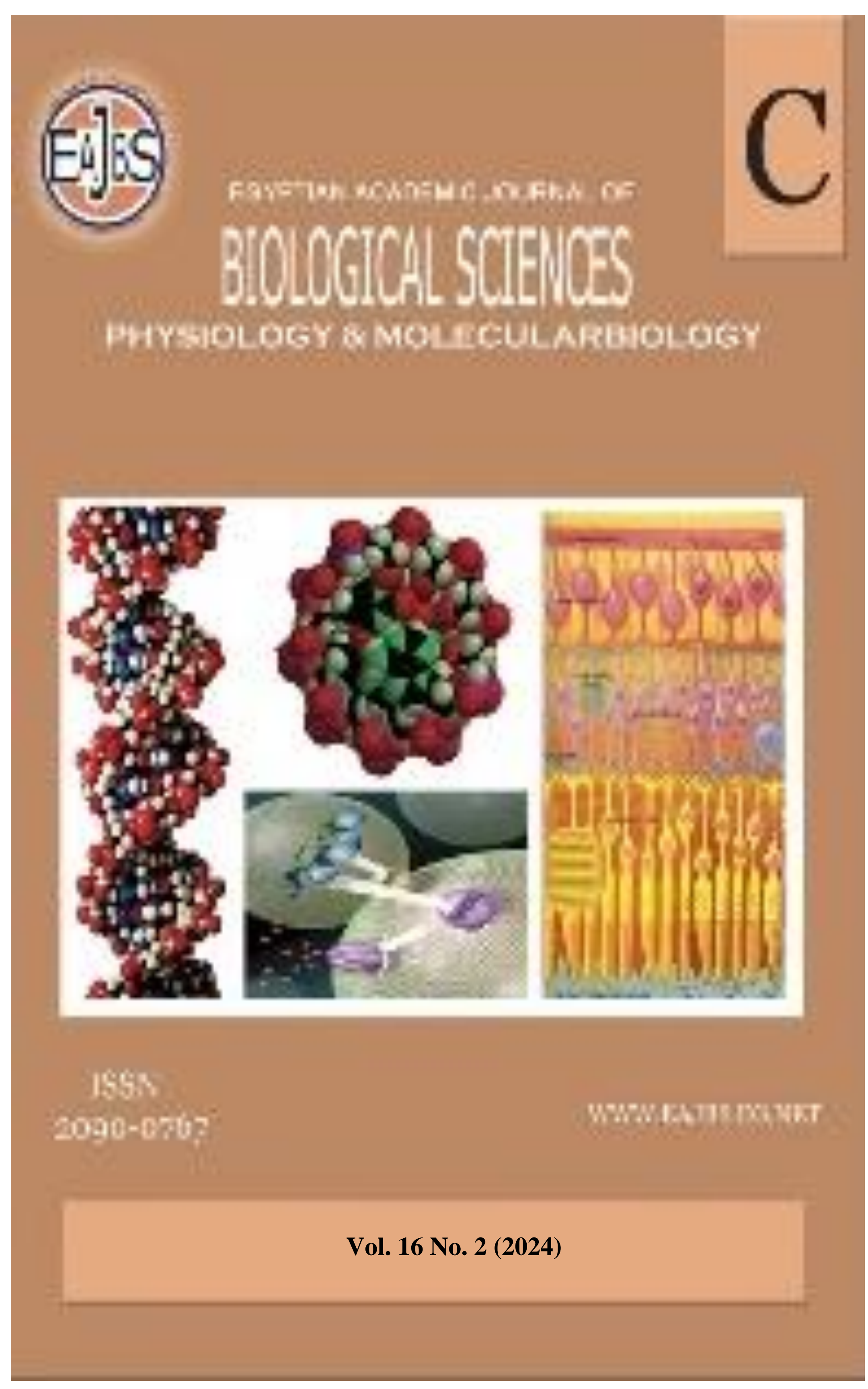

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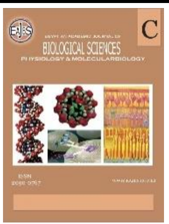

Monitoring and Bio-Controlling of *Salmonella sp* Contaminant in Home-Made Cheeses in Fayoum Government

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ABSTRACT

Salmonella typhi is a summer born suspected to be contaminated home-made cheeses in addition *E. coli, Pseudomonas arugonosa*, and *Staphylococcus aureus*. This study aims to monitoring *Salmonella sp* and it control Chlorella extract is a nutritional supplement to improve the taste and shelf life of home-made cheeses. The current study *Salmonella sp.* detected and prevalence percentage contamination in Home Made Cheeses in Fayoum government into seven locations (Fayoum – Tamya- Ebshaway – Yousef El-sdek –Etsaa –senores – Bander El Fayoum) at five years 2018 to 2022. As well as *E. coli* and *Staphylococcus spp.* of food poisoning detected and prevalence percentage contamination in Home Made Cartage Cheeses preservation and extend the shelf life of home-made cheeses. For all results considered unacceptable or potentially hazardous due to the presence of spoilage pathogenic isolates, E. *coli, Ps. aeruginosa, S. typhi and Staph. aureus.* The applied Chlorella aqueous extract natural antimicrobials effectively inhibited and reduced the spoilage microorganisms, with antioxidant, and anti-virulence activities. As well as the usage of Chlorella aqueous extract based natural antimicrobials can be alternatives for chemicals used in preservation to improve the taste and extend the shelf life of meat products.

INTRODUCTION

The risk of contracting a foodborne illness through everyday meals is a concern that affects everyone. According to the World Health Organization (WHO), 'Food Safety' refers to the assurance that food will not harm consumers when it is prepared and consumed as intended. In addition, 'Food Hygiene' involves all necessary actions to maintain the safety, integrity, and quality of food from its production or manufacturing to its final consumption (WHO, 2010).

According to the Centers for Disease Control and Prevention (CDC), foodborne pathogens can be traced as the source of millions of illnesses worldwide each year. Contaminated meat and dairy products have emerged as the most common causes of human salmonellosis globally (Herikstad *et al.*,2002).

Microbiological food quality has become a major worldwide concern regarding food safety. Foodborne diseases caused by food-poisoning bacteria have significant economic and public health consequences.

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These illnesses continue to be responsible for high morbidity and mortality, especially among vulnerable groups like infants, the elderly, and the immunocompromised. Cases of foodborne diseases occur daily across both developed and developing nations. Consuming foods tainted by foodborne pathogenic microorganisms and their toxins can lead to fatalities, illnesses, hospitalizations, and economic losses. In industrialized countries, up to 30% of the population may suffer from foodborne diseases annually (WHO, 2007).

The epidemiology of foodborne illnesses has shifted over the past two decades, with an increase in bacterial infections from emerging organisms as well as a considerable rise in the incidence of illnesses from well-recognized pathogens like *Salmonella* (Forsythe & Hayes, 2002). Bacteria account for approximately 60% of foodborne illness cases requiring hospitalization. *Salmonella*, *Campylobacter*, Pseudomonas aeruginosa, *Bacillus subtilis*, *Aspergillus niger*, and *Shigella* are among the most commonly documented foodborne pathogens (FoodNet, 2003), which are ubiquitous in nature. In the context of foodborne illnesses, *Salmonella* contributes to 31% of deaths, while Listeria follows with 28%, Campylobacter with 5%, and Escherichia coli with 3% (Marriott & Gravani, 2006; Bhunia, 2008; Scharff, 2012).

Recently, there has been increased emphasis in the exploration of innovative food packaging materials that are derived from natural plants and exhibit antimicrobial properties. In response to consumer preferences for food products devoid of chemical preservatives, food manufacturers are increasingly utilizing naturally sourced antimicrobial agents as substitutes for conventional chemical preservatives. The use of these natural plant extracts for food preservation can effectively extend the shelf life of products (Abdallah, 2011; Ravishankar *et al.,*2008; Ravishankar *et al.,*2010). Plant extracts, with their antioxidative and antimicrobial activities, have become essential for a wide range of applications, notably in the preservation of both raw and processed food items (Lis-Balchin and Deans, 1997). Aim of the work: Monitoring and bio controlling of *Salmonella* sp contaminant in home-made Cheeses aqueous by *Chlorella vulgaris* extract in Fayoum government. The aqueous *Chlorella vulgaris* extract was assessed for antimicrobial activity to inhibit pathogenic and contaminant bacteria for representing a good alternative to the use of traditional antimicrobials in food preservation. In addition, the study of antioxidants, as a free radical scavenger, various ant virulence.

MATERIALS AND METHODS

Collection and Preparation of Cheese Products Samples:

During period in 2018 to 2023, a total of 750 home-made cheese products, Cottage cheese, Salt cheese and Cream cheese (Table 1) were randomly collected from different at Fayoum governorates. In addition, 1340 cottage cheese samples were collected in June and August (at the beginning and the summer end) for five years 2018 to 2023 from different places in each El Fayoum governorate to investigate suspected to be contaminated with *E. coli, Salmonella typhi*, and *Staphylococcus aureus* (Tables 2&3). All samples were labeled, recorded, and transported in an ice box to microbiology at Fac. of Science, Fayoum Univ., and analyzed as soon. The aseptic manner for the collected samples, for self-protection gloves and a face mask were worn during the sample collection.

Table 1: Assessment of total S*almonella* spp counts in positive sample of cheese products**.**

Region of Collected sample	Total aerobic bacterial count									
	Cottage cheese (n=250)			Salt cheese(n=250)			Cream cheese(n=250)			Positive samples %
	Positive samples	Prevalence (%)	Average CFU/ml	Positive samples	Prevalence (%)	Average CFU/ml	Positive samples	Prevalence (%)	Average CFU/ml	
Bandar El Fayoum (n=103)	6	2.4	175	2	0.8	25	2	0.8	17	10/9.7
El Fayoum (n=141)	8	3.2	172	2	0.8	22	2	0.8	17	12/8.5
Snores (n=110)	6	2.4	165	1	0.4	20	3	1.2	29	10/9.1
Tamaya (n=92)	5	2.0	154	2	0.8	25	3	1.2	15	10/10.9
Abshaway (n=91)	6	2.4	112	1	0.4	24	3	1.2	15	10/11.0
Etsaa (n=116)	5	2.0	170	2	0.8	15	2	0.8	15	9/7.8
Yousef El sadik (n=97)	6	2.4	185	2	0.8	17	3	1.2	12	11/11.3
Total (n=750)	42	16.8	1133	12	4.8	184	18	7.2	120	72/9.6

Enrichment of Contaminated Bacteria:

After transportation, the sample was mixed with Buffered Peptone Water (in 1:9 ratio), homogenized in a sterile mixer in test tube, and kept at 37 °C ± 1 °C for 18 h ± 2 h.

