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Control of biofilm-producing *Aeromonas* bacteria in the water tanks and drinkers of broiler poultry farms using chitosan nanoparticle-based coating thyme oil

A.N. Mohammed[1] and A.S. Attia[2]

[1]Department of Hygiene, Zoonoses and Epidemiology, Faculty of Veterinary Medicine, Beni-Suef University, Beni-Suef, [2]Department of Veterinary Public Health, Faculty of Veterinary Medicine, Zagazig University, Zagazig, Egypt

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Correspondence:
A.S. Attia
amy_talat20002@yahoo.com

Abstract

In a drinking water distribution system, biofilm-producing bacteria are considered an alarm bell for increased emergence of waterborne pathogens. This study aims to monitor the prevalence of biofilm-forming *Aeromonas* species in the drinking water distribution systems in different broiler chicken farms. The antimicrobial activity of thyme essential oil (TEO), thyme essential oil nano-emulsion (TEO-N), chitosan (CS), chitosan nanoparticles (CS-NPs), and both CS and CS-NP-based coating TEO against the different *Aeromonas* spp. was evaluated using the broth microdilution and agar well diffusion assay. The overall prevalence rate of *Aeromonas* spp. was 49.3% (74.0/150). The highest rate of *Aeromonas* isolates was noted in water drinkers and tanks 75.0% (30/40) and 62.5% (25/40), respectively) followed by feedstuff 40.0% (12/30). In contrast, the highest percentage of biofilm-producing *Aeromonas* spp. was *Aeromonas hydrophila* 70.0% (14/20) followed by *Aeromonas caviae* 30.0% (6/20). The fatal effect of CS-NPs against all isolated *Aeromonas* spp. was achieved 100% at 1.5 and 2.0 µg/mL. Moreover, chitosan nanoparticles coating thyme essential oil (CS-NPs/TEO) verified the lethal effect 100% on both *A. hydrophila* and *A. caviae* at the ratio of 1:1 and 1:0.75 µg/mL. In conclusion, the main source of *Aeromonas* spp. in the drinking water distribution system was the unhygienic status of water tanks and drinkers that allowed biofilm to produce due to aggregation of *Aeromonas* bacteria on the inner surface of that equipment. Both CS-NPs and CS-NPs/TEO could be applied as a sanitizer and/or disinfectant for *Aeromonas* biofilm control.

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Introduction

The existence of biofilms forming bacterial organisms in drinking water systems has received inadequate consideration (1). In addition, the assessment of biofilm microbial isolates of drinking water distribution systems remains ambiguous, and there is negligible literature demonstrating that certain bacteria are integral parts of biofilm in the distribution systems of water (2). The occurrence of biofilm in a drinking water system could enhance the provision of nutrients and carbon required for bacterial biosynthesis. This could permit the persistence and propagation of diverse pathogenic bacteria, such as *Aeromonas hydrophila* and *Pseudomonas aeruginosa*, and other fungi, viruses, and protozoa (3). These organisms are related to a variety of infections and symptoms, such as diarrhea, gastroenteritis, food poisoning, typhoid fever, chronic sinusitis, chronic wound infection, endocarditis, osteonecrosis, and severe periodontal diseases (4) besides to the microorganism has been isolated from several

environmental such as aquatic one (5). According to the United States Environmental Protection Agency (6) *Aeromonas* bacteria are listed as emerging waterborne pathogens that can grow in chlorinated water distribution systems and form biofilm. Furthermore, *A. hydrophila* has been identified as a contributing agent to intestinal and extraintestinal diseases in humans, including septic arthritis, fulminating septicemia, diarrhea, gastroenteritis, wound infections, and meningitis. The pathogenicity of *Aeromonas* has been associated with several known virulence factors, such as aerolysin, hemolysin, proteases, lipases, and DNases (7). These toxins play a foremost role in disease progression (8). Nowadays, the investigation of new antimicrobial agents to control different infections in animals and on poultry farms has become an urgent need. Therefore, the application of natural material and/or some essential oils (EOs) against a wide variety of microorganisms is quite imperative (9). There is literature verifying that EOs containing a high content of phenolic derivatives (such as thyme and carvacrol) target the bacterial membrane transport system, causing disrupting at the cytoplasmic homeostasis, affecting the microbial enzyme system (10). Additionally, the hazardous growth of microbial resistance has increased hope that replacing antibiotics with EOs could potentially become a safe way to use natural growth promoters for farmed animals in their diets to improve the quality of gut microbiota. Thus, results could show good growth performance of animals and eventually contribute to consumer safety (11). Chitosan (CS) is a natural cationic polysaccharide obtained from crustacean shells such as crabs and shrimp using either chemical or microbiological procedures (12). It has unique biological characteristics, being both biodegradable and non-toxic (13). Many applications have been found, either alone or in combination with other natural polymers in food, textiles, water treatment, and other industries. CS has proved its activity against foodborne pathogens, pathogenic viruses, and fungi (14). CS has also proven its ability to load sensitive bioactive composites or compounds such as lipophilic drugs and polyphenolic compounds (15). However, to the best of our knowledge, the creation of novel composites (chitosan nanoparticle-based coating with thyme essential oil (CS-NPs/TEO) using CS particles at a nanoscale range as an outer shell has not been studied.

Therefore, the present work was conducted to monitor the prevalence of *Aeromonas* spp. in the drinking water of broiler chicken farms and assess the antibacterial and/or disinfectant properties of thyme essential oil (TEO), thyme essential oil nano-emulsion (TEO-N), CS, and chitosan nanoparticles (CS-NPs). Additionally, we evaluated the effectiveness of both CS and CS-NP-based coating TEO on isolated *Aeromonas* spp. to seek an alternative method for establishing an efficient control strategy for biofilm-forming *Aeromonas* spp.

Materials and methods

Ethical approve

The present study was approved by Institutional Animal Care and Use Committee with issue number: 9215, date:10 December, 2020, Faculty of Veterinary Medicine, Beni-Sue University, Egypt.

