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Antimicrobial activity of microencapsulated nisin with ascorbic acid and ethylenediaminetetraacetic acid prepared using double emulsion and freeze-drying technique against *Salmonella* Enteritidis ATCC 13076 in culture broth and minced fish

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Abstract

Importance of the work: Nisin, a 'generally recognized as safe' natural antimicrobial agent, has poor antimicrobial activity against Gram-negative bacteria and is easily combined with food components.

Objectives: To enhance the antimicrobial activity of nisin against Gram-negative bacteria and to prolong its antimicrobial activity using an encapsulating-technique.

Materials & Methods: Nisin (N) was combined with ascorbic acid (A) and ethylenediaminetetraacetic acid (EDTA, E) at final concentrations of 500 parts per million (ppm), 2,000 ppm and 250 ppm, respectively. Subsequently, double emulsion and freeze-drying techniques were applied to encapsulate the N-A-E mixture to decelerate their release and prolong their antimicrobial activity in culture broth and a minced fish sample.

Results: Besides Gram-positive bacteria, the N-A-E mixture reduced Gram-negative bacteria by 24.74–100%. The obtained microcapsules (MCs) had a smooth surface and spherical morphology with values for moisture content, water activity and production yield of 1.33–5.51%, 0.248–0.402% and 84.00–98.76%, respectively. The MCs prepared using a ratio of maltodextrin-to-gum arabic of 1:1, with a ratio of primary emulsion-to-outer aqueous phase of 1:9 (weight per weight), produced the highest encapsulation efficiency ($p < 0.05$) of nisin (44.40%). The MCs could completely inhibit *Salmonella* Enteritidis ATCC 13076 in culture broth (4.85 log CFU/mL) after 4 d of incubation and also exhibited gradual release. In addition, the MCs had an antibacterial effect on minced fish by reducing *S.* Enteritidis ATCC 13076 by 1.5 log CFU/g after storage at 4 °C for 8 d.

Main finding: The N-A-E mixture using an encapsulation approach showed potential as an alternative scheme to address the confined application of nisin in the food industry.

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Introduction

Contamination of fish and shellfish with *Salmonella* is a major public health concern and *Salmonella* Typhimurium together with *Salmonella* Enteritidis are important serovars in human infection (Fernandes et al., 2018). *Salmonella* frequently contaminates various kinds of food, including fish and shellfish (Novoslavskij et al., 2016). While numerous physical and chemical preventative techniques have been proposed to control *Salmonella* contaminated in food, one of reliable alternative approaches for controlling *Salmonella* is to focus on the application of natural antimicrobial agents (Mahmud et al., 2018).

Nisin, a bacteriocin produced from a safety strain of *Lactococcus lactis* subsp. *lactis*, is widely used as a food preservative and has been certificated by the Joint Expert Committee on Food Additives (JECFA), the US Food and Drug Administration (FDA) and the European Union (EU) as generally recognized as safe (GRAS) (EFSA, 2017). It can inhibit the growth of major Gram-positive foodborne pathogenic bacteria, such as *Clostridium botulinum, Listeria monocytogenes* and *Staphylococcus aureus* (Ibarra-Sánchez et al., 2020). The advantages of utilizing nisin in the food industry are: it is colorless, and odorless, non-toxic to the consumer and has high heat resistance under acidic conditions (Tong et al., 2014). However, nisin cannot inhibit the growth of Gram-negative bacteria due to its limited penetration through the outer membrane of Gram-negative bacteria (Martin-Visscher et al., 2011). In addition, its antimicrobial efficiency gradually reduces during food processing and storage (Aasen et al., 2003). Nisin easily combines with food components, with the remaining nisin providing inefficient inhibition of bacterial growth (Gharsallaoui et al., 2016). These limitations can be diminished by mixing nisin with other antimicrobial compounds and subsequently entrapping the mixture using encapsulation techniques to retard the reaction between nisin and the food components, while maintaining the antimicrobial activity against the target bacteria of nisin (Ibarra-Sánchez et al., 2020).

Previously, nisin was applied in various fishery products using different approaches, such as: 1) individual use (Behnam et al., 2015); 2) combining with lactic acid (Shirazinejad et al., 2010), plant extract and partially purified bacteriocin produced by *Bacillus velezensis* (Nimrat et al., 2021) and grape seed extract (Zhao et al., 2020); and 3) integrating with ethylenediaminetetraacetic acid (EDTA) in food packaging (Chang et al., 2021). The current research aimed to determine potential antimicrobial agents to enhance nisin efficiency in inhibiting the growth of target foodborne pathogens and spoilage bacteria. Subsequently, an encapsulation technique was used to encapsulate nisin and the target antimicrobial agent mixture to retard their release, as well as prolong antimicrobial efficiency when applied in a minced fish sample. The proposed scheme could be an alternative approach to expand the application of nisin in controlling foodborne pathogens and spoilage bacteria in the food and related industries.

Materials and Methods

Chemicals and culture media

Chemicals: Nisin (Nisaplin®) was purchased from Danisco (Grindsted, Denmark), ascorbic acid from Riedel-Graham (Barrington, IL, USA), ethylenediaminetetraacetic acid (EDTA) from Ajax (Taren Point, Australia), maltodextrin DE 10 (Stardri® 10S) from Tate & Lyle (Decatur, IL, USA), gum arabic (KB-120) from Jumbo Trading Co., Ltd. (Chachoengsao, Thailand), soybean oil (A-Ngoon®) from Thai vegetable oil Co., Ltd. (Nakhon Pathom, Thailand), span 60 (sorbitan monostearate) from Merck (Darmstadt, Germany), Tween 20 (polyoxyethylene (20) sorbitan monolaurate) from Sigma (Saint Louis, MO, USA), silicon dioxide (SiO_2, Tokusil FC®) from Tokuyama (Rayong, Thailand). All other reagents used in this study were of analytical grade.

