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Influence of reduced levels or suppression of sodium nitrite on the outgrowth and toxinogenesis of psychrotrophic *Clostridium botulinum* Group II type B in cooked ham

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ABSTRACT

Outgrowth and toxinogenesis of *Clostridium botulinum* Group II (non-proteolytic) type B were studied in cooked ham prepared with different $NaNO_2$ (ranging from 0 to 80 mg/kg) and sodium chloride (NaCl, ranging from 12 to 19 g/kg) incorporation rates. Cured ground pork batters were inoculated with a cocktail of 3 strains of *C. botulinum* Group II type B at 3.5 $\log_{10}$ CFU/g, portioned and samples of 50 g were vacuum packed then cooked and cooled based on thermal processing employed by the meat processing industry. These cooked ham model samples were stored under reasonably foreseeable conditions of use and storage i.e. for 14 days at 4 °C, followed by a cold chain break for 1 h at 20 °C then up to 33 days at 8 °C. Storage times and temperatures were used to mimic those commonly encountered along the supply chain. Enumeration of *C. botulinum* and detection of the botulinum neurotoxin type B (BoNT/B) were performed in triplicate at different storage times. Under these experimental conditions, incorporation rates of $NaNO_2 \geq 30$ mg/kg prevented the outgrowth and toxinogenesis of *C. botulinum* Group II type B in the cooked ham model, regardless of the NaCl concentrations tested. In contrast, total removal of nitrite allowed outgrowth and toxin production during storage of the processed meat product. Results showed that the maximum ingoing amount of nitrite (i.e. 150 mg/kg) that may be added according to the EU legislation (Regulation (EC) No 1333/2008) can be reduced in cooked ham while still ensuring control of *C. botulinum* Group II type B. According to the multiple factors that could affect *C. botulinum* behavior in processing meat products, outgrowth and toxin production of *C. botulinum* should be evaluated on a case by case basis, depending on the recipe, manufacturing process, food matrix and storage conditions.

1. Introduction

Botulism is a severe disease that still occurs sporadically in Europe. The last surveillance reports edited from the European Centre for Disease Prevention and Control (European Centre for Disease Prevention and Control, 2018, 2014) mentioned that 91 and 146 confirmed cases of botulism were reported in 2014 and 2015, respectively. The Surveillance Atlas of Infectious diseases announced 95 cases in 2018 in the European Union (http://atlas.ecdc.europa.eu/public/index.aspx). These data include all forms of botulism (i.e. foodborne, infant, wound, inhalation) but foodborne origin constitutes one of the most common forms. In France, an average annual incidence rate of 0.37 per one million population was obtained over the period 1991–2016 (https://www.santepubliquefrance.fr). Foodborne botulism poisoning may occur in case of the ingestion of *C. botulinum* neurotoxins through improperly processed foods initially contaminated with the pathogenic bacteria/spores. Thirty nanograms of neurotoxin is sufficient to induce illness and death (Peck, 2006). *Clostridium botulinum* is a Gram-positive spore-forming anaerobic bacteria present in the environment (soil and water) and in the intestinal tract of farm animals. As a consequence, it could be found in a variety of foods, including vegetables (such as soybean, spinach, mushrooms etc.), fish and meat products after carcass contamination linked to inappropriate slaughtering or handling practices. Their implication differs between countries and depends on local eating habits and food preservation practices.

Four physiological Groups (I-IV) have been described but Group I

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Table 1
Details of the 3 experiments conducted using the cooked ham model.

Experiment	Nitrite (N) (mg/kg) and sodium chloride (S) (g/kg) combinations tested	Reducing agent tested (mg/kg)	Dates of *C. botulinum* enumeration
No. 1	N0S0, N30S12, N30S16, N60S12, N60S16[a]	Ascorbate (2500)	D0B[b], D0A[c], D14, D34[c], D40 and D47[c] (=DE)
No. 2	N0S0, N0S17, N0S19	Ascorbate (2500)	D0B, D0A[c], D14, D21, D26, D34[c] (=DE)
No. 3	N0S0, N0S13.5, N0S18, N30S13.5, N30S18, N60S13.5, N60S18, N80S13.5	Erythorbate (500)	D0B, D0A[c], D26[c], D47[c] (=DE)

[a] An erythorbate concentration of 500 mg/kg was also tested in experiment no. 1 for N60S12 and N60S16 only.
[b] D0B: Day 0 Before thermal processing; D0A: Day 0 After thermal processing; DE: Day end of shelf-life.
[c] Toxin detection analysis.

(proteolytic) and Group II (non-proteolytic) are responsible for most cases of human foodborne botulism (Peck, 2006). Proteolytic *C. botulinum* strains from Group I are able to grow at a minimal temperature of 10 °C, at pH values ranging from 4.6 to 9.0 and at a minimal a_w value of 0.94; they produce type A, B and F neurotoxin forms. In comparison, non-proteolytic *C. botulinum* strains from Group II are able to grow at a minimal temperature of 2.5 °C, at pH values ranging from 5.0 to 9.0 and at a minimal a_w value of 0.97; they produce type B, E and F neurotoxin forms (ANSES, 2019, 2010). Especially, Group II (type B) strains were isolated from all processed meat products implicated in foodborne botulism in France between 2013 and 2016 (Mazuet, 2017). Those meat products, mostly homemade, accounted for 70% of the foodborne botulism cases registered in France during this period of time. Physicochemical characteristics of charcuteries such as cooked ham (pH around 6.0 and a_w values ranging from 0.97 to 0.99) as well as storage conditions (4–8 °C for several weeks) are suitable for growth and toxin production of these non-proteolytic *C. botulinum* strains.

