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ORIGINAL RESEARCH

Control of Virulent *Listeria monocytogenes* Originating from Dairy Products and Cattle Environment Using Marine Algal Extracts, Silver Nanoparticles Thereof, and Quaternary Disinfectants

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Introduction: *Listeria monocytogenes* is an important foodborne pathogen of public- and animal-health concern globally. The persistence of *L. monocytogenes* in the dairy-processing environment has multifactorial causes, including lack of hygiene, inefficient cleaning, and improper disinfection practices.

Materials and Methods: A total of 300 dairy-product and environmental samples were collected from dairy-cattle facilities and local dairy shops and vendors in Qena, Egypt. Samples were screened for the incidence of *Listeria* spp. and to detect virulence determinants and disinfectant-resistance genes. Three marine algal species — *Caulerpa racemosa*, *Jania rubens*, and *Padina pavonica* — were collected from Hurghada on the Red Sea coast. Algal extracts were screened using gas chromatography–mass spectrometry. The antimicrobial activity of some marine algal extracts, nanoparticles derived therefrom, and some disinfectants against *L. monocytogenes* strains were assessed in vitro using agar-well diffusion and liquid-broth methods. The impact of *P. pavonica* extract on the growth and survival of virulent *L. monocytogenes* in cheese and whey were clarified.

Results and Discussion: The incidence of *L. monocytogenes* in dairy products and environmental samples was 15.5% and 19%, respectively. The most common toxigenic gene profile found among the isolates was $hlyA^{+}$–$inlA^{+}$–$prfA^{+}$. The sensitivity pattern of *L. monocytogenes* strains to disinfectant containing alkyl ($C_{12–16}$) dimethyl BAC was high compared to other tested quaternary ammonium compounds (QAC) disinfectants tested, which showed lower log reductions against resistant strains. The QAC disinfectant–resistance gene *qacH* was detected in 40% of the isolates. Potent bactericidal activity of a petroleum ether extract of *P. pavonica* and silver nanoparticles of *P. pavonica* were obtained against the virulent *L. monocytogenes* strain. The population of *L. monocytogenes* in cheese curd and whey after 14 days was reduced at a rate of 9 log CFU/g and 8 log CFU/mL, respectively due to the effect of *P. pavonica* extract. After 28 days of storage, *L. monocytogenes* was completely inactivated in those dairy products.

Conclusion: *P. pavonica* extract showed promising antimicrobial properties, calling for further comprehensive studies prior to it being applied in the food industry to enhance the safety, quality, and shelf life of products and protect public health.

Keywords: *Listeria monocytogenes*, virulence genes, cheese, *qacH* gene, disinfectants, algae, antimicrobial activity

Introduction

The genus *Listeria* is ubiquitous in nature and occurs frequently on farms and in food processing, handling, and storage environments.[1] *L. monocytogenes* causes

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listeriosis, which is one of the most serious foodborne diseases in humans, especially in pregnant women, neonates, and immunocompromised adults.[2]

The virulence potential of *L. monocytogenes* relies on several molecular determinants, including virulence genes responsible for the invasion of host cells (*inlA*, *inlB*, and *iap*), phagosomal escape (*hlyA*, *plcA*, and *plcB*) and positive regulatory factor A (*PrfA*).[3] All these facilitate the intracellular growth and spread of the bacterium within a mammalian host.[4] Another trait of *L. monocytogenes* is its colonization on working surfaces and transmission to dairy products,[5] even after cleaning and disinfection, due to subinhibitory concentrations of disinfectants on different scales.[6] Disinfectants based on quaternary ammonium compounds (QACs) are widely used in the food industry, and are known to be effective against *L. monocytogenes*.[7] Tolerance to such QACs as benzalkonium chloride (BAC) has been observed in many *L. monocytogenes* strains. Multiple genetic markers have been identified that confer *L. monocytogenes* resistance to QACs, including the *qacH* gene of transposon Tn6188.[8]

Chemical or physical preservatives have traditionally been used to prevent *L. monocytogenes* degradation of food causing microbial resistance and the emergence of human- and animal-health risks.[9,10] Biopreservation is an alternative technologies that enhances food safety, stability, and quality using natural additives, and has gained increased attention.[11,12] Marine algae could prove to be promising agents to replace synthetic antimicrobial agents used in the food industry, because of their substantial natural bioactive compounds with broad antimicrobial bioactivity.[13] Antimicrobial action of marine algae against foodborne pathogens, including *L. monocytogenes*, has been confirmed.[14] As such, utilization of algal extracts and nanoparticle (NP) derivatives thereof as antimicrobial agents for food preservation could be an interesting alternative to physical and chemical methods.

As raw milk and ready-to-eat food products, including soft or semisoft cheese, are majorly involved in listeriosis outbreaks,[15,16] and the ability of *L. monocytogenes* to resist hostile conditions, including low temperature, osmotic stress, high salt content, and mild preservation treatment, its ability to form biofilms,[17] and the antimicrobial and antioxidant properties of microalga-derived compounds that have been used as food ingredients,[12,14] the present study aimed to investigate the incidence of *Listeria* spp., especially *L. monocytogenes*, in dairy-product (cheese and dairy desserts) and dairy-cattle environmental samples in Qena, Egypt, and the presence of some virulence genes in the obtained *L. monocytogenes* isolates, detect *L. monocytogenes* resistance to some surface disinfectants and molecular detection of the *qacH* resistance gene, and control *L. monocytogenes* propagation in both the dairy environment and ripened soft white cheese using some marine algal extracts, NP derivatives thereof, and QACs disinfectants.

Materials and Methods

Ethics Approval

Ethics approval was not required for this study.

Collection of Samples

A total of 300 dairy-product (200) and environmental (100) samples were collected from dairy-cattle facilities and local dairy shops and vendors in Qena. From dairy shops and vendors, 25 milk-product samples were collected: cheese (feta, kareish, talaga, processed) and dairy desserts (ice cream, custard, mahalabia, rice milk). Fifty drinking-water troughs and udder water-wash samples were collected as per the WHO,[18] as well as 25 cattle-bedding and 25 manure samples as per Rendos et al.[19]

Isolation and Identification of *Listeria* Species

Isolation of *Listeria* spp. was carried out as per Roberts and Greenwood[20] using *Listeria*-selective enrichment broth (CM0862, Oxoid) supplemented with the *Listeria*-selective enrichment agent SR0141 (Oxoid) and Oxford *Listeria*-selective agar (CM0856, Oxoid) supplemented with the *Listeria*-selective supplement SR0140 (Oxoid). Presumptive *Listeria* spp. isolates were confirmed according to Gram reactions and biochemical identification.[21] The isolated and characterized *L. monocytogenes* strains were confirmed using a Microbact Listeria 12L kit system (Oxoid) as per Chye et al.[22]

Detection of Virulence and Disinfectant-Resistance Gene *qacH* in *L. monocytogenes* by PCR

Isolated *L. monocytogenes* strains underwent PCR to detect *hly*A, *inlA*, and *prfA* virulence genes, while disinfectant resistance in *L. monocytogenes* isolates was detected using *qac*H. Primer sequences and amplicon size are presented in Table 1.[23,24] Equipment and material used comprised a QIAamp DNA mini kit (51304),

Table 1 PCR protocol: primer sequences, amplicon size, and amplification reactions for virulence genes

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
Virulence genes		174	94°C 5 minutes	(35 cycles)			72°C 7 minutes	Deneer and Boychuk[23]
hlyA	GCATCTGCATTCAATAAAGA			94°C 30 seconds	50°C 30 seconds	72°C 30 seconds		
	TGTCACTGCATCTCCGTGGT							
inlA	ACGAGTAACGGGACAAATGC	800	94°C 5 minutes	94°C 30 seconds	55°C 40 seconds	72°C 50 seconds	72°C 10 minutes	Liu et al.[3]
	CCCGACAGTGGTGCTAGATT							
prfA	TCTCCGAGCAACCTCGGAACC	1,052	94°C 5 minutes	94°C 30 seconds	50°C 40 seconds	72°C 1 min.	72°C 10 minutes	Dickinson et al.[24]
	TGGATTGACAAAATGGAACA							
Disinfectant-resistance gene		366	95°C 15 minutes	(30 cycles)			72°C 10 minutes	Müller et al.[8]
qacH	ATGTCATATCTATATTTAGC			94°C 30 seconds	56°C 90seconds	72°C 30 seconds		
	TCACTCTTCATTAATTGTAATAG							

DreamTaq green PCR master mix (2×), K1081; Thermo FisherScientific), and agarose-gel electrophoresis.[25]

Evaluation of Antibacterial Activity of Disinfectants Against *L. monocytogenes*

W aimed to evaluate the bactericidal activity of three disinfectants — BAC (Sigma-Aldrich), Vanoquat (alkyl [$c_{12–16}$] dimethyl BAC, $c_{12–15}$ alcohol ethoxylate; Evans Vanodine International), and BioSentry 904 QAC base (Hacco) — against *L. monocytogenes* strains.