Detection of Bacteria Contaminated Cheeses:

The detected bacteria contaminated cheeses were used selective medium. It was performed by streaking 0.1 ml of adequately diluted samples of each cheese onto Eosin Methylene blue agar plates (FDA, 2002) for *E. coli*, Baird parker media agar plates enriched with egg yolk and potassium tellurite (FDA, 2002) for *Staph aureus*, XLD agar medium plates (FDA, 2002) for *Salmonella typhi* and incubated at 37°C for 24h. Subsequently, Typical colonies of each bacteria were isolated and placed to nutrient agar slants and incubated at 37°C for 24h for further identification.

Isolation and identification of *Salmonella typhi* (FDA, 2002):

Salmonella sp. was isolated on selective platting by inoculating 0.1 mL of broth solution sample on XLD agar, Buffered Peptone Water, Eosin Methylene blue (EMB), Baird parker media brain heart infusion agar Salmonella, Shigella (SS) agar and xylose lysine deoxycholate agar (XLD plates were incubated at 37°C for 24h. Suspected colonies were observed to be creamy on SS agar, with or without the presence of black centers, and displayed a red color on XLD agar, similarly with or without black centers.

Table 2: Prevalence of pathogenic food borne bacteria contaminated Quraish cheese samples in five seasons (2018 – 2022).

No. of samples /Seasons	Incidence of foodborne bacteria										
	Eshirashia sp			*Salmonella* sp			*Staphylococcus* sp			Total Positive samples	Positive samples %
	Positive samples	Incidence percentage	No. of log. average	Positive samples	Incidence percentage	No. of log. average	Positive samples	Incidence percentage	No. of log. average		
6–8/2018 (n=256)	27	10.4	1.41	16	6.2	1.2	15	5.8	1.18	**58**	**22.65**
6–8/2019 (n=239)	38	15.9	1.58	37	15.5	1.5	35	15	1.54	**110**	**40.03**
6–8/2020 (n=246)	19	7.7	1.28	12	4.9	1.0	15	6.1	1.18	**46**	**18.69**
6–8/2021 (n=**279**)	23	8.2	1.36	14	5.0	1.1	17	6.1	1.22	**54**	**19.35**
6–8/2022 (n=320)	16	5	1.20	12	3.75	1.0	14	4.4	1.14	**42**	**13.13**
Total (n=1340)	123	9.2	2.09	91	6.8	1.9	96	7.14	1.98	**310**	**23.13**

Table 3: Prevalence of pathogenic food borne bacteria in five seasons (2018 – 2022).

No of sample / regions	Incidence of foodborne bacteria										
	Eshirashia sp			*Salmonella* sp			*Staphylococcus* sp			Total Positive samples	Positive samples %
	Positive samples	Incidence percentage	No. of log. average	Positive samples	Incidence percentage	No. of log. average	Positive samples	Incidence percentage	No. of log. average		
Bandar ElFayoum (n=184)	20	1.08	1.30	12	6.5	1.08	12	6.5	1.08	44	23.78
El Fayoum (n=242)	28	1.16	1.44	32	8.7	1.70	10	8.3	1.30	70	28.92
Snores (n=202)	15	7.4	1.17	19	4.4	0.95	13	6.4	1.10	37	23.27
Tamaya (n=170)	17	1.0	1.23	12	7.0	1.08	11	6.5	1.04	40	23.53
Abshaway (n=144)	12	8.3	1.08	18	5.5	0.89	10	11.7	1.00	44	27.59
Etsaa (n=250)	28	1.1	1.44	22	8.8	1.34	10	8.4	1.31	50	24.00
Yousef Elsadik (n=148)	9	4.0	0.95	7	4.7	0.84	9	6	0.95	25	16.78
Total (n=1340)	129	9.6%	2.11	122	6.8%	1.95	75	7.1%	1.96	310	23.13

Microbiological Identification *of Salmonella* spp Isolates:

Salmonella sp. colonies derived from the aforementioned media were selected and isolated according to variations in their cultural traits and colony structure. Pure isolates were stored on slants of the same medium at a temperature of 4°C for later identification. Biochemical identification of non-fastidious *Salmonella* spp isolates by 5 set tubes according to Bergey's manual, (2009), Collins and Lyne (2004) and Cheesbrough (2006).

1-Triple sugar iron agar (TSI) (FDA, 2002): The pure *Salmonella* spp isolates were cultured in peptone broth containing 0.5% of sterilized TSI by filtration and bromothymol blue.

2-Lycine iron egar (LIA) (FDA, 2002): The pure *Salmonella* spp isolates were cultured in LIA medium which contains lycine and Phenol red.

3-Motility Indole Ornithine agar (MIO) (FDA, 2002): The pure *Salmonella* spp isolates were cultured in sterilized MIO Motility medium in test tubes and incubated for 1 to 2 days at 35°C.

4- Citrate agar (FDA, 2002): The pure *Salmonella* spp isolates were cultured in Nutrient broth medium containing sodium citrate, ammonium salt, and bromothymol blue.

5-Urea agar (FDA, 2002): The pure *Salmonella* spp isolates were cultured in urea agar medium, and Phenol red.

6- Haemolysis on blood agar medium (FDA, 2002): Blood haemolysis ability of the pure *Salmonella* spp isolates were tested using tryptic soy agar obtained from (Difco, USA), supplemented with 5% sterile human blood.

Antibiotic Sensitivity Test for *Salmonella* spp Isolates:

For this investigation, we selected 18 commercial antibiotic discs (6 mm diameter) from different antibiotic groups to evaluate their effectiveness against food-isolated bacteria. The antibiotic discs were sourced from Oxoid, UK, with their properties detailed in Table (4).

Table 4: Antibiotics for sensitivity test**.**

Scientific Name	Trade Name	Symbol	Disc Potency (mcg)	Scientific Name	Trade Name	Symbol	Disc Potency (mcg)
Clindamycin	Augmentin	DA	2	Aztreonam	Meronam	ATM	1
Levofloxacin	Lee flox	LEV	5	Gentamicin	Gentamicin	CN	10
Kanamycin	Kanatrex	K	30	Norfloxacin	Noroxin	NOR	10
Tobramycin	Nebcin	TOB	10	Gatifloxacin	Lincocin	GAT	5
Flucloxacillin	Keflin	FL	5	Cephradine	Velocef	CE	30
Ofloxacin	Tarivid	OFX	5	Oxacillin	Oxacillin	OX	1
Rifamycin	Remactan	RF	30	Tetracycline	Tetracycline	TE	30
Ampicillin	Ampicillin	Am	10	Ciprofloxacin	Tarivid	CIP	5
Vancomycin	Vancocin	VA	30	Erythromycin	Erythromycin	E	15

The antimicrobial susceptibility testing employed the standard Kirby-Bauer disk diffusion technique (Bauer *et al.,*1966). For each *Salmonella sp*. isolate, 4-5 similar colonies underwent aseptic transfer into sterile distilled water and were vigorously mixed to achieve turbidity matching the 0.5 McFarland standard (approximately 108 CFU/ml), following protocols by D'Amato and Hochstein (1982). Researchers measured the inhibition zone diameters to the nearest millimeter and interpreted results according to National Committee for Clinical Laboratory Standards (NCCLS) guidelines, categorizing them as resistant (R), intermediate sensitive (I), or sensitive (S) (Hindler, 1998; NCCLS/CLSI, 2007).

Widal Test:

The *Salmonella sp* was identified Serologically by Widal test according to Abraham *et al.,* (1981) and Kim *et al.,* (2019) used a slide with reaction circles, marked as O, H, AH, BH, PC and NC. Put one drop of *Salmonella sp* broth culture and incubation at 35°C for 24h in each circle. The antigen, i.e., O, H, AH, BH in both PC and NC were added to *Salmonella sp* in well and mixed and touch the slide. Also, one mixture should not mix with another, as it can influence the test results. Finally, rotated the slide in a slow circular motion to ensure a proper mixture of sample and reagent.

Upon completion of the procedure, the results will be observable. A positive test result will resemble the positive control circle (PC), while a negative result will resemble the negative control circle (NC). In essence, the presence of agglutination indicates a positive result, whereas its absence signifies a negative outcome.

Identification by *Inv A gene*- PCR:

-Preparation of Template DNA: A 1 mL aliquot (10^8 CFU/ml) of pre-enriched *Salmonella sp.* cultures was subjected to centrifugation at 15,000 × g for 5 minutes. The resulting pellet was utilized for DNA extraction via the lysis–guanidine isothiocyanate (GuSCN) method as previously outlined (Kawasaki *et al.,* (2010).

-Amplification of *Inv A gene*: using Primer (F:5′-ACAGTGCTCGTTTACGACCTGAA T-3′ and R:5′-AGACGGCTGGTACTGATT ATAAT-3′) (Rozen and Skaletsky). The optimal efficiency of PCR was realized through a cycling profile that commenced with a denaturation phase at 95°C for 30 seconds, then 40 cycles of 5 seconds at 95°C and 30 seconds at 60.0°C. Each PCR reaction contained a positive control (*S. enterica* DNA), a no template control (which consisted of master mix and sterile DW), and a negative DNA control (*Escherichia coli* DNA). The PCR procedure was executed in duplicate and confirmed via 1.0% agarose gels. The resulting PCR products were stained with EZ view mixed with a 6x loading buffer and visualized using ultraviolet light with a gel documentation system (Gel Doc Chemidoc+, Bio-Rad, USA).

-Sequencing of *Inv A* Gene: The sequencing of the Inv A gene involved purifying PCR products using a QIAGEN PCR purification kit (Hilden, Germany). Sequencing was performed on a Genetic Analyzer system (3130xl, Applied Biosystems) with BigDye Terminator v3.1 Cycle Sequencing kit. Sequence analysis utilized NCBI-BLAST, and phylogenetic relationships were examined through MEGA software (version 11) using UPGMA and bootstrap methods (2000 replications).

