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An overview of the Role of *Clostridium botulinum* in Honey: Pathogenesis, Detection, and Public Health Implications

Clostridium botulinum a mézben: kórokozó szerep, kimutatás és közegészségügyi vonatkozások

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Abstract

Clostridium botulinum produces botulinum neurotoxins causing a rare but serious illness known as botulism. Botulism continues to pose significant public health risks, particularly in the context of foodborne outbreaks and infant botulism. This thesis investigates the occurrence of *C. botulinum* contamination in honey, highlighting the pathogen's association with infant botulism and the challenges in detecting botulism-causing strains. While honey is a valuable natural product, its microbiological safety is compromised by the potential presence of *C. botulinum* spores, which, when ingested by infants, can germinate in the gut and produce neurotoxins. The study reviews the current diagnostic methods for detecting botulism, including conventional PCR techniques and emerging metagenomic approaches that offer improved sensitivity and specificity in food safety testing. A key focus is placed on infant botulism, which remains a significant concern due to the vulnerability of this population. Furthermore, this research examines the broader implications of *C. botulinum* contamination on public health, food safety, and regulatory practices, emphasising the importance of stringent monitoring and preventive measures in honey production. As botulism continues to be an issue of global importance, advancements in diagnostic technologies and international collaboration are crucial to mitigating the risks associated with this deadly disease.

Összefoglaló

A Clostridium (C.) botulinum által okozott neurotoxikózis, a botulizmus, ritka, ugyanakkor végzetes kimenetelű humán bántalomként is megjelenhet, így közegészségügyi jelentősége egyáltalán nem elhanyagolható. A botulizmus főleg az igen fiatal gyermekekben okozhat megbetegedést, amely forrásaként főleg a mézfogyasztás tehető felelőssé. Jelen dolgozat *C. botulinum* által okozott méz kontamináció kérdését vizsgálja, rámutatva a gyermekeben előforduló botulizmus oktani aspektusaira és a *C. botulinum* egyes típusai spóráival szennyezett mézből a kóroki kimutatás diagnosztikai nehézségeire. A méz értékes és természetes tápanyagforrás, élelmiszerbiztonsági szempontból azonban aggályosak lehetnek a mátrixban esetlegesen életképesen fennmaradó spórák, amelyek elfogyasztva a bélben való germinációt követően szisztémás tüneteket okozó neuro-toxinokat termelnek. Jelen dolgozat a szakirodalom elemzésével a botulizmus diagnosztikai lehetőségeit vizsgálja, beleértve a hagyományos polimeráz láncreakciót és az újabban használatos metagenom-elemzés lehetőségeit. A kisgyermekekben előforduló botulizmus mellett a dolgozat, szélesebb

merítéssel, a botulizmus közegészségügyi, élelmiszerbiztonsági és törvényi szabályozási vonzatait is vizsgálja, kiemelve a méztermeléssel- és vizsgálattal kapcsolatos megelőző- és szabályozó intézkedések lehetőségeit. Mivel a botulizmus az emberi egészséget is érintő, globális probléma, a diagnosztikai módszerek fejlesztése továbbra is fontos szakmai feladat marad, azért, hogy a humán botulizmusos eseteket a lehető leghatékonyabban meg tudjuk előzni.

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1. Introduction

In September 2023, a botulism outbreak occurred in Bordeaux, France. No less than 15 individuals developed foodborne botulism, with eight requiring intensive care and six necessitating invasive mechanical ventilation, after consuming contaminated canned sardines [1]. This outbreak underscores the vital need for prompt detection, reporting, and mitigation strategies in cases of botulism to prevent widespread health crises.

Clostridium botulinum, a spore-forming anaerobic bacterium, is a significant public health concern due to its capacity to produce botulinum toxin, one of the most potent biological toxins known. This pathogen is not only a critical focus in food safety but also an emerging concern in the context of apiculture and honey consumption. Indeed, while honey is celebrated for its health benefits, nutritional value, and medicinal use, it can occasionally harbour *Clostridium botulinum* spores, posing a risk for foodborne botulism, particularly in infants. Understanding the interplay between this pathogen, honey as a natural product, and its potential public health implications underscores the relevance of this study.

Honey, often perceived as a safe and beneficial food product, is a well-established reservoir for *Clostridium botulinum* spores. Infant botulism, first identified in the 1970s, remains strongly associated with honey consumption, which has fuelled scientific interest in this field. The increasing demand for natural and authentic foods has heightened the necessity to ensure honey's quality, authenticity and microbiological safety [2]. As new uses for honey and advanced detection technologies evolve, understanding and mitigating its potential risks, particularly for vulnerable populations, becomes imperative [3].

Despite the rich history of research on *Clostridium botulinum*, systematic reviews reveal gaps in the literature, particularly regarding its occurrence in honey and its implications for both public and bee health (Figure 1). The first scientific paper on *C. botulinum* was published in 1922, but it yielded to the 1970s to have a growing interest for the bacteria, more for the fact of the discovery of the botox component in chirurgical and esthetical treatment. Since 1995, the quantity of papers being published has been relatively constant (~100 articles/years). Scientific concerns for botulism with infants and presence of the bacteria in honey started in the 1970s (1971 and 1976, respectively) and has been

maintained until now. Research on cases with infants represents around 6% of the publications since 2015 and interest on this is slowly growing more important.

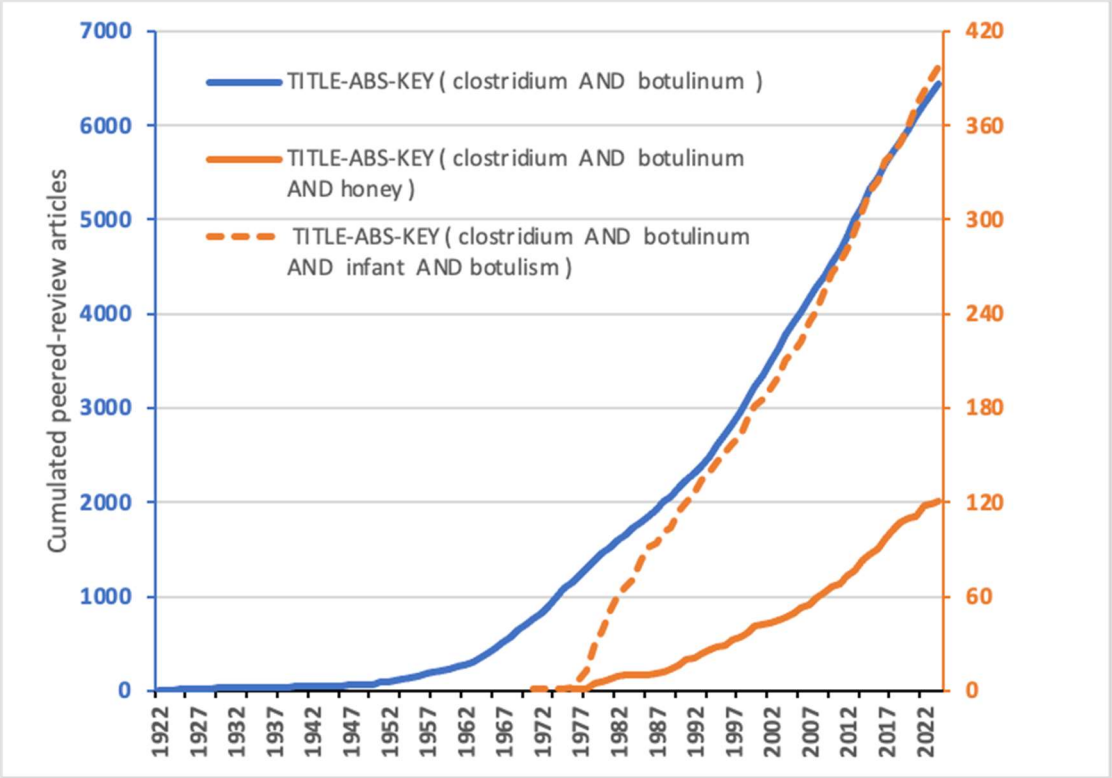

Figure 1. Cumulative peer-reviewed research articles on *Clostridium botulinum* between 1922 and 2024 from systematic review in Scopus using a filter combination of keywords in the title (TITLE), abstract (ABS) and keyword (KEY) of the publication. Blue lines: filter combination yielding to a cumulative number of articles of 7000. Orange lines: filter combination yielding to a cumulative number of articles lower than 420.

This thesis explores the occurrence of *Clostridium botulinum* contamination in honey, the most effective methods for detecting its presence, and strategies to prevent contamination and associated risks, with a particular emphasis on infant botulism. The study begins with an analysis of honey as a potential medium for *Clostridium botulinum* contamination, examining both the pathogens present and the physico-chemical properties that may influence bacterial survival. This is followed by a description of *Clostridium botulinum*, including the key characteristics of the bacteria and the disease it causes.

Following this, a review of diagnostic methods used in botulism outbreaks is presented, with special emphasis on advanced molecular techniques, such as Polymerase Chain Reaction (PCR) and metagenomics, which allow for faster and more precise analysis of

honey and other foods linked to botulism cases. A primary focus of this research is infant botulism and its association with honey consumption.

The final section explores the broader implications of *Clostridium botulinum* contamination for public health and food safety, emphasising the importance of awareness, prevention, and policy measures to protect vulnerable populations, particularly infants. This structure aims to provide a comprehensive understanding of the relationship between *Clostridium botulinum* and honey, while contributing actionable insights to improve detection and mitigate public health risks.

2. Contamination Route and Honey Properties

Honey is a natural product cherished for its flavour, nutritional value, and health benefits. It also stands out as a food of animal origin that has not been commonly linked to major microbiological foodborne illnesses, with the notable exception of infant botulism. Understanding the sources of potential contamination and the diversity of pathogens present in honey is crucial for assessing its safety and quality. While honey can contain a wide range of bacteria, viruses, fungi, and even parasites, many of these microorganisms seem to pose minimal risk, as direct infections transmitted through honey have not been documented. Nevertheless, the potential for opportunistic infections from honey's microflora remains an area of interest. This chapter will explore the sources of pathogens, the variety of microorganisms found in honey, and its inherent properties that influence contamination and safety.

2.1. Sources of Pathogens

Honey is a sweet paste produced by honeybees (*Apis mellifera*) using nectar from flowers (Blossom Honey) or the secretions of plant-sucking insects (Honeydew Honey), as defined by the Codex Alimentarius [4]. Honeybees collect these substances and bring them back to the hive, while mixing them with specific enzymes produced in their bodies. The modified substance is then stored in wax cells of the honeycomb. During this process, the bees help reduce the water content of the mixture through dehydration, often fanning it with their wings to speed up evaporation, until it reaches about 83% sugar and 17% water [5]. Once the honey is fully mature, the bees seal the cells with a thin layer of wax, preserving it until they need to feed. Honey’s main components are sugars, predominantly

fructose and glucose, along with small amounts of organic acids, enzymes, and particles collected during the process. For human consumption, honey is harvested from hives by extracting it from the honeycombs. The beekeeper first uncaps the sealed wax cells using a heated knife. The exposed honeycombs are then placed in an extractor, where centrifugation forces the honey out of the combs through gravity [5].

Microbial contamination of honey can occur at various stages of production and extraction. During foraging, honeybee workers inadvertently collect a wide range of microorganisms from the environment, which can then be transferred into the honey as it is produced. These are considered primary sources of microbial contamination. Secondary sources, which are often similar across different food products, can further impact honey, particularly during and after the extraction process [6]. In fact, microbial contamination is generally more likely to occur during handling and processing stages than within the hive itself [7].

Primary sources of microbial contamination in honey include the honeybees themselves, environmental exposure, and certain hive management practices. Honeybees, during their foraging activities, inadvertently collect not only nectar but also microorganisms (bacteria, yeasts, fungi) from plants, dust, air, and soil. Additionally, the digestive tracts of honeybees harbour a natural microbiota that can be introduced into the honey during its processing. These pathogenic sources are challenging to control, as they are integral to the honeybees' environment [6]. Moreover, the biologic compounds present in the nectar collected by bees are transferred to the honey, contributing to the distinctive organoleptic, nutritional, and biological properties of each honey variety [2].These microorganisms and plant-derived compounds can influence the initial microbial load and unique characteristics of honey. Research is still going on about these primary sources of microbes in honey, to understand the microbial ecology of the honeybee.