Testing was performed as per European Norm 1276,[26] which specifies a quantitative suspension test for evaluation of bactericidal activity of chemical disinfectants used in the food industry. Bacterial suspensions were prepared from fresh *L. monocytogenes* strains that had been isolated. The bacterial log count was adjusted to 9 $\log_{10}$ using spectrophotometry and McFarland standards. The mixture was maintained at 20°C for 2 minutes. Then, the disinfectant solutions were added and the mixtures maintained at 20°C for 5 and 10 minutes. After this, an aliquot was taken and the bactericidal activity in this portion immediately neutralized or suppressed by the dilution-neutralization method using a mixture of polysorbate 80 30.0 g/L, saponin 30 g/L, lecithin 3 g/L, and histidine 1g/L for QAC-based disinfectants.[27,28] After neutralization for 5 minutes at 20°C, 1 mL of the neutralized test mixture was immediately taken and diluted with diluent to 10^{-7} dilution. The log count of *L. monocytogenes* was determined after plate counting on specific media.

Antibacterial Activity of Algal Extracts and Nanoparticle Derivatives Against *L. monocytogenes*

Algal Collection and Extraction Preparation

Three marine algal species — *Caulerpa racemosa* (Chlorophyta), *Jania rubens* (Rhodophyta), and *Padina pavonica* (Phaeophyta) — were collected from Hurghada on the Red Sea coast during March 2019. These species were identified according to standard taxonomic keys[29,30] Samples were collected in sterilized polyethylene bags and kept in an icebox for transport to the laboratory. Sample preparation and extraction were done as per to Ahmed et al.[31] The weighted crude extracts were suspended in dimethyl sulfoxide (DMSO) to a final concentration of 50 mg/mL, then stored in airtight bottles in a refrigerator until used for experiments.

Analysis of Algal Extracts

Algal extracts were analyzed using gas chromatography–mass spectrometry. technique. A Thermo Fisher Scientific Trace 1310 with capillary column TG-5 (30×250×0.25 µm) was used. A mass detector was used in split mode, and helium gas with flow rate 1.5 mL/min was used as a carrier. The injector was operated at 230°C and oven temperature for initial setup was 60°C for 2 minutes, ramping up by 10°C/min to 300°C for 8 minutes. Mass spectra were taken at 70 eV, and total GC run time was 35 minutes.

Biosynthesis and Characterization of Silver Nanoparticles of *P. pavonica* (Ag-NPs–*P. pavonica*)

Biosynthesis and characterization of Ag-NPs–*P. pavonica* were performed: 1 g algal powder was dissolved in 100 mL distilled water in a 250 mL Erlenmeyer flask and heated in a water bath at 60°C for 1 hour. The heated extract was filtered through Whatman No. 1 filter paper. The filtrates were collected, and the algal extracts were stored in fridge at 4°C. Ag-NPs were synthesized by adding 10 mL of pure algal extracts to 90 mL of $AgNO_3$ (1 mM) in a 250 mL Erlenmeyer flask, heated in water bath at 60°C for 1 hour with continuous stirring, then incubated in the dark for 24 hours at room temperature.[31]

Characterization of Ag-NPs–*P. pavonica*

Characterization of Ag-NPs was performed as described previously by Saber et al[32] and El-Sheekh et al.[33] Ag-NP morphology was measured with transmission electron microscopy (TEM) JEOL 2010. Dynamic light scattering (DLS) was used for measuring size distribution, and potential was assessed using Zetasizer Nano compact scattering (Malvern Instruments).

Anti–*L. monocytogenes* Activity of Algal Extracts and Ag-NPs–*P. pavonica*

The anti–*L. monocytogenes* activity of algae extracts and Ag-NPs–*P. pavonica* against virulent and QAC-resistant strains was detected using agar-well diffusion as per Zainol et al[34] and the liquid-broth method described by Ahmed et al.[31] In the latter method, data are expressed as log CFU/mL as a function of alga-extract concentration in the assay medium. All assays were performed in triplicate, and the results are given as means of three independent experiments.

Impact of *P. pavonica* Extract on Growth and Survival of Virulent *L. monocytogenes* in Soft Cheese and Whey

The initial suspension of *L. monocytogenes* was prepared using the 0.5 McFarland standard. The strain used was carrying all the virulence and disinfectant-resistance genes for which it was being investigated. Cheese was prepared at the lab from UHT milk warmed to 40°C and then 5% NaCl and rennet extract (1:6,000) added. Cheese was manufactured as per Abou-Donia,[35] with some modification. Then, an inoculum of *L. monocytogenes* was added to obtain a load of 5 log CFU/mL. After thorough mixing of the milk-bacteria suspension, *P. pavonica* extract was added at a concentration of 750 µg/mL. The mixture was left to coagulate for 2–3 hours at 40°C, then whey was removed completely from the curd. Portions (1 g and 1 mL) were taken from the curd and whey, respectively, at once to enumerate *L. monocytogenes*, and counting was done for the first 3 days and then weekly during the ripening period. The manufactured cheese was stored at 10°C in soldered tins. *L. monocytogenes* was enumerated by surface plating using Oxford *Listeria*-selective agar supplemented with *Listeria*-selective supplement. Cheese kept as control was manufactured and examined with the same procedure without addition of the algal extract. Results are expressed as CFU/g. Logarithmic bacterial load reduction was calculated:

$$\log_{10}\left[\frac{A}{B}\right]$$

where A is the *L. monocytogenes* count in cheese kept as control and B the *L. monocytogenes* count in cheese with *P. pavonica* extract, both in CFU/g.

Results

Incidence of *L. monocytogenes* Isolated from Dairy Products and Environmental Samples

Overall 38%, 45% and 46% of samples yielded *Listeria* spp.: cheese, dairy desserts, and environmental samples, respectively. Of these 13%, 18%, and 19% of samples were positive for *L. monocytogenes*, respectively. From the 100 cheese samples, 16%, 20%, 16%, and 0 of feta, kareish, talaga, and processed cheese were contaminated with *L. monocytogenes*, while *L. monocytogenes* was isolated from 20%, 16%, 8%, and 28% of ice cream, custard, mahalabia, and rice milk samples, respectively (Table 2). Among the tested environmental samples, 46% were positive for *Listeria* spp., of which 41% were identified as *L. monocytogenes*. Manure samples contaminated with

Table 2 Incidence of *Listeria* spp. in the examined samples

Type of sample			Total No. of *Listeria* spp.		*L. monocytogenes*		*L. welshimeri*		*L. innocua*		*L. seeligeri*		*L. ivanovi*		^{2}(nonparametric)	*P*
			No.	%	No.	%	No.	%	No.	%	No.	%	No.	%		
Milk products samples	Cheese	Feta cheese	12	48	4	16	2	8	3	12	2	8	1	4	2.167	0.705
		Kareish cheese	13	52	5	20	1	4	2	8	3	12	2	8	1.231	0.873
		Talaga cheese	10	40	4	16	2	8	1	4	2	8	1	4	3.00	0.558
		Processed cheese	3	12	0	0.0	1	4	0	0.0	1	4	1	4	0	1.00
	Sub-Total (n=100)		38	38	13	13	6	6	6	6	8	8	5	5	5.421	0.247
	Dairy desserts	Ice cream	9	36	5	20	2	8	1	4	0	0.0	1	4	4.778	0.189
		Custard	11	44	4	16	2	8	2	8	2	8	1	4	2.182	0.702
		Mahalabia	9	36	2	8	3	12	2	8	1	4	1	4	1.556	0.817
		Rice Milk	16	64	7	28	2	8	3	12	2	8	2	8	5.875	0.209
	Sub-Total (n=100)		45	45	18	18	9	9	8	8	5	5	5	5	12.67	0.13
Total (n=200)			83	41.5	31	15.5	15	7.5	14	7	13	6.5	10	5	16.46	0.002**
Water and environmental samples		Cattle manure	16	64	7	28	3	12	1	4	2	8	3	12	6.50	0.165
		Cattle bedding	13	52	5	20	1	4	2	8	3	12	2	8	3.538	0.472
		Udder water wash	8	32	3	12	0	0	2	8	1	4	2	8	1.00	0.801
		Drinking water troughs	9	36	4	16	2	8	1	4	1	4	1	4	3.778	0.437
Total (n=100)			46	46	19	19	6	6	6	6	7	7	8	8	13.35	0.010*
Overall Total (300)			129	43	50	16.6	21	7	20	6.7	20	6.7	18	6	28.56	0.000*

Notes: *P value ≤ 0.05 is significant; **P value ≤ 0.01 is significant.

