tween 60, medium-molecular-weight chitosan, acetic acid, DPPH, ABTS, FRAP reagents, Bradford reagent, and trichloroacetic acid were of analytical or food grade. All aqueous solutions were prepared with double-distilled water (13).

2.2. Protein extraction and enzymatic hydrolysis

The by-products of the quail abattoir were washed with cold distilled water and homogenised in a ratio of 1:3 (w/v). The homogenate was centrifuged at 10,000 × g, 4 °C and the supernatant was collected as crude protein extract (5). The concentration of total protein was determined by the Bradford method using bovine serum albumin as the standard (Bradford, 1976) (14). The enzymatic hydrolysis was performed using alkaline protease from Bacillus subtilis (Sigma-Aldrich, USA) at an enzyme to substrate ratio of 1.5% (w/w) for 8 h. The enzyme was boiled after hydrolysis to inactivate it. The degree of hydrolysis (DH %) was calculated with the trichloroacetic acid-soluble nitrogen method using the following equation: DH (%) = [TCA-soluble nitrogen/total nitrogen] × 100 (Hoyle and Merritt, 1994) (15). The supernatant was then filtered through a 3-kDa Amicon ultrafiltration membrane (Merck Millipore, Darmstadt, Germany) after cooling and centrifugation. The obtained peptide fraction was freeze-dried and stored at −20 °C for further analysis (6, 10, 13).

2.3. Preparation of nanocarriers

Two peptides loaded nanocarrier formulations were prepared: chitosan coated nanoliposomes and nanoniosomes. Then a nanoliposomal formulation (5 mg/mL) of the peptide fraction with molecular weight less than 3 kDa was prepared to obtain the chitosan-coated nanoliposomes. The formulation was composed of sunflower oil, soy lecithin and 1.2% glycerol and was hydrated in distilled water, heated to 60–80 °C and stirred at 60 °C for 1 h. The resultant nanoliposomes were then coated with 0.5% (w/v) chitosan solution prepared in 1% acetic acid. The suspension was stirred at 200 rpm for 1 h at room temperature and sonicated at 25 °C with a UP200S probe sonicator (Hielscher Ultrasonics; nominal power: 200 W; frequency: 24 kHz). Nanoniosomes were prepared by thin film hydration method. Cholesterol, Span 60 and Tween 60 were dissolved in a chloroform-methanol (2:1) mixture and the organic solvents were evaporated under reduced pressure using a rotary evaporator at 60 °C and 150 rpm to obtain a thin lipid film. The film was hydrated in 10 mL of phosphate buffer with 10 mg of peptides (1 mg/mL) at 60 °C and rotated at 120 rpm for 30 min. The obtained suspension was sonicated for 7 min to reduce the vesicle size (11).

2.4. Encapsulation efficiency (EE%)

Encapsulation efficiency was determined by measuring the concentrations of total and non-encapsulated peptides. Briefly, 10 mL of the vesicle suspension was centrifuged at 5000 rpm for 15 min, and the supernatant containing non-encapsulated peptides was collected. A separate aliquot of the vesicle suspension was treated with chloroform to disrupt the vesicular membrane and release the encapsulated peptides. Free and total peptide concentrations were measured spectrophotometrically at 765 nm. Encapsulation efficiency was calculated using the following equation:

EE (%) = [(Ctotal − Cfree) / Ctotal] × 100

where Ctotal represents the total peptide concentration and Cfree represents the concentration of non-encapsulated peptides (16).

2.5. Physicochemical characterization

Particle-size distribution was assessed by dynamic light scattering (DLS) using a Nano Q instrument. The available sample was diluted 1:50 with deionized water and analyzed at 25 °C. The recorded DLS analysis provided a single particle-size distribution, from which the D10, D50, and D90 values were obtained. Separate particle-size measurements for each nanocarrier formulation were not available, and the polydispersity index and zeta potential were not measured. The morphology of the nanoniosomes and chitosan-coated nanoliposomes was examined using scanning electron microscopy (SEM).

2.6. *In vitro* release in simulated gastrointestinal fluids

The release of encapsulated peptides was evaluated in simulated gastric fluid (SGF, pH 1.2) and simulated intestinal fluid (SIF, pH 6.8). Aliquots of each nanocarrier formulation (20 µL) were diluted to a final volume of 1 mL with the corresponding simulated fluid and incubated at 37 °C under continuous mixing. The incubation periods were 2 h for SGF and 1 h for SIF. At predetermined intervals, samples were centrifuged to separate the nanovesicles, and the concentration of released peptides in the supernatant was determined using the biuret assay at 595 nm (13).