Collection and Extraction Algae Preparation:

The powder extract of *Chlorella vulgaris was provided from the algae lab. Institute of Agr*iculture Microbiology, Agriculture Research Center (ARC) Giza, Egypt.

Aqueous Extracts Preparation: The powdered extract was immersed in sterile distilled water at a ratio of 1:3 (w/v) for the purpose of aqueous extraction. This mixture was maintained at room temperature for a duration of 24 hours, with continuous agitation provided by a magnetic stirrer. Following this period, the mixture was filtered using cheesecloth, and the extraction process of the residue was repeated 3 to 5

times until a clear, colorless supernatant was obtained. The resulting aqueous extracts were then dried until a consistent dry weight was achieved for each extract. Subsequently, the aqueous extract underwent sterilization using a 0.22µm Millipore filter. The remaining residues were stored at a temperature of 4°C for future applications (Woo *et al.,*1977).

Preparation of Ethanol *Chlorella vulgaris* Extract (EChE):

Powdered *Chlorella vulgaris* (50 g) soaked into 70% ethanol as 1:5 volumes. The mixture was held at room temperature in tightly sealed jars for 24 hours (Yusof and Saat, 2017) and then exposed to rotary evaporation to remove the ethanol, and the residual was weight.

GC-MS Condition: The GC-MS analysis of the crude extract was performed out utilizing Perkin Elmer system (GC clarus 600, USA) with AOC-20i autosampler at the Regional Center for Mycology and Biotechnology, Al-Azhar University.

Bioactivity of EChE :

-Disc Diffusion Assay: Discs of paper, saturated with MChE at a concentration of 100 mg/ml, were applied to the surface of agar plates that had been inoculated with *Salmonella* isolates. The agar layer, approximately 4 mm thick, was incubated at 37°C for 24 hours. A negative control was conducted using sterile distilled water instead of the active compounds. The sensitivity of the bacterial strains to each extract was determined by measuring the diameters of the inhibition zones, and the results were reported as the average of three separate trails (NARMS, 2002).

Mean *Salmonella* Isolates Growth Concentration EChE (MBC):

MBC of *Salmonella* isolates was determined by ELISA reader microdilution method (NCCLS/CLSI, 2007). *Salmonella* isolates suspension equivalent to the turbidity of 0.5 McFarland standard (10^8 cfu/ml) in Mueller Hinton Broth (MHB). *Salmonella* isolates (100 µL) were introduced into each well of a sterile 96-well flat-bottom microtiter plate that contained 100 mg/ml of EChE. After 24 h at 37°C, the optical density was obtained at 620 nm utilizing an ELISA reader. Ampicillin was designated as the standard antibiotic for Gram-positive bacteria, while Gentamicin served the same purpose for Gram-negative bacteria. The calculation of bacterial growth reduction percentage (GR %) was performed by referencing the control treatment (without extract) as a reference, according to the formula: GR% = (C - T) / C x 100, Where C represents the concentrations of the control treatment and T denotes the concentrations under the extract treatment. The results were documented as means ± SE from the triplicate experiments.

Determination of Minimum Inhibitory Concentration (MIC) of EchE:

MIC of EChE was established using the broth microdilution method, in accordance with the guidelines set forth by the Clinical and Laboratory Standards Institute (NCCL/CLSI, 2007) as described before.

Antimicrobial Activity Mode of MChE Action:

Mode of EChE action was determined based on MIC and MBC obtained results, the MBC/MIC ratio was calculated. The MBEE had a bactericidal effect when the ratio's value was equal to or larger than 4. Whereas the ratio values equal to 2 or less are considered static action 25 (Galal *et al.,* 2021 and Ogidi *et al*., 2021).

$$\text{Antimicrobial agent mode of action} = \frac{\text{Minimum Bacteria concentration (MBC)}}{\text{Minimum inhibition concentration (MIC)}}$$

Antioxidant Activity Assay:

The antioxidant activity of MChE was assayed in triplicate using the DPPH method for free radical scavenging (Yen and Duh, 1994), and mean values were considered.

DPPH Radical Scavenging Activity: Freshly prepared methanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical at 0.004% (w/v) and left at 10 °C in the dark. A 40µL aliquot of MChE was mixed with 3 (ml) of DPPH solution. The absorbance rate was

determined right away using a UV-visible spectrophotometer (Milton Roy, Spectronic 1201). Continuous absorbance readings were taken at 515 nm, with data recorded at one-minute intervals until stabilization was achieved at 16 minutes. Additionally, the absorbance of the DPPH radical without any antioxidant (control) and that of the reference compound ascorbic acid were also assessed. All measurements were conducted in triplicate and averaged. The inhibition percentage (PI) of the DPPH radical was evaluated using the following formula:

$$PI= [\{(AC-AT)/AC\} \times 100]$$

Where, AC= Absorbance of control at t=0 min and AT = absorbance of the sample +DPPH at t = 16 min (Yen and Duh, 1994) .

Anti Virulence Activity:

-Biofilm Inhibition Assay Using Tissue Culture Plate Method:

Biofilm formation of *Salmonella* isolates was assayed by utilizing the tissue culture plate method as established by Christensen *et al*. (1995). The biofilm production was interpreted using the parameters established by (Stepanovic *et al.,*2007). The efficacy of EChE in inhibiting biofilm formation by *Salmonella typhi*, a strong biofilm producer, was evaluated at a sub-MIC concentration of 0.781 mg/ml. A series of two-fold dilutions were prepared in a 96-well microtiter plate containing trypticase soy broth supplemented with 2% glucose, resulting in the desired concentrations (100µL). Subsequently, 50 µL of bacterial suspensions (5×10^5 cfu/ml, final concentration) were added to the plate. Trypticase soy broth (TSBGlc) with distilled water served as the negative control, while inoculated TSBGlc without EChE acted as the positive control. After 24 hours of residence at 37°C, biofilm inhibition was assessed using a microplate reader at an optical density of 570 nm, as described by Lin *et al.,*2011.

-Application on Cheese Preservation:

The fresh home-made cartage cheese was cut into cubes (weighing 20 g/cub) and dived into three sets (10 cubes/set) for the preservation experiment. Two sets were artificially inoculated with isolated *Salmonella typhi* at a level of 25 (cfu/g cartage cheese), one set was treated with the aqueous Chlorella vulgaris extract at concentration 12.5 (mg/ml) and the other set served as positive control The third set untreated as negative control. The Three sets were kept at 4°C for 8 weeks and regularly gathered for the assessment of bacteriological quality throughout the storage period.

-Bacterial Enumeration:

Cubes of treated cartage cheese and non-treated ones were tested at intervals (0 day, 2, 4, 6, and 8 weeks) for count total bacteria and *Salmonella typhi* as follows: Cheese sample (10g) of each set was taken aseptically on the sampling intervals days and placed to a sterile stomacher bag. An aliquot of 90 ml of 0.1M sterile sodium phosphate buffer solution (pH 7.0) was introduced, followed by homogenization of the sample for one minute using a laboratory blender (Seward Stomacher® 400, Seward Medical, UK). Subsequently, a series of decimal dilutions were executed in accordance with established bacteriological protocols (Spencer and Regout de Spencer 2001). In order to bacteria examination, (1ml) of serially diluted samples was spread in duplicate for total bacteria on nutrient ager medium plates and *Salmonella typhi* on XLD agar medium plates (FDA, 2002). The count plates underwent aerobic incubation for a duration of 24 hours at 37°C. After the incubation, the colonies were counted, and the findings were presented as colony-forming units per gram (cfu/g).

Statistical Analysis:

Statistical analysis employed five independent replicates, with significance determined using Least Significant Difference (LSD) at $p < 0.05$ using CoStat statistics software version 6.400 (Keselman & Rogan, 1977).

RESULTS

Microbiological Assessment of Cheese Products Samples:

-Total S*almonella* spp count in Positive Cheese Products Samples:

Seven hundred and fifty cheese products samples collected from Bandar El

Fayoum, El Fayoum , Snores , Tamaya , Abshaway , Etsaa , Yousef and El sadik regions included, 250 of each of Cartage cheese , Salt cheese and Cream cheese were investigated S*almonella spp* counts in positive samples (table, 4). The results revealed that , 42 , 12 , 18 positive cheese contaminant with S*almonella* spp out of 250 of each Cartage cheese , Salt cheese and Cream cheese respectively . The average of CFU /ml was ranged according to the type of cheese products and region of collected sample . Cartage cheese recorded the high total S*almonella spp* were 175, 172, 164, 154, 112, 170, 185/ml out of 6, 8. 6, 5, 7, 5, 6 positive samples respectively**.** Compared to the lowest S*almonella spp* count in Salt cheese recorded 25, 22, 20, 25,13, 15, 17 CFU /ml out of 2, 2, 1, 2, 1, 2, 2 positive samples respectively and Cream cheese which recorded 17, 17, 29, 15, 15, 15, 12 CFU /ml out of 2, 2, 3, 3, 3, 2, 3 positive samples respectively (Table 2).

