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Detection and inhibition of *Clostridium botulinum* in some Egyptian fish products by probiotics cell-free supernatants as bio-preservation agents

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ABSTRACT

The detection and inhibition of *Clostridium botulinum* by probiotics' cell-free supernatants, as a bio-preservation strategy, were investigated in some Egyptian fish-stuffs *i.e.,* canned sardines, fesikh, moloha, renga, and anchovy. The conventional and multiplex polymerase chain reaction were used to detect *C. botulinum*, while the inhibitory and antioxidant capacity of four probiotics' cell-free supernatants were assessed using disc diffusion assay (DDA) and radical scavenging activity approaches, respectively. The results revealed that the *C. botulinum* was isolated from 41.6% of the examined products, where the type E. was the most predominant genotyping. The DDA showed that *Lactobacillus bulgaricus* EMCC 1102 and *L. paracasei* EMCC 1103 expressed the highest inhibitory action against *C. botulinum* type E. Meanwhile, *L. bulgaricus* EMCC 1102 has higher *in vitro* antioxidant activity (IC_{50}) than *L. paracasei* EMCC 1103, mostly due to its high bioactive constituent. Consequently, *L. bulgaricus* EMCC 1102 was used to control the growth of *C. botulinum* type E in stored anchovy fillets. *L. bulgaricus* EMCC 1102 supernatant showed a dose-dependent inhibitory effect on *C. botulinum* type E. These data provided an innovative perspective on the inhibitory potential of probiotics, as a bio-preservation strategy, to guarantee fish products safety for further applications in the food industry.

1. Introduction

Botulism, a neuroparalytic illness, is caused mainly by a potent neurotoxin produced by *C. botulinum;* a gram-positive, obligatory anaerobic, and spore-forming rod bacteria (Doyle, 1989). Ingestion of preformed toxins and/or development of *C. botulinum* in anaerobic tissues can cause botulism with many symptoms *i.e.,* paralysis, nausea, vomiting, abdominal cramps, cantankerousness, droopy eyelids, fatigue, hard feeding, and swallowing (Alizadeh et al., 2020). Seven types of *botulinum* toxins have been identified from A to G. The types A, B, E, and F are well-known to cause humans illness. *C. botulinum* could be categorized according to their antigenic specificity of the relatedness of DNA or toxins produced (Hannett, Stone, Davis, & Wroblewski, 2011). The majority of human infections caused by *C. botulinum* E-type which have been linked to the ingestion of fish and aquatic animals (Lalitha & Surendran, 2002). Although this severe form of food poisoning is uncommon, it has a significant death rate. Outbreaks of foodborne botulism have been reported globally (Czerwiński, Czarkowski, & Kondej, 2016; Fleck-Derderian et al., 2017).

Fishery products *i.e.,* sardines, fesikh, moloha, smoked fish (renga),

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and anchovy, constitute a key economic resource for Egyptian consumers. Pickled fish, which is a ready-to-eat fish product without heat treatment, is a semi-preserved for a definite shelf life by adding organic acids and salt to hinder the microbial growth and enzymatic deterioration (Fuentes, Fernández-Segovia, Barat, & Serra, 2010).

Recently, the consumer requirement for natural foods without chemical additives has strengthened, especially the demand for probiotics and their use in food bio-preservation, both for their high antimicrobial and antioxidant potential (Cizeikiene, Juodeikiene, Paskevicius, & Bartkiene, 2013). At present, the food industries are the principal food sector where the highest number and diversity of probiotics are used to improve of both final product quality and safety (Gao et al., 2021). These probiotics are most *Lactobacillus* sp. Bacteria (Verschuere, Rombaut, Sorgeloos, & Verstraete, 2000).

Probiotics have an immense potential in the detoxification of environmental pollutants. Lactic acid bacteria have a strong affinity for many toxic metals and organic pesticides, which makes it possible to keep them from getting into the bloodstream and tissues before they get adsorbed (Reid, 2015). Furthermore, probiotic cultures have been shown to have strong antimicrobial and antioxidant properties, suggesting that they could be used as biological agents against clostridial disease (Düz, Doğan, & Doğan, 2020; Hamad, Abdelmotilib, Darwish, & Zeitoun, 2020; Monteiro et al., 2019; Song, Jang, Kim, & Paik, 2019).

To date, there is a lack of literature which investigate the anti-clostridial activity of various commercial probiotics against *C. botulinum*. Therefore, this study aims to I) determine the occurrence of *C. botulinum* in some Egyptian fish products, II) evaluate the anti-clostridial and antioxidant abilities of cell-free supernatants of some probiotics *i.e., L. bulgaricus* EMCC 1102, *L. paracasei* EMCC 1103, *L. fermentum* EMCC 1346, and *L. plantarum* EMCC 1027, III) assess their inhibition effect on *C. botulinum* in the anchovy fillets.

2. Materials and methods

2.1. Sample's collection

5 types of Egyptian fish products, including canned sardines, fesikh (non-eviscerated, salted mullet fish), moloha (fermented salted fish), renga (smoked herring fish), and anchovy were studied and 25 samples from each type were analyzed, with a totally 125 samples were arbitrarily collected from the local fish factories, retail stores, and supermarkets in the cities of Alexandria and Borg El-Arab, Egypt. The samples were refrigerated until being evaluated.