2.7. Assays of antioxidant activity

The antioxidant activities of free and encapsulated BPs were evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging assay, the 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical-scavenging assay, and the ferric reducing antioxidant power (FRAP) assay. Ascorbic acid was used as a positive control. Before the DPPH and ABTS assays, encapsulated samples were heated at 100 °C for 5 min to release the peptides.

2.7.1. DPPH radical-scavenging assay

A 2-mL aliquot of each sample was mixed with 2 mL of 0.16 mM DPPH solution prepared in 95% ethanol. The mixture was incubated in the dark at room temperature for 30 min, and absorbance was measured at 517 nm. Radical-scavenging activity and the half-maximal inhibitory concentration (IC50) were calculated.

2.7.2. ABTS radical-scavenging assay

The ABTS radical cation was generated by mixing 7 mM ABTS solution with 2.45 mM potassium persulfate and incubating the mixture in the dark at room temperature for 16 h. The resulting solution was diluted to an absorbance of 0.70 ± 0.02 at 734 nm. Subsequently, 980 µL of the ABTS solution was mixed with 20 µL of each sample, and absorbance was

measured at 734 nm. Radical-scavenging activity and IC50 values were determined.

2.7.3. FRAP assay

Ferric reducing antioxidant power was determined using a freshly prepared reagent containing 2,4,6-tripyridyl-s-triazine (TPTZ), and absorbance was measured at 593 nm. Results were expressed as milligrams of Trolox equivalents per gram of sample. All measurements were performed in triplicate (5, 13, 17).

2.8. Antibacterial activity

Antibacterial activity against *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), and *Salmonella enteritidis* (ATCC 13076) was evaluated using the broth microdilution method in sterile 96-well plates. Free peptides and peptide-loaded nanocarrier formulations were serially diluted in nutrient broth and inoculated to obtain a final bacterial concentration of 5×10^5 cfu/mL. Following incubation at 37 °C for 24 h, resazurin was used as a bacterial-viability indicator. MIC was defined as the lowest sample concentration at which no color change was observed, indicating the absence of detectable bacterial growth. To determine MBC, 100 µL aliquots from wells showing no growth were cultured on nutrient agar plates and incubated at 37 °C for 24 h. The MBC was defined as the lowest concentration resulting in a reduction of at least 99.9% in the initial viable bacterial population.

2.9. SDS–PAGE analysis

The molecular-weight distribution of proteins and peptides and the extent of protein hydrolysis were evaluated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under denaturing conditions according to the method described by Laemmli (1970). Samples were analyzed alongside a molecular-weight marker using a 5% stacking gel and a resolving gel. Electrophoresis was performed at 100 V for 2 h. Following electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 and destained with an ethanol–acetic acid solution to visualize the protein bands (18).

2.10. Statistical analysis

Each of the experiments was done in triplicate (n = 3). Data are represented in the form of mean and standard deviation. SPSS v.22 was used to make statistical analyses. Where normality was not observed, the Kruskal-Wallis test followed by the Mann-Whitney test with Dunn-Bonferroni correction was used. The level of difference was regarded as significant at $p<0.05$.

2.11. Ethical considerations

No animal or human experiments were involved in the study, and only animal by-products were used. A check of all the procedures involved was made in relation to the ethical requirements of the Islamic Azar University and the local requirements in the handling of animal by-products.

3. Results

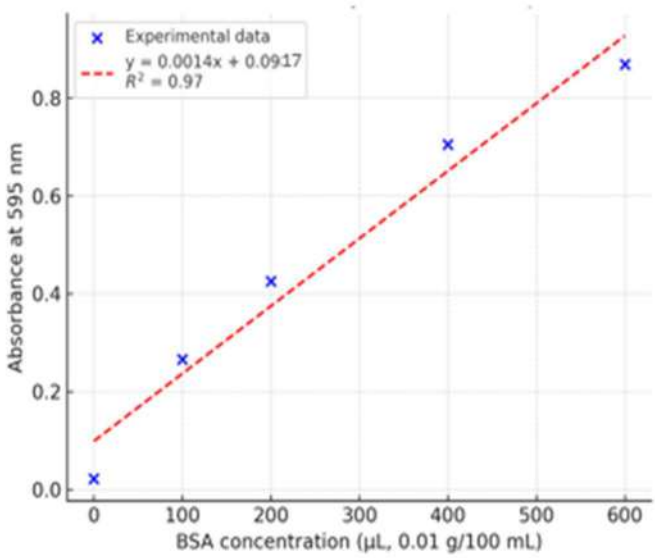
3.1. Protein extraction and enzymatic hydrolysis

The protein concentration of the crude extract obtained from quail slaughterhouse by-products was 5.166 ± 0.08 mg/mL, as determined by the Bradford assay. The calibration curve was described by the equation $y = 0.0014x + 0.0917$, with a coefficient of determination of $R^2 = 0.97$ (Fig. 1).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) demonstrated a reduction in the intensity of high-molecular-weight protein bands, particularly in the approximate range of 100–140

kDa, following enzymatic treatment, indicating protein hydrolysis and peptide formation (Fig. 2).