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Listeria spp. had incidence of 64% and 28% pathogenic *L. monocytogenes* strains, which represented about 43.8% of *Listeria* spp. Furthermore, *Listeria* spp. existed in about 36% of drinking water–trough samples, and 44.4% of these isolates were found to be *L. monocytogenes* (Table 2 and Table S1). Other *Listeria* isolates were identified as *L. welshimeri* (7%), *L. innocua* (6.7%), *L. seeligeri* (6.7%), and *L. ivanovi* (6%; Table 2).

Incidence of Virulence Genes in Isolated *L. monocytogenes*

hlyA, *inlA*, and *prfA* were found in 54.8%, 67.7%, and 61.3%, respectively, of *L. monocytogenes* isolates of dairy products and 52.6%, 73.7%, and 57.9% in environmental samples, respectively (Table 3 and Figure 1). Specific virulence-gene profiles turned out to be more common than others in the current study. Eight virulence-gene profiles were detected in our survey, which covered a total of 50 isolates. The most common virulence profile found among the isolates was toxin profile 1 ($hlyA^+/inlA^+/prfA^+$), where strains isolated from most examined samples were found, followed by toxin profile 4 ($inlA^+/prfA^+$) and toxin profile 2 ($hlyA^+/inlA^+$). Other profiles are shown in Figure 2 and Table S2. In almost all profiles, isolated strains in cheese, dairy desserts, and environmental samples were involved.

Antibacterial Activity of Disinfectants and QAC-Resistance Genes

All strains of *L. monocytogenes* (50) isolated from dairy and environmental samples were screened for susceptibility to/resistance against QAC disinfectants. The overall prevalence of susceptible strains was 30 of 50 (60%): 20 isolates from dairy products and the remaining ten from environmental samples. A total of 20 *L. monocytogenes* isolates showed resistance against QAC disinfectant: eleven isolated from dairy products and nine isolates recovered from environmental samples.

Table 3 Virulence-gene profile of *L. monocytogenes* strains screened by PCR[a]

Type of sample			*hlyA*		*inlA*		*prfA*		P value	^{2}(nonparametric)
			No.	%	No.	%	No.	%		
Milk products samples	Cheese	Feta cheese (n=4)	2	50	4	100	3	75	0.667	0.717
		Kareish cheese (n=5)	3	60	3	60	4	80	0.200	0.905
		Talaga cheese (n=4)	2	50	2	50	3	75	0.286	0.867
	Sub-Total (n=13)		7	53.8	9	69.2	10	76.9	0.538	0.764
	Dairy desserts	Ice cream (n=5)	3	60	3	60	2	40	0.250	0.883
		Custard (n=4)	2	50	2	50	3	75	0.286	0.867
		Mahalabia (n=2)	1	50	2	100	1	50	0.500	0.779
		Rice Milk (n=7)	4	57.1	5	71.4	3	42.9	0.500	0.779
	Sub-Total (n=18)		10	55.6	12	66.7	9	50	0.452	0.798
Total (n=31)			17	54.8	21	67.7	19	61.3	0.421	0.810
Water and environmental samples		Cattle manure (n=7)	3	42.9	4	57.1	5	71.4	0.500	0.779
		Cattle bedding (n=5)	4	80	4	80	2	40	0.800	0.670
		Udder water wash (n=3)	1	33.3	2	66.7	1	33.3	0.500	0.779
		Drinking water troughs (n=4)	2	50	4	100	3	75	0.667	0.717
Total (n=19)			10	52.6	14	73.7	11	57.9	0.743	0.690
Overall Total (n=50)			27	54	35	70	30	60	1.065	0.587

Notes: [a]All processed cheese samples were devoid of *L. monocytogenes*.

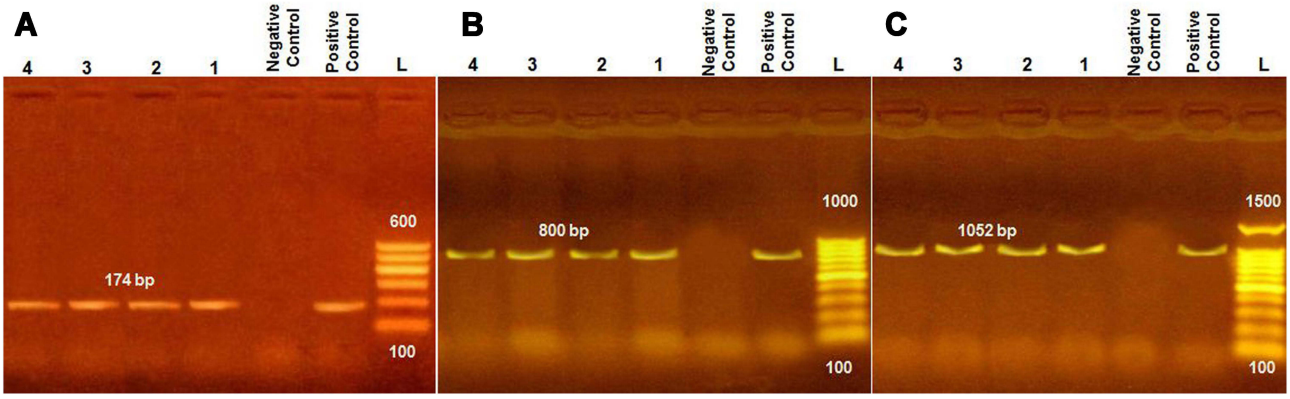

Figure 1 PCR products of amplified virulent genes identified in *L. monocytogenes* and visualized with agarose-gel electrophoresis. Molecular size of amplified DNA: 174 bp for *hlyA* (**A**), 800 bp for *inlA* (**B**), and 1052 bp for *prfA* (**C**). Lanes 1–4, samples; L, 100 bp DNA ladder.

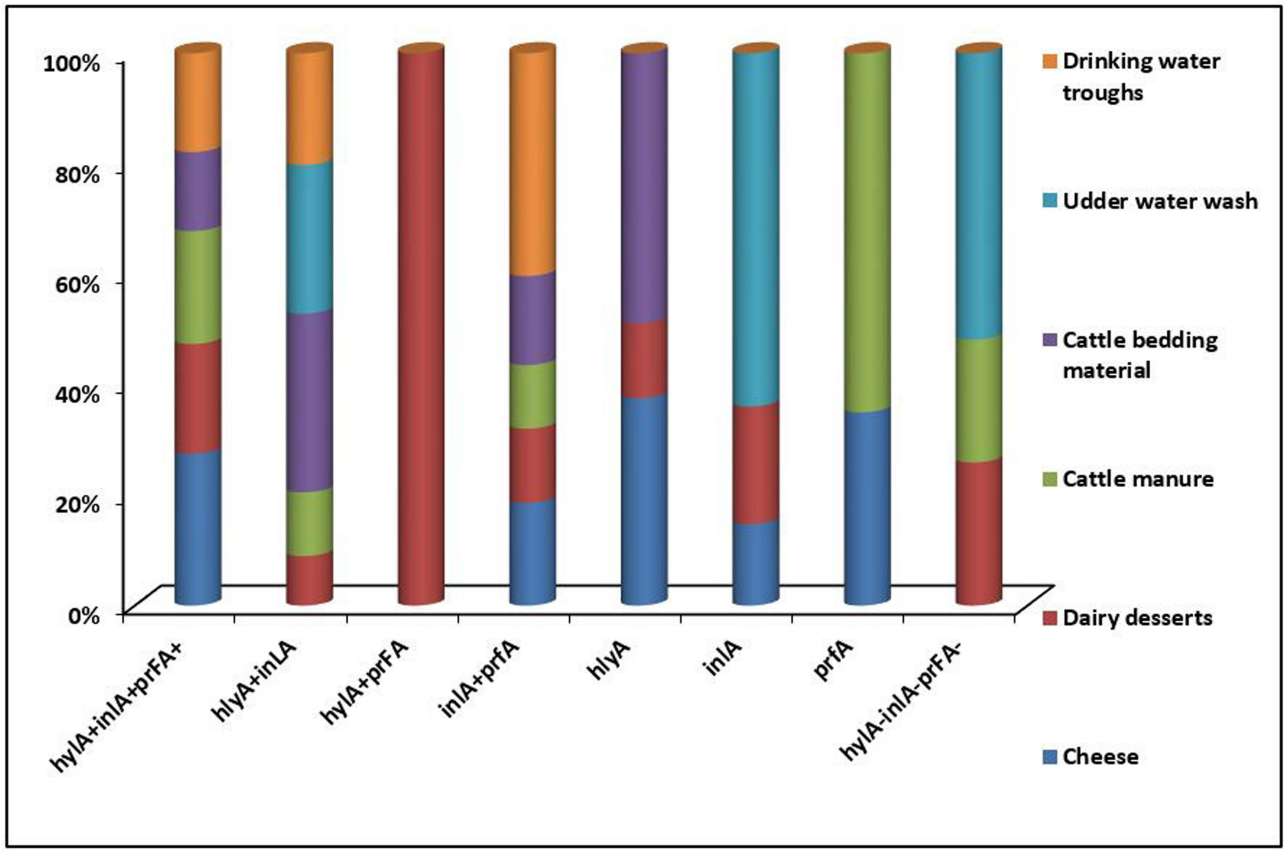

Figure 2 Distribution of *hlyA*, *inlA*, and *prfA* virulence genes in *L. monocytogenes* isolates (n=50) recovered from cheese, dairy desserts, and environmental samples.