Sodium nitrite ($NaNO_2$) is commonly used in meat processing for its antimicrobial effect against *Clostridium* spp. (Majou and Christieans, 2018; Sindelar and Milkowski, 2011). Furthermore, $NaNO_2$ plays a key role in the development of color, flavor and in the control of lipid oxidation (Sindelar and Milkowski, 2011). Until now, no single substitute is able to procure all the functionalities of nitrite (Alahakoon et al., 2015). Some food producers use vegetables extracts with high content of nitrate (such as beet, celery, arugula and spinach (Majou and Christieans, 2018)) as an alternative to the addition of sodium nitrite; however, nitrate is reduced to nitrite during food processing via the nitrate reductase activity of starter cultures (Alahakoon et al., 2015; Hammes, 2012). In Europe, scientific experts from EFSA recommend an addition of between 50 and 150 mg/kg nitrite to prevent the outgrowth of *C. botulinum* in meat products with a low sodium chloride content and having a prolonged shelf-life (EFSA, 2004). The use of nitrite may nevertheless contribute to the formation of nitrosamines, which are considered as carcinogenic substances (EFSA, 2010, 2004; Majou and Christieans, 2018; Mortensen et al., 2017). Additionally, high sodium chloride consumption is correlated with a higher risk of cardiovascular diseases (Cappuccio, 2013; Kong et al., 2016). A maximal consumption of 5 g of sodium chloride a day is recommended by the World Health Organization (2012). According to the European Commission (European Union, 2015), the current daily sodium chloride consumption is between 8 and 12 g in most European countries. Professionals in accordance with consumer expectations want to propose clean label processed meat products, i.e. nitrite-free or containing reduced concentrations of nitrite (< 150 mg/kg for cooked ham) w/wo lower sodium chloride levels (e.g. a 25% reduction). However, these new practices may alter microbiological safety of these products and their impact must be evaluated with regard to the risk of *C. botulinum* outgrowth and toxin production.

The objective of the present study was to investigate the outgrowth and toxin production of psychrotrophic *C. botulinum* Group II (non-proteolytic) type B during manufacture and storage of cooked ham prepared with different ingoing amounts of $NaNO_2$ (ranging from 0 to 80 mg/kg) and sodium chloride (NaCl, ranging from 12 to 19 g/kg). The generated data will help producers and governmental authorities to define critical incorporation rates of $NaNO_2$ and NaCl necessary to achieve the required preservative effect against *C. botulinum*.

2. Material and methods

2.1. Cooked ham model samples preparation

The outgrowth and toxin production of *C. botulinum* Group II type B were assayed during manufacture and storage of cooked ham. In this attempt, a cooked ham model derived from previous work of Redondo-Solano et al. (2013) was used in this study. This model is composed of 50 g ground pork supplemented with a specific brine solution as described below. The use of this model permits homogeneous inoculation with *C. botulinum* as well as homogeneous curing process of samples. Another benefit of this approach is to ensure optimal safety conditions that are required when handling samples contaminated with *C. botulinum*.

Three independent experiments, including various recipes that could be used in the meat processing industry, were carried out (Table 1). The cooked ham model samples were made in a pilot-scale unit. They were prepared with fresh pork (*biceps femoris* muscle) which was ground twice through a 4.5 mm plate of a grinder (TX 98 Compact, Dadaux, France). In each experiment, the ground pork was divided into as many recipes as necessary, then mixed with appropriate concentrations of $NaNO_2$ (0, 30, 60 or 80 mg/kg), NaCl (0, 12, 13.5, 16, 17, 18 or 19 g/kg), sodium erythorbate (500 mg/kg) or sodium ascorbate (2500 mg/kg) and dextrose (5 g/kg). According to the French "Code des usages de la charcuterie, de la salaison et des conserves de viandes", the maximal incorporation rate of erythorbate may not exceed 500 mg/kg in heat-treated processed meat products. When higher amounts of antioxidant are used, sodium erythorbate is replaced with sodium ascorbate which can be used *quantum satis*. A control recipe (N0S0) without nitrite and sodium chloride was included in all 3 experiments. In all recipes, water was added in order to have a 9% brine rate.

2.2. Clostridium botulinum Group II type B strains and inoculation

A cocktail of 3 *C. botulinum* Group II (non-proteolytic) type B strains i.e. BL7 (reference strain, UK), 300.05 (isolated from a homemade dry cured ham in 2005, France) and 815.12 (isolated from a homemade dry cured ham in 2012, France) was used to inoculate the cured ground pork batters. The use of several strains mixed in a cocktail is recommended by the ISO 20976-1 standard for estimation of growth potential so that variations among strains are taken into account. All these strains were provided by the CNR Bactéries anaérobies et Botulisme (Pasteur Institute, Paris, France) and previously selected based on their toxin production levels in raw ground pork incubated under permissive growth conditions of the pathogen. The BL7, 300.05 and 815.12 strains displayed toxin production levels equal to 120, 3800 and 4800 Lethal Dose/g, respectively. This reflects variations of toxin levels naturally encountered within foods incriminated in foodborne botulism cases (Popoff, 2016; personal communication).

These strains were maintained at −80 °C on cryobeads. For each strain, spores were *produced* from growing cells in BHI broth after

anaerobic incubation at 30 °C for 12 weeks. Vegetative forms were eliminated by heating suspensions at 60 °C for 20 min (Canada, 2010). Spores were harvested following centrifugation at 4500*g* for 20 min and washed three times using saline solution (9 g/l NaCl). Before inoculation, the spore suspension of each strain was enumerated on tryptone sulfite agar. A spore cocktail of the three *C. botulinum* Group II type B strains in equal proportions was prepared in saline solution, which was used to inoculate the different cured ground pork batters at 3.5 $\log_{10}$ CFU/g (mean inoculum value ± standard deviation (s.d.) of 3.6 ± 0.39 $\log_{10}$ CFU/g, all 3 experiments included). Afterwards, each inoculated cured ground pork batter was portioned and samples of 50 g were vacuum packed (Cryovac, CN 300, 60 µm, oxygen permeability: 13 cm^3/m^2). They were then stored at 4 °C for 4–5 h to simulate vacuum tumbling before thermal processing.

2.3. Thermal processing and storage conditions

Vacuum packed samples of 50 g were subjected to thermal treatment classically used in the meat processing industry. Cooking and cooling processes were performed in an oven Spakomat 550 KRB (Spako, Netherlands). Temperature of the cooking and cooling processes was monitored using two temperature probes (CTN, Testo AG 06102217-803), one inserted into a 50 g sample and another one placed in ambience of the oven chamber. All values were registered by using a datalogger Testo 171 (Testo, Allemagne). The core temperature of the processed meat product reached 67 °C after 520 min followed by a cooling process that required 820 min from 67 °C to 4 °C.

The cooked ham model samples were then stored under reasonably foreseeable conditions (time and temperature) that could be encountered along the supply chain i.e. 14 days at 4 °C (cold storage chamber) + 1 h at 20 °C (rupture in the cold chain relative to the transport of products by consumers) + 20 (Experiment no. 2) or 33 (Experiments nos. 1 and 3) days at 8 °C (distributor shelving and domestic storage in refrigerator).

