Study of an acid phosphatase test for the confirmation of *Clostridium perfringens* directly from CHROMagar™ C.perfringens agar medium

CONFIDENTIAL

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This report contains 15 pages, including 4 pages of appendices.

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Appendices

1. Introduction

The aim of this study is to evaluate the effectiveness of an acid phosphatase test to differentiate *Clostridium perfringens* from other potentially false positive bacteria in the CHROMagar™ C.perfringens medium.

The ISO 14189:2017 standard, allowing the enumeration of *Clostridium perfringens* in water by membrane filtration, provides an acid phosphatase test for the confirmation of the characteristic colonies.

The study aims at verifying that the replacement of the FAST Blue B salt by the FAST Blue RR salt in the acid phosphatase reagent optimizes the interpretation of the test on CHROMagar™ C.perfringens agar.

2. Materials and methods

2.1. Materials

2.1.1. Devices

The devices used in this study are the ones usually encountered in a laboratory. The culture media were incubated in anaerobic jars containing anaerogenic generators (Anaerogen ThermoScientific, ref: AG0025A) and an anaerobic control (Anaerotest Merck, ref: 15112). The paper used to carry out the acid phosphatase test is white calligraph type blotting paper at a grammage of 100 g/m^2.

2.1.2. Culture media

The culture media used were as follows:

- Tryptone Sulfite Cycloserine agar (OXOID - ref: CM0587)
- CHROMagar™ C.perfringens agar (Base ref: PF652(B) / Supplements ref: PF652(S1) and ref: PF652(S2))
- Trypto caseine Soja Agar (BioMérieux – ref : 41466)
- Columbia 5% Sheep Blood Agar (BioMérieux – ref: 43041)

2.1.3. Reagents

The acid phosphatase reagent used was as follows:

- 1-Naphthylphosphate monosodium salt (CAS N°81012-89-7) 0,4 g
- FAST Blue RR salt (4-Benzoylamino-2,5-dimethoxybenzenediazonium chloride hemi (zinc chloride) salt) (CAS N°14726-29-5) 0,8 g

- Acetate buffer (pH 4,6 ± 0,2) 20 ml
  - Glacial acetic acid (CAS N°64-19-7)
  - Sodium acetate (CAS N°127-09-3)

  Acetate buffer has been prepared by dissolving 0,3 ml glacial acid acetic and 0,4 g sodium acetate in deionized water qsp to 1000 ml.

The acid phosphatase reagent was obtained by dissolving the ingredients in the acetate buffer and allow to stand for 60 ± 5 min at 5 ± 3 °C to allow precipitation. The solution was passed through a fluted filter to remove the precipitate.

2.2. Methods

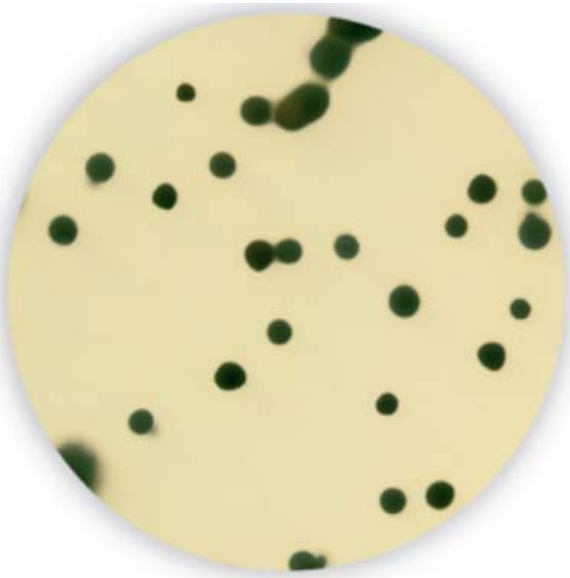
2.2.1. Strain culture protocol

During this study, 15 strains of *Clostridium perfringens* from different origins were tested, as well as 7 *Clostridium* spp. exclusive of *Clostridium perfringens* strains. Eighteen strains which may have characteristics close to *Clostridium perfringens* on CHROMagar™ C.perfringens agar were also tested. All of these strains were isolated on TSC agar and on CHROMagar™ C.perfringens agar.