Study location and farm description

A cross-sectional design was applied using 12 private broiler chicken farms situated in Beni-Suef (coordinates, 29° 04′ N-31° 05′E) and El-Faiyum Governorate (coordinates, 29° 308374′ N-30° 844105′E). The broiler chickens in these farms were raised on a deep litter system and kept on wood shaving litter at a stocking rate of 7 birds/ m^2. Building dimensions were 10.5 x 40.4 meters. Inside the farms, the main water supply was tap water. In addition, the water supply was not treated at all. Water was available from drinkers. Before a production cycle began, all drinkers and water tanks were cleaned and disinfected once per cycle.

Collecting samples

In each city, 150 samples were collected from (water supply, n = 40; water tanks, n = 40; drinkers, n = 40; feedstuff, n = 30) once per week from 6 different locations. All water samples were aseptically collected in sterilized glass bottles (250 mL capacity) from different broiler chicken farms. We collected 25 g of feedstuff samples in sterilized plastic bags; then, they were homogenized in 225 mL of peptone water. The samples were properly labeled and transported in an icebox to the laboratory for further microbiological analysis. Samples were collected for 4 months. All collected samples were used for the selective isolation of *Aeromonas* bacteria based on standard microbiological procedures (16).

Aeromonas spp. isolation and molecular identification

Tenfold serial dilutions of water and feedstuff samples were prepared; then, 0.1 mL of samples were inoculated on *Aeromonas* enrichment broth (BD, Becton, Dickinson and Company, Sparks, MD 21152, USA). Thereafter, 10 µL of enrichment broth was aseptically streaked on *Aeromonas* ampicillin base media (Oxoid, CM 833, SR136) and incubated at 37°C for 24 h. All colonies of green and yellow color were sub-cultured on nutrient agar and incubated again at 37°C for 24 h for further investigation (17). The primary identification of *Aeromonas* spp. was achieved using morphological characteristics under microscopic examination and motility test (18). Furthermore, polymerase chain reaction (PCR) was used to identify specific virulent genes of aerolysin (*aerA*) and hemolysin (*hylH*) of *Aeromonas* spp. (19,20). PCR assay was performed using the oligonucleotide primer. Furthermore, the PCR cycling program was started with denaturation of DNA at 95°C for 5

min, followed by 30 cycles for 2 min at 94°C, then 55°C and 72°C for 1 min, and, finally, final extension at 72°C for 10 min to amplify both *aerA* and *hylH* genes (Table 1).

Screening of biofilm-forming *Aeromonas* spp.

Biofilm-forming by *Aeromonas* spp. was qualitatively detected using the tube method (21). The isolated strains of *Aeromonas* spp. were inoculated into 5 mL of Tryptone Soy Broth (TSB) tubes (Oxoid, UK) and then incubated at 37° C for 48 h. Thereafter, the content of the tubes was decanted and washed with phosphate buffer saline (pH 7) and air-dried. Subsequently, all tubes were stained with 1% crystal violet (% w/v); to ensure uniform staining, tubes were then gently rotated. The stain was removed, and tubes were washed with distilled water and dried in an inverted position. Biofilm formation was considered positive when a visible stained film was observed to adhere to the wall and bottom of the tube. The testing was done in triplicate, and for clarity, results were compared with each other.

Table 1: Oligonucleotide primer sequences of target genes in *Aeromonas* spp

Target gene	Primer sequences (5'-3')	Amplified segment	Reference
aer A	Aer 2F: AGCGGCAGAGCCCGTCTATCCA Aer 2R: AGTTGGTGGCGGTGTCGTAGCG	416 bp	(18)
hyl H	Hyl 2F: GGCCCGTGGCCCGAAGATGCAGG Hyl 2R: CAGTCCCACCCACTTC	597 bp	(19)

Chitosan and chitosan nanoparticles preparation

CS is a powder material (low molecular weight, crab shells, poly-1.4-B-D-glucopyranosamine; 2-amino-2-deoxy-(1 ≥ 4)-B-D-glucopyranan, Kochi 682005, India). CS solution was formulated by dissolving CS 2% (w/v) in 1% (v/v) acetic acid and then stirring the solution for 3 h on a magnetic stirrer at 23°C-25°C to ensure complete dispersal. pH of the solution was adjusted 5.9 by adding a solution of 10N NaOH (22). Thereafter, different concentrations of CS solution were prepared 2, 1.5, 1.25, 1.0, and 0.5 µg/mL. CS-NPs were spontaneously formed upon dropwise addition of an aqueous tripolyphosphate solution (0.25%, w/v) to different CS concentrations with magnetic stirring. CS-NPs were purified by centrifugation at 6000 rpm for 30 min. Supernatants were discarded, and CS-NPs were rinsed with distilled water several times to remove any sodium hydroxide and then freeze-dried before further use (22).

Thyme oil and thyme essential oil nano-emulsion preparation

At different testing concentrations, TEO (100% pure; Sigma-Aldrich, St. Louis, MO, USA) was mixed with Tween 80 (polyoxymethylene sorbitan monolaurate; Sigma-Aldrich) at a concentration of 0.3% to completely dissolve TEO. In addition, the TEO-N was prepared by ultrasonication method where the emulsion of thyme was formulated by joining oil phase (TEO) with aqueous phase (deionized water and Tween® 80 at 3%); then, TEO was slowly added to the aqueous phase at 25°C with a magnetic stirrer at 500 rpm for 15 min. Thereafter, TEO-N was formed using ultrasonicator bath (ASU-10D, AS ONE, Japan) at various temperatures (25°C-30°C) for 15 min (23). TEO and TEO-N were prepared at different concentrations 0.15, 0.25, 0.5, 0.75, and 1% for further application.

Chitosan-based coating with thyme oil preparation

CS at 2% (w/v) was prepared in acetic acid 1% (v/v). To ensure a complete scattering of CS, the CS solution was stirred for 3 h at 25°C with a magnetic stirrer. The CS solution was placed in a beaker with glycerol at 0.75 mL/g and stirred for 10 min. It was then filtrated using a Whatman filter paper to eliminate undissolved particles. Then, TE mixed with Tween 80 at a concentration of 0.3 was added to the CS solution. The final coating solution consisted of the following: CS, 2%; acetic acid, 1%; glycerol, 0.75%; Tween 80, 0.3%; and TE, 1.0%. Various amounts of TEO were used to prepare different weight ratio of CS to TEO (w/w) of 1:0.15, 1:0.25, 1:0.5, 1:0.75, and 1:1, respectively. Under aseptic conditions, the final CS-based coating oil was homogenized for 2 min at 600 rpm. The solution was prepared without adding TEO as a control (24).