2.2. Sample's preparation

The cans were opened with a sterile can opener. The cans surfaces were cleaned, dried, and their tops were covered with EtOH (96%; Thermo Fisher Scientific, USA) and left for 2 min. The EtOH-residual was decanted and flamed off. Lastly, 20 g of each fish sample was mixed under aseptic conditions with peptone water (10 mL, 0.1%) for 2 min. Further, 2 g of the prepared samples were inoculated into two screw-capped bottles; one contained of Trypticase Peptone Glucose Yeast broth (TPGY; Oxoid, UK) in a volume of 15 mL, and the other one was contained of Cooked Meat Medium (CMM; obtained from Oxoid, UK) up to 15 mL. The inoculated media was incubated using the AnaeroGen™ gas generating kit (Oxoid, UK) in anaerobic conditions, for 7–10 d. TPGY broth was incubated (New Brunswick™ Galaxy® 170 R CO2 Incubator Series, Eppendorf, Spain) at 28 °C, where CMM was incubated at 37 °C.

Each broth culture was studied for acidity, gas production, turbidity, and proteolytic activity. The acidity was determined using the method of Cao et al. (2017) by the titration with NaOH (0.1 mol/L), where phenolphthalein was used as an indicator. The gas amount production was estimated by measuring the linear displacement (mm) of media by gas in the Durham tube according to Montville (1983). The growth was determined by the appearance of visible turbidity. The proteolytic activity was analyzed using protease activity which was detected by hydrolysis of casein and gelatin in a dual-substrate plate diffusion assay (Montville, 1983). For purification purpose, the procedure of Ahmed, Badary, Mohamed, and Elkhawaga (2011) was followed, where subcultures on TPGY broth at 30 °C for 16 h were used to purify *C. botulinum*, after which the bacterial suspension in this overnight culture was utilized for its isolation, and a DNA template was generated for detection and genotyping by multiplex polymerase chain reaction (PCR).

2.3. Isolation and identification of C. botulinum using conventional methods

2.3.1. Isolation of pure culture of C. botulinum

To acquire a pure culture of *C. botulinum*, adding the same EtOH-volume to 2 mL of the enrichment culture significantly improved isolation. Then, the mixture was incubated at 20–22 °C for 1 h. One loopful of the enrichment treated culture from each sample was marked onto 2 Clostridial Agar (HIMEDIA, USA) plates by the plating out method. One of the straked plates was consequently incubated at 37 °C to isolate the proteolytic strains (group I) of *C. botulinum*-types, A, B, and F, while the other plate was incubated at 28 °C to isolate its non-proteolytic strains (group II) types, B, E, and F. The streaked plates were incubated for 3–5 d in anaerobic conditions using the AnaeroGen™ (Oxoid, UK) gas generation kit. *C. botulinum* colonies were examined on all culture plates after 3–5 d (Küplülü, Göncüoğlu, Özdemir, & Koluman, 2006).

2.3.2. Multiplex PCR detecting and genotyping technique of C. botulinum types (A, B, E, and F) neurotoxin genes

Genotyping and detecting of *C. botulinum*types; A, B, E, and F neurotoxin genes were completed using 4 pairs of primers, as shown in Table 1S. Multiplex PCR (Agilent Technologies, SureCycler 8800 Thermal Cycler, USA) approach was performed with a 50 μL of the blend containing 2x multiplex PCR master mix (Qiagen, Hilden, Germany), 0.3 μM of separately primer (Bioneer, USA), and 3.0 μL of DNA purified template. The amplification programme was showed on the thermal cycler (Biometra, USA). The reaction mixture was heated for 15 min at 95 °C to activate the HotStarTaq DNA polymerase, and then subjected to 35 cycles, each consisting of 30 s of denaturation at 95 °C, 30 s of annealing at 51 °C, and 90 s of extension passed at 72 °C, following by a final extension at 72 °C for 7 min. PCR products were then examined by agarose (2%) gel electrophoresis at 90 V for 70 min and examined under an ultraviolet transilluminator. The size of the multiplex PCR products was compared to a standard 100bp DNA ladder (Nippon, Genetics, Düren, Germany) (Ahmed et al., 2011; De Medici et al., 2009).

2.4. Inhibition of isolated C. botulinum using probiotics cell-free supernatant

2.4.1. Probiotics and culture conditions

A total of 4 strains commonly used in food products manufacturing, *L. paracasei* EMCC 1103, *L. bulgaricus* EMCC 1102, *L. fermentum* EMCC 1346, and *L. plantarum* EMCC 1027 were gotten from the Microbiological Resources Center, MERCIN, Ain Shams University, Egypt. The strains were full-grown aerobically on deMan Rogosa Sharpe (MRS) Agar (TM Media, India) at 37 °C for 48 h, while *C. botulinum* type E was grown on TPGY agar under anaerobic conditions.

2.4.2. Cell-free supernatant preparation from probiotic cultures

Preparation of cell-free supernatant from probiotic cultures was done following the method of Hamad, Abu-serie, Ali, and Hafez (2018). The suspensions of the strain in the phosphate buffer saline (PBS; bought from Sigma Aldrich, USA) were adjusted to 0.220 of optical density (OD) at 600 nm. A 1 mL of the suspension was then inoculated into MRS broth

Table 1
Prevalence of *C. botulinum* types (A, B, E and F) in samples of fish products.

Types of fish products	No. of samples Analyzed	Positive samples		*C. botulinum* types			
		No.	%	A No. of isolates	B No. of isolates	E No. of isolates	F No. of isolates
Canned sardines	25	8	32	8	2	1	2
Fesikh	25	10	40	0	10	0	0
Moloha	25	9	36	7	3	2	1
Renga	25	12	48	0	2	12	3
Anchovy	25	13	52	2	1	13	2
Total	125	52	41.6	17	18	28	8

(9 mL) and incubated for 24 h at 37 °C. The cultures were independently enriched in MRS broth (200 mL), incubated at 37 °C for 48 h with a continuously shaking till the OD of 600 ranged from 0.4 to 1.6, and centrifuged (Micro centrifuge, SELECTA, Spain) at 10000 g for 10 min. The supernatant was then collected, filter-sterilized using a membrane syringe filter (0.2 mm, Thermo Fisher Scientific, USA), transferred-aseptically into a conical tube, and stored at (−80 °C). The culture supernatant was freeze-dried (Lyophilizer, Model FDF 0350, Korea), and the obtained powder was weighed.

