Middle East Journal of Science

https://dergipark.org.tr/mejs **MEJS**

e-ISSN:2618-6136

Research Article

ASSESSING THE POTENTIAL PUBLIC HEALTH RISK OF *Clostridium botulinum* TOXIN GENES IN CANNED FOOD: A LABORATORY EXPERIENCE IN TÜRKİYE

Hisamettin DURMAZ [1] ***Akın YİGİN*** [2] ***Serap Kılıç ALTUN*** [1] ***Mehmet Emin AYDEMİR*** [1] ***

[1]Dep. of Food Hygiene and Technology, Faculty of Veterinary Medicine, Harran University, Sanlıurfa, Türkiye
[2]Department of Genetics, Faculty of Veterinary Medicine, Harran University, Sanlıurfa, Türkiye.
*Corresponding author: aydemiremin23@harran.edu.tr

Abstract*: Due to the ability to synthesize a highly potent neurotoxin, Clostridium botulinum (C. botulinum) poses an important risk to food safety. Foodborne botulism is a neuroparalytic disease caused by the ingestion of neurotoxins produced by C. botulinum. Botulism commonly occurs as a result of consuming canned foods. In this study, the aim was to investigate the presence of C. botulinum and detect the genes producing A, B, E, and F toxins in C. botulinum isolates in homemade and commercial canned foods using the rapid, sensitive, and reliable Real-Time PCR technique. A total of 81 canned samples were collected, including 51 commercial and 30 homemade cans. The canned samples were initially enriched in a tryptone peptone glucose yeast (TPGY) medium. Subsequently, DNA isolation was performed using the foodproof® StarPrep Two Kit. After the isolation process, Real-Time PCR was conducted using the foodproof® C. botulinum Detection LyoKit and 5' Nuclease (Biotecon, R60240) kit. Among all samples, only 2 samples were positive. Both positive samples were found to contain neurotoxin type A, were commercially canned foods, and were packaged in glass containers. It was also determined that one was a mixed vegetable side dish and the other was roasted red pepper. Consequently, it was concluded that commercially available canned foods in Türkiye could still pose a potential risk to public health due to the presence of C. botulinum. Therefore, it was recommended to perform quality control analyses through food inspections in companies involved in canned food production and sales.*

Keywords: *Canned food, Clostridium botulinum, BoNT, Real-time PCR*

Received: February 16, 2024 *Accepted: June 5, 2024*

1. Introduction

Since *Clostridium botulinum* (*C. botulinum*) is a spore-forming bacterium that thrives in low-oxygen settings, canned foods provide the ideal conditions for it to flourish and produce toxins. When consumed, the bacterium's powerful neurotoxin can cause severe neurological symptoms, paralysis, and, in severe cases, respiratory failure. Botulism is a relatively rare neuroparalytic disease that poses a life-threatening risk to humans and animals. It is caused by botulinum neurotoxins (BoNTs) produced by organisms belonging to the *Clostridium* genus [1]. BoNTs are among the most potent toxins known, with lethal doses as low as 30-100 ng. Botulism, which develops through the consumption of food contaminated with neurotoxin, remains a serious type of food poisoning that can result in death [2]. The disease occurs when a food containing the toxin of one or more of the seven immunological types (A-

G) of *C. botulinum* is consumed [3]. It is known that botulism cases in humans are primarily caused by types A, B, E, and F [4].

In botulism cases, early symptoms typically include fatigue, weakness, nausea, vomiting, diarrhea, and dizziness. These symptoms are followed by manifestations such as blurred vision, mydriasis, ptosis, dry mouth, difficulty swallowing and speaking, bladder atony, and constipation. In progressive stages, the disease can lead to muscle weakness in the limbs, respiratory paralysis, and death [4, 5]. The responsible foods are usually products that undergo certain treatments for preservation, such as canning, pickling, and smoking, but where the elimination of *C. botulinum* spores is not guaranteed. When the preservation method applied to the food is inadequate, and it continues to be stored under conditions that allow the growth of *C. botulinum*, this contaminated food can harbor *C. botulinum* toxin, one of the deadliest toxins known to humans [6].

Canned foods have long been considered a convenient and popular choice for consumers due to their extended shelf life and ease of storage. However, concerns regarding the presence of *C. botulinum*, the bacterium responsible for causing botulism, have raised questions about the safety of these products. Botulism is a severe and potentially life-threatening form of food poisoning, primarily associated with improperly processed or stored canned goods.

In the early years of commercial canning, many problems related to *C. botulinum* existed, but intensive studies on processing procedures resulted in significant improvements in the 1920s and 1930s [7]. However, cases of foodborne botulism still occur today. The frequency of these cases varies depending on countries' food preferences, food preparation methods, food safety regulations, and even the available treatment options [8, 9]. Although botulism has been reported to arise from commercial canned foods, the majority of cases recorded since 1899 have been attributed to home-canned foods [10, 11]. However, since home-canned foods are often produced in rural areas and are not well-documented, the full extent of the problem associated with home canning is not known.

In developed countries, the presence and distribution of bacteria and toxins in high-risk foods are regularly monitored, and a reliable database is established to shed light on their development and support epidemiological studies, leading to intensified measures for control. However, it is notable that in Türkiye, comprehensive studies on this subject are limited, mainly consisting of case reports. A study by Güran and Öksüztepe [4] revealed that *C. botulinum* requires an acidic pH level, usually above 4.6, to produce toxins. According to initial research by Stumbo et al. [7], several *C. botulinum*-related concerns were seen in the early years of using tin cans for commercial canning.