The degree of hydrolysis (DH) increased with increasing enzyme-to-substrate ratio and incubation time, whereas the calculated mean peptide chain length (CL) decreased correspondingly (Fig. 3 and 4). The highest DH, 5.00%, and the lowest calculated CL, 20, were obtained at an enzyme-to-substrate ratio of 1.5 (w/w) after 8 h of incubation. Therefore, these conditions were selected for subsequent experiments. Because the hydrolysis-screening measurements were performed once for each experimental condition, no inferential statistical comparisons were conducted for these data.



standard (BSA, 0.01 g/100 mL)	0	100	200	400	600
Absorbance at 595 nm	0.22	0.266	0.4	0.705	0.869

Figure 1. Bradford calibration curve and bovine serum albumin standard absorbance values used to determine the protein concentration of the quail by-product extract

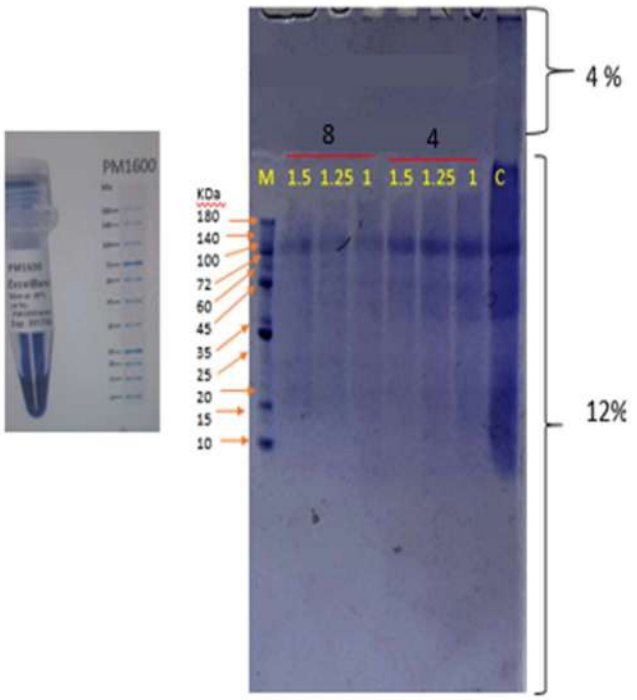


Figure 2. SDS–PAGE profiles of quail by-product proteins following enzymatic hydrolysis at different enzyme concentrations and incubation times

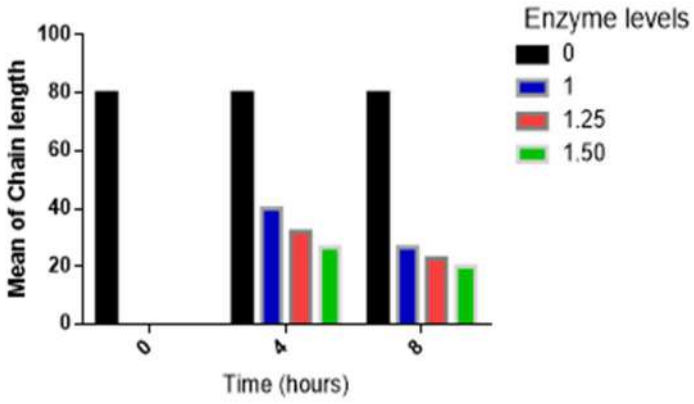


Figure 3. Calculated peptide chain length at different enzyme concentrations and incubation times