Culture media: Mueller Hinton broth (MHB), tryptic soy agar (TSA), tryptic soy broth (TSB), yeast extract, plate count agar (PCA) and xylose lysine desoxycholate agar (XLD agar) were acquired from Difco (Sparks, MD, USA) and glycerol from Merck (Darmstadt, Germany).

Bacterial strains preparation

Escherichia coli ATCC 25922, *Listeria innocua* ATCC 33090, *Micrococcus luteus* IFO 12708, *Morganella morganii* ATCC 25830, *S.* Enteritidis ATCC 13076, *Salmonella* Weltevreden DMST 3380 and *S. aureus* ATCC 25923 were used as the target strains. Each target strain was stocked in TSB+0.6% (weight per volume, w/v) yeast extract containing 20% (volume per volume, v/v) glycerol at -18 °C. Before use, all target strains were cultured in TSB+0.6% (w/v) yeast extract and incubated at 35 °C for 18 h. After incubation, each strain was serially diluted with TSB+0.6% (w/v) yeast extract to a final population of approximately 7.0 log CFU/mL.

Preparation of nisin, ascorbic acid and ethylenediaminetetraacetic acid solution

Stock solution of nisin (commercial nisin containing 1×10^6 IU of pure nisin/g), ascorbic acid and EDTA were prepared by dissolving each antimicrobial agent in distilled water at final concentrations of 25,000 parts per million (ppm), 100,000 ppm and 12,500 ppm, respectively. Then, the solutions were sterilized using filtration through a syringe filter with a membrane pore size of 0.22 µm (Minisart; Göttingen, Germany).

Screening on antimicrobial activity of nisin, ascorbic acid and ethylenediaminetetraacetic acid against target tested strains in culture broth

Stock solutions of nisin (N), ascorbic acid (A), EDTA (E), as well as a mixture of nisin-ascorbic acid (N-A), nisin-EDTA (N-E), ascorbic acid-EDTA (A-E) and nisin-ascorbic acid-EDTA (N-A-E) were added into MHB to yield final concentrations of 500 ppm, 2,000 ppm and 250 ppm for nisin, ascorbic acid and EDTA, respectively. Subsequently, 100 µL of log phase growth of each target strain was separately inoculated into 10 mL of each antimicrobial agent-treated MHB to a final population of 5.0 log CFU/mL, with the same target strain grown in MHB without any antimicrobial agent used as the control. Then, the samples were incubated at 35 °C for 24 h. After incubation, the viable cell number was determined using a spread plate technique on TSA+0.6% (w/v) yeast extract and a incubated agar plate at 35 °C for 48 h (Suksathit et al., 2013). Reduction of the target bacteria number (%) in each antimicrobial agent-treated MHB was calculated by comparing with the same target bacteria number grown in MHB without any antimicrobial agent.

Preparation of dried double emulsion microcapsules

The N-A-E mixture was added to a water/oil/water (W/O/W) emulsion using the modified two-step emulsification method (Khalid et al., 2013). The primary emulsion (W_1/O) was prepared using the inner aqueous phase (W_1) and oil phase (O) with a ratio of 20:80 (weight per weight, w/w). The ratios of maltodextrin (MD) and gum arabic (GA), which were used as the outer aqueous phase (W_2), as well as W_1/O and W_2, are shown in Table 1. W_1 was prepared by dissolving nisin, ascorbic acid and EDTA in distilled water at final concentrations of 0.05%, 0.2% and 0.025% (w/w), respectively, then mixing using a magnetic stirrer for 30 min (Global Lab; GLHPS-G; Gyeonggi-do, Korea). O was prepared by dispersing 0.2% (w/w) hydrophobic emulsifier Span 60 into soybean oil and heating to 60 °C for 10 min. The W_1/O emulsion was prepared by mixing W_1 and O using a high-speed homogenizer (IKA, T25 digital Ultra-Turrax; Baden-Württemberg, Germany) at 15,000 rpm for 15 min and further passing the solution through a high-pressure homogenizer (15 MR-8TA; APV-Gaulin Inc.; Wilmington, MA, USA) at 20,684.27 kPa. W_2 was prepared by dissolving MD and GA in distilled water, containing 1% (w/w) Tween 20 (hydrophilic emulsifier) together with 2% (w/w) SiO_2 and mixed using magnetic stirring for 30 min. Then, the W_1/O was gradually dispersed into the W_2 and mixed using a high-speed homogenizer at 15,000 rpm for 15 min, followed by passing through a high-pressure homogenizer at 20,684.27 kPa to produce the W_1/O/W_2 double emulsion. The W_1/O/W_2 double emulsion preparation was performed at room temperature. After preparation, the W_1/O/W_2 double emulsion was placed in a Petri dish and frozen at -40 °C. After 24 h, the frozen emulsion was dried at -40 °C under a pressure of 0.1 mbar for 24 h using a laboratory scale freeze dryer (Scan Vac, Coolsafe Pro; Hovedstaden, Denmark) to obtain the encapsulated powder (microcapsules; MCs).