Chitosan nanoparticle-based coating with thyme essential oil preparation

The CS solution at 2% (w/v) in acetic acid 1% (v/v) was prepared by the same previously mentioned method; then, Tween 80 at 0.3% concentration was added as a surfactant to this solution and stirred for 2 h using a magnetic stirrer at 45°C to obtain a homogenous mixture. TEO was then gradually dropped into the CS solution under the stirring condition to obtain oil-in-water emulsion. Various amounts of TEO were used to prepare different weight ratio of CS to TEO (w/w) of 1:0.15, 1:0.25, 1:0.5, 1:0.75, and 1:1%, respectively. The solution of TEO-loaded CS-NPs could be obtained by dropwise addition of an aqueous tripolyphosphate solution (0.25%, w/v) into the oil-in-water emulsion under the stirring condition at room temperature for 1 h (25).

Characterization of chitosan nanoparticles and thyme essential oil nano-emulsion and its loaded form

CS-NPs, TEO-N, and its loaded form (CS-NPs/TEO) were characterized using the Fourier transform infrared (FT-IR) spectrophotometer (VERTEX, 70) at the region of 3500-500 cm^{-1} with a spectral resolution of 4 cm^{-1}. Moreover, the morphological shape and average diameter of CS-NPs, TEO-N, and CS-NPs/TEO were determined using transmission electron microscopy (TEM; a JEOL JEM 2000EX) at the National Research Center (NRC; Central Labs, Egypt).

Testing the antimicrobial activity using the broth microdilution and agar well diffusion assay

The biocidal effects CS, CS-NPs, TEO-N, and its loaded form at different concentrations on the growth rate of *Aeromonas* spp. isolates (n = 45) were tested. In addition, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were defined using the broth microdilution assay (26). The freshly prepared dilutions of CS, CS-NPs, TEO-N, and its loaded form in Mueller-Hinton Broth were tested in a 96-well microtiter plate (Nunc, Copenhagen, Denmark). Then, 100 µL of bacterial suspensions (1 x 10^{-8} CFU/mL) were inoculated in each well. The contents of microtiter plates were mixed by shaking for 10 min and incubated for 24 h at 37°C. The optical density (OD) of each well was monitored using a microplate reader at 600 nm during the incubation time. The difference between OD of each testing sample was compared with negative control (without CS, CS-NPs, TEO-N, and its loaded form); then, MIC and MBC values were assessed. The MIC was revealed as the least concentration of each testing material that avoided visible turbidity in microtiter wells after 24 h. To assess the MBC, 100 mL of the testing sample was transferred from each well without obvious growth to a Mueller-Hinton agar plate and incubated at 37°C for 24 h to confirm the absence of microbial growth. Furthermore, the antibacterial activity of all testing compounds and their loaded form was evaluated using the agar well diffusion method with Mueller-Hinton agar (22).

Statistical analysis

All collected data were prepared in Microsoft Excel Spreadsheet for statistical analysis using the Statistical Package for Social Sciences (SPSS software, version 26). The prevalence rate and distribution of *Aeromonas* bacterial isolates were analyzed using the Chi-square test (nonparametric test). Meanwhile, one of the parametric tests (one-way ANOVA) was used to determine the diameter of inhibition zone (mm) of testing compounds against *Aeromonas* spp. isolates. A *P*-value of < 0.05 was considered statistically significant.

Results

Frequency and distribution rate of *Aeromonas* spp. in the water distribution system

In the water distribution system and feedstuff of investigated broiler chicken farms, the prevalence rate of *Aeromonas* spp. was 49.3% (74.0/150). The highest rate of *Aeromonas* spp. was recorded in water drinkers and tanks 75.0% (30/40) and 62.5% (25/40), respectively), followed by feedstuff and water supply 40.0% (12/30) and 17.5% (7/40), respectively), as shown in Table 2. Additionally, the ability to form a biofilm was confirmed in 20 of 74 positive samples of *Aeromonas* spp. 27.03% (20/74) at $\chi 2 = 17.2$ (P<0.01) (Figure 1).

Table 2: Prevalence of *Aeromonas* spp. in the investigated broiler chicken farms

Examined samples	Total		Prevalence rate (%)
	Examined no.	Positive no.	
Water supply	40	7.0	17.5
Water tanks	40	25.0	62.5
Drinkers	40	30.0	75.0
Feedstuff	30	12.0	40.0
Total	150	74.0	49.3
Biofilm-forming bacteria	74.0	20.0	27.03

The association between positive isolated *Aeromonas* bacteria and other examined samples is significantly different at Chi-square ($\chi 2$) = 17.2, P<0.01.

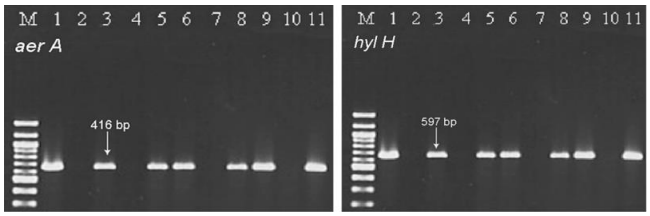

Figure 1: PCR amplification of the 416-bp fragment of aerolysin gene *(aerA)* detected in *A. hydrophila* (lanes 1, 3, 5, 6, 8, 9, and
11) and hemolysin gene (*hylH*) amplified at 597-bp fragment (lanes 1, 3, 5, 6, 8, 9, and 11) and control negative at lane 2; DNA: ladder.