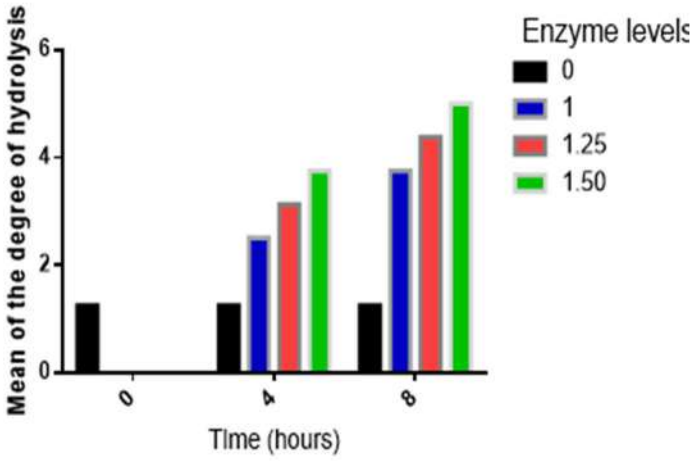


Figure 4. Degree of protein hydrolysis at different enzyme concentrations and incubation times

3.2. Encapsulation efficiency

The encapsulation efficiencies of peptide-loaded uncoated nanoliposomes, chitosan-coated nanoliposomes, and nanoniosomes are presented in Table 1. Peptide concentration was determined using a calibration curve described by the equation y = 488.96x − 137.13, with $R^2 = 0.99$.

Chitosan-coated nanoliposomes exhibited the highest encapsulation efficiency, followed by nanoniosomes and uncoated nanoliposomes. The overall difference among the three formulations was statistically significant (Kruskal–Wallis test, p = 0.02). Dunn–Bonferroni post hoc analysis showed a significant difference between chitosan-coated and uncoated nanoliposomes (p = 0.007). However, the difference between chitosan-coated nanoliposomes and nanoniosomes was not statistically significant (p = 0.17)

3.3. Physicochemical characterization

Dynamic light scattering (DLS) analysis of the available sample demonstrated a particle-size distribution characterized by D10, D50, and D90 values of 77.91, 93.73, and 129.55 nm, respectively. The D50 value indicated a median particle diameter of 93.73 nm, whereas 90% of the measured particles were smaller than 129.55 nm (Fig. 5).

Only one particle-size distribution was available; therefore, formulation-specific particle-size values could not be reported. The polydispersity index and zeta potential were not measured and are not reported. Scanning electron microscopy (SEM) provided representative images of the peptide fraction and nanocarrier preparations, allowing visual assessment of their morphology (Fig. 6).

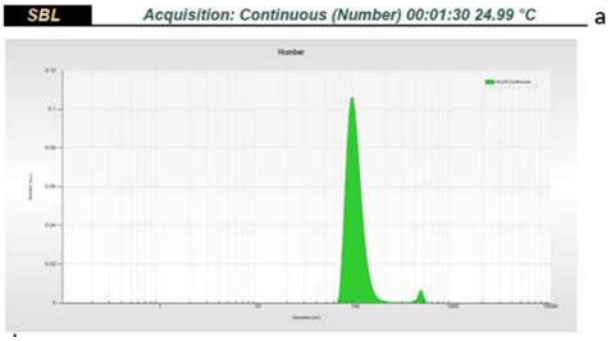
3.4. *In vitro* peptide release in simulated gastrointestinal fluids

Peptide release from uncoated nanoliposomes, chitosan-coated nanoliposomes, and nanoniosomes was evaluated in simulated gastric fluid (SGF; pH 1.2) and simulated intestinal fluid (SIF; pH 6.8) at 37 °C. The incubation times were 2 h for SGF and 1 h for SIF. Chitosan-coated nanoliposomes exhibited the lowest peptide release in both media, whereas uncoated nanoliposomes exhibited the highest release (Table 2). The overall differences among formulations were statistically significant in SGF (p = 0.02) and SIF (p = 0.04). Post hoc analysis demonstrated significant differences between uncoated and chitosan-coated nanoliposomes in SGF (p = 0.007) and SIF (p = 0.01). However, the differences between chitosan-coated nanoliposomes and nanoniosomes were not statistically significant in SGF (p = 0.18) or SIF (p = 0.10).

Table 1. Encapsulation efficiency of peptide-loaded uncoated nanoliposomes, chitosan-coated nanoliposomes, and nanoniosomes

Formulation	Encapsulation efficiency (%)
Uncoated nanoliposome, formulation control	68.65 ± 0.19[a]
Chitosan-coated nanoliposome	73.70 ± 0.22[b]
Nanoniosome	70.51 ± 0.25[ab]

Values are presented as mean ± standard deviation (n = 3). Different superscript letters indicate statistically significant differences according to the Dunn–Bonferroni post hoc test ($p < 0.05$). Overall Kruskal–Wallis test: $p = 0.02$.