2.4. Microbiological analysis

The cooked ham model samples were analyzed at different sampling dates, including Day 0 before and after thermal processing (noted D0B and D0A, respectively), intermediate points and Day end (end of shelf-life, noted DE) as described in Table 1.

At each sampling date, three samples per recipe were analyzed for the enumeration of *C. botulinum*. Briefly, each 50 g sample was five-fold diluted in buffered peptone water and stomacher at medium speed for 2 min. One milliliter of this suspension and/or of the appropriate ten-fold serial dilutions was plated on tryptone sulfite agar plates which were incubated anaerobically at 30 °C during 48 h. Colonies of *C. botulinum* are black and develop a black halo around them. Absence of natural contamination of raw materials with quantifiable *C. botulinum* cells was checked at D0B, D0A and DE by using the above protocol on 3 samples of the control recipe (N0S0) prepared for each 3 experiments. Total aerobic mesophilic microbiota was enumerated in triplicate (from 3 different samples/recipe) at the same 3 sampling dates by plating appropriate dilutions on PCA after a 72 h-incubation at 30 °C. Lactic acid bacteria counts were determined in triplicate (from 3 different samples/recipe) at D0A and DE following plating of appropriate dilutions on MRS medium after a 48 h-incubation at 30 °C. The quantification limit of the different microbiological analysis was of 5 CFU/g.

2.5. C. botulinum Type B neurotoxin (BoNT/B) detection

Toxin detection was performed by using the gold standard mouse bioassay (Centers for Disease Control, 1978) with approval from ethics commission of Sciensano for animal experiments (ethical file numbers 20190319-01 and 20140808-01). The absence of the toxin was checked at D0A in 5 cooked ham model samples of the control recipe (N0S0) prepared for each three experiments. Then, bioassay analyses were carried out on 3 samples/recipe at 2 sampling dates (during shelf-life and DE, experiments nos. 1 and 3) or 1 (only DE, experiment no. 2). Briefly, 25 g test portions were cut into small pieces and homogenized in 25 ml of phosphate gelatin buffer (0.4% Na_2HPO_4, 0.2% gelatin, pH 6.2), centrifuged and supernatants were passed over a 0.45 µm filter. Extracts were administered into two mice by intraperitoneal (ip) injection of 400 µl/mouse. Botulism symptoms were observed during the 4 following days. In case botulism symptoms and/or mortality were observed, a seroneutralization was performed in order to confirm the presence of BoNT/B. To this end, two control mice were injected ip with the food extract (400 µl/mouse) and two more mice were injected ip with a mixture of food extract (400 µl) and BoNT/B neutralizing antibodies (NIBSC; 100 µl at 10 IU/ml). A sample was considered positive for BoNT/B when control mice showed botulism symptoms and/or mortality and in parallel mice receiving BoNT/B neutralizing antibodies were protected. For ethical reasons, one sample per triplicate was first tested. In case BoNT/B was detected in this sample, the other replicates were not tested and this recipe was considered positive at this sampling date. In case the first sample showed a negative result, the other two replicates were tested using the mouse bioassay for confirmation.

2.6. Physico-chemical analysis

Water activity (a_w) and pH were measured on 1 cooked ham model sample per recipe at D0A and DE according to the NF ISO 21807:2005 and ISO 2917:1999 standards, respectively. A_w and pH measurements were performed by using a Thermoconstanter TH200 (Novasina, Swiss) and a 765 CALIMATIC® (Knick, Allemagne) apparatus connected to a puncture electrode LoT406-M6-DXK-S7/25 (Mettler-Toledo, Suisse), respectively. At the same sampling dates, residual nitrite and nitrate contents in meat products were determined, using a Spectronic Genesys 2PC (Namur, Belgium), on 1 sample per recipe according to the ISO 2918:1975 (F) and ISO 3091:1975 standards, respectively. The quantification limits of the method were 0.26 mg nitrite and 0.41 mg nitrate per kg of meat. For each sample analyzed, results were expressed as total nitrite content (mg $NaNO_2$ per kg of meat) obtained by adding measured amounts of nitrite and nitrate once converted to nitrite. Since both additives interact with biochemical components from meat in complex ways and taking into account the measurement uncertainty, it is recommended not to dissociate measured amounts of nitrite and nitrate, according to the French “Code des usages de la charcuterie, de la salaison et des conserves de viandes”. The sodium chloride content analysis was carried out in 1 sample per recipe at D0A according to the ISO 1841-1:1996 (F) standard.

2.7. Statistical analysis

Growth potential (δ) was calculated according to the equation from the ISO 20976-1:2019 standard: $(\delta) = \overline{\log_{10} t} - \overline{\log_{10} i}$ where $\log_{10} t$ is the mean value obtained at a given sampling date and $\log_{10} i$ is the mean value obtained at day 0 after thermal processing (D0A). When δ was higher than 0.5 $\log_{10}$ CFU/g the cooked ham model samples were considered permissive to the growth of *C. botulinum* Group II type B.

The effect of erythorbate (500 mg/kg) and ascorbate (2500 mg/kg) input levels on growth potentials of *C. botulinum* Group II type B in the cooked ham model was assessed for each nitrite/sodium chloride combinations (compiling data from all three experiments) by using a nonparametric Kruskal Wallis test. Different groups of nitrite/sodium chloride combinations were also defined (i.e. N0S0; N30S12 and 13.5; N30S16 to 19; N60S12 and 13.5; N60S16 to 19) prior statistical analysis in order to increase sample size per group and then the power of the test.

The relationship between the NaCl contents measured at D0A and their respective ingoing amounts was assessed with a simple linear regression model using the lm() function.

Table 2
Mean pH and a_w values of the cooked ham model calculated at Day 0A (after thermal processing) and Day end (end of shelf-life), all recipes included. Results were expressed as mean ± standard deviation (n = 18 for each mean value).

	Day 0A	Day end
pH	6.11 ± 0.115	6.06 ± 0.088
a_w	0.994 ± 0.0050	0.993 ± 0.0048

All statistical analyses were performed using the RStudio statistical software (version 1.1.456—2009–2018).

