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Enumeration medium study of *Clostridium perfringens* in food products

January 2018 report

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I. MOTIVATION

CHROMagar requested ACTALIA to study a *Clostridium perfringens* enumeration medium for the analysis of food products. This study follows a preliminary study (SMI 2015 651 1). The previous study made it possible to examine the selectivity and fertility of the *Clostridium perfringens* enumeration medium as well as the application of this medium to artificially contaminated samples (water, food) in comparison with the TSC (tryptone-sulphite-cycloserine) medium and potentially naturally contaminated foodstuffs.
Since the first study was completed, a new version of the medium has been developed in which the colours for the detection and counter-staining of strains have been modified.
In this new version, colonies of *C. perfringens* are detected in orange (and no longer in blue-green) and the other strains are either blue, colourless or inhibited.
The objective of this study is to verify the selectivity of the medium and its application to the analysis of food samples.

II. DESCRIPTION OF STRAINS

The strains to be studied were selected in consultation with CHROMagar.

II.2.1. description of C perfringens strains

Five strains of *C. perfringens* will be selected from the 16 strains previously studied: two strains from international collection, and three strains from different categories of food products (meat, poultry, dairy products).

Table1: list of *Clostridium perfringens* strains studied

Code	Identification	origin
ATCC 13124	*C. perfringens*	
ATCC 12916	*C. perfringens*	
Ad 246	*C. perfringens*	poultry
214	*C. perfringens*	environment
1221	*C. perfringens*	poultry

II.2.2. description of Clostridium sp.

Five strains of *Clostridium* non-*perfringens* commonly found in samples in which *C. perfringens* are sought will be selected from the nine previously tested strains. The most commonly encountered species in food products will be favoured, for example, *C. sporogenes, C. bifermentans, C. pasteurianum, C. tyrobutyricum* and *C. beijerinckii*.

Table2: List of *Clostridium* non-*perfringens* strains studied

Code	Identification	origin
Act74-001	*C. sporogenes*	Milk product
Act74-019	*C. pasteurianum*	Milk product
Act74-065	*C. bifermentans*	Milk product
Act74-198	*C. bifermentans*	Milk product
Act74-014	*C. tyrobutyricum*	Milk product

■ *II.2.3. description of strains belonging to genera other than Clostridium*

Five strains belonging to genera other than *Clostridium* and commonly found in samples in which *C. perfringens* are sought will be selected, the following genera may be selected: *E. coli, Serratia* or *Citrobacter, Bacillus* (strains previously studied) as well as *Lactobacillus* and *Staphylococcus* (genera identified on CHROMagar medium during the analysis of food naturally contaminated).

Table3: List of strains studied belonging to bacterial genera other than *Clostridium*

Code	Identification
LMG 8063	*Escherichia coli*
CNRZ 134	*Enterococcus faecalis*
ATCC 8454	*Citrobacter freundii*
ADQP 407	*Bacillus cereus*
LMG 8195	*Staphylococcus aureus*
ATCC 8014	*Lactobacillus plantarum*

■ III. DESCRIPTION OF CULTURE MEDIA

Three media were used: CHROMagar, the TSC medium recommended by ISO 7937 (applied to food) and ISO 14189 (applied to water analysis), and the RCM medium recommended for the cultivation of *Clostridium*.

The description of the media and the appearance of *C. perfringens* colonies on these media are given below:

- TSC medium (Biokar, Ref: BK031HA, Batch: 0007856) + D-cycloserine (Biokar, Ref: BS0060, Batch: 00012214) on this medium, the characteristic colonies are black with black halo.

- Medium CHROMagar™ C. perfringens X203B-5 (batch: P001013, expiry 30/03/2019) + supplement 1 (X203S1-5, batch: P001014, expiry 30/03/2019) + supplement 2 (X203S2-5, batch: P001015; expiry 30/03/2019): characteristic colonies are orange.

■ IV. STUDY OF STRAINS ON CULTURE MEDIA

The fifteen pure strains (five *C. perfringens* and 10 other species) were analysed on CHROMagar medium and on TSC medium, in order to verify that they present the distinctive morphological characters sought. A culture on TSA was performed in parallel (positive growth control).