Log reductions for various disinfectants against susceptible and resistant *L. monocytogenes* clarified the marked lower efficacy of all the tested disinfectants against resistant strains. Increasing the contact time to 10 minutes did not improve the inhibitory power of disinfectants, eg, BioSentry 904 at its highest concentration 1:32 showed log reduction of 3.97, which slightly improved to 4.23 after increasing the contact time to 10 minutes at the same concentration. The same phenomena were observed for BAC and Vanoquat disinfectants against resistant *L. monocytogenes* strains. In contrast, the most potent disinfectant against *L. monocytogenes*-susceptible/sensitive strains was Vanoquat, with log reductions of 6.64–8.42 $\log_{10}$, followed by BioSentry 904 with 6.47–8.14 $\log_{10}$, and the least powerful was BAC: 5.21–7.76 $\log_{10}$ (Figure 3 and Table S3). Screening of *L. monocytogenes*

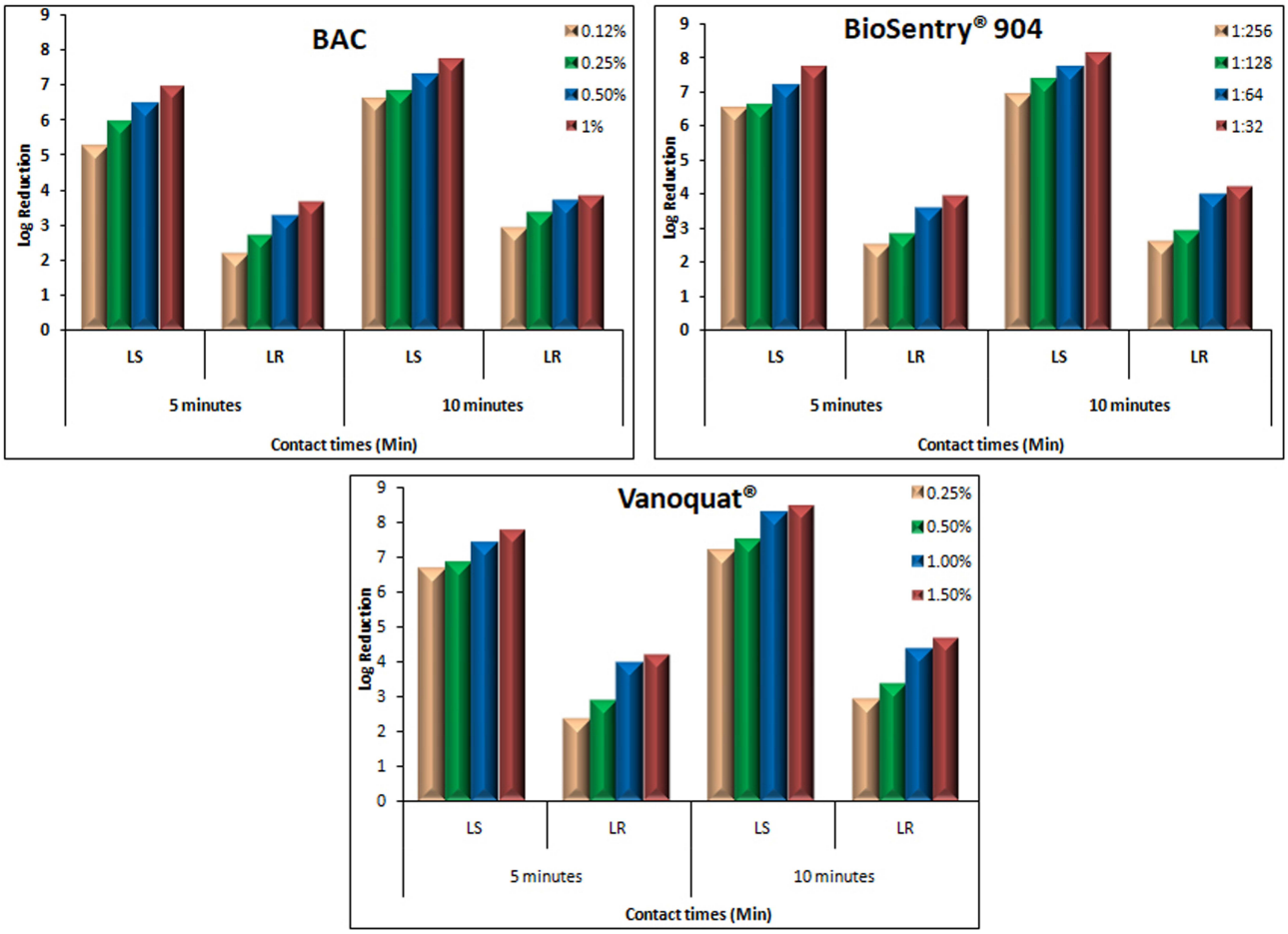

Figure 3 Log-reduction values of various QAC disinfectants (BAC, BioSentry 904, and Vanoquat) against *L. monocytogenes*-susceptible and -resistant strains isolated from dairy products using European suspension at various contact times.

strains for QAC-resistance genes revealed that 20 of 50 (40%) carried *qacH* (Figure 4).

Characterization of Ag-NPs–*P. pavonica*

TEM showed the shape of Ag-NPs biosynthesized by *P. pavonica* extract (Figure 5). The image reveals that most NPs are spherical and some agglomerates in ellipsoidal form. Size, count, and aggregation of Ag-NPs were estimated by DLS. The total count of Ag-NPs was 5×10^5 NPs/mL and average diameter 46.21 nm. Ag-NPs synthesized by *P. pavonica* extract showed high negative -potential: −25.4 mV. The polydispersity index (PDI) value of Ag-NPs was 0.638 and *Z*-average 142.6 (d•nm). The PDI value shows that the particles were polydispersed.

Antibacterial Activity of Algal Extracts

Acetone extracts of *C. racemosa* and *J. rubens* and petroleum ether extract of *P. pavonica* were potentially effective in inhibiting *L. monocytogenes* growth, with variable potency. The phytochemical constituents of those algal extracts were identified by GC-MS (Figure 7). The active compounds with retention time, molecular formula, molecular weight, and relative concentration (%) for the algal extracts are presented in Table 5 and Table S4. The acetone extract of *J. rubens* and petroleum ether extract of *P. pavonica* were the most effective suppressors of *L. monocytogenes* at 3 mg/mL, with inhibition zones of 7.4 and 9.2 mm, respectively (Table 4). Moreover, the antibacterial activity of algal extracts and Ag-NPs–*P. pavonica* against *L. monocytogenes* was confirmed using liquid dilution (Figure 6A and B). When incubated with *L. monocytogenes* for 24 hours, all tested materials exhibited strong bactericidal activity in a dose-dependent manner. The petroleum ether extract of *P. pavonica* was very effective against *L. monocytogenes*, resulting in severe reduction in bacteria CFU (13 $\log_{10}$ orders of killing) at 3 mg/mL; however, the acetone extracts of *J. rubens* and *C. racemosa* showed much less log reduction (8 $\log_{10}$ orders of killing) of *L.*

