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Report of the Scientific Committee of the Spanish Agency for Food Safety and Nutrition (AESAN) on the risk assessment of botulism resulting from the consumption of vacuum-packed or modified atmosphere-packed foods

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Abstract

Foodborne botulism is caused by the ingestion of a neurotoxin (BoNT) primarily produced by *Clostridium botulinum*. This bacterium is classified into six phenotypic Groups, with Groups I and II being associated with human disease. BoNT are divided into seven main types with different antigenic potentials, of which types A, B, E, and F are responsible for botulism in humans. These toxins are produced by *C. botulinum* Group I (mesophilic, with toxin production temperatures between 30 and 37 ºC) and Group II (psychrotrophic, capable of producing toxins at temperatures as low as 3-4 ºC). The formulation of food products (pH, water activity (a_w), NaCl concentration, addition of preser-

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vatives) is critical for assessing botulism risk, and thermal processing (sterilization) remains the primary control measure. For this reason, the risk of botulism has traditionally been associated with deficiencies in the thermal treatment of canned foods.

Additionally, V-range foods (cooked, packaged, lightly pasteurized, and ready-to-eat products that require reheating before consumption), especially those that are refrigerated and vacuum-packed or stored in modified atmospheres (known as Refrigerated Processed Foods of Extended Durability, REPFED), are particularly vulnerable. Depending on their composition, these products may permit the growth of *C. botulinum* Group II at temperatures above 3.3 ºC, with subsequent toxin production that cannot be inactivated during reheating before consumption.

To mitigate risks in this type of food, adherence to good hygienic practices throughout the production process is essential. Similarly, the formulation of the product should be designed to prevent pathogen growth (e.g., through pH control, a_w reduction, NaCl concentration, or the use of antimicrobial agents). Strict control of storage temperatures (below 4 ºC, ideally below 3.3 ºC) is also crucial, as is ensuring that consumers follow the storage and consumption instructions provided by the manufacturer.

Key words

Botulism, *Clostridium botulinum*, botulinum toxin, refrigerated V-range foods.

Suggested citation

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1. Introduction

Botulism is a serious, but rare, food-borne disease that occurs after the ingestion of a neurotoxin (BoNT) developed after the germination and multiplication of spores mainly of *Clostridium botulinum* and some other *Clostridium* species, such as *C. argentinense*, *C. baratii* or *C. butyricum*.

C. botulinum is a Gram-positive, strict anaerobic and sporulated bacillus. The spores of this microorganism are widely distributed in the soil and in aquatic sediments, and can contaminate different types of food. Based on its serological properties, seven main BoNT produced by *C. botulinum* (A, B, C, D, E, F and G) have been described. Types A, B, E and F are mainly those associated with botulism in humans. *C. botulinum* strains are also classified into four Groups (I-IV) based on their different biochemical properties, especially in relation to their proteolytic capacity. *C. botulinum* strains that produce botulism in humans belong to Group I (proteolytic and mesophilic strains that produce toxins A, B and F) and Group II (non-proteolytic and psychrotrophic strains that produce toxins B, F and E). Therefore, to evaluate the risk of botulism derived from food consumption, it is very important to consider not only the aspects related to *C. botulinum*, but also to know the conditions that lead to the development of the toxin.

During the months of June and July 2023, an increase in cases of botulism was detected in Spain, which suggested the association between the consumption of prepared dishes packaged under vacuum or in a modified atmosphere and the development of the disease. This association was not confirmed microbiologically as the presence of *C. botulinum*, or botulinum toxin, was not detected in the microbiological analysis carried out on the dishes prepared from the batches potentially linked to the outbreak. However, considering the severity of the pathology, as well as the fact that not following the instructions for preservation and use indicated on the labelling of certain foods or prepared dishes packaged under vacuum or in modified atmospheres, may pose a serious risk, it is necessary to evaluate the risk of botulism and its relationship with the consumption of these foods. On this basis, the Scientific Committee of the Spanish Agency for Food Safety and Nutrition (AESAN) has been asked to prepare a report determining:

1. The growth of *C. botulinum* in different food matrices, taking into account the factors that influence it such as oxygen content, pH, water activity (a_w), preservatives, temperature and storage time, as well as possible indications of treatment or cooking in the labelling.
2. The risk of botulism related to the consumption of foods that are packaged under vacuum or in modified atmospheres, whether or not subjected to post-packaging pasteurisation treatments, and stored at refrigeration temperatures.

2. Background

2.1 *Clostridium botulinum*: taxonomic aspects

The genus *Clostridium* is a member of the *Clostridiaceae* family, in the order *Clostridiales*, class *Clostridia* and phylum *Bacillota* (formerly *Firmicutes*) (Finegold et al., 2002). This genus consists of approximately 200 Gram-positive, spore-forming species, including notable pathogenic species such as *Clostridium difficile* (recently reclassified as *Clostridiodes difficile*), *C. botulinum*, *C. tetani*, and *C. perfringens*. The original classification was based on the phenotypic characterisation of

the isolates as strict anaerobes, Gram-positive and endospore-forming. The most recent genomic information, however, shows that many of the species classified within the genus *Clostridium* are distantly related according to the most current taxonomic principles (Lawson and Raney, 2016).