Pre-cultures were prepared under the following conditions.

- A cryo-crystal was taken from a cryotube and cultured in 9 mL BHI broth and then incubated at 37 °C under anaerobic conditions (in a jar) for 16 hours.
- Pre-cultures were left overnight (16 to 18 hours) for the sixteen strains in BHI broth incubated at 37 °C.

Before beginning the experiment, a wet mount hanging drop preparation was observed microscopically for absence of spores in the different stock suspensions of *Clostridium*.

The pure strains were diluted in Tryptone Salt (TS) diluent and enumerated with two types of inoculation, "pour plate technique" within the medium, and “surface plate” (incubation under strict

anaerobic conditions). The TSC medium was inoculated with pour plate technique in a double layer without supplement and with pour plate technique in the CHROMagar medium **without a double layer**.

Enumeration of the different strains was carried out for both TSC and CHROMagar media, on the surface and with pour plate technique as follows:

- Surface: for *Clostridium*, 100 µL of dilution -5 was spread on the media, and 100 µL of dilution -6 for the other genera.
- Pour plate technique: for *Clostridium*, 100 µL of dilution -6 was spread on the media, 100 µL of the dilution -7 and for other genera.

The media were incubated anaerobically (in a jar) at 37 °C for 20 hours ± 2 hours (incubation conditions as recommended in NF ISO 7937 (Horizontal method for the enumeration of *Clostridium perfringens* in food).
The selectivity of the medium was verified using the *Serratia* AR5569 strain.

The results in CFU/mL are provided in Appendix 1 and log CFU/mL in Table 4.

Table 4: Results of enumerations on CHROMagar and TSC media (log CFU/ml)

Clostridium perfringens	CHROMagar		TSC	
	Surface	Pouring	Surface	Pouring
ATCC 12916	8.32	8.25	8.30	8.20
AD 246	8.46	8.18	8.04	7.99
214	7.74	7.61	7.73	7.71
1221	7.77	7.87	7.72	7.83
ATCC 13124	8.40	8.18	7.98	8.04
Clostridium* non-*perfringens	CHROMagar		TSC	
	Surface	Pouring	Surface	Pouring
C. sporogenes		8.25	8.48	8.43
C. pasteurianum		7.66	8.00	7.88
C. bifermentans 065		7.70		7.78
C. bifermentans 198	7.78	8.00	8.00	8.11
C. tyrobutyricum	7.88	8.01	7.97	8.00
Other genera	CHROMagar		TSC	
	Surface	Pouring	Surface	Pouring
Escherichia coli				
Enterococcus faecum			9.04	9.00
Citrobacter freundii			8.32	8.58
Bacillus cereus				7.48
Staphylococcus aureus				
Lactobacillus plantarum			8.30	8.2

Legend: shaded boxes: no growth

The results of the enumerations show that the results are comparable for *C. perfringens* on CHROMagar and TSC (enumeration difference < 0.5 log).

With regard to the morphological characteristics of the strains studied, Table 5 describes the characteristics of the colonies according to the medium and the type of inoculation.

Macroscopic observation on the medium CHROMagar™ *C. perfringens* showed that with surface or at-depth inoculation, the colonies of *C. perfringens* present an orange coloration. On the TSC medium, for surface inoculation, *C. perfringens* is distinguished by cream coloured star colonies, while with pour plate inoculation, black colonies with a black halo are visible on this medium.

The strains of *Clostridium* non-*perfringens* develop less well on the surface of the CHROMagar medium and provide atypical colonies with pour plate technique (white or beige with green blue halo). These strains also provide atypical colonies on TSC.

Strains belonging to other genera do not develop on CHROMagar medium.