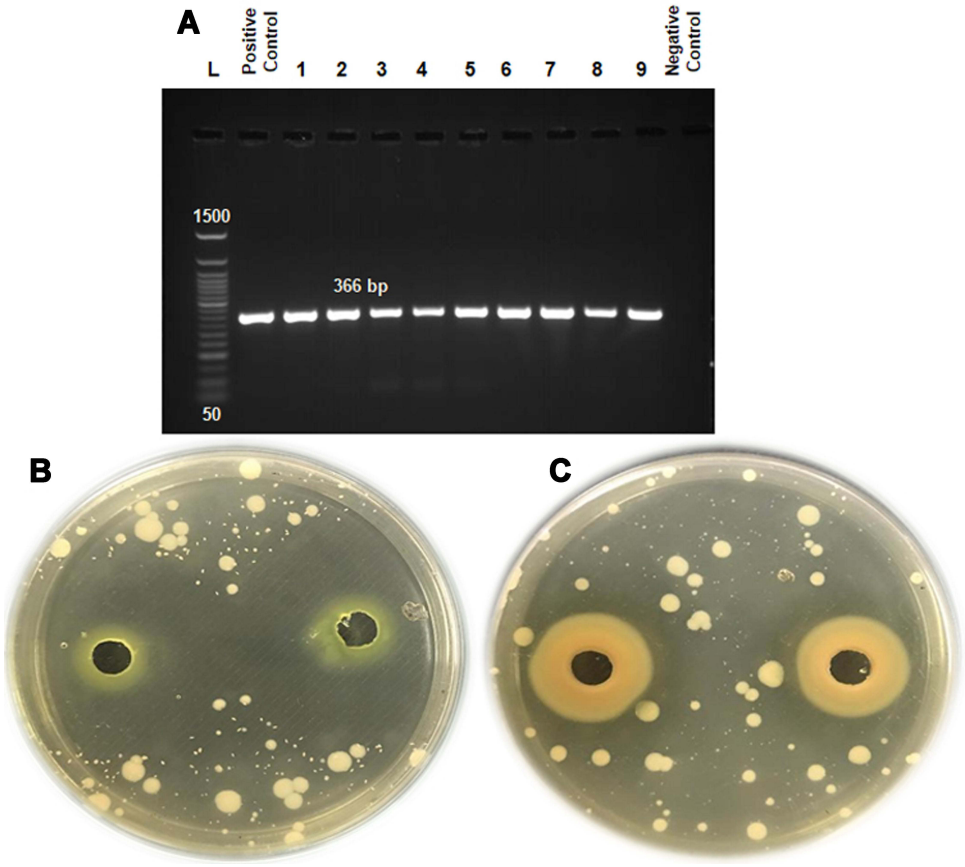

Figure 4 (**A**) The *qacH* gene in *L. monocytogenes*-tolerant strains conferred resistance to QAC disinfectants. (**B**, **C**) Agar-well diffusion assay showed inhibition zones for susceptible and resistant *L. monocytogenes* strains.

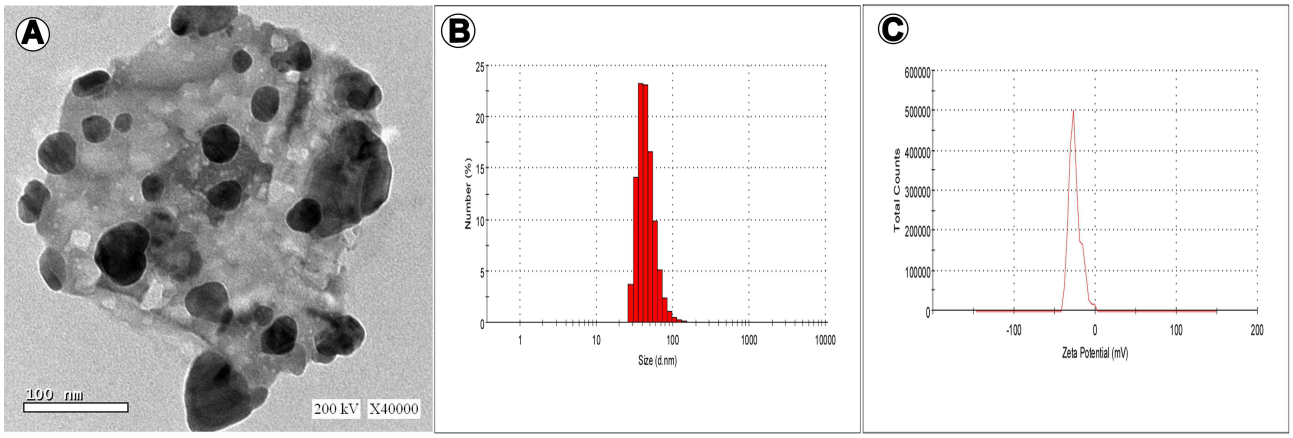

Figure 5 TEM (**A**), size distribution (**B**), and -potential (**C**) of Ag-NPs biosynthesized by *Padina pavonica*.zzzz

monocytogenes at the same concentration of 3 mg/mL (Figure 6A). Also, Ag-NPs–*P. pavonica* exhibited effective bactericidal action (7–8 $\log_{10}$ orders of killing) at concentration of 1.6×10^5 NPs–mL (Figure 6B).

The results of GC-MS analysis showed 10, 23, and 24 compounds in the acetone extracts of *C. racemosa* and *J. rubens* and petroleum ether extract of *P. pavonica*, respectively. The most prevalent compounds in *C. racemosa* acetone extract were 9-octadecenoic acid (*Z*)-, methyl ester (48.31%), β-sitosterol (16.23%), and hexadecanoic acid methyl ester (3.94%), while those of *J. rubens* acetone extract were 11-octadecenoic acid, methyl ester

Table 4 Antibacterial-screening tests of algal extracts (1.5 and 3 mg/mL) and disinfectants (various concentrations*) against *L. monocytogenes* determined by zone of inhibition using agar-well diffusion[a,b]

(A) Algae	Extract	Inhibition-zone diameter (mm)	
		1.5 mg/mL	3 mg/mL
Caulerpa racemosa	Acetone	3.1±0.44	6.5±0.35
Jania rubens	Acetone	5.8±0.03	7.4±0.15
Padina pavonica	Petroleum ether	6.3±0.41	9.2±0.47
DMSO		No inhibition	No inhibition

(B) Disinfectant*	Inhibition-zone diameter (mm)			
	Various concentrations*			
BAC	18.3±0.88	19.3±0.88	2.7±0.33	27.0±0.58
BioSentry 904	21.2±0.38	22.5±0.29	27.0±0.12	30.6±0.35
Vanoquat	28.0±0.58	32.6±0.34	37.3±0.88	40.0±0.15

Notes: [a]Means± SE of three replicates. Diameter of well (7 mm) not included. [b]Only positive values of the examined algae extracts were recorded. *BAC 0.12%, 0.25%, 0.5%, 1%; BioSentry 904 1:32, 1:64, 1:128, 1:256; Vanoquat 0.25%, 0.25%, 0.5%, 1.5%).

Table 5 Major bioactive chemical compounds identified in the most effective algal extracts

Algae	Extract	Retention time (minutes)	Compound name	Molecular formula	Molecular weight	Peak area (%)
Caulerpa racemosa	Acetone	32.29	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	3.94
		36.95	9-Octadecenoic acid (Z)-, methyl ester	$C_{19}H_{36}O_2$	296	48.31
		41.28	Heptacosanoic acid, methyl ester	$C_{28}H_{56}O_2$	424	2.8
		45.23	Docosanoic acid, methyl ester	$C_{23}H_{46}O_2$	354	2.08
		61.17	β-Sitosterol	$C_{29}H_{50}O$	414	16.23
Jania rubens	Acetone	3.46	13-Tetradecynoic acid, methyl ester	$C_{15}H_{26}O_2$	238	4.34
		6.36	17-Octadecynoic acid	$C_{18}H_{32}O_2$	280	3.26
		15.12	Tetradecanoic acid, methyl ester	$C_{15}H_{30}O_2$	242	3.23
		17.67	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	26.54
		18.38	Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	284	2.37
		20.36	11-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	296	38.51
		21.09	Ethyl Oleate	$C_{20}H_{38}O_2$	310	2.55
Padina pavonica	Petroleum ether	18.90	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	3.95
		22.22	Ethanone, 1-(7,8-dihydro-3-hydroxy-4-propyl-2-naphthalenyl)-	$C_{15}H_{18}O_2$	230	1.99
		22.47	Erucic acid	$C_{22}H_{42}O_2$	338	7.47
		26.34	Octacosane	$C_{28}H_{58}$	394	38.41
		28.10	15-Nonacosanone	$C_{29}H_{58}O$	422	33.05
		29.03	1-Heptatriacotanol	$C_{37}H_{76}O$	536	7.51

(38.51%), hexadecanoic acid methyl ester (26.54%), 13-tetradecynoic acid methyl ester (4.34%), 17-octadecynoic acid (3.26%), and tetradecanoic acid methyl ester (3.23%). The most abundant compounds in the *P. pavonica* petroleum ether extract were octacosane (38.41%), 15-nonacosanone (33.05%), 1-heptatriacotanol (7.51%), erucic acid (7.47%), and *n*-hexadecanoic acid (3.95%; Table 5, Table S4, and Figure 7).