The necessity to consider public health hazards beyond conventional canning methods is highlighted by the latest results in our study, which show positive samples in glass jars, demonstrating that the potential threat extends not only to canned items but also to canned products packaged in glass containers. The link between botulism cases and vegetable canning has been supported by a number of Turkish studies. According to Karsen et al. [2], green bean canning was a factor in the majority of Türkiye's botulism incidents between 1983 and 2017. In a comparable way, all botulism cases documented in Türkiye from 2017 to 2021 were linked to vegetable preserves, according to Bıcakcıoglu et al. [12], Daşkaya and Pek [5], and Botan et al. [8]. The precise type of neurotoxin that caused these cases, however, was not routinely recorded.

Between 2008 and 2019, 55 cases of foodborne botulism were reported in Iran by Soltan et al. [13]; 19 of these cases were connected to Type A neurotoxin. It is crucial to educate canning businesses and retailers about the possible risk of *C. botulinum* infection in canned foods to address this worrying issue. Rigorous quality control procedures must be put in place, including routine food inspections and adherence to strict hygiene standards throughout the production process. Additionally, teaching people about the proper handling, storage, and consumption of canned products helps reduce the number of instances of botulism. We intend to provide useful insights into the potential public health dangers

associated with *C. botulinum* in this study by conducting it and building on previous literature. Studies conducted in other countries also support our findings. According to the Centers for Disease Control and Prevention [9], Type A neurotoxin was the primary cause of the majority of botulism occurrences in the United States during 2016 and 2017. Therefore, the aim of this study is to use the rapid, sensitive, and reliable Real-Time PCR technique to detect the presence of *C. botulinum* in homemade and commercial canned foods and to identify the A, B, E, and F toxin-producing genes in positive samples.

2. Materials And Methods

2.1. Sample collection

In this study, 51 commercial canned foods and 30 homemade canned foods were used as study material. The commercial canned foods were obtained from national supermarkets located in Şanlıurfa province, while the homemade canned foods were sourced from various regions of Türkiye. For commercial canned food collection, at least one sample was obtained from each brand sold in chain markets throughout Türkiye. Homemade canned foods were collected with at least one sample from regions where canned food production is predominant in Türkiye. The main criteria for selecting samples were that commercial canned foods had no expiration date when purchased, and that homemade canned foods did not have a production date of more than 6 months. Information regarding the collected samples is provided in Table 1.

Ethical Statement

The laboratory was used as material in the study, ethics committee permission is not required.

Table 1. Details of canned food samples

Type of Can	Can content	Number of Cans (pieces)	Can Material	City (Number of samples)
Commercial Cans	Garniture	4	Glass jar	Şanlıurfa
	Kidney bean	4	Tin	
	Roasted Eggplant	3	Glass jar	
	Boiled chickpeas	4	Tin	
	Tomatoes	4	Tin	
	Boiled fresh Bean	4	Tin	
	Okra	3	Glass jar	
	Mushroom	2	Glass jar	
	Roasted red pepper	3	Glass jar	
	Pea	5	Glass jar	
	Pea	3	Tin	
	Boiled dry bean	2	Tin	
	Sweetcorn	10	Tin	

Table 1. Continued

Type of Can	Can content	Number of Cans (pieces)	Can Material	City (Number of samples)
Home-made Cans	Mixed tomatoes and peppers	8	Glass jar	Elazığ (3) Malatya (1) Diyarbakır (1) Şanlıurfa (1) Gaziantep (1) Adıyaman (1)
	Tomatoes	8	Glass jar	Elazığ (3) Adana (1) Samsun (1) Şanlıurfa (1) Balıkesir (1) Adıyaman (1)
	Boiled fresh beans	5	Glass jar	Mardin (1) Sivas (2) Kayseri (2)
	Mixed Tomato Bean	5	Glass jar	Ankara (2) Elazığ (2) Erzurum (1)
	Mixed Roasted Eggplant and Capia Pepper	4	Glass jar	Şanlıurfa (2) Adıyaman (1) Mardin (1)
Total		81		

2.2. Enrichment of samples

Before performing PCR, an enrichment process was conducted on the canned samples. 1 mL of the homogenized canned samples was taken and added to Falcon tubes containing 9 mL tryptone peptone glucose yeast (TPGY) broth (Sigma-Aldrich). The tubes were then incubated under anaerobic conditions at 30°C for 96 hours. After the enrichment process, DNA isolation was performed.

2.3. DNA isolation

For DNA isolation, the manual method using the foodproof® StarPrep Two Kit - bulk purification (Biotecon, Catalog No. S40008) commercial kit was employed. Two hundred microliters (200 µL) of liquid or homogenized material from the pre-enriched canned samples were used for DNA isolation. Initially, the elution solution was heated to +70°C, and 200 µL of the sample material was added to a nuclease-free 1.5 mL microcentrifuge tube, along with 200 µL of Binding Buffer and 40 µL of Proteinase K. The mixture was incubated at +70°C for 10 minutes and then thoroughly mixed with the addition of 100 µL of isopropanol. Subsequently, the mixture was transferred to a filter collection tube. In the next step, the filter tube was centrifuged at 8,000 × g for 1 minute. After centrifugation, the bottom collection tube was discarded, and a new collection tube was placed under the filter tube. Five hundred microliters (500 µL) of inhibitor removal solution was added, and the tube was centrifuged at 8,000 × g for 1 minute. The discarded collection tube was replaced with a new one. The same process was repeated by adding 500 µL of wash solution and centrifuging at 8,000 × g for 1 minute. After repeating the wash step with 500 µL of wash solution, the tube was centrifuged at maximum speed for 10 seconds. The elution solution (previously heated) was then distributed to the samples in the microcentrifuge tube (200 µL). The tube was centrifuged at 8,000 × g for 1 minute. Subsequently, the filter tube was discarded, and the accumulated DNA was stored at -20°C in the collection tube.