2.4.3. Assessment of anti-clostridial activity of the probiotic cell-free supernatants

The obtained probiotic cell-free supernatants ability to inhibit *C. botulinum*type E was evaluated through a DDA. Briefly, *C. botulinum* type E was enriched (100 μL) on TPGY broth at 37 °C for 48 h. Broth cultures were adjusted with a sterile saline to obtain a final concentration of 10^6 CFU/mL, and were then spread over the TPGY plates using sterile cotton swabs. After drying, each culture supernatant (20 μL) was preloaded onto sterile plain discs, and the plates were maintained at 4 °C for 30 min before incubation under anaerobic conditions at 37 °C for 24 h. The clear zones formed were logged in (mm), considering the anti-clostridial activity. Six antibiotic discs were used as a control: tetracycline (30 mg/mL), amoxicillin (25 mg/mL), metronidazole (50 mg/mL), doxycycline (30 mg/mL), chloramphenicol (30 mg/mL), and clindamycin (20 mg/mL) (Weese et al., 2004).

The minimum inhibitory concentrations (MICs) of probiotic supernatants against *C. botulinum* were determined using descending concentrations. The *C. botulinum* suspensions were prepared in a sterile saline and the density were adjusted to 10^6 cells/mL. After that, TPGY media was inoculated with *C. botulinum* suspensions using a sterile cotton swab. Then, the plates were dried at RT for 15 min before applying the disks. Seven sterile disks were placed on the medium surface. The probiotic supernatants were serially diluted in H_2O (Milli-Q, Siemens Ultra Clear, Germany) and 10 μL of each dilution was separately used to saturate the disks. The plates were incubated at 37 °C for 24 h. Finally, the MICs values were recorded (Hamad et al., 2020).

2.5. Antioxidant activity of the probiotic supernatants

This antioxidant capacity method measures the free radical scavenging capacity of the probiotics cell-free supernatant (PCFS), on the radical diphenyl picryl-hydrazyl (DPPH; Sigma-Aldrich, USA). The radical scavenging activity at numerous concentrations *i.e.,* 5, 10, 20, 30, 40, 50, 60, 70, 80, and 100 mg/mL of *L. paracasei* EMCC 1103, *L. bulgaricus* EMCC 1102, *L. fermentum* EMCC 1346, and *L. plantarum* EMCC 1027 was assessed. The similar ascorbic acid concentrations were used as a standard. The absorbance was measured at 517 nm by a T80 UV/VIS spectrophotometer (PG Instrument Ltd. UK) (Hamad et al., 2020).

2.6. Total phenolic content of the probiotic's supernatants

The total phenolic content (TPC) of the probiotic supernatants was analyzed by the Folin-Ciocalteu spectrophotometric assay (Hamad, Taha, El-Deeb, & Alshehri, 2015). A 0.1 mL of Folin-Ciocalteu reagent (Sigma-Aldrich, USA) was added to 1% of probiotic cell-free supernatant (2 mL). The mixture was left for 15 min at RT. A 3 mL of 2% Na_2Co_3 (Thermo Fisher Scientific, USA) was added. The mixture was allowed at RT for 30 min before determining TPC at 760 nm and comparing it to gallic acid as a standard by UV/Vis spectrophotometer, and expressed in mg of gallic acid equivalents per mL of sample.

2.7. Total flavonoid content of probiotic supernatants

The total flavonoid ratio analyses of probiotic supernatants were determined by a UV/Vis spectrophotometer at 510 nm. The contents were expressed as mg of quercetin equivalent per mL of the sample (Sakanaka, Tachibana, & Okada, 2005).

2.8. Analysis of phenolic and flavonoid compounds in the probiotic supernatants by HPLC

The phenolic compounds analysis was performed with Agilent 1260 Infinity HPLC Series (Agilent, USA) set with a Quaternary Pump, a ZORBAX Eclipse Plus C18 column (100 mm × 4.6 mm i. d. Agilent Technologies, USA) and operated at 25 °C. The separation was achieved using a ternary linear elution gradient with (A): HPLC-grade water-0.2% H_3PO_4 (v/v); purchased from Sigma-Aldrich, USA, (B) methanol (HPLC-grade, Thermo Fisher Scientific, USA), and (C) acetonitrile; (HPLC-grade, Thermo Fisher Scientific, USA). The injected volume was 20 μL. Detection: VWD detector set at 284 nm according to Hamad et al. (2020), following Agilent Application Note Publication Number 5991–3801 EN, 2016.

2.9. Inhibitory effect of L. bulgaricus EMCC 1102 supernatant against C. botulinum type E in anchovy samples

The probiotic L. *bulgaricus* EMCC 1102 was chosen for the application due to its antibacterial and antioxidant properties. The procedure was performed in the manner described by Testa et al. (2019) with some modifications. All anchovy fillets were marinated in a solution containing acetic acid 2% (v/v) and NaCl (10%, w/v), with adjusted pH at (2.32) using pH meter (Hanna Instruments, USA). The fish-to-solution ratio was 1:1.5 (w/v). The marinating time was 30 h at 20 ± 1 °C. After marinating, the fish fillets were removed from the marinating solution and drained on a sterile stainless steel mesh screen for 5 min.