The appearance of the characteristic colonies on these 2 media is as follows:

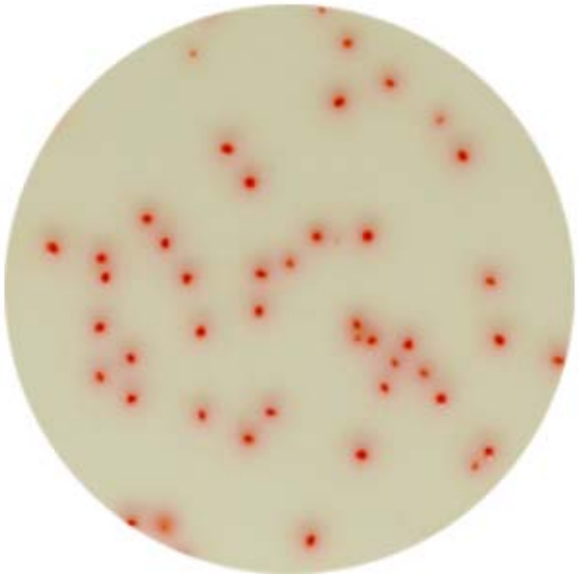
Figure 1:
appearance of the characteristic colonies on TSC agar media and CHROMagar™ C.perfringens agar.

TSC agar media



Clostridium perfringens : black colonies

CHROMagar™ C.perfringens agar



Clostridium perfringens : red colonies

Table 1 shows all the strains used for the tests and their aspect on the selective agar media.

Table 1: list of the different bacterial strains isolated on TSC agar media and on CHROMagar™ C. perfringens agar.

Number	Strain	Origin	TSC	CHROMAGAR
1	*Clostridium perfringens*	Tap water	Black colonies	Red colonies
3	*Clostridium perfringens*	WDCM00007	Black colonies	Red colonies
6	*Clostridium perfringens*	Composite food product	Black colonies	Red colonies
7	*Clostridium perfringens*	Spirulina	Black colonies	Red colonies
8	*Clostridium perfringens*	Duck meat	Black colonies	Red colonies
9	*Clostridium perfringens*	Poultry meat	Black colonies	Red colonies
10	*Clostridium perfringens*	Seaweed Dulse	Black colonies	Red colonies
13	*Clostridium perfringens*	Sea bean seaweed	Black colonies	Red colonies
14	*Clostridium perfringens*	Seaweed Dulse	Black colonies	Red colonies
15	*Clostridium perfringens*	Spirulina powder	Black colonies	Red colonies
16	*Clostridium perfringens*	Water sewage treatment plant	Black colonies	Red colonies
17	*Clostridium perfringens*	Seaweed Laminaria digitata powder	Black colonies	Red colonies
18	*Clostridium perfringens*	Water sewage treatment plant	Black colonies	Red colonies
19	*Clostridium perfringens*	Pond water	Black colonies	Red colonies
20	*Clostridium perfringens*	Seaweed Asco powder	Black colonies	Red colonies
2	*Clostridium butyricum*	River water	Black colonies	Red colonies
4	*Clostridium sp. (butyricum or glycolicum)*	Outlet water by lagoon treatment	Black colonies	Red colonies
5	*Clostridium glycolicum*	Pond water	Black colonies	Red colonies
11	*Clostridium sordelii*	Thyme	Black colonies	Red colonies
12	*Clostridium bifermentans*	Composite food product	Black colonies	Red colonies
N	*Paeniclostridium sordelii*	DSMZ collection	Black colonies	Red colonies
P	*Clostridium septicum*	DSMZ collection	White colonies	No growth
A	*Leuconostoc mesenteroides*	Zucchini flan	No growth	Blue colonies
B	*Lactobacillus sakei*	Smoked salmon	White colonies	Red colonies
C	*Lactobacillus gasseri*	Probiotic	White colonies	Blue colonies
D	*Lactobacillus reuteri*	Probiotic	No growth	Red colonies
E	*Bifidobacterium lactis*	Probiotic	White colonies	Blue colonies
F	*Lactobacillus paracasei*	Dairy product	No growth	No growth
G	*Leuconostoc mesenteroides*	Meat product	No growth	Red colonies
H	*Streptococcus thermophilus*	Probiotic	No growth	Red colonies
I	*Lactobacillus sakei*	Dairy product	White colonies	Red colonies
J	*Enterococcus faecalis*	Bathing water	White colonies	Red colonies
K	*Enterococcus faecalis*	Water sewage treatment plant	White colonies	Red colonies
L	*Enterococcus faecium*	Outlet water by lagoon treatment	White colonies	Red colonies
M	*Weissella viridescens*	Composite food product	No growth	No growth
O	*Pediococcus pentosaceus*	DSMZ collection	No growth	No growth
Q	*Lactobacillus plantarum*	DSMZ collection	No growth	Blue colonies
R	*Enterobacter cloacae*	Outlet water by lagoon treatment	No growth	No growth
S	*Serratia marescens*	Pond water	No growth	No growth
T	*Citrobacter freundii*	Outlet water by lagoon treatment	No growth	No growth