-Prevalence and Count of Pathogenic Bacteria at Five Seasons:

E. coli , *Salmonella* sp and *Staphylococcus* sp contaminant Cortege cheese which collected from Bandar El Fayoum , El Fayoum , Snores , Tamaya , Abshaway , Etsaa , Yousef El sadik regions at five seasons 2018 , 2019 , 2020, 2021 & 2022 (table,2 & 3) were detected in 310 (23.13%) out of one thousand three hundred and forty-three cartage cheese samples distributed by 58, 110, 46, 54, 42 with prevalence 22.63 ,40.03 , 18.69 , 19.35 and 13.13 % respectively **.** The results emphasized that cottage cheese samples contaminated and pathogenic bacteria included:

E. coli was found in 123 out of 1250 samples with prevalence 9.2 % with the average log 2.09 CFU/g . *Salmonella* sp. found in 91 out of 1250 samples with prevalence 6.8 % with the average log 1.9 CFU/g . ***Staphylococcus* sp** found in 96 out of 125 samples with prevalence 7.14 % with the average log 1.98 CFU/g . **.**

-Prevalence and Count of Pathogenic Bacteria in Different Regions:

The pathogenic bacteria contaminated Cortege cheese are *E. coli* , *Salmonella sp* and *Staphylococcus sp* which collected at five seasons 2018 , 2019 , 2020, 2021 & 2022 from Bandar El Fayoum , El Fayoum , Snores , Tamaya , Abshaway , Etsaa , Yousef El sadik regions (table,4 & 5) were detected in 310 (23.13%) out of one thousand three hundred and forty cartage cheese samples distributed by 44, 70 , 37, 40, 44, 50 & 25 with prevalence 23.78, 28.92, 23.27, 23.53, 27.59, 24.00 and 16.78 % respectively. The results emphasized that cottage cheese samples contaminated, and pathogenic bacteria included:

E. coli was found in 129 out of 1340 samples with prevalence 9.6 % with the average log 2.11 CFU/g. *Salmonella* sp. found in 122 out of 1340 samples with prevalence 6.8 % with the average log 1.95 CFU/g. *Staphylococcus sp* found in 75 out of 1340 samples with prevalence 7.14 % with the average log 1.96 CFU/g

-Detection of Pathogenic Bacteria of Cheese Samples:

E. coli was Isolated of each cartage cheese, salt cheese and cream cheese and enumerated on Eosin Methylene blue (EMB) which showed Typical colonies (characterized by a greenish metallic appearance with a dark purple center). One colony selected and transferred on nutrient agar slants. These slants were then incubated at 37°C for a duration of 24 hours for subsequent preservation.

Staphlylococcus spp. was Isolated of each cheese, salt cheese and cream cheese and enumerated on Baird parker media which showed typical colonies, which appears gray-black coloration, characterized by a shiny and convex surface, bordered by a narrow white margin and an adjacent clearing zone. Isolated *Staphlyococcus spp.* caused coagulase plasma to clot by converting fibrinogen to fibrin. The results were interpreted to reveal a significant organized clot, with the entire content of the tube coagulating and remaining stationary upon inversion. This outcome was deemed positive evidence of coagulase production. One colony was isolated, transferred to nutrient

agar slants, and incubated for a duration of 24 hours at 37°C for further maintenance.

Salmonella spp. was Isolated of each cartage cheese, salt cheese and cream cheese and enumerated. The *Salmonella typhi* cultured showed the typical colony characteristics growth, which appears gray-black, shiny and convex with a narrow white margin surrounded by a clearing zone. One colony picked up and transferred on nutrient agar slants and incubated at 37°C for 24h for further keeping showed black head colony on the XLD plate (Fig. 1).

-Identification of *Salmonella typhi* Isolates:

The *Salmonella typhi* culture was studied by the colony characteristics growth showed black head colony on the XLD plate which showed typical colonies, which appears gray-black, shiny, and convex with a narrow white margin surrounded by a clearing zone. The pre-enrichment of samples was performed according to the Rappaport-Vassiliadis medium with soya (RVS broth).

The media plates, *Salmonella typhi* isolates Suspected colonies were creamy with or without black centers on SS agar and red in color with without black centers on (XLD) agar. After incubation, the culture was studied by its colony characteristics, observation of black head colony on the plate was supposed to represent *Salmonella* further identification and conformation gram staining, coagulate test. Catalase Test. biochemical reaction and Detection of *Salmonella spp* by PCR were performed.

In the present work, three *Salmonella sp* isolated from cartage cheese (SCC-1), salt cheese (SSC-2) and Cream cheese (SCrC-3) were determined based on their morphological, cultural characteristics and consumption of broth manual some biochemical analyses according to (Bergey's manual, 2009) . These three different *Salmonella sp* isolates were more prevalence and high count for confirming identification by 16s DNA gene by PCR.

Morphological and Biochemical Identification: The findings of morphological and biochemical features of three *Salmonella sp* isolates were given in Table (4).

Salmonella sp isolates exhibited Low convex and entire of Shape of colony, Smooth Texture, no Pigmentation, positive Motility and facultative anaerobic of O2 requirements on selective XLD medium.

It was found negative Gram reaction , short rods of cell shape and found singly or in pairs , no sporulation and no capsulation by microscopic examination :.

-Biochemical Characteristics of Non-Fastidious Gram Negative Basilli: *Salmonella sp* isolates gave pisitive results witk 5 set tubes (Fig. 1) . *Salmonella sp* isolates exhibited different non-significant in some biochemical characteristics as showing in Table (6).

1-Triple sugar iron agar (TSI) which detected by color change of the medium to yellow.

2-Lycine iron egar (LIA) which shown by change in color of the indicator to red-pink .

3-Motility Indole Ornithine agar (MIO), the *Salmonella* sp were diffused growth through the medium from the point of inoculation is considered motility positive.

4-Citrate agar which showed growth The *Salmonella* sp in the medium by turbidity and a change in color of the indicator from light green to blue due to citrate utilization.

5-Urea agar which showed *Salmonella* sp isolates cultured and the indicator Phenol red. Pure bacterial cultures containing urease enzyme will hydrolyze the urea to give ammonia that turns the pH alkaline as shown by change in color of the indicator to red-pink. Positive Catalase and Coagulase , negative Oxidase , Urease , Gelatin liquefaction, Starch hydrolysis , Phenyl alanine deaminize , positive H2S production . Hemolysis on blood agar, positive Nitrate reduction, Methyl red and negative Indole formation, Voges-Proskauer, Citrate utilization and Alpha Hemolysis on blood agar.

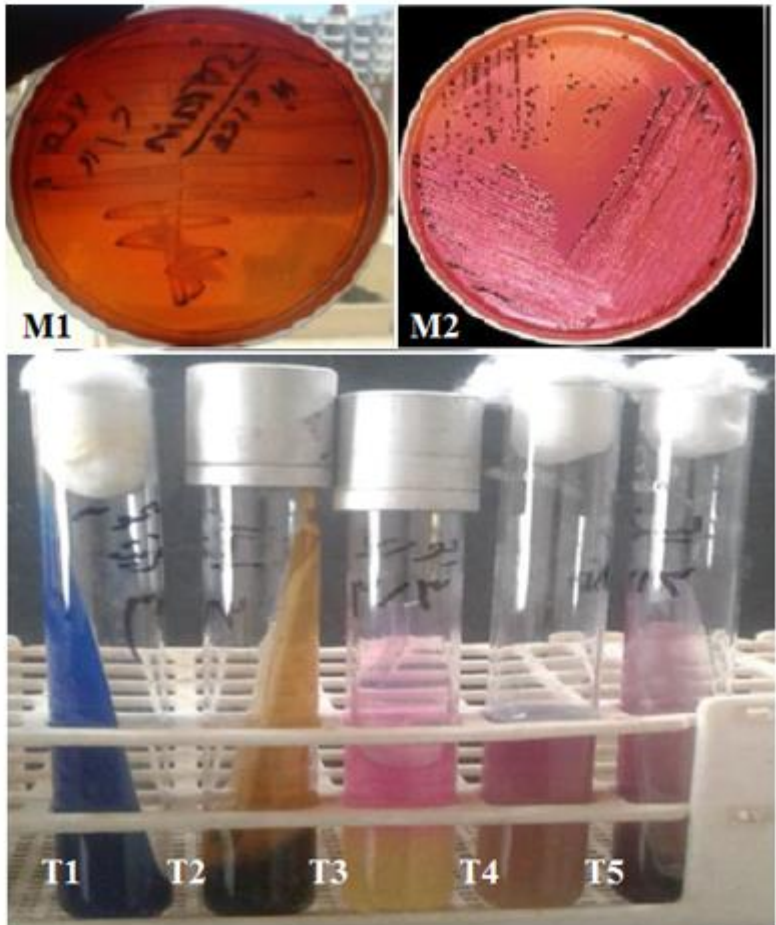

Fig. 1: XLD ager plate (M1) and S.S. ager plate (M1) showing different *Salmonella sp.* growth Characteristic colonies . Biochemical Reaction of isolated *Salmonella sp*. where: T1(Citrate agar test), T2(Triple sugar iron agar (TSI) test) T3 (Urea agar test) T4 (Motility Indole Ornithine agar (MIO) test) and T5 (Lycine iron agar (LIA), test)

Fermentation of Sugar : positive / negative D-glucose, Mannose, negative Sucrose, Lactose and Mannitol.

The fermentation of sugars is a key biochemical test used to characterize bacterial isolates. In this study, three distinct Salmonella sp. isolates (from cartage cheese, salt cheese, and cream cheese) were subjected to sugar fermentation tests. The findings revealed specific patterns of metabolic activity for these isolates:

- **Positive fermentation** was observed for **D-glucose** and **Mannose**, indicating that these bacteria possess the enzymatic machinery to metabolize these sugars for energy production, yielding acidic by-products detectable in the medium.
- **Negative results** were reported for **Sucrose, Lactose**, and **Mannitol**, suggesting the absence or inactivity of the enzymes necessary to process these sugars into fermentable substrates.

These results are consistent with the typical biochemical characteristics of *Salmonella sp.,* which preferentially ferment specific sugars, aiding in their differentiation from other gram-negative bacilli.

Antibiotic Sensitivity of Isolated *Salmonella sp.*:

The examination of antibiotic sensitivity among the three *Salmonella typhi* isolates demonstrated a range of susceptibilities, including sensitive, intermediate, and resistant responses to the various antibiotics evaluated (Table 5 and Fig 2).

Table 5: Antibiotics sensitivity profile of isolated *Salmonella typhi*

Antibiotics	***Salmonella sp*. isolates**			Antibiotics	***Salmonella sp*. isolates**		
	(SCrC-3)	**(SSC-2)**	**(SCC-1)**		**(SCrC-1)**	**(SSC-2)**	**(SCC-3)**
Clindamycin	R	S	S	Aztreonam	S	S	I
Levofloxacin	S	I	I	Gentamicin	I	I	R
Kanamycin	I	R	R	Norfloxacin	R	R	S
Tobramycin	R	S	S	Gatifloxacin	S	S	S
Flucloxacillin	R	S	S	Cephradine	S	S	R
Ofloxacin	S	R	R	Oxacillin	R	R	R
Rifamycin	R	R	R	Tetracycline	R	R	R
Ampicillin	I	R	R	Ciprofloxacin	R	R	R
Vancomycin	R	R	S	Erythromycin	R	R	S
Cream cheese (SCrC-3) , Salt cheese (SSC-2) , Cartage cheese (SCC-1)							

Fig. 2: Agar plate gram detected antibiotics sensitivity profile of isolated *Salmonella typhi* R; Resistant, I; Intermediate sensitive, S; Sensitive.