2.4. Real-time PCR analysis

For the target bacterium *C. botulinum*, the foodproof® *C. botulinum* Detection LyoKit, 5' Nuclease (Biotecon, Catalog No. R60240) kit was utilized. This commercially available kit is capable of detecting various *Clostridium spp*., including *C. sporogenes*, *C. botulinum*, and *C. butyricum*, along with toxin genes A, B, E, and F (Table 2).

Table 2. Real-Time PCR mix (master mix)

Chemicals	**µL**
PCR-pure ddH2O	25 µL
foodproof® *C. botulinum* Detection LyoKit	25 µL
foodproof® *C. botulinum* Detection Control Template (PK)	25 µL
Isolated DNA	25 µL
Total	**100 µL**

The melt curves observed in the real-time PCR monitor are presented in Figure 1. The reaction mixture was prepared in 100 µL tubes, and the neurotoxins were detected using the Real-Time PCR instrument (Qiagen RotorGene Q, USA) (Table 3). The sample, positive control (PK), and negative controls (ddH2O- 25 µL) were prepared in the same manner.

Table 3. Real-Time PCR Protocol

Programs	**Denaturation**	**Cycle**	**Melt Curve**	**Cooling**
Analysis Mode	-	Quantitation Mode	Melt Curve Mode	-
Cycle	1	50	1	
Target (ºC)	95	95 60	95 37	40
Condition (ss:dd:sn)	00:04:00	00:05:00	00:00:05	00:00:50
Acceleration (°C/sn)	20	20	20	0.2
Reading Mode	-	Single	Continuous	--

2.5. Melting curve analysis (Tm)

Melting Curve analysis was employed to identify the A, B, E, and F neurotoxins (refer to Table 4). Fluorescence emission was monitored for 10 seconds at the end of each binding cycle. In case the sample tested positive, the amplified products underwent melting curve analysis (Tm). Denaturation was conducted at 95°C for 50 seconds, followed by annealing at 37°C for 20 seconds. Subsequently, the temperature was gradually increased at a rate of 0.2°C per second until reaching 85°C, while continuously collecting and recording fluorescence measurements. This process facilitated the determination of the presence of A, B, E, and F neurotoxins in the DNA of the positive samples.

3. Results and Discussion

The amplification curves (Cp values) of all samples (n=81), along with the corresponding positive and negative results are illustrated in Figure 1.

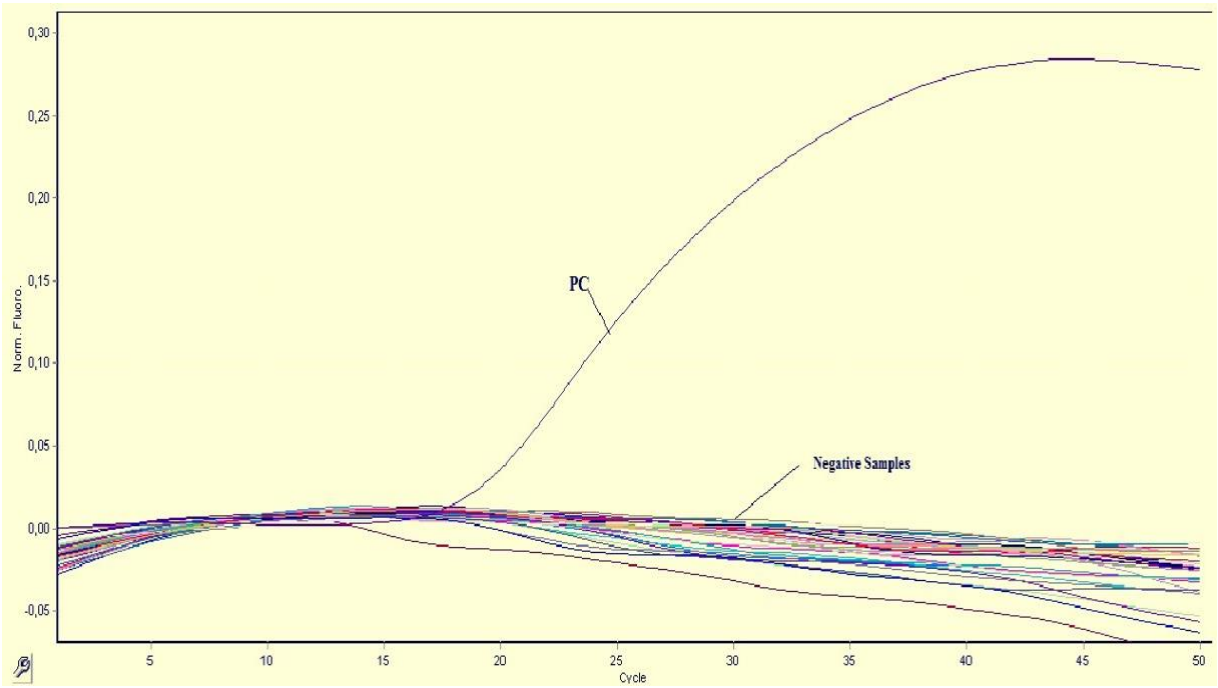

Figure 1. Re-run of negative samples and positive control (PC)

Figure 2 shows the amplification curves of the positive samples. Only two of the samples tested were positive for C. botulinum. The neurotoxin species of the positive samples was identified as BoNT A. One of these samples was commercial garnish canned and the other was a commercial roasted red pepper canned and in both cases, glass was the type of can used.

