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Clostridium botulinum Spores and Toxin in Mascarpone Cheese and Other Milk Products

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ABSTRACT

A total of 1,017 mascarpone cheese samples, collected at retail, were analyzed for *Clostridium botulinum* spores and toxin, aerobic mesophilic spore counts, as well as pH, a_w (water activity), and Eh (oxidation–reduction potential). In addition 260 samples from other dairy products were also analyzed for spores and botulinum toxin. Experiments were carried out on naturally and artificially contaminated mascarpone to investigate the influence of different temperature conditions on toxin production by *C. botulinum.* Three hundred and thirty-one samples (32.5%) of mascarpone were positive for botulinal spores, and 7 (0.8%) of the 878 samples produced at the plant involved in an outbreak of foodborne botulism also contained toxin type A. The chemical–physical parameters (pH, a_w, Eh) of all samples were compatible with *C. botulinum* growth and toxinogenesis. Of the other milk products, 2.7% were positive for *C. botulinum* spores. Growth and toxin formation occurred in naturally and experimentally contaminated mascarpone samples after 3 and 4 days of incubation at 28°C, respectively.

Manufactured milk products have seldom been implicated in episodes of foodborne illness since pasteurization and sterilization procedures were introduced in the dairy industry to eradicate most of the microbial pathogens from raw milk *(22).* However, milk pasteurization temperatures do not kill spore-forming organisms. This is the case with *Clostridium* and *Bacillus* species that still challenge the safety of pasteurized, processed products *(17, 20).* In particular, *C. botulinum* raises special concerns because of the serious disease it causes. Spores themselves are not harmful (except for infants and rare adults) *(13),* but once in food, where they may occur as environmental contaminants owing to improper processing or storage, they can germinate under suitable conditions and synthesize highly lethal, though thermolabile, neurotoxins. The ingestion of the botulinum toxin with uncooked contaminated food leads to the flaccid paralysis known as foodborne botulism *(25).* The growth requirements of *C. botulinum* include a low-oxygen atmosphere, certain chemical–physical characteristics of the food, and values of minimum temperature, pH, and a_w (water activity) that are slightly different for proteolytic and nonproteolytic strains: T = 10°C, pH 4.6, a_w = 0.93 for the former; T = 3.3°C, pH 5.0, a_w = 0.97 for the latter *(15).*

While pasteurized milk has been demonstrated not to support *C. botulinum* growth and toxin production as a result of the competitive action of the microorganisms that survive the high-temperature short-time treatment, pasteurized, processed cheese may pose this risk because of its intrinsic and extrinsic properties *(4–6, 18).* Yet, pasteurized, processed milk products have so far accounted for very few cases of foodborne botulism *(6),* probably because their production and storage are usually regulated to ensure the application of one or more barriers controlling the growth of *C. botulinum* and other contaminating microorganisms *(21, 26–29).* The few existing surveys of dairy products for *C. botulinum* contamination show a very low incidence of the microorganism in these kinds of foods *(16, 26).* Therefore, pasteurized milk products are generally regarded as safe from botulism. However, mascarpone cream cheese containing preformed type A botulinum toxin was the cause of a recent outbreak of botulism in Italy *(3).* According to the Italian Institute for Standardization (UNI), mascarpone is a soft spread cheese (not ripened) produced by the thermal-acidic coagulation of milk cream *(32).* Hence, the raw materials for its production are: milk cream containing >80% dry weight lipids, 2.8 to 6% protein, and acidifying substances (single or mixed) such as acetic, citric, tartaric, or lactic acids and vinegar or lemon juice. The final pH of this cheese ranges from 5.7 to 6.6. The coagulation of the cream is achieved by the combined action of heat (>92 to 95°C) and acidity that causes the denaturation of the proteins. This typically Italian cheese was once produced domestically by the farmers of some northern regions and consumed immediately after production because hand manufacturing did not allow for an extended shelf-life of the product. More recently, it has been industrially produced to satisfy the increasing demand.