Serological Characteristic of Isolated *Salmonella* sp.:

Typhoid fever, commonly termed enteric fever, is a critical illness induced by *Salmonella* bacteria. It influences the digestive system when food contaminated with fecal material is ingested. The *Salmonella* food contaminated were detected and identified by A Widal test using primary antigen *S. typhi* O (TO), secondary antigen *S. typhi* H (TH), *S. Paratyphi* A and *S. Paratyphi* B. It was found that, some isolated *Salmonella* sp gave positive serology reaction with TO, (TH) which identified *Salmonella typhi* and other *isolated Salmonella* sp negative antigen TO. On the other hand, some isolated *Salmonella* gave positive serology reaction with AH, (BH) which identified *Salmonella Paratyphi* and other *isolated Salmonella sp* negative antigen AH (Table 6).

Table 6: Serology agglutination reaction of isolated *Salmonella sp* by A Widal test

Salmonella sp	Antigens			
S. typhi	Typhe H O		Typhe H O	
	+Ve (++++)	+Ve (+++)	+Ve (++)	-Ve (----)
S. Paratyphi	Para typhi A B		Para typhi A B	
	+Ve (++++)	+Ve (++)	-Ve (--)	+Ve (++)
Serology reaction : Strong agglutination (++++) , Moderate agglutination (+++) , Low agglutination (++) no agglutination (---) , +Ve = positive serology reaction and –Ve=negative serology reaction				

Molecular Characteristics of *Salmonella sp.* by my- invA gene PCR:

-Genetic Variability Analysis of *Salmonella sp* Isolates:

The isolated DNA from the three *Salmonella sp* (SCC-1), (SSC-2) and (SCrC-3)) isolates were amplified by PCR conventional method after reaching the sufficient concentration (35 & 46 mg/mL) and high purity (1.6 & 1.8 , A260/280 nm) respectively and using specific primers sets for my- inv A gene DNA gene of *Salmonella sp*. The DNA amplicons returned as electropherogram files showed distinct fragments expected 1224 bp for *Salmonella sp* three isolates as shown in Figure (3).

-my- *invA Gene* Sequence:

PCR amplicons from two *Salmonella sp.* isolates (SCC-1 and SSC-2) underwent sequencing through the cycle sequencing method. The identification of primers was straightforward in both the forward and reverse directions across each sequence fragment, enabling the effective compilation of the individual sequences. Each primer's sequences for the respective isolates demonstrated sufficient overlap, which was instrumental in creating a continuous sequence (contig). The analysis of three sequence charts (Appendix 1) using Finch TV program version 1.4 (Geospiza, USA) resulted in the generation of two contigs, one for each isolate, revealing the partial nucleotide sequences of the my-invA gene. The two contig (one for each isolate) were obtained by analysis of three sequences charts (Appendixes 1) using Finch TV program version 1.4 (Geospiza, USA) and showed the partial nucleotide sequences of my- invA gene. DNAMAN program comparison with Genbank bacterial species identified the sequences as *Salmonella enterica* subsp enterica serovar blackley strain and *Salmonella enterica* subsp enterica serovar dublin strain for isolates SCC-1 and SSC-2, respectively, as depicted in Figure (3). These sequences were subsequently submitted to the gene bank under Accession BSeq#1 with culture collection="ATCC:13311".

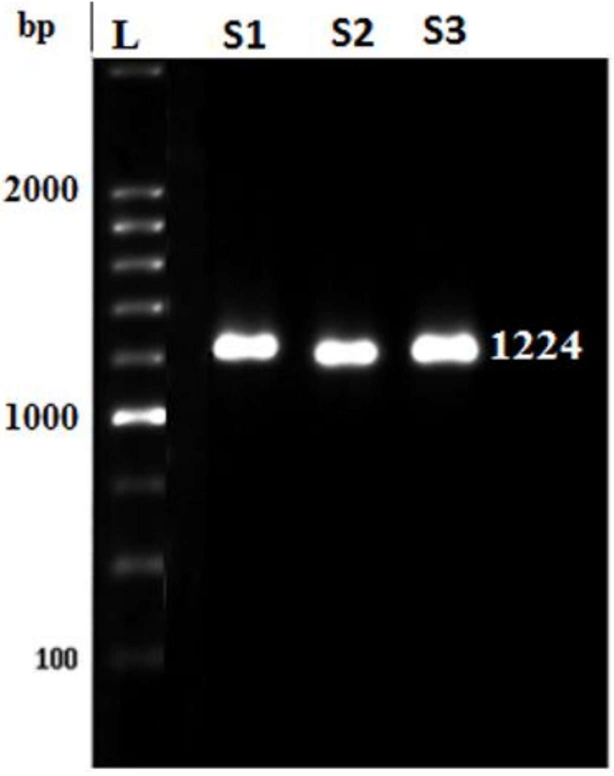

Fig. 3: Agarose gel electrophoresis (1%) showing PCR products amplified fragment of my-invA gene isolated from two *Salmonella sp* isolates (Scc1. Ssc-2 & SCrC-3) using specific set, and L (DNA leader).

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Sequence Variability of my- *invA Gene*:

Isolate -1: DNA sequence variability analysis employed DNAMAN and MEGA.4 programs (Wisconsin, Madison, USA). The *Salmonella sp.* sequence underwent alignment to determine GenBank registration and phylogenetic relationships between the two isolates. The Multiple Sequence Alignment (MSA) displayed corresponding nucleotides in aligned columns, with gaps (represented by "-") indicating deletion events. The alignment revealed conserved sites and conservation percentages for each position, with aligned residues demonstrating shared evolutionary origins and sequence similarities.

Phylogenetic Tree: MSA analysis generated a phylogenetic tree revealing five distinct clusters. The *Salmonella enterica* subsp enterica serovar blackley strain exhibited high homology (95%), while *Salmonella enterica* subsp enterica serovar dublin strain showed lower homology (38%). Among the strains, *Salmonella enterica* subsp enterica serovar dublin strain JQ796859.1, KC121042.1, and HM067869.1 demonstrated 94% homology. Further analysis revealed 98% homology between strains JQ796859.1, KC121042.1, and HM067869.1, while strains KC121042.1, HM067869.1, and JQ659920.1 showed 99% homology, forming a separate cluster as shown in Figure (4).

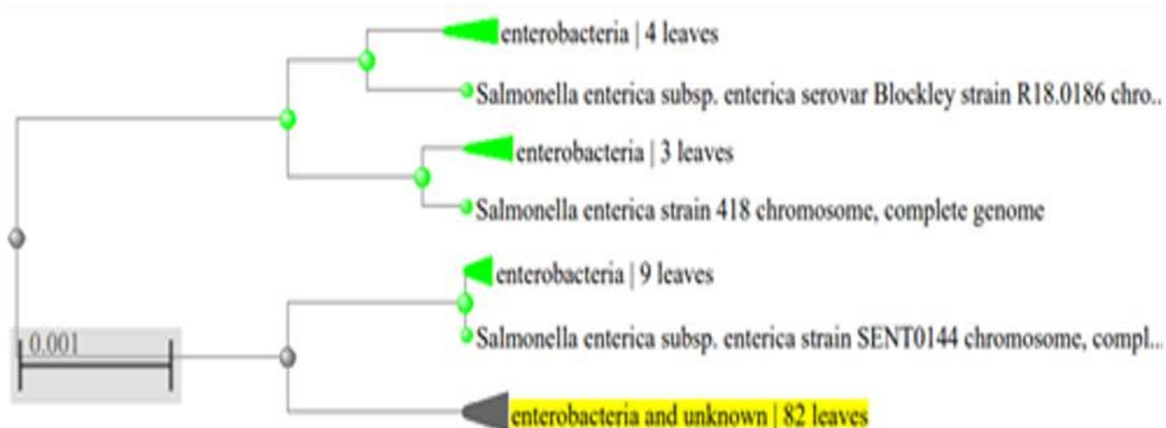

Fig. 4: Phylogenetic tree Based on MSA analysis, S1 isolate identified as *Salmonella enterica* subsp enterica serovar Blackley strain was found to be highly homologous with percentage (95%) while the *Salmonella enterica* subsp enterica serovar dublin strain showed distant homology.

Isolate -2: Similar DNA sequence analysis using DNAMAN and MEGA.4 programs examined sequence variability. The two *Salmonella sp.* sequences underwent GenBank registration alignment using MSA, with corresponding nucleotides arranged in columns. Gap representation and conservation analysis followed the same methodology as Isolate-1, revealing evolutionary relationships and sequence similarities.

Phylogenetic Tree: Following the MSA analysis, a phylogenetic tree was constructed, revealing five clusters. Notably, the *Salmonella enterica* subsp. enterica serovar Blackley strain was found to have a high homology percentage of 95%, whereas the *Salmonella enterica* subsp. enterica serovar Dublin strain exhibited a lower homology of 38%. Additionally, the strains JQ796859.1, KC121042.1, and HM067869.1 of *Salmonella enterica* subsp. enterica serovar Dublin showed a high homology percentage of 94%. The homology percentage rose to 98% among these strains, and a striking 99% homology was noted between the strains KC121042.1, HM067869.1, and JQ659920.1, resulting in their representation as a distinct cluster, as depicted in (Fig. 5).