Parameter	Value (nm)
D10	77.91
D50 (average size)	93.73
D90	129.55
Median diameter	108.84

Figure 5. Particle-size distribution of the analyzed preparation determined by dynamic light scattering. The D10, D50, and D90 values were 77.91, 93.73, and 129.55 nm, respectively.

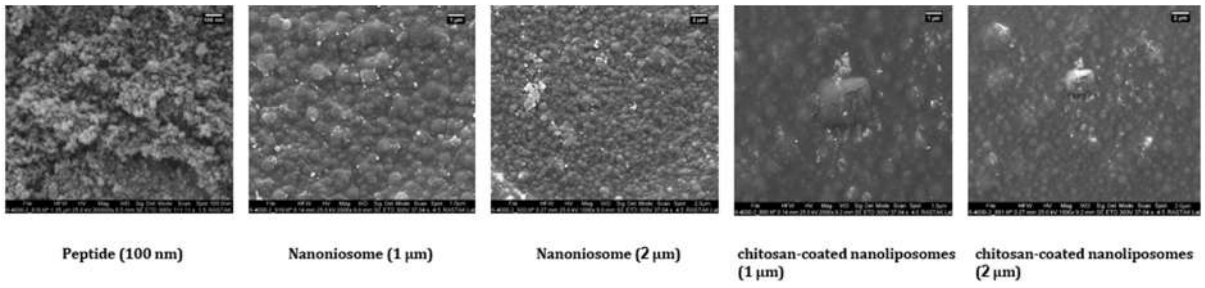


Figure 6. Representative scanning electron microscopy images of the peptide fraction and nanocarrier preparations. Scale bars correspond to 100 nm for the peptide image and 1–2 µm for the nanocarrier images

Table 2. Peptide release from uncoated nanoliposomes, chitosan-coated nanoliposomes, and nanoniosomes under simulated gastrointestinal conditions.

Formulation	SGF release after 2 h (%)	SIF release after 1 h (%)
Uncoated nanoliposome, formulation control	58.29 ± 1.01[a]	74.42 ± 1.01[a]
Chitosan-coated nanoliposome	42.15 ± 1.01[b]	57.31 ± 0.56[b]
Nanoniosome	53.56 ± 0.48[ab]	73.44 ± 1.01[ab]

Values are presented as mean ± standard deviation (n = 3). SGF, simulated gastric fluid, pH 1.2; SIF, simulated intestinal fluid, pH 6.8. Different superscript letters within the same column indicate statistically significant differences according to the Dunn–Bonferroni post hoc test ($p < 0.05$). Overall Kruskal–Wallis test: $p = 0.02$ for SGF and $p = 0.04$ for SIF

Table 3. Antioxidant activity of peptide-loaded nanocarrier formulations and the ascorbic acid positive control, evaluated using DPPH, ABTS, and FRAP assays

Formulation or control	DPPH IC_{50} (mg/mL)	ABTS IC_{50} (mg/mL)	FRAP (mg/g)
Uncoated nanoliposome, formulation control	2.90 ± 0.04	2.80 ± 0.03	1.79 ± 0.01
Chitosan-coated nanoliposome	1.55 ± 0.01	1.67 ± 0.01	2.13 ± 0.01
Nanoniosome	1.80 ± 0.02	2.60 ± 0.02	1.99 ± 0.01
Ascorbic acid, positive control	122.76 ± 0.63	0.04548 ± 0.00035	91.05 ± 2.02

Values are presented as mean ± standard deviation (n = 3). DPPH, 2,2-diphenyl-1-picrylhydrazyl; ABTS, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid); FRAP, ferric reducing antioxidant power; IC_{50}, half-maximal inhibitory concentration. The ABTS IC_{50} of ascorbic acid was converted from 45.48 ± 0.35 µg/mL to 0.04548 ± 0.00035 mg/mL. The original experimental record reports the DPPH IC_{50} of ascorbic acid in µg/mg; this unit requires verification before direct comparison with values expressed in mg/mL.

Table 4A. Minimum inhibitory concentrations of free peptides, peptide-loaded and peptide-free nanocarriers, and nisin against foodborne bacterial pathogens