Marinated anchovy fillets were divided into 4 parts; a negative control and 3 treatments (T1, T2, and T3) were inoculated with (25, 50, and 100) mg/g of the cell-free supernatant of *L. bulgaricus* EMCC 1102, respectively. A 50 g were inoculated with *C. botulinum*-type E (10^4 CFU/g) for each sample. After homogenization, the samples were stored in polyethylene bags within firmly sealed glass containers, refrigerated at 6 ± 1 °C, and examined for the presence of *C. botulinum* type E at 0, 1, 2, 4, 6, 8, 10, 12, and 15 d.

For microbial enumeration purposes, the cooled samples were homogenized (1 min), and incubated at 37 °C for 24 h in a CO_2 incubator. A

1 mL was added to 9 mL of peptone broth and incubated at 37 °C for 24 h in a CO_2 incubator. The counts were taken on TPGY agar under anaerobic conditions.

2.10. Statistical analysis

The experimental design was completely randomized with 3 replicates. Data analysis began by obtaining frequency distribution and descriptive statistics for most variables. The means ± standard error (SE) was used to express the data. The date was analyzed *via* multiple comparison, also by one-way analysis of variance (ANOVA) using the Duncan test and the IBM SPSS Statistics software (ver. 23), where the probability was considered statistically significant ($P < 0.05$).

3. Results and discussion

3.1. Prevalence and isolation of C. botulinum types in fish products

As shown in Table (1), *C. botulinum* could be isolated from 41.6% of examined fish products, with different incidences of each product as follows: (8) 32%, (10) 40%, (9) 36%, (12) 48%, and (13) 52% from canned sardines, fesikh, moloha, renga, and anchovy, respectively. Moreover, the study of Ahmed et al. (2011) concerning prevalence of the hazardous microorganisms in some Egyptian foods including fish products that demonstrated the number of isolated strains mainly *C. botulinum* type E to be 25% in faseikh, 15% in canned sardine, 15% in moloha, 10% in canned tuna, and 5% in packaged smoked fish, and could present a potential threat for infant botulism and foodborne. The high prevalence of *C. botulinum* in the examined fish products in our study may be attributed to the unhygienic production of such products. In the past, *C. botulinum* was found in only 13% of India's fresh and cured fish products (Lalitha & Surendran, 2002). In addition, many surveys regarding *C. botulinum* prevalence in ready-to-eat smoked seafood in sealed packaging have been conducted by Gilbert, Lake, Hudson, and Cressey (2006). The authors have identified *C. botulinum* spores in food products, particularly fish (Salted carp, Smoked fish, Salmon, and Haddock fillets). The findings on the incidence of *C. botulinum* in seafood overseas have been worldwide summarized *i.e.,* USA, Denmark, Finland, Japan, UK, France, Norway, and Indonesia, the high contamination prevalence of *C. botulinum* spores in the seas or oceans in these regions (up to 100%) seems to reflected in the many fish products (Gilbert et al., 2006). Foodborne botulism, although rare, remains a public health emergency because of its high mortality rate and its severity (Fleck--Derderian et al., 2017).

Genotyping of *C. botulinum* (A, B, E, and F) was performed by multiplex PCR, and the results revealed that *C. botulinum* type E was the most prevalent type among the obtained isolates (Table 1). In this regard, the total number of isolated type E strains was 28 in the fish products examined, compared to 17, 18, and 8 for the other clostridial types, A, B, and F, respectively. In another study, a lower finding was reported in which the authors found that the total number of isolated *C. botulinum* strains in examined fish products was 14 for type E, 5 for type B, and zero for types A and F (Ahmed et al., 2011). *C. botulinum* type E strains were notably found in anchovy and renga, with their numbers reaching to 13 and 12, respectively, while they were extremely low in canned sardines and moloha at 1 and 2, respectively. A varied result has been previously reported (Ahmed et al., 2011). The authors reported that 3 of the isolated strains of *C. botulinum* type E strains were found in canned sardines, 5 in fesikh, 1 in moloha, and 3 in smoked fish. In addition, *C. botulinum* type B was most common in fesikh, with an isolated number of 10, while other Clostridial types could not be detected in fesikh. The number of isolated *C. botulinum* type B strains in canned sardines, moloha, renga, and anchovy was 2, 3, 2, and 1, respectively. This result is higher than those reported elsewhere by Ahmed et al. (2011), who found that the number of *C. botulinum* type B in canned sardines, fesikh, and smoked fish was 1, 2, and 1, respectively. Furthermore, *C. botulinum* type A was the most detectable in canned sardines and moloha at 8 and 7, respectively. *C. botulinum* type F had the minimum detection rate (2 ± 1) in all fish products examined. The results indicated that using multiplex PCR with a specialized primer, as shown in Table 1S, to confirm the presence of clostridial neurotoxins in fish product samples was an effective. From Fig. 1S, clear bands were formed at definite base-pairs of 101, 205, 389, and 543 pb for clostridial strains types A, B, E, and F, respectively.

C. botulinum type E is commonly found in fish and fishery products, and its ability to grow at temperatures as low as 3–5 °C, while there is little or no visible evidence of spoilage is of particular concern. *C. botulinum* type E has been implicated in outbreaks in Egypt and Canada due to the consumption of contaminated fesikh (Walton et al., 2014). In addition, it has been associated with outbreaks in North America, Europe, and Asia. Of 197 foodborne outbreaks caused by *C. botulinum* reported between 1920 and 2014, type E was involved in 17% (Fleck-Derderian et al., 2017). They also found that types C and D were the most prevalent ones in the examined fish products (Lalitha & Surendran, 2002).

In general, *C. botulinum* strains can secrete their neurotoxins in foods under favourable niches, such as contaminated foods with bacterial spores, insufficient heat treatment, re-contamination after processing, and anaerobic products (canned foods) (Bremner, Fletcher, & Osborne, 2003). Hence, this may explain the variation in clostridial types among the examined fish products reported in this study.