C. botulinum is a strict anaerobic bacterium, Gram-positive, sporulated, bacillus-shaped (approximately 3-8 µm long by 0.4-1.2 µm wide) (Corsalini et al., 2021) and whose cells are presented individually or grouped (in pairs or short chains). *C. botulinum* is widely distributed in nature, being able to be present in soil, dust, water and marine and freshwater sediments (Long and Tauscher, 2006) (Espelund and Klaveness, 2014). Spores can persist in soils and sediments for decades, since they present a high resistance to different environmental factors, such as desiccation, chemical agents, radiation or high concentrations of oxygen (Liu, 2024). The microorganism is also occasionally present in the intestinal content of asymptomatic animals (fish, birds and mammals) (Espelund and Klaveness, 2014).

Based on the combination of molecular analyses and metabolic profiles, six phenotypic Groups have been established within the species *C. botulinum* (I-VI) (Liu, 2024), with Group I (proteolytic) and II (non-proteolytic) strains being the most commonly associated with cases of human botulism (Artin et al., 2008).

Group I strains have proteolytic activity. Since they are mesophilic and their minimum growth temperature is 10 ºC, they have a limited importance in refrigerated foods, unless there is a break in the cold chain (Parker et al., 2015). These strains are mainly associated with cases of human botulism due to canned foods, given the high heat resistance of their spores, requiring treatments of at least 121 ºC for 3 minutes to make them inactive (Carter and Peck, 2015) (Rawson et al., 2023). Group II strains are generally psychrotrophic (they grow at temperatures below 7 ºC), producing spores of lower heat resistance than those of Groups I and III. They are non-proteolytic strains, which can grow and produce toxins at refrigeration temperatures, some even do so at temperatures below 4 ºC, so they are of great importance in foods that are refrigerated or that have been subjected to a gentle heat treatment (mainly fish and seafood) and packaged in anaerobiosis.

Group III strains are mesophilic or slightly thermophilic and produce spores of moderate heat resistance (Portinha et al., 2022). Group IV strains are proteolytic and non-glycolytic, Group V strains are non-proteolytic strains capable of fermenting glucose, and finally, Group VI strains are partially proteolytic strains that do not ferment glucose (Liu, 2024). Tables 1, 2, 4, 5, 6 and 7 show the main characteristics of each of the Groups of *C. botulinum* according to different authors consulted.

Table 1. Characteristics of the different phenotypic Groups of *Clostridium botulinum*

Properties	Phenotypic Groups of *Clostridium botulinum*					
	Group I	**Group II**	**Group III**	**Group IV**	**Group V**	**Group VI**
Types of toxins	A, B, F, AB, BF, FA	B4, E, F6	C, CD, DC	G	F7	E4/5
Proteolysis	+	-	+/-	+	-	+/-
Glucose fermentation	+	+	-	-	+	-

Table 1. Characteristics of the different phenotypic Groups of *Clostridium botulinum*

Properties	Phenotypic Groups of *Clostridium botulinum*					
	Group I	**Group II**	**Group III**	**Group IV**	**Group V**	**Group VI**
Group of genes encoding the toxin	*orfX* +, *ha* +	*orfX* +, *ha* +	*ha* +	*ha* +	*orfX* +	*orfX* +
Affected group	Humans	Humans	Animals	n.a.[a]	Humans	Humans
Proximate non-toxigenic types	*C. sporogenes* serotype B		*C. haemolyticum* *C. novyi* type A	Renamed *C. argentinense*	Renamed *C. barati* serotype F	Renamed *C. butyricum* serotype E

[a]Not available. **Source:** (Liu, 2024).

The differences between strains of different phenotypic Groups of *C. botulinum* have a genetic basis, as has been recently confirmed by comparative genomics studies (Smith et al., 2020). The location of the genes encoding the different toxins is also different. Depending on the strain, the genes of Groups I and II are encoded on the chromosome or on plasmids, while the genes of Group III strains are located in a bacteriophage and, those of Group IV, in plasmids (Moore and Lacey, 2019). The genomes of the different strains also vary widely in size (between 2.4 and 4.5 Mb), which indicates large differences in their genetic potential.

2.2 Botulinum toxin

C. botulinum produces botulinum toxin (BoNT). This is probably the toxin with the highest known biological activity; it is also the causative agent of botulism (Rawson et al., 2023). Another 15 species of clostridia, including *C. baratii*, *C. argentinense* and *C. butyricum* are also capable of producing BoNT (Poulain and Popoff, 2019). BoNT are metalloproteases that specifically cleave soluble proteins from postsynaptic terminals, preventing the release of the neurotransmitter and blocking the transmission of the neuronal signal to the effector muscles (Rossetto et al., 2021). However, BoNT are not able to cross the blood-brain barrier and, therefore, only affect the motor nerves. BoNT share a similar structure and are synthesized as a 150 kD protoxin (Meurens et al., 2023). The original protoxin is cleaved into two asymmetric polypeptide chains: a heavy chain (100 kD) and a light chain (50 kD). These two chains constitute the active toxin and are covalently linked by disulphide bridges. In proteolytic strains, the cellular protease is responsible for activation, while, in non-proteolytic strains, the toxin is activated by the degradative action of other proteolytic microorganisms present in the medium (Popoff and Brüggemann, 2022). They all have a similar mode of action: the heavy chain of the activated toxin binds to peripheral neuronal cells and allows light chain endocytosis (Rawson et al., 2023). Inside the cells, the light chain binds to the proteins responsible for acetylcholine exocytosis, so that acetylcholine is not released and the nerve activity used to control muscles is lost.