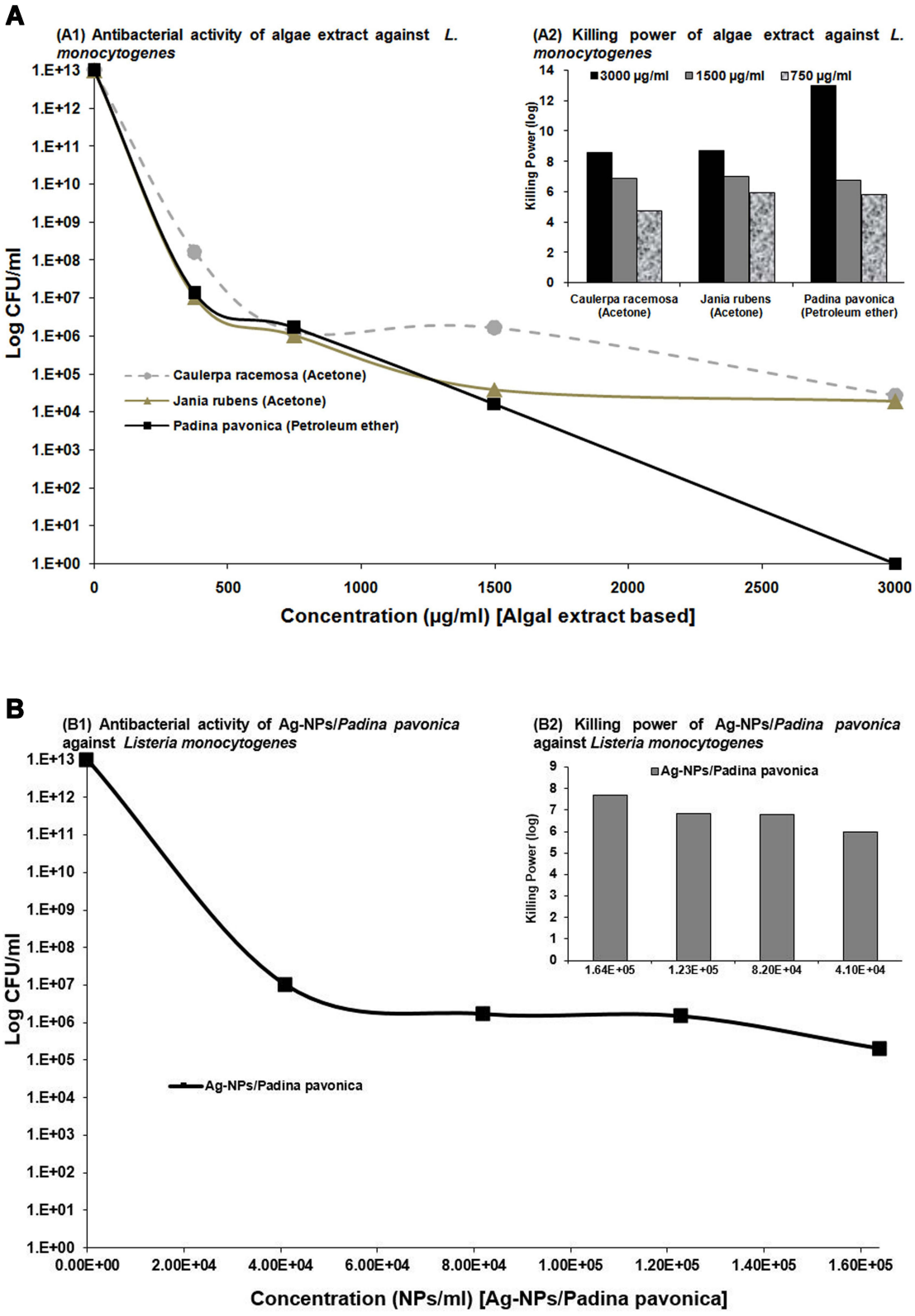

Figure 6 Antibacterial activity and killing power of algae extracts (**A**) and 1.64×10^5, 1.23×10^5, 8.2×10^4, and 4.10×10^4 Ag-NPs–*Padina pavonica* (**B**) against *L. monocytogenes* at concentrations of 750, 1,500, and 3,000 µg/mL. Data presented as log CFU/mL. Only positive values of the examined algal extracts and NPs were recorded. Assays were performed in triplicate.

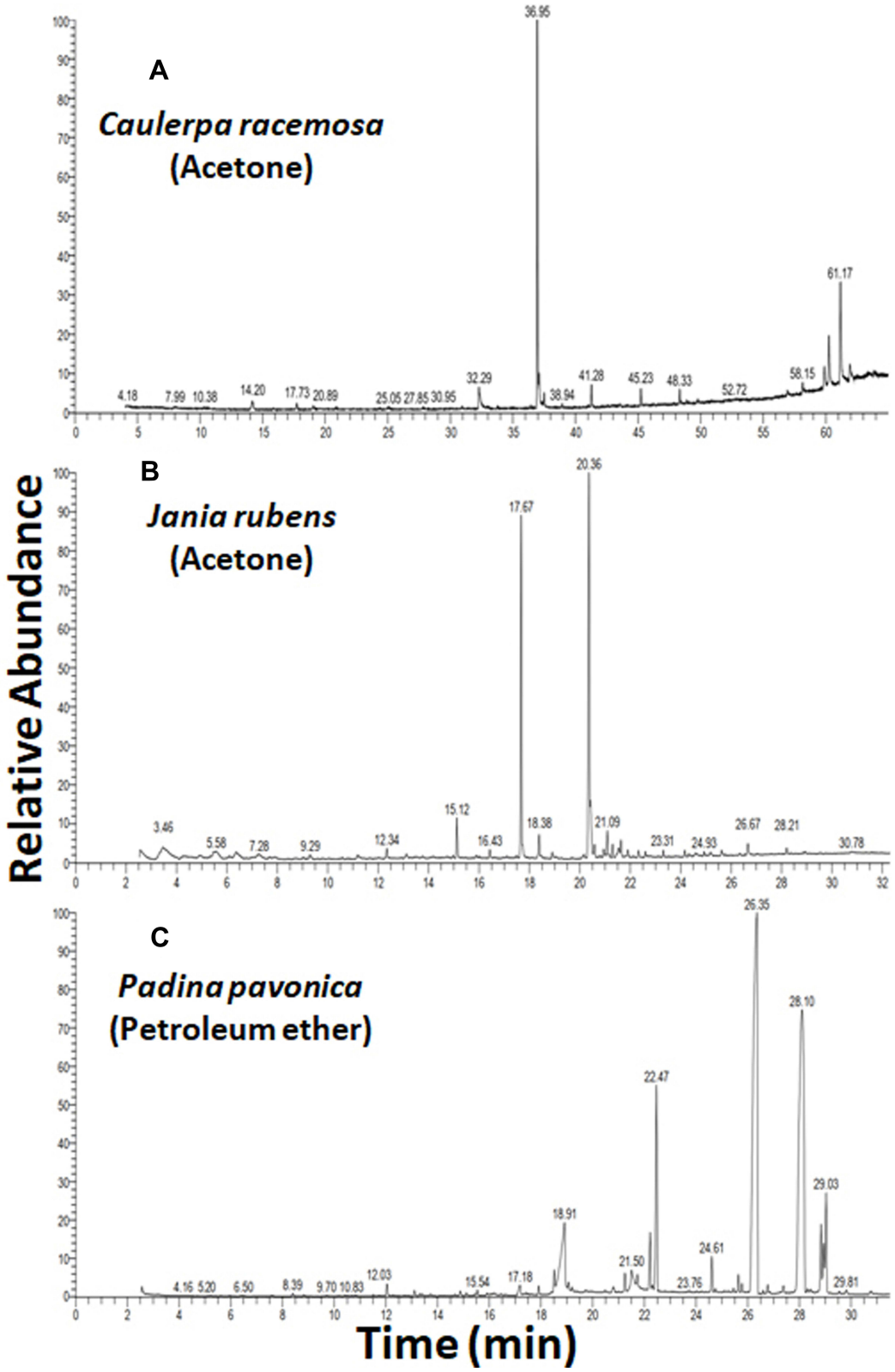

Figure 7 GC-MS of the most effective algal extracts.