Anchovy showed the highest contamination level with *C. botulinum* in the present study. This result may be associated with the conventional manufacturing implemented to process anchovy and with the elevated salt content that may reach 5%. Anchovies are a conventional product made by gutting and salting anchovy fillets in brine, allowing them to mature and packing them in oil or salt into small tins or jars (Dewi, 2002). Moreover, C. *botulinum* strains and their spores are abundant in marine water, deposits, guts, and gills. Even if anchovies might be cleaned, gutted, and packed, the possibility of botulism is still present. This is because *C. botulinum* spores could transfer from the exterior of

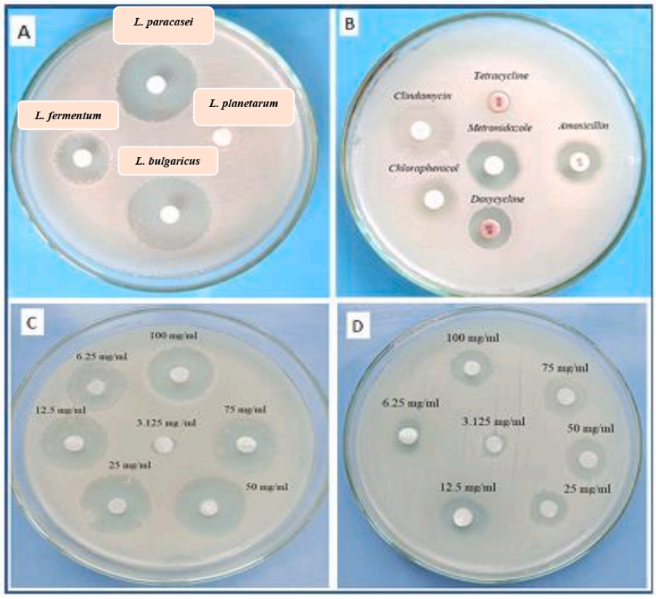

Fig. 1. Anti-clostridial effect of probiotic cell-free supernatants against *C. botulinum* type E vs. tetracycline, amoxicillin, metronidazole, doxycycline, chloramphenicol and clindamycin, and doxycycline antibiotics. **(A)**: Anti-clostridial effect of probiotic supernatants. **(B)**: Anti-clostridial effect of antibiotics. **(C)**: Minimum Inhibitory concentration (MIC) of *L. bulgaricus* EMCC 1102 supernatant against *C. botulinum* type E (3.10 mg/mL). **(D)**: Minimum Inhibitory concentration (MIC) of *L. paracasei* EMCC 1103 supernatant against *C. botulinum* type E (3.10 mg/mL).

anchovy into muscles during processing, which could provide an anaerobic condition for spore germination, cell vegetation, and toxin production (Artin, 2008). In addition, *C. botulinum* can resist a 5–10% NaCl concentration in brine (Lindström, Kiviniemi, & Korkeala, 2006).

3.2. Inhibitory effect of the probiotic supernatants on isolated *C. botulinum type E*

The inhibitory effects of the probiotic supernatants against *C. botulinum* type E are shown in Fig. 1A–D and Table 2. Fig. 1A and B showed the anti-clostridial activity of probiotic cell-free supernatants against *C. botulinum* compared to tetracycline, amoxicillin, metronidazole, doxycycline, chloramphenicol, and clindamycin antibiotics. At the same time, Fig. 1C and D explained the anti-clostridial activity of different concentrations *L. bulgaricus* EMCC 1102 supernatant vis. *L. paracasei* EMCC 1103 supernatant for evaluating their MIC. The cell-free supernatants of *L. bulgaricus* EMCC 1102 and *L. paracasei* EMCC 1103 (100 mg/mL) exhibited the maximum activity against *C. botulinum* type E with inhibition zones of 32 ± 1.15 and 36 ± 0.57 mm, respectively (Table 2). This result is significantly different ($P < 0.05$) from the inhibition zone values of antibiotics and other probiotic supernatants. Furthermore, these results (Table 2) were proved by the findings of MICs, where *L. bulgaricus* EMCC 1102 and *L. paracasei* EMCC 1103 strains demonstrated the lower MIC of 3.10 mg/mL. The results of the DDA showed that cell-free supernatants of *L. fermentum* EMCC 1346 expressed a low inhibitory action against *C. botulinum* type E with an inhibition zone value of 21 ± 0.88 mm.

In contrast, *L. plantarum* EMCC 1027 failed to exhibit any anti-clostridial activity. This finding disagrees with that obtained by Monteiro et al. (2019), who reported that cell-free supernatant of *L. plantarum* ATCC 8014 achieved the maximum inhibition zone values against *C. butyricum* (17 mm), *C. difficile* (13 mm), and *C. perfringens* (13 mm), compared with *L. bulgaricus* ATCC 9649, *L. paracasei* ATCC 335, and *L. fermentum* ATCC 23271. Several *Lactobacillus* strains can yield active metabolites with antimicrobial potentials, such as organic acids and peptides with bactericidal or bacteriostatic activities, including bacteriocins (do Carmo et al., 2018). It is observed that bacteriocins have a higher inhibitory activity against gram-positive bacteria involving *Clostridium* species than against gram-negative bacteria. The possible bactericidal or bacteriostatic activity depends on the nature of the compound generated by the *Lactobacillus* strain (Salminen, Von Wright, & Ouwehand, 2004). Concerning the maximum anti-clostridial activity, *L. bulgaricus* EMCC 1102 and *L. paracasei* EMCC 1103 cell-free supernatants were investigated for their active compounds as phenolic and flavonoids and their antioxidant activity.