Moreover, at all frequency distribution, *A. hydrophila* was significantly higher than *A. caviae* in the investigated farms of broiler chickens 74.3% (55/74) and 25.7% (19/74), respectively). The highest frequency of *A. hydrophila* was detected in water drinkers and feedstuff 80.0% (24/30) and 75.0% (9/12), in comparison with *A. caviae*, which was detected in the highest rate in water supply and water tanks 42.9% (3/7) and 28.0% (7/25), respectively (Table 3). The

distribution of *A. hydrophila* in water tanks and water supply was 72.0% (18/25) and 57.1% (4/7), respectively, while *A. caviae* was isolated from feedstuff and water drinkers at 25.0% (3/12) and 20.0% (6/30), respectively. From the total positive biofilm-forming *Aeromonas* spp., it was found that the highest percentage of biofilm-producing *Aeromonas* spp. was *A. hydrophila* 70.0% (14/20) and then *A. caviae* 30.0% (6/20).

Table 3: Frequency distribution of different *Aeromonas* spp. in the investigated samples

Investigated samples	Total positive no.	Frequent distribution of *Aeromonas* spp. no. (%)	
		A. hydrophila	*A. caviae*
Water supply	7.0	4.0 (57.1)	3.0 (42.9)
Water tanks	25.0	18.0 (72.0)	7.0 (28.0)
Drinkers	30.0	24.0 (80.0)	6.0 (20.0)
Feedstuff	12.0	9.0 (75.0)	3.0 (25.0)
Total	74.0	55.0 (74.3)	19.0 (25.7)
Biofilm-forming bacteria	20.0	14.0 (70.0)	6.0 (30.0)

The distribution rate of different *Aeromonas* spp. in the investigated samples is statistically significant at Chi-square ($\chi 2$) = 19.3, P<0.01.

Characterization of chitosan nanoparticles, thyme essential oil nano-emulsion, and its loaded form

High-resolution transmission electron microscopy (HR-TEM) images of CS-NPs showed that the nanoparticle (NP) shape was fine spherical and slightly elongated (Figure 2a). In addition, the diameter of NPs ranged from 16.8 to 18.4 nm, as shown in Figure 2b. HR-TEM images of TEO-N showed that the NP shape of thyme oil was typically spherical and elongated and distributed in the field (Figure 3a), and the size of NPs ranged from 150 to 220 nm in diameter, as shown in Figure 3b. HR-TEM images of CS-NPs/TEO showed the spherical and oval shape of NPs distributed in the microscopic field (Figure 4a). The NP diameter ranged from 2.39 to 8.64 nm (Figure 4b). FT-IR spectra of TEO-N (Figure 5a-b) showed the widened peak at 3331.25 cm^{-1} that approved hydrophilic interaction (H-OH) in TEO-N. Moreover, other peaks were noticed at 1646.34, 1086.55, and 619.29 cm^{-1}. FT-IR spectra of TEO, CS-NPs, and CS-NPs/TEO, as shown in Figure 6a-c, showed that a noticed peak of thyme oil (Figure 6a) was obvious at 2955.2, 1706, 1438, 1225, 808, and 586 cm^{-1}. FT-IR spectra of CS-NPs (Figure 6b) showed characteristic peaks that appeared at 3289.6, 2351.9, 1638, 1053, and 600.9 cm^{-1}. The FT-IR spectra of CS-NPs/TEO approved the formation of CS nanoparticle-based coating with thyme oil where characteristic peaks formed at 3272.4, 1642.8, 1045, and 610.4 cm^{-1} (Figure 6c).

Antimicrobial activity of chitosan, thyme oil, and nanocomposites

To evaluate the antibacterial activity of TEO, TEO-N, CS, CS-NPs, chitosan-based coating with thyme oil (CS-TEO), and CS-NPs/TEO, the MIC and MBC of these compounds were determined, as exhibited in Table 4. The MIC for all *Aeromonas* spp. was CS > CS-NPs > TEO > TEO-N (1.25, 1.0, 0.25, and 0.15 µg/mL, respectively). The MIC value of CS-TEO was barely higher (1:0.25 µg/mL) than that of CS-NPs/TEO (1:0.15 µg/mL). MBC values for all *Aeromonas* spp. isolates were 2.0, 1.25, 0.75, and 0.5 µg/mL, respectively, in all testing CS > CS-NPs > TEO > TEO-N, while MBC values of both CS-TEO and CS-NPs/TEO were 1:0.5 and 1.025 µg/mL, respectively. In addition, the inhibition zone diameter was significantly evident in table 4 and figure 7. The diameter (mm) of inhibition zone for CS-NPs/TEO and CS-NPs was cleared at 34.6 ± 1.5 and 31.5 ± 0.4 mm, followed by TEO-N and CS-TEO (28.9 ± 2.6 and 26.1 ± 2.2 mm, respectively), compared with TEO and CS (24.0 ± 1.3 and 9.3 ± 1.0 mm, respectively) at P≤0.05.

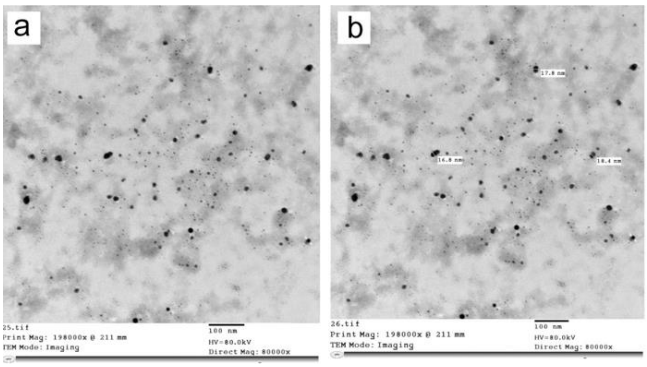

Figure 2: HR-TEM images of CS-NPs show the morphological shape of NPs in CS-NPs (a) that appear as fine spherical and slightly elongated shapes. Additionally, the diameter of NPs (b) ranged from 16.8 to 18.4 nm.