Table 5: Description of the morphological characteristics of colonies according to the medium used and the type of inoculation (shaded boxes: no growth)

Clostridium perfringens	CHROMagar		TSC	
	Surface	Pouring	Surface	Pouring
ATCC 12916	orange/regular	orange/light halo	white irregular	Black, white outline
AD 246	orange/regular	orange/light halo	white regular	white with black halo
214	orange/diffuse	orange/light halo	white irregular	Black, white outline
1221	orange/regular	orange/light halo	white regular	Black, white outline
ATCC 13124	orange/diffuse	orange/light halo	white irregular	white with black halo
Clostridium* non-*perfringens	CHROMagar		TSC	
	Surface	Pouring	Surface	Pouring
C. sporogenes 001		white	transparent with white pinhead	black
C. pasteurianum -019		white	white pinhead	black
C. bifermentans -065		beige with green halo		large, black
C. bifermentans -198	white	beige	white	black
C. tyrobutyricum -014		white	small white regular	small white
Other genera	CHROMagar		TSC	
	Surface	Pouring	Surface	Pouring
Escherichia coli				white
Enterococcus faecium			white	white
Citrobacter freundii			white	white
Bacillus cereus				white
Staphylococcus aureus				
Lactobacillus plantarum			white	white

■ V. ANALYSES OF POTENTIALLY NATURALLY CONTAMINATED AND ARTIFICIALLY CONTAMINATED FOOD SAMPLES

Seven samples, likely to have a natural contamination with presumptive *C. perfringens* (double positive samples in the laboratory), were analysed.

For these samples, enumerations were carried out according to NF EN ISO 7937 on CHROMagar and on TSC seeded on the surface and with pour plate technique.

- 3 samples correspond to non-inoculated products tested in point VI
- 1 sample of offal purchased commercially
- Three samples correspond to frozen double-samples having been detected positive for *C. perfringens* by laboratory analysis at 37 °C in jars according to NF EN ISO 7937 or for SRAs (sulphite-reducing anaerobes) at 46 °C in tubes (other samples) according to NF V08 061 by the microbiological control laboratory.

The double-samples were thawed overnight at 4 °C. The test sample (10 to 100 g) was diluted with peptone water. This suspension was used for enumerations on TSC and CHROMagar media after incubation at 37 °C for 20 h ± 2 h.

Four samples showed a level of contamination above the detection limit of the method (Table 6).

- Minced beef: for inoculation with pour plate technique, the enumeration is slightly higher on CHROMagar medium (non-significant difference, less than 0.5 log). For surface inoculation, the results confirm that TSC does not allow the characteristic black colonies to be observed,

- Mincing beef: for inoculation with pour plate technique the enumeration is slightly higher on CHROMagar medium (non-significant difference, less than 0.5 log). For surface inoculation on CHROMagar medium, the enumeration is higher.

- Stuffed veal parcel: estimated number ranging from 10 to 40 CFU/g, no significant difference between media, a substantial annex flora is observed on TSC medium surface and on CHROMagar medium (small blue colonies).

- Raspberry tart: the contamination is close to the limit of quantification and is detected on the TSC medium (1 black colony) and on CHROMagar medium (1 atypical non-*perfringens* colony). The black colony present on the medium TSC was isolated on the CHROMagar™ *C. perfringens* medium and provides one atypical colony (cream) corresponding to a *Clostridium* non-*perfringens*.

Table 6: Results obtained for the different matrices analysed according to NF EN ISO 7937 (results expressed in CFU/g).

	CHROMagar		TSC		Results from the laboratory
	Surface	Pouring	Surface	Pouring	SRAB on TSC at 46°C
Chopped meat [(1)] (90 g diluted to 1/2)	4	18	< 2[(2)]	10	10
Meat chopping Meat [(1)] (45g diluted to 1/2)	440	120	-	52	50
Stuffed veal parcel (10 g diluted 1:10)	40 10 < 10	10 10 < 10	-	10 < 10 20	
Minced beef (10 g diluted to 1/10)	< 10	< 10[(3)]	-	< 10	
Poultry meat frankfurter (10 g diluted 1:10)	< 10	< 10	-	< 10	
Raspberry tart (45 g diluted 1:2) [(1)]	< 2	< 2[(4)]	-	2[(5)]	30
Beef heart (10 g diluted 1:10)	10	< 10	-	< 10	