3. Results and discussion

3.1. Physico-chemical characteristics of the cooked ham model

The cooked ham model displayed typical pH (mean value of 6.11 right after cooking/cooling treatment) and a_w (mean value of 0.994 right after cooking/cooling treatment) values, which remained steady over the storage period (Table 2). These results were consistent with those reported in other studies in which cooked ham exhibited pH values of between 5.9 and 6.25 units and a_w values of between 0.98 and 0.99 (FDA, 2001; Redondo-Solano et al., 2013; Tomović et al., 2013). All pH and a_w values measured during the present study were permissive to the growth of *C. botulinum* Group II type B (ANSES, 2019).

Levels of residual nitrite measured in the cooked ham model samples right after cooking/cooling treatment were typically lower than the corresponding inputs, depending on the ingoing amounts of ascorbate/erythorbate (Table 3). Honikel (2008) mentioned that the largest depletion of nitrite (about 65% of the ingoing nitrite concentration) is observed during manufacturing up to the end of the cooking process, then depletion slightly continues during cold storage. This depletion is due to the high reactivity of nitrite, which can participate in multiple reactions such as nitrosylation/nitrosation (promoting the formation of nitrosomyoglobin, nitrosamines, nitrosothiols and other nitrosated compounds), conversion to nitrate, generation of some gaseous derivatives (nitrogen, nitric oxide, nitrous oxide) and/or formation of prooxidative peroxynitrite. All these reactions may be affected by several factors including structure and composition of meat, recipe, pH, heat treatment and storage conditions (time and temperature) (Govari and Pexara, 2018; Merino et al., 2016; Sofos et al., 1979a).

In our study, an ascorbate input equal to 2500 mg/kg was linked to absence or almost total absence of measurable nitrite contents at D0A. The presence of ascorbate or erythorbate that acts as a reducing agent is known to enhance the rate of decrease of nitrite in meat products (Govari and Pexara, 2018; Honikel, 2008); the higher the ingoing amount of reducing agent is, the more depleted nitrite will be (Brown et al., 1974; Izumi et al., 1989). In comparison, residual nitrite levels recovered at D0A in the cooked ham model samples made with 500 mg erythorbate per kg of meat ranged from 3.8 to 7.5 mg/kg, with no marked difference between the different ingoing amounts of nitrite tested (30, 60 and 80 mg/kg) (control recipes w/o nitrite were not taken into account in this analysis) (Table 3).

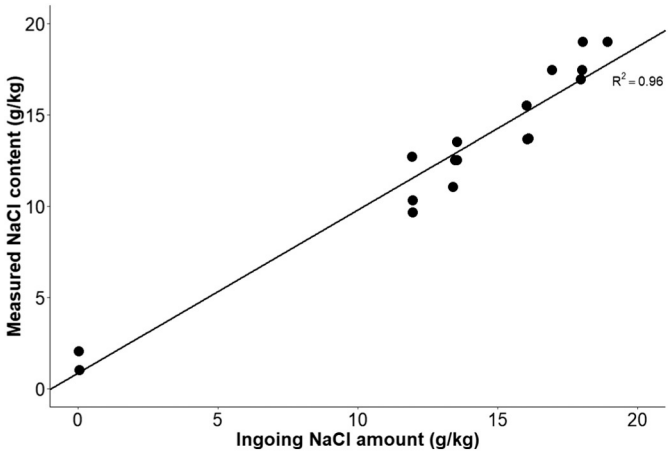

Fig. 1. Correlation between NaCl contents measured at Day 0A (after thermal processing) and the ingoing amounts of NaCl (g/kg) used in the different cooked ham recipes (data compiled from all 3 experiments, n = 18). The solid line represents the fitted linear regression line (R^2 = 0.96).

Regarding the NaCl contents, significant positive correlation was obtained between those measured in the meat samples right after cooking/cooling treatment and their respective ingoing amounts (linear regression coefficient R^2 = 0.96, $P < 0.0001$) (Fig. 1). Standard deviations values calculated from the NaCl contents measured in the meat samples ranged from 0.90 to 1.30 g/kg, which reflected those observed in an industrial context.

3.2. Outgrowth and toxin production of C. botulinum gr. II type B in the cooked ham model

For each experiment, raw materials used for preparation of the cooked ham model samples did not naturally contain quantifiable *C. botulinum* cells (i.e. < 0.7 Log_{10} CFU/g) since no characteristic colonies were observed on tryptone sulfite agar plates. Results showed that contamination levels of raw materials with total aerobic mesophilic microbiota at D0B was within an acceptable range i.e. 3.5 (s.d. = ± 0.53) log_{10} CFU/g. This indigenous microbiota was mostly inactivated by the cooking process with populations below the quantification limit of 0.7 log_{10} CFU/g at D0A; no regrowth was then observed throughout the shelf life of the product. Similar trends were obtained for the lactic acid microbiota.

Table 3
Total nitrite contents measured in the cooked ham model at Day 0A (after thermal processing) and Day end (end of shelf-life) according to the ingoing amounts of nitrite and reducing agent (500 mg erythorbate/kg or 2500 mg ascorbate/kg), regardless of the NaCl concentrations tested. Numbers of observations (n) are entered in parentheses.

Ingoing amounts of nitrite (mg/kg)	Ingoing amounts of reducing agent (mg/kg)	Total nitrite contents (mg/kg)	
		Day 0A	Day end
0	500	Absence[a] (n = 3)	Absence (n = 3)
	2500	Absence (n = 4)	Absence (n = 4)
30	500	3.8–5.9 (n = 2)	3.4–5.1 (n = 2)
	2500	Absence – 0.7 (n = 2)	Absence (n = 2)
60	500	4.3–6.0 (n = 4)[b]	2.0–5.9 (n = 4)[b]
	2500	Absence – 0.7 (n = 2)	Absence (n = 2)
80	500	7.5 (n = 1)	4.1 (n = 1)

[a] All nitrite and nitrate measures were inferior to the quantification limits of the analytical method used in the present study i.e. 0.26 and 0.41 mg/kg, respectively.
[b] These are minimum and maximum values. Mean values (± s.d.) of 5.3 (± 0.73) and 3.5 (± 1.69) were obtained at Day 0A and Day end, respectively.