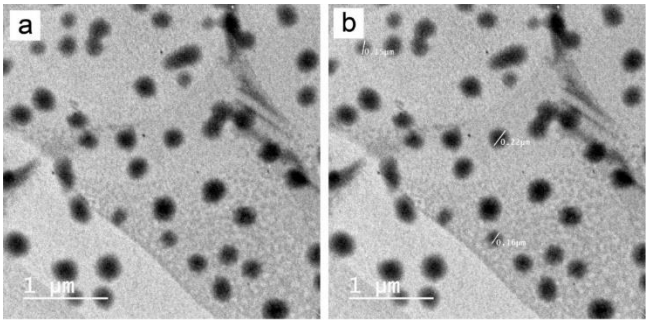

Figure 3: HR-TEM images of TEO-N exhibited the NP shape of thymol oil that appeared as spherical and bean-shaped (a) distributed in the field, and the size of NPs (b) ranged from 150 to 220 nm in diameter.

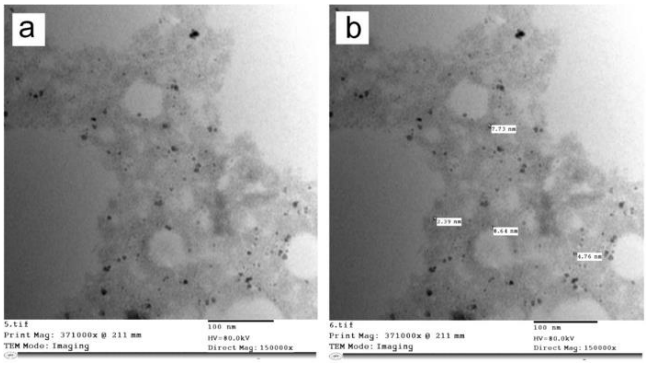

Figure 4: HR-TEM images of CS-NPs/TEO show the spherical and oval shape (a) of NPs distributed in the microscopic field, and the nanoparticle diameter ranged from 2.39 to 8.64 nm (b).

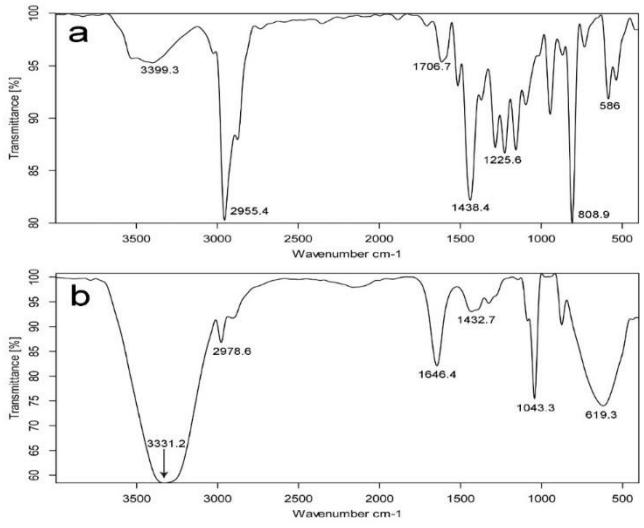

Figure 5: FT-IR spectra of thyme oil (a) and TEO-N (b).

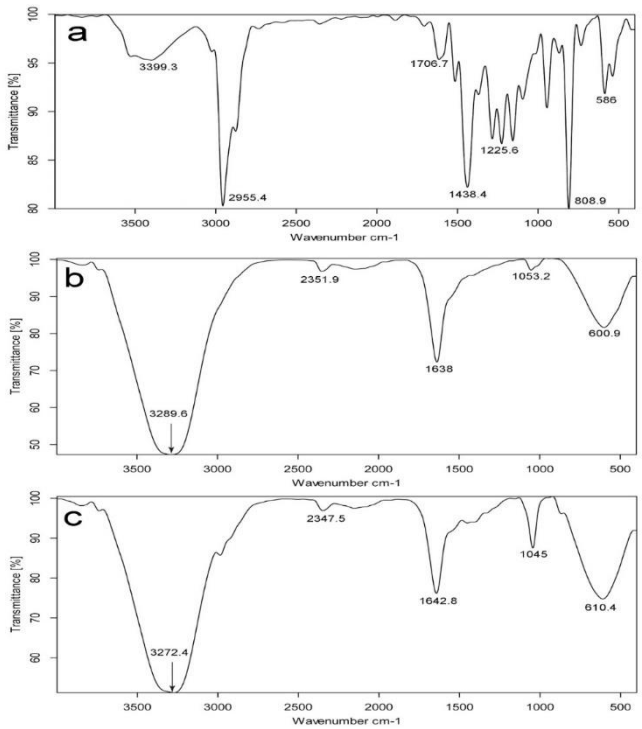

Figure 6: FT-IR spectrum of TEO (a), CS-NPs (b), and CS-NPs/TEO (c).

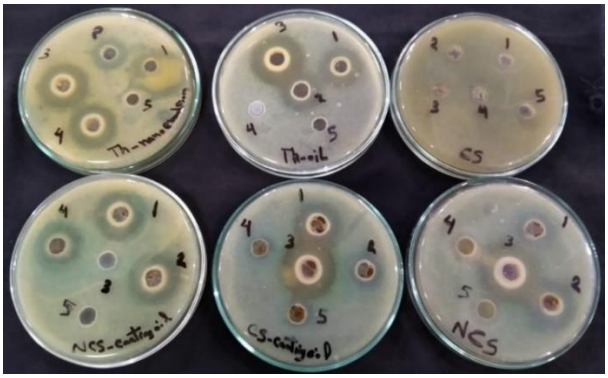

Figure 7: The antibiofilm activity of CS, CS-NPs, TEO, TEO-N, CS coating TEO, and CS-NPs/TEO using the well diffusion method shows the effectiveness of TEO-N *Aeromonas* spp. that is noticeably clear at different testing concentrations. The inhibition zone diameter was 28.9 ± 2.6 mm compared with thymol oil. CS-NPs exhibited the lethal effect against *Aeromonas* spp. at 1.25, 1.5, and 2 µg/ mL, respectively, and the inhibition zone was 31.5 ± 0.4 mm in diameter. In addition, CS-NPs/TEO exhibited the lethal effect on biofilm-forming bacteria at a ratio of 1:0.25 µg/ mL, and the inhibition zone was 34.6 ± 1.5 mm, followed by chitosan-based coating with thymol oil at the testing concentration 1:0.5 µg/ mL.