An industrial manufacturing flow-chart of mascarpone cheese that includes the ultrafiltration process meant to increase the protein and fat content is reported in Figure 1 as an example. Variations in the manufacturing process include the use of condensed milk added with milk cream, bactofugation of the raw cream, different technologies for

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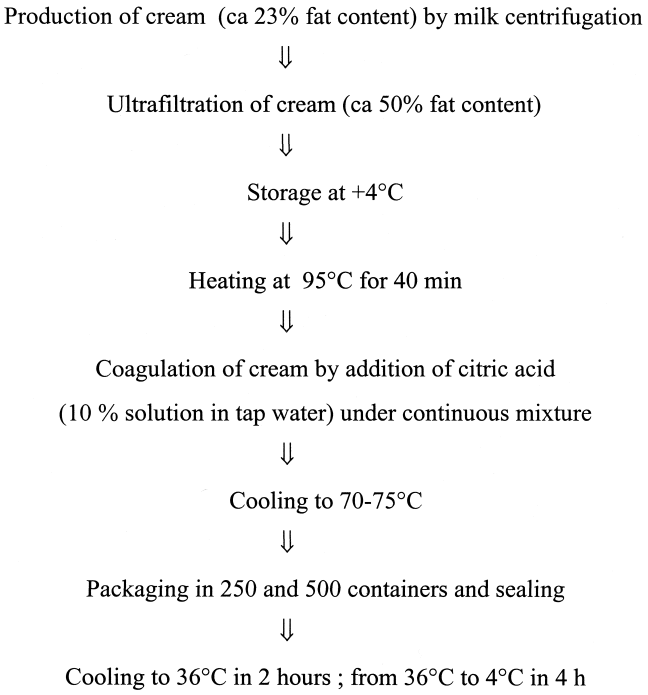

FIGURE 1. *Flow chart of mascarpone manufacture.*

concentrating the milk cream, the heat treatment (times and temperatures), and the concentration of citric acid for coagulation.

The worldwide lack of data on the incidence of *C. botulinum* in dairy products has probably contributed to the exclusion of this pathogen from the routine control measures applied in most dairy plants according to the hazard analysis and critical control points system.

Here, we contribute to the need for data by describing the actual level of *C. botulinum* contamination of milk products marketed in Italy. Furthermore, some inoculation experiments with mascarpone cheese samples were carried out in order to elucidate the influence of the time and temperature combination of storage on the growth of *C. botulinum* type A and production of toxin in mascarpone cheese.

MATERIALS AND METHODS

Mascarpone cheese samples. A total of 1,017 mascarpone cheese samples collected at retail were analyzed. Eight hundred and seventy-eight samples were from the industrial plant that had produced the mascarpone implicated in the outbreak of foodborne botulism in Italy *(3).* These samples represented 34 diverse lots produced over a period of 4 months and identified by the expiration date printed on each package. They were packaged in plastic containers of two sizes (250 g and 500 g), sealed with low-oxygen-permeable films (polyester–polyethylene), capped with a plastic lid, and labeled with the bold-faced indication ''Keep the product at +4°C.'' Each production cycle yielded 12,000 to 25,000 units.

The commercial shelf-life of the cheese was 45 days. The remaining 139 mascarpone cheese samples belonged in 56 lots produced in the same period by 13 plants, 12 in Italy and 1 in Germany.

Sample size was also 250 g and 500 g; packaging materials were the same as described above, except for a few units of a single manufacturer in which the liner was film-coupled with aluminum. The same instructions for refrigerated storage (+4°C) were reported on the labels.

TABLE 1. *Dairy products contaminated with* C. botulinum *spores*

Dairy product	No. positive for *C. botulinum* spores/no. samples tested	Toxin type
Mozzarella cheese	5/30	A
Soft cheese	1/81	A
Processed cheese	1/1	A
Ricotta cheese	0/33	
Raw milk	0/35	
Pasteurized milk	0/13	
Clotted milk	0/3	
Butter	0/2	
Pasteurized cream	0/62	

Other milk products. Two hundred and sixty samples of dairy products other than mascarpone were collected from retailers and included raw and pasteurized milk, pasteurized cream, butter, mozzarella and ricotta cheese, soft cheese, and spread processed cheese. The relative number of samples for each product is reported in Table 1. Sixty of these samples had been produced at plants that also marketed mascarpone cheese. Upon arrival, all samples were stored refrigerated (+4°C) before the analyses.

Detection of *C. botulinum* toxin and spores. All samples were screened for contamination with *C. botulinum* toxin and spores by conventional methods *(12).*

***C. botulinum* toxin.** Approximately 100 g of the solid samples or 100 ml of the liquid ones were mixed with appropriate volumes of 0.2 M gelatin phosphate buffer and kept at 4°C overnight. Aliquots of the mixtures were then centrifuged at 12,000 rpm for 20 min under refrigeration, and 0.8 ml aliquots of the supernatants were intraperitoneally injected into two mice (0.4 ml each). Simultaneously, 1 ml of the extracts was mixed with 0.25 ml of polyvalent antiserum (750 IU botulinum type A antitoxin, 500 IU botulinum type B antitoxin, and 50 IU botulinum type E antitoxin, Behringwerke AG, Marburg, Germany) and inoculated in pairs of mice (0.5 ml of the mixtures for each mouse). When the mice injected with the untreated supernatants died and those inoculated with the extract-polyvalent antiserum mixture survived, the same experiment was performed for toxin typing by adding monospecific antisera (anti-A, anti-B, and anti-E, kindly provided by Dr. Hatheway, Botulism Laboratory, Centers for Disease Control, Atlanta, Ga.) to the extracts instead of polyvalent antiserum.

***C. botulinum* spores.** About 25 to 50 g of the solid samples, including mascarpone, or 50 to 100 ml of the cream or milk samples were inoculated either in 50 to 100 ml of cooked meat broth (Unipath, Basingstoke, England) or in trypticase-peptone-glucose-yeast extract broth *(1),* heat-shocked at 70°C for 10 min, and incubated under anaerobic conditions (Whitley anaerobic cabinet MKIII) at 37°C for 5 days.

The broth cultures were then subjected to the mouse neutralization test for the presence of any botulinum toxin. When the toxin was found in the enrichment cultures, the neurotoxigenic organisms were isolated from egg yolk agar plates by testing single colonies for the presence of neurotoxin A, B, or E genes by polymerase chain reaction, as described elsewhere *(9, 10).* Spores

in the positive samples were enumerated by the three tubes most probable number method *(1).*

Other microbiological analyses. Anaerobic and aerobic mesophilic spore counts of mascarpone samples were performed following the Bacteriological Analytical Manual procedures *(1).*

Measurement of pH, a_w, and Eh. The pH, a_w, and Eh values of mascarpone cheese were measured immediately after the samplings for the microbial tests. The pH was determined by means of a digital pH meter with slope control (Crison 2001, Barcelona, Spain). Measurements were made by inserting the electrode (Crison, electrode 52.32) into the samples.

The a_w was determined by a water activity analyzer LUFFT model (a_w Wert-Messer, Stuttgard, Germany) standardized against a saturated solution of barium chloride to get a value of 0.9 at 20°C *(4).* Determinations were made after placing the sample on the bottom of the stainless steel chamber of the instrument and letting it equilibrate for 3 h at 20°C.

Oxidation–reduction potential measurements were made with a combination platinum redox electrode that contained a calomel reference electrode (Crison electrode 52.61) housed in the pH meter. The electrode was calibrated against a standard redox solution ranging 200 to 275 mV at 25°C (Hanna, Padova, Italy). The redox electrode was inserted into the product inside the package. The redox potential in millivolts was read directly from the meter at appropriate intervals until the readings reached a plateau.

Toxinogenesis experiments with mascarpone cheese. Two series of experiments were carried out to investigate the influence of normal and abused temperature conditions of storage on the production of toxin by *C. botulinum* type A in mascarpone cheese.

i) All samples of *mascarpone* cheese positive for *C. botulinum* spores but negative for toxin were examined for spore count. Some samples (A samples) were incubated in sterile bags. Five samples were incubated at 15°C and another 5 at 28°C. Samples were tested after 3, 5, 7, 10, or 20 days of incubation.

ii) In the second series of experiments, we used mascarpone cheese samples (composition: fat content 47%, protein concentration 4.5%, humidity 42.8 to 44.2%, pH 6.3) prepared in a plant that subjects cream to the bactofugation process *(30)* (B samples). Some samples were tested for contamination with *C. botulinum* spores as described above, while others were incubated at 28°C as negative controls and tested for the presence of botulinum toxin after 45 days (the total duration of the experiment).

Scalar concentrations of spores were inoculated into the mascarpone cheese by the postprocess or ''cold'' method *(18).* Spore crops of the outbreak strain of *C. botulinum* type A were prepared as described by Doyle *(8)* and enumerated by the three tubes most probable number method *(1).* After heat-shock treatment at 80°C for 10 min, the stock solution was 10-fold diluted with sterile distilled water. One hundred-microliter aliquots of the dilutions were dispensed by microsyringe in 10 different spots (10 μl each) into the hermetically closed samples (size 250 g) to obtain the following final concentrations: ~10, 100, 1,000 spores/g of product. *C. botulinum* spores were enumerated in some cheese samples immediately after inoculation. The inoculated samples were incubated at either 15°C or 28°C until toxin production was revealed and in no case for more than 45 days. These experiments were repeated three times.