Table 1 Composition of water-in-oil-in-water (W_1/O/W_2) emulsion

Treatment	W_1/O emulsion							W_1/O/W_2 emulsion						
	W_1:O ratio (w/w)	W_1 phase				O phase		W_2 phase					Distilled water	W_1/O:W_2 ratio (w/w)
		Nisin	Ascorbic acid	EDTA	Distilled water	Soybean oil	Span 60	MD:GA ratio (w/w)	MD	GA	Tween 20	SiO_2		
1	1:4	0.05	0.20	0.025	1.725	7.80	0.20	1:1	13.50	13.50	1.00	0.726	63.000	1:9
2	1:4	0.10	0.40	0.050	1.450	15.60	0.40	1:1	12.00	12.00	1.00	0.832	57.620	2:8
3	1:4	0.15	0.60	0.075	1.175	23.40	0.60	1:1	10.50	10.50	1.00	0.936	52.238	3:7
4	1:4	0.20	0.80	0.100	0.900	31.20	0.80	1:1	9.00	9.00	1.00	1.042	46.858	4:6
5	1:4	0.05	0.20	0.025	1.725	7.80	0.20	5:1	22.50	4.50	1.00	0.363	63.000	1:9
6	1:4	0.10	0.40	0.050	1.450	15.60	0.40	5:1	20.00	4.00	1.00	0.416	57.620	2:8
7	1:4	0.15	0.60	0.075	1.175	23.40	0.60	5:1	17.50	3.50	1.00	0.468	52.238	3:7
8	1:4	0.20	0.80	0.100	0.900	31.20	0.80	5:1	15.00	3.00	1.00	0.521	46.858	4:6

EDTA = ethylenediaminetetraacetic acid; MD = maltodextrin; GA = gum arabic;
Each composition in emulsion expressed as percentage by weight (%, w/w)

Determination of physical and chemical properties of microcapsules

The moisture content of the MCs was determined according to Association of Official Analytical Chemists (2005) and water activity (a_w) was analyzed using a water activity meter (Aqualab, 4TEV; Riverside, CA, USA). The color was analyzed using a chroma meter (Konica Minolta, CM-3500d; Osaka, Japan) and expressed in values of L* (darkness/whiteness), a* (greenness/redness) and b* (blueness/yellowness). The microscopic aspects of the MCs were observed using the scanning electron microscopy (SEM; Hitachi, SU8020; Tokyo, Japan). The concentrations of nisin, ascorbic acid and EDTA were determined as described below. The encapsulation efficiency (EE) of the MCs was calculated according to Equation 1 as described by Piacentini (2016):

$$EE\ (\%) = (\text{Analyzed concentration} / \text{Initial concentration}) \times 100 \quad (1)$$

Concentrations of nisin, ascorbic acid and ethylenediaminetetraacetic acid in microcapsules

A sample of MCs (2 g) was mixed with 10 mL of 0.02 N HCl and allowed to dissolve in a sonicator for 20 min. The supernatant was separated using centrifugation at 7,000 rpm for 10 min and then sterilized by passing through a syringe filter with a membrane pore size of 0.22 µm.

The concentration of nisin was determined using agar well diffusion assay (Lalpuria et al., 2012) and *M. luteus* IFO 12708 was used as an indicator strain. Each agar well was filled with 100 µL of nisin standard solution or the supernatant of dissolved MCs. After incubation, the inhibition zone diameter was measured in millimeters and used to calculate the concentration of nisin by comparing to the nisin standard curve (coefficient of determination = 96.38%). The nisin concentration in milligrams per gram was calculated using Equation 2:

$$\text{Nisin} = [(X + 0.2091) / 4.4801] / 1{,}000 \quad (2)$$

where X is the diameter of inhibition zone (in millimeters) and 0.2091 and 4.4801 are the correlation coefficients obtained from the regression equation of the standard nisin concentration and inhibition zone diameter, respectively.

The concentration of ascorbic acid was determined according to the 2,6-dichlorophenol indophenol titrimetric method (Association of Official Analytical Chemists, 2005) with modifications. A sample (2 mL) of the supernatant of dissolved MCs was mixed with 5 mL of metaphosphoric acid-acetic acid solution and then slowly titrated with indophenol solution. The ascorbic acid concentration in milligrams per gram was calculated using Equation 3:

$$\text{Ascorbic acid} = [(X - B) \times F \times V] / E \times Y \quad (3)$$

where X and B are the volumes of indophenol solution in milliliters used for the sample and blank titration, respectively, F is the amount of ascorbic acid in milligrams equivalent to 1.0 mL indophenol standard solution obtained from standardization, E is the amount of sample in grams or milliliters and V and Y are the volumes in milliliters of the initial test solution and test solution titrated, respectively.

The concentration of EDTA was determined using the zinc chloride titrimetric method (Hamano et al., 1995; The Japan Food Chemical Research Foundation, 2009). Briefly, supernatant of dissolved MCs (2 mL) was mixed with 20 mL of 0.1 M ammonium buffer solution (pH 8.5) and 0.1 mL of eriochrome black T, followed by titration with 0.01 M zinc chloride. The EDTA concentration in milligrams per gram was calculated using Equation 4:

$$\text{EDTA} = V \times 3.7224 \quad (4)$$

where V is the volume of zinc chloride in milliliters used for the sample titration.

Antimicrobial effect of microcapsules against S. Enteritidis ATCC 13076 in culture broth

The antimicrobial activities against *S.* Enteritidis ATCC 13076 of MC and non-encapsulated N-A-E mixture in culture broth were compared. Non-encapsulated N-A-E mixture was suspended in 10 mL of MHB to obtain final concentrations of nisin, ascorbic acid and EDTA of 500 ppm, 2,000 ppm and 250 ppm, respectively. The MCs in treatment 1 that had the highest EE of nisin (44.40%) was selected for the MC treatment. The amount of MCs that could be suspended in MHB was calculated based on an initial nisin concentration added into the double emulsion before freezing. Then, 3.7 g of MCs were dispersed in 10 mL of MHB to obtain a final concentration of nisin of 500 ppm. The log phase growth of *S.* Enteritidis ATCC 13076 was inoculated into treated MHB at a final population of 5.0 log CFU/mL and incubated at 35 °C for 5 d. At selected time intervals, the number of *S.* Enteritidis ATCC 13076 was

enumerated on TSA plates using a spread plate technique. After incubation, colonies of target strains were counted, calculated and expressed as log colony forming units per milliliter (Suksathit et al., 2013).