[(1)] double sampling [(2)] 3,300 CFU/g but no black colonies [(3)] 1 blue colony, no annex flora, [(4)] one non-typical colony, [(5)] one unconfirmed black *C. perfringens* colony after isolation on CHROMagar™ *C. perfringens*

■ VI. ANALYSIS OF ARTIFICIALLY CONTAMINATED FOOD SAMPLES

Three matrices with an annex flora (minced beef, poultry frankfurter, stuffed veal parcel etc.) were artificially contaminated with 1 strain of *C perfringens* (strain 214) at a target concentration between 50 CFU/g and 500 CFU/g.

For these 6 samples, enumerations were carried out according to NF EN ISO 7937 on CHROMagar (with pour plate technique and on the surface) and on TSC medium (with pour plate technique).

a) Experimental protocol

Pre-culture of the strain

A cryo-crystal was taken from a cryotube and cultured in 9 ml BHI broth and then incubated at 37 °C under anaerobic conditions (in a jar) for 16 hours.
Overnight pre-culture (16 to 18 hours) was performed for the sixteen BHI broth strains incubated at 37 °C.

Inoculation of food matrices

Samples of 100 g were inoculated with a volume of 4 mL of the bacterial suspension (vegetative cells) of *C. perfringens* strain 214, isolated from the environment. The theoretical inoculation rates obtained as a function of the concentration of the stock suspension are close to 1,000 CFU/g. This relatively high level relative to natural contamination levels of food by *C. perfringens* (generally < 10

to 100 CFU/g) was chosen in light of the detection limits of the enumeration methods, in order to compare the two enumeration media in terms of recovery of *C. perfringens*. During the enumeration of SRAs (sulphite reducing anaerobes) on the TSC medium, enumerations > 100 CFU/g were sometimes obtained in the laboratory.

Sample analysis

From the 100 g sample, 3 test portions of 10 g were analysed, after dilution at 1:10 in peptone water, on day 0 after 30 min at room temperature (duration of the microbiological analysis and allowing the adsorption of bacterial cells into the food matrix).

The remaining part was conditioned under a modified atmosphere (50% CO_2/50% N_2) then incubated at 8°C overnight to better approach the conditions of preservation of food products after manufacture.

The next day, 3 samples of 10 g were analysed as before, using two methods (with or without thermic shock (10 min at 80°C beforehand), in order to determine the quantity of vegetative cells or spores present in case of sporulation during the night.

Enumerations were carried out with a double layer with pour plate technique on TSC and without a double layer with pour plate technique on CHROMagar (1-mL suspension) and on the surface on CHROMagar (100-mL suspension) after incubation at 37 °C for 20 h ± 2 h. (see experimental design, Figure 1).

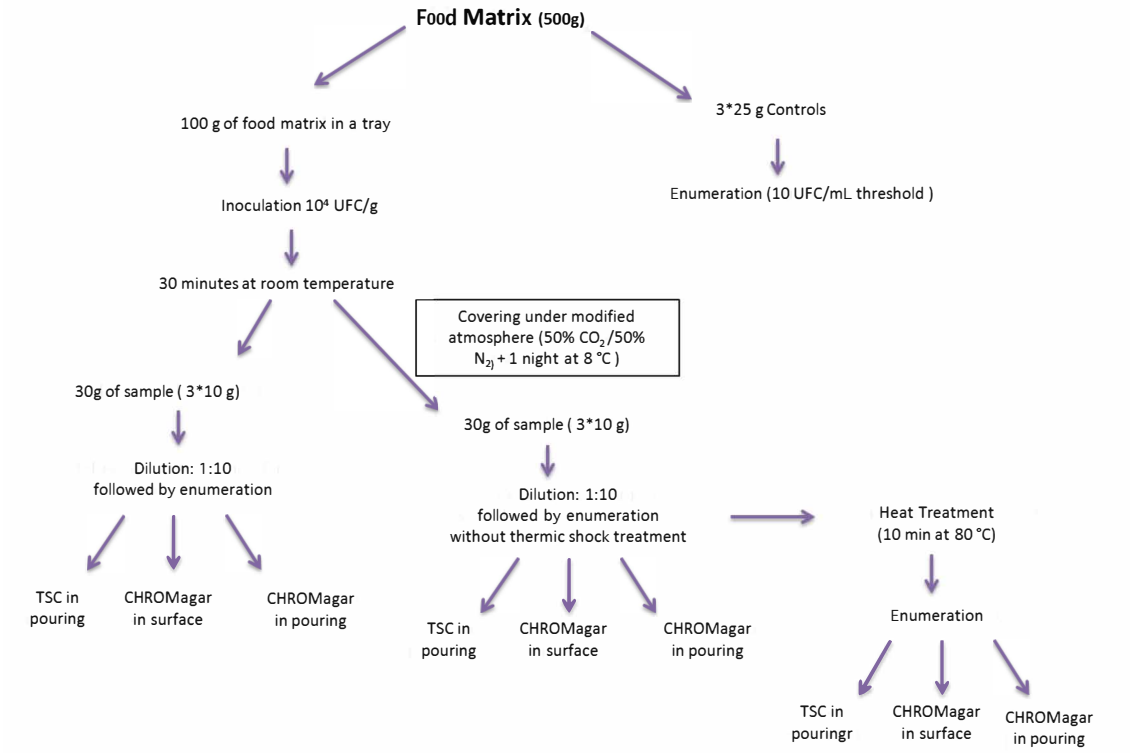

Figure 1: Experimental design for the study of artificially contaminated samples

b) Results

Three food matrices known as potential sources of *C. perfringens* contamination have been studied to date: minced beef, poultry frankfurters and stuffed veal parcels. The analysis of the non-artificially contaminated control samples revealed no natural contamination by *C. perfringens* or sulphite-reducing anaerobes (Table x).

The results are provided in Tables 7 to 9.

The results of the enumerations obtained on day 0 (the day of inoculation) are consistent with the theoretical inoculation concentrations of the products.

These results show a lack of growth of *C. perfringens* in the foodstuffs studied during overnight storage at 8 °C. This is logical, insofar as the minimum growth temperature for *C. perfringens* is 12 °C. There was no sporulation at 8°C during this time.

The results show that the enumerations obtained on TSC and CHROMagar media are generally comparable at D0 (difference in enumeration results < 0.5 log unit).

After 1 night at 8 °C for minced beef, the enumerations are comparable on TSC and CHROMagar media (Table 7).
For poultry frankfurters, enumerations are slightly lower on CHROMagar medium but with a mean log difference < 0.3 (Table 8).
For the stuffed veal parcel, enumerations are comparable on TSC and CHROMagar media (Table 9).

The reading of the CHROMagar™ *C. perfringens* plates is easy (the orange colour is easier to distinguish than the green colour of the previous version). In addition, the colonies retain the orange colour once the pates are removed from the jar, whereas colonies on TSC medium tend to lose their black colour once exposed to the air.

Table 7: Results of inoculation of minced beef with *Clostridium perfringens* strain 214 (initial concentration 4.7×10^7 CFU/mL) (results expressed as mean log CFU/g of 3 replicates).

Types of media inoculated	Controls	Minced beef artificially contaminated at 1,000 CFU/g		
		D0	After 1 night at 8°C	
			Before thermic shock	After thermic shock
TSC with pour plate technique	< 1	3.39 ± 0.05	3.41 ± 0.05	< 1
CHROMagar with pour plate technique	< 1	3.46 ± 0.12	3.43 ± 0.03	< 1
CHROMagar on surface	< 1	3.43 ± 0.14	3.44 ± 0.09	< 1

Table 8: Results of inoculation of poultry frankfurter with *Clostridium perfringens* strain 214 (initial concentration 4.7×10^7 CFU/mL) (results expressed as mean log CFU/g of 3 replicates).

Types of media inoculated	Controls	poultry frankfurter artificially contaminated at 1,000 CFU/g		
		D0	After 1 night at 8°C	
			Before thermic shock	After thermic shock
TSC with pour plate technique	< 1	3.08 ± 0.16	3.00 ± 0.14	< 1
CHROMagar with pour plate technique	< 1	3.01 ± 0.18	2.69 ± 0.02	< 1
CHROMagar on surface	< 1	3.02 ± 0.17	2.79 ± 0.10	< 1

Table 9: Results of inoculation of stuffed veal parcel with *Clostridium perfringens* strain 214 (initial concentration 4.7×7 CFU/mL) (results expressed as mean log CFU/g of 3 replicates).

Types of media inoculated	Controls	Stuffed veal parcel artificially contaminated at 1,000 CFU/g		
		D0	After 1 night at 8°C	
			Before thermic shock	After thermic shock
TSC with pour plate technique	1.15 ± 0.21*	3.27 ± 0.15	3.33 ± 0.10	< 1
CHROMagar with pour plate technique	1 ± 0.00*	3.33 ± 0.21	3.25 ± 0.10	< 1
CHROMagar on surface	1.3 ± 0.42*	3.10 ± 0.09	3.21 ± 0.15	< 1

* mean of 2 replicates (1 result < 10 CFU/g)

APPENDIX 1

Results of enumerations on CHROMagar and TSC media (CFU/mL)

Clostridium perfringens	CHROMagar		TSC	
	Surface	Pouring	Surface	Pouring
ATCC 12916	$2.1x10^8$	$1.8x10^8$	$2x10^8$	$1.6x10^8$
AD 246	$2.9x10^8$	$1.5x10^8$	$1.1x10^8$	$9.8x10^7$
214	$5.5x10^7$	$4.1x10^7$	$5.4x10^7$	$5.1x10^7$
1221	$5.9x10^7$	$7.5x10^7$	$5.3x10^7$	$6.8x10^7$
ATCC 13124	$2.5x10^8$	$1.5x10^8$	$9.5x10^7$	$1.1x10^8$
Clostridium* non-*perfringens	CHROMagar		TSC	
	Surface	Pouring	Surface	Pouring
C. sporogenes		$1.8x10^8$	$3.0x10^8$	$2.7x10^8$
C. pasteurianum		$4.6x10^7$	$1.0x10^8$	$7.6x10^7$
C. bifermentans		$5.0x10^7$		$6.0x10^7$
C. bifermentans	$6x10^7$	$1x10^8$	$1x10^8$	$1.3x10^8$
C. tyrobutyricum		$2.8x10^7$	$1.2x10^8$	$8.5x10^7$
Other genera	CHROMagar		TSC	
	Surface	Pouring	Surface	Pouring
Escherichia coli				
Enterococcus faecum			$1.1x10^9$	$1.0x10^9$
Citrobacter freundii			$2.1x10^8$	$3.8x10^8$
Bacillus cereus				$3.0x10^7$
Staphylococcus aureus				
Lactobacillus plantarum			$2.0x10^8$	$1.6x10^8$

Legend: shaded boxes: no growth

APPENDIX 2: Microbiological criteria for *C. perfringens*

Regulation (EC) 2073: 2005 relating to microbiological criteria for foodstuffs)

Criteria of the *Féderation du Commerce et Distribution* - FCD (Microbiological criteria applicable to private labels, first price brands and raw materials in their initial industrial packaging)

The *C. perfringens* criterion applies to:

- Certain delicatessen products: microbiological limit m = 30/g
- Crustaceans, raw or cooked molluscs, anchovy paste, marinated and pickled products, crabsticks: microbiological limit m = 30/g
- Catering products:
  - Cooked under vacuum in packaging or deep-frozen: microbiological limit m = 10/g
  - Cooked out of packaging, raw products made from meat products: microbiological limit m = 30/g
  - Pasteurised foie gras: microbiological limit m = 30/g
- Whole or cut poultry raw, offal, rabbit: microbiological limit m = 100/g
- Raw poultry products: microbiological limit m = 30/g