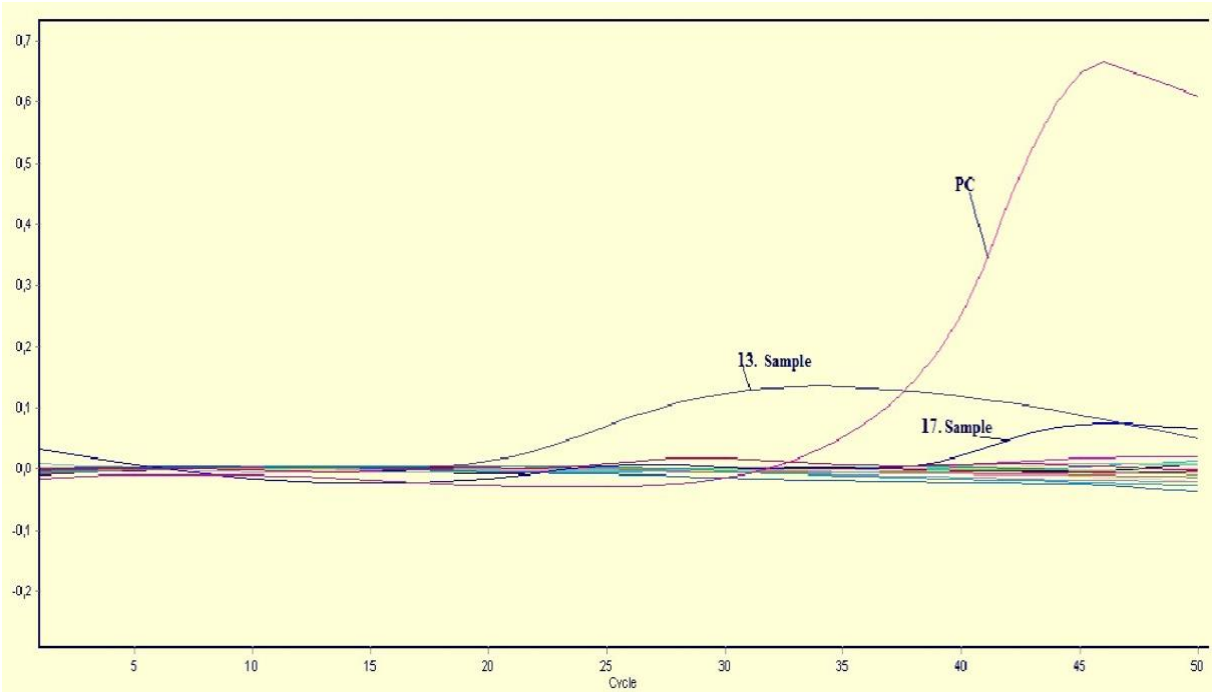

Figure 2. Positive samples and Positive control (PC)

The amplification curves of the Internal controls (IC) are presented in Figure 3 to demonstrate the accuracy of the PCR studies for all working samples.

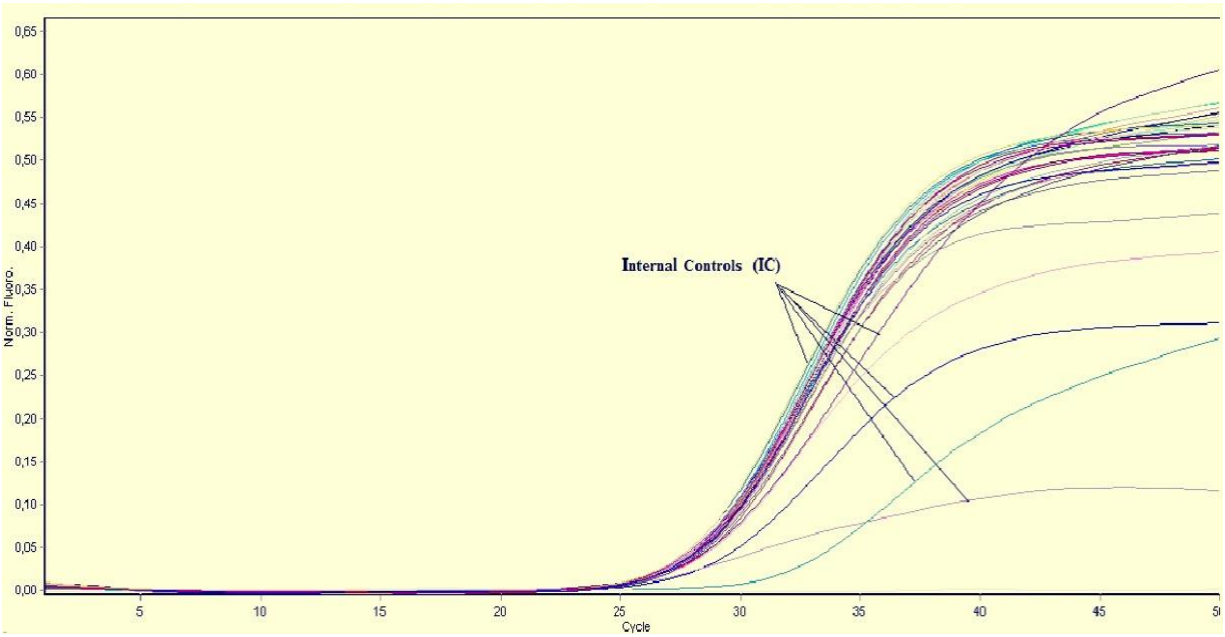

Figure 3. Internal controls (IC)