Antimicrobial effect of microcapsules containing N-A-E mixture on target bacterial number in minced fish sample

The minced fish sample was composed of 70.25% (w/w) fresh fish meat, 2.5% (w/w) salt, 1.35% (w/w) egg white powder, 0.3% (w/w) sugar, 2.35% (w/w) spice, 6.75% (w/w) soybean oil, 3% (w/w) tapioca starch and 13.5% (w/w) crushed ice. The fresh fish meat was minced in a blender (Panasonic, MK-5080M; Pataling Jaya, Malaysia) and combined with all the ingredients. After blending, the minced fish was separated into two portions. The first portion was mixed with MCs to obtain a final concentration of nisin at 500 ppm, while the other half was used as the control (without any antimicrobial agent). Subsequently, each portion was divided into two treatments: with and without *S.* Enteritidis ATCC 13076 at a final population of approximately 6 log CFU/g based on the optical density of the cell suspension before inoculating to the minced fish sample, according to a preliminary trial. All treatments were stored in sterile Petri dishes at 4 °C for 8 d. On days 0, 2, 4, 6 and 8, the total viable count (TVC) was enumerated using a pour plated technique and PCA agar, with the *S.* Enteritidis ATCC 13076 number enumerated using a spread plate technique on *Salmonella* selective media (XLD agar). All plates were incubated at 35 °C for 48 h. After incubation, the TVC and *S.* Enteritidis ATCC 13076 were determined (Suksathit et al., 2013).

Statistical analysis

Each experiment was conducted in three replications and the results were expressed as mean ± SD. Analysis of variance was performed and means were compared using Duncan's multiple range tests (significance level at $p < 0.05$).

Results and Discussion

Screening on antimicrobial activity of nisin, ascorbic acid and ethylenediaminetetraacetic acid against target tested strains in culture broth

After 24 h incubation, the N-A-E mixtures at final concentrations of 500 ppm, 2,000 ppm and 250 ppm, respectively, had the highest antimicrobial efficiency against all seven target tested strains (Table 2). Based on the calculated ratio of total number reduction, the N-A-E mixture could completely inhibit the growth of *E. coli* ATCC 25922, *L. innocua* ATCC 33096 and *M. luteus* IFO 12708 compared to the same strain grown in MHB without any antimicrobial agent (control),

Table 2 Reduction of target bacterial number in Mueller Hinton broth treated with nisin, ascorbic acid and ethylenediaminetetraacetic acid

Tested strains	Reduction in bacterial number (%)						
	Nisin (N)	Ascorbic acid (A)	EDTA (E)	N-A	N-E	A-E	N-A-E
E. coli ATCC 25922	0.91±0.85^{dC}	0.84±1.35^{dE}	-0.11±0.84^{dE}	0.44±0.81^{dC}	33.68±0.22^{bB}	8.00±1.30^{eE}	100.00±0.00^{aA}
M. morganii ATCC 25830	-2.45±6.14^{eC}	6.62±0.72^{cdC}	5.44±0.58^{cdD}	1.38±0.64^{deC}	0.02±1.02^{eE}	18.55±0.23^{bD}	24.74±0.96^{aE}
S. Weltevreden DMST 3380	-0.91±1.62^{cdC}	-2.22±2.07^{deF}	-4.78±1.07^{fF}	-4.18±1.00^{efD}	0.63±1.57^{cdE}	18.05±0.61^{bD}	29.25±0.94^{aD}
S. Enteritidis ATCC 13076	0.94±0.74^{dC}	2.64±1.96^{dDE}	0.49±1.02^{dE}	1.26±2.26^{dC}	16.76±2.30^{bD}	7.25±0.77^{eE}	39.45±1.29^{aC}
L. innocua ATCC 33096	100.00±0.00^{aA}	4.81±0.32^{dCD}	51.20±0.72^{bB}	100.00±0.00^{aA}	100.00±0.00^{aA}	44.21±1.01^{cC}	100.00±0.00^{aA}
M. luteus IFO 12708	100.00±0.00^{aA}	100.00±0.00^{aA}	100.00±0.00^{aA}	100.00±0.00^{aA}	100.00±0.00^{aA}	100.00±0.00^{aA}	100.00±0.00^{aA}
S. aureus ATCC 25923	28.65±1.31^{dB}	19.65±1.43^{eB}	45.63±0.85^{cC}	44.38±1.22^{cB}	29.56±1.07^{dC}	63.51±0.18^{bB}	81.06±1.62^{aB}

N = nisin; A = ascorbic acid; E = ethylenediaminetetraacetic acid (EDTA).

Values (mean ± SD) in the same row with different lowercase superscripts are significantly ($p < 0.05$) different and different uppercase superscripts indicate significantly ($p < 0.05$) different among means in the same column.

Negative mean values indicate that after 24 h incubation, bacterial numbers were higher than for the control.

while *S. aureus* ATCC 25923 was reduced by 81.06%. The N-A-E mixture could reduce *S.* Enteritidis ATCC 13076, *S.* Weltevreden DMST 3380 and *M. morganii* ATCC 25830 by 39.45%, 29.25% and 24.74%, respectively. For the same period, treatment with only nisin, N-E or N-A could completely inhibit the growth of only some target Gram-positive bacteria (*L. innocua* ATCC 33096, *M. luteus* IFO 12708). These results suggested that *L. innocua* ATCC 33096 and *M. luteus* IFO 12708 were sensitive to nisin. Notably, treatment with only nisin, ascorbic acid, EDTA, N-A, N-E or A-E did not inhibit growth or only showed poor antimicrobial activity against the target Gram-negative bacteria (*E. coli* ATCC 25922, *S.* Weltevreden DMST 3380 and *S.* Enteritidis ATCC 13076). However, the N-A-E mixture had much higher antimicrobial efficiency against these target strains. This evidence suggested that there was a synergistic interaction among nisin, ascorbic acid and EDTA that enhanced antimicrobial activity against these target Gram-negative bacteria.