3.3. Antioxidant effect of the probiotic cell-free supernatants

The antioxidant activity of the probiotic cell-free supernatants with their half-maximal inhibitory concentration (IC_{50}, mg/mL) is tabulated in Table 3. The results showed that the antioxidant activity increased in a concentration-dependent manner based on the type and concentrations of active compounds in the probiotic cell-free supernatants. *L. bulgaricus* EMCC 1102 exhibited the highest DPPH activity at all concentrations compared to *L. paracasei* EMCC 1103. The DPPH values varied among strains, and the radical scavenging potential ranged from 145.34 to 129.83% at 100 μg/mL of supernatant, 22.37 and 15.47% at 5 μg/mL of supernatant for *L. bulgaricus* EMCC 1102 and *L. paracasei* EMCC 1103, orderly. It was found that the range of DPPH radical scavenging potentials of ascorbic acid used as an antioxidant was between 37.15 and 246.53%. Furthermore, *L. bulgaricus* EMCC 1102 supernatant showed a lower half-maximal inhibitory concentration (IC_{50}) of 25.14 μg/mL than *L. paracasei* EMCC 1103 and control with IC_{50} values of 36.40 and 8.86 μg/mL, respectively. *Lactobacilli* were found to be powerful as DPPH radical scavengers (Xing et al., 2015). This finding agrees with that obtained by Kocak, Sanli, Anli, and Hayaloglu (2020), who found that the highest antioxidant activity was linked to the water-soluble extract of *L. bulgaricus* EMCC 1102 in ripened feta cheese compared to extracts of *L. paracasei* EMCC 1103 or *L. plantarum* EMCC 1027. The study of Yousefi, Dovom, Najafi, and Mortazavian (2021) investigated that the highest DPPH radical scavenging of *L. brevis* in the supernatant of ripened cheese was 31.45%. Moreover, Osuntoki and Korie (2010) found that the highest DPPH scavenging activity was observed with *L. brevis* (33.7%), followed by *L. fermentum* (30.8%) and *L. casei* (16.2%), while the minimum effect was achieved by *L. bulgaricus* (6.3%). Furthermore, Ji, Jang, and Kim (2015) revealed the DPPH radical values of 11 *Lactobacillus* sp. Supernatant was 50%, while Zhang et al. (2011) found that the DPPH radical scavenging value of *L. casei* subsp. *Casei* SY13 and *L. bulgaricus* LJJ strains were 23.99 and 27.50%, respectively. In addition, Düz et al. (2020) reported that the DPPH radical scavenging potential of *L. bulgaricus* is between 56.3 and 77.7%. Among reactive oxygen species, –OH–radical is the most reactive and it

Table 2
Anti-clostridial potentials and minimum inhibitory concentrations (MICs)* of probiotic cell free supernatants against *C. botulinum* type E.

Anti-clostridial			Minimum Inhibitory Concentrations (MICs)			
Probiotic supernatant/ antibiotics	Concentration (mg/mL)	Inhibition zone diameter (mm ± *Standards error*) **	Probiotic cell free supernatant	Concentration (mg/mL)	Inhibition zone diameter (mm)	MICs
Lactobacillus paracasei EMCC 1103	100 mg/mL	36 ± 1.15[a]	*Lactobacillus* paracasei EMCC 1103	100	36 ± 0.95[a]	3.10
Lactobacillus planetarum EMCC 1027	100 mg/mL	NZ***		50	30 ± 0.75[b]	
Lactobacillus bulgaricus EMCC 1102	100 mg/mL	32 ± 0.57[b]		25	25 ± 0.47[c]	
Lactobacillus fermentum EMCC 1346	100 mg/mL	21 ± 0.88[c]		12.5	20 ± 0.37[d]	
Tetracycline	30 mg/mL	NZ		6.25	16 ± 0.84[e]	
Amoxicillin	25 mg/mL	18 ± 0.51[d]		3.1	10 ± 0.71[f]	
Doxycycline	30 mg/mL	19 ± 0.53[c]		1.5	ND#	
Chloramphenicol	30 mg/mL	15 ± 0.50[e]	*Lactobacillus bulgaricus*	100	32 ± 0.25[a]	3.10
Clindamycin	20 mg/mL	NZ	EMCC 1102	50	26 ± 0.37[b]	
Metronidazole	50 mg/mL	20 ± 0.58[c]		25	21 ± 0.78[c]	
				12.5	15 ± 0.37[d]	
				6.25	11 ± 0.79[e]	
				3.1	8 ± 1.01[f]	
				1.5	ND	

*MICs = Minimum Inhibition Concentration.
**Data represented are the means of triplicates & [a, b, c] Data in the same column followed by different superscript letters differ significantly ($P < 0.05$).
***NZ= No Zone and # ND= Not detected.

Table 3
Antioxidant activity of probiotic cell free supernatants (IC_{50} mg/mL) using DPPH with ascorbic acid as standard.

Conc. (μg/mL)	Ascorbic acid		*Lactobacillus bulgaricus* EMCC 1102 supernatant		*Lactobacillus paracasei* EMCC 1103 supernatant	
	Inhibition (%)	IC_{50} (μg/mL)	Inhibition (%)	IC_{50} (μg/mL)	Inhibition (%)	IC_{50} (μg/mL)
5	37.15	8.86	22.37	25.14	15.47	36.40
10	56.43		36.82		21.51	
20	72.82		47.15		33.36	
30	85.64		59.66		42.62	
40	99.71		68.59		54.93	
50	125,63		82.14		67.72	
60	152.57		97.23		86.13	
70	176.38		106.51		97.54	
80	192.43		118.26		105.17	
90	219.14		131.63		119.34	
100	246.53		145.34		129.83	

can react with all bio macromolecules resulting in severe damage to the adjacent macro-molecules. It reacts with polyunsaturated fats of cell membrane phospholipids and causes cell damage. The neutral exopolysaccharide which isolated from *L. plantarum* had a great scavenging capability on the radicals (Zhang et al., 2013). In addition, the different concentrations of probiotic species of *Lactobacillus* sp. And *Bifidobacterium* sp. exhibited a strong radical scavenging activity (Venkatesan, Kirithika, Roselin, Ganesan, & Muthuchelian, 2012).