It was observed that all samples ran smoothly. Upon examining the results of the melting curve analysis, as intended, the melting curve of the positive control at 64.0°C ± 2.0°C in the FAM channel and 55.0°C ± 2.0°C and 62.5°C ± 2.0°C in the HEX channel is depicted in Figure 4 and Figure 5.

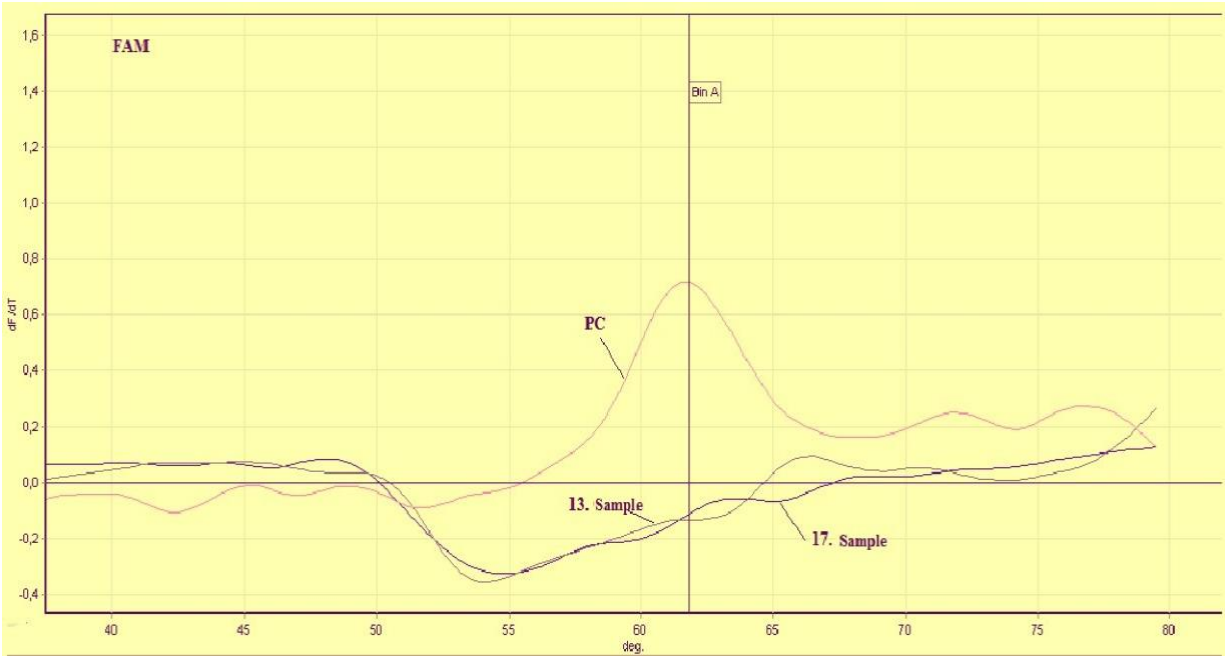

Figure 4. Melting curve analyzes of the FAM channel of positive control (64 °C ± 2 °C)