RESULTS

Mascarpone cheese samples. Three hundred and thirty-one samples out of the 1,017 analyzed were positive for *C. botulinum* spores. Of these, 327 were from the same plant: most of them (325 samples) were contaminated with *C. botulinum* type A. Proteolytic *C. botulinum* type B was also recovered from 2 samples with the same manufacturing code; another 23 samples of the same lot were contaminated with *C. botulinum* type A. All the samples except for those containing preformed botulinum toxin, were contaminated with less than 10 spores/g of product. The samples positive for *C. botulinum* spores were randomly distributed among the different production lots (expiration date between the end of August and the end of November 1996).

TABLE 2. *The effects of storage temperature and initial spore contamination on time (in days) to toxin production*

Samples	Inoculum (spores/g)	Storage temperature (°C)	Toxin production (days)
A[a]	~10	15	0
	~10	28	3
B[b]	~10	15	0
		28	4
	~100	15	34
		28	3
	~1,000	15	27
		28	3

[a] Naturally contaminated mascarpone.
[b] Experimentally contaminated mascarpone.

Moreover, 4 (3%) of the 139 mascarpone cheese samples produced by different manufacturers were contaminated with <10 spores of *C. botulinum* type A/g of product. Two of these samples had been produced at the same factory, and one at a plant located in Germany (pH 5.7).

Seven (0.81%) of the 878 samples of mascarpone cheese from the same plant were also contaminated with botulinum toxin type A. All of them had been collected from the two markets where the mascarpone involved in the outbreak had been purchased. They belonged in two different production lots, one of which had been implicated in the outbreak. None of the other 139 mascarpone cheese samples produced by different manufacturers contained botulinum toxin.

Our results show that the other contaminating bacteria in the products were essentially mesophilic aerobic and anaerobic spores, ranging from <10 to 1.6×10^4 and from <10 to 3.6×10^3, respectively.

The results of the chemical–physical analyses ranged from 5.40 to 6.60 for pH, from 0.945 to 0.988 for a_w, and from +78 to +285 for Eh.

Other dairy products. Of the 260 samples of dairy products that we tested, 7 (2.5%) were contaminated with <10 spores of *C. botulinum* type A/g of product (Table 1). Five of these were mozzarella cheese samples, one soft cheese and one spread processed cheese. This latter sample came from the manufacturing plant implicated in the botulism outbreak that indirectly confirmed the use of milk ingredients contaminated at the source.

Toxinogenesis experiments. Times and temperatures required for toxin formation in naturally and artificially contaminated mascarpone samples are shown in Table 2.

One sample out of five of the naturally contaminated mascarpone cheese (2 spores/g) exhibited *C. botulinum* spore growth (2.1×10^4/g) and toxin formation (4 minimal lethal dose/g) after 3 days of incubation at 28°C; the other samples were negative for both *C. botulinum* growth and toxin.

Production of type A botulinum neurotoxin in samples inoculated with about 100 and 1,000 spores/g occurred after 3 days of incubation at 28°C. When the initial inoculum was about 10 spores/g, botulinum toxin type A was detected in the product after 4 days of incubation at the same temperature.

No botulinum toxin was revealed either in the samples inoculated with about 10 spores/g or in those originally contaminated after 45 days of incubation at 15°C. At this temperature, 34 and 27 days were necessary for toxin formation in the samples inoculated with about 100 and 1,000 spores of *C. botulinum* type A per gram of product. No toxin was found in any of the negative controls of mascarpone cheese after 45 days of incubation at 28°C. No variation was observed in the triplicate experiments.

The analyses for the presence of *C. botulinum* spores in noninoculated samples were also negative. Hence, the mascarpone manufactured with bactofugated cream and used for these experiments was likely to have been free originally from botulinum spores.

DISCUSSION

We found a relatively high incidence of proteolytic spores of *C. botulinum* in mascarpone cheese samples. Failure to adopt adequate treatments to eliminate *Clostridium* spores from the ingredients or inactivate them altogether and the lack of microbial criteria for the selection of the cream to be used in the production process have contributed to the frequent detection of these organisms that can also survive the successive heating phases.

The consistent and intermittent contamination of the product with low levels of *C. botulinum* spores suggests that the raw milk and/or the cream obtained from the skimming of milk and meant for cheese production was the likely source of the microorganism. This hypothesis seems to be confirmed by the failure to detect *C. botulinum* from (i) the environmental swabs taken at the incriminated plant, (ii) the packaging materials, or (iii) the other ingredients used in the preparation of the cheese (data not shown). Moreover, recovery of both *C. botulinum* type A and B from different samples packaged during the same production cycle further indicates the involvement of raw materials as the source of spores.