2.2.2. Confirmation of *Clostridium perfringens*

2.2.2.1. General

It is recommended, for counts of 1 to 10 colonies, to submit for confirmation all colonies and, for counts greater than 10 colonies, at least 10 colonies randomly chosen.
The ISO 14189:2017 standard indicates that the confirmation of the characteristic colonies on TSC agar must be carried out after subculturing on a blood agar, a Columbia agar or a nutrient-rich agar (for example: Tryptone Soy Agar). These subcultures are then subjected to the acid phosphatase test.

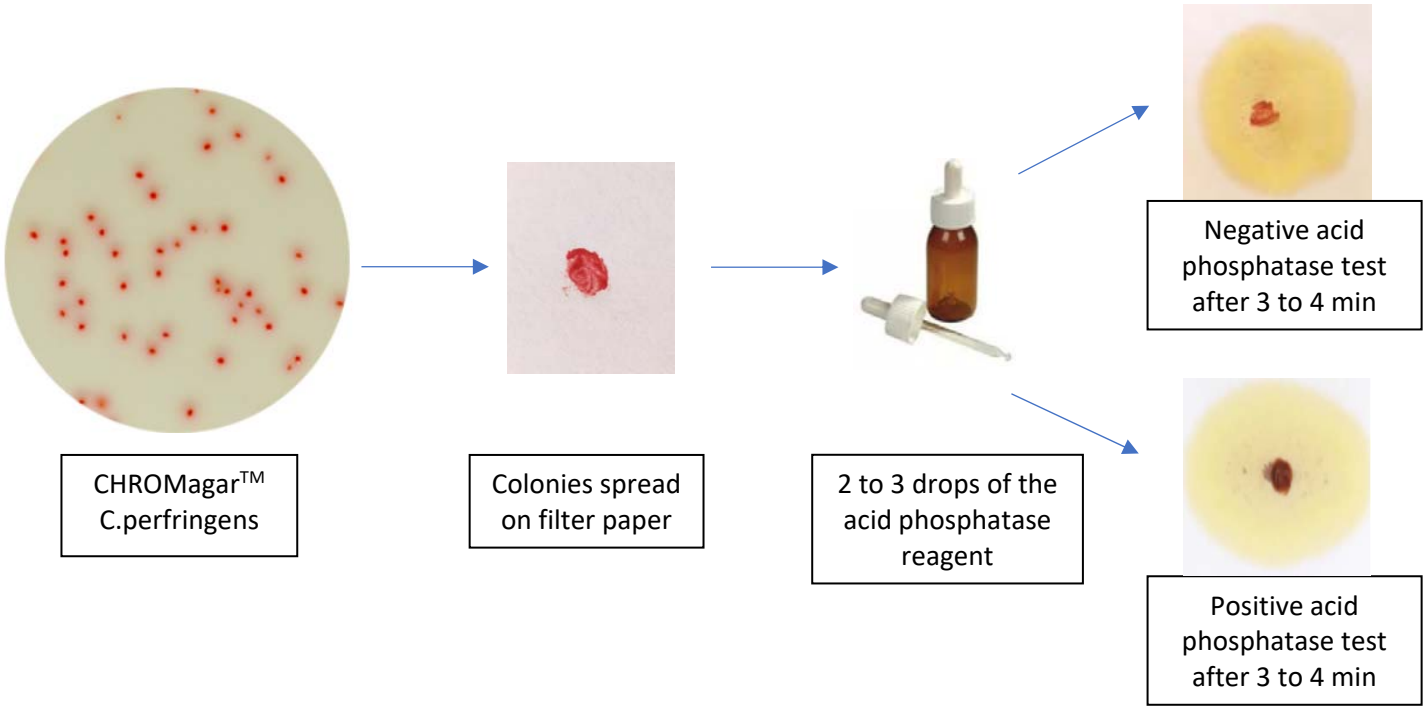
The objective of this study is to determine the effectiveness of the new acid phosphatase reagent directly on the red colonies present on the CHROMagar™ C.perfringens agar.

2.2.2.2. Acid phosphatase test

Colonies grown anaerobically are spread on filter paper and 2 to 3 drops of the acid phosphatase reagent are placed onto the colonies. A purplish colour developed within 3 minutes to 4 minutes is considered as a positive reaction. The replacement of the FAST Blue B salt by the FAST Blue RR salt, gives a yellow color to the reagent and allows an easier reading contrast of the positive colonies.

Clostridium perfringens produces black colonies on TSC agar, red colonies on CHROMagar™ C.perfringens agar and has an acid phosphatase activity.

Figure 2: principle of the acid phosphatase test



3. Results

3.1. Raw results

All the strains of *Clostridium* for which growth has been observed on CHROMagar™ C.perfringens agar (red colonies or blue colonies) were tested with the acid phosphatase reagent. In parallel, an isolation on a Columbia agar as provided for in the standard ISO 14189:2017 was carried out. An acid phosphatase test (as in ISO 14189:2017 but with FAST Blue RR salt) was then carried out on the colonies present on the Columbia agar. The different results are presented in table 2.

The strains not belonging to the genus *Clostridium* but which may have similar growth characteristics, were isolated on CHROMagar™ C.perfringens agar and tested by the acid phosphatase reagent. In parallel, an isolation on TSA agar was carried out. The results are presented in table 3 and in appendices A and B.

Table 2: results of the acid phosphatase tests on *Clostridium* strains

Number	Strain	Origin	TSC	CHROMAGAR		Final results (CHROMAGAR and phosphatase)	COLUMBIA		Result
				Aspect	Phosphatase result		Aspect	Phosphatase	
1	*Clostridium perfringens*	Tap water	Black colonies	Red colonies	Positive	*Clostridium perfringens*	White colonies	Positive	*Clostridium perfringens*
3	*Clostridium perfringens*	WDCM00007	Black colonies	Red colonies	Positive	*Clostridium perfringens*	White colonies	Positive	*Clostridium perfringens*
6	*Clostridium perfringens*	Composite food product	Black colonies	Red colonies	Positive	*Clostridium perfringens*	White colonies	Positive	*Clostridium perfringens*
7	*Clostridium perfringens*	Spirulina	Black colonies	Red colonies	Positive	*Clostridium perfringens*	White colonies	Positive	*Clostridium perfringens*
8	*Clostridium perfringens*	Duck meat	Black colonies	Red colonies	Positive	*Clostridium perfringens*	White colonies	Positive	*Clostridium perfringens*
9	*Clostridium perfringens*	Poultry meat	Black colonies	Red colonies	Positive	*Clostridium perfringens*	White colonies	Positive	*Clostridium perfringens*
10	*Clostridium perfringens*	Seaweed Dulse	Black colonies	Red colonies	Positive	*Clostridium perfringens*	White colonies	Positive	*Clostridium perfringens*
13	*Clostridium perfringens*	Sea bean seaweed	Black colonies	Red colonies	Positive	*Clostridium perfringens*	White colonies	Positive	*Clostridium perfringens*
14	*Clostridium perfringens*	Seaweed Dulse	Black colonies	Red colonies	Positive	*Clostridium perfringens*	White colonies	Positive	*Clostridium perfringens*
15	*Clostridium perfringens*	Spirulina powder	Black colonies	Red colonies	Positive	*Clostridium perfringens*	White colonies	Positive	*Clostridium perfringens*
16	*Clostridium perfringens*	Water sewage treatment plant	Black colonies	Red colonies	Positive	*Clostridium perfringens*	White colonies	Positive	*Clostridium perfringens*
17	*Clostridium perfringens*	Seaweed Laminaria digitata powder	Black colonies	Red colonies	Positive	*Clostridium perfringens*	White colonies	Positive	*Clostridium perfringens*
18	*Clostridium perfringens*	Water sewage treatment plant	Black colonies	Red colonies	Positive	*Clostridium perfringens*	White colonies	Positive	*Clostridium perfringens*
19	*Clostridium perfringens*	Pond water	Black colonies	Red colonies	Positive	*Clostridium perfringens*	White colonies	Positive	*Clostridium perfringens*
20	*Clostridium perfringens*	Seaweed Asco powder	Black colonies	Red colonies	Positive	*Clostridium perfringens*	White colonies	Positive	*Clostridium perfringens*
2	*Clostridium butyricum*	River water	Black colonies	Red colonies	Negative	No *Clostridium perfringens*	White colonies	Negative	Not *Clostridium perfringens*
4	*Clostridium sp. (butyricum ou glycolicum)*	Outlet water by lagoon treatment	Black colonies	Red colonies	Negative	No *Clostridium perfringens*	White colonies	Negative	Not *Clostridium perfringens*
5	*Clostridium glycolicum*	Pond water	Black colonies	Red colonies	Negative	No *Clostridium perfringens*	White colonies	Negative	Not *Clostridium perfringens*
11	*Clostridium sordelii*	Thyme	Black colonies	Red colonies	Negative	No *Clostridium perfringens*	White colonies	Negative	Not *Clostridium perfringens*
12	*Clostridium bifermentans*	Composite food product	Black colonies	Red colonies	Negative	No *Clostridium perfringens*	White colonies	Negative	Not *Clostridium perfringens*
N	*Paeniclostridium sordelii*	DSMZ collection	Black colonies	Red colonies	Negative	No *Clostridium perfringens*	White colonies	Negative	Not *Clostridium perfringens*
P	*Clostridium septicum*	DSMZ collection	White colonies	No growth	∅	No *Clostridium perfringens*	White colonies	Positive	*Possible Clostridium perfringens*

Table 3: results of the acid phosphatase tests on strains not belonging to the genus *Clostridium*.

Number	Strain	Origin	TSC	CHROMAGAR		Final result (CHROMAGAR and phosphatase)	TSA	
				Aspect	Phosphatase		Aspect	Phosphatase
A	*Leuconostoc mesenteroides*	Zucchini flan	No growth	Blue colonies	Negative	No *Clostridium perfringens*	White colonies	Negative
B	*Lactobacillus sakei*	Smoked salmon	No growth	Red colonies	Positive	Possible *Clostridium perfringens*	White colonies	Positive
C	*Lactobacillus gasseri*	Probiotic	No growth	Blue colonies	Negative	No *Clostridium perfringens*	White colonies	Negative
D	*Lactobacillus reuteri*	Probiotic	No growth	Red colonies	Negative	No *Clostridium perfringens*	White colonies	Negative
E	*Bifidobacterium lactis*	Probiotic	No growth	Blue colonies	Negative	No *Clostridium perfringens*	White colonies	Negative
F	*Lactobacillus paracasei*	Dairy product	No growth	No growth	∅	No *Clostridium perfringens*	White colonies	Positive (very weak)
G	*Leuconostoc mesenteroides*	Meat product	No growth	Red colonies	Negative	No *Clostridium perfringens*	White colonies	Negative
H	*Streptococcus thermophilus*	Probiotic	No growth	Red colonies	Negative	No *Clostridium perfringens*	White colonies	Negative
I	*Lactobacillus sakei*	Dairy product	No growth	Red colonies	Negative	No *Clostridium perfringens*	White colonies	Negative
J	*Enterococcus faecalis*	Bathing water	White colonies	Red colonies	Negative	No *Clostridium perfringens*	White colonies	Negative
K	*Enterococcus faecalis*	Water sewage treatment plant	White colonies	Red colonies	Negative	No *Clostridium perfringens*	White colonies	Negative
L	*Enterococcus faecium*	Outlet water by lagoon treatment	White colonies	Red colonies	Negative	No *Clostridium perfringens*	White colonies	Negative
M	*Weissella viridescens*	Composite food product	No growth	No growth	∅	No *Clostridium perfringens*	White colonies	Negative
O	*Pediococcus pentosaceus*	DSMZ collection	No growth	No growth	∅	No *Clostridium perfringens*	White colonies	Negative
Q	*Lactobacillus plantarum*	DSMZ collection	No growth	Blue colonies	Negative	No *Clostridium perfringens*	White colonies	Negative
R	*Enterobacter cloacae*	Outlet water by lagoon treatment	White colonies	No growth	∅	No *Clostridium perfringens*	White colonies	Negative
S	*Serratia marescens*	Pond water	No growth	No growth	∅	No *Clostridium perfringens*	White colonies	Positive
T	*Citrobacter freundii*	Outlet water by lagoon treatment	No growth	No growth	∅	No *Clostridium perfringens*	White colonies	Positive

3.2. Comments

22 strains belonging to the genus *Clostridium* and 18 other strains of different genera were tested during this study. 21 strains out of the 22 *Clostridium* strains developed characteristic red colonies on CHROMagar™ C.perfringens agar medium and 15 of these strains gave a positive reaction to the modified acid phosphatase test, both from CHROMagar™ C.perfringens agar and COLUMBIA agar. All the strains of *Clostridium* spp. exclusive of *Clostridium perfringens*, gave a negative result with the acid phosphatase test.

Among the 18 strains of bacteria not belonging to the genus *Clostridium*, 8 of them gave characteristic colonies on the CHROMagar™ C.perfringens agar medium:

- Strain B: *Lactobacillus sakei*
- Strain D: *Lactobacillus reuteri*
- Strain G: *Leuconostoc mesenteroïdes*
- Strain H: *Streptococcus thermophilus*
- Strain I: *Lactobacillus sakei*
- Strain J: *Enterococcus faecalis*
- Strain K: *Enterococcus faecalis*
- Strain L: *Enterococcus faecium*

For all these strains except for *Lactobacillus sakei* (B), the acid phosphatase test was negative. A second strain of *Lactobacillus sakei* (I) from a different origin has been tested and gave a negative result.

4. Interpretation and conclusion

The present study involved 40 strains analyzed with the CHROMagar™ C.perfringens agar media and confirmed with the modified acid phosphatase test. No difference was observed between the confirmation results by the modified acid phospahatase test carried out directly from the CHROMagar™ C.perfringens agar or after purification on Columbia or TSA. A single strain of *Lactobacillus sakei* gave a false positive result, another strain of *Lactobacillus sakei* having given a negative result on both CHROMagar™ C.perfringens and Columbia agar media.
Particular attention must be paid when reading the acid phosphatase test when it is carried out directly from the red colonies on CHROMagar™ C.perfringens agar. Indeed, the appearance of a purple coloration for a positive sample is obtained after 3 to 4 minutes and a training or a warning specific to the operator must be specified to avoid the false positive results of a red colony.

In conclusion, it appears that the replacement of the FAST Blue B salt by the FAST Blue RR salt has no impact on the results obtained with the CHROMagar™ C.perfringens agar media and that the reading contrast is satisfactory for the detection of *Clostridium perfringens*.

This conclusion is only valid in the operating conditions of the study and with the bacterial strains tested.

Le Lion d'Angers, May 25, 2020
Guillaume MESNARD, Deputy Manager

APPENDICES

Raw results of study

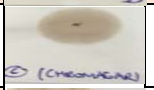
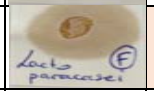
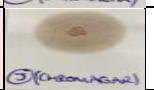
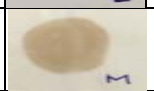
APPENDIX A: Raw results for strains of *Clostridium*

Number	Strain (identified by MALDI-TOF)	Origin	TSC	CHROMAGAR			Final result (CHROMAGAR and phosphatase)	COLUMBIA		Result
				Aspect	Phosphatase	Phosphatase result		Aspect	Phosphatase	
1	*Clostridium perfringens*	Tap water	Black colonies	Red colonies		Positive	*Clostridium perfringens*	White colonies	Positive	*Clostridium perfringens*
3	*Clostridium perfringens*	WDCM00007	Black colonies	Red colonies		Positive	*Clostridium perfringens*	White colonies	Positive	*Clostridium perfringens*
6	*Clostridium perfringens*	Composite food product	Black colonies	Red colonies		Positive	*Clostridium perfringens*	White colonies	Positive	*Clostridium perfringens*
7	*Clostridium perfringens*	Spirulina	Black colonies	Red colonies		Positive	*Clostridium perfringens*	White colonies	Positive	*Clostridium perfringens*
8	*Clostridium perfringens*	Duck meat	Black colonies	Red colonies		Positive	*Clostridium perfringens*	White colonies	Positive	*Clostridium perfringens*
9	*Clostridium perfringens*	Poultry meat	Black colonies	Red colonies		Positive	*Clostridium perfringens*	White colonies	Positive	*Clostridium perfringens*
10	*Clostridium perfringens*	Seaweed Dulse	Black colonies	Red colonies		Positive	*Clostridium perfringens*	White colonies	Positive	*Clostridium perfringens*
13	*Clostridium perfringens*	Sea bean seaweed	Black colonies	Red colonies		Positive	*Clostridium perfringens*	White colonies	Positive	*Clostridium perfringens*
14	*Clostridium perfringens*	Seaweed Dulse	Black colonies	Red colonies		Positive	*Clostridium perfringens*	White colonies	Positive	*Clostridium perfringens*
15	*Clostridium perfringens*	Spirulina powder	Black colonies	Red colonies		Positive	*Clostridium perfringens*	White colonies	Positive	*Clostridium perfringens*
16	*Clostridium perfringens*	Water sewage treatment plant	Black colonies	Red colonies		Positive	*Clostridium perfringens*	White colonies	Positive	*Clostridium perfringens*
17	*Clostridium perfringens*	Seaweed Laminaria digitata powder	Black colonies	Red colonies		Positive	*Clostridium perfringens*	White colonies	Positive	*Clostridium perfringens*
18	*Clostridium perfringens*	Water sewage treatment plant	Black colonies	Red colonies		Positive	*Clostridium perfringens*	White colonies	Positive	*Clostridium perfringens*
19	*Clostridium perfringens*	Pond water	Black colonies	Red colonies		Positive	*Clostridium perfringens*	White colonies	Positive	*Clostridium perfringens*

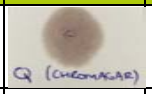
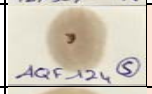
APPENDIX A: Raw results for strains of *Clostridium*

Number	Strain (identified by MALDI-TOF)	Origin	TSC	CHROMAGAR			Final result (CHROMAGAR and phosphatase)	COLUMBIA		Result
				Aspect	Phosphatase	Phosphatase result		Aspect	Phosphatase	
20	*Clostridium perfringens*	Seaweed Asco powder	Black colonies	Red colonies		Positive	*Clostridium perfringens*	White colonies	Positive	*Clostridium perfringens*
2	*Clostridium butyricum*	River water	Black colonies	Red colonies		Negative	Not *Clostridium perfringens*	White colonies	Negative	Not *Clostridium perfringens*
4	*Clostridium sp. (butyricum or glycolicum)*	Outlet water by lagoon treatment	Black colonies	Red colonies		Negative	Not *Clostridium perfringens*	White colonies	Negative	Not *Clostridium perfringens*
5	*Clostridium glycolicum*	Pond water	Black colonies	Red colonies		Negative	Not *Clostridium perfringens*	White colonies	Negative	Not *Clostridium perfringens*
11	*Clostridium sordelii*	Thyme	Black colonies	Red colonies		Negative	Not *Clostridium perfringens*	White colonies	Negative	Not *Clostridium perfringens*
12	*Clostridium bifermentans*	Composite food product	Black colonies	Red colonies		Negative	Not *Clostridium perfringens*	White colonies	Negative	Not *Clostridium perfringens*
N	*Paeniclostridium sordelii*	DSMZ collection	Black colonies	Red colonies		Negative	Not *Clostridium perfringens*	White colonies	Negative	Not *Clostridium perfringens*
P	*Clostridium septicum*	DSMZ collection	White colonies	No growth	∅	∅	Not *Clostridium perfringens*	White colonies	Positive	Possible *Clostridium perfringens*

APPENDIX B: Raw results for strains other than *Clostridium*

Number	Strain (identified by MALDI-TOF)	Origin	TSC	CHROMAGAR			Final result (CHROMAGAR and phosphatase)	TSA		
				Aspect	Phosphatase	Phosphatase result		Aspect	Phosphatase	Phosphatase result
A	*Leuconostoc mesenteroides*	Zucchini flan	No growth	Blue colonies		Negative	Not *Clostridium perfringens*	White colonies		Negative
B	*Lactobacillus sakei*	Smoked salmon	No growth	Red colonies		Positive	Possible *Clostridium perfringens*	White colonies		Positive
C	*Lactobacillus gasseri*	Probiotic	No growth	Blue colonies		Negative	Not *Clostridium perfringens*	White colonies		Negative
D	*Lactobacillus reuteri*	Probiotic	No growth	Red colonies		Negative	Not *Clostridium perfringens*	White colonies		Negative
E	*Bifidobacterium lactis*	Probiotic	No growth	Blue colonies		Negative	Not *Clostridium perfringens*	White colonies		Negative
F	*Lactobacillus paracasei*	Dairy product	No growth	No growth	∅	∅	Not *Clostridium perfringens*	White colonies		Positive (very weak)
G	*Leuconostoc mesenteroides*	Meat product	No growth	Red colonies		Negative	Not *Clostridium perfringens*	White colonies		Negative
H	*Streptococcus thermophilus*	Probiotic	No growth	Red colonies		Negative	Not *Clostridium perfringens*	White colonies		Negative
I	*Lactobacillus sakei*	Dairy product	No growth	Red colonies		Negative	Not *Clostridium perfringens*	White colonies		Negative (weak coloration after 4 min)
J	*Enterococcus faecalis*	Bathing water	White colonies	Red colonies		Negative	Not *Clostridium perfringens*	White colonies		Negative (weak coloration after 4 min)
K	*Enterococcus faecalis*	Water sewage treatment plant	White colonies	Red colonies		Negative	Not *Clostridium perfringens*	White colonies		Negative
L	*Enterococcus faecium*	Outlet water by lagoon treatment	White colonies	Red colonies		Negative	Not *Clostridium perfringens*	White colonies		Negative
M	*Weissella viridescens*	Composite food product	No growth	No growth	∅	∅	Not *Clostridium perfringens*	White colonies		Negative
O	*Pediococcus pentosaceus*	DSMZ collection	No growth	No growth	∅	∅	Not *Clostridium perfringens*	White colonies		Negative

APPENDIX B: Raw results for strains other than *Clostridium*

Number	Strain (identified by MALDI-TOF)	Origin	TSC	CHROMAGAR			Final result (CHROMAGAR and phosphatase)	TSA		
				Aspect	Phosphatase	Phosphatase result		Aspect	Phosphatase	Phosphatase result
Q	*Lactobacillus plantarum*	DSMZ collection	No growth	Blue colonies		Negative	Not *Clostridium perfringens*	White colonies		Negative
R	*Enterobacter cloacae*	Outlet water by lagoon treatment	White colonies	No growth	∅	∅	Not *Clostridium perfringens*	White colonies		Negative
S	*Serratia marescens*	Pond water	No growth	No growth	∅	∅	Not *Clostridium perfringens*	White colonies		Positive
T	*Citrobacter freundii*	Outlet water by lagoon treatment	No growth	No growth	∅	∅	Not *Clostridium perfringens*	White colonies		Positive