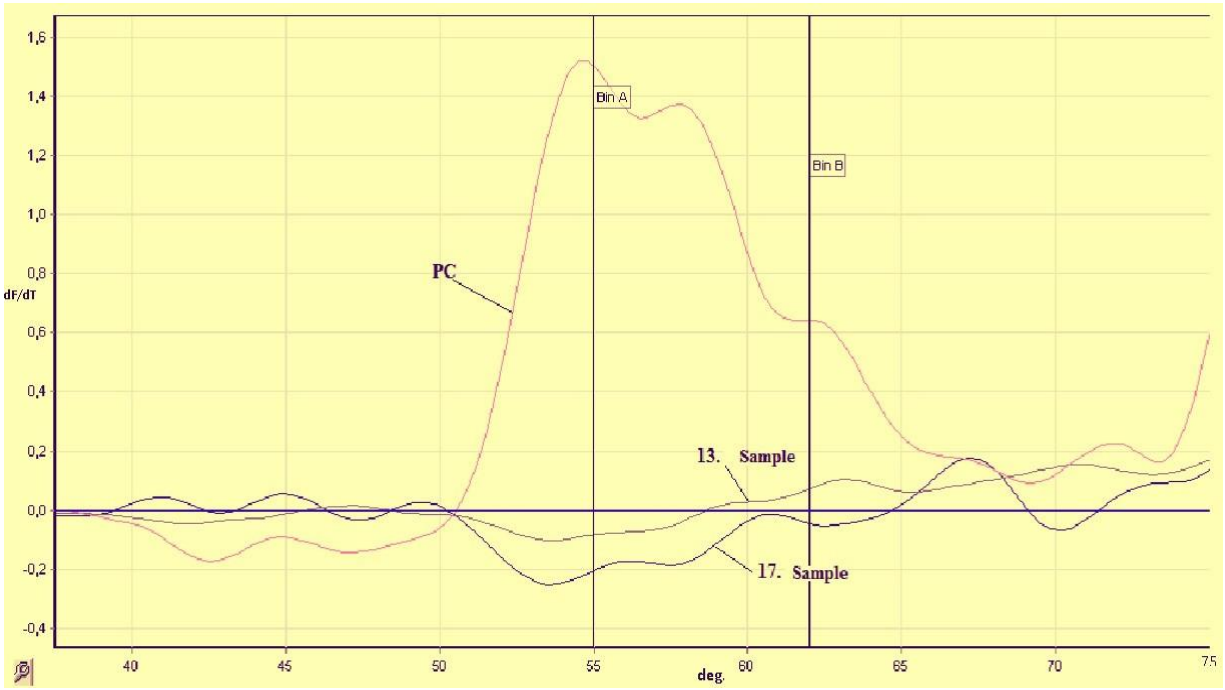

Figure 5. Melting curve analyzes of the HEX channel of positive control (55 °C ± 2 °C and 62.5 °C ± 2 °C)

Upon re-examining the positive samples, Real-time PCR analysis was conducted before and after enrichment, revealing no amplification in the samples before enrichment. After enrichment, both samples were found to be positive again (Figure 6).

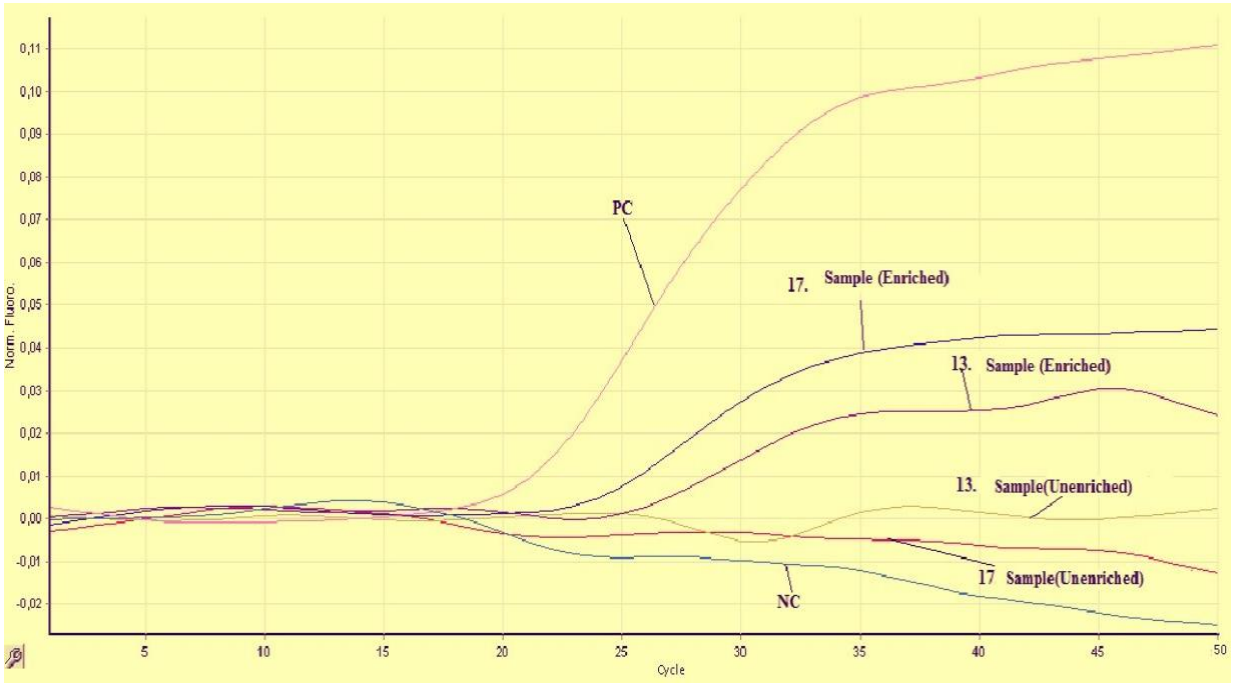

Figure 6. Real-time PCR results of positive samples (before and after enrichment)

C. botulinum spores are commonly found in nature. As a soil-borne bacterium, this pathogen naturally occurs in many fruits and vegetables. Canned foods made from contaminated fruits and

vegetables play a significant role in the production and transmission of the toxin produced by this pathogen. To prevent human exposure to *C. botulinum* and ensure effective canning, it is necessary to prevent the contamination of raw foods with *C. botulinum* and to perform the canning processes properly [14].

Despite the parameters for canning being established and implemented many years ago, botulism cases are still reported. In 2014, the European Centre for Disease Prevention and Control reported 91 cases of botulism from 16 European Union countries, mostly in Romania, Poland, and Hungary [15]. In the United States, it was reported that 245 people were diagnosed with botulism in 2016, with 29 cases being foodborne; in 2017, 182 people were diagnosed with botulism, with 19 cases being foodborne; and in 2018, 231 people were diagnosed with botulism, with 18 cases being foodborne [9]. In Romania, between 2012 and 2018, 130 cases of foodborne botulism were reported [16]. In France, the annual incidence of botulism from 2010 to 2016 was reported as 17.4 cases per year, with foodborne botulism being the main cause [17]. In a hospital in Tehran, Iran, between 2008 and 2019, 61 cases of botulism were diagnosed, with 55 cases being foodborne [13]. Karsen et al. [2] reported 95 cases of foodborne botulism in Türkiye between 1983 and 2017, with 18 deaths. These findings support the idea that canned products still pose a public health risk for botulism.