The antimicrobial effect of nisin on Gram-positive bacteria is due to the binding of the nisin molecule to lipid II in the bacterial cytoplasmic membrane in pore formation followed by the efflux of cellular constituents and interruption of cell wall biosynthesis (Zhou et al., 2016). Gram-negative bacteria have a selective permeability barrier, composing of phospholipids and lipopolysaccharide that prevent the nisin molecule binding with lipid II in the cytoplasmic membrane (Li et al., 2018). Several studies have reported on enhancing the antimicrobial activity of nisin by combining it with EDTA (Belfiore et al., 2007; Martin-Visscher et al., 2011; Prudêncio et al., 2015). Normally, EDTA can destabilize the outer membrane of Gram-negative bacteria by releasing a lipopolysaccharides layer and allowing nisin to attack the cytoplasmic membrane (Gomes et al., 2021). However, the combination of nisin and EDTA in the current study had rather low antimicrobial effects against the Gram-negative tested strains. The antimicrobial activity of ascorbic acid was due to its pH lowering effect (Mousavi et al., 2019). When the pH of the solution lower than the pKa of ascorbic acid (4.10), the proportion of undissociated form increases (Bevilacqua et al., 2023). Only its undissociated form is able to pass across the plasma membrane into the cytoplasm, resulting in acidification (Seekles et al., 2022). In the current study, the pH values of MHB with only ascorbic acid, N-A, A-E or N-A-E mixtures were 5.37, 5.38, 5.40 and 5.42, respectively, (data not shown), which were higher than the pKa value of ascorbic acid. However, the MHB with ascorbic acid still showed strong antimicrobial effects against some tested strains, indicating that besides the environmental pH there were other factors, such as the final concentration (Verghese et al., 2017; Tajkarimi and Ibrahim, 2011) and sensitivity of the target strain (Przekwas et al., 2020) involved with the antimicrobial activity of ascorbic acid.

Other researchers have reported on the effects of a combination of nisin with only an individual antimicrobial compound. However, in the current study, the N-A-E mixture proved to be highly effective against all of seven tested strains. This synergistic antimicrobial activity could be explained by two major reactions. First, it is known that the pH affects the solubility of nisin (Fang et al., 2017), with the solubility high at a low pH. At pH 2, nisin solubility is 57 mg/mL and decreases to 1.5 mg/mL at pH 6 (Liu and Hansen, 1990). In the current study, ascorbic acid increased the stability of nisin by binding to the nisin molecule and also increasing nisin solubility by decreasing the pH of solution (Adhikari et al., 2012). Secondly, EDTA chelated the Ca and Mg salts that then interacted with lipopolysaccharide, resulting in denaturation of the lipopolysaccharide structure in the outer membrane of Gram-negative bacteria and allowed a nisin molecule to be inserted into the cytoplasmic membrane (Helander and Mattila-Sandholm, 2000).

Determination of physical and chemical properties of microcapsules

The physical and chemical properties of MCs prepared at different MD-to-GA ratios and W_1/O emulsion volumes are shown in Table 3. The moisture content and a_w were the most important factors preventing the growth of microorganisms in the MCs during storage. The MCs moisture content and a_w should be below 6% and 0.6, respectively (Conde-Islas et al., 2019). In the current study, the moisture content of all samples varied in the range 1.33–5.51%. The moisture content significantly increased with an increased W_2 phase and GA content. The MCs produced with GA had the higher moisture content due to the high hygroscopicity of GA which has hydrophilic groups and therefore binds to water molecules, which prevents it from escaping (Esmaeili et al., 2022). The a_w is an essential index to determine the shelf life of MCs and it is related to the moisture content. In Table 3, the a_w of the MCs was in the range 0.248–0.402, which is considered microbiologically stable (Ashwar, et al., 2018). Nawi et al. (2015) reported that MCs produced using a combination of MD and GA had the lowest a_w, followed by those produced using MD alone and GA alone, respectively.

Table 3 Physical properties of microcapsules containing nisin, ascorbic acid and ethylenediaminetetraacetic acid

Treatment	Moisture (%)	Water activity	L*	a*	b*	Yield (%)
1	5.51±0.11^{A}	0.402±0.011^{A}	93.71±0.09^{B}	0.36±0.05^{B}	7.46±0.13^{A}	98.76±0.01^{A}
2	3.90±0.05^{C}	0.388±0.217^{A}	90.71±0.46^{D}	0.45±0.10^{AB}	7.02±0.34^{B}	91.18±0.01^{G}
3	2.41±0.02^{E}	0.380±0.002^{AB}	90.35±0.20^{D}	0.44±0.09^{AB}	6.89±0.21^{B}	91.82±0.01^{F}
4	2.17±0.12^{F}	0.271±0.001^{C}	89.86±0.24^{D}	0.52±0.09^{A}	7.62±0.19^{A}	97.93±0.01^{C}
5	4.20±0.15^{B}	0.396±0.003^{A}	95.08±1.22^{A}	0.09±0.04^{C}	4.54±0.22^{C}	92.34±0.01^{E}
6	2.84±0.10^{D}	0.331±0.000^{B}	92.52±0.78^{C}	0.04±0.04^{C}	4.16±0.31^{D}	98.49±0.01^{B}
7	1.77±0.09^{G}	0.256±0.004^{C}	90.59±0.86^{D}	-0.41±0.05^{D}	3.38±0.30^{E}	94.42±0.01^{D}
8	1.33±0.06^{H}	0.248±0.004^{C}	90.68±1.01^{D}	-0.49±0.05^{D}	3.52±0.42^{E}	84.00±0.01^{H}

Detail of treatments are in Table 1; L*, a* and b* values represent lightness (0 to 100), greenness (-) to redness (+) and blueness (-) to yellowness (+), respectively (Zhang et al., 2022).