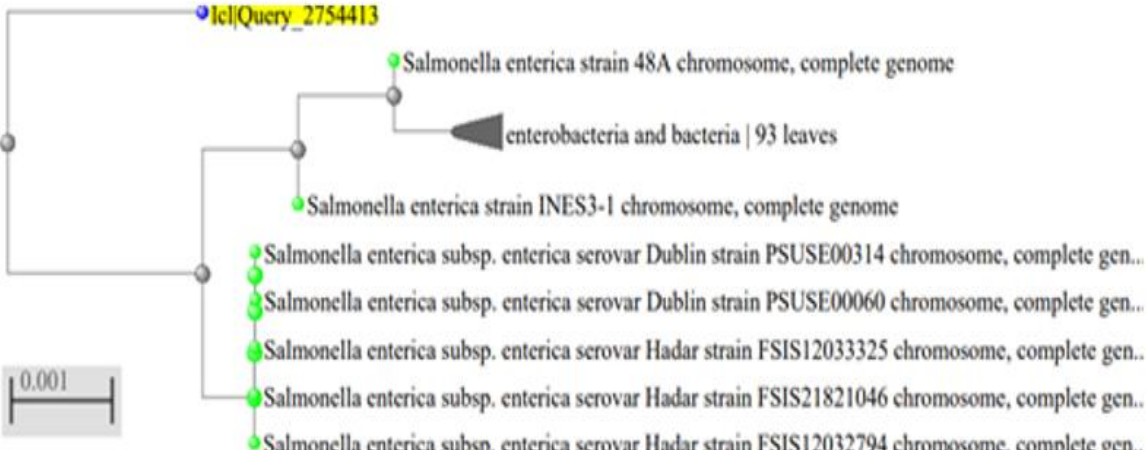

Fig. 5: Phylogenetic tree Based on MSA analysis, S2 isolate identified as *Salmonella enterica* subsp enterica serovar Dublin strain was found to be highly homologous with percentage (95%) while the *Salmonella enterica* subsp enterica serovar dublin strain showed distant homology.

Active Ingredient Compounds of Chlorella Methanolic Extract (ChME):

The TLC analysis of methanol chlorella extract (ChME) appeared nine active ingredient compounds act as antioxidant and antibacterial based on chemical formula and determinate groups were Ferruginol (4.53 %), Camphor (3.88 %), Cineole (2.66 %), Verbenone (2.50 %) & Borneol (1.56 %) and αcaryophyllene, Terpinen-4-ol, 2-methyl-4-vinylphenol, Eugenol ranged from (0.40 to 0.09 %) (Table 7 and Fig. 6).

Table 7: Phytochemical (Active compounds gradient) of chlorella ethanol extract

Retention Time	Area (%)	Compounds	Molecular weight	Pub-Chem	Pub-Med
14.099	0.56	Ethylacridine	204	Phenol	Antimicrobial
19.704	9.69	Octadienal	152	Phenol	Antimicrobial Antioxidant
20.106	2.32	Hexane	154	Phenol	Antimicrobial
28.583	2.32	Triazolo pyrimidine-	154	Phenol	Antimicrobial Antioxidant
28.583	2.52	Pentasiloxane	150	Phenol	Antimicrobial
29.327	1.25	Silane	134	Phenol	Antimicrobial Antioxidant
33.183	7.07	Octadecyne	Phenol	Phenol	Antimicrobial
33.697	5.25	Cyclotrisiloxane	286	Phenol	Antimicrobial Antioxidant
21.213	4.85	Cyclohexasiloxane	204	Phenol	Antimicrobial

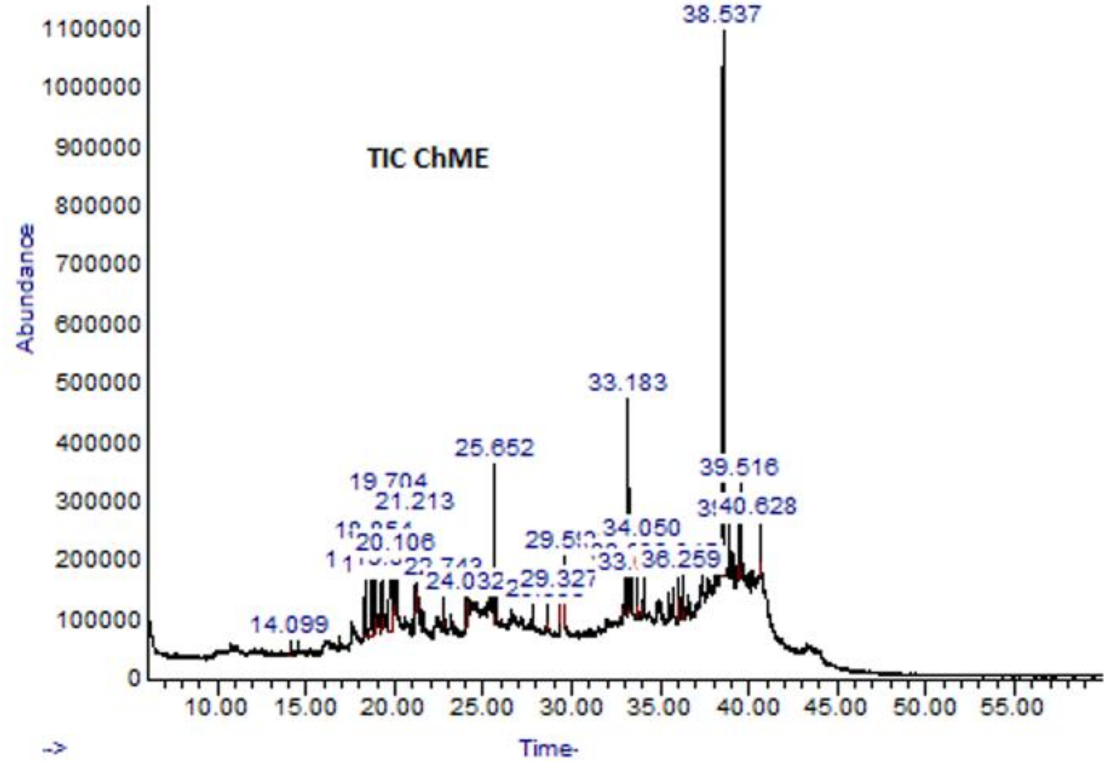

Fig. 6: Chart peak of TLC analysis of Chlorella methanol extract (ChME) showing Retention Time and Area (%) of Phytochemical (Active compounds gradient) .

Bioactivity of Used of **ChAE :** Chlorella aqueous extract (ChAE) showed most potential antibacterial activity was assessed to inhibit pathogenic two *Salmonella typhi* (S1&S2) for representing a good alternative to the use of traditional antimicrobials in food preservation. In addition, the study of antioxidants, as a free radical scavenger, various anti virulence. **Antibacterial activity:** The results of the clear zones diameter of inhibition and mean growth inhibition percentages in Table (8) demonstrated the antibacterial activity of ChAE against two *S. typhi* isolates. The clear zones diameter of inhibition was 20.03 20.60 mm for *S. typhi* (SCC-1) & *S. typhi* (SSC-2) respectively and the mean growth inhibition was 100 % for two *S.typhi* isolates (Table 8) .

Minimum Inhibitory Concentrations (MICs) of ChAE tested *S. typhi* isolates: -

In the presence of ChAE at concentrations 0.781 (mg/ml), the growth of S. *typhi* isolates were inhibited. The Log CFU/ml number of *S. typhi* (S1) & (S2) growth was 0.48 and 0.75 CFU/ml respectively (Table 9).

Table 8:The antibacterial activity of Chlorella aqueous extract (100mg/ml/disc) against two *S. typhi* based on (inhibition zones diameter mm) and the mean growth inhibition .

***S. typhi* isolates**	**T. vulgaris plant aqueous extract (100mg/ml/disc)**			**Means**	**MGI**
S. typhi (SCC-1)	20.24 ±0.55	24.34 ±0.46	15.52 ±0.68	20.03	100±00
S, typhi (SSC-2)	21. 23 ±0.66	18.16±0.74	22.42±0.76	20.60	100±00

(MGI) = Mean growth inhibition
Each value is mean of 3 replicates ± standard error

Table 9: MICs (mg/ml) of ChAE against bacterial isolates.

Isolates	**ChAE Conc (mg/ml)**	**Log CFU/ml bacteria growth**
S. typhi (S1)	0.781	0.48
S. typhi (S2)	0.781	0.75

Microdilution method (one-fold serial dilution with 100 mg/ml at initial conc.)

Bactericidal Activity of ChAE: The MIC values of ChAE ranged from 250 to 1000 µg/mL against the tested two *Salmonella typhi* isolates. It was 250 µg/mL ChAE for *S. typhi* (S1 & S2) isolates. The results clearly show 100% spectrum activity of ChAE at doses between 500 to 1000 µg/ml, whereas at concentration of 250 µg/mL the activity was 83.3%. In addition, concentration of 125 µg/mL did not show any antibacterial activity against the tested pathogen strains. The MBC values of ChAE ranged from 500 to 1000 µg/mL against *S. typhi* (S1 & S2) revealed an MBC value of 1000 µg/mL. The findings

clearly show 100% of the antibacterial spectrum activity of of ChAE at dose of 1000 µg/mL, whereas at concentration of 500 µg/ml the activity was 50.0% whereas at concentrations ranged of 125-250 µg/ml did not score antibacterial influence against *S. typhi* (S1 & S2) pathogenic isolates (Table10).

Table 10: Minimum inhibitory concentration (MIC) , Minimum bactericidal concentration (MBC and spectrum activity of ChAE against *S. typhi* pathogenic isolates after incubation at 37°C for 24 h.

Salmonella / **MPEE (µg/ml)**	**(MIC)**		**Spectrum activity of ChAE**		**MBC**		**Spectrum activity of ChAE**	
	(S1)	(S2)	Activity	(%)	(S1)	(S2)	Activity	(%)
1000 (control)	-	-	6/6	100	-	-	6/6	100
500	-	-	6/5	100	-	-	3/6	50.0
250	+	+	5/6	83.3	+	+	0/6	0.00
125	+	+	0/6	0.00	+	+	0/6	0.00
MIC value (µg/ml)	250	250	-------------------		------------		--------------------	
MBC value (µg/ml)	-------------		-------------------		500		500	--------------------

(MIC)=Minimum inhibitory concentration, (MBC)=Minimum bactericidal concentration
- = No growth, + = growth, (S1&S2) = *S. typhi* isolates

The Maximum Non-Toxic Concentration MNTD of ChAE on Vero Cell Culture Using by MTT Assay:

Before studying the application of ChAE Conc.(ug/ml) was imperative to determine . The maximum nontoxic Dose MNTD, concentration tested in the study was 250 ug/ml treated Vero cells did not show any morphological and **cytotoxicity** difference when compared with control one.