BoNT are classified into seven types with different antigenic potential (A, B, C, D, E, F and G) (Peck et al., 2017). Toxins A, B, E and F are mainly responsible for botulism in humans (65 % due to type A

toxin, 25 % to type E toxin and 7 % to type B toxin). They are produced by *C. botulinum* genotypes I (A, B and F) and II (B, F and E). Due to the differences in growth temperatures of the different genotypes (Tables 2, 4, 5, 6 and 7), the optimum temperature of formation of BoNT A, produced by *C. botulinum* I (mesophilic) ranges between 30 and 37 ºC, but BoNT B, E and F, which can be produced by *C. botulinum* II (psychrotroph), can develop at temperatures as low as 3-4 ºC. Therefore, it is possible that, in the case of low acidity, non-sterile foods, stored under anaerobic conditions and under refrigeration, BoNT may develop.

Table 2. Characteristics of neurotoxin-producing strains of *Clostridium* spp. (BoNT)

Characteristics	Group I (Proteolytic *C. botulinum*)	Group II (Non-proteolytic *C. botulinum*)	Group III (*C. botulinum*)	Group IV (*C. argentinense*)	Group V (*C. butyricum*)	Group VI (*C. baratii*)
Types of neurotoxins produced	A B (proteolytic strains) F H	E B (non-proteolytic strains) F	C D	G	E	F
Proteolysis	+	-	-	+	-	-
Optimum growth temperature	35-40 ºC	18-25 ºC	40 ºC	37 ºC	30-37 ºC	30-45 ºC
Associated botulism cases	Humans (usually canned foods)	Humans (usually refrigerated or mildly heat-treated foods)	Animals (C poultry; D cattle)	Unreported outbreaks; environmental isolations	Humans	Humans

Source: (Rawson et al., 2023)

BoNT are produced after the sporulation and germination phases (Shen et al., 2019). There is evidence that, in the case of toxins produced by non-proteolytic strains, a time of at least 10 days at 8 ºC is needed for them to develop (Peck et al., 2006). *In vitro*, the highest levels of neurotoxins occur at the end of the exponential phase of growth and in the early phases of stationary development (Popoff and Brüggemann, 2022). The different types of BoNT have different stability to heat and other environmental conditions (Tables 4 and 7). In general, BoNT denature after 10 minutes at 100 ºC or 30 minutes at 80 ºC and remain stable for more than 3 cycles of freezing and thawing (Munir et al., 2023). As a common feature, they are all very stable in extremely acidic environments.

2.3 Botulism

Botulism is a paralysing, rare, but serious illness that becomes fatal if it is not diagnosed quickly and treated with botulinum antitoxin (ANSES, 2019), requiring, in some cases, supportive treatment, especially mechanical ventilation.

Depending on the characteristics of the host and the route of infection, there are five main types of botulism: food-borne botulism, wound botulism, infant botulism, iatrogenic botulism, and adult

intestinal toxemia (CDC, 2024), of which food-borne botulism is the most common. As mentioned above, the cause of the latter is the ingestion of food contaminated with BoNT. In a study prepared by the RIVM (*Rijksinstituut voor Volksgezondheid en Milieu*), it was estimated that values above 0.06 ng/kg body weight could cause adverse health effects, while values between 0.004 and 0.008 ng/kg body weight could be considered safe (RIVM, 2000). Human botulism is characterised by a bilateral weakness of the descending muscles, the symptoms usually begin in the cranial nerves and present as blurred or double vision, dry mouth and difficulty speaking (Johnson et al., 2008). The classic early symptomatology of botulism can be remembered using the "four D" mnemonic: dysarthria, diplopia, dysphonia and dysphagia (Rawson et al., 2023). The symptoms of botulism last from a few days to 8 months, although full recovery could last for years.

2.4 Prevalence of *C. botulinum* in food

C. botulinum is a microorganism that can be found in certain foods, both of plant and animal origin. Rhodehamel et al. (1992), Dodds (1993) and Chai et al. (2013) mention its presence in spinach, potatoes, corn, onion peel, mushrooms, garlic, cabbage, honey and both fresh and processed meats. *C. botulinum* is also common in certain fish and fishery products, such as farmed trout (Hielm et al., 1998). The incidence in meats is low (Tompkin, 1980) (Hauschild, 1989), although it has been detected in pork (Abrahamsson and Riemann, 1971) (Roberts and Smart, 1976).

However, there are not much data on the prevalence in foods of the V-range, refrigerated and packaged under vacuum or in modified atmospheres. Pernu et al. (2020) revealed a high prevalence (32 %) of *C. botulinum* Group II in sausages of plant origin (74 samples from 7 different producers in the German and Finnish markets), that had been subjected to mild heat treatment and vacuum-packed.

In the case of the prevalence in raw materials intended to be used in REPFED foods (Refrigerated Processed Foods of Extended Durability), this is frequently low (data given as most likely number/kg): 2-3/kg (102 samples); meat, 2-4/kg (143 samples); dairy products, 2-5/kg (26 samples); thickeners, 2-4/kg (143 samples); flavours and sauces, 0.3-0.6/kg (25 samples); spices and herbs, <0.6/kg (65 samples), only BoNT types A and B being detected (Carlin et al., 2004).