Contamination of honey with *Clostridium botulinum* is from primary source as it is ubiquitous in nature and the spores are occasionally found in honey. Huhtanen *et al.* (1981) [8] did an experiment where honeybees were fed syrup, inoculated with *C. botulinum* spores. All the spores initially ingested by the honeybees were eventually incorporated into the honey [8]. Interestingly, the spores were not detected in the bees' intestinal tracts two weeks after feeding, suggesting that the honeybees do not retain these ingested organisms for long and do not seem to be affected by it [8]. This finding

underscores the potential for honey to serve as a reservoir for certain microorganisms, even if they do not persist in the honeybees themselves.

Secondary sources of microbial contamination in honey arise during the stages of extraction, processing, and packaging. Unlike primary sources, which are difficult to control due to their environmental nature, secondary contamination can often be mitigated through proper hygiene and good manufacturing practices. Potential sources include humans, equipment, containers, wind, dust, insects, animals, and water [6]. Improperly cleaned harvesting equipment, for instance, can introduce bacteria and fungi to the honey, while human handling can result in contamination from skin microbiota. Additionally, inadequate storage conditions, such as exposure to moisture, can facilitate the growth of certain microorganisms like *Saccharomyces cerevisiae* or other osmotolerant yeasts [6]. Cross-contamination from air, buildings, and food handlers further elevates the risk. Good manufacturing practices are crucial to minimise these contamination risks and ensure the microbial safety of honey across all stages of production and processing.

2.2. Pathogen Diversity

Honey is known to harbour a variety of microorganisms, that often emerge from symbiose between the honeybee and the microorganisms, with antimicrobial properties associated with the honey types [3, 9] (see section 2.3). Among the most common pathogens found in honey (see Table 1) are bacterial spores (77 gena, [3]), particularly from the *Bacillaes* and *Lactobacillaes* families (13 gena, [3]). However, bacteria do not replicate in honey due to its inhospitable environment [6]. For the hive, it is worth noting that some commonly detected bacteria in honey are probiotic associated with the honeybee gut microbiota (*Lactobacillus*, *Bifidobacterium* and some *Bacillum* spp.) and have antagonistic actions against other pathogens like *Paenibacillus* and fungal growth.

A total of 84 genus of fungi (i.e., moulds and yeast) were detected in honey [3]. Moulds, introduced through honeybees, the hive, or the surrounding environment, can survive in honey but generally do not grow [6]. Yeasts, on the other hand, can thrive in the acidic, high-sugar conditions of honey, with osmophilic varieties fermenting sugars into alcohol and carbon dioxide. Honey from humid regions is especially prone to yeast proliferation, potentially leading to spoilage [10]. Despite the presence of these microbes, honey's antimicrobial properties significantly restrict microbial diversity, allowing only moulds

and yeasts to grow, while vegetative bacteria typically do not survive for long unless stored at cool temperatures [6].

While viruses affect the honeybees, studies of viruses are scarce (11 gena, [3]) and risks from the virus presence are mostly related to bee colonies' health, with a contamination from the honey to the bee [3].

Table 1. Pathogens commonly detected in honey.

Group	Family	Genus
Bacteria	Acetobacteraceae	Parasaccharibacter, Asaia, Saccharibacter, Bombella, Gluconobacter
	Enterobacteriacea	Enterobacter, Escherichia, Citrobacter, Cedecea, Vagococcus, Raoultella, Salmonella, Shiggela, Klebsiella
	Bifidobacteriaceae	Bifidobacterium
	Microbacteriaceae	Microbacterium
	Bacillaceae	Bacillus, Lysinibacillus, Oceanobacillus
	Paenibacillaceae	Paenibacillus[&]
	Lactobacillaceae	Lactobacillus, Pediococcus, Fructobacillus, Leuconostoc, Oenococcus, Weissella
	Enterococcaceae	Melissococcus[&]
	Clostridiaceae	Clostridium[#]
Fungi	Ascosphaeraceae	Ascosphaera[&]
	Aspergillaceae	Aspergillus[(&)], Penicillium
	Pichiaceae	Candida
	Nectriaceae	Fusarium
	Malasseziaceae	Malassezia
Virus	Unclassified DNA Viruses	Apis mellifera filamentous virus (AmFV)[&]

[#] Genus of interest in the present study. [&] Pathogens causing diseases in honeybees, listed in the WOAH terrestrial animal health code.

2.3. Honey properties

Honey, as one the oldest traditional medicines, is renowned for its antimicrobial properties and wound-healing activities. Serving as a nutrient, drug, and ointment, honey exhibits broad-spectrum antibacterial activity against pathogenic and oral bacteria. The antimicrobial property of most types of honey (blossom monofloral or polyfloral and honeydew) is due to the production of hydrogen peroxide when diluted, which results from the activation of the enzyme glucose oxidase. This enzyme oxidises the glucose $C_6H_{12}O_6$ to gluconic acid $C_6H_{12}O_7$ and hydrogen peroxide H_2O_2 ($C_6H_{12}O_6 + O_2 + H_2O \rightarrow C_6H_{12}O_7 + H_2O_2$) [5]. However, this enzymatic peroxide activity can be easily destroyed by heat (activity affected by temperature over 60ºC) or the presence of catalase. The antibacterial potency of honey can vary significantly, up to 100-fold, due to differences in hydrogen peroxide H_2O_2 concentration [11]. The rationale beneath these variations in the antimicrobial activity is attributed to spatiotemporal differences in nectar sources.

Interestingly, several non-peroxide factors (like polyphenolic components that are plant-dependent) are also responsible for honeys' unique antibacterial activity. The manuka honey is the most curative honey, known up to date and produced from the nectar of the *Leptospermum scoparium* (commonly named tea tree). It has been reported to have an inhibitory effect on around 60 species of bacteria. Among them are included aerobes and anaerobes, gram-positives and gram-negatives [11].

Currently, several honeys are sold with standardized antibacterial activity levels, with manuka (*Leptospermum*) and Tualang (*Koompassia excelsa*) honeys being the most notable. Manuka, jelly bush and pasture honeys, containing glycosylated proteins, stimulate monocytes, the precursors to macrophages, to secrete TNF-alpha, a cytokine crucial for wound repair mechanism. Furthermore, honey immunomodulatory properties can be associated to its potential to reduce reactive intermediates release, limiting tissue damage by early activation of macrophages during wound healing.

Its medicinal properties include not only antimicrobial actions but also stimulation of healing. Honey’s hygroscopic properties help dehydrate bacteria, preventing their growth due to its high sugar content and low pH. Additionally, honey maintains a moist wound condition, aiding in repair, and its high viscosity acts as a barrier against new pathogen infections.

Honey promotes the regeneration of damaged intestinal mucosa, stimulates new tissue growth, and acts as an anti-inflammatory agent. Raw honey contains antioxidants compounds, like flavonoids and polyphenols. Previous studies have reported reduced inflammation symptoms when honey is applied to wounds. The antimicrobial activity of honey ranges from concentrations less than 3% to over 50% [11], making it effective on wounds that do not respond to conventional therapy.

Spores of *Clostridium botulinum* may persist in honey for extended periods, but they do not germinate or produce toxins under honey's conditions. While *C. botulinum* spores have been detected in honey from various countries, the lack of comprehensive data on the contamination pathways prevents the implementation of effective measures to reduce their presence during honey production [12]. Additionally, applying heat treatments capable of destroying these spores would compromise the flavour and texture of honey [12].

3. *Clostridium botulinum* and Botulism

In 1897, the Belgian bacteriologist Emile Pierre Marie van Ermengem published a paper following a botulism outbreak that occurred in 1895 among 23 musicians who had consumed a preserved, uncooked ham [13]. He concluded that botulism is not an infection but an intoxication caused by a specific bacterium, which produces a potent neurotoxin responsible for the disease. Van Ermengem initially named it *Bacillus botulinus*, it is now classified as *Clostridium botulinum* due to its anaerobic, spore-forming properties. The illness was also known as Kerner's disease in honour of Justinus Kerner (1786-1862), one of the first persons to study and to give a critical description of botulism, hypothesising the existence of a toxic agent in spoiled food.

Clostridium botulinum are gram positive, obligate anaerobic, spore-forming bacteria present in the environment, usually found in soils. Under anaerobic conditions, and more specifically in the presence of decaying organic matter, this bacterium proliferates and can release a neurotoxin, known to be one of the most potent toxins of the world. This botulinum neurotoxin is responsible for a severe and sometimes fatal neurological illness called botulism in humans and vertebrate animals.

3.1. Characteristics of *Clostridium botulinum*

Clostridium botulinum is now known as a heterogeneous group of bacteria within the *Clostridium* genus and characterised by their ability to produce the botulinum neurotoxin [13]. Genomic analysis suggests that *C. botulinum* are fit to thrive as saprophytic bacteria in both soil and water based environments. It appears that this pathogen relies on its potent toxin to swiftly incapacitate a wide range of species, and releases a large number of extracellular enzymes to break down and degrade decaying tissues, facilitating access to nutrient sources [13].

Based on phenotypic and physiological properties, we can divide those bacteria into four main groups (Table 2), with the addition of some atypical strains of *Clostridium baratii* and *Clostridium butyricum* able to form botulinum neurotoxin F and E respectively [14]. Some articles mention six phenotypically distinct groups of clostridia capable of producing botulinum neurotoxin [15].

The bacteria producing botulinum neurotoxin are extraordinarily diverse, and this diversity extends also to the neurotoxins they produce [14]. We can distinguish seven serotypes of toxin producing *Clostridium botulinum*, based on the serological properties of the toxins they produce, they are called toxinotypes A, B, C, D, E, F and G [16]. Those seven toxinotypes are classified according to their neutralisation by monoclonal antibodies [15, 17].

However, we can observe a discordant phylogeny between the botulinum neurotoxin genes and the host bacterial genomes which indicates that horizontal gene transfer plays a crucial role in shaping the diversity of neurotoxins within the species [18]. This transfer of the botulinum neurotoxin gene clusters contributes to the occurrence of similar toxins across different clostridial groups, complicating the traditional classification based purely on toxin type. For instance, type B strains can be found in both Group I and Group II, yet they differ significantly in terms of metabolic capabilities and genetic makeup[15].

Phylogenetic analyses based on 16S rRNA gene sequences closely mirror the phenotypic subdivisions (Groups I-IV), indicating a strong correlation between phenotype and genetic lineage [15]. Within each phenotypic group, strains tend to produce a restricted set of neurotoxin types. However, these neurotoxin genes appear to have been acquired independently via horizontal gene transfer, highlighting the role of gene exchange in

shaping toxin diversity across different clostridial lineages [19]. Interestingly, *Clostridium botulinum* types C and D are phylogenetically close, sharing approximately 99% 16S rRNA sequence similarity, suggesting a recent common ancestor [18].

This genetic diversity among *Clostridium botulinum* is highlighted in a phylogenetic dendrogram of strains producing the seven different serotypes of botulinum neurotoxin and other related clostridia (NT – nontoxigenic strain) (Figure 2).