Assessment of Antioxidant Activity of ChAE using DPPH Scavenging:

The oxidation inhibition system of (2,2-diphenyl-1-picrylhydrazyl) radical was determined to ascertain its antioxidant effect as a natural preservative in food. Furthermore, the ChAE showed *in vitro* antioxidant activity by neutralizing the DPPH radical and the results were presented in Table (11) and designated in Figure (7).

DPPH Free Radical-Scavenging Activity of the ChAE demonstrated an increase with higher concentrations, achieving a notable scavenging activity of 84.46% at a concentration of 2560 µg/ml. However, a decrease in radical-scavenging percentages was observed at concentrations with an IC50 value of 289.5 µg (Table 11 & Fig. 7).

Absorbance values were translated into scavenging effects in percentage form, and the data were illustrated as the mean scavenging effect percentages derived from triplicate measurements.

Table 11: Antioxidant activity of ChAE using DPPH scavenging.

ChAE conc. (µg)	DPPH scavenging %
2560	84.46
1280	78.9
640	63.92
320	52.23
160	40.54
80	23.11
40	12.50
0	0

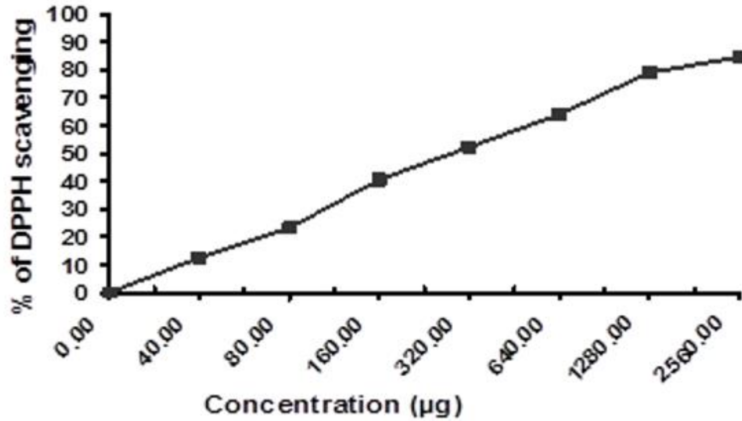

Fig.7: Curve of antioxidant activity of ChAE was assessed through its capacity to scavenge free radicals.

Application of *Chlorella* Aqueous Extract (ChAE) Preservation Of Cartage Cheese :

The Application treatment cartage cheese samples artificially inoculated with *Salmonella typhi* with ChAE die to significantly reduction of *Salmonella typhi*. The results given in Table (12) & Figure (8) showed that, *Salmonella typhi* and total bacteria counts in non-treated cartage cheese as a control sample were increased as the storage period increases significantly. On the other hand, cartage cheese samples incorporated with the *ChAE* at concentration 12.5 (mg/ml), *Salmonella typhi* and total bacteria counts were decreased as the storage period decreases significantly where at zero day of storage period was 1.40 and 4.15 CFU/g /g respectively. While, throughout the 2nd, the 4th, the 6th and the 8th weeks of storage were, 0.25, 0.00, 0.00 and 0.00 *Salmonella sp* as well as 3.15, 2.10 ,0.45 and 0.0 CFU/g total bacteria respectively . These results clearly indicated that, the preservation effectiveness of *ChAE* at 12.5 (mg/ml) against artificially inoculated *Salmonella typhi* in cartage cheese sample exhibited complete Bactericidal effect with no recovery of the pathogen *Salmonella typhi*.

Table 12: Viable count of *Salmonella typhi* on cartage cheese Treated with ChAE storage period (0-8 weeks) at 4°C) .

Storage time (weeks)	Viable count of bacteria (Log CFU/g /g)			
	Untreated sample (Control)		Treated sample (12.5mg/ml)	
	Salmonella sp.	Total bacteria	*Salmonella sp.*	Total bacteria
0 day	1.40 ± 1.5	4.15 ± 1.8	1.40 ±0.98	4.15 ±0.98
2nd week	2.45 ± 0.76	4.35 ± 0.25	0.25 ±0.00	3.15 ±0.00
4th week	2.85 ±1.54	4.65 ±1.13	0.00 ±0.00	2.10 ±0.00
6th week	3.45 ±1.47	4.95 ±1.52	0.00 ±0.00	0.45 ±0.00
8th week	3.92 ±1.73	5.12 ±1.36	0.00 ±0.00	0.00 ±0.00

Each reported value for viable bacterial count indicates the mean standard error calculated from three experimental replications.

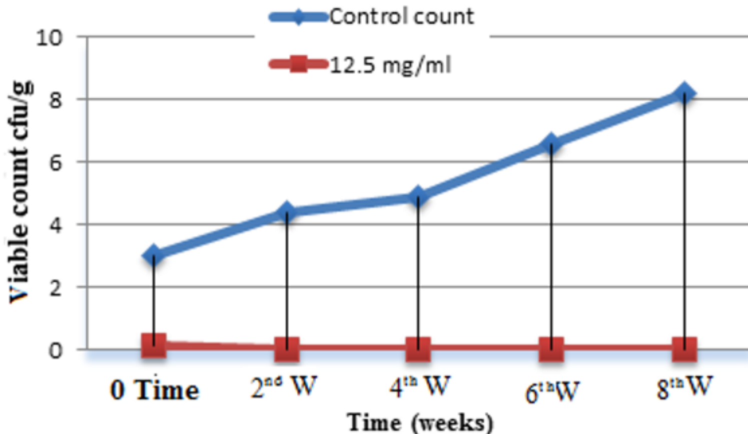

Fig. 8: Inhibition curve of *Salmonella* tophi inoculated in cortege cheese treated with Chlorella aqueous extract at concentration 12.5 (mg/ml) and refrigerated at 4 °C for 8 weeks.

DISCUSSION

Food poisoning is recognized as any illness that develops from the intake of food or drinks that have been contaminated with disease-causing microorganisms. *Salmonella enteritis*'s the second most common intestine infection, cause of salmonellosis which is the second major appear in a wide variety of food ingredients, thus posing a great problem to the food industry cause of foodborne disease, leads episodes of hospitalization and death. This study has been focused on *Salmonella* in time to show that, home-made cheeses such as, Cartage cheese, Salt cheese and Cream cheese is free from food poisoning by *Salmonella* during five seasons (2018 – 2022) on 2000 samples collected from Bandar El Fayoum, El Fayoum, Snores, Tamaya, Abshaway, Etsaa, Yousef and El sadik regions. The principal cause of enteric fever in homo sapiens is *Salmonella typhi*. *Salmonella* species are known to be facultative intracellular pathogens (Jantsch, *et al.*,2011). Various cell types, including epithelial cells, M cells, macrophages, and dendritic cells, can be invaded by *Salmonella*. (LaRock *et al.*,2015). The topic of the concern now a day is the emerging multidrug resistance. Despite increased public awareness and strengthened federal initiatives aimed at controlling infections, the transmission of foodborne pathogens continues to be a significant concern. The mechanisms of infection encompass the ingestion of preformed toxins, the production of toxins after consumption, and the direct invasion of intestinal epithelial cells by the pathogenic organism. Diagnosis is generally established through a comprehensive history, given that culture tests often yield minimal results (Cangemi, 2011).

Prevalence and count of *E. coli, Salmonella* sp and *Staphylococcus sp* were contaminant Cortege cheese which collected from different regions in Fayoum government at five years with prevalence ranged 40.03 - 18.69 % respectively . The results emphasized that cottage cheese samples contaminated with pathogenic bacteria. E. coli was found with prevalence 9.6 % with the average log 2.11 CFU/g . *Salmonella sp.* found with prevalence 6.8 % with the average log 1.95 CFU/g. *Staphylococcus sp* found with prevalence 7.14 % with the average log 1.96 CFU/g

Isolation of pathogenic bacteria from cartage cheese samples such as E. coli isolated on Eosin Methylene blue medium. Typical colonies exhibit a a greenish metallic color accompanied by a dark purple center. *Staphlylococcus sp* were isolated on the surface of Baird-Parker media, which was supplemented with egg yolk and potassium tellurite solution. The resulting colonies are noted for their gray-black coloration, shiny and convex structure, and a narrow white margin that is surrounded by a zone of clearing. *Staphlyococcus sp* gave coagulase positive (Coagulase causes plasma to clot by converting fibrinogen to fibrin). The results

were interpreted with 3+ (large organized clot), 4+ (entire content of the tube coagulates and is not displaced when the tube is inverted).The results were considered positive evidence of coagulase production . *Salmonella* spp.: The pre-enrichment of samples was performed according to the Rappaport-Vassiliadis medium with soya (RVS broth) .

The *Salmonella typhi* culture was studied by the colony characteristics showed black head colony on the XLD plate. The Buffered Peptone Water, count agar plates of Eosin Methylene blue (EMB) , Baird parker media brain heart infusion agar *Salmonella* and Shigella (SS) agar and xylose lysine deoxycholate agar (XLD). The suspected colonies appeared creamy, exhibiting either the presence or absence of black centers on SS agar, while on XLD agar, they displayed a red coloration, also with or without black centers. After incubation, the culture was studied by its colony characteristics, observation of black head colony on the plate was supposed to represent *Salmonella* further identification and conformation gram staining, coagulate test. Catalase Test. biochemical reaction Identification of isolated *Salmonella sp*.

Three *Salmonella sp* different isolates, more prevalence and high count, (SCC-1) from cartage cheese, (SSC-2) salt cheese and (SCrC-3) Cream cheese identified morphologically, cultural features and consumption of broth manual some biochemical analyses according to (Bergey's manual, 2009) and confirming by 16s DNA gene by PCR. as the following

The results of morphological and biochemical features of three *Salmonella* sp isolates. belonging to Bacillaceae family exhibited Low convex and entire of Shape of colony , Smooth Texture, , no Pigmentation, positive Motility and facultative anaerobic of O2 requirements on selective XLD medium .

It was found negative Gram reaction , short rods of cell shape and found singly or in pairs , no sporulation and no capsulation .

Biochemical characteristics of non-fastidious Gram negative basilli *Salmonella sp* isolates. *Salmonella sp* isolates exhibited different non-significant in some Biochemical characteristics gave positive results with 5 set tubes.