3. Alerts and outbreaks associated with *C. botulinum* in foods packaged under vacuum or in modified atmospheres

In Europe, about 100 confirmed cases of botulism are reported each year by national surveillance systems (ECDC, 2023). This number has remained stable from 2017 to 2021, with an average of 85 cases per year (ECDC, 2023). In 2022, 7 food transmission outbreaks were reported in the European Union, affecting an average of 20 people, mainly in Italy (32), Romania (11), France and Spain. In the United States, an average of 110 cases of botulism occur per year, and 25 % of them are confirmed cases of food-borne illness (CDC, 2024). Recent outbreaks declared in Europe have been linked to canned homemade vegetables (Italy), canned pork and ham products (Poland and Romania), canned fish and fish products, and in canned foods and homemade products (France) (ECDC, 2024).

Since 2020, the European Rapid Alert System for Food and Feed (RASFF) has notified 7 alerts relat-

ed to the presence or suspicion of BoNT in foods, mainly in prepared dishes (Spain in 2023 and Italy in 2024), brine mushrooms (Russia in 2024), preserves (France in 2023), fish and derivatives (Turkey in 2022), and cream cheese (United Kingdom in 2020).

Table 3. Recent outbreaks of food botulism related to non-proteolytic strains

Year	Country	Product	Toxin	Cases (deaths)	Cause outbreak	Reference
2001	Australia	Reheated chicken	E	1	Temperature control failure	Mackle et al. (2001)
2001	United States	Homemade fermented beaver tail and paws	E	3	Temperature control failure	CDC (2001)
2001	Canada	Homemade fermented salmon eggs	E	4	n.a.[a]	Anon (2002)
2002	United States	Homemade beluga skin and fat	E	12	n.a.	McLaughlin et al. (2004)
2003	Germany	Homemade salted fish	E	3	Temperature control failure	Eriksen et al. (2004)
2004	Germany	Vacuum packaged commercial smoked salmon	E	1	Consumed after expiry	Dressler (2005)
2016	Germany and Spain	Dried, salted fish	E	5	n.a.	ECDC/EFSA (2016)
2023	France	Homemade canned sardines	B	15 (1)	n.a.	Meurice et al. (2023)

[a]n.a.: not available.

Recent outbreaks of food-borne botulism associated with proteolytic *C. botulinum* are primarily related to improperly processed or preserved food preserves. In the case of outbreaks of botulism transmitted by non-proteolytic *C. botulinum*, these have been mainly associated with refrigerated foods in which the commercial shelf life or storage temperature has not been respected prior to consumption (Table 3).

4. Factors influencing the survival of *C. botulinum* in food

4.1 Effect of oxygen content

The main characteristics of the microorganism, as well as the conditions necessary for its growth and production of toxins, are included in Tables 1, 2, 4, 5 and 6, based on the data offered by the different authors consulted. *C. botulinum* is an anaerobic bacterium (in the absence of oxygen it can germinate and produce the botulinum toxin, for example in plant-based preserves). However, the presence of high levels of oxygen alone is not a sufficient barrier to prevent the growth or production of toxins (Camerini et al., 2019). On the one hand, it has been verified that the microorganism can tolerate traces of oxygen given its production of superoxide dismutase (Liu, 2024). In addition, it

is possible that in the food, micro-environments exist in which the bacteria are protected from the effects of oxygen and, therefore, from oxidative stress (ACMSF, 2024). In this regard, under laboratory conditions, growth and production of proteolytic *C. botulinum* toxins have been detected in high humidity bakery products (water activity (a_w)= 0.990) packaged in a modified atmosphere (15 % oxygen) at 25 ºC (Daifas et al., 1999) and in mushrooms packaged in a semi-permeable plastic film (Whiting and Naftulin, 1992). Similarly, Cai et al. (1997) and Erickson et al. (2015) have also described the production of BoNT type E in catfish and salmon, respectively, packaged in oxygen-permeable materials.

4.2 Effect of pH

With regard to pH, *C. botulinum* strains can grow with pH values of 4.6 or higher, so they pose a risk in low-acid foods (Peck, 2009). Although some outbreaks of botulism have been attributed to foods with a pH below 4.6, it has been verified that these products were also contaminated with moulds, which had increased the pH of the food, allowing *C. botulinum* to grow and produce toxins (Peck, 2009).

Table 4. Conditions necessary for the survival, growth and toxin production of *Clostridium botulinum* and other neurotoxigenic Clostridia under laboratory conditions

	***C. botulinum* Group I Proteolytic**			***C. botulinum* Group II Non-proteolytic**			***C. botulinum* Group III Non-proteolytic**			***C. botulinum* Group IV Proteolytic**			***C. butyricum* Group V**			***C. baratii* Group VI**		
Types of neurotoxins produced	A, B, F			B, E, F			C, D			G			E			F		
Clostridia not producing closely related neurotoxins	*C. sporogenes*			None at the time			*C. novyii*			*C. argentinense*			*C. butyricum*			*C. baratii*		
Growth	**Min[a]**	**Opt[b]**	**Max[c]**	**Min**	**Opt**	**Max**	**Min**	**Opt**	**Max**	**Min**	**Opt**	**Max**	**Min**	**Opt**	**Max**	**Min**	**Opt**	**Max**
Temperature (ºC)	10	35-40	48	2.5	18-25	45	15	37-40	n.a.[d]	n.a.	37	n.a.	12	3-37	n.a.	10	30-45	n.a.
pH	4.6	n.a.	9	5	7	9	5.1	6.1-6.3	9	4.6	7	n.a.	4.8	7	n.a.	3.7	7	n.a.
Water activity (a_w)	0.94	n.a.	n.a.	0.97	n.a.	n.a.	0.97	n.a.	n.a.	0.94	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
% NaCl that inhibits growth	10			5			5			10			n.a.			8.5		
Toxin production																		
Minimum temperature (ºC)	10			3			15			n.a.			12			10		
Minimum water activity (a_w)	0.94			0.97			0.97			0.94			n.a.			n.a.		

[a]Min: minimum. [b]Opt: optimum. [c]Max: maximum. [d]n.a.: not available. **Source:** (ANSES, 2019).

4.3 Effect of water activity (a_w)

The minimum value of a_w that allows the growth and toxin production of *C. botulinum* strains is shown in Tables 4, 5, 6 and 7. The minimum a_w for growth is 0.94 for proteolytic strains and 0.97 for non-proteolytic strains, although the value may be affected by the type of substrate (lower with glycerol than with NaCl) or food.

Table 5. Marginal conditions for the growth of *Clostridium botulinum*

Conditions	Proteolytic strains	Non-proteolytic strains
Minimum water activity value (a_w)	0.935	0.970
Minimum pH value	4.6	5.0
Maximum pH value	9.0	9.0
Maximum NaCl concentration	10 %	5 %
Minimum temperature	10-12 ºC	3.3 ºC
Maximum temperature	50 ºC	45 ºC

Source: (Chaidoutis et al., 2022).

4.4 Effect of food preservatives

To control the risk of botulism, it could be of interest to resort to the use of certain food additives whose use, in addition to being effective, must be permitted by the applicable legislation, and will be conditioned, among other reasons, by the type of food in question. For example, in the case of some meat products, such as ham, the use of nitrates and nitrites has proven to be an effective strategy. However, and despite the advantages of these compounds, their use poses some drawbacks, such as the fact that they can react with secondary amines, resulting in the formation of N-nitrosamines, substances with potential carcinogenic activity (Chaidoutis et al., 2022) (Munir et al., 2023). Therefore, the use and maximum use concentration for these additives is limited by law.

In addition to nitrates and nitrites, there are other additives that, as noted above, depending on the food in question, could be used as preservatives, understood as those substances that prolong the shelf life of food, protecting it from deterioration caused by microorganisms or that protect it from the growth of pathogenic microorganisms.

The authorized food additives and their conditions of use in food processing are established in Regulation (EC) No. 1333/2008 on food additives (EU, 2008). More specifically, the conditions of use are identified in Part E of Annex II of that regulation, which is organized into food categories, in such a way that to know the additives whose use is allowed in a particular food, it is essential to know the food category in which that food is classified.

4.5 Effect of temperature

The optimum growth temperature of *C. botulinum* Group I is 35-40 ºC (Liu, 2024). For strains in this Group, the minimum growth temperature is 10 ºC (12 ºC, according to some authors) (Tables 2, 4, 5, 6 and 7). Group II strains have an optimum growth temperature of 26-28 ºC, and the peculiarity that they can grow and produce toxin at 3 ºC (and even at lower temperatures, Table 7). For this reason, these strains constitute the main risk in relation to *C. botulism* in refrigerated foods stored in anaerobic conditions.

Table 6. Characteristics of *Clostridium botulinum* Groups I and II

Characteristic	***C. botulinum* type I (proteolytic *C. botulinum*)**	***C. botulinum* type II (non-proteolytic *C. botulinum*)**
Neurotoxins formed	A, B, F, H[a]	B, E, F
Proteolysis[b]	+	-
Minimum growth temperature	12 ºC	3 ºC
Optimum growth temperature	37 ºC	30 ºC
Minimum pH value for growth	4.6	5.0
NaCl concentration that prevents growth	10 %	5 %
Minimum water activity (a_w) (with NaCl) for growth	0.94	0.97
Minimum water activity (a_w) (with glycerol) for growth	0.93	0.94
Spore heat resistance[c]	D_{121}= 0.21 minutes	$D_{82.2}$= 2.4/231 minutes[d]
Non-neurotoxigenic *Clostridium* equivalent	*Clostridium sporogenes*	

[a]More than one type of toxin may be formed; neurotoxin H type not verified at the time of table editing. [b]Proteolysis denotes the ability to degrade native proteins (e.g., coagulated egg white, cooked meat, casein). Both *C. botulinum* Group I and *C. botulinum* Group II can degrade gelatin. [c]Decimal reduction time (D value, i.e., the time to achieve a decimal reduction in viable spores) at a specified given temperature in a phosphate buffer (pH 7.0). [d]D-value without/with lysozyme during recovery. **Source:** (Carter y Peck, 2015).

Table 7. Conditions for the growth, survival and production of toxins in *Clostridium botulinum* strains of interest in food safety

Characteristic	Environmental conditions	Group I strains	Group II strains
Growth of vegetative cells	Optimum temperature (ºC)	35-40	18-25
	Temperature range (ºC)	10-48	1.5-45
	Optimal pH value	7.0	7.0
	Range of pH values	4.6-9.0	5.0-9.0
	Minimum water activity (a_w) with NaCl	0.94	0.97
	Minimum water activity (a_w) with glycerol	0.93	0.94
	% NaCl that prevents growth	≥10	≥5
Spore resistance	Heat	D_{121}= 0.04-0.2	D_{80}= 0.23-2.63
		Z ~ 10 ºC	
	Freezing	Yes	Yes
Toxin stability	Heat	Denaturation after 10 minutes at 100 ºC or 30 minutes at 80 ºC	
	Freezing	Stable even after 3 thaws and freezes	
	pH	More stable under strongly acidic conditions[a]	

[a]In general, toxins are more stable in acidic foods, such as tomato sauce at pH 4.2, than in low-acid foods, such as canned corn at pH 6.2. **Source:** (Munir et al., 2023).