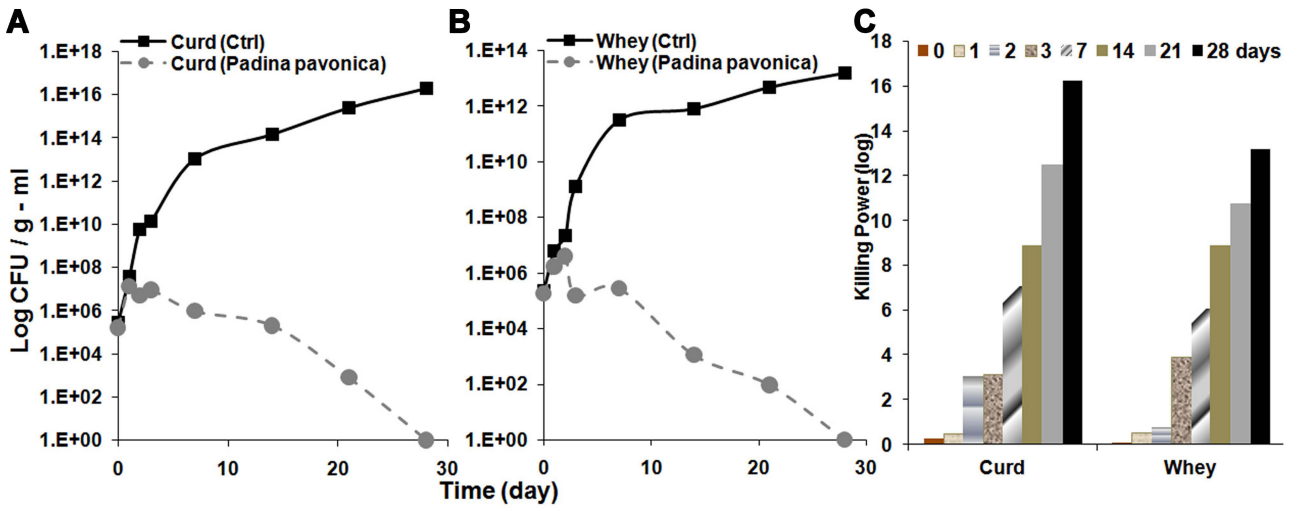

Figure 8 Effect of *Padina pavonica* extract (750 µg/mL) on the growth of *L. monocytogenes* in experimentally manufactured ripened white cheese: (**A**) cheese curd and (**B**) cheese whey. (**C**) Killing power of *Padina pavonica* extract (750 µg/mL) against *L. monocytogenes* in comparison with time (days) in both curd and whey of experimentally manufactured ripened white cheese.

Impact of *P. pavonica* Extract on Growth and Survival of Virulent *L. monocytogenes* in Cheese and Whey

This part of the study was designed based on the results exhibited by *P. pavonica* in the in vitro trials for antimicrobial activity against *L. monocytogenes*. An isolate of *L. monocytogenes* with toxin-virulence profile 1 (Table s2) and phenotypically and genotypically resistant to QACs had been inoculated in raw milk. The behaviour of *L. monocytogenes* during cheese manufacturing and storage in both the presence and absence of the *P. pavonica* extract is illustrated in Figure 8. Initial numbers of *L. monocytogenes* in control cheese curd and whey were 5.46 and 5.36 log CFU/g, respectively, while in *P. pavonica* extract cheese curd and whey were 5.2 and 5.2 log CFU/mL, respectively. In control cheese curd and whey, *L. monocytogenes* grew well, reaching 10, 14, and 16 log CFU/g in cheese curd and 9, 11, 13 log CFU/mL in cheese whey after 3, 14, and 28 days, respectively, under refrigeration. On the other hand, *L. monocytogenes* loads were reduced at different levels for curd and whey where the cheese had been treated with *P. pavonica* extract. After 3 and 14 days of ripening, *L. monocytogenes* counts in cheese curd were reduced by more than 4 and 9 log CFU/g; while in cheese whey counts were reduced by more than 4 and 8 log CFU/mL compared with the cheese inoculated with *L. monocytogenes* without *P. pavonica* extract. Pathogen levels continued to decline during storage, and finally at the 28th day of storage in either cheese curd or whey *L. monocytogenes* could not be detected, indicating the bactericidal power of *P. pavonica* extract against *L. monocytogenes* in cheese curd and whey.

Discussion

Rice milk and kareish cheese had the highest isolation rate of *L. monocytogenes*, followed by feta cheese and custard. The high prevalence of *L. monocytogenes* in these dairy products could be due to environmental pollution or unsanitary production methods and food storage. However, it could also be due to the use of unpasteurized milk. Also, *Listeria* spp. can continue to grow in the refrigerator and low salt–concentration environments. Moreover, cheese is the perfect environment for the growth of microorganisms.[36] On the other hand, no processed cheese was found to be contaminated with *Listeria* spp., emphasizing the fact that processed cheese is considered and classified as one that does not support the growth of *L. monocytogenes* under reasonable foreseeable conditions of distribution and storage.[37] However, postprocessing contamination of the product should be strictly avoided, as the pathogen can survive in the product for extended periods, particularly under refrigeration (4°C).

In animals, listeriosis is mainly a disease of ruminants.[38] Animals are commonly asymptomatic intestinal carriers, frequently shedding the organism and maintaining its populations in the environment. Especially, bovine hosts may amplify ingested *L. monocytogenes* and thus serve as a critical factor in maintaining high prevalence of the pathogen

on cattle farms.[39] Farm-specific factors, such as using untreated livestock manure as fertilizer, contamination of irrigation water through runoff from livestock facilities,[40] large group sizes, unhygienic practices during milking, and cattle drinking from a trough, might greatly affect the prevalence of *L. monocytogenes*. Good farm-level practices can thus be utilized to reduce the prevalence of this pathogen on the farm and possibly further in the food chain.[41]

In the present study, virulent *L. monocytogenes* strains harboring *hlyA*, *inlA*, and *prfA* were detected in 21 of 31 (67.7%) and 14 of 19 (73.7%) *L. monocytogenes* isolates obtained from dairy products and environmental samples, respectively (Table 3). This finding was less than previous studies in which virulence genes were detected in *L. monocytogenes* isolated from dairy products.[42,43] More than half the *L. monocytogenes* isolated from dairy products (61.3%) and environmental samples (68.4%) was positive for more than one virulence-associated gene, while none of 31 (29%) and 5 of 19 (26.3%) from dairy products and environmental samples, respectively, harbored only one virulence-associated gene (Figure 2 and Table S2) and were likely less virulent than those with multiple virulence-associated genes.

The current study revealed QAC disinfectants exhibited clear inhibition zones with complete lack of growth for some *L. monocytogenes* strains, while others showed tolerance against the QACs and had the ability to survive with complete growth inside the inhibition zones (Table 4 and Figure 4). All strains were further checked using the European suspension test to clarify the killing power/log reduction of the various disinfectants. This revealed a marked difference in killing power/log reductions of all disinfectants between susceptible *L. monocytogenes* strains and resistant. To confirm the findings, we used the QAC disinfectants at various concentrations and variable contact times against both a single sensitive strain and another single resistant strain of *L. monocytogenes* selected from those isolated from dairy products. Even after exposure of the resistant *L. monocytogenes* strains to higher concentrations and prolonged contact times, the log reduction was still very low for resistant strains compared to susceptible ones (Table S3 and Figure 3).

Our data revealed that BAC-QAC disinfectants had good bactericidal activity against *L. monocytogenes*–susceptible strains at all the tested concentrations for both contact times. However, against resistant strains, log reductions were much lower at all concentrations for both contact times. The association of the lower log reductions with resistant *L. monocytogenes* strains was observed among all the tested QAC disinfectants, including BioSentry 904 and Vanoquat (Table S3 and Figure 3).

Resistance to QACs disinfectants, such as BAC, is especially relevant to *Listeria*'s adaptations in food-related environments, as these compounds are used extensively in food processing, in retail, and for household or personal use.[44] BAC-resistant strains of *L. monocytogenes* have been implicated in multistate outbreaks of listeriosis, and have frequently been isolated from food-processing plants. However, the genetic basis for BAC resistance in *L. monocytogenes* remains poorly understood. The tolerance of *L. monocytogenes* to BAC varies between strains.[45] Resistance to BAC has been observed in different countries.[8] BAC resistance of *L. monocytogenes* isolated from food and the processing-plant environment has been found to range from 10%[7] to as much as 42%–46%.[46]

Different genetic markers have been identified that confer *L. monocytogenes* resistance to QACs, including the *qacH* gene of transposon Tn6188.[8] The presence and distribution of these genes have been anticipated to have a role in the survival and growth of *L. monocytogenes* in food-processing environments where QAC-based disinfectants are in common use. Some studies have shown that *L. monocytogenes* harbouring the QAC-resistance gene *qacH* are prevalent in the food industry and that residuals of QAC may be present after sanitation that result in a growth advantage for bacteria with such resistance genes.[47]

All strains were screened for the presence of *qacH*. Resistance to QACs was associated with *qacH* in 40% of the tested strains, while the remainder (60%) did not harbor that genetic determinant and were classified as sensitive to QACs (Figure 4). Previous research has shown that resistance to BAC was associated with *qacH* in a majority (80%) of the tested strains.[48] Furthermore, *qacH* has been found in 22% of *L. monocytogenes* isolates.[47] *L. monocytogenes* strains harbor the transposon Tn6188, responsible for increased tolerance against QAC through *qacH*, and it has been suggested that this contributes to survival and persistence.[49]

The persistent strains of *L. monocytogenes* in food-processing environments after cleaning and disinfection contribute to many factors, the most important being the presence of organic material.[50] Inadequate disinfection may produce resistance to the disinfectant as a result of selection or adaptation through regular exposure to sublethal concentrations.[7] In dairy/food-processing environments, *L. monocytogenes* is exposed to different disinfectants and sanitizers, sometimes at subinhibitory concentrations. This is particularly true for disinfectants that are not fully biodegradable and may persist in sewage for long periods. QACs are considered to have poor

biodegradability, so the contact between bacteria and QACs may be prolonged and consequently cause frequent exposure of microbial communities to subinhibitory concentrations of QACs, facilitating the development of resistance of certain strains over time.[51,52]

It has become increasingly difficult to protect human health from the adverse effects of *L. monocytogenes* pathogens. Limited use of chemical preservatives, susceptibility, toxicity, microbial resistance, and adverse effects on human health increase the need for pure, healthier, safer, and potentially successful antibacterial agents. Therefore, the antibacterial activity of algal extracts can provide key materials to be used as natural preservatives to ensure healthy and safe food. Potent antibacterial activity was observed for *P. pavonica* in petroleum ether extract against *L. monocytogenes*, resulting in a severe reduction in the CFU of bacterial load (13 $\log_{10}$ orders of killing) at 3 mg/mL. However, 3 mg/mL acetone extract of *C. racemosa* and *J. rubens* showed much less log reduction (8 $\log_{10}$ orders of killing) of *L. monocytogenes*.

Various studies have evaluated the antimicrobial activity of marine seaweed, ie, *P. pavonica*, *C. racemosa*, and *J. rubens*. The solvents petroleum ether, acetone, chloroform, methanol, ethanol, hexane, ethyl acetate, and water have been used for algal extraction to explore antibacterial activity against both Gram-positive and Gram-negative bacteria, including *L. monocytogenes*.[31] Globally, *P. pavonica* ethanolic extract exhibits the highest activity against *L. monocytogenes* growth, with an inhibition zone of 14 mm[53] and 13 mm against other *Listeria* spp.[54] In addition, methanolic ethanol, chloroform, acetone, ethyl acetate, and hexane extracts of *P. pavonica* show have shown activity against the growth of *L. monocytogenes* with 16±0.27, 14±0.11, 13±0.19, 11±0.22, 15±0.27, and 9±0.09 mm inhibition zones, respectively.[55] *Caulerpa* spp. exhibit antibacterial activity against the growth of *L. monocytogenes*, with a 20.6±0.6 mm inhibition zone.[56] Moreover, it has been reported that *C. racemosa* extract exhibits significant antimicrobial activity against pathogenic bacteria in human food.[57] The great efficacy of marine seaweed extracts against *L. monocytogenes* could be due to active phytochemicals and metabolite compounds, in addition to fatty acids and their derivatives.[13]

The potent antibacterial effects of *P. pavonica* petroleum ether extract against *L. monocytogenes* could be attributed to the most abundant detected phytochemical bioactive compounds: octacosane (38.41%), 15-nonacosanone (33.05%), 1-heptatriacotanol (7.51%), erucic acid (7.47%), and *n*-hexadecanoic acid (3.95%).

In vitro data presented in previous literature has proved that octacosane and 15-nonacosanone have potent antimicrobial activity against a wide range of bacterial species, including *L. monocytogenes*, show a nontoxic, nonmutagenic, and nontumorigenic properties, and could be potential antibacterial drugs. Moreover, octacosane and 15-nonacosanone show such antimicrobial properties as antibacterial, antifungal, and antiviral activity, which can be used to prevent microbial growth in wide-ranging applications such as food, cosmetics, and drug molecules.[58]

It could be concluded that those molecules identified have potential antimicrobial activities, and notably that the extracts of the algae where they are abundant also show potent antimicrobial activities. These algal species could be more effective against bacterial infection than traditional bactericidal agents. Therefore, natural preservatives can be considered to provide healthy, safe food via their stable biologically active compounds without the unpleasant effects of chemical ones. Also, this study provides insights into designing novel antibacterial agents for food preservation or clinical use.

Ripened soft white cheese is the most popular dairy product in Egypt and other Middle Eastern countries, and because of its popularity, upon contamination it can be a major cause of listeriosis, as *L. monocytogenes* has been reported to be the most common foodborne pathogen associated with cheese[7,59] and cheese is a potential reservoir for *L. monocytogenes* due to higher nutrient quality and greater contamination risk because increased handling. In Egypt, the incidence of *L. monocytogenes* has been evaluated in locally produced dairy products, such as cheese, and its higher incidence in dairy products, especially cheese, may be attributed to traditional unhygienic manufacturing techniques, resulting in linkage of these products to several outbreaks of listeriosis.[42,60] These results indicate the need for control strategies to prevent the dispersion of *L. monocytogenes* through cheese and the public-health hazards linked to consumption of these products. El Shinaway et al[61] described the ability of *L. monocytogenes* to survive and resist acidic, refrigeration, or freezing conditions during manufacturing, distribution, and storage of dairy products. We cannot rely solely upon these processes to control *L. monocytogenes* to provide safe products for human consumption. Green, brown, and red algae with antioxidant activity can be safely consumed by humans.[62,63]

The present research enabled us to discover the ability of algal species as antibacterial agents against *L. monocytogenes* and their ability to control *L. monocytogenes* in artificially inoculated white cheese, thereby offering strong reasons for the development of antimicrobial formulations for food

preservation. The data clarified that in the absence of *P. pavonica* extract, *L. monocytogenes* grew well, reaching 16 log CFU/g after 28 days under refrigeration. The excessive growth of *L. monocytogenes* in the control cheese is consistent with previous studies illustrating high growth potential of bacterial pathogens in white cheeses.[64]

Our results showed that the *P. pavonica* extract reduced the population of *L. monocytogenes* in white cheese by more than 4 and 9 log CFU/g after 3 and 14 days of storage, respectively, following the addition of the algal extract, and finally at the 28th day of storage, *L. monocytogenes* could not be detected. There have been few previous studies in relation to algal extracts incorporated into model food systems to challenge their antimicrobial efficacy against food microorganisms, especially *L. monocytogenes*. Cox et al[12] reported antimicrobial activity of *Himanthalia elongata* extract at varying concentrations (1%, 5%, and 10%) against *L. monocytogenes* in protein and carbohydrate model food systems. The extract provided complete bacterial inhibition, with bactericidal and bacteriostatic effects in carbohydrate and protein model food systems, respectively. Such extracts may have a multipurpose functionality, including antimicrobial and antioxidant bioactivity, which could potentially increase shelf life, safety, and quality of a wide range of food products.

Cho et al[65] investigated making a functional drinkable milk product using algal extract powder (*Chlorella* spp.), with evaluation of quality maintenance. They demonstrated that preservation of the dairy products with the addition of *Chlorella* extract was relatively good at 4°C° for 15 days. The results of sensory evaluation indicated that color and taste were significantly acceptable. In the current study, the addition of *P. pavonica* extract to cheese not only increased its microbiological safety but also increased the quality and smoothness of the cheese-curd texture, and the pH of the cheese by the end of the experiment was 4.32. Our results are in accordance with Jeon,[66] who investigated the effect of addition of *Chlorella* algal extract on the microbial and sensory quality of processed cheese. Their results suggested that processed cheese with improved microbial quality can be made by the addition of microalgae, due to its ability to inhibit microorganisms. Moreover, processed cheese prepared with *Chlorella* was higher on descriptive analysis scores for color, mouth feel, hardness, and springiness.

Conclusion

In the current study, the petroleum ether extract of the seaweed tested showed strong antimicrobial efficacy against *L. monocytogenes* in cheese. At a concentration of 3 mg/mL, *L. monocytogenes* was remarkably inhibited upon exposure to *P. pavonica* in petroleum ether extract, resulting in 13 $\log_{10}$ orders of killing. QAC disinfectant (alkyl [$c_{12\text{-}16}$] dimethyl BAC) at a concentration of 1.5% for 5 minutes' contact resulted in a 7.7 log reduction. However, efficacy was improved to 8.4 log reduction through increasing the contact time to 10 minutes at the same concentration. On the in vitro level, the study provides promising findings for using the algal extract as an antimicrobial agent in food or drink products. Further investigations for the identification of promising algal species, standardization of analytical methods, isolation of compounds through bioassay-guided fractionation, detailed chemical characterization and evaluation of their safety, evaluation of synergistic effects among the components, and efforts to enhance yields and lower extraction costs are needed. Detection of disinfectant-resistance genes will help us in understanding the mechanisms of resistance and to avoid persistence of *L. monocytogenes*–resistant strains in the dairy environment.

Abbreviations

BAC, benzalkonium chloride; CFU, colony-forming unit; DMSO, dimethyl sulfoxide; QAC, quaternary ammonium compound.

Data Sharing Statement

All data sets generated or analyzed during this study are included in the manuscript.

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Author Contributions

All authors contributed to data analysis, drafting or revising the article, have agreed on the journal to which the article will be submitted, gave final approval to the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors declare that they have no conflicts of interest for this work.

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