Formulation or control	*S. aureus* MIC (mg/mL)	*E. coli* MIC (mg/mL)	*S. enteritidis* MIC (mg/mL)
Free peptide, nonencapsulated control	12.50 ± 0.00	20.83 ± 7.21	20.83 ± 7.21
Uncoated nanoliposome, peptide-free	41.66 ± 14.43	66.66 ± 28.86	83.33 ± 28.86
Uncoated nanoliposome, peptide-loaded	10.41 ± 3.60	12.50 ± 0.0001	16.66 ± 7.21
Chitosan-coated nanoliposome, peptide-free	33.33 ± 14.43	33.33 ± 14.43	33.33 ± 14.43
Chitosan-coated nanoliposome, peptide-loaded	8.33 ± 3.60	10.41 ± 3.60	12.50 ± 0.0001
Nanoniosome, peptide-free	33.33 ± 14.43	33.33 ± 14.43	41.66 ± 14.43
Nanoniosome, peptide-loaded	8.33 ± 3.60	16.66 ± 7.21	12.50 ± 0.0001
Nisin, positive control	0.01 ± 0.005	0.02 ± 0.0001	0.02 ± 0.01
Overall p value	0.007	0.006	0.005

Values are presented as mean ± standard deviation (n = 3). MIC, minimum inhibitory concentration. Overall p values were obtained using the Kruskal–Wallis test for the seven originally analyzed nanocarrier and nisin groups. The free-peptide control was evaluated separately and was not included in the reported overall statistical comparisons.

Table 4B. Minimum bactericidal concentrations of free peptides, peptide-loaded and peptide-free nanocarriers, and nisin against foodborne bacterial pathogens

Formulation or control	*S. aureus* MBC (mg/mL)	*E. coli* MBC (mg/mL)	*S. enteritidis* MBC (mg/mL)
Free peptide, nonencapsulated control	25.00 ± 0.00	50.00 ± 0.00	50.00 ± 0.00
Uncoated nanoliposome, peptide-free	No bactericidal effect	No bactericidal effect	No bactericidal effect
Uncoated nanoliposome, peptide-loaded	25.00 ± 0.0001	41.66 ± 14.43	50.00 ± 0.0001
Chitosan-coated nanoliposome, peptide-free	83.33 ± 28.86	83.33 ± 28.86	100.00 ± 0.0001
Chitosan-coated nanoliposome, peptide-loaded	16.66 ± 7.21	20.83 ± 7.21	25.00 ± 0.0001
Nanoniosome, peptide-free	66.66 ± 28.86	83.33 ± 28.86	100.00 ± 0.0001
Nanoniosome, peptide-loaded	12.50 ± 0.0001	25.00 ± 21.65	25.00 ± 21.65
Nisin, positive control	0.01 ± 0.005	0.02 ± 0.01	0.03 ± 0.01
Overall p value	0.007	0.01	0.007

Values are presented as mean ± standard deviation (n = 3). MBC, minimum bactericidal concentration; NR, not reported in the original experimental record. Overall p values were obtained using the Kruskal–Wallis test for the six originally analyzed nanocarrier and nisin groups with available MBC data. Overall, peptide incorporation was associated with improved antibacterial activity relative to the corresponding peptide-free formulations. However, the results indicate formulation- and microorganism-dependent effects rather than consistent superiority of chitosan-coated nanoliposomes over nanoniosomes.

3.5. Antioxidant activity

The antioxidant activities of the peptide-loaded formulations were evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and ferric reducing antioxidant power (FRAP) assays. Ascorbic acid was included as the positive control.

Among the nanocarrier formulations, chitosan-coated nanoliposomes exhibited the lowest DPPH and ABTS half-maximal inhibitory concentration (IC_{50}) values and the highest FRAP value (Table 3). Overall differences among the study groups were statistically significant for DPPH ($p = 0.01$), ABTS ($p = 0.01$), and FRAP ($p = 0.02$).

Post hoc analysis showed significant differences between chitosan-coated and uncoated nanoliposomes for DPPH ($p = 0.04$), ABTS ($p = 0.04$), and FRAP ($p = 0.03$). However, differences between chitosan-coated nanoliposomes and nanoniosomes were not statistically significant for DPPH ($p = 0.30$), ABTS ($p = 0.30$), or FRAP ($p = 0.27$)

3.6. Antimicrobial activity

The antibacterial activities of peptide-loaded and peptide-free nanocarriers were evaluated against *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella enteritidis* using MIC and MBC assays. Free peptides were included as a nonencapsulated control, and nisin served as the positive antimicrobial control.

Overall differences in MIC values among the originally analyzed formulation and nisin groups were statistically significant for *S. aureus* ($p = 0.007$), *E. coli* ($p = 0.006$), and *S. enteritidis* ($p = 0.005$). The corresponding overall differences in MBC values were also significant

for *S. aureus* (p = 0.007), *E. coli* (p = 0.01), and *S. enteritidis* (p = 0.007).