5. Risk assessment of *C. botulinum* related to the consumption of foods packaged under vacuum or in modified atmospheres, whether or not subjected to post-packaging pasteurisation treatments

Food-borne botulism occurs after ingestion of a certain amount of BoNT generated once *C. botulinum* spores germinate and begin to multiply in the food. Due to the severity of the illness, an acceptable level of *C. botulinum* toxin in food is not contemplated in practice. And, although toxin production is associated with the proliferation of *C. botulinum*, it is difficult to accurately determine the final concentration in the food so, generally, risk management is based on establishing safety margins based on the probability of survival, germination and growth of *C. botulinum* spores. In this sense, the formulation of the food, as well as the conditions of distribution and storage, are key factors in reducing the risk of *C. botulinum*.

In general, products classified as V-range are marketed after applying a heat treatment and usually require heating prior to consumption. Some of these prepared dishes can be stored at room temperature, so they are associated with a longer shelf life and, therefore, depending on the different control measures applied, they could be more susceptible to *C. botulinum* growth (Membré et al., 2015). Under refrigerated conditions, the generation of toxin is caused only by non-proteolytic strains, which can also constitute a food safety problem (Peck et al., 2006). Although botulinum toxin has a moderate heat resistance (80 ºC, 30 minutes) compared to the spore, heating prior to consumption is often insufficient for the inactivation of the toxin, with the consequent risk of food poisoning if a sufficiently high amount is ingested. This means that heat treatments at the domestic level are not an effective measure for the mitigation of the risk associated with botulism.

Hauschild and Simonsen (1986) estimated the margin of safety at levels of probability of germination and growth of proteolytic *C. botulinum* spores in cured meat products to be between $<10^{-7}$ and 10^{-8}. In their study, the authors relied on the use of data from industries regarding the number of contaminated units per batch over the years, together with epidemiological information on cases of food-borne botulism. This information can provide a meaningful assessment of the actual risks of those products in the past and, in the event that no cases of botulism have been reported, could demonstrate that the controls used were valid and led to a safe product. Likewise, Pflug (1987) estimated a probability of 10^{-9} in those foods preserved at room temperature that may favour the growth and production of toxin by *C. botulinum*.

Peck et al. (2006) used the same approach for non-proteolytic *C. botulinum* in refrigerated foods, including fresh meat packaged in a modified atmosphere and under vacuum, with a maximum shelf life of 10 days at ≤8 ºC and without other additional specific controls (pH, a_w, NaCl, etc.), apart from storage temperature and shelf life. In this study, it was established that between 1986 and 2005 a total of 8.3×10^9 units had been marketed under these conditions (maximum shelf life of 10 days at ≤8 ºC) without cases of botulism having been reported, which means that the probability of the appearance of a unit with the presence of toxin was equal to or less than 1.58×10^{-10} for refrigerated foods with a maximum shelf life of 10 days at ≤8 ºC. These conditions have served as the basis for the establishment of the shelf life under the principle of the "10-day rule", since the formation of toxin by non-proteolytic strains of *C. botulinum* originates in a time of at least 10 days and at

a temperature ≤8 ºC (ACMSF, 2006). This fact was subsequently demonstrated by Peck et al. (2020) in fresh meat packaged and stored at a temperature of between 3 and 8 ºC. Their results showed that the amount of toxin was below the detection limit in those samples stored for more than 25 days.

According to the information available in the risk assessment studies, the proportion of contaminated units in a batch is normally very low, which considerably decreases the risk associated with the production of toxin by *C. botulinum*. Therefore, when considering monitoring of good hygiene practices, together with the quality control of raw materials, and adequate storage conditions (time and temperature), the risk should be low or very low. However, it is necessary to consider the probability of survival of *C. botulinum* based on environmental factors associated with the formulation of the product and storage conditions.

Given the existence of multiple factors associated with the production of toxin by *C. botulinum*, to date there are few studies that address a complete risk assessment for this pathogen, most of them related to non-proteolytic strains.

One of the first approaches was established by Barker et al. (2002), who developed models for estimating *C. botulinum* growth based on various factors such as temperature, pH, NaCl, dormancy phase, inoculum concentration and storage time. This approach was subsequently exemplified in a non-proteolytic *C. botulinum* exposure assessment in a pasteurized potato-based food (80 ºC, 1 minute) (Barker et al., 2005). Based on this, different safety margins were established. A probability of the presence of toxin of 10^{-9} was obtained in containers stored for 75 days at 20 °C in the presence of sorbic acid. In the case of storage at temperatures of 8 and 12 ºC, this probability is reduced to values below 10^{-11}.

On the other hand, Peck et al. (2008) carried out a review of the factors associated with the growth and production of toxin by non-proteolytic *C. botulinum* in short shelf life refrigerated foods. The authors concluded that the risk of botulism is related to the control of factors that are difficult to quantify, and that they present a high variability depending on the strain, type of food and processing conditions, such as the hygienic state of the raw materials, inactivation or cell damage caused by heat treatment, or storage conditions.