Table 4: Inhibition zone formation using TEO, TEO-N, CS, CS-NPs, CS/TEO, and CS-NPs/TEO against isolated *Aeromonas*

Testing compound	Concentration (µg/mL)	Broth microdilution method		Well diffusion assay
		MIC (µg/ mL)	MBC (µg/ mL)	Inhibition zone diameter (mm)
Thyme oil	0.15 0.25 0.5 0.75 1.0	0.25	0.75	24.0 ± 1.3^{c}
Thyme oil nano-emulsion	0.15 0.25 0.5 0.75 1.0	0.15	0.5	28.9 ± 2.6^{b}
Chitosan	0.5 1.0 1.25 1.5 2.0	1.25	2.0	9.3 ± 1.0^{ab}
Chitosan nanoparticles	0.5 1.0 1.25 1.5 2.0	1.0	1.25	31.5 ± 0.4^{a}
Chitosan-based coating with thyme oil	1:0.15 1:0.25 1:0.5 1: 0.75 1:1.0	1:0.25	1:0.5	26.1 ± 2.2^{b}
Chitosan nanoparticle-based coating with thyme essential oil	1:0.15 1:0.25 1:0.5 1: 0.75 1:1.0	1:0.15	1:0.25	34.6 ± 1.5^{a}

The mean values of inhibition zone diameter (mean ± SE) with different superscript letters $^{(a,b,c)}$ in the same column are significantly different at $P \leq 0.05$.

The sensitivity pattern of *Aeromonas* spp. to different tested compounds and nanocomposites after 24 h of exposure (Table 5) clarified that the effectiveness of TEO on both *A. caviae* and *A. hydrophila* was significantly obvious (73.3% and 66.7%, respectively) at the highest tested concentration 1 µg/mL compared with the lowest (0.75, 0.5, 0.25, and 0.15 µg/mL, respectively). The resistance of *Aeromonas* spp. to TEO exceeded 46.0% at the different tested concentrations (0.5, 0.25, and 0.15 µg/mL, respectively), whereas the resistant percentage was 46.7% (14/30), 53.3% (16/30), and 63.3% (19/30), respectively, compared with resistant isolates of *A. caviae* at the same tested concentrations (40.0% (6/15), 40.0% (6/15), and 33.3% (5/15), respectively). Furthermore, the susceptibility of *Aeromonas* spp. to TEO-N exhibited a significant effect at 86.7% (13/15) and 70.0% (21/30) for *A. caviae* and *A. hydrophila* at the highest tested concentration (1 µg/mL) compared with other concentrations. The susceptibility of different isolates to CS did not exceed 50.0% (15/30) in the case of *A. hydrophila* compared with *A. caviae* (66.7% (10/15)) at a concentration of 2.0 µg/ mL. CS-NPs showed the lethal effect (100%) against all isolated *Aeromonas* spp. at 1.5 and 2.0 µg/mL compared with the lowest concentrations (1.25, 1.0, and 0.5 µg/ mL, respectively). In contrast, CS-TEO recorded the highest antimicrobial effect on *A. caviae* (80.0% (12/15)) followed by *A. hydrophila* (76.7% (23/30)) at a ratio of 1:1 µg/ mL compared with other tested concentrations. On the other hand, the CS-NPs/TEO proved the lethal effect (100% (30/30) and 100% (15/15), respectively) on both *A. hydrophila* and *A. caviae* at the ratio of 1:1 and 1:0.75 µg/ mL, respectively. The susceptibility of *A. hydrophila* was 83.3% (25/30) and 73.3% (22/30), while that of *A. caviae* was 93.3% (14/15) and 86.7% (13/15), respectively, at ratios of 1:0.5 and 1:0.25 µg/ mL.

Table 5: Antimicrobial activity of TEO, TEO-N, CS, CS-NPs, CS-TEO, and CS-NPs/ TEO against *Aeromonas* spp