Values (mean ± SD) in the same column with different uppercase superscripts are significantly ($p < 0.05$) different.

Product color information of MCs is needed for their further application in colorless or white food products. In the current study, the MCs color was determined based on L*, a* and b* values, representing lightness (0–100), greenness (-) to redness (+) and blueness (-) to yellowness (+), respectively (Zhang et al., 2022). The obtained MC powders were off-white in color with the L*, a*, and b* values in the ranges 89.86–95.08, -0.49 to 0.52, and 3.38–7.62, respectively (Table 3). These findings were consistent with Tan et al. (2015) who reported that the L* value of MCs with MD and GA was 92.41 and with Dayal et al. (2018) who also reported that the L* value increased with an increasing W_2 phase content. In addition, the a* and b* values of the obtained MCs tended to significantly decrease along with a higher ratio of MD-to-GA in the W_2 phase. The distinction between the b* values of the MCs prepared using different ratios of MD-to-GA in the W_2 phase was possibly related to the light yellow color of gum arabic.

Normally, the production yield of MCs is related to the total solid content in the emulsion. However, the production yields for all MCs in the current study were in the range 84.00–98.76% and not related to the ratios of MD-to-GA or W_1/O-to-W_2. Treatment 1 with ratios of MD-to-GA and W_1/O-to-W_2 of 1:1 and 10:90, respectively, had the highest production yields. The obtained MCs had a smooth surface and spherical shape when observed using SEM (Fig. 1) as a result of the phase isolation of substances during freeze-drying, leading to consequent microcapsule formation under freeze-drying conditions (Degobert and Aydin, 2021).

The EE of nisin and related combined compounds in the MCs is required for their further application. The final concentrations of nisin, ascorbic acid, EDTA and the EEs of each antimicrobial agent in the MCs are displayed in Table 4. The EEs of nisin, ascorbic acid and EDTA in MCs prepared using the different treatments were in the ranges 0.62–44.40%,

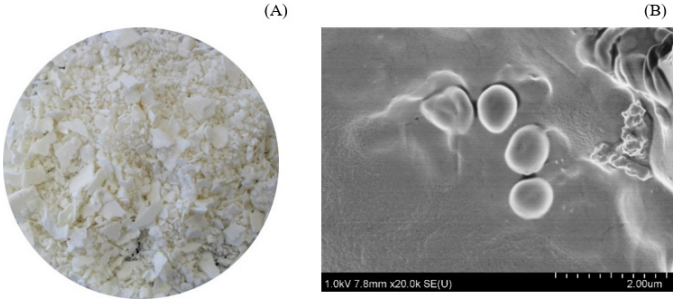

Fig. 1 Encapsulated powder of nisin with ascorbic acid and ethylenediaminetetraacetic acid: (A) after freeze drying; (B) under a scanning electron microscope

Table 4 The concentrations of nisin, ascorbic acid and ethylenediaminetetraacetic acid in microcapsules (MCs) and calculated encapsulation efficiency (EE)

Trt	Nisin (mg/g MC)			Ascorbic acid (mg/g MC)			EDTA (mg/g MC)		
	Initial	Analyzed	EE (%)	Initial	Analyzed	EE (%)	Initial	Analyzed	EE (%)
1	1.35	0.60±0.04^{B}	44.40±3.03^{A}	5.40	0.59±0.05^{H}	10.99±0.98^{G}	0.6757	0.56±0.00^{B}	83.37±0.64^{A}
2	2.36	0.02±0.00^{F}	0.99±0.00^{F}	9.44	1.45±0.17^{F}	15.37±1.81^{F}	1.1798	0.51±0.03^{C}	43.12±2.91^{B}
3	3.14	0.02±0.00^{F}	0.74±0.00^{F}	12.56	2.13±0.12^{E}	16.94±0.92^{EF}	1.5703	0.39±0.01^{D}	24.57±0.76^{D}
4	3.76	0.02±0.00^{F}	0.62±0.00^{F}	15.05	3.10±0.16^{C}	20.56±1.08^{D}	1.8818	0.67±0.02^{A}	35.61±1.30^{C}
5	1.35	0.54±0.03^{C}	40.27±2.54^{B}	5.40	1.04±0.11^{G}	19.17±2.11^{DE}	0.6757	0.07±0.00^{G}	10.65±0.64^{F}
6	2.36	0.38±0.07^{E}	15.97±2.90^{D}	9.44	2.44±0.21^{D}	25.92±2.21^{C}	1.1798	0.04±0.01^{H}	3.37±1.01^{H}
7	3.14	0.68±0.04^{A}	21.71±1.17^{C}	12.56	6.26±0.10^{B}	49.83±0.83^{A}	1.5703	0.18±0.01^{F}	11.69±0.72^{F}
8	3.76	0.50±0.04^{D}	13.22±0.93^{E}	15.05	6.94±0.24^{A}	46.08±1.60^{B}	1.8818	0.27±0.01^{E}	14.24±0.52^{E}

Detail of treatments are in Table 1; Values (mean ± SD) in the same column with different uppercase superscripts are significantly ($p < 0.05$) different.

10.99–49.83% and 3.37–83.37%, respectively. Treatment 1 had the significantly highest EE for nisin (44.40%) and EDTA (83.37%), while treatment 7 had the highest EE for ascorbic acid (49.83%). Several studies have reported on the EE of nisin in liposome prepared using different wall materials, such as phosphatidylcholine liposome (EE 77–87%) (Lopes et al., 2019), 5% soy lecithin (EE 47%) (Imran et al., 2015) and pectin-chitosan polyelectrolyte complex (EE 65.9%) (Wang et al., 2017). However, encapsulation of nisin with MD and GA has not been reported. Nevertheless, the EEs of catechins and total phenolic compound encapsulated using a combination of MD and GA were in the ranges 78–81% and 26.83%, respectively (Pudziuvelyte et al., 2020; Ruengdech and Siripatrawan, 2022). Based on the maximum EE of nisin and the production yield together with other physical properties, MCs prepared according to treatment 1 condition was selected for the next experiment.

Antimicrobial effect of microcapsules on S. Enteritidis ATCC 13076 in culture broth

S. Enteritidis is a major foodborne serovar in human infection (Fernandes et al., 2018) and a preliminary study found that *S*. Enteritidis ATCC 13076 was the most resistant strain against the N-A-E mixture among all the target Gram-negative bacteria (data not shown). Consequently, *S*. Enteritidis ATCC 13076 was selected as a target indicator strain to investigate the antimicrobial efficiency of the MCs. The *in vitro* study indicated that on the first day of incubation period, the growth of *S*. Enteritidis ATCC 13076 in MHB containing the selected MC (treatment 1) increased from 4.85 to 8.10 log CFU/mL. Subsequently, the number of *S*. Enteritidis ATCC substantially decreased until there was none detected after incubation for 4 d (Fig. 2). The growth of the target bacteria in MHB without any antimicrobial agent (control) increased from 4.88 to 8.78 log CFU/mL after incubation for 1 d and was maintained at almost the same level (8.44–8.78 log CFU/mL) throughout the incubation period. In addition, MHB treated with the non-encapsulated N-A-E mixture had a decreased number of *S*. Enteritidis ATCC 13076 from the first day (4.57 log CFU/mL) until no detection level after incubation for 3 d. Even though growth inhibiting rate of the MCs was slightly lower than for the non-encapsulated N-A-E mixture, it was clear that encapsulated N-A-E mixture could completely inhibit the growth of *S*. Enteritidis ATCC 13076 in MHB and that it maintained its antimicrobial activity as it was gradually released from the MCs. This finding indicated its major evidence for prolonging the stability and antimicrobial activity against target bacteria of nisin in a culture media environment.

Similar to our findings, Gruskiene et al. (2021) reported that the minimum inhibitory concentration of encapsulated nisin in ulvan particles (50 µg/mL) against *E. coli* was higher than for non-encapsulated nisin (37.5 µg/mL). In addition, encapsulated nisin with EDTA in a niosome structure had a lower impact on the growth of *E. coli* than direct application during the early incubation period. However, after extending the incubation time to more than 24 h, the inhibition efficiency of the encapsulated nisin and EDTA in niosome was higher than for the direct application (Kopermsub et al., 2012). The main rationale for the gradual release of nisin from MCs was the emulsifier property due to its molecular structure which is composed of hydrophilic and hydrophobic parts (Neves et al., 2016). The isoelectric point (pI) of nisin is in the alkaline range; therefore, the level of total ion charge of nisin under acid condition is higher than under neutral condition and will be substantially lower when close to the pI of nisin (Tai et al., 2008). In addition, under neutral conditions, nisin becomes more hydrophobic, leading to an increase in the surface tension between nisin and the oil particle surface of MCs (Neves et al., 2016). Generally, the antimicrobial activity of encapsulated antimicrobial agents varies with the type of the antimicrobial agent, the tested strain and the encapsulation materials (Rahnemoon et al., 2021). In addition, environmental factors, such as the pH, ionic strength and sequestering ions can impact on the release of the core materials (Butstraen and Salaün, 2014; Corrêa-Filho et al., 2019; Choudhury et al., 2021).

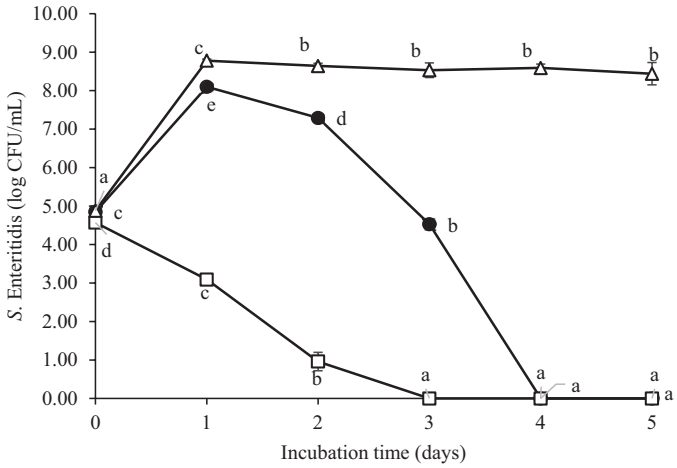

Fig. 2 Numbers of *Salmonella* Enteritidis ATCC 13076 in Mueller Hinton broth, with encapsulated N-A-E mixture (●), non-encapsulated N-A-E mixture (□), without any antimicrobial agent (control) (△) and different lowercase letters indicate significant ($p < 0.05$) difference between means in same treatment and error bars indicate ± SD

Antimicrobial effect of MCs containing N-A-E mixture on target bacterial number in minced fish sample

The antimicrobial effects of MCs containing the N-A-E mixture on the TVC in minced fish are shown in Fig. 3A. During 8 d of storage, the TVCs in the MC-treated minced fish with and without *S*. Enteritidis ATCC 13076 inoculation were 5.73–6.04 log CFU/g and 4.86–5.62 log CFU/g, respectively, whereas in the control, the TVC in non MC-treated minced fish with and without *S*. Enteritidis ATCC 13076 inoculation increased from 6.08 to 7.99 and from 5.53 to 8.15 log CFU/g, respectively. These findings suggested that MCs containing the N-A-E mixture could retard the TVC increase in minced fish both with and without *S*. Enteritidis ATCC 13076 inoculation throughout 8 d of storage at 4 °C.