Malakar et al. (2011) developed a quantitative risk assessment of non-proteolytic *C. botulinum* in refrigerated dairy desserts. Among other factors, variability in spore destruction due to heat treatment was considered. The results showed that the probability of obtaining units with the presence of spores was very low (9.4×10^{-5} and 8.0×10^{-6}), considering D values of 0.03 and 0.24 minutes at 95 ºC, respectively. For those contaminated units, the toxin production time depended on the storage temperature, ranging between 8 and 26 days at 4 ºC, and between 3 and 9 days at 7 ºC. The decimal reduction time and storage temperature by the consumer were the most determinant factors on the risk associated with non-proteolytic *C. botulinum* in this type of product.

Other studies related to *C. botulinum* proteolytic strains were developed by Membré et al. (2015), who carried out a quantitative risk assessment on canned pâté, stable at room temperature. The model was constructed using a sufficiently representative heat treatment for that product (with a lethality value or F_0 set at 0.5 minutes, with or without addition of nitrite). The results obtained showed that the probability of disease per inhabitant per year was very low (8.0×10^{-10}), corroborated by

the analysis of epidemiological data. Therefore, the industrial practice of producing canned *foie gras* was considered adequate to control the risk of proteolytic *C. botulinum*, with equivalent heat treatments at a temperature of 105 ºC.

By combining the information obtained from the risk assessments, together with epidemiological data, the so-called Food Safety Objectives (FSO) can be established, defined as "the maximum frequency and maximum concentration of a hazard in a food at the time of consumption, which provides or contributes to the Appropriate Level Of Protection (ALOP)" (ICMSF, 2002). In this sense, Anderson et al. (2011) considered that, taking into account an average contamination level of 10^3 *C. botulinum* spores per unit of product, and an inactivation treatment that guarantees the elimination of 12 logs, the FSO can be established at -9.0 logs/unit of product, that is, in a batch of 10^9 units, there is no spore capable of surviving, germinating and growing to produce toxin. Using this reasoning, the authors established a series of metrics related to FSO values in certain heat-treated foods (canned legumes, meat tenderloin, pesto sauce and spreadable cheese), depending on the intensity of the heat treatment and formulation (Table 8). To do this, they applied the inequality proposed by ICMSF (2002), where H_0 reflects the initial contamination of each of the units of the batch (log N_0/unit), ΣR is the sum of the reductions as a result of the inactivation treatments (log N_0/N_R), and ΣI is the sum of the increases in the concentration of the pathogen (log N_i/N_0'):

$$H_0 - \Sigma R + \Sigma I \leq FSO \qquad \textbf{Equation 1}$$

Using this formula, FSO values can be proposed that can be translated into the probability of the appearance of units in a batch that may be related to cases of botulism.

Table 8. Application of the inequality proposed by ICMSF (2002) for estimating Food Safety Objectives (FSO) and control measures in heat-treated foods with respect to *C. botulinum*

Product	H_0	ΣR	ΣI	FSO	Control measures
Canned beans	1.2	7.2	0.0	-6.0	F_0*= 1.3 (minutes)
Canned beans	1.2	10.2	0.0	-9.0	F_0= 1.9 (minutes)
Cold meat	-1.7	3.0	-3.3	-8.0	F_0= 0.6 (minutes); pH= 7.0; NaCl= 4.5 %; nitrite= 150 ppm
Cold meat	-1.7	3.0	-4.3	-9.0	F_0= 0.6 (minutes); pH= 6.5; NaCl= 5.5 %; nitrite= 150 ppm
Pesto sauce	2.3	0.0	-7.0	>-4.7	pH= 4.6
Pesto sauce	2.3	0.0	-2.0	0.3	pH= 4.75
Cheese spread	-1.6	0.0	-4.4	-6.0	pH= 5.6; NaCl+Na_2PO_4= 4.5 %; moisture= 56 %
Cheese spread	-1.6	0.0	-4.4	-6.0	pH= 6.0; NaCl+Na_2PO_4= 5.0 %; moisture= 54 %

*F_0= lethality value (minutes) equivalent to a treatment of 121 ºC. **Source**: (Anderson et al., 2011).

In any case, the values obtained in the *C. botulinum* risk assessments published to date indicate a low or very low probability of occurrence of contaminated units, assuming that adequate industrial practices are followed at the level of control of raw materials, formulation, inactivation treatments and distribution and storage conditions. However, the characteristics of *C. botulinum* strains that can pro-

liferate in foods subjected to pasteurisation and preserved by refrigeration, as well as the variability in consumer behaviour patterns, must be taken into account in order to establish adequate prevention measures against botulism. The knowledge of the factors that induce the survival, germination, growth and production of toxin by *C. botulinum* in each type of particular food seems essential in order to develop effective risk mitigation strategies.

6. Control measures

6.1 Heat treatment

Heat treatment is the most effective method for the inactivation of *C. botulinum* spores. However, the heat tolerance of these spores varies both between Groups and within them. In general, *C. botulinum* spores are among the most heat-resistant of those produced by pathogenic microorganisms. In fact, in commercially sterile products, the treatment is established with the aim of achieving 12 decimal (12D) reductions of *C. botulinum* (ANSES, 2021).