Testing compound	concentration (µg/ mL)	Susceptibility profile of *Aeromonas* spp. after 24 h of exposure					
		A. hydrophila (*n*=30)			*A. caviae* (*n*=15)		
		Susceptible	Intermediate	Resist	Susceptible	Intermediate	Resist
Thyme oil	0.15	5 (16.7)	6 (20.0)	19 (63.3)	4 (26.7)	5 (33.3)	6 (40.0)
	0.25	9 (30.0)	5 (16.7)	16 (53.3)	6 (40.0)	3 (20.0)	6 (40.0)
	0.5	11(36.7)	5 (16.7)	14 (46.7)	8 (53.3)	2 (13.3)	5 (33.3)
	0.75	17 (56.7)	3 (10.0)	10 (33.3)	9 (60.0)	2 (13.3)	4 (26.7)
	1.0	20 (66.7)	2 (6.7)	8 (26.7)	11 (73.3)	1 (6.7)	3 (20.0)
Thyme oil nano-emulsion	0.15	10 (33.3)	5 (16.7)	15 (50.0)	7 (46.7)	4 (26.7)	4 (26.7)
	0.25	13 (43.3)	5 (16.7)	12 (40.0)	9 (60.0)	4 (26.7)	2 (13.3)
	0.5	17 (56.7)	3 (10.0)	10 (33.3)	9 (60.0)	3 (20.0)	3 (20.0)
	0.75	17 (56.7)	3 (10.0)	10 (33.3)	11 (73.3)	2 (13.3)	2 (13.3)
	1.0	21(70.0)	1 (3.3)	8 (26.7)	13 (86.7)	0 (0.0)	2 (13.3)
Chitosan	0.5	4 (13.3)	3 (10.0)	23 (76.7)	5 (33.3)	4 (26.7)	6 (40.0)
	1.0	6 (20.0)	3 (10.0)	21 (70.0)	6 (40.0)	3 (20.0)	6 (40.0)
	1.25	9 (30.0)	2 (6.7)	19 (63.3)	7 (46.7)	3 (20.0)	5 (33.3)
	1.5	13 (43.3)	2 (6.7)	15 (50.0)	9 (60.0)	2 (13.3)	4 (26.7)
	2.0	15 (50.0)	0 (0.0)	15 (50.0)	10 (66.7)	1 (6.7)	4 (26.7)
Chitosan nanoparticles	0.5	10 (33.3)	7 (23.3)	13 (43.3)	10 (66.7)	1 (6.7)	4 (26.7)
	1.0	15 (50.0)	5 (16.7)	10 (33.3)	12 (80.0)	1 (6.7)	2 (13.3)
	1.25	19 (63.3)	4 (13.3)	7 (23.3)	14 (93.3)	0 (0.0)	1 (6.7)
	1.5	30 (100)	0 (0.0)	0 (0.0)	15 (100)	0 (0.0)	0 (0.0)
	2.0	30 (100)	0 (0.0)	0 (0.0)	15 (100)	0 (0.0)	0 (0.0)
Chitosan-based-coating with thyme oil	1:0.15	10 (33.3)	2 (6.7)	18 (60.0)	6 (40.0)	4 (26.7)	5 (33.3)
	1:0.25	13 (43.3)	2 (6.7)	15 (50.0)	7 (46.7)	3 (20.0)	5 (33.3)
	1:0.5	14 (46.7)	1 (3.3)	15 (50.0)	9 (60.0)	2 (13.3)	4 (26.7)
	1: 0.75	17 (56.7)	1 (3.3)	12 (40.0)	9 (60.0)	2 (13.3)	4 (26.7)
	1:1	23 (76.7)	0 (0.0)	7 (23.3)	12 (80.0)	0 (0.0)	3 (20.0)
Chitosan nanoparticle-based coating with thyme essential oil	1:0.15	17 (56.7)	4 (13.3)	9 (30.0)	12 (80.0)	1 (6.7)	2 (13.3)
	1:0.25	22 (73.3)	3 (10.0)	5 (16.7)	13 (86.7)	0 (0.0)	2 (13.3)
	1:0.5	25 (83.3)	2 (6.7)	3 (10.0)	14 (93.3)	0 (0.0)	1 (6.7)
	1: 0.75	30 (100)	0 (0.0)	0 (0.0)	15 (100)	0 (0.0)	0 (0.0)
	1:1	30 (100)	0 (0.0)	0 (0.0)	15 (100)	0 (0.0)	0 (0.0)

The association of susceptibility testing of *Aeromonas* spp. and different testing compounds (TEO, TEO-N, CS, CS-NPs, and its loaded form) is statistically significant at $P \leq 0.05$.

Discussion

It is very imperative to combat *Aeromonas* spp. as an emerging water pathogen. Most *Aeromonas* strains could produce different putative virulence factors, such as enterotoxins, cytotoxins, or hemolysins (26,27). Furthermore, both fish and chicken play a serious role in pathogen transmission to human beings (28). The present work clarified that the prevalence rate of *Aeromonas* spp. isolates was significantly high in water drinkers and tanks, followed by feedstuff, at the broiler chicken farm level at $P<0.01$. In addition, they confirmed their ability to produce biofilm in water tanks and drinkers. Moreover, the frequency distribution of *A. hydrophila* was significantly higher than *A. caviae* in the investigated farms. Mailafia and Agbede (2) found that there were a wide variety of bacteria, including *A. hydrophila* 6.67% and *P. aeruginosa* 25%, in drinking water supplies. The existence of *A. hydrophila* within the water distribution system provided the opportunity to multiply (28) and they could mutate in the water supply that provided the appropriate conditions to the microorganisms to produce virulent genes (3,6). *Aeromonas hydrophila* is a potential waterborne pathogen that could cause an increase of infection in livestock, laboratory animals, fishes, wildlife, and chickens (5). There are some hazardous factors, such as ingestion of contaminated drinking water and food, presented by such microorganisms that are predisposed to cause several human diseases (29). Additionally, *A. hydrophila* is considered an important human pathogen linked with foodborne disease outbreaks (30). Previous

literature reported that *A. hydrophila* could be horizontally transmitted through an oral route, including unhygienic feed sources and contaminated drinking water sources (31). The ability of *Aeromonas* bacteria to produce biofilm was recorded in this study, where the highest percentage of biofilm-producing *Aeromonas* spp. was *A. hydrophila*, followed by *A. caviae*, that was isolated from water tanks and drinkers. These findings could be attributed to the unhygienic water source and/or accumulation of biofilm-forming bacteria on the inner surface of the water tanks and drinkers. This could occur when hygienic and sanitation rules are not applied in the broiler chicken farms to protect the birds from the risk of exposure to such bacterial contaminants through drinking contaminated water and feed. Scwab and Straus (32,33) found that the accumulation of biofilms on the inner surface of water distribution systems led to additional contamination of water in the pipes, and an increase in the concentration of *Aeromonas* bacteria in the water was attributed to factors including inadequate water treatment and unhygienic water sources (34).

The effectiveness of TEO on both *A. caviae* and *A. hydrophila* was significantly low at the tested concentration of 1.0 µg/mL, but the resistance of *Aeromonas* spp. to TEO exceeded 46.0% at the different tested concentrations during this study. Donsì and Ferrari (35) clarified that the use of EOs was considered a promising alternative to chemical sanitizers. Puvaca *et al.* (7) stated that in the treatment of bacterial infections, there are a viable alternative to synthetic drugs involve many aromatic and medicinal plants, and herbs have been proposed as a significant source of natural antimicrobials. Regarding the sensitivity pattern of *Aeromonas* spp. to CS, it has been discovered that the efficiency of CS did not exceed 50% in the case of *A. hydrophila* compared with *A. caviae* at the highest tested concentration of 2.0 µg/ mL. Chavez de Paz *et al.* (36) found that low molecular weight CS had a high antibacterial effect of more than 95%, especially against *Streptococcus mutans* that produce biofilms. The germicide activity of CS can be attributed to a change in cell permeability due to interactive action between the amine groups of CS and the electronegative charges on the exterior of the microbial cell.