The contamination of raw meats with *C. botulinum* spores is poorly documented but according to previously published work it is considered to be very low (occasional incidence of 1 spore per 0.5–3 kg of meat) (Sofos et al., 1979b). Contamination of raw meats with *C. botulinum* is most likely to occur during slaughtering process, especially through contact with intestinal contents following inappropriate evisceration practices. The work of Dahlenborg et al. (2001) showed that faecal load of *C. botulinum* Gr. II type B spores in pigs was less than 4 spores per g, which is consistent with the above-mentioned low-level contamination of raw meats. In this study, a relatively high inoculum level of *C. botulinum* (mean value ± s.d. of 3.6 ± 0.39 $\log_{10}$ CFU/g before cooking and cooling processes) was deliberately chosen in order to draw safe conclusions. It is in accordance with the ISO 20976-1:2019 instructions which dictate that the inoculation rate must be not more than 4 $\log_{10}$ CFU/g and more than 5 times the limit of quantification (i.e. 5 CFU/g in this study). Moreover, this allowed to begin the storage period with relevant *C. botulinum* population levels (mean value ± s.d. of 2.4 ± 0.5 $\log_{10}$ CFU/g) since the cooking and cooling process resulted in 1 to 2 Log_{10} reduction of *C. botulinum* cells (destruction of vegetative cells formed by germination of spores during the early stage of the cooking process), regardless of the recipes tested.

In any cases (based on individual observations or following nitrite/sodium chloride clustering in various groups as described in the material and methods section), results showed that the ingoing amount of ascorbate (2500 mg/kg) or erythorbate (500 mg/kg) did not significantly impact the outgrowth of *C. botulinum* Group II type B (as well as toxin production as deduced from the results) in the cooked ham model (Kruskal test, $P > 0.05$). Consequently, data obtained for a same nitrite/sodium chloride combination were compiled regardless of the ascorbate/erythorbate inputs and shown accordingly in Figs. 2, 3 and 4. The role of sodium ascorbate/erythorbate regarding the outgrowth and toxin production by *C. botulinum* is under debate in the scientific literature. Depending on conditions, it was sometimes demonstrated that these compounds may enhance the nitrite's inhibition of *C. botulinum* by sequestering metal ions in cured meats (Robinson et al., 2007; Tompkin et al., 1979). However, others found that sodium ascorbate did not alter or potentiate the inhibition of *C. botulinum* toxin formation by sodium nitrite (Bowen et al., 1974).

The data set suggested that *C. botulinum* Group II type B will not be able to significantly grow ($\delta < 0.5$) and to produce BoNT/B toxins in the cooked ham model samples prepared with an incorporation rate of nitrite ≥ 30 mg/kg and NaCl concentrations ≥ 12 g/kg after 47 days

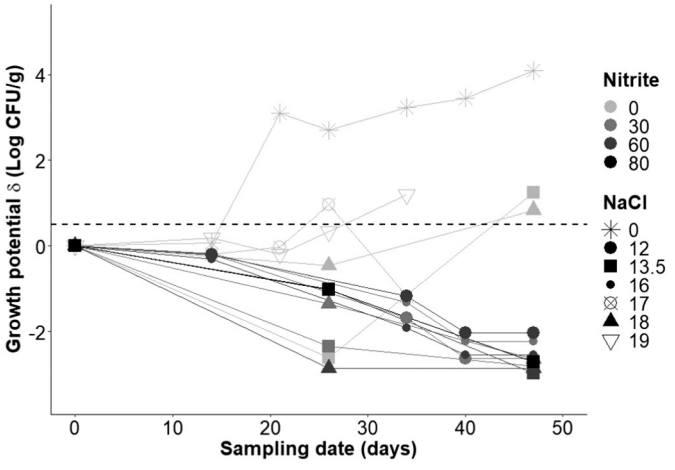

Fig. 2. Growth potentials (δ) of *C. botulinum* group II type B obtained at the different sampling times during shelf-life of the cooked ham model in relation to nitrite (mg/kg) and NaCl (g/kg) input levels (data compiled from all 3 experiments). Results were based on triplicate analysis. The dashed line represents the limit value of 0.5 Log_{10} CFU/g above which the cooked ham model samples support the growth of *C. botulinum*.

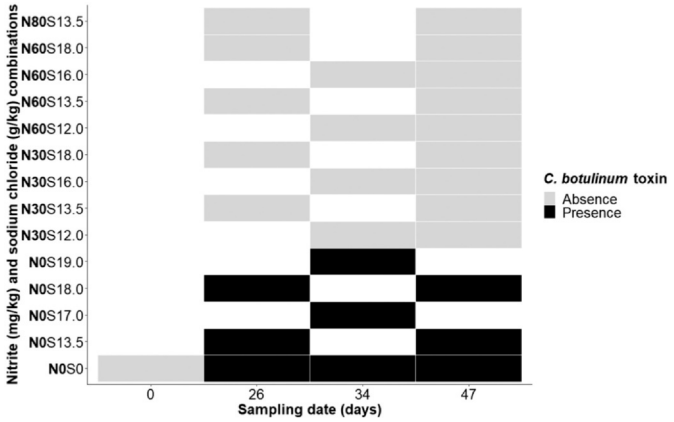

Fig. 3. Detection of the *C. botulinum* neurotoxin type B (BoNT/B) at the different sampling times during shelf-life of the cooked ham model according to the various nitrite and NaCl combinations tested (N, nitrite in mg/kg; S, sodium chloride in g/kg) (data compiled from all 3 experiments). Results were based on triplicate analysis (in case the first sample showed a positive result, the other two replicates were not tested). All N0S0 samples ($n = 5$) from each 3 experiments analyzed at D0A were negative for the toxin BoNT/B. Blank boxes indicate absence of toxin detection analysis.