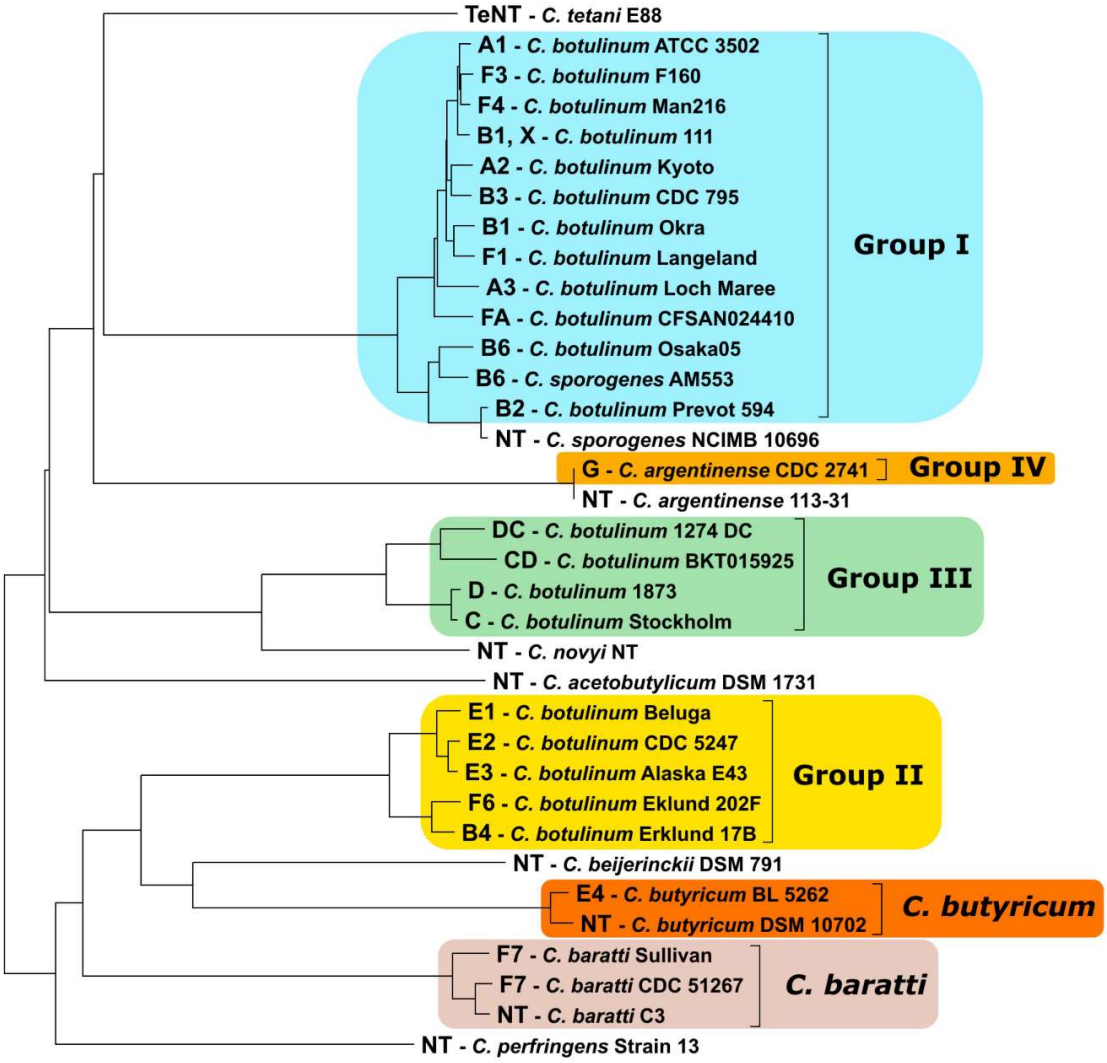

Figure 2. Classification of Clostridium constructed by average nucleotide and neighbour-joining tree method. Information of group (I-IV) and neurotoxin type (A-F). Source: Rawson *et al.* (2023) [20].

Group I consists of mesophilic and proteolytic bacteria, characterised by their ability to break down proteins. Their optimal growth temperature is around 37°C [21]. Typically, they produce toxins A, B and/or F, with the potential to possess combinations of

botulinum neurotoxin gene clusters that may be either silent or variably expressed (bivalent strains). These strains can exhibit major and minor neurotoxin production, designated as Ab or Ba, for example, depending on the dominant toxin, or dual toxin types AB, AF and BF [15]. Notably, these bacteria produce highly heat-resistant spores, and the neurotoxin gene is primarily located on the chromosome, contributing to the stability of their toxicity [13].

In contrast, Group II is composed of the non-proteolytic but psychotrophic bacteria which thrive in colder environments. The optimal growth temperature for these bacteria is around 25°C [13]. They typically contain a single neurotoxin gene (B, E or F), often located on plasmids, and are primarily linked to foodborne cases. Unlike Group I, the spores of Group II are less heat resistant and can be inactivated by exposure to 80-85°C for a few minutes [13].

Group III comprises proteolytic strains producing toxins C and/or D, which are primarily associated with botulism outbreaks in animals. These mesophilic bacteria have an optimal growth temperature of about 40°C [13]. The neurotoxin gene in this group is predominantly located on bacteriophage DNA, making the bacteria more prone to losing their toxic capabilities [13].

Group IV includes only toxinotype G, which has been reclassified as *Clostridium argentinense* [12]. These bacteria are mesophilic and proteolytic, with an optimal growth temperature of 37°C. Notably, this group is the only one that lacks lipase activity.

Table 2. Characterization of *Clostridium botulinum* groups I-IV, adapted from: Nevas (2006) [12].

	Group			
Property	I	II	III	IV
Neurotoxin type	A, B, F	B, E, F	C, D	G
Optimal growth temperature	35-40 ºC	18-25 ºC	40 ºC	37 ºC
Proteolysis	+	-	-/(+)	+
Lipase production	+	+	+	-
Spore heat resistance [a]	1.23/112 ºC	0.6/80 ºC	0.1/104 ºC	0.8/104ºC

[a] Example of the D-value of a certain temperature

The predominant bacteria behind most human botulism cases are strains affiliated with groups I and II, whereas group III strains are commonly responsible for botulism occurrences in animals. There is no confirmation, or publication, of clinical botulism type G in humans or in animals [12, 13].

A major concern with these bacteria is their ability to produce spores - a dormant, highly resistant form that can withstand various food processing methods. These spores are resistant to heat, desiccation, high pressure, low pH, and UV light [13]. Once favourable conditions return, the spores present in food can germinate, allowing the bacteria to multiply and produce toxins. Toxin production and sporulation occur simultaneously during the shift from exponential growth to stationary phase, indicating potential coregulation of these processes [22].

3.2. Neurotoxins

Botulinum neurotoxins (BoNTs) are potent proteins produced primarily by the anaerobic, gram-positive bacteria *Clostridium botulinum* and, in rare cases, by related species such as *Clostridium baratii* and *Clostridium butyricum* [23]. These neurotoxins are classified into seven antigenically distinct serotypes (A to G), with variations of 35% to 70% in their amino acid sequences [14, 17]. Within each serotype, BoNTs are further divided into around 40 subtype variants, identified numerically based on their order of discovery. These subtypes exhibit unique amino acid sequences, with minimum 2.6% variability, resulting in differences in their biological activity [14]. Moreover, naturally occurring mosaic toxins, also referred to as chimeric toxins, are single botulinum neurotoxins composed of domains originating from different serotypes, including BoNT/CD, BoNT/DC, and BoNT/FA. For example, the BoNT/DC toxin features a C-terminal heavy chain resembling that of BoNT/C toxins, while its light chain and N-terminal heavy chain are nearly identical to those of BoNT/D [20].

Structurally, BoNTs are 150 kDa proteins with zinc endopeptidase activity, acting as cholinergic, heat-labile neurotoxins that specifically inhibit acetylcholine release at the neuromuscular junction, leading to flaccid muscle paralysis without affecting sensory functions [16, 24].

Botulinum neurotoxin is a dipeptide chain initially produced as a single soluble 150 kDa polypeptide (Figure 3. c.), which is later cleaved by proteases either of bacterial origin,

or from external proteases like trypsin in non-proteolytic strains (group II), to become active [16]. This active toxin consists of a light chain (50 kDa) and a heavy chain (100 kDa) linked by a disulfide bond [14] (Figure 3). The light chain houses a zinc-dependent metalloprotease, cleaving components of the Soluble N-ethylmaleimide-Sensitive-Factor Attachment Protein Receptors (SNARE) complex, thereby inhibiting the release of acetylcholine into the synaptic cleft, disrupting neurotransmission [25]. The heavy chain is further subdivided into two domains: the carboxyl-terminal domain (Hcc), which binds specific receptors on presynaptic motor neurons and facilitates endocytosis of the neurotoxin, and the amino-terminal domain (Hcn), which assists in translocating the light chain into the cytoplasm. The synergistic activity of these domains ensures the efficient intoxication of neural tissue [14, 26].

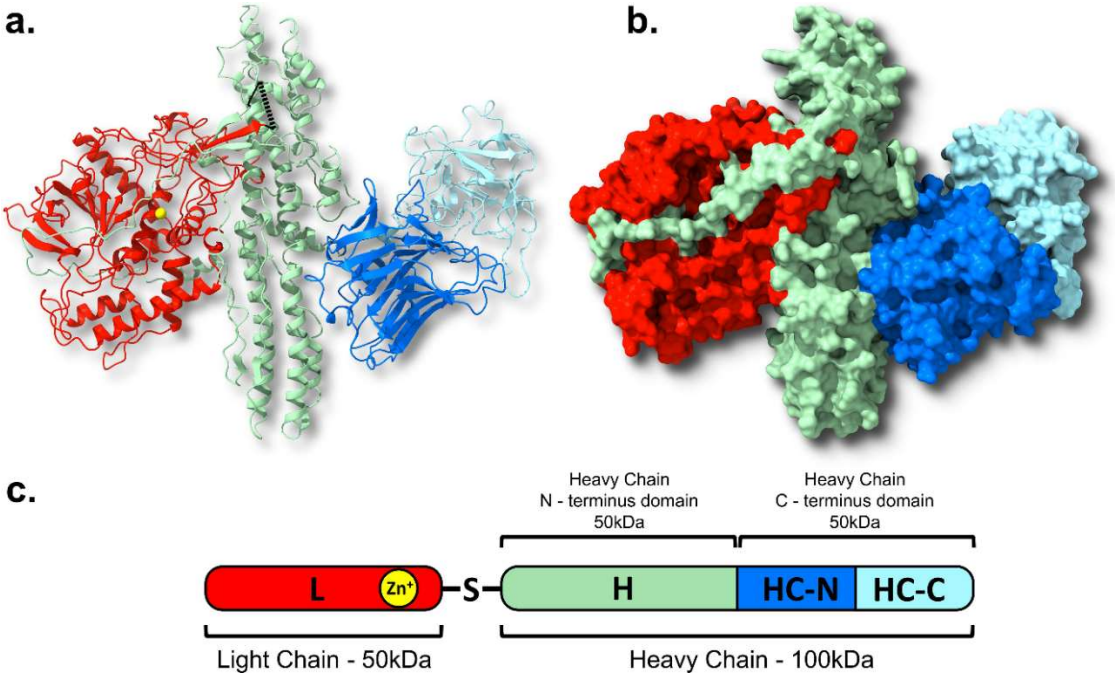

Figure 3. Molecular structure of the botulinum neurotoxin A1, **a.** displayed in ribbon format and **b.** a spacefill representation. The bontA1 gene is translated into a single polypeptide (**c.**), which is cleaved into a light chain (L, coloured red) and a heavy chain joined by a disulphide bridge (shown in black, **a.**). The heavy chain contains a N-terminal translocation domain (HN, coloured light green) and a C-terminal receptor binding domain, which is formed of two subunits (HC-N and HC-C coloured dark and light blue respectively). The light chain is the catalytic domain and consists of a zinc metalloprotease (Zn^{2+} atom shown in yellow). Source: Rawson *et al.* (2023) [20].

Despite their diversity, all BoNTs share a common mechanism of action: it begins with the absorption of the neurotoxin into the bloodstream and lymphatic system, from where it travels to the motor nerve endings of peripheral neurons [16]. At the presynaptic terminal of the neuromuscular junction, the heavy chain (Hcc) binds to specific neuronal receptors, facilitating the toxin's internalisation through vesicle endocytosis. The amino-terminal domain (Hcn) then mediates the translocation of the light chain into the neuronal cytoplasm [14, 19, 26]. Once inside, the light chain hydrolyses a distinct site on one or more components of the SNARE complex, including SNAP-25 (synaptosomal-associated protein 25), VAMP (vesicle-associated membrane protein), and syntaxin, which are critical for vesicle docking and fusion [19, 25]. The various BoNT serotypes target different components of the SNARE complex at distinct sites. BoNT/A, C, and E specifically cleave regions of SNAP-25, located on the cytosolic face of the presynaptic membrane. BoNT/C has the unique ability to cleave both SNAP-25 and syntaxin. Meanwhile, BoNT/B, D, F, and G act on specific regions of VAMP (synaptobrevin) [19] (Figure 4). This enzymatic activity blocks the exocytosis of acetylcholine vesicles, leading to an absence of neurotransmitter release. This inhibition stops signal transmission to the muscle, resulting in flaccid paralysis [25, 26].

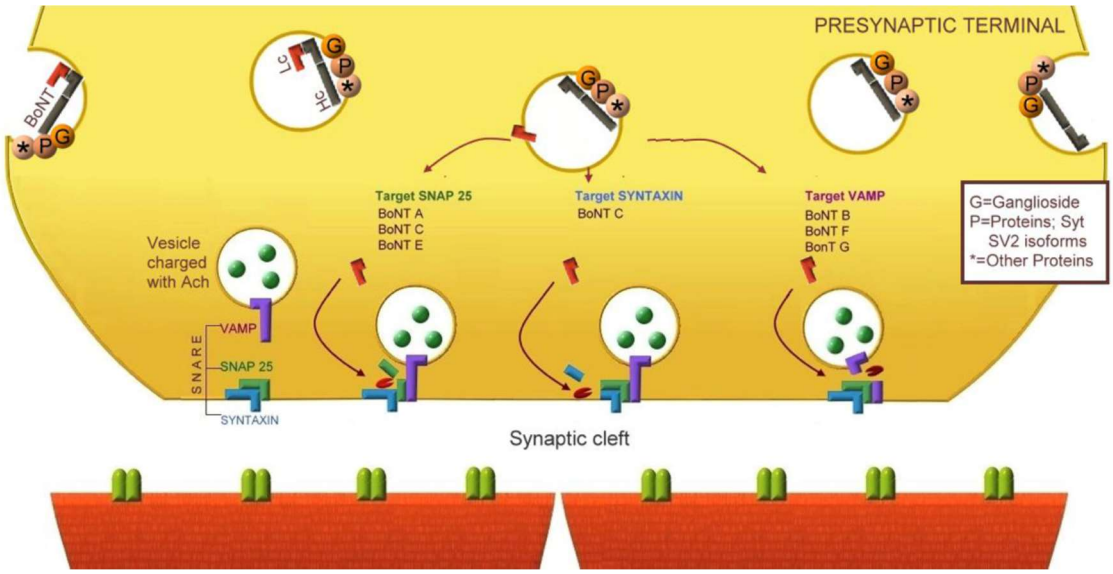

Figure 4. Actions of the botulinum neurotoxin in a neuronal cell. Source: Peng Chen Z *et al*. (2012) [19].

Additionally, BoNTs are often found in association with some nontoxic neurotoxin associated proteins (NAPs), forming what is known as the progenitor complex (PC) through non-covalent interactions [20]. These complexes, which vary in size depending

on the BoNT serotype and can reach up to 900 kDa, serve to enhance the stability and functionality of the neurotoxin [13]. Those non-toxic proteins, are encoded by genes located in a cluster adjacent to the botulinum neurotoxin gene, either located on the bacterial chromosome or on extrachromosomal elements (example: plasmids) [14]. Among the NAPs, there are always non-toxic non-hemagglutinin (NTNH) proteins that are structurally similar to BoNT but lack the zinc-binding motif required for catalytic activity [20]. NTNH proteins are believed to protect the neurotoxin from chemical and enzymatic degradation, such as the low pH and proteases encountered in the host (e.g., gastric acidity) or decaying matter [13, 14]. In addition, the NAPs may include hemagglutinin (HA) proteins, which are thought to assist in neurotoxin attachment to the small intestine for absorption, or OrfX proteins, whose exact role remains uncertain but is presumed to be similar to that of hemagglutinin [20]. The distribution of these accessory proteins differs across BoNT serotypes: hemagglutinins are typically found in complexes with BoNT/B, C, D, G, and some BoNT/A serotypes, while OrfX proteins are present in BoNT/E, F, and other BoNT/A serotypes such as BoNT/A2 [14, 20].

The onset of paralysis is typically symmetrical and descending, with the toxin active at extremely low concentrations (30-100 ng depending on the producing strain), highlighting its high potency [13, 24]. The lethal dose of type A BoNT in humans is estimated between 0.1 and 1.0 mg, corresponding to 300-30 000 mouse intraperitoneal LD50 doses [27]. *C. botulinum* type A is associated with the highest mortality rate in human botulism cases [19].

Invertebrates lack these specific receptors on their neuronal cell surface and are assumed resistant against the botulinum neurotoxins. Although, it was found recently that one strain of *Clostridium botulinum* can affect mosquitoes [28].

3.3. Botulism

Botulism is a rare but serious non-contagious illness caused by exposure to botulinum neurotoxin, produced by the bacteria *Clostridium botulinum*. Transmission occurs when a person, or an animal, ingests the preformed toxin, or when spores or vegetative cells of the bacterium enter the body and subsequently produce the toxin. The primary routes of transmission include contaminated food, open wounds, or, mainly in the case of infants, ingestion of spores that germinate in the gut.

Botulism symptoms are characterised by flaccid paralysis and can lead to sudden death if not treated immediately and appropriately. The botulinum neurotoxin damages nerve cells and tissues, resulting in severe complications such as heart failure and paralysis of the respiratory muscles. The toxin can enter the body through the gastrointestinal tract or via mucous membranes, including those of the eyes and respiratory system [16]. Clinical symptoms across all forms of botulism typically begin with cranial nerve involvement, presenting as blurred or double vision, dilated pupils, dry mouth, slurred speech, and difficulty swallowing or speaking. As the condition progresses, muscle weakness spreads to the torso, arms and limbs, eventually causing paralysis of the respiratory muscles, although sensation remains intact. Without immediate intervention and assisted respiration, respiratory failure may lead to death [16]. Given the severity of these symptoms, botulism poses significant public health risks, highlighting the need for a thorough understanding and effective management strategies.

Botulism treatment focuses primarily on neutralising the neurotoxin before it binds to nerve endings, as once clinical symptoms appear, the toxin has irreversibly attached to receptors and entered the cells, inhibiting acetylcholine release [19]. The administration of antitoxin can help neutralise unbound neurotoxin, but it cannot reverse symptoms that have already developed. The effectiveness of antitoxin therapy depends greatly on how quickly it is administered; if the toxin has already entered the nerve endings and cleared from the bloodstream, the treatment becomes ineffective. Additionally, there is a risk of anaphylactic shock associated with antitoxin use [16]. Recovery from botulism is a lengthy process, requiring the sprouting of new, temporary nerve endings while the original synaptic activity regenerates. The duration of recovery, which can range from several weeks to months, depends on the amount and type of toxin ingested, with type A toxin typically causing the most severe and prolonged symptoms [16]. In severe cases, patients may experience prolonged muscle paralysis, leading to disabilities that can last for months or even years [29].

In human botulism, we can distinguish five types of contamination routes : foodborne, intestinal, wound, iatrogenic and inhalational. The last three being rare, we could say that botulism is mainly related to food poisoning.

Foodborne botulism is the oldest and most common form of botulism in humans, resulting from the ingestion of food containing preformed botulinum neurotoxin. Historically

linked to food poisoning from contaminated meat products, it remains a significant public health concern worldwide [30-35]. The bacteria *Clostridium botulinum* can germinate and produce toxin in inadequately processed foods, especially homemade items subjected to insufficient heat treatment, which fails to destroy heat-resistant spores [16]. While hermetically sealed packaging can extend product shelf life, it also creates an anaerobic environment conducive to spore growth. After consuming a significant amount of the toxin, as little as 50 ng of botulinum toxin being enough to cause illness [17], symptoms may develop within hours, though the typical incubation period ranges from 12 to 72 hours, depending on the toxic dose [16, 36]. Initial symptoms, appearing within 6 to 36 hours of ingestion, often include nausea, vomiting, and constipation, followed by neurological signs such as blurred vision, dry mouth, and muscle weakness, making diagnosis challenging due to similarities with other neurological conditions [37]. Outbreaks are usually sporadic and localised, often affecting families who consume contaminated homemade foods with mild heat treatments, allowing the survival of heat-resistant spores. Although commercial food products are less commonly implicated, when involved, they can result in large outbreaks and cause substantial economic losses [16].

Unlike foodborne botulism, which involves ingestion of preformed toxin, infant botulism is an infection caused by the ingestion of *Clostridium botulinum* spores, which can germinate, grow, and produce neurotoxin within the intestinal lumen [38]. This form of botulism primarily affects infants under one year of age, as their gut microflora is not fully developed, allowing the spores to germinate and establish a toxin-producing bacterial culture. The condition often begins with constipation lasting several days, followed by characteristic symptoms of flaccid paralysis, such as feeding difficulties due to weakened mouth and throat muscles, facial paralysis, ptosis (drooping eyelids), and generalised weakness [16].

While rare, cases of intestinal botulism have been reported in adults and children older than one year [44, 45]. This condition is typically associated with individuals who have disrupted intestinal microflora, often due to factors like abdominal surgery, prolonged antimicrobial therapy, or gastrointestinal wounds and abscesses [16]. Structural abnormalities, such as anatomical defects or changes from surgery or inflammatory bowel disease, can also increase susceptibility. Broad-spectrum antibiotic use is a key contributor to altered gut microflora, reducing the natural biodiversity that normally prevents *C. botulinum* spore germination and toxin production [46]. The development of

adult intestinal botulism involves the ingestion and germination of spores, leading to bacterial colonisation in the gut, in situ neurotoxin production, and subsequent systemic absorption. Symptoms are similar to those of infant botulism, including constipation, lethargy, and feeding difficulties [46]. Since the first documented case in 1986, 33 cases of intestinal toxaemia botulism have been reported in the literature [46].

Other forms of human botulism, such as wound botulism, involve an infection where toxin production occurs in vivo [16]. The first case of wound botulism in the United States of America was reported in 1943 and formally described in 1951 [39]. This condition arises when *Clostridium botulinum* colonises injured tissue and produces botulinum neurotoxin directly at the infection site. The bacteria germinate and proliferate in deep wounds or abscesses that provide an anaerobic environment, releasing toxin that is absorbed into the bloodstream and disseminated systemically. Experimental studies have shown that only 10 spores of *C. botulinum* type A can induce wound botulism in mice [40]. The median incubation period for wound botulism is about a week and its estimated case-fatality rate is 15% [16]. Treatment typically involves respiratory support, thorough surgical debridement, antibiotics, and the administration of antitoxin [16]. Despite its rarity, wound botulism should be considered in patients presenting with neurological symptoms following trauma, surgery, injection drug use, or sinusitis linked to cocaine sniffing [41]. Cases have become increasingly common among injecting drug users, particularly those using contaminated needles or impure heroin, especially black tar heroin, injected subcutaneously or intramuscularly [16]. The largest known cluster of wound botulism among drug users in Europe occurred in early 2015, with 40 cases reported in Scotland [42]. Since 2016, the incidence of wound botulism in the U.S.A. has risen sharply, with 82% of cases reported occurring in California, a region where black tar heroin use is prevalent [43].

Iatrogenic botulism refers to botulism cases resulting from therapeutic or cosmetic use of botulinum toxin (BoNT). Since its approval by the U.S. Food and Drug Administration (FDA) in 1989 for treating conditions like blepharospasm and strabismus, BoNT applications have expanded significantly to include treatments for neurological syndromes involving muscle, nerve, and gland hyperactivity, and cosmetic procedures. The therapy results in the prevention of muscle contractions and/or gland secretion, which can help in situation like dystonia, spasticity, and hyperhidrosis (excess sweating) [19, 47]. Despite its therapeutic benefits, improper administration can lead to severe

complications. A notable outbreak of iatrogenic botulism occurred in November 2004, when four patients in Florida developed botulism after receiving unlicensed, highly concentrated cosmetic botulinum toxin injections [48]. BoNT is primarily used as a focal injection therapy, minimizing systemic side effects, yet in rare cases, the toxin can spread beyond the injection site, reaching lymphatic circulation, causing muscle weakness, allergic reactions, and typical symptoms of botulism [47]. The risk of such adverse effects has increased with the widespread use of BoNT across a variety of medical and cosmetic fields, often involving off-label applications of more than fifty conditions in the areas of ophthalmology, neurology, plastic surgery, orthopaedics, gastroenterology, urology and gynaecology (conditions like chronic anal fissures, tension headaches, and lower limb spasticity) [19, 47]. In March 2023, a large outbreak of iatrogenic botulism was reported among patients treated with BoNT for weight reduction in Türkiye, affecting 87 individuals across Europe and highlighting the risks associated with improper use [47]. Positive effects of BoNT treatments generally last for 3–6 months, depending on the toxin type, dose, and administration method, but the potential for serious side effects underscores the need for careful handling and regulation [47].