3.4. Phenolic and flavonoid compounds of the probiotic supernatants

The findings in Table 4 showed that the supernatant from *L. bulgaricus* EMCC 1102 had a higher total phenolics and flavonoids content than the supernatant from *L. paracasei* EMCC 1103. The antioxidant activity of *L. bulgaricus* EMCC 1102 can be attributed to the higher phenolic and flavonoid content of 0.583 ± 0.002 and 0.151 ± 0.001 mg/mL, respectively. Similarly, the total phenolic and flavonoid contents in the cell-free supernatant of *L. paracasei* EMCC 1103 were 0.468 ± 0.001 and 0.143 ± 0.001 mg/mL, respectively, with no significance ($P > 0.05$) among the tested supernatants. *Lactobacillus* strains exhibited a potential to scavenge DPPH radicals based on the phenolic and flavonoid compounds (Talib et al., 2019). This study observed that probiotic strains and their supernatants that were evaluated *in vitro* had a great antioxidant activity against DPPH radicals.

Table 4
Total phenolic and flavonoid compounds (mg/mL) and their profile using HPLC (μg/mL) of probiotic cell free supernatants.

Items	*Lactobacillus bulgaricus* EMCC 1102 supernatant	*Lactobacillus paracasei* EMCC 1103 supernatant
Total phenolics (mg/mL)	0.583 ± 0.002[a]	0.468 ± 0.001[a]
Total flavonoids (mg/mL)	0.151 ± 0.001[a]	0.143 ± 0.001[a]
Phenolic and flavonoid compounds (μg/mL)		
Gallic acid	4973.32 ± 1.22[a]	4117.89 ± 2.38[b]
Chlorogenic acid	347.49 ± 2.19[b]	1211.53 ± 0.89[a]
Catechin	76.61 ± 1.40[b]	200.06 ± 1.88[a]
Methyl gallate	99.84 ± 0.79[b]	105.36 ± 0.45[a]
Caffeic acid	335.98 ± 0.87[a]	185.81 ± 1.77[b]
Syringic acid	107.88 ± 1.21[a]	38.62 ± 0.99[b]
Pyro catechol	ND*	ND
Rutin	ND	12.51 ± 0.89[a]
Ellagic acid	7.62 ± 0.33[a]	6.67 ± 0.47[a]
Coumaric acid	7.28 ± 0.19[a]	5.26 ± 1.21[a]
Vanillin	ND	ND
Ferulic acid	ND	ND
Naringenin	436.54 ± 1.73[a]	179.36 ± 0.39[b]
Taxifolin	147.48 ± 1.59[a]	ND
Cinnamic acid	ND	3.29 ± 0.89
Kaempferol	ND	ND

*ND: Not detected.
[a, b] Data in the same row followed by different superscript letters differ significantly ($P < 0.05$).

The HPLC results in Table 4 displayed that the portfolio of phenolic and flavonoid compounds for *L. bulgaricus* EMCC 1102 and *L. paracasei* EMCC 1103 supernatants contained a variety of active compounds, in which gallic and chlorogenic acids have been peaked. Accordingly, gallic acid, caffeic acid, syringic acid, and naringenin contents were 4973.32 ± 1.22, 335.98 ± 0.87, 107.88 ± 1.21, and 436.541.73 μg/mL, respectively, in the cell-free supernatant of *L. bulgaricus* EMCC 1102, which were higher than those of *L. paracasei* EMCC 1103 supernatant. On the other hand, the contents of chlorogenic acid, catechin, and methyl gallate were 1211.53 ± 0.89, 200.06 ± 1.88, and 105.36 ± 0.45 μg/mL, respectively, in the cell-free supernatant of *L. paracasei* EMCC 1103, which was greater than the supernatant of *L. bulgaricus* EMCC 1102. Some undetectable compounds in the examined supernatants are pyrocatechol, vanillin, ferulic acid, and kaempferol. Rutin and cinnamic acid failed to be detected in the cell-free supernatant of *L. bulgaricus* EMCC 1102, while taxifolin was undetectable in the cell-free supernatant of *L. paracasei* EMCC 1103. Previous studies have shown that probiotics can be used *in vitro* to control *Clostridium* species (Hamad et al., 2020; Monteiro et al., 2019). These different results could be due to differences in the *Lactobacillus* species and the enzymatic activities among the examined strains, which have been displayed to produce various types and concentrations of phenolic acids based on the microorganisms (Barthelmebs, Diviès, & Cavin, 2001). It has been observed that *Lactobacilli* have different abilities to produce and assimilate phenolic and flavonoid compounds depending on the strain type; thereby the increase in phenolic and flavonoid compounds is consequently associated with the increase in their antioxidant potential (Rupasinghe, Parmar, & Neir, 2019). Other variations in phenolic compounds, including interactions with other dietary elements and changing chemical composition or solubility, may also occur (Pavez-Guajardo et al., 2020). Based on its antimicrobial activity against *C. botulinum* type E, *L. bulgaricus* EMCC 1102 was the most promising strain among the probiotic strains examined in this study. This can be attributed to the antimicrobial metabolites produced by such strains.