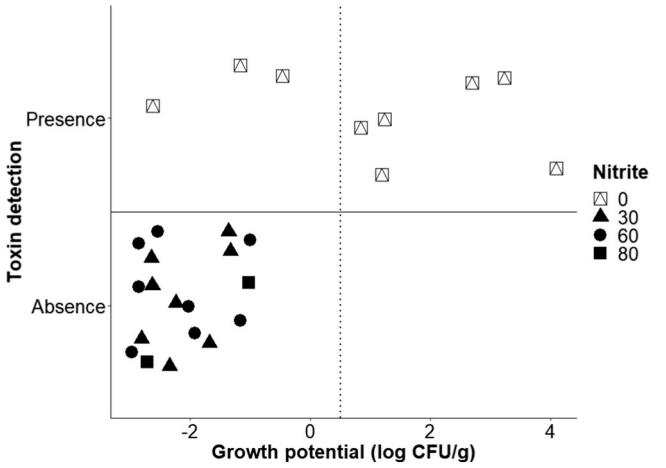

Fig. 4. Relationship between detection results for the neurotoxin type B (BoNT/B) and growth potentials of *C. botulinum* group II type B obtained from all the cooked ham model samples analyzed (data compiled from all 3 experiments) according to the nitrite input levels (ranging from 0 to 80 mg/kg). The dotted line represents the limit value of 0.5 Log_{10} CFU/g above which the cooked ham model samples support the growth of *C. botulinum*. Points were voluntarily showed using the geom_jitter function (package ggplot2, R), which add a small amount of random variation to the location of each point, in order to avoid overplotting.

storage under defined temperature conditions (Figs. 2 and 3). However, it is interesting to note that one sample tested positive for BoNT/B toxin in cooked ham formulated with 18 g/kg NaCl and 30 mg/kg $NaNO_2$ after 9 weeks of storage at 8 °C (data not shown). This input level of nitrite is probably closed to the threshold value associated with an anti-botulinum effect in cooked ham, which could lead under long-term storage at abusive refrigerated temperature to outgrowth of *C. botulinum* and toxin production. Both nitrite and sodium chloride exhibit inhibitory activities against *C. botulinum* germination and its subsequent outgrowth as described earlier. Majou and Christieans (2018) suggested that the antimicrobial activity of nitrite is likely attributed to the level of oxidative stress caused by the formation of peroxynitrite ($ONOO^-$) and peroxynitrous acid (ONOOH). Notably, this oxidative stress is enhanced by the chloride anion coming from dissociation of NaCl as well as the presence of H_2O_2 that *C. botulinum* cannot

metabolize because is devoid of catalase. Additional factors may interact with production and reaction kinetics of these chemical compounds and consequently affect the behavior of *C. botulinum* in processed meat products such as pH, a_w, iron content, reductants, redox potential, heat treatment, storage time and temperature (Govari and Pexara, 2018; Lee et al., 2018; Sofos et al., 1979b). Therefore, microbiological safety of processed meat products must be evaluated while considering combinations of all these factors, which may interact with each other to produce synergistic effect on inhibition of *C. botulinum*.

In contrary, *C. botulinum* toxin BoNT/B was detected during storage of the cooked ham model samples devoid of nitrite (Fig. 3). Based on results of the present study only, it is not possible to appreciate precisely the time and temperature conditions necessary for supporting production of detectable quantities of *C. botulinum* toxin in cooked ham. A storage at temperatures ≤ 4 °C for few weeks seems to be efficient in controlling the risk of outgrowth of *C. botulinum* Group II type B strains in products that contained enough sodium chloride. Using the same cooked ham model formulated without nitrite and with 18 g sodium chloride/kg we showed that an 11-weeks storage at 4 °C did not permit the outgrowth and toxigenesis of the same cocktail of *C. botulinum* as that employed in this study (unpublished data). This corroborates results obtained by Hyytiä-Trees et al. (2000) on different vacuum packed processed products for which toxigenesis was detected only in samples stored at 8 °C (those stored at 4 °C for up to 37 days were all negative for the toxins). Nevertheless, supplemental experiments are required to reinforce this point, especially by using an extended panel of *C. botulinum* Group II strains characterized for their cardinal temperatures. These results emphasized that temperature plays a key role in growth control of psychrotrophic *C. botulinum*, and that the cold supply chain must be vigorously maintained, especially for cooked hams formulated with reduced-level of nitrite or w/o nitrite. Conversely, results from the present study highlighted that a 2 weeks storage at 8 °C led to outgrowth and toxinogenesis of *C. botulinum* in the cooked ham model w/o nitrite, regardless of the NaCl concentrations tested (based on results obtained at D26, experiment no. 3). This finding is in accordance with previous works of Peck et al. (2008), which showed that non-proteolytic *C. botulinum* can grow and form toxin in ≤10 days at ≤8 °C in foods/food materials in laboratory tests. In comparison, other authors demonstrated that cooked ham (1.8% of sodium chloride) formulated without addition of nitrite supported the outgrowth of *C. botulinum* after 3 weeks storage at 8 °C, whereas other products supplemented with nitrite at 75 or 120 mg/kg did not display any outgrowth and toxin production during the 5-weeks storage period (Keto-Timonen et al., 2012). This is of particular importance since a recent study in France showed that 51% of the household fridges were between 5.0 °C and 8.0 °C and 20% were over 8.0 °C (ANSES, 2017). Similar results were obtained from a study conducted in UK, in which 28% of the domestic fridges were between 7.0 and 8.9 °C and 9% over 9.0 °C (Peck et al., 2008).

In most cases, there was a positive correlation between growth potentials and BoNT/B toxin detection results obtained for a same sample. However, in few cases, the presence of botulinum toxin BoNT/B was not associated with a significant growth of the pathogen ($\delta < 0.5$ $\log_{10}$ CFU/g) (Fig. 4). There is no consensus on the minimum populations of *C. botulinum* or the log increase required to produce detectable toxin levels in the scientific literature. Based on previous studies, toxin can be detected for increases in *C. botulinum* numbers ranging from 10 to 1000-fold, and in some cases prior to any increase in plate counts (Glass and Marshall, 2013). The fact that toxin can be detected in absence of significant growth of the pathogen could be explained by: (i) growth of vegetative cells to a high number followed by cell death (absence of further sporulation during storage was previously demonstrated, unpublished work); consequently the increase in cell count was missed between sampling points, (ii) growth of small numbers of cells leading to toxin; heterogeneous growth dynamics of single cells is likely to be important at stressed growth conditions as often found in foods (Hashimoto et al., 2016), (iii) technical issues making it easy to extract toxin but not viable and cultivable cells. This finding highlighted that toxin analysis is an obligate parameter to be considered when *C. botulinum* behavior in foods is investigated.