In a study conducted in northern Italy between 2013 and 2020, 2187 food samples were collected, and the presence of *C. botulinum* toxin was investigated using multiplex real-time PCR. The toxin was detected in 16 samples, most of which were industrial products [18]. This study provides evidence that not only homemade canned foods but also commercial canned products may pose a risk to public health. Especially the detection of BoNT A genes in positive samples makes this situation even more risky. The presence of positive samples in commercial products is thought to be due to a failure to comply with the necessary parameters during the canning and storage stages. In contrast to our study, Davidson et al. [19] conducted a microbiological analysis of swollen cans and reported no detection of toxigenic strains. This discrepancy may be attributed to insufficient heat treatment of the products or post-processing contamination. The pathogen can withstand high temperatures and different environmental conditions and can cause intoxication through food [20]. It has been reported that consumption of commercial foods, which are the main cause of foodborne botulism, after the expiry date and storage at room temperature rather than refrigeration increase the risk of poisoning [11]. In the present study, although the positive samples were not problematic in terms of expiry date, the fact that they were stored at room temperature poses a potential risk to public health. This is because, depending on the storage conditions, the toxin level will increase until the canned food is consumed, causing more severe poisoning.

The absence of positive samples in homemade canned foods was surprising. This may be attributed to inadequate hermetic sealing during the production stage. Toxin production by bacteria requires the presence of a suitable anaerobic environment [21]. Additionally, the examined homemade canned foods in this study were mainly tomato-based, which may also contribute to the absence of positive samples. Tomato-based canned foods have low pH values, and pH is crucial for toxin production by the pathogen. The pH should be non-acidic (above 4.6) for the pathogen to produce the toxin [4]. For many acidic foods, including fermented and acidified vegetables, salsas, salad dressings, and others, keeping the pH below 4.6 is a critical control to prevent botulism [22]. It has been reported that there were many problems related to *C. botulinum* in the early years of commercial canned production using tin cans [7]. However, in our study, it is observed that both positive samples were found in glass jars. The findings of this study indicate that not only canned foods in tin cans but also those in glass jars may pose a risk to public health.

It is seen that one of the positive samples in the study was mixed vegetable canned food, and the other was roasted red pepper. Karsen et al. [2] reported that most cases of botulism in Türkiye between

1983 and 2017 were caused by green bean canned foods. This shows that canned vegetables sold and consumed in Turkey pose a risk for *C. botulinum.*

Considering the potential use of BoNTs as a biological weapon, foodborne botulism can still be considered an emerging risk disease. The detection of BoNT A genes in positive samples indicates that canned foods in Turkey still pose a risk to public health. However, heating can also inactivate BoNTs, depending on various factors such as medium composition, strain type, process temperature, etc. Different types of BoNT have different heat stability and some can even survive normal pasteurisation [23]. Rasooly and Do [24] reported that low-temperature pasteurisation of milk (63 °C, 30 min) inactivated BoNT of serotype A but not serotype B. Therefore, heat treatment of canned foods to inactivate BoNTs before consumption may reduce the risk in these products.

4. Conclusion

In conclusion, our study highlights the potential danger to public health posed by commercially available canned foods in Türkiye, as they may contain *C. botulinum*. This finding emphasizes the need for stringent quality control measures through regular food inspections in both canning companies and retail markets. It is crucial to prioritize effective hygiene practices and provide comprehensive education to producers and consumers regarding canning production procedures. It is crucial for canning businesses to implement and follow stringent hygiene measures throughout the whole production process in order to prevent food contamination and reduce the danger of foodborne botulism. This entails meticulous pH level monitoring, careful handling of chemicals, and extensive equipment cleanliness to prevent the growth of *C. botulinum*. Additionally, educating consumers about the possible dangers of canned foods and giving them instructions on how to handle, store, and consume them safely can make a big difference in reducing the number of cases of botulism. The right storage conditions, expiration dates, and the significance of checking cans for damage or bulging before consumption should all be explained to consumers. To ensure adherence to hygienic and safety standards, government regulatory bodies, food safety organizations, and public health authorities should work closely with canning businesses. To confirm that best practices are being followed and to quickly identify and address any possible problems, routine inspections and audits should be carried out. Prioritizing these actions will help canned food products be safer overall and of higher quality, reducing the risk of foodborne botulism and preserving the general public's health.

Ethical Statement

The laboratory was used as material in the study, ethics committee permission is not required.

Acknowledgments

This work was financially supported by the Harran University Scientific Research Projects Unit.

Compliance with the Research and Publication Ethics

This study was carried out in accordance with the rules of research and publication ethics.

Conflict of Interest

The authors declare no conflict of interest.

Financial Disclosure

Harran University Scientific Research Projects Unit

Author Contribution

H.D: Writing - Original draft preparation

A.Y., S.K.A., M.E.A: Methodology
A.Y., M.E.A: Formal analysis, Writing
S.K.A., M.E.A: Conceptualization, Methodology
H.D., S.K.A: Investigation
Resources All authors read and approved the final manuscript.