Between 2008 and 2018, 100 human botulism outbreaks were recorded in France. Among these, 82 cases (89.8%) were foodborne, 17 cases (9.6%) involved infant intestinal botulism, and 1 case (0.6%) was attributed to wound botulism following an open leg fracture caused by a road traffic accident in 2008. No cases of adult infectious botulism (intestinal colonisation) were reported during this period [49]. Human botulism with type B toxin was implicated in the majority of cases (49.7%), followed by type A (44.3%) and type E (2.5%), according to the Centers for Disease Control and Prevention (CDC) data of 2019 [50].

Botulism outbreaks also occur in animals, with birds being the most commonly affected, followed by cattle. In cattle, clinical signs of botulism result from ingesting preformed toxins, whereas in birds, outbreaks are caused by the ingestion of spores, which then produce toxins within the caecum. Transmission is primarily oral. According to Lebouquin *et al.* (2022) [49], an average of 10 outbreaks are recorded annually in the bovine sector, 30 in the poultry sector, and 20 among wild birds, with each outbreak potentially affecting several thousand birds.

4. Diagnostic Methods of Botulism

Given that botulism is a life-threatening condition, rapid diagnosis is crucial. Although foodborne botulism outbreaks are relatively rare, they most often involve home-prepared foods, including cured meats, canned vegetables, and fermented fish products. These outbreaks are typically sporadic and limited to family units. While commercial foods are rarely implicated in foodborne botulism, their outbreaks tend to be more extensive and can result in significant economic, reputation and trust losses for the food industry.

4.1. Conventional Diagnostic Methods

Laboratory testing is critical in confirming cases of botulism, verifying that administered antitoxins are effective, and identifying sources of contamination in suspected food to prevent further outbreaks [23]. Testing focuses on detecting botulinum neurotoxin (BoNT) or identifying *Clostridium* species capable of producing this toxin. Clinical samples commonly tested include serum, stool, and gastric fluid, while suspected food samples may also be analysed. Confirmation typically involves detecting BoNT in biological or food samples, or BoNT-producing species (*C. botulinum*, *C. baratii*, *C. butyricum*) in stool or wound cultures [23]. Timely sample collection and proper storage are important for reliable detection. BoNT levels decrease rapidly in biological samples as the toxin is internalized by neurons. Delays or storage at temperatures above 2-8°C risk degradation and false negatives [20]. Furthermore, while identifying *C. botulinum* or related species in clinical samples supports the diagnosis, it is not definitive due to potential confounding factors such as the presence of multiple toxin-producing strains [16].

The Mouse lethality Bioassay (MBA) is the FDA-approved gold standard for laboratory confirmation of botulism [23]. This assay involves intraperitoneal injection of a sample, diluted in phosphate buffer, into laboratory mice (with addition of trypsin for the strains of Group II as they are non-proteolytic). If the sample contains the toxin, the mice exhibit typical symptoms of botulism (usually within a day but can take longer), such as fuzzy hair, muscle weakness, and respiratory failure [16]. The specific toxinotype is identified through neutralisation with antitoxins : mice injected with the matching neutralising antitoxin survive, while those without develop botulism. The MBA is highly sensitive, capable of detecting toxin levels as low as 0.01 ng/mL of sample eluate, with an LD50

(median lethal dose causing the death of 50% of a population of Swiss Webster Mice) range of 0.5 to 5 ng/kg depending on the serotype [16, 19]. It is also effective in identifying the wide spectrum of BoNT subtypes reported in the literature [37]. However, it cannot differentiate individual BoNT subtypes. Despite its accuracy, the MBA raises significant ethical issues due to animal suffering and mortality. In 2019, an approximation stated that 400000 animals were used annually for batch testing in the EU, contributing to a global total of 600000 animals, including 70000 in the UK alone [51]. The MBA is labour-intensive, costly, and slow (at least 4 to 6 days), making it unsuitable for urgent cases [52]. Significant advancements in alternative testing methods to detect and identify botulinum neurotoxin and botulinum neurotoxin producing species of *Clostridium* have emerged over the past decade and can support a clinical diagnosis of botulism, but face challenges with validation and acceptance as a replacement standard [16].

An alternative mouse assay to investigate the potential of botulinum neurotoxins for therapeutic purposes, consist in a local muscle paralysis as the endpoint following subcutaneous injection of botulinum toxin type A [53]. This nonlethal approach matches the conventional bioassay in both sensitivity and specificity in addition to preventing the causes of distress or impaired movement in the animals. However, it is primarily designed for testing the potency of purified neurotoxins of therapeutic preparations and has not been validated for use in microbiological laboratories that analyse complex sample matrices, like faeces, blood, pus, and foods, which may interfere with test reactions [16].

Cultivation and isolation is also possible for the detection of *Clostridium botulinum*, but it requires strict anaerobic conditions for growth which creates challenges for laboratory work. All culture media, glassware and plastic supplies must be deoxygenated before any contact with the bacteria. Reducing agents like thioglycolate can be included in media to sustain anaerobic conditions during culture and incubation [16].

The cultivation of *Clostridium botulinum* relies on conventional methods involving liquid media, followed by toxin detection in the culture supernatant, using the mouse bioassay. Positive samples are then streaked onto solid media, and toxin production by individual colonies is traditionally confirmed through additional mouse testing [16]. However, isolating and identifying *C. botulinum* can be challenging, and its toxicity must be confirmed through mouse bioassays. Complications arise in environmental and food samples due to the presence of proteolytic and nonproteolytic nontoxigenic strains that

closely resemble *C. botulinum* both phenotypically and genetically, complicating differentiation from their toxigenic counterparts [54]. For clinical samples, such as serum and faeces, direct cultivation is possible, though pretreatment methods like ethanol exposure are often employed to eliminate vegetative bacteria while allowing the recovery of bacterial spores. Alternatively, heat treatment can be used to remove non-spore-forming bacteria, but careful temperature selection is critical, as group II spores are less heat-resistant than group I spores. Heating at 60°C for 10-20 minutes is considered a safer approach for recovering group II spores [16]. The most widely used nonselective broth for *C. botulinum* cultivation is tryptone peptone glucose yeast extract (TPGY). Blood agar and egg yolk agar (EYA) serve as common nonselective plating media, with EYA enabling the detection of the lipase reaction typical of *C. botulinum*. Colonies displaying positive lipase activity (an iridescent sheen on the colony surface) and lecithinase activity (an opaque precipitate surrounding the colony) must be collected and screened individually to detect botulinum neurotoxin (BoNT) genes or the colony's ability to produce BoNTs [16]. However, all of these media are nonselective, allowing the growth of various bacterial species, which complicates the isolation process. This challenge is exacerbated by the differing physiological requirements of *C. botulinum* strains. Group I strains grow optimally at 35–37°C, while group II strains prefer 25–30°C. A compromise incubation temperature of 30°C has been proposed, though parallel incubation at 26–30°C and 35–37°C is recommended to ensure optimal growth for both groups [16]. In vitro identification of BoNT in and around colonies grown on agar plates could significantly help the identification and isolation of *C. botulinum*, as well as neurotoxin-producing *C. butyricum* and *C. baratii*, from among competitive microflora. Immunological techniques such as immunodiffusion or immunoblotting have been proposed for this purpose. A single *C. botulinum* colony can produce up to 10^5 minimal lethal doses (MLD) of toxin within 24 hours, and the colony immunoblot assay, capable of detecting as little as 10-25 MLD_{50} (50% MLD) of toxin per spot, provides sufficient sensitivity for colony identification. However, the application of these techniques to naturally contaminated clinical or food samples, or to mixed bacterial populations, has not yet been reported [16].

The isolation and identification of *Clostridium botulinum* are inherently laborious and time-consuming, especially when dealing with samples containing high levels of competitive bacterial flora, such as faecal and environmental specimens. Multiple rounds of broth cultures and platings are often required to obtain a pure culture, with no guarantee

of success. Furthermore, the presence of nontoxigenic *C. botulinum*-like strains frequently complicates the process, as they can outcompete or obscure the growth of toxigenic strains [16]. Due to these limitations, molecular methods such as PCR have gained importance as complementary techniques. By amplifying the bacterial population during incubation, PCR improves detection sensitivity, particularly in cases of low bacterial contamination, offering a more reliable and efficient alternative to traditional methods [16].

4.2. Advances in Diagnostic Technology

The rapid onset of botulism, the extreme toxicity of BoNTs, and the lack of treatments to reverse paralysis necessitate a detection method that is not only highly sensitive and specific but also versatile enough to analyse food and environmental samples effectively [52]. Numerous detection platforms have been developed to identify botulinum neurotoxins, all with the shared objective of replacing the mouse bioassay (MBA) while adhering to the principles of the 3Rs: replacement, refinement, and reduction of animal use in research [52]. Among these, many in vitro or cell-based assays have been designed to detect BoNTs or characterise BoNT-producing organisms using PCR, offering alternatives that reduce or potentially eliminate the need for animal testing. However, despite these advancements, many recent assays are not fully validated for detecting all toxinotypes in clinical specimens or food matrices, highlighting ongoing challenges in achieving comprehensive and reliable testing [37].

Compared to the mouse test, immunoassays are technically simpler and faster to perform and interpret. However, many early immunoassays, such as radioimmunoassay, gel diffusion assay, passive hemagglutination assay, and initial versions of enzyme-linked immunosorbent assay (i.e., ELISA), suffer from poor sensitivity or specificity [16]. A significant limitation of immunological tests is the general unavailability of high-quality antibodies. Additionally, inactivated toxins (e.g., those subjected to heat treatment) can lead to false-positive results, while genetic variations within different neurotoxin serotypes may reduce monoclonal antibody affinity, potentially causing false negatives [16].

Immunoassays represent a rapid, sensitive, and reproducible approach to detect and quantify botulinum neurotoxin proteins across various sample types [37]. These methods,

including ELISA and its advanced variant, the Luminex assay, utilise antibodies to target BoNT proteins. The Luminex assay, using microsphere beads conjugated to antibodies, has demonstrated superior detection limits compared to the traditional mouse bioassay [37]. Despite their advantages, such as ease of transferability and potential for high-throughput analysis, immunoassays are limited by their dependency on the availability of high-quality reagents like antibodies and their inability to determine the functional activity of the toxin [37].

In contrast, mass spectrometry coupled with endopeptidase activity assays, particularly the Endopep-MS approach, offers unparalleled sensitivity and specificity for detecting both active and inactive BoNTs while also distinguishing between toxinotypes [23]. This method identifies BoNT cleavage products, knowing that each substrate sequence is uniquely attributed to a specific toxinotype, making it highly effective for use in complex food matrices and clinical samples [55]. The introduction of an immunoaffinity enrichment step has further optimized the Endopep-MS format, achieving detection limits comparable to or exceeding those of the mouse bioassay [55]. Despite these advancements, challenges remain. Robust testing of peptide substrates against all BoNT subtypes is critical to avoid false negatives during botulism outbreaks, particularly when the specific subtype is unknown. Additionally, further refinement is necessary to enhance detection and differentiation across the numerous BoNT subtypes. Recent modifications to the Endopep-MS protocol, such as the inclusion of a salt washing step and a protease inhibitor cocktail, have significantly improved sensitivities for BoNT/C and C/D, which are prominent in animal botulism diagnostics for cattle, horses, and avian species. While promising, the broader implementation of Endopep-MS in reference laboratories requires additional inter-laboratory validation across diverse clinical, food, and environmental matrices to ensure its widespread applicability [37].