Biofilm-forming bacteria are a foremost problem in food production due to their resistance to disinfectants. EO nano-emulsions could reduce biofilms that are formed via the accretion of microorganisms on the surface. Moreover, nano-emulsion efficiency can inhibit the biofilm-forming activity by preventing the bacterial attachment on the surface (37). It was recently found that EO encapsulation in nanoscale exhibited a potential to enhance EOS bioactivity via the activation of the cell absorption mechanism. Due to subcellular size, nanoscale encapsulation can increase the bioactive compound concentration in food zones where microbes are preferably situated (38). Li *et al.* (39) noticed that the use of TEO-N led to a reduction in the biofilms produced by foodborne pathogens on food surfaces of romaine lettuce and blueberries within 60 s of washing with oil nano-emulsion.

Creating a new formula based on CS was aimed at enhancing the hydrophilic properties of polymer (40). In this context, the application of CS-NPs alone or in combination as a coating material (CS-NPs/TEO) proved the lethal effect on bacterial isolates (100%) at two of five tested concentrations. Therefore, the coating of TEO with CS-NPs was highly effective for inhibiting the growth of all *Aeromonas* spp. isolates at a ratio of 1:1 and 1:0.75 µg/ mL, respectively, compared with the least tested concentrations. In contrast, Ibrahim *et al.* (41) stated that CS-NPs could prevent the growth of both Gram-positive and Gram-negative bacteria. El-Wafai *et al.* (42) found that, in the case of *Aeromonas veronii*, the diameter of inhibition zone increased when the CS-NP concentration was increased at 2.0 µg/mL. Previous literature stated that the small-sized droplets of nano-emulsion enhanced the antimicrobial efficacy due to increased surface areas that allowed its penetration into the outer cell wall of bacteria (35). In the current study, the diameter of TEO-N ranged from 150 to 200 nm, while the NP diameter ranged from 16.8 to 18.4 nm in CS-NPs. On the other hand, Mohammadi *et al.* (25) found that using cinnamon oil/CS nanoparticle coating enhanced the physicochemical and microbial features of cucumbers and lowered microbial count during storage. Sessa *et al.* (43) observed that the effectiveness of nano-emulsion-based coatings (modified CS containing lemon oil nano-emulsion) on rucola leaf shelf-life was much better than when using lemon oil and/or CS coating alone, extending the shelf-life up to 7 days.

Conclusion

The results obtained in this study are considered promising, with a product that can be exploited for the control of biofilm-producing *Aeromonas* spp. The use of CS-NPs alone and/or the coating of TEO with CS-NPs was quite effective in inhibiting growth (100%) of all *Aeromonas* spp. isolates at a ratio of 1:1 and 1:0.75 µg/ mL. Additionally, it can be applied as a disinfectant product and/or antimicrobial agent for the treatment of a drinking water distribution system; it also acts as a decontaminator for water tanks and drinkers at the poultry farm level.

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Conflict of interest

The authors declare that there are no competing interests.

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السيطرة على بكتيريا الأيروموناس المنتجة للبيوفيلم في خزانات المياه والمساقي لمزارع الدجاج اللاحم باستخدام زيت الزعتر المحمل على جزيئات الكيتوزان النانو

أسماء نادى محمد[١] و أميرة سمير عطية[٢]

[١]قسم الصحة والأمراض المشتركة والوبائيات، كلية الطب البيطري، جامعة بني سويف، بني سويف، [٢]قسم الصحة العامة البيطرية، كلية الطب البيطري، جامعة الزقازيق، الزقازيق، مصر

الخلاصة

تعد البكتيريا المنتجة للغشاء الحيوي (البيوفيلم) في نظام توزيع مياه الشرب بمثابة جرس إنذار لزيادة ظهور مسببات الأمراض المنقولة بالمياه. لذا تهدف الدراسة إلى رصد مدى انتشار أنواع الأيروموناس المكونة للغشاء الحيوي في أنظمة توزيع مياه الشرب في مزارع الدجاج اللاحم المختلفة. كما تم تقييم النشاط المضاد للميكروبات لكل من زيت الزعتر العطري، مستحلب النانو من زيت الزعتر، الكيتوزان، جزيئات الكيتوزان النانوية، زيت الزعتر المحمل على كلا من الكيتوزان وجزيئات الكيتوزان النانوية ضد جميع أنواع معزولات الأيروموناس باستخدام التخفيف الكلي في المرق ومقايسة انتشاره في الأجار. وأظهرت النتائج أن معدل الانتشار لميكروب الأيروموناس هو ٤٩,٣٪ (١٥٠/٧٤). كما لوحظ أن أعلى معدل لمعزولات الأيروموناس في خزانات ومساقي المياه ٧٥% (٤٠/٣٠) و ٦٢,٥% (٤٠/٢٥) على التوالي تليها الأعلاف ٤٠% (٣٠/١٢). وفي المقابل، فإن أعلى نسبة مئوية من الأيروموناس المنتجة للغشاء الحيوي كانت الأيروموناس هيدروفيلا ٧٠% (٢٠/١٤) تليها الأيروموناس كافي ٣٠% (٢٠/٦). ووجد أن التأثير المميت لـ جزيئات الكيتوزان النانوية ١٠٠% ضد جميع أنواع الأيروموناس المعزولة عند ١,٥ و ٢,٠ ميكروغرام / مل. علاوة على ذلك، أثبتت جزيئات الكيتوزان النانوية التي تغطي زيت الزعتر العطري تأثيرها المميت ١٠٠% على كل من الأيروموناس هيدروفيلا وكافي بنسبة ١: ١ و ١: ٠,٧٥ ميكروغرام / مل. ولقد أثبتت الدراسة أن المصدر الرئيسي لـميكروب الأيروموناس في نظام توزيع مياه الشرب هى الحالة غير الصحية لخزانات المياه والمساقي التي سمحت للأغشية الحيوية بالإنتاج بسبب تراكم بكتيريا الأيروموناس على السطح الداخلي لتلك المعدات. كما يمكن استخدام كل من جزيئات الكيتوزان النانوية وزيت الزعتر العطري المحمل على جزيئات الكيتوزان النانوية كمطهر للسيطرة على الغشاء الحيوي المنتج بميكروب الأيروموناس.