References

[1] Scalfaro, C., Auricchio, B., De Medici, D., Anniballi, F., "Foodborne botulism: an evolving public health challenge", *Infectious Diseases*, 51(2), 97-101, 2019. https://doi.org/10.1080/23744235.2018.1524584

[2] Karsen, H., Ceylan, M.R., Bayındır, H., Akdeniz, H., "Foodborne botulism in Turkey, 1983 to 2017", *Infectious Diseases*, 51(2), 91-96, 2019. https://doi.org/10.1080/23744235.2018.1524582

[3] Kanaan, M. H. G., Tarek, A. M., "Clostridium botulinum, a foodborne pathogen and its impact on public health", *Annals of Tropical Medicine and Public Health*, 23, 49-62, 2020. https://doi.org/10.36295/ASRO.2020.2357

[4] Güran, H.Ş., Öksüztepe, G., "Gıda kaynaklı botulizm ve önemi", *Fırat Üniversitesi Sağlık Bilimleri Veteriner Dergisi*, 26(3), 191-195, 2012.

[5] Daşkaya, H., İpek, H., "First case of botulism type A in Turkey", *Turkish Journal of Medical Sciences*, 49(4), 1349-1351, 2019.

[6] Rawson, A. M., Dempster, A. W., Humphreys, C. M., Minton, N. P., "Pathogenicity and virulence of *Clostridium botulinum", Virulence*, 14(1), 2205251, 2023. https://doi.org/10.1080/21505594.2023.2205251

[7] Stumbo, C. R., Gross, C. E., Vinton, C., "Bacteriological studies relating to thermal processing of canned meats", *Food Research*, 10, 260-272, 1945.

[8] Botan, Y., Öztürk, E., Şahin, O. T., Uğurlu, G., "An outbreak of foodborne botulism due to consumption of homemade roasted eggplant paste in Turkey", *Foodborne Pathogens and Disease*, 18(2), 118-121, 2021.

[9] CDC., "Botulism in the United States, 2016–2018", *Morbidity and Mortality Weekly Report*, 69(34), 1150-1154, 2020.

[10] Sofos, J. N., "Botulism in Home-Processed Foods", *In Food Poisoning, Routledge*, 171-203, 2019.

[11] Rasetti-Escargueil, C., Lemichez, E., Popoff, M. R. "Public health risk associated with botulism as foodborne zoonoses" *Toxins*, 12(1), 17, 2019. https://doi.org/10.3390/toxins12010017

[12] Bıcakcıoglu, M., Sırıken, B., Çelik, M., İnce, E., Ener, B., "Three clustered cases of botulism associated with canned green beans contaminated with *Clostridium botulinum" Foodborne Pathogens and Disease*, 13(2), 119-121, 2016.

[13] Soltan Dallal, M. M., Najar, F., Karbalaei Shabani, A., Ezoji, K., Shabani, M., Montazer Khorasan, M. R. "Investigation of the frequency of foodborne botulism in patients referred to Loghman Hospital in Tehran City, Iran, From 2008 to 2019", *International Journal of Medical Toxicology and Forensic Medicine*, 10(2), 28348, 2020. https://doi.org/10.32598/ijmtfm.v10i2.28348

[14] Lúquez, C., Edwards, L., Griffin, C., Sobel, J., "Foodborne botulism outbreaks in the United States, 2001–2017", *Frontiers in Microbiology*, 12, 713101, 2021. https://doi.org/10.3389/fmicb.2021.713101

[15] ECDC., "Annual epidemiological report 2016 – Botulism", *ECDE*, 2016. https://www.ecdc.europa.eu/en/publications-data/monitoring/all-annual-epidemiological-reports

[16] Negrut, N., et al., "Epidemiology of botulism in the north-western Romania 7-year survey", *Environmental Science and Pollution Research*, 28(45), 64234-64240, 2021. https://doi.org/10.1007/s11356-021-15576-w

[17] Rasetti-Escargueil, C., Lemichez, E., Popoff, M. R., "Human botulism in France, 1875–2016", *Toxins*, 12(5), 338, 2020. https://doi.org/10.3390/toxins12050338

[18] Benevenia, R., et al., "Foodborne botulism survey in Northern Italy from 2013 to 2020: Emerging risk or stable situation", *Food Control*, 132, 108520, 2021. https://doi.org/10.1016/j.foodcont.2021.108520

[19] Davidson, P. M., Pflug, I. J., Smith, G. M., "Microbiological analysis of food product in swelled cans of low-acid foods collected from supermarkets" *Journal of Food Protection*, 44(9), 686-691, 1981.

[20] Lake, D. E., Lesniewski, R. S., Anderson, J. E., Graves, R. R., Bremser, J. F., "Enumeration and isolation of mesophilic anaerobic sporeformers from cannery post-processing equipment", *Journal of Food Protection*, 48(9), 794-798, 1985. https://doi.org/10.4315/0362-028X-48.9.794

[21] Johnson, E. A., "*Clostridium botulinum*", *Food Microbiology: Fundamentals and Frontiers*, 487-512, 2019.

[22] Breidt, F., "BufferCapacity3 an interactive GUI program for modelling food ingredient buffering and pH", *SoftwareX*, 22, 101351, 2023. https://doi.org/10.1016/j.softx.2023.101351

[23] Munir, M. T., Mtimet, N., Guillier, L., Meurens, F., Fravalo, P., Federighi, M., Kooh, P., "Physical treatments to control Clostridium botulinum hazards in food", *Foods*, 12(8), 1580, 2023. https://doi.org/10.3390/foods12081580

[24] Rasooly, R., Do, P. M., "Clostridium botulinum neurotoxin type B is heat-stable in milk and not inactivated by pasteurization", *Journal of Agricultural and Food Chemistry*, 58(23), 12557-12561, 2010. https://doi.org/10.1021/jf1028398