Cell-based assays (CBAs) represent a significant advancement in botulinum neurotoxin detection and potency testing, offering a reduction in animal testing while mimicking the toxin's biological activity. These assays involve neuronal networks that replicate the key steps of BoNT activity: binding, endocytosis, translocation, and cleavage of SNARE substrate proteins. As a result, CBAs can achieve sensitivity levels comparable to the traditional mouse bioassay (MBA) while also allowing for upscaling with unlimited cell sources. They are particularly useful for assessing neutralizing antibodies, testing toxoids, and evaluating antitoxin preparations, with endpoints such as SNARE cleavage products

measurable through Western blot, ELISA, or immunofluorescence [37]. The first FDA-approved CBA for BoNT testing was developed by Allergan in 2011, followed by approvals in the EU in 2012. Merz and Ipsen later developed their own CBAs, receiving EU approvals in 2015 and 2018, respectively [20]. However, these assays were designed specifically for potency testing of purified single serotypes, like BoNT-based drug products, and are not suitable for diagnostic applications in clinical or food matrices due to their inability to handle complex sample types [20]. Despite their potential, CBAs face technical challenges that limit their robustness and applicability. Maintaining well-differentiated neuronal cells derived from embryonic or induced pluripotent stem cells is complex, and high batch-to-batch variability impact the reproducibility. Furthermore, these assays are sensitive to interference from sample matrices, making them less suited for certain diagnostic applications. Nonetheless, neuronal CBAs remain a promising tool for BoNT testing and continue to evolve through ongoing research and development [37].

Polymerase Chain Reaction (PCR) has emerged as a powerful tool for detecting botulinum neurotoxin genes with high sensitivity and specificity, offering rapid results compared to conventional methods. PCR-based techniques focus on identifying the presence of the botulinum neurotoxin gene rather than detecting the toxin itself or its activity, making them particularly useful for screening bacterial colonies, pure cultures, and sample enrichments for *Clostridium botulinum* or related toxin-producing clostridia. This gene-focused approach eliminates the need for laboratory animals and is ideal for epidemiological studies, such as tracing outbreak strains and analysing phylogeny [16, 37]. Despite its advantages, PCR detection faces challenges in analysing complex sample types like food and clinical materials, where factors such as bile salts, immunoglobulins, or high protein and fat content can inhibit the reaction, drastically reducing sensitivity. These limitations necessitate thorough optimization of PCR protocols and sample preparation for each material type [16]. Greater sensitivity is typically achieved when extracted DNA, rather than crude cell lysates, is used as a template, although DNA extraction can be labour-intensive and time-consuming. In contrast, cell lysates are quicker to prepare but may compromise detection efficiency [16]. Another critical consideration is the inherently low levels of *C. botulinum* in natural samples, often ranging from 10 to 1000 spores per kilogram, coupled with the organism's spore-forming nature [16]. Direct PCR detection may fail under these conditions, necessitating enrichment steps to germinate spores and increase target cell concentrations. However,

enrichment must be carefully managed, as too short incubation can allow competitive bacteria to dominate, while excessive incubation may lead to lysis or sporulation of *C. botulinum* cells. Ideally, PCR should be performed on cultures over multiple days to identify the optimal enrichment duration for each sample type and medium [16]. These refinements ensure the reliability of PCR as a diagnostic tool for *C. botulinum* in diverse food, clinical, and environmental matrices, although it does not confirm the actual toxicity of the sample [37].

Several types of Polymerase Chain Reaction methods have been developed for the detection of *Clostridium botulinum* and its neurotoxin genes, each with distinct advantages and limitations. Conventional PCR is widely used for detecting specific BoNT genes, but it identifies the presence of DNA rather than the active toxin, leading to potential false positives when silent toxin genes are present [23, 56]. To address this, reverse transcription PCR (RT-PCR) targets gene expression instead of DNA, ensuring that only produced neurotoxins and live cells are detected. However, RT-PCR requires high-quality RNA, which can be labour-intensive and time-consuming, particularly during outbreaks [16]. Multiplex PCR represents a significant advancement, allowing for the simultaneous amplification of multiple target genes in a single reaction. This method is particularly useful for traceback investigations in foodborne botulism cases, as it generates comprehensive data while reducing assay time, labour, and costs. The resulting PCR products can be detected through conventional gel electrophoresis or by hybridization [54, 56]. Real-time PCR enhances traditional PCR by enabling real-time monitoring of DNA amplification during each cycle, providing rapid and quantitative data about the target sequence. This approach reflects the level of *C. botulinum* present but requires careful standardization, appropriate reagent selection, and knowledge of the target gene's copy number in the genome for reliable quantification [16].

Despite these innovations, PCR methods remain indirect tools, as they detect genetic material rather than the active neurotoxin, necessitating complementary assays for definitive confirmation such as a mouse bioassay [23].

5. PCR and Metagenomics in Honey Analysis

Botulinum neurotoxins (BoNTs) exhibit significant immunological and genetic diversity. This stress the need for rapid, accurate, and reliable detection methods to effectively monitor outbreaks and maintain botulism surveillance. In recent years, culture-independent methods have revolutionized the study of honey microbiota by enabling the direct analysis of microbial DNA, RNA, or protein patterns without the need for traditional cultivation techniques. Among these, PCR-based molecular methods, including quantitative PCR (qPCR) and multiplex PCR, have been pivotal in providing insights into microbial diversity. Additionally, advances in next-generation sequencing technologies have significantly enhanced the resolution of honey microbiota composition, surpassing the capabilities of conventional culture methods. These cutting-edge techniques have facilitated the development of interdisciplinary fields such as metagenomics, metatranscriptomics, and metaproteomics, opening new avenues for exploring the complex microbial communities in honey [3].

5.1. Real-Time PCR and Multiplex PCR

PCR, or Polymerase Chain Reaction, is a fundamental technique in molecular biology that amplifies a specific segment of DNA. It is widely used in various scientific disciplines, including genetics, forensics, medical diagnostics, and evolutionary biology. This technique is based on the enzymatic amplification of DNA and allows the selective amplification of a specific DNA sequence from a complex mixture [54]. PCR offers high sensitivity and specificity for detecting microorganisms, including *Clostridium botulinum*, by targeting the botulinum neurotoxin gene. However, since PCR detects the gene rather than the toxin itself, a positive result does not necessarily indicate the presence of the toxin in the sample [16].

Real-time PCR (RT-PCR) and multiplex PCR are both variations of the Polymerase Chain Reaction technique, but they serve different purposes and have distinct characteristics.

Real-time PCR, also known as quantitative PCR (qPCR), measures the amplification of DNA during each cycle in real-time. It allows for the quantification of the initial amount of DNA or RNA in the sample by monitoring the fluorescence emitted by fluorescent dyes or probes. This method is particularly valuable for pathogens like *Clostridium botulinum*,

where traditional culture techniques face significant challenges. Due to the low prevalence of *C. botulinum* in naturally contaminated samples (10-1000 spores/kg) and the lack of selective media, direct quantification is difficult. Instead, enrichment in liquid media is often required prior to detection [16]. Real-time PCR assays typically employ toxin type-specific primers, but their application is limited to detecting one serotype at a time, which increases costs and time when screening for multiple serotypes [54]. Moreover, designing universal primers for BoNT genes is complicated by their nucleotide diversity (identity ranges from 56% to 76%) and data bank collection of all available DNA sequences encoding the target genes is necessary [57]. Despite these challenges, assays targeting conserved genes such as BoNT and NTNH (parts of the toxin complex), which are associated with all BoNT-producing strains, provide an effective solution. Using enrichment cultures, real-time PCR can rapidly and sensitively screen for *C. botulinum* in food specimens, streamlining the detection process [57].

Multiplex PCR is a versatile molecular technique that allows the simultaneous amplification of multiple DNA sequences in a single reaction by including multiple primer sets specific to different targets sequences. Each primer set amplifies a distinct target, allowing for the detection of multiple targets in a single reaction tube. Detection typically occurs at the end of the reaction using gel electrophoresis, where the amplified products are separated based on size and visualized under UV light. This approach is particularly useful for the detection of *Clostridium botulinum*, enabling the simultaneous identification of multiple botulinum neurotoxin serotypes, such as A, B, E, and F, through primers designed for nonhomologous regions of their respective genes [54]. A combination of multiplex PCR and two-step enrichment has proven highly sensitive, capable of detecting low spore counts in complex sample types. However, enrichment conditions must be tailored to optimize detection for specific materials. Advances in whole-genome sequencing and comparative genomic tools have further refined multiplex PCR assays, allowing for accurate characterization of *C. botulinum* species and subgroups, even within mixed cultures [58]. For example, a multiplex PCR targeting the NTNH gene - a gene associated with the botulinum neurotoxin gene cluster and conserved marker of BoNT-producing strains - can be paired with markers for identifying members of *C. botulinum* group I, *C. sporogenes*, the *C. botulinum* group II E subgroup, and the *C. botulinum* group II BEF subgroup [58], which can efficiently identify and classify isolates, providing both serotype and taxonomic context. This makes multiplex PCR a

rapid, cost-effective tool for screening food, clinical, and environmental samples. Multiplex PCR can also be combined with real-time detection methods for simultaneous amplification and quantification of multiple targets.

The analysis of honey using PCR methods for the detection of *Clostridium botulinum* spores poses significant challenges due to the complex composition of honey and its natural properties. Honey's high sugar content (at least 80%) can inhibit PCR reactions and prevent *C. botulinum* spores from germinating, complicating the direct detection of this pathogen [59]. Its viscosity further interferes with standard laboratory protocols, while its antibacterial properties and the presence of DNA polymerase inhibitors, such as alkaloids and polyphenols, can impair enzymatic activity and DNA extraction [2]. It also seem that darker honeys, which contain higher levels of phenolic compounds, tend to show greater PCR inhibition than lighter varieties, reflecting the influence of plant-derived secondary metabolites [60]. Given these barriers, pre-treatment steps are essential to prepare honey for analysis. Methods often involve diluting honey with distilled water and a 1% Tween 80 solution, followed by centrifugation or filtration to separate potential inhibitors from the sample. Some protocols use the supernatant for PCR, while others rely on the pellet sediment obtained after centrifugation. Furthermore, an enrichment culture under anaerobic conditions is often required to amplify the low levels of *C. botulinum* spores present, as contamination levels are generally minimal but still capable of producing harmful toxins [12]. The complex matrix of honey, containing DNA from plants, bees, bacteria, and other microorganisms, complicates the specificity of PCR-based detection methods. Efficient DNA extraction, ensuring high-quality and pure genetic material, is critical to overcome these issues. Advances in sequencing technologies, such as metagenomic approaches, have been employed to bypass PCR inhibitors and directly analyse environmental DNA (eDNA) from honey. However, the multi-kingdom DNA present in honey further underscores the need for precise methods to distinguish *C. botulinum* DNA from other sources while mitigating inhibitory factors [2].

5.2. Metagenomic Approaches

Metagenomics is a field of study within genetics and molecular biology that involves the analysis of genetic material recovered directly from environmental samples. Unlike traditional genomics, which focuses on the genomes of individual organisms,

metagenomics explores the collective genomes of entire communities of microorganisms present in an environment. These microorganisms can include bacteria, viruses, fungi, and other microscopic organisms. Environmental samples are collected from various sources such as soil, water, air, food or the human body. Genetic material (usually DNA) is extracted from the collected samples. This DNA represents the combined genomes of all microorganisms present in the sample. The extracted DNA is then sequenced using high-throughput sequencing technologies, which determine the order of nucleotides (the building blocks of DNA) in the genetic material. The sequenced data is analysed using bioinformatics tools and techniques. This analysis involves tasks such as genome assembly, where the short DNA sequences obtained from sequencing are stitched together to reconstruct the genomes of the microorganisms present in the sample, and taxonomic classification, where the organisms are identified and classified based on their genetic sequences. In addition to identifying the organisms present, metagenomic analysis can also reveal the functional capabilities of the microbial community. This involves predicting the functions encoded by the genes in the metagenome, such as metabolic pathways or antibiotic resistance genes.

Metagenomics has applications in various fields, including environmental microbiology, biotechnology, medicine, and ecology.

Metagenomics, particularly through shotgun sequencing, offers a transformative approach to analysing honey by enabling the direct sequencing of environmental DNA (eDNA) without prior PCR enrichment [61]. This method provides a comprehensive view of the microbial and environmental DNA present in honey, which reflects the colony ecosystem and the landscapes from which honeybees collect nutrients. The honey core microbiome overlaps with the microbial communities found in nectar, pollen, and the bee stomach, illustrating the interconnectedness of the hive environment [62]. The process typically involves meticulous preparation steps to extract DNA from honey, which includes washing, centrifugation, and bead-based disruption to isolate high-quality genetic material [61]. This is critical given the challenges posed by honey's antimicrobial properties, such as hygroscopicity, hyperosmolarity, and antibiotic content, which limit the survival of most microorganisms in their active forms.