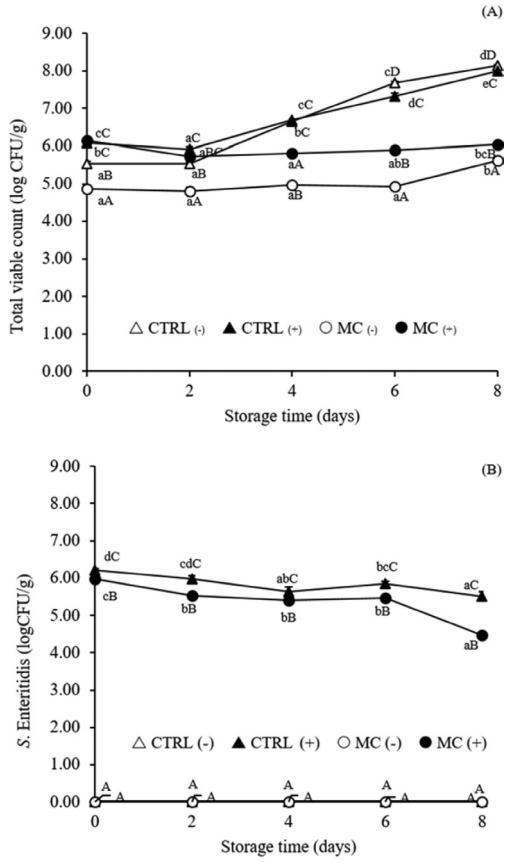

Fig. 3 Changes in total viable count (A); and *Salmonella* Enteritidis ATCC 13076 (B) in minced fish treated with encapsulated N-A-E mixture, where CTRL (△, ▲) is the control minced fish, MC in minced fish treated with encapsulated N-A-E mixture (○,●), (+) and (-) are inoculated and non-inoculated *S.* Enteritidis ATCC 13076, respectively, different lowercase letters indicate significant ($p < 0.05$) difference between means in same treatment, different uppercase letters indicate significant ($p < 0.05$) difference between means on same day of storage and error bars indicate ± SD

The antimicrobial effects of MCs containing the N-A-E mixture against *S*. Enteritidis ATCC 13076 in minced fish are shown in Fig. 3B. The numbers of *S*. Enteritidis ATCC 13076 in MC-treated minced fish significantly declined from 5.98 log CFU/g to 4.48 log CFU/g, while the numbers of *S*. Enteritidis ATCC 13076 in minced fish without MCs (control) remained at almost the same level (5.51–6.20 log CFU/g) throughout 8 d of storage. These results indicated that MCs containing the N-A-E mixture had a bactericidal mode of action by reducing *S*. Enteritidis ATCC 13076 (1.50 log CFU/g) in minced fish samples.

Similar to the current findings, adding encapsulated nisin in nanoparticle of alginate-chitosan to beef samples at a final concentration of 800 IU/g was able to control *L. monocytogenes* growth during 17 d of storage and the numbers of *L. monocytogenes* slightly increased from 1.3 log CFU/g to 1.6 log CFU/g (Zimet et al., 2018). In addition, nisin in liposome prolonged the spoilage time of pasteurized milk from 4 d to 14 d (Tafreshi and Mirdamadi, 2015). Overall, the current findings suggested that the N-A-E mixture and the encapsulation procedure under the designed conditions provided a practical approach to overcoming limitations on the further application of nisin in the control of *Salmonella* and other Gram-negative bacteria that contaminate fish and fishery products, as well as another related food.

The combination of nisin (N) with ascorbic acid (A) and EDTA (E) at final concentrations of 500 ppm, 2,000 ppm and 250 ppm, respectively, could completely reduce total number of *E. coli* ATCC 25922, *L. innocua* ATCC 33096 and *M. luteus* IFO 12708 in culture broth. Furthermore, this mixture could reduce the *S. aureus* ATCC 25923 population by 81.06%. Notably, numbers of *S.* Enteritidis ATCC 13076, *S.* Weltevreden DMST 3380 and *M. morganii* ATCC 25830 decreased by 39.45%, 29.25% and 24.74%, respectively. All the MCs containing the N-A-E mixture prepared using W/O/W emulsion followed by freeze-drying had smooth surfaces and a spherical morphology, with the product yield of the MCs in the range 84.00–98.76% and moisture content and water activity ranges of 1.33–5.51% and 0.248–0.402, respectively. Among all the investigated MCs, the one with the ratios of maltodextrin-to-gum arabic and W_1/O-to-W_2 were 1:1 and 1:9 (w/w), respectively, had the highest production yield (98.76%) with EE values for nisin, ascorbic acid and EDTA of 44.40%, 10.99% and 83.37%, respectively. In addition, this MC had high efficiency regarding its antimicrobial effect against *S*. Enteritidis ATCC 13076 by substantially decreasing the bacterial numbers until they were undetectable after incubation for 4 d in culture broth. It could retard increases in the TVC and reduced 1.50 log CFU/g of *S*. Enteritidis ATCC 13076 inoculated in minced fish sample after storage at 4 °C for 8 d.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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