As demonstrated under the specific conditions of this study, nitrite levels could be substantially reduced in cooked ham, while still ensuring control of *C. botulinum* Group II type B. Since inhibition of *C. botulinum* is multifactorial, this should be evaluated on a case by case basis, depending on the recipe, manufacturing process and storage conditions. Nevertheless, it is important to note that the absence of added sodium nitrite in the cooked ham model resulted in *C. botulinum* outgrowth and toxin production. This finding was obtained using an inoculum level higher than that expected on naturally encountered raw pork. This represents a worst-case situation which offers further perspectives on reducing ingoing amounts of nitrite in this processed meat product. Additional antibotulinal hurdles and their interactions have to be implemented to ensure safety of nitrite-free products. This can be reached by using optimal combination of various factors including for example lower pH, higher NaCl concentrations, a well-managed cold supply chain and by reducing shelf-life of the products.

CRediT authorship contribution statement

Lebrun Sarah: Methodology, Validation, Investigation, Resources, Writing - original draft, Writing - review & editing, Visualization and Project administration. Van Nieuwenhuysen Tom: Methodology, Validation, Investigation, Resources and Supervision. Crèvecoeur Sébastien: Resources. Vanleyssem Raphaël: Resources. Thimister Jacqueline: Resources. Denayer Sarah: Supervision. Jeuge Sabine: Formal analysis. Daube Georges: Supervision. Clinquart Antoine: Supervision. Fremaux Bastien: Conceptualization, Formal analysis, Data curation, Writing - original draft, Writing - review & editing, Project administration and Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Alahakoon, A.U., Jayasena, D.D., Ramachandra, S., Jo, C., 2015. Alternatives to nitrite in processed meat: up to date. Trends Food Sci. Technol. 45, 37–49. https://doi.org/10.1016/j.tifs.2015.05.008.

ANSES, 2010. *Clostridium botulinum* and neurotoxigenic Clostridia. URL. https://www.anses.fr/en/system/files/MIC2010sa0234FiEN.pdf, Accessed date: 16 May 2019 (WWW Document).

ANSES, 2017. Publication d'INCA3 (Étude individuelle nationale des consommations alimentaires 3). CERIN URL. https://www.cerin.org/rapports/publication-dinca3-etude-individuelle-nationale-consommations-alimentaires-3/, Accessed date: 19 December 2019 (WWW Document).

ANSES, 2019. *Clostridium botulinum*, *Clostridium* neurotoxinogènes. URL. https://www.anses.fr/fr/system/files/BIORISK2016SA0074Fi.pdf, Accessed date: 11 February 2020 (WWW Document).

Bowen, V.G., Cerveny, J.G., Deibel, R.H., 1974. Effect of sodium ascorbate and sodium nitrite on toxin formation of *Clostridium botulinum* in wieners. Appl. Microbiol. 27, 605–606.

Brown, C.L., Hedrick, H.B., Bailey, M.E., 1974. Characteristics of cured ham as influenced by levels of sodium nitrite and sodium ascorbate. J. Food Sci. 39, 977–979. https://doi.org/10.1111/j.1365-2621.1974.tb07290.x.

Canada, H., 2010. *Clostridium botulinum* challenge testing of ready-to-eat foods. URL