Peptide-loaded formulations generally exhibited lower MIC and MBC values than their corresponding peptide-free carriers. Among the peptide-loaded formulations, chitosan-coated nanoliposomes and nanoniosomes exhibited identical MIC values against *S. aureus*, 8.33 ± 3.60 mg/mL, and *S. enteritidis*, 12.50 ± 0.0001 mg/mL. Against *E. coli*, chitosan-coated nanoliposomes exhibited the lowest MIC among the peptide-loaded formulations, 10.41 ± 3.60 mg/mL, compared with 12.50 ± 0.0001 mg/mL for uncoated nanoliposomes and 16.66 ± 7.21 mg/mL for nanoniosomes (Table 4A).

For bactericidal activity, peptide-loaded nanoniosomes exhibited the lowest MBC against *S. aureus*, 12.50 ± 0.0001 mg/mL, whereas chitosan-coated nanoliposomes exhibited the lowest MBC against *E. coli*, 20.83 ± 7.21 mg/mL, among the peptide-loaded formulations. Both chitosan-coated nanoliposomes and nanoniosomes exhibited an MBC of 25.00 mg/mL against *S. enteritidis* (Table 4B). Direct comparisons between peptide-loaded chitosan-coated nanoliposomes and peptide-loaded nanoniosomes were not statistically significant for MIC or MBC outcomes.

4. Discussion

The present study investigated the encapsulation of BPs obtained from enzymatically hydrolyzed quail slaughterhouse by-products in three formulations: uncoated nanoliposomes, chitosan-coated nanoliposomes, and nanoniosomes. The findings indicate that chitosan-coated nanoliposomes exhibited the highest encapsulation efficiency and the lowest peptide release under simulated gastrointestinal conditions. However, their advantages were statistically significant primarily in comparison with uncoated nanoliposomes, whereas differences relative to nanoniosomes were generally not statistically significant.

Enzymatic hydrolysis was influenced by both incubation time and enzyme-to-substrate ratio. The highest degree of hydrolysis, 5.00%, and the lowest calculated peptide chain length, 20, were obtained after 8 h at an enzyme-to-substrate ratio of 1.5 (w/w). The reduction in the intensity of high-molecular-weight protein bands observed by SDS–PAGE further supported the occurrence of protein hydrolysis. These findings are consistent with previous studies reporting the conversion of animal-derived proteins into smaller peptide fractions following enzymatic treatment (19). Nevertheless, the available electrophoretic data do not establish the exact amino acid sequences or molecular identities of the resulting peptides.

Maximum encapsulation efficiency was found to be for chitosan coated nanoliposomes 73.70 ± 0.22% followed by nanoniosomes 70.51 ± 0.25% and uncoated nanoliposomes 68.65 ± 0.19%. The overall difference among formulations was statistically significant, but a significant difference was shown only between chitosan-coated and uncoated nanoliposomes in post hoc analysis. Therefore, the numerically higher encapsulation efficiency of chitosan-coated nanoliposomes than nanoniosomes should not be taken as a proof of statistical superiority. The enhanced performance compared to uncoated nanoliposomes is consistent with previous reports indicating that chitosan coating may reduce the escape of encapsulated

compounds and improve the retention of vesicles (10, 20, 21).

The results of the dynamic light scattering analysis showed that the analysed preparation contained particles in the nanometre range with D10, D50 and D90 values of 77.91, 93.73 and 129.55 nm, respectively. Particle size differences between the formulations could not be assessed however, as only one particle size distribution was available. Besides, the polydispersity index and zeta potential were not measured. Thus, claims of formulation-specific particle size uniformity, surface-charge modification or confirmation of successful chitosan coating based on zeta potential cannot be supported by the available data.

Chitosan-coated nanoliposomes released a lower proportion of encapsulated peptides than uncoated nanoliposomes in simulated gastrointestinal conditions. The percentage of peptide release in simulated gastric fluid was 42.15 ± 1.01% for chitosan coated nanoliposomes and 58.29 ± 1.01% for uncoated nanoliposomes. The values were 57.31 ± 0.56% and 74.42 ± 1.01% in simulated intestinal fluid, respectively. The differences were statistically significant. Release of nanoniosomes was numerically higher than chitosan-coated nanoliposomes. However, the difference between these two formulations was not statistically significant. The lower release seen for coated nanoliposomes may be due to the presence of an additional polymeric layer that reduces peptide diffusion, as indicated in previous studies with chitosan-based delivery systems (8, 22, 23). Release was, however, assessed at defined endpoints rather than by complete kinetic modelling, so the results indicate reduced release under the tested conditions, but do not demonstrate complete release mechanism or *in vivo* gastrointestinal protection.