3.5. Inhibitory and bio-preservation effect of the L. bulgaricus EMCC 1102 supernatant against C. botulinum type E in anchovy fillets

The inhibitory activity of *L. bulgaricus* EMCC 1102 supernatant against *C. botulinum* type E in anchovy fillets during 15 d of cold storage is described in Fig. 2 with the packaging image provided in Fig. 2S for control and treated anchovy fillets. The results showed that the counting value of injected *C. botulinum* in all samples started at 4.0 $\log_{10}$ CFU/g and significantly increased to 8.5 $\log_{10}$ CFU/g on the 15th d of cold storage in untreated anchovies. Based on the DDA results, *L. bulgaricus* EMCC 1102 supernatant was used to control *C. botulinum* type E in anchovy fillets. The results also show that cell-free supernatant from *L. bulgaricus* EMCC 1102 can be used to treat anchovy fillets that have

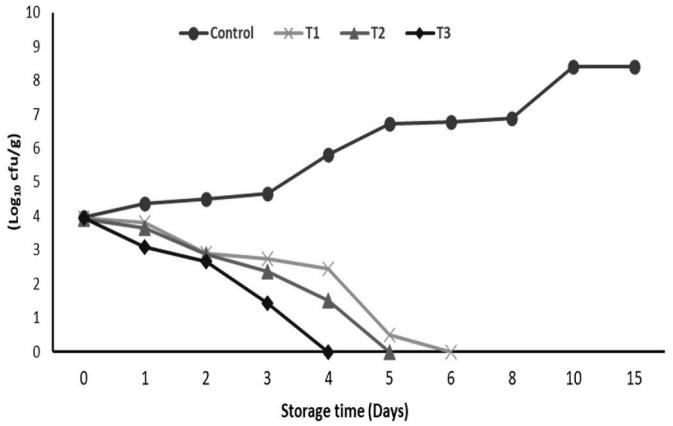

Fig. 2. Inhibitory effect of *L. bulgaricus* EMCC 1102 cell free supernatant against *C. botulinum* type E in stored anchovy fillets. Control = samples without addition of supernatant; T1 = samples treated with 25 mg/g supernatant; T2 = samples treated with 50 mg/g supernatant and T3 = samples treated with 100 mg/g supernatant.

been artificially contaminated with *C. botulinum* type E at different concentrations, and the percentage of inhibition is a concentration-dependent.

The initial *C. botulinum* type E count in anchovy fillets, at zero time, was 3.97 ± 0.005 $\log_{10}$ CFU/g. However, the count reached 6.71 ± 0.008 Log_{10} CFU/g after 5 d of incubation of anchovy fillets in control samples. Samples treated with *L. bulgaricus* EMCC 1102 supernatant showed extremely low (in samples treated with 25 mg/g (T1) supernatant) or no counts (in samples treated with 50 mg/g (T2) or 100 mg/g (T3) supernatant). Treatment T3 revealed the greatest results, with a considerable elimination of *C. botulinum* type E counting values compared with control, T1, and T2. Comparable findings of the inhibitory activity of *Lactobacillus* species, including *L. bulgaricus*, against clostridial species were investigated early (De Vuyst et al., 2004; Golić et al., 2017). Concerning the manufacturing conditions implemented in this study, we found that *C. botulinum* type E was completely inhibited by increasing the total phenols and flavonoids' average content after adjusting the pH at 2.32. In addition, the complete elimination of *C. botulinum* type E may be attributed to the active compounds of the supernatants, which can enhance the release of phenolic and flavonoid compounds related to fibers and other elements of the food matrix, including anchovy fillets. Similarly, an extra work by Vivek, Mishra, Pradhan, and Jayabalan (2019) reported that the total phenolics, flavonoids, and DPPH radical scavenging potential of Sohiong juice increased in the presence of *L. plantarum* and its metabolites. Based on the marinating procedure of the anchovy fillets, we could indicate that the elevated level of 10% NaCl and the existence of 2% acetic acid may be have an inhibitory activity against many microorganisms, including *C. botulinum*, as previously reported (Gökoglu, Cengiz, & Yerlikaya, 2004; Sen & Temelli, 2003). Regarding the storage condition of anchovy fillets applied in this study, we found that treatments T1, T2, and T3 completely eliminated *C. botulinum* type E within 4, 5, and 6 d of the cold storage, respectively. To avoid the production of botulinic toxins by *C. botulinum* type E, the FDA recommends refrigerated storage (4 °C or less), salting (not less than 5%), water activity (below 0.97), and pH (below 5) for salted fishery products, including anchovy fillets (FDA, 2001). *C. botulinum* type E and other clostridial types can grow less when these changes are made during storage and distribution.

Control of *C. botulinum* type E is a critically important due to food safety aspects. The results of our study indicated that among the studied probiotic strains, *L. bulgaricus* EMCC 1102 showed the potential as a biological control agent against *C. botulinum* type E in anchovy fillets, which could be explained by the high content and the diversity of active compounds produced by this strain.

4. Conclusions

To sum up, this study highlighted the high occurrence of *C. botulinum* in the examined fish products, which is a significant health risk. The stored anchovy fillets were *C. botulinum* type E -free within 6 d at 100 mg/g concentration of *L. bulgaricus* EMCC 1102 cell-free supernatants. Cell-free supernatants from all tests exhibited inhibitory and antioxidant activities on *C. botulinum* type E. Consequently, these probiotic cell-free supernatants can be suggested for decontamination with *C. botulinum* and prevent neurotoxin production, ensuring food safety. Finally, the use of probiotic cell-free supernatants can help fight off *C. botulinum* and as bio-preservation agents to keep fish products fresh.

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CRediT authorship contribution statement

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Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2022.113603.

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