aemhttps://www.canada.ca/en/health-canada/services/food-nutrition/legislation-guidelines/policies/clostridium-botulinum-challenge-testing-ready-foods-2010.html, Accessed date: 6 May 2020 (WWW Document).
Cappuccio, F.P., 2013. Cardiovascular and other effects of salt consumption. Kidney Int. Suppl. 3, 312–315. https://doi.org/10.1038/kisup.2013.65.
Centers for Disease Control, 1978. Botulism in the United States, 18991973: Handbook for Epidemiologists, Clinicians, and Laboratory Workers. CDC.
Dahlenborg, M., Borch, E., Rådström, P., 2001. Development of a combined selection and enrichment PCR procedure for *Clostridium botulinum* Types B, E, and F and its use to determine prevalence in fecal samples from slaughtered pigs. Appl. Environ. Microbiol. 67, 4781–4788. https://doi.org/10.1128/aem.67.10.4781-4788.2001.
EFSA, 2004. Opinion of the scientific panel on biological hazards (BIOHAZ) related to the effects of nitrites/nitrates on the microbiological safety of meat products. EFSA J. 2, 14. https://doi.org/10.2903/j.efsa.2004.14.
EFSA, 2010. Statement on nitrites in meat products. EFSA J. 8, 1538. https://doi.org/10.2903/j.efsa.2010.1538.
European Centre for Disease Prevention and Control, 2014. Annual epidemiological report 2014 –food- and waterborne diseases and zoonoses. URL. https://ecdc.europa.eu/sites/portal/files/media/en/publications/Publications/food-waterborne-diseases-annual-epidemiological-report-2014.pdf, Accessed date: 15 May 2019 (WWW Document).
European Centre for Disease Prevention and Control, 2018. Botulism - annual report for 2015. URL. https://www.ecdc.europa.eu/sites/portal/files/documents/AER_for_2015-botulism.pdf, Accessed date: 15 May 2019 (WWW Document).
European Union, 2015. Implementation of the EU salt reduction framework: results of member states survey.
FDA, 2001. Evaluation and definition of potentially hazardous foods.
Glass, K., Marshall, K., 2013. Chapter 27. *Clostridium botulinum*. In: Elsevier Enhanced Reader, https://doi.org/10.1016/B978-0-12-416041-5.00027-5. (WWW Document).
Govari, M., Pexara, A., 2018. Nitrates and nitrites in meat products. J. Hell. Vet. Med. Soc. 66 (127). https://doi.org/10.12681/jhvms.15856.
Hammes, W.P., 2012. Metabolism of nitrate in fermented meats: the characteristic feature of a specific group of fermented foods. Food Microbiol. 29, 151–156. https://doi.org/10.1016/j.fm.2011.06.016.
Hashimoto, M., Nozoe, T., Nakaoka, H., Okura, R., Akiyoshi, S., Kaneko, K., Kussell, E., Wakamoto, Y., 2016. Noise-driven growth rate gain in clonal cellular populations. Proc. Natl. Acad. Sci. 113, 201519412. https://doi.org/10.1073/pnas.1519412113.
Honikel, K.-O., 2008. The use and control of nitrate and nitrite for the processing of meat products. Meat Sci. 78, 68–76. https://doi.org/10.1016/j.meatsci.2007.05.030.
Hyytiä-Trees, E., Skyttä, E., Mokkila, M., Kinnunen, A., Lindström, M., Lähteenmäki, L., Ahvenainen, R., Korkeala, H., 2000. Safety evaluation of sous vide-processed products with respect to nonproteolytic *Clostridium botulinum* by use of challenge studies and predictive microbiological models. Appl. Environ. Microbiol. 66, 223–229. https://doi.org/10.1128/aem.66.1.223-229.2000.
Izumi, K., Cassens, R.G., Greaser, M.L., 1989. Reaction of nitrite with ascorbic acid and its significant role in nitrite-cured food. Meat Sci. 26, 141–153. https://doi.org/10.1016/0309-1740(89)90037-5.
Keto-Timonen, R., LindströM, M., Puolanne, E., Niemistö, M., Korkeala, H., 2012. Inhibition of toxigenesis of Group II (nonproteolytic) *Clostridium botulinum* Type B in meat products by using a reduced level of nitrite. J. Food Prot. 75, 1346–1349. https://doi.org/10.4315/0362-028X.JFP-12-056.
Kong, Y.W., Baqar, S., Jerums, G., Ekinci, E.I., 2016. Sodium and its role in cardiovascular disease – the debate continues. Front. Endocrinol. 7. https://doi.org/10.3389/fendo.2016.00164.
Lee, S., Lee, H., Kim, S., Lee, J., Ha, J., Choi, Y., Oh, H., Choi, K.-H., Yoon, Y., 2018. Microbiological safety of processed meat products formulated with low nitrite concentration — a review. Asian-Australas. J. Anim. Sci. 31, 1073–1077. https://doi.org/10.5713/ajas.17.0675.
Majou, D., Christieans, S., 2018. Mechanisms of the bactericidal effects of nitrate and nitrite in cured meats. Meat Sci. 145, 273–284. https://doi.org/10.1016/j.meatsci.2018.06.013.
Mazuet, C., 2017. Le botulisme humain en France, 2013–2016/Human botulism in France, 2013-2016 9.
Merino, L., Darnerud, P.O., Toldrá, F., Ilbäck, N.-G., 2016. Time-dependent depletion of nitrite in pork/beef and chicken meat products and its effect on nitrite intake estimation. Food Addit. Contam. Part Chem. Anal. Control Expo. Risk Assess. 33, 186–192. https://doi.org/10.1080/19440049.2015.1125530.
Mortensen, A., Aguilar, F., Crebelli, R., Domenico, A.D., Dusemund, B., Frutos, M.J., Galtier, P., Gott, D., Gundert-Remy, U., Lambré, C., Leblanc, J.-C., Lindtner, O., Moldeus, P., Mosesso, P., Oskarsson, A., Parent-Massin, D., Stankovic, I., Waalkens-Berendsen, I., Woutersen, R.A., Wright, M., van den Brandt, P., Fortes, C., Merino, L., Toldrà, F., Arcella, D., Christodoulidou, A., Abrahantes, J.C., Barrucci, F., Garcia, A., Pizzo, F., Battacchi, D., Younes, M., 2017. Re-evaluation of potassium nitrite (E 249) and sodium nitrite (E 250) as food additives. EFSA J. 15, e04786. https://doi.org/10.2903/j.efsa.2017.4786.
Peck, M.W., 2006. *Clostridium botulinum* and the safety of minimally heated, chilled foods: an emerging issue? J. Appl. Microbiol. 101, 556–570. https://doi.org/10.1111/j.1365-2672.2006.02987.x.
Peck, M.W., Goodburn, K.E., Betts, R.P., Stringer, S.C., 2008. Assessment of the potential for growth and neurotoxin formation by non-proteolytic *Clostridium botulinum* in short shelf-life commercial foods designed to be stored chilled. Trends Food Sci. Technol. 19, 207–216. https://doi.org/10.1016/j.tifs.2007.12.006.
Redondo-Solano, M., Valenzuela-Martinez, C., Cassada, D.A., Snow, D.D., Juneja, V.K., Burson, D.E., Thippareddi, H., 2013. Effect of meat ingredients (sodium nitrite and erythorbate) and processing (vacuum storage and packaging atmosphere) on germination and outgrowth of *Clostridium perfringens* spores in ham during abusive cooling. Food Microbiol. 35, 108–115. https://doi.org/10.1016/j.fm.2013.02.008.
Robinson, A., Gibson, A., Roberts, T., 2007. Factors controlling the growth of *Clostridium botulinum* types A and B in pasteurized, cured meats. V. Prediction of toxin production: non-linear effects of storage temperature and salt concentration. Int. J. Food Sci. Technol. 17, 727–744. https://doi.org/10.1111/j.1365-2621.1982.tb00233.x.
Sindelar, J., Milkowski, A., 2011. Sodium nitrite in processed meat and poultry meats: a review of curin and examining the risk and benefit of its use. In: White Pap. Ser. No 3, pp. 1–14.
Sofos, J., Busta, F., Allen, C.E., 1979a. Sodium nitrite and sorbic acid effects on *Clostridium botulinum* spore germination and total microbial growth in chicken frankfurter emulsions during temperature abuse. Appl. Environ. Microbiol. 37, 1103–1109.
Sofos, J., Busta, F., Allen, C.E., 1979b. Botulism control by nitrite and sorbate in cured meats: a review. J. Food Prot. 42, 739–770. https://doi.org/10.4315/0362-028X-42.9.739.
Tomović, V.M., Jokanović, M.R., Petrović, L.S., Tomović, M.S., Tasić, T.A., Ikonić, P.M., Šumić, Z.M., Šojić, B.V., Škaljac, S.B., Šošo, M.M., 2013. Sensory, physical and chemical characteristics of cooked ham manufactured from rapidly chilled and earlier deboned M. semimembranosus. Meat Sci. 93, 46–52. https://doi.org/10.1016/j.meatsci.2012.07.015.
Tompkin, R.B., Christiansen, L.N., Shaparis, A.B., 1979. Iron and the antibotulinal efficacy of nitrite. Appl. Environ. Microbiol. 37, 351–353.
World Health Organization, 2012. Guideline: Sodium Intake for Adults and Children. World Health Organization.

Web references

https://atlas.ecdc.europa.eu/public/index.aspx.
https://www.santepubliquefrance.fr/maladies-et-traumatismes/maladies-infectieuses-d-origine-alimentaire/botulisme/documents/donnees/caracteristiques-epidemiologiques-du-botulisme-chez-l-homme-en-france-en-2017.