Chitosan-coated nanoliposomes were among the nanocarrier formulations with the lowest half-maximal inhibitory concentration values in the DPPH and ABTS assays and the highest value in the FRAP assay. These results demonstrate a higher antioxidant activity than uncoated nanoliposomes, with significant differences between the two formulations. But the variations between the chitosan-coated nanoliposomes and nanoniosomes were not statistically meaningful. Hence, chitosan-coated nanoliposomes should be called as having numerically desirable antioxidant properties, rather than being definitively better than nanoniosomes. The observed activity may be attributed to preservation of the encapsulated peptides and possible chitosan contributions. However, the individual contributions of these factors were not directly quantified (22-24).

Antibacterial tests showed that the minimum inhibitory concentration and minimum bactericidal concentration values for the peptide-loaded nanocarriers were typically lower than their corresponding peptide-free formulations. Chitosan-coated nanoliposomes and nanoniosomes had the same minimum inhibitory concentrations against *Staphylococcus aureus* and *Salmonella enteritidis*, whereas chitosan-coated nanoliposomes had a numerically lower minimum inhibitory concentration against *Escherichia coli*. In contrast, the minimum bactericidal concentration of peptide-loaded nanoniosomes against *S. aureus* was numerically lower than that of chitosan-coated nanoliposomes. Direct comparisons between the two peptide-loaded formulations showed no statistical

significance. Thus, the results do not support the claim that chitosan-coated nanoliposomes consistently exhibited superior antibacterial activity than nanoniosomes.

Peptide-loaded formulations exhibited lower minimum bactericidal concentrations than corresponding peptide-free carriers, suggesting that the encapsulated peptides contributed to the antibacterial effects. Previous studies indicated that chitosan could interact with bacterial cell surfaces through electrostatic interactions, and could also influence the activity of the encapsulated antimicrobial compounds (21, 25). However, no dedicated mechanistic or synergy assays were conducted in the current study. Thus, the available data do not allow us to establish synergistic interactions between chitosan and the peptides.

From an application point of view, quail abattoir by-products represent an undervalued source of proteins that can be converted into hydrolysates containing peptides and integrated into nanocarrier formulations. The reported encapsulation efficiencies, antioxidant activities and antibacterial effects indicate potential importance for the development of functional ingredients and delivery systems in relation to food. BPs from poultry, fish and other protein-rich by-products have been proposed for applications (25-28). However, applications in foods, nutraceuticals, pharmaceuticals, or antimicrobial packaging still remain prospective since the formulations were not evaluated in real product matrices, animal models, or clinical settings.

5. Conclusion

The present study has identified the possibility of using chitosan-modified Nanoliposomes as a viable delivery system of BPs formed by quail slaughterhouse by-products. Encapsulation was found to enhance the stability of peptides, their antioxidant properties, and their antimicrobial properties against significant foodborne pathogens. It provided the ability to control release under simulated gastrointestinal conditions. These results suggest that these nanocarriers have the potential to modify the functional characteristics of BPS, presenting a potentially practical application in the food, nutraceutical, and pharmaceutical industries. However, additional research is needed on toxicity, sensory characteristics, and scalability.

6. Limitations

Some limitations of this study must be admitted. First, the antioxidant and antimicrobial properties of antimicrobial peptides encapsulated were promising; however, their potential toxicity, hemolytic toxicity on eukaryotic cells, and enzymatic hydrolysis in the human body were not effectively considered in the given work. Second, antimicrobial peptides have also been reported to develop resistance to some pathogens, primarily due to alterations in the cell membranes of these pathogens, which may limit the general use of these compounds. Third, the present study was performed in vitro conditions; therefore, the results cannot be wholly applied to in vivo systems, where physiological dynamics may affect stability and bioactivity. Additionally, the encapsulation process, in itself, can generate heterogeneity in peptide release rates and efficacy that will need to be further refined. Lastly, the paper failed to assess the long-term stability and large-scale applicability of the research nanocarriers, which should be evaluated in future studies.

Funding

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors

Author Contributions

Najme Ebrahimi: Investigation, Data curation, Formal analysis, Visualization, Writing – original draft.

Leila Golestan: Conceptualization, Methodology, Supervision, Project administration, Writing – review and editing.

Amir Ali Anvar: Formal analysis, Validation, Supervision, Writing – review and editing.

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All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no known financial or personal relationships that could have influenced the work reported in this manuscript.

Data Availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Acknowledgment

The authors would like to thank Mizan Sanjesh Pasargad Laboratory for providing laboratory services and technical support during this study.

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