In general, the resistance of vegetative cells and spores of proteolytic strains (Group I) is higher than those of non-proteolytic strains (Group II) (ANSES, 2010). Traditionally, a time of 0.21 minutes at 121.1 ºC (Esty and Meyer, 1922) has been established as necessary to reduce the load of *C. botulinum* by 1 logarithmic cycle ($D_{121.1}$). More recent studies have confirmed the value. A meta-analysis carried out using 23 scientific articles (394 D values collected) determined a mean $D_{121.1}$ value of 0.20 minutes, with a standard deviation of 0.11 minutes, and individual values of up to 0.48 minutes (Diao et al., 2014). Additionally, based on this value, the concept of "minimum botulinum cooking" is established, which involves a heat treatment at the coldest point of the food for 3 minutes at 121.1 ºC, or any combination of equivalent lethality (F). These time-temperature combinations result in 12 logarithmic reductions of *C. botulinum* (Group I), which is currently the standard scale established for the sterilization of low-acid preserves (12D concept). In the case of *C. botulinum* Group II, a heat treatment of 90 ºC for 10 minutes (6D) is sufficient (ACMSF, 1992) to achieve commercial sterilisation. This heat treatment is recommended for the preservation of refrigerated V-range foods packaged under vacuum or in modified atmospheres (REPFED) (ACMSF, 1992).

Botulinum toxins have a lower thermal stability, being inactivated after heat treatments (temperatures and times) that vary, according to the authors, with the values collected in the literature being 85 ºC for 5 minutes (Chaidoutis et al., 2022) (Rawson et al., 2023), 100 ºC for 10 minutes or 80 ºC for 30 minutes (Poulain and Papoff, 2019). It should be noted that this variability can be due to several factors, such as the composition of the medium or the type of strain (Munir et al., 2023).

6.2 Effect of food preservatives

For centuries, the preservation of meat and fish has been based on the use of preservatives (nitrites and sodium nitrates, mainly). However, due to concerns about the possible formation of carcinogenic compounds such as N-nitrosamines from derivatives of these compounds, alternatives have been identified, from potassium sorbate, certain polyphosphates (Nelson et al., 1983) and sodium lactate (Meng and Genigeorgis, 1993) (Houtsma et al., 1994). Notermans et al. (1985) demonstrated that a combination of ascorbic acid and citric acid could inhibit the formation of toxins in vacuum-packed

cooked potatoes, improving safety even under conditions of temperature abuse. Together, these studies highlight the potential of various additives to develop effects that contribute to controlling the growth of *C. botulinum* and the production of toxins in different foods. The use of food additives during food processing is strictly regulated in the European Union through the aforementioned Regulation (EC) No. 1333/2008 (EU, 2008).

6.3 Other treatments

In the last 30 years, a major effort has been made in research on alternative technologies to heat treatment for food preservation. Such is the case with High Pressure Processing (HPP), Pulse Electrical Field (PEF), Ionizing Radiation (IR) and, recently, Cold Plasma (CP).

Currently, the degree of development of each of them is variable. In the case of High Pressure Processing, the technology is fully implemented at the industrial level as a process for the pasteurization of food (elimination of vegetative cells of pathogenic microorganisms). However, the ability of this technology to inactivate bacterial spores with current industrial developments is limited. Pressure values above those currently established by the industry or in combination with temperatures of more than 80 ºC are required to be able to find considerable logarithmic reductions of bacterial spores from a commercial point of view (Munir et al., 2023).

In the case of Pulse Electrical Field, the research carried out shows the ineffectiveness of this technology to inactivate microbial spores (Soni et al., 2020) (Qiu et al., 2022).

Ionizing Radiation has shown effectiveness for the inactivation of bacterial spores, and its effects can be observed in the form of structural damage, spilled cytoplasmic content, reduction of membrane integrity and fragmentation of genomic DNA (Fietser et al., 2012). However, its application as a preservation technology is a controversial issue in the European Union, due to concerns about its safety, the possible effects on the nutritional and sensory quality of food, and the impact on human health. Its use is authorized in Spain and in the rest of the European Union Member States for aromatic herbs, spices and (dry) plant seasonings, although some European Union countries also allow the irradiation of other products.

The application of Cold Plasma to food preservation is currently under study. The results described so far reveal the potential of this technology for the inactivation of bacterial spores (Liao et al., 2019) (Valdez-Narváez et al., 2024), however, no studies have been found focused on the inactivation of *C. botulinum* spores, so it is not yet possible to establish specific conclusions in this regard.

Conclusions of the Scientific Committee

Botulism risk management is based on establishing safety margins based on the probability of survival, germination and growth of BoNT-forming *C. botulinum* spores.

In the case of sterilized (canned) foods, the heat treatment applied is sufficient to inactivate *C. botulinum* spores. Therefore, as long as there are no processing or packaging failures, they are safe foods regardless of their formulation, temperature and storage time.

In the case of foods with milder heat treatments (cooking and/or thermal pasteurisation), such as those of the V-range, which are refrigerated, vacuum-packed, ready for consumption or that

require only a slight previous heating, the risk of botulism will largely depend on the monitoring of good hygienic practices throughout the production process. It is also of particular importance to establish control measures that prevent the development of *C. botulinum* both at the level of the food formulation (pH, a_w, NaCl concentration or some type of antimicrobial agent), as well as strict control of the time and temperature of food storage (below 4 ºC, ideally below 3.3 ºC). In addition, the consumer must adhere to the preservation and consumption instructions provided by the producer.

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