The presence of *C. botulinum* spores in foods of different origin is not rare, as this microorganism is widespread in the environment *(25).* Hence, the raw milk can become contaminated even during harvesting at the farm (e.g., from silage, dust, feces, etc.) *(23).* Studies at the University of Guelph, Canada, suggest that the naturally occurring contamination of raw milk with *C. botulinum* is around 1 spore/liter *(6).*

The higher number of *C. botulinum* spores that we detected in different mascarpone cheese lots was partly due to the use of creams produced by centrifugation or the skimming process in some hard cheese production plants. This process entails the microbial enrichment of milk/cream and consequently the concentration of *Clostridium* spores. The presence of a high number of *Clostridium* spores, especially *C. tyrobutyricum*, causes the ''late blowing'' phenomenon, well known in the hard cheese technology *(2).*

Further concentration of the spores in the mascarpone cheese probably occurred during the cream concentration phase carried out by the ultrafiltration, which was also likely to retain the bacterial spores.

The high prevalence of *C. botulinum* type A spores recovered from the samples is attributable to their wider distribution in central and northern Italy *(7).* So far, type B has been the toxin mostly involved in foodborne botulism cases in Italy, generally as a result of the consumption of home-canned vegetables that are traditionally prepared in the southern areas.

During the manufacturing of mascarpone cheese, the temperatures and times used for the thermal treatment of milk cream were not sufficient to kill the proteolytic spores of *C. botulinum*, which are more heat resistant than nonproteolytic ones *(14).* Indeed, only *C. botulinum* type A and proteolytic B were recovered from the samples analyzed.

However, the time–temperature combination used did eliminate the viable cells of other microorganisms (e.g., lactic acid bacteria), well-known competitors of *C. botulinum (11),* thus promoting its multiplication under favorable conditions.

The pH of the mascarpone brand involved in the botulism outbreak ranged from 5.98 to 6.25, which did not prevent *C. botulinum* growth *(15).* Other intrinsic factors such as water activity and redox potential affect the limiting pH. At an $a_w > 0.96$, as observed in experimental cheese samples, a critical pH of 5.6 is sufficient to ensure inhibition of *C. botulinum* outgrowth for up to 3 months *(29).* Furthermore, the acidulant used for the coagulation of the milk cream was citric acid, which has been demonstrated to be a less active preservative against *C. botulinum* than other acids, even at lower pH values *(24).*

Finally, the production process and packaging at high temperatures (70 to 75°C) with low-gas-permeable films probably produced quite a hermetic packaging that, combined with a decrease in air (and oxygen), probably created a suitable environment for *C. botulinum* growth and toxin production.

The only parameter prescribed that would have acted as a factor controlling *C. botulinum* growth in the product was the storage temperature of +4°C, recommended by the manufacturer and clearly printed on each package. At this temperature, the proteolytic strains of *C. botulinum* surviving the heat treatment do not multiply *(15).* However, any possible breakdown in the cold chain for long periods of time from storage, distribution, and retail to the final consumer may be very risky if the conditions within the products are suitable for spore outgrowth and toxin production. Either elimination of the naturally occurring spores from the raw milk/cream used for mascarpone production or in-

hibition of their growth in the product are necessary to ensure the safety of the final product.

According to the ''hurdle concept'' *(19)*, several different barriers should be set up in order to control the potential occurrence of foodborne pathogens in processed cheeses. Hence, although refrigeration temperature of storage would be sufficient alone to ensure the safety of the final products considered in this study, the botulism episode in Italy clearly shows that the products may undergo temperature abuse because they are probably regarded as unperishable. In consideration of the impossibility of modifying those factors that influence pH and a_w and render the product unacceptable to most consumers, the use of nisin has recently been authorized (10 mg/kg) in order to control the hazard posed by these temperature abuse events *(31)*.

The presence of *C. botulinum* spores in other cheeses is kept under control by refrigeration and intrinsic factors. Furthermore, any temperature abuse entails a swift and marked deterioration of the organoleptic characteristics of the product (consistency, odor, color, and flavor) that sets the consumer on the alert. Moreover, the high load of lactic acid bacteria that characterizes Italian mozzarella cheese has been demonstrated to inhibit *C. botulinum* outgrowth and toxin production *(11)*.

Finally, none of the raw or pasteurized milk samples were spoiled with *C. botulinum* spores, confirming a very low incidence of this microorganism in large volumes of milk, as reported by other authors *(6)*. In conclusion, to reduce the risk of botulism from mascarpone and similar dairy products, dairies should monitor the efficacy of their heat process or any other inhibitory barrier used to control *C. botulinum* and observe the hazard analysis critical control point program.

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