Shotgun sequencing generates a large volume of data, with millions of reads requiring sophisticated bioinformatics tools for analysis. Techniques such as supervised binning,

which aligns sequences to databases like NCBI using BLAST, enable annotation and identification of microbial and environmental DNA. However, the high computational demand of these analyses highlights the need for robust data processing pipelines [61].

By capturing a broad spectrum of DNA, metagenomic analysis extends the utility of honey beyond food safety, enabling its use as a biomonitoring tool. It provides insights into biodiversity, the environmental conditions of the hive's surroundings, and potential pathogens or contaminants, showcasing honey's value in ecological and environmental studies.

6. Infant Botulism

Infant botulism is an intestinal toxaemia (i.e., imbalance in internal flora) that manifests with paralysis progressing from the upper part of the body to the limbs (i.e., descending paralysis), bowel dysfunction (i.e., constipation), and, in some cases, respiratory failure [63]. The underdeveloped and immature intestinal flora in infants provide a conducive environment for ingested spores to germinate and generate botulinal neurotoxins within the intestinal tract.

6.1. History and Occurrence

The primary zoonotic pathogen associated with honey consumption is *Clostridium botulinum*, which is notably linked to cases of botulism in infants under one year of age. Until 2005, honey was the only food item linked to infant botulism, when Brett *et al.* (2005b) [64] reported a suspected case attributed to infant milk formula. First identified in 1976 [65, 66], this disease has been documented in numerous countries, with the United States reporting the highest number of cases, albeit with a low incidence rate. 1345 cases of infant botulism were reported in the USA from 1976 to 2016, with 45 of the 58 California counties being represented [67].

In 2019, in the USA, health departments reported 215 cases of botulism, out of which 152 cases were infant botulism (71%) [50]. Infant botulism has been reported over four continents. The highest number of cases have been documented in the USA, Canada, Argentina, Australia, Japan and Italy [68]. Most infected individuals were breastfed infants residing in rural areas and/or peri-urban areas.

Honey is a well-known resource which carries *Clostridium botulinum* spores and is considered as an important food related risk factor for infant botulism [69]. The spores of *Clostridium botulinum* can survive in honey at low temperatures (4°C) and are spore-forming microorganisms. Infant botulism results from spores' germination in the intestine with in-vivo toxin production [70].

Honey samples were collected from different parts of the world and tested positive for botulinum toxins [70]. In many studies, the association between honey and infant botulism has been demonstrated. Honey consumption was associated with 15-35% of reported cases of infant botulism in the literature [71]. Consequently, it is recommended to exclude honey from infants' diets and the consumption of honey, or any foods containing it, is prohibited for children under 12 months of age.

6.2. Pathophysiology and detection

Infant botulism is an acute reversible neuroparalytic infection [68], yet a rare condition with a peak incidence in infants of 2 to 8 months of age. However, Avila *et al*. (2023) [68] reported incidences in infants as early as one week old. When it manifests in an infant, it is often from the botulinum neurotoxin (BoNT) serotypes A and B, rarely from serotypes E and F [63]. Type C has been reported to have caused botulism in a single case of infant botulism [72].

Infants ingest the spores of *Clostridium botulinum* due to exposure to contaminated soil or farmed products, especially honey when the bacteria develop and release the toxins in the intestines [63, 68]. In Argentina, a link has been established between the presence of *Clostridium botulinum* spores in the soil and the occurrence of infant botulism in various regions [68]. Avila *et al*. (2023) [68] reported that Type A toxin was predominant, being detected in soil samples and all documented cases of the illness. Several studies have identified potential sources of spore contamination contributing to infant botulism cases, including honey, milk formulas, and certain herbs like chamomile, which are likely to be contaminated.

The severity of infant botulism ranges from mild hypotonia to life-threatening sudden flaccid paralysis, with severe cases often requiring conventional mechanical ventilation. An estimate of 3-5% of infant death is diagnosed with a sudden death syndrome [68]. Unlike other forms of botulism, infant botulism has a mortality rate of just 1%. However,

it is associated with significant morbidity, including prolonged hospitalisation, extended use of medical ventilation and nasoenteral tube feeding. Current available treatment for infant botulism comprises supportive care during the period of muscle weakness and specific antitoxin therapy. Limited evidence exists regarding ear, nose, and throat complications linked to prolonged mechanical ventilation, as well as the frequency and potential benefits of tracheostomy in these patients [68].

Laboratory criteria for diagnosing botulism in infant include detecting BoNT in stool or serum or isolating *Clostridium botulinum* from stool samples [68]. The mouse bioassay is regarded as the gold standard for botulism diagnosis. This method involves injecting mice with samples from suspected patients and observing the development of botulism symptoms in the laboratory mice. The specific type of toxin is identified by neutralising it using specific antitoxins.

Infant botulism is a potentially severe disease that may require intensive care, including conventional mechanical ventilation. Avila et al. (2023) [68] highlighted challenges in administering specific treatments within the recommended timeframe using the current diagnostic strategy. While timing may be improved by optimising preliminary typing tests, the process faces additional obstacles, including cost-related concerns and ethical dilemmas surrounding the use of live animals. To address these issues and reduce diagnostic time to less than 24-hours, a highly sensitive polymerase chain reaction (PCR) method for detecting and typing toxins in stool samples has been under development since the 2000s [68]. However, conventional PCR methods have limitations, such as an inability to distinguish between biologically active toxin genes and silent ones. Although significant advancements have been made in botulinum neurotoxin detection technologies, none have yet fully been able to replace the mouse bioassay.

Administering equine botulinum antitoxin (EqBA) significantly reduces the duration of conventional mechanical ventilation and hospital stays [68]. EqBA is most effective when given early, while the toxin remains in the plasma and before it is internalised into cholinergic presynaptic terminals. However, anaphylaxis has been reported in 1-2% of patients receiving EqBA [68]. In the USA, a human-derived botulinum antitoxin has been developed and approved by the US Food and Drug Administration (FDA) for treating infant botulism [68]. Despite its benefits, this treatment can be costly and challenging to obtain.

In many cases, antitoxin administration is based on clinical suspicion rather than confirmed diagnostic evidence of infant botulism. Electrodiagnostic techniques, such as electromyoneurography (EMNG), can be helpful while awaiting for diagnostic confirmation. However, the sensitivity of EMNG can vary, and it is not readily available in all healthcare facilities.

7. Implications for Public Health and Safety

This chapter examines the broader impact of this rare but significant disease, called botulism, affecting multiple species, including humans, birds, and cattle. In humans, the disease is exceedingly rare, with an annual occurrence of approximately 10 cases reported in France [49]. In Europe, the incidence rate stands at about 0.02 cases per 100 000 inhabitants, mirroring rates in France. Countries such as Italy, the United Kingdom, Poland, Romania, and France report the highest confirmed case numbers. Historical data reveals variations in disease manifestations: in Italy, 93% of the 466 cases documented between 1986 and 2015 were foodborne, while infant and wound botulism accounted for only 6% and 1%, respectively. Conversely, in Turkey, home-canned vegetables were identified as the primary source in 95 cases recorded from 1983 to 2017. Meanwhile, Ukraine reported 8614 cases from 1955 to 2018 [49]. In the United States of America, infant botulism is the predominant form, contributing 80% of global childhood cases since its recognition in 1976, with an annual incidence of 2.1 cases per 100 000 live births. Similarly, Canada observed an average annual incidence of 4.3 cases per million live births from 1979 to 2019 [49]. While surveillance data for animals remains sparse globally, the disease is known to affect birds and cattle at much higher rates, posing a notable challenge to both animal health and food safety systems. This epidemiological overview underscores the importance of addressing the disease's implications for public health, food security, and veterinary practices.

7.1. Public Health Risks

First of all, diagnosing *Clostridium botulinum* during an outbreak presents numerous challenges, starting with the detection of BoNT-producing strains in samples with low and uneven contamination [49]. Environmental and food samples often contain proteolytic and nonproteolytic nontoxigenic strains that closely resemble *C. botulinum*

phenotypically and genetically, making it difficult to distinguish toxigenic strains [54]. Conventional PCR, a widely used detection method, identifies DNA rather than active toxin, which can lead to false positives if silent toxin genes are present [16, 23]. Furthermore, PCR's effectiveness can be hampered by the complexity of food and clinical sample matrices, as components like honey, bile salts, immunoglobulins, or high protein and fat content inhibit the reaction, reducing sensitivity [56]. Designing universal primers for BoNT genes is also complicated due to their genetic diversity, necessitating a comprehensive database of all target gene sequences [57]. Enrichment cultures under anaerobic conditions are often required to detect the low spore counts present in suspect samples, but even these steps can be hindered by factors such as the high sugar content of honey, which inhibits spore germination and PCR enzymatic activity [59]. Advances in genomic tools and multiplex PCR have improved the ability to differentiate *C. botulinum* species and subgroups, even in mixed cultures, but challenges remain in analysing diverse sample types with high sensitivity and specificity [58].

Adding to these difficulties, the various forms of botulism - foodborne, wound, infant, iatrogenic, and intestinal - require tailored diagnostic and treatment approaches, complicating a unified public health response. Each form involves distinct sources and pathways of contamination, necessitating the analysis of diverse matrices, such as food, stool, serum, and environmental samples, each requiring specific preparation and enrichment protocols. For instance, foodborne botulism typically involves testing food products for toxin presence, while infant botulism relies on stool sample analysis due to the colonization of the gut. Wound botulism and iatrogenic cases require clinical samples like wound swabs or injected material, and intestinal botulism presents further challenges as the toxin is produced in situ. The heterogeneity of matrices and clinical presentations highlights the complexity of managing botulism outbreaks and underscores the need for specialised diagnostic and response strategies for each form.

Moreover, working with *Clostridium botulinum* presents significant dangers due to the extreme potency of its botulinum neurotoxin, which is one of the most toxic substances known. The lethal dose (LD50) of BoNT depends on the route of entry into the body, with estimates for an average 70 kg adult being approximately 70 μg if ingested, 0.7-0.9 μg if inhaled, and as little as 0.09-0.15 μg if administered intravenously [20]. This necessitates strict safety protocols to protect laboratory personnel and prevent accidental exposure. Handling *C. botulinum* should only be conducted by trained personnel within Biosafety

Level 2 (BSL-2) facilities, which provide the necessary containment measures for moderate-risk agents that pose hazards through accidental inhalation, ingestion, or skin exposure [16]. These facilities mandate controlled access, specialised safety equipment, and rigorous practices to minimise risks.

Given the potential use of botulinum neurotoxin as a bioterrorism agent, additional precautions are often implemented to restrict access to laboratories housing toxic cultures, safeguarding public health and national security. The most notorious example of a BoNT bioterrorism attempt was by the Japanese religious cult "Aum Shinrikyo", which failed to release aerosolised BoNT preparations on three separate occasions in Tokyo before resorting to the release of sarin nerve gas on the Tokyo subway in March 1995 [20]. To mitigate such risks, the U.S. Department of Health and Human Services categorises biological agents into four biosafety levels [73]. While BSL-2 is sufficient for *C. botulinum* under controlled conditions, BSL-3 or higher may be warranted when handling aerosolized forms or conducting research involving higher-risk scenarios. BSL-3 and BSL-4 facilities are designed to contain agents that pose greater risks of airborne transmission and severe disease, underscoring the critical importance of selecting the appropriate biosafety level based on the nature of the work and the agent being studied.

Finally, diagnosing botulism presents significant challenges due to its rarity and the potential for its symptoms to mimic other neurological and toxic conditions. Clinical diagnosis relies heavily on the presentation of characteristic features, including descending symmetrical flaccid paralysis, cranial nerve dysfunction, and gastrointestinal symptoms, as well as the epidemiological context. However, initial symptoms often overlap with other conditions such as Guillain-Barré syndrome, myasthenia gravis, Lambert-Eaton syndrome, or toxic exposures (e.g., organophosphates, snake venom), which complicates prompt recognition [74]. Laboratory confirmation involves detecting botulinum neurotoxin or identifying *Clostridium botulinum*, but this process is time-consuming and prone to delays, particularly if samples are not collected or stored properly at 2-8°C to preserve toxin integrity [20].

Despite the diagnostic challenges, early administration of antitoxin is critical to neutralize circulating toxins and prevent further neuromuscular damage. The antitoxin cannot reverse paralysis but can block BoNT from binding to presynaptic receptors, making timely treatment essential [20]. Given the rapid progression of symptoms and the

potential for fatal outcomes, treatment should begin based on clinical suspicion without awaiting laboratory confirmation. Awareness among healthcare providers and rapid consultation with specialised laboratories, such as the CDC, are crucial for managing this life-threatening condition and preventing delays in care [23, 46].

7.2. Safety Measures and Regulations

The resilience of *Clostridium botulinum* spores represents a significant challenge in food safety, particularly for minimally processed foods like honey. Traditional sterilization techniques require extreme conditions, such as the "botulinum cook" (121°C for 3 minutes), which may be unsuitable for preserving the qualities of certain food products. The "botulinum cook" is a thermal processing standard developed to ensure the safety of low-acid canned foods by effectively eliminating *Clostridium botulinum* spores, which are highly heat-resistant. This process involves heating food to 121°C for 3 minutes, a treatment designed to achieve a 12-log reduction (12-D process) in the number of spores of proteolytic *C. botulinum*, thereby reducing the risk of botulism to negligible levels [13, 24]. The origin of the botulinum cook dates back to studies by Esty and Meyer (1922) [75], who evaluated the heat resistance of 109 *C. botulinum* strains and determined that heat resistance varied depending on the strain and the composition of the media used. Their work laid the foundation for the canning industry's adoption of this rigorous heat treatment, which has ensured the safe production of low-acid canned foods for decades. However, botulism outbreaks may still occur if the heat treatment is not correctly applied or if post-process contamination occurs [13]. This highlights the critical importance of adhering to this safety standard in food processing.

Understanding the germination process of *Clostridium botulinum* spores offers significant opportunities to enhance food safety, particularly in challenging matrices like honey [20]. Inducing germination under controlled conditions, followed by the destruction of the more vulnerable vegetative cells, would present a promising alternative to traditional high-temperature sterilization methods. Such strategies could preserve the sensory and nutritional qualities of food while reducing the risk of botulinum toxin production. However, the germination process is highly strain-dependent, with significant differences observed between proteolytic and non-proteolytic *C. botulinum*. Specific germinants, such as L-alanine and L-lactate, have shown effectiveness in enhancing germination, but their success often depends on combinations of germinants and precise environmental

conditions, including pH and heat-shock activation [24]. Further research into germinant receptors, mechanisms, and the lag phase could pave the way for tailored interventions to either block germination entirely or promote controlled germination for targeted spore elimination [13]. Honey's unique properties, such as its low water activity and complex composition, may influence the germination process, necessitating adjustments to protocols. By leveraging insights into strain-specific germination mechanisms and optimising treatment combinations, it may become possible to develop novel strategies for controlling *C. botulinum* in the food industry, reducing the risk of contamination and improving food safety [20, 24].

The World Trade Organization (WTO) has established food safety regulations to safeguard public health and ensure the global safety of food products. Complementary measures have been adopted worldwide to promote safe food processing practices and minimize the incidence of foodborne illnesses. Among the significant concerns in food safety is *Clostridium botulinum* Group I, which is prevalent in the environment and poses a serious risk of foodborne botulism when pre-formed botulinum neurotoxin contaminates food. The spores produced by Group I strains are highly heat-resistant, making them a particular challenge in the safe production of low-acid canned foods [37]. These regulations are reinforced by standards like the ISO norms and the Codex Alimentarius [4], which provide a framework for ensuring food safety and quality in international trade.

The International Organization for Standardization (ISO) has developed a technical specification addressing the detection of *Clostridium botulinum* in various matrices, including products for human consumption, animal feed, and environmental samples. This specification, titled "Microbiology of the food chain - Polymerase chain reaction (PCR) for the detection of food-borne pathogens - Detection of botulinum type A, B, E, and F neurotoxin producing clostridia", outlines the use of Polymerase Chain Reaction (PCR) as a method for identifying specific neurotoxin-producing clostridia in complex samples [76]. Although the full technical details of the standard are proprietary, its existence underscores the importance of internationally recognised methods for ensuring food safety. By providing a consistent framework for the detection of these pathogens, ISO/TS 17919:2013 facilitates global standardisation, promotes best practices, and supports regulatory compliance. This specification is particularly relevant for industries where the risk of botulinum contamination poses significant public health concerns, such

as honey production. The adoption of such standards can play a vital role in enhancing food safety measures, enabling more precise and reliable pathogen detection while fostering international collaboration in addressing the risks associated with *Clostridium botulinum*.

In France, botulism is notifiable in both humans and animals, with mandatory reporting to health authorities. Human botulism has been monitored since 1978 by the National Reference Centre for Anaerobic Bacteria and Botulism (NRC), and its notification to Santé Publique France (SPF) has been compulsory since 1986. Animal botulism became regulated in 2006, with surveillance expanded to wild birds and cattle by the National Reference Laboratory (NRL) from 2011 and 2017, respectively [49]. At the European level, botulism is part of List B in Annex I of the zoonoses directive, yet notification requirements vary, particularly for animals. Effective management requires systemic, transdisciplinary approaches, as human cases are often linked to the pig sector (e.g., ham) or canned vegetables, emphasizing the need for integrated data collection across food, animal, and environmental sectors [49]. Additionally, botulinum neurotoxin is a critical focus in biodefense, classified as a Category A bioweapon by the U.S. Centers for Disease Control and Prevention (CDC), necessitating robust detection and prevention measures [37].

The regulation and safety of botulism management involve stringent measures across human, animal, and environmental health sectors. We could also add the food safety regulation with the definitions stated in the Codex Alimentarius [4]. More specifically, honey is defined as a natural product that must not contain any added food ingredients, including food additives, nor have any foreign substances introduced. Honey must remain free from objectionable matter, flavours, aromas, or taints absorbed during processing and storage, and it should not show signs of fermentation nor effervescence. Pollen or other inherent honey constituents may only be removed if unavoidable during the removal of foreign organic or inorganic matter. Processing and heating should not alter the essential composition or impair the quality of the honey. Chemical or biochemical treatments intended to influence crystallization are strictly prohibited. The moisture content must not exceed 20%, and the combined fructose and glucose content should be at least 60% (or 45% for honeydew honey). Sucrose content should not exceed 5%, although lavender honey may reach up to 15%, provided the sucrose originates naturally from nectar rather than being artificially added or derived from syrup [4].

In societies where complementary medicine is widely embraced, and traditional systems of medicine play a central role, honey is often regarded as a miracle remedy with numerous benefits. It is believed to promote circulation, relieve stomach, intestinal, and colic pain, and can serve as a topical antibiotic [71]. In these contexts, convincing people that honey can pose a serious health risk to infants and lead to life-threatening illnesses presents a significant challenge [70]. Infant botulism is relatively unknown in these communities which makes the situation even more challenging. Bamumin *et al.* (2023) [69] explored, in 2022, the knowledge, attitude, and practice, concerning the relationship between honey and infant botulism, among mothers in Hail city, Saudi Arabia. The result of this study showed that only 48% of the mothers did heard about infant botulism, 40% of them knew the relation between honey ingestion and the disease and only 6.5% knew the causative agent [69]. The prevalence of feeding honey to infants before 12 months was 52%. In these societies such as Saudi Arabia, the limited reported cases of infant botulism do not necessarily indicate the rarity of the disease but might indicate a lack of awareness and, by consequence, an under-reporting due to misdiagnosis, given that infant botulism symptoms are quite varied and, in most cases, resemble other more common diseases such as sepsis [69]. Alerting public health professionals and researchers about the importance of infant botulism, its relationship with honey and the prevalence of honey-feeding to infants among Saudi mothers, could help implement appropriate maternal health education and awareness towards the specific symptoms of this disease [69]. Conducting surveillance studies targeting infant health could also be beneficial to determine infant botulism prevalence in a community [69].

8. Conclusion

Clostridium botulinum, the causative agent of botulism, represents a significant public health concern due to the severity of its neurotoxic effects. This rare, non-contagious, but potentially fatal illness results from exposure to botulinum neurotoxins, produced by various *Clostridium* species, including *C. botulinum* groups I-IV, *C. baratii*, and *C. butyricum*. Characterized by flaccid paralysis, botulism can lead to respiratory failure and death without prompt treatment. Diagnosing C. botulinum remains a challenge due to the need to differentiate toxigenic strains from phenotypically similar nontoxigenic ones, the genetic diversity of BoNT genes, and the inhibitory properties of sample matrices like honey. While advancements in molecular and genomic tools have enhanced diagnostic

accuracy, significant hurdles persist, including sensitivity in complex samples and the need for universal detection standards. These challenges underscore the importance of ongoing research and improved diagnostic methodologies to better manage and mitigate botulism outbreaks.

Honey, often viewed as a natural and healthful product, harbours a complex microbiota influenced by environmental factors, including the nectar sources and hive conditions. Among the microbial communities identified in honey are osmotolerant and acidotolerant bacteria (77 gena, [3]), as well as diverse fungal gena (84 gena [3]) such as *Bettsia* and *Zygosaccharomyces*. Although many of these microorganisms are benign or beneficial, honey can also serve as a reservoir for bacterial spores, including those of *Clostridium botulinum*, posing a unique risk of infant botulism. This condition, most commonly affecting infants under one year old, occurs when ingested spores germinate and produce neurotoxins in the immature gut. The connection between honey and infant botulism highlights the dual role of honey as both a valuable natural product and a potential vector for pathogens, emphasising the need for improved safety measures in honey production and consumption.

Botulism, particularly foodborne and infant botulism, continues to present significant challenges to global food safety and public health. With a total of 6932 cases reported worldwide in the literature between January 2000 and January 2023 [77], underreporting remains a major issue due to inconsistent diagnostic capabilities and a lack of coordinated international surveillance [77]. Regulatory frameworks such as the Codex Alimentarius [4] and ISO standards [76] play a critical role in promoting safe food processing practices and mitigating risks. However, the resilience of *C. botulinum* spores, particularly in minimally processed foods like honey, demands continuous improvement in detection methods and global collaboration to strengthen food safety measures. Raising awareness among healthcare professionals, implementing harmonized reporting mechanisms, and advancing research in diagnostic technologies will be essential to reducing the global incidence of botulism and safeguarding public health.

Despite its long-standing recognition in the scientific literature, animal botulism remains an understudied and challenging disease to manage. While efforts have been made to develop diagnostic tools, significant gaps persist in understanding its epidemiology and in creating molecular epidemiological methods to track and mitigate outbreaks [78].

Unlike human botulism, which has been extensively characterized, animal outbreaks often involve different BoNT types, such as BoNT C/D in poultry and wild birds and BoNT D/C in cattle, highlighting the distinct ecological and epidemiological dynamics of the disease in animals [49]. However, the overlap in BoNT sensitivity between humans and animals exist, like BoNT E which regularly causes sporadic cases in wild fish-eating birds or some BoNT B outbreaks in cattle which have also been described in the literature [49]. Those cases highlight the potential for zoonotic risks, necessitating greater vigilance in animal surveillance to prevent cross-species contamination and safeguard public health. Integrating animal botulism into broader disease surveillance frameworks is critical, particularly in the context of the One Health approach, emphasising the importance of collaborative efforts to address diseases with shared impacts. For example, routine monitoring of botulism in both humans and animals, as demonstrated in France [49], could provide a model for integrative surveillance that can identify potential zoonotic risks and enhance our understanding of botulism's broader ecological impact. Expanding research on animal botulism seems essential to bridging these knowledge gaps and mitigating its public health implications. Enhancing epidemiological studies, developing advanced molecular tools, and fostering international collaboration can deepen our understanding of animal botulism's occurrence and its potential zoonotic significance.

Early detection of zoonotic pathogens through the improvement of laboratory capabilities and intensifying surveillance at the interface between animals and humans are essential measures in the control and prevention of zoonoses. Strengthening the connection between human and animal surveillance systems is imperative, particularly considering the omnipresence of *Clostridium botulinum* in the environment and its potential to induce illness in both human and animal populations.

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