1-Triple sugar iron agar (TSI) which detected by color change of the medium to yellow. 2-Lycine iron egar (LIA) which shown by change in color of the indicator to red-pink. 3-Motility Indole Ornithine agar (MIO) The *Salmonella sp* were diffused growth through the medium from the point of inoculation is considered motility positive. 4-Citrate agar which showed growth The *Salmonella sp* in the medium by turbulence and an alteration in color of the indicator from light green to blue caused by citrate utilization. 5-Urea agar which showed *Salmonella sp* isolates cultured and the indicator Phenol red. Bacteria that possess the urease enzyme hydrolyze urea, producing ammonia that elevates the pH, as indicated by a color change to red or pink. The assessment of antibiotic sensitivity among three *Salmonella typhi* isolates demonstrated a range of susceptibilities, including sensitive, intermediate, and resistant responses to various antibiotics. Also, from these results we note that *Salmonella* growing in Fayoum is too weak. The samples also showcased emerging resistance against azithromycin (Gohar Shah *et al.,*2022).

Salmonella food contaminated were serologically detected and identified by A Widal test using primary antigen *S. Typhi* O (TO), secondary antigen *S. typhi* H (TH), *S. Paratyphi* A and *S. Paratyphi* B. It was found that, some isolated *Salmonella* sp gave positive serology reaction with TO, (TH) which identified *Salmonella typhi* and other isolated *Salmonella sp* negative antigen TO. On the other hand, some isolated of *Salmonella* gave positive serology reaction with AH, (BH) which identified *Salmonella Paratyphi* and other isolated *Salmonella sp* negative antigen AH

The molecular modeling and analysis of the active site of the SdiA homolog, which is a potential quorum sensor associated with the pathogenicity of *Salmonella typhi*murium, elucidate the distinct binding patterns of AHL transcriptional regulators (Garai *et al.,*2012). The virulence gene spvB, found in

Salmonella plasmids, is recognized for its role in increasing bacterial virulence by suppressing autophagy (Li *et al.,*2016).

In this study *Salmonella* sp was identified molecularly by my- invA gene PCR Using isolated DNA from the three *Salmonella sp* (SCC-1), (SSC-2) and (SCrC-3) isolates and amplified by PCR conventional method after reaching the sufficient concentration and high purity using specific primers to my- invA gene. PCR product returned as electropherogram files demonstrated distinct fragments with expected size 1224 bp for three isolates.

Amplicons of two *Salmonella sp* (SCC-1) and (SSC-2) isolates were subjected to sequencing reactions utilizing the cycle sequencing method. The sequences generated for each primer from the respective isolates demonstrated sufficient overlap, which facilitated the assembly of a continuous sequence, referred to as a contig. The two contig (in Appendix) were obtained by analysis of three sequences charts using Finch TV program version 1.4 (Geospiza, USA) and showed the partial nucleotide sequences of my- invA gene. The resulted sequences were compared with bacterial species recorded on the Genbank using DNAMAN program and identified as *Salmonella enterica* subsp enterica serovar blackley strain and *Salmonella enterica* subsp enterica serovar dublin strain and submitted and recorded on gene bank under Submitted with ACCESSION BSeq#1 and culture collection="ATCC:13311" (in Appendix).

Sequence variability of my- invA gene based on multiple sequence alignment for two *Salmonella* sp (SCC-1) and (SCrC-2) isolates were subjected to variability analysis using DNAMAN and MEGA.4 programmers (Wisconsin, Madison, USA). The sequences of two isolates were aligned for determining the sequence registered in GenBank, as well as phylogenetic relationships among the two isolates. Based on MSA analysis, Phylogenetic tree of *Salmonella sp* (SCC-1) was performed and shows five clusters in which the *Salmonella enterica* subsp enterica serovar blackley strain was found to be highly homologous with percentage. *Salmonella enterica* subsp enterica serovar dublin strain JQ796859.1; KC121042.1 and HM067869.1 were found to be highly homologous with percentage (94%), while percentage was (98%) between *Salmonella enterica* subsp enterica serovar dublin strain JQ796859.1; KC121042.1 and HM067869.1, also percentage was (99%) between *Salmonella enterica* subsp enterica serovar dublin strain strains KC121042.1; HM067869.1 and JQ659920.1 so it was represented as a separate cluster

As well as phylogenetic tree of *Salmonella sp* (SCC-2) isolate shows five clusters in which the *Salmonella enterica* subsp enterica serovar blackley strain was found to be highly homologous with percentage while the *Salmonella enterica* subsp enterica serovar dublin strain showed distant homology . *Salmonella enterica* subsp enterica serovar dublin strain JQ796859.1; KC121042.1 and HM067869.1 were found to be highly homologous with percentage (94%), while percentage was (98%) between *Salmonella enterica* subsp enterica serovar dublin strain JQ796859.1; KC121042.1 and HM067869.1, also percentage was (99%) between *Salmonella enterica* subsp enterica serovar dublin strain strains KC121042.1; HM067869.1 and JQ659920.1 so it was represented as a separate cluster .

Active ingredient compounds of Chlorella methanolic extract (ChME) were determined by GC-mass analysis appeared nine active ingredient compounds act as antioxidant and antibacterial based on chemical formula and determinate groups. *Chlorella* is consumed as a dietary supplement. Certain manufacturers have erroneously claimed that it possesses health benefits (RCS, 2024), including the treatment of cancer (ACS, 2011). The American Cancer Society has stated that "available scientific studies do not support its effectiveness for preventing or treating cancer or any other disease in humans." (Correll. 2020) .

Aqueous extract of *Chlorella* showed potential antibacterial activity to

inhibit two *Salmonella typhi* (SCC-1) and (SCrC-2) isolates for providing an appropriate substitution to the use of conventional antimicrobials in food preservation. It revealed clear zones inhibition with diameter 20.03 and 20.60 mm for *S. typhi* (SCC-1) & (SSC-2) isolates respectively and the mean growth inhibition was 100 % for two *S. typhi* isolates. In addition, the study of antioxidants, as a free radical scavenger, various anti virulence. The biochemical profile of microalgae is notably rich, encompassing significant compounds such as polyphenols, flavonoids, and antioxidants. These substances have drawn considerable attention owing to their potential health benefits and usefulness in industrial contexts (Barkia, *et al.,* 2019). Traditional methods for extracting bioactive compounds from microalgae generally utilize volatile organic solvents (VOCs) sourced from petroleum or employ water-based techniques (David and Niculescu 2021).

Evaluation of MIC and MBC of ChAE against two *S. typhi* isolates. The results clearly show 100% of the antibacterial spectrum activity of ChAE at doses between 500 to 1000 µg/ml, whereas at concentration of 250 µg/mL the activity was 83.3%. The MBC values of ChAE against *S. typhi* (S1 & S2) revealed 1000 µg/mL which show 100% of the antibacterial spectrum activity of ChAE, whereas at concentration of 250 µg/ml the activity was 50.0%. Whereas the concentrations 125 µg/ml did not score antibacterial influence against *S. typhi* (S1 & S2) pathogenic isolates. MIC 250 ug/ml of ChAE showed non-toxic concentration on Vero cell culture using by MTT assayed and did not show any morphological and cytotoxicity difference when compared with control one.

The DPPH free radical-scavenging activity of ChAE demonstrated an increase with higher concentrations, achieving a notable scavenging activity of 84.46% at a concentration of 2560 µg/ml. Additionally, the radical-scavenging percentages recorded at concentrations of 1280, 640, 320, 160, 80, 40, and 0 µg/ml were 78.9%, 63.92%, 52.23%, 40.54%, 23.11%, 12.50%, and 0%, respectively, with an IC50 value of 289.5 µg.

In the last few years, many researchers have explored the synthesis of extracellular antibiotic metabolites by marine algae, revealing that green microalgae extracts contain a significant quantity of antifungal agents (Kuda *et al.,*2007). In conclusion, microalgae represent a nearly inexhaustible source of natural antioxidants, owing to their extensive biodiversity, which surpasses that of terrestrial plants. These organisms encompass a variety of biologically potent compounds that are employed in nutrition, animal feed, and pharmaceutical products (Fedorov *et al.,*2013). Microalgae are proposed as a substitute for the molecular pharming system (Specht *et al.,*2010). Natural antioxidants demonstrated a higher level of antioxidant activity compared to their synthetic counterparts (Sarkar and Ghosh 2016). Algae are recognized for their potential as a source of natural antioxidants, comprising carotenoids, vitamins, phenolic compounds, and phycobilins, which are utilized in various fields such as pharmaceuticals, nutraceuticals, medicine, and the food sector. Microalgae serve as a crucial source of pharmacologically potent metabolites, demonstrating activities that are antineoplastic, antitumor, antibacterial, antifungal, and antiviral in nature (Munir *et al.,*2013). Chlorella is regarded as one of the most ancient microorganisms on the planet, characterized by its spherical shape and remarkably stable cell wall. *Chlorella sp.* represents a category of unicellular eukaryotic green microalgae (Nick, 2003). Application ChAE of cartage cheese die to long life preservation and significantly reduction of *Salmonella typhi* . On the other hand, cartage cheese incorporated with ChAE at concentration 12.5 (mg/ml), *Salmonella typhi* counts at zero day of storage period was 1.85 CFU/g /g then decreased at 2nd, the 4th, the 6th and the 8th weeks of storage .These results clearly indicated that, the preservation effectiveness of ChAE at Minimum Bactericidal Concentration (MBC) 12.5 (mg/ml) exhibited complete cidal effect with

no recovery of the pathogen *Salmonella typhi*. In East Asian countries, microalgae are commonly included in the daily diet. Their bioactive properties render them an appealing area of study for potential cancer therapies (Ye, *et al.,*2008). Microalgae are commonly utilized as a food additive to enhance the nutritional value of food and promote the health of living organisms, owing to their rich phytochemical content and bioactive compounds. The primary focus on microalgae lies in their protein composition, which serves as an unconventional source of protein (Fan, *et al.,*2014). Furthermore, microalgae possess the ability to synthesize essential amino acids for both humans and animals, offering a more advantageous profile compared to other protein sources (Guil-Guerrero, *et al.,*2004).

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