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Effect of Sanitizers and Glucose on *Clostridium perfringens* Biofilm Formation and Growth

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A B S T R A C T

Clostridium perfringens enterotoxigenic strain is one of the responsible of food poisoning; this is caused by cpe enterotoxin. Cpe is produced during sporulation, a resistance mechanism. It is described that *C. perfringens* can form biofilm under stressed environments and this depends of carbohydrates availability. The aim of this work was to evaluate the ability of enteroxigenic (E^+) and no enterotoxigenic (E^-) *C. perfringens* strains to form biofilm at different concentrations of carbon source and the inhibitory effect of various Poli P over biofilm formation and the expression of *cpe* and *ccpa* genes. Our results indicate that E^+ *C. perfringens* biofilm reach the more complex structure with high glucose concentrations. Even more current sanitizers used in food industry like SAPP and TSPP, showed to stimulate biofilm formation and to inhibit the growth of planktonic cells. On the other hand, morphological changes were observed during biofilm formation under low glucose concentration and in presence of sanitizers. We propose that the change from a bacillary to coccoid form is related to a resistance to stressed environments. Finally the *cpe* expression is not related to biofilm formation.

Introduction

Clostridium perfringens is a Gram-positive anaerobic, spore forming, rod-shaped bacteria that commonly found in the gastrointestinal tract of both humans and animals. The microorganism causes toxin-mediated diseases, including gas gangrene (clostridial myonecrosis), food poisoning and necrotizing enterocolitis in humans (Radhika *et al.,* 2016). Also, necrotic enteritis or pigbel of the tribes of Papua New Guinea (Smedley *et al.,* 2004).

C.perfringens poisoning can be caused by ingestion of food containing ≥ 5 log vegetative cells/g of food of an enterotoxigenic strain. The poisoning is characterized by diarrhea and abdominal pain, symptoms that are detected 6-24 h after ingestion of contaminated food (Shandera *et al.,* 1983).

This pathogen is classified based on the production of 4 major toxins: type A (α),

type B (α, β, ε), type C (α, β), type D (α, ε) and E (α, ι). The 5% of the strains produces an enterotoxin (CPE) encoded by the *cpe* gene (Czeczulin *et al.,* 1993). This gene can be located in the chromosome or in a plasmid, and its location could define the type of disease produced. (Sparks *et al.,* 2001; Shandera *et al.,* 1983; Brynestad *et al.,* 2001; Collie and McClane, 1998; Cornillot *et al.,* 1995). Strains with enterotoxin in chromosomal location, both vegetative cells and spores, display enhanced resistance to high temperatures, osmotic and nitrites stress than those with plasmidic *cpe* gene (Sarker *et al.,* 2000; Li and McClane, 2006). CPE production is associated with sporulation both in vivo and in vitro (Tórtora, 1984; Tórtora *et al.,* 1986; Rood and Cole, 1991; Fach and Popoff, 1997).

Previously studies performed with strains isolated from food in our laboratory, confirm that vegetative cells and endospores of enterotoxigenic strains are significantly more resistant to heat and osmotic/nitrite-induced stress than non-enterotoxigenic strains of *C. perfringens* (Abbona and Stagnitta 2016). Even more, it is know that *C. perfringens* can develop another resistant form: biofilm (Charlebois *et al.,* 2014; Donelli *et al.,* 2012). This gives the microorganism some of the resistance provided by the endospores, but the biofilm cells can adapt more quick to changes in the environment (Varga *et al.,* 2008). Bacterial biofilms are communities of cells adhered to surfaces and embedded in a matrix of extracellular polymeric substances, nucleic acids and proteins (Yantorno *et al.,* 2010). Bacteria that are part of the biofilm are termed sessile and are physiologically distinct from those which are suspended, called planktonic. Several studies suggest that biofilm maturation affects antibiotic resistance and sanitizers through different mechanisms (Wang *et al.,* 2014, Charlebois

et al., 2014). It has been reported that biotypes A of ATCC 13124, 13, and SM101, as well as type C, D, and E strains of *C. perfringens* can form biofilm (Varga *et al.,* 2008) and the persistence in the intestine forming biofilm could explain the establishment of Antibiotic Associated Diarrhea (AAD) after damage in the normal flora by antibiotics. There are evidence that shows that *C. perfringens* biofilm is present in gut mucosal Microbiotas of Ulcerative Colitis patients. (Fite *et al.,* 2013)

The regulation of biofilm production in Gram-positive bacteria is due to many factors, one of which is the availability of carbohydrates in the medium (Varga *et al.,* 2008).

The catabolite control protein A (CcpA) belongs to the family of regulators LacI/GalR and acts as a global transcriptional regulator in response to limitations of carbohydrates. *C. perfringens* CcpA protein is a protein of 332 amino acids, whose gene coding exhibits the highest level of identity (70%) with CcpA orthologous genes from other clostridia (Bruggemann *et al.,* 2003).

The sanitizers are used to reduce food spoilage by microorganisms. Sanitation reduces the number of microorganisms to a safe level to health. The presence of biofilm or spores makes hard to sanitize since they become resistant to chemical attack by the detergent. In addition, biofilm may attract other pathogenic bacteria. Although a large number of chemical preservatives have been proved to protect edible products, only a relatively small number of them are allowed to be used as such. It is due to antimicrobial activity that they keep in *in vitro* assays, but not when they are added to the food (Jay *et al.,* 2005). FDA (Food and Drug Administration) regulates this issue allowing

the Compounds Generally Recognized As Safe (GRAS) to be used as additives and sanitizers.

Inorganic polyphosphates (Poly P) are GRAS compounds and are widely used as food additives in meat and dairy industry to improve functional properties, retard oxidative rancidity, protect flavor, increase productivity due to its water binding ability and improve emulsification properties of the food. There are several studies establishing that Poly P are growth inhibitors of various pathogenic microorganisms (Akhtar *et al.,* 2008). The suggested inhibition mechanism indicates that Poly P would bind to the cell wall, chelating metals such as Ca^{2+} and Mg^{2+} held together without releasing them, this would cause bactericidal or bacteriolytic effects. At high temperatures Poly P lose its chelating ability and it turns into orthophosphate (Molina Ubeda and Diaz; 2002). Indirectly, Poly P block the dynamics of Z ring formation and in this way they act directly on bacterial cell division inhibition (Maier *et al.,* 1999).

The aim of this work was to evaluate the ability of enteroxigenic and no enterotoxigenic *C. perfringens* strains to form biofilm at different concentrations of carbon source and to evaluate the inhibitory effect of various Poly P on biofilm already formed and the expression of *cpe* and *ccpa* genes.

Materials and Methods

Strains, Media and Culture conditions

Enterotoxigenic (E^+) and non-enterotoxigenic (E^-) *C. perfringens* strains isolated from meat foods in San Luis, Argentine were used in this study. The strains were previously characterized by our group by reversed passive latex agglutination (RPLA) and PCR for enterotoxin (CPE) and *cpe* gene detection respectively (Corigliano *et al.,* 2011; Stagnitta *et al.,* 2006).

The strains were conserved in cooked meat medium (CMM) at room temperature, and biofilm formation were obtained in clostridia optimized medium (COM) (p/v); (i) , 2% peptone , 0.5% yeast extract, 0.15% $MgCl_2$, 0.075% cysteine hydrochloride, (ii). Glucose 0.5% (iii). 0.5% dipotassium phosphate, pH 7.2.

Biofilm

Biofilm formation was determined by measuring sessile/planktonic cells ratio (Abs570/OD580). For each experiment 10 Petri dishes provided with 5 coverslips (1.8 x 1.8 cm) placed in their base were used with 10 ml of COM. The inocula were obtained in COM at logarithmic phase of growth (5-7 h of incubation) with an initial concentration of OD580nm = 0.2 (10^8 CFU/ml). The plates were incubated at 37°C in anaerobic jar under an atmosphere of 9%5 N_2:5% CO_2. The biofilm growth was determined at different incubation periods (24 to 216h).

The quantification of sessile cells was determined with 0.1% crystal violet for 20 min and after 30 min discoloration with cold methanol. The Abs of the extracted dye was measured at 570 nm in spectrophotometer. Planktonic cells were determinated by measurements of OD 580nm.

Viable count of sessile and planktonic cells

Viable count cells adhered to the coverslips were washed with saline solution (SS) and removed by vortex. The cells were seeded in Sodium Sulphite-Polymyxin-Sulfadiazine

Agar (SPS). The plates were incubated at 37 °C in anaerobic atmosphere for 24 h and the results were expressed as CFU/cm². Dilutions of planktonic cells were seeded in SPS medium and cultured in the same conditions previously described and the results expressed as CFU/ml.

Most probable number (MPN)

The viability of planktonic cells was also quantified by the MPN technique, using milk-Fe^{2+} medium. The cultures were incubated for 16 to 18 h at 45 °C. The reaction was considered positive when milk coagulation and iron oxidation was observed. The total number of viable bacteria was determined using the table of Man (Man 1983 Corrected)

Biofilm observation

Optical microscopy

The coverslips were washed, fixed and colored with 0.1% crystal violet for 20 min. It was observed by light microscopy at 40 and 100x amplification.

Scanning electron microscopy (SEM)

Biofilm coverslips were fixed with methanol for 30 min and dehydrate for 24 h at 37 °C and were mounted on aluminum stubs, covered with gold with a standard sputter coater and then photographed with a Zeiss LEO 1450VP. Photos of scanning electronic microscopy (SEM) were conducted at 'Laboratorio de Microscopía Electrónica y Microanálisis' (LABMEM) of Universidad Nacional de San Luis (Argentina).

Effect of glucose concentration on the formation of biofilm

The effect of the concentration of the carbon source was carried out using COM and modified using 0.1 % and % 1 glucose and other culture conditions previously described (Varga *et al.,* 2008).

Preparation of polyP solutions

Tetra Sodium Pyrophosphate (TSPP), Sodium Tripolyphosphate (STPP) and Sodium Acid Pyrophosphate (SAPP) from Sigma, all were dissolved in distilled water to a final concentration of 10% (w/v), adjusted to pH 7.0, filter sterilized and stored at 4 ℃ for no more than 1 month.

Inhibitory effect of sanitizers SAPP, STPP and TSPP on biofilm growth

The inhibitory effect of SAPP, STPP and TSPP on the biofilm obtained after 48h of incubation was determined. The experiments were carried out in COM medium with 0.4, 0.6 and 0.8% of polyphosphates. The effect of sanitizers on biofilm at 6, 24 and 48h of treatment was determined.

Effect of Mg^{2+}

The effect of the concentration of Mg ions in the inhibitory mechanism of sanitizers was determined in the absence and presence of 0.15% $MgCl_2$ in COM added of 0.8% SAPP and 0.8% TSPP.

Expression of *ccpA* and *cpe* in sessile and planktonic cells by RT-PCR

RNA extraction was performed by a rapid method for Gram positive bacteria (Oh and So 2003); using glass beads (106 microns, Sigma). RNA integrity was determined by gel electrophoresis in 1% agarose at 80volt. And RNA was quantified in Beckam DU-600

The cDNA was obtained using Cloned Avian Myeloblastosis Virus (AMV) Reverse

Transcriptase (RT) (Invitrogen). The reaction mixture: 2.8mM RNase -free water, 0.1-0.5 ug RNA, 0.02mM hexamer primers, final volume of 20ul was used and incubated during 5 min at 70 °C. Buffer 5X reverse transcriptase kit, 1 mM dNTPs, 0.25mm ribonuclease inhibitor and ddH2O was then added. It was incubated for 5 min at 25 ° C. 1 ul of AMV reverse transcriptase enzyme was added and incubated for 10 min at 25 °C, 60 min at 42 °C and 10 min at 70 °C.

For the following PCR reaction mixture was used: 1X buffer, 0.2 mM dNTP, 1.5-3 mM Mg^{2+}, 0.2 mM Primer forward, 0.2 mM Primer reverse, ddH2O, 1.5 U of Taq polymerase, and 5 ul of cDNA. Primer sequence is listed in Table 1.

The protocol used was: 94 °C for 5 min, 40 cycles of 94 °C for 45 sec, 50 °C for 1 min and 72 °C for 1 min. The final extension was at 72 °C for 7 min. The PCR products were determined by gel electrophoresis in 1.8 % agarose at 80 volts for 40 min and compared with Molecular Wight Ladder.

Quantitative Data Analysis

Data are expressed as mean ±SEM from three independent experiments. Statistical analysis was performed using the Infostat software version 2012 and GraphPad Prism5 software version 5.01. p≤ 0.05 and p≤ 0.001 compared to controls no treated, were considered as significant difference (Di Renzo *et al.,* 2012).

Results and Discussion

Effect of glucose concentration on biofilm formation

A significant increase in Abs/OD ratios of E^+ strain until 120h incubation at three glucose concentrations was observed. These

ratios diminished after incubations longer that 120 h. Biofilm formation significantly decreased with glucose concentration 1% vs 0.5 % (P ≤ 0.05) and vs 0.1 % (P ≤ 0.01) at 120 h (Fig. 1A, 1B). Statistical analysis using Pearson coefficient established an inverse correlation between glucose concentration and biofilm formation (-0.59). (Two way ANOVA, GraphPad Prism 5 and Infostat. A similar effect was seen in E⁻ strain (Fig. 1C, 1D).

In order to elucidate if the glucose-dependent biofilm growth is related to *ccpA* expression, we analyzed the *C. perfringens* mRNA by RT-PCR. The results indicate that *ccpA* is expressed at the beginning of biofilm formation (24h) and two days later its expression decreases. Interestingly, planktonic cells do not express *ccpA* at 24h, but at 72h of incubation (Fig. 2).

Effect of sanitizers SAPP, STPP and TSPP on E^+ biofilm and planktonic cells growth

The inhibitory effect of sanitizers was studied on both planktonic and sessile cells. OD decreased on planktonic cells treated with 0.6, 0.8% SAPP (p ≤0.05) and 0.8% TSPP (p ≤0.05) until 48h. Any sanitizer caused a total inhibition of the planktonic cells growth. Interestingly STPP did not caused OD diminution of the *C. perfringens* cells.

The inhibitory effect of the sanitizers on sessile cells was determined on mature biofilms. The effect of 0.4, 0.6 y 0.8% SAPP, TSPP and STPP after 120 h of treatment was determined. The ratio Abs/OD averages obtained after all treatment were as follows 0.83, 0.8, 0.28, and 0.22 (TSPP, SAPP, STPP and control, respectively) (Fig. 3).

This effect was confirmed by Bonferroni test for TSPP and SAPP (0.4 % and 0.8 %) meanwhile after treatment with STPP no significant differences with control were observed.

On the other hand the increased growth seen with Crystal Violet stains was correlated with the Abs/OD ratios. Interestingly some sanitizers stimulated biofilm growth, and decreased OD planktonic measures. This suggest that planktonic cells in suspension attach to the biofilm through time. (Fig. 4)

To rule out an unwanted effect by the sanitizers-Mg^{2+} complexation, we performed an experience in absence of Mg^{2+}. Statistical analysis on Abs/OD ratio showed no significant difference between the treatments and controls through time (Data not shown).

Viability of planktonic and sessile cells

In order to see if the planktonic and sessile cells were alive, we preformed two methods to measure this**.** The counted cell assay of treatments with 0.8% TSPP and 0.8% SAPP showed a big inhibition on sessile and planktonic cells, while 0.6% SAPP did not affect *C. perfringens* viability at 72h. Surprisingly 0.8% SAPP increased CFU counted at 72h (Fig. 5) and OD at 120h (Fig. 3). This could be explained by sanitizer metabolization and loss of effect.

Therefore SAPP stimulated biofilm growth and affected bacteria viability in a concentration and time-dependent way. On the other hand TSPP stimulated biofilm growth, but diminished *C. perfringens* viability with all concentration tested for a long time. It is notable to mention that TSPP affects planktonic CFU two times more than sessile CFU (Fig. 5). These results were confirmed with a more sensitive method (MPN) (Data not shown). Changes of *C.*

perfringes morphology in biofilm. During biofilm formation, *C. perfringens* cells showed morphological changes from bacillary to coccoid forms depending on the glucose concentrations assayed.

In Crystal Violet staining, biofilm showed increased number of coccoid forms with 0.1% glucose treatment through time; 28% and 39 % at 24 and 72h respectively. The most remarkable morphological changes were observed at 0.5% glucose 8.7% and 81.2% at 24 and 72h respectively. While at 1% glucose the number of bacteria with coccoid forms was significant lower than those with bacillary forms, 3.9% and 2.7 % at 24 and 72 h.

The E$^-$ strain cultured with 0.1% glucose presented 41% and 66% of coccoid forms at 24 and 72hs respectively. With 0.5% glucose coccoid forms were significatively represented with a 71% and 56 % at 24 and 72h respectively. And with 1% glucose the biofilm presented 4.06% and 2.9% of coccoid forms at 24 and 72h.

There was not significative difference between coccoid percentages in both strains. But coccoid forms of E$^+$ strain increased through the time with 0.5% glucose.

The formation of a gel layer on the biofilm in the presence of SAPP 0.8 % and TSPP 0.4 % and 0.8 % was detected, distorting classical palisade arrangement as time increased (Fig. 4A).

Scanning electron microscopy (SEM) showed the presence of coccoid forms in control as well as in treated biofilm (Fig. 6A, 6B and 6D). At 48h biofilm treated with TSPP presented cells with the following lengths: 5,298 and 4,625 microns. Also the no treated photograph showed a flat surface biofilm and coccoid forms with the

following lengths: 3,474 microns and 1,399 microns (48h); 4,126 x 1,411 microns and 2,397 x 1,610 microns (24h).

To elucidate if the sanitizer precipitates on cells surface, turning them into characteristic forms, we observed by SEM the sanitizer in absence of bacteria, demonstrating the crystals formation (Fig. 6C). Surprisingly, the measure of the crystals were bigger than those seen with bacteria; indicating that sanitizer is deposited on the bacteria surface besides preforming specific structures.

On the other hand, the counted cells in SEM photographs showed that *C. perfringens* treated with TSPP and control biofilm presented 50% and 27% of coccoid forms respectively.

Monte *et al.,* 2014 saw changes in Gram negative walls induced by vegetables extracts, so we suggest that stressful environments as glucose consumption and sanitizer treatment could affect *C. perfringens* wall. This could turn the common morphology on a resistant structure to keep the bacteria alive (Monte *et al.,* 2014).

cpe expression in *C. perfringes* biofilm

Alternatively, to further inquire if biofilm formation is related to *C. perfringens* pathogenesis, we observed *cpe* expression in planktonic and sessile cells. There was no expression of *cpe* (Fig. 2). It is known that cpe production and secretion occurs in a sporulation-dependent way. We do not have indication that sporulation occurs during biofilm formation. But Pantaléon *et al.,* proposed that the germination rate is lower in the spores isolated from the biofilm of *Clostridium difficile* in comparison with the spores purified in planktonic culture. This lower rate of germination may be due to the

presence of a shroud present around the spores formed within the biofilm. This may prevent germination in the biofilm (Pantaléon *et al.,* 2014).

The present study suppose that biofilm formation, sporulation and initiating TFP-dependent gliding motility are all three responses to stressed environments. This could be CcpA dependent or CcpA independent. We think that in particular environments *C. perfringens* will decide which process is the most advantageous or possible combinations of them to survive.

On the other hand, sporulation process need a big consumption of energy and does not allow a quick adaptation to the environmental changes, as biofilm do.

It is important to note that *cpe* expression is absence in biofilm cells of 72h growth. But it does not indicate necessarily the germination activation and cpe protein release.

Finally, Varga *et al.,* 2004, reported that CcpA inhibits *cpe* expression during exponential phase of *C. perfringens* and under sporulation conditions CcpA leads to Sig E and Sig K synthesis stimulation *cpe* transcription during stationary phase. This may explain the absence of *cpe* expression in planktonic cells.

The present study results suggest that there is an inverse correlation between glucose concentration and biofilm formation in E[+] *C. perfringens*.In another hand *CcpA* expression decreases with time in biofilm but planktonic cells. This result could be explained by two hypotheses: 1. Taking in account bibliography, the availability of glucose activates CcpA and glucose availability decreases over time. This is due to increased glucose consumption, and the

activation of CcpA is not essential. So it is not expressed at 72h of biofilm formation. But it does not happen with planktonic cells, it seems to be that CcpA delays it expression, starting between 24 and 72h.

The hypothesis 2 is based on previous studies of Varga *et al.,* (2008) which revealed that *ccpa* is expressed both in planktonic and sessile cells, being this

essential for maximal biofilm formation of *C. perfringens*. Other studies suggest that CcpA negatively regulates its expression in *Staphylococcus aureus* strains (Canova *et al.,* 2014). All this together suggest that the CcpA expression in biofilm since 24h is expressed corresponding with maximal biofilm formation from *C. perfringens*; then it start to diminish its expression by negatively regulation.

Table.1

Primers	Segment amplified
a) Fw5`-AGAAGCTGGAGTGTCAATAGCACCA- 3` Rv 5 `-GTTAGCTACGTCCTCAGCACCTCT- 3 `	*ccpA* (232 bp) (Primer- BLAST program)
b) Fw5`-TGTTAATACTTTAAGGATATGTATCC-3` Rv 5 `- TCCATCACCTAAGGACTG -3 `	*Cpe* (935 bp) (Aguilera *et al.,* 2005)
c) Fw5`-TTTCGAAAGGAAGATTAATACC-3` Rv5`-CAACTTAATGGTAGTAACTAAC-3`	*16S rRNA* (965pb)

Primers sequences used to amplified *ccpA*, *cpe* and *16s rRNA* genes.

Fig. 1 *Clostridium perfringens* biofilm formation (Abs/OD) at different glucose concentration (0.1%, 0.5%, 1%) in a enterotoxigenic strain E + (A) and non enterotoxigenic strain E- (C). Crystal Violet 0.1% stain of biofilm formation from E+ (B) and E- (D) * (P < 0.05) ** (P < 0.01%).

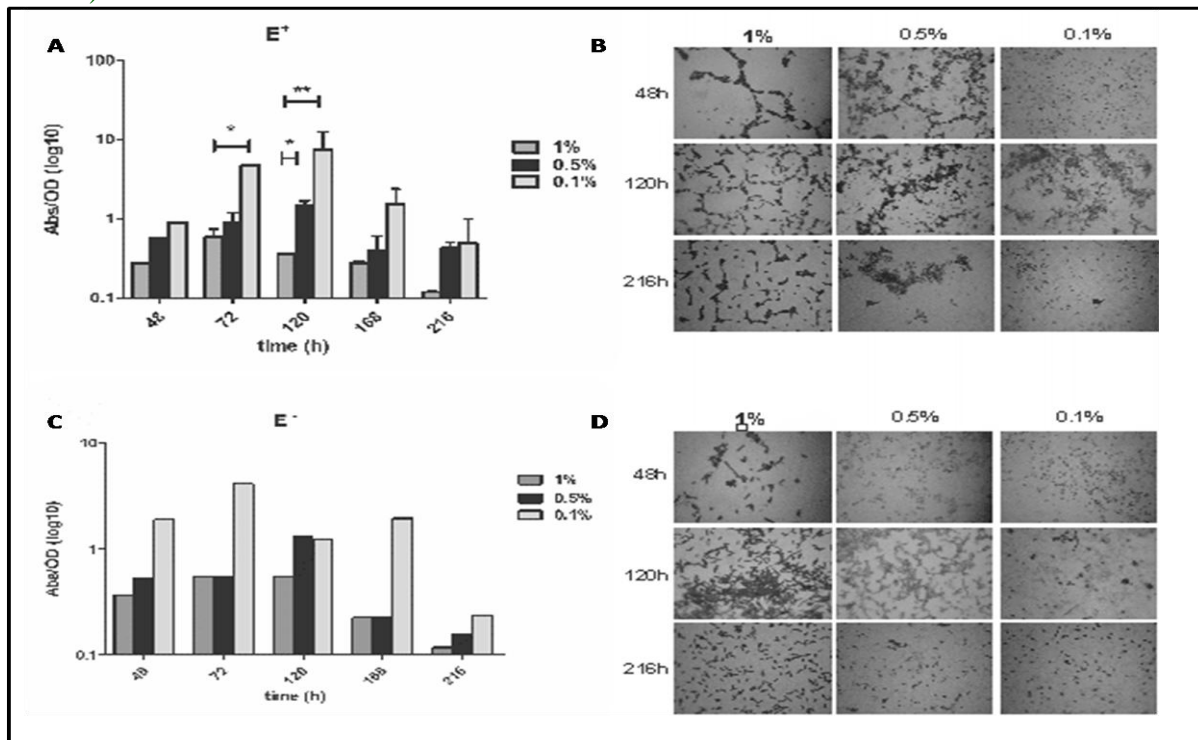**Fig. 2** RT-PCR from Planktonic (P) and sessile (B) cells mRNA at 24 and 72h of biofilm formation.
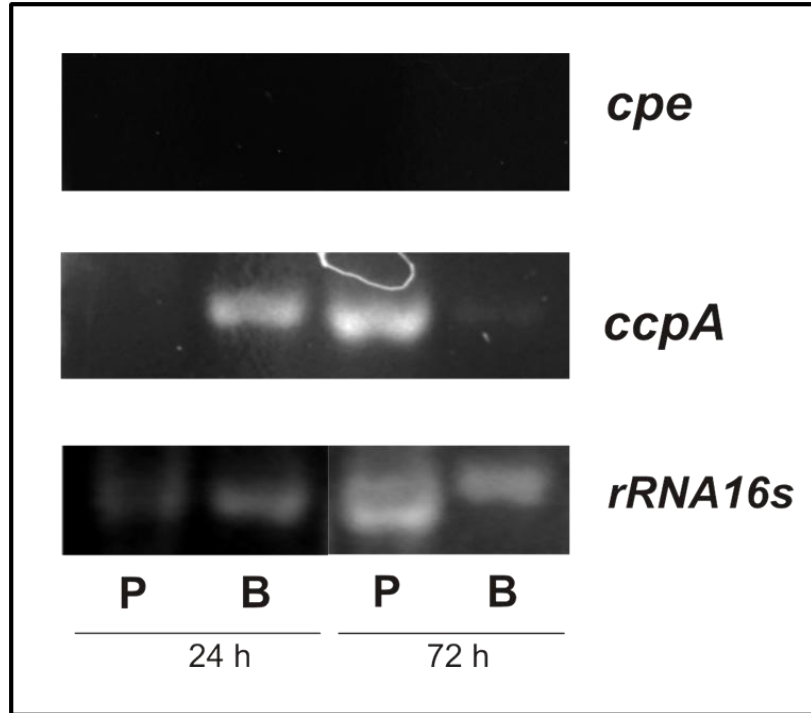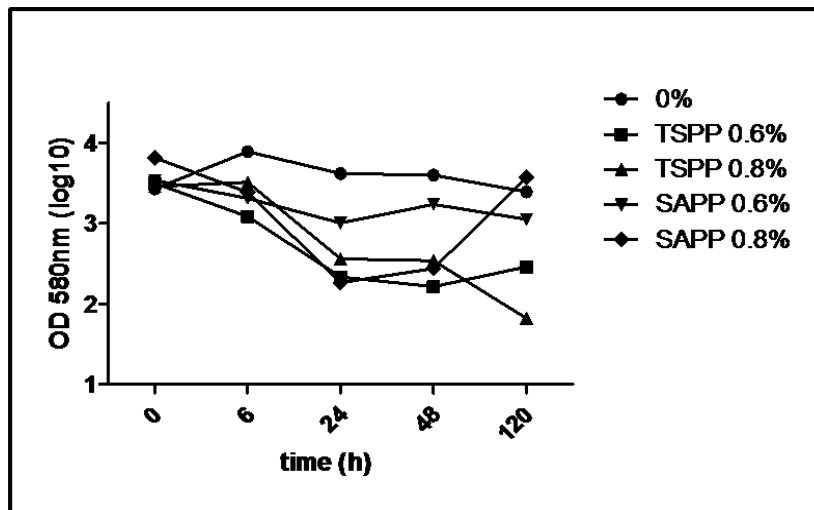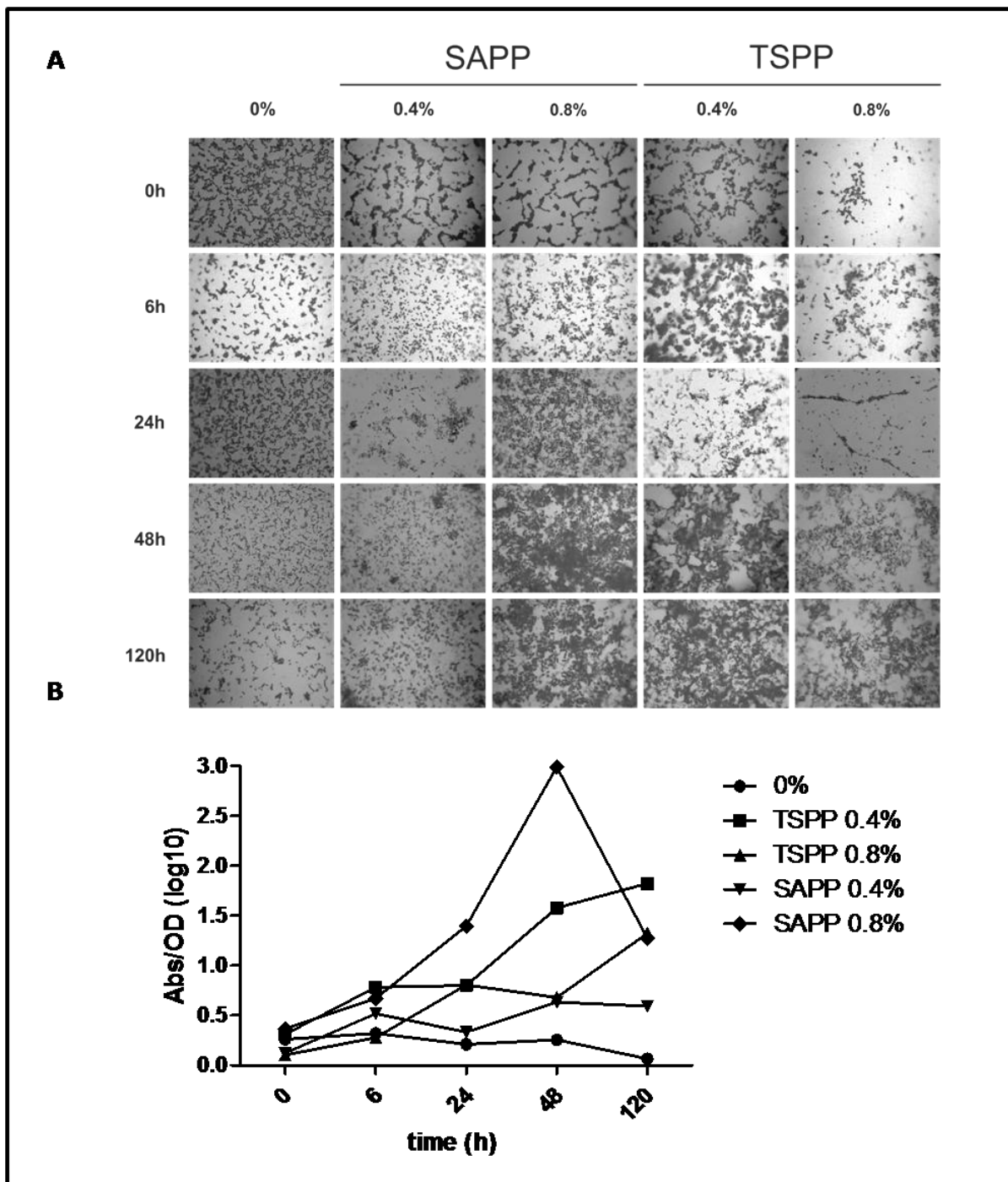

Fig. 3 Effect of sanitizers TSPP and SAPP on Optical Density (OD) of planktonic cells from *C. perfringens* E$^+$ strain

Fig. 4 Effect of sanitizers TSPP and SAPP on mature biofilm from *C. perfringens* E[+] strain.
Crystal Violet 0.1% stain (A). Abs/OD ratio (B)

Fig. 5 CFU from sessile and planktonic cells from a enterotoxigenic *C. perfringens* strain at 72h.
* p≤ 0.05 compared to no treated planktonic cells
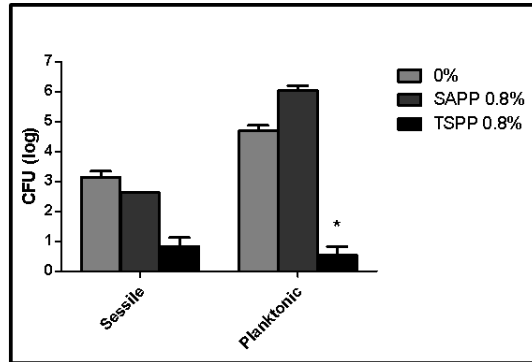
Fig. 6 SEM of a mature biofilm from an enterotoxigenic strain of *C. perfringens* **A**. 48h TSPP
0.8% 5,298 and 4,625 microns **B.** 48h 0% 3,474 and 1,399 microns **C.** 72h TSPP **D.** 24h 0%
2,397 x 1,610 microns **E.** 24h 0% 4,126 x 1,411 microns.
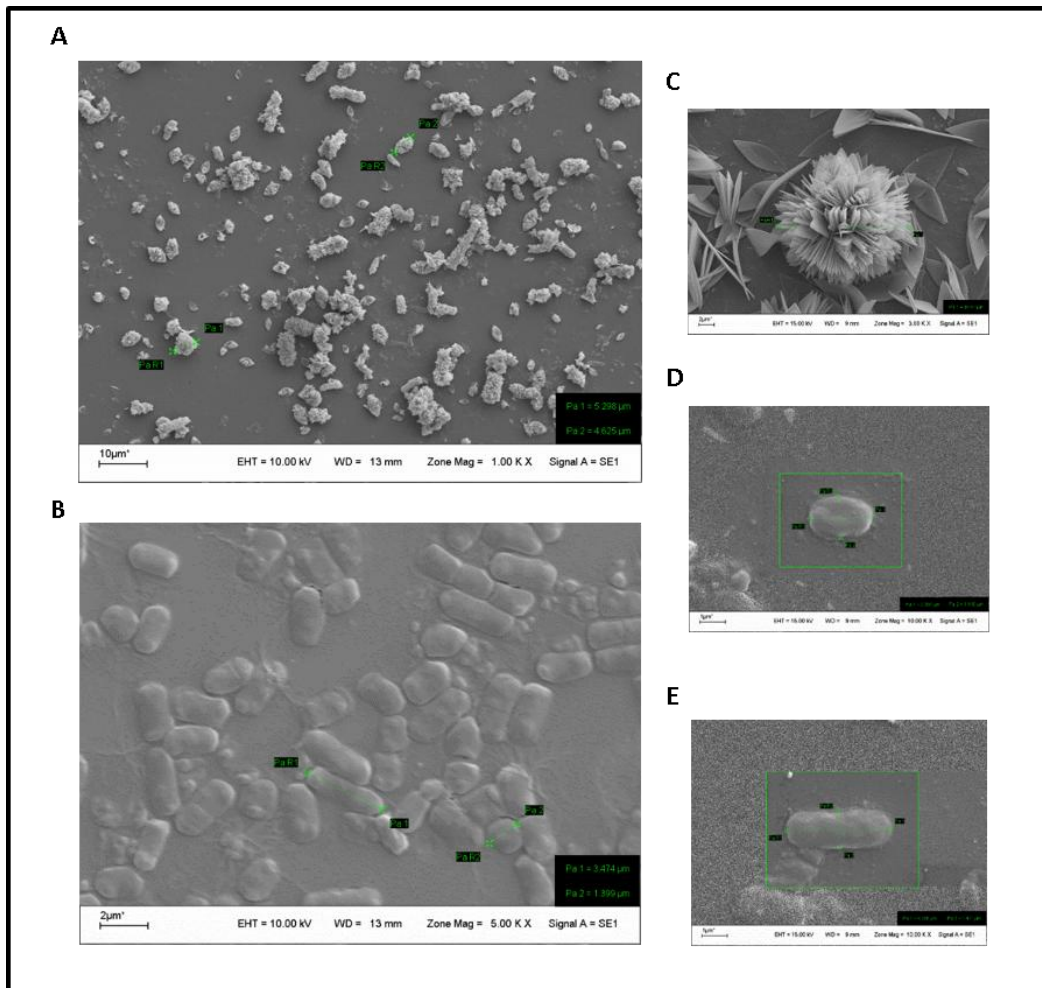
None of both hypotheses indicates any clue to elucidate why planktonic cells express *ccpa* before sessile cells. We think biofilm retains most nutrients, including glucose, being this necessary to survive in the sessile resistance form; so in this way when environmental conditions improve for its normal growth, it could be easy and fast to restore *Clostridium perfringens* metabolism.

On the other hand, only SAPP and TSPP produced a decrease in OD, but interestingly they stimulated biofilm structure growth. Furthermore, the viability of biofilm cells diminishes with SAPP and TSPP, so we suggest that the high values of Abs from biofilm treated with SAPP and TSPP correspond to the whole biofilm structure and the gel layer of sanitizer that could recruit some planktonic cells. Despite of this, we think this uncommon biofilm structure situated in an environment with other bacteria could recruit them and increase its pathological features. This is an important feature in biofilm pathogenesis, because this could provoke other bacteria attachment, and an increase in pathogenesis. Interestingly, the morphological changes observed at low glucose concentration for both strains (E^+ and E^-) could be due to a glucose consumption resistance, and could be related in some way with the formation of biofilm. Even more, we demonstrate that sanitizers promote coccoid forms. Glucose consumption and treatment with sanitizer are both features of a stressed environment for *C. perfringens*. Finally we suggest that the absence of *cpe* enterotoxin in planktonic and sessile cells can be explained to the absence of sporulation; which is essential for cpe release.

All this together, remark the importance of biofilm formation and complex structures in presence of sanitizers used to improve food quality, suggest a failure in this method used in industry. This could lead to increase pathogenesis of *C. perfringens*, helping to form mixed biofilm with other bacteria.

Acknowledgments

References

Abbona, C.C., and P.V. Stagnitta. 2016. "Clostridium perfringens: Comparative effects of heat and osmotic stress on non-enterotoxigenic and enterotoxigenic strains." *Anaerobe* .39: 105-113. doi: 10.1016/j.anaerobe.2016.03.007.

Aguilera, M.O., P.V. Stagnitta, B. Micalizzi, and A.M. de Guzmán. 2005. "Prevalence and characterization of Clostridium perfringens from spices in Argentina." *Anaerobe,* 11(6): 327-34. doi: 10.1016/j.anaerobe.2005.05.003.

Akhtar, S., D. Paredes-Sabja, and M.R. Sarker. 2008. "Inhibitory effects of polyphosphates on Clostridium perfringens growth, sporulation and spore outgrowth." *Food Microbiol.,* 25(6): 802-8. doi: 10.1016/j.fm.2008.04.006.

Bruggemann, H., S. Baumer, W.F. Fricke, A. Wiezer, H. Liesegang, I. Decker, C. Herzberg, R. Martinez-Arias, R. Merkl, A. Henne, and G. Gottschalk. 2003. "The genome sequence of Clostridium tetani, the causative agent of tetanus disease." *Proc. Natl. Acad. Sci. U S A,* 100(3): 1316-21. doi: 10.1073/pnas.0335853100.

Brynestad, S., M.R. Sarker, B.A. McClane, P.E. Granum, and J.I. Rood. 2001. "Enterotoxin plasmid from Clostridium perfringens is conjugative." *Infect. Immun.,* 69(5): 3483-7. doi: 10.1128/IAI.69.5.3483-3487.2001.

Canova, M.J., G. Baronian, S. Brelle, M. Cohen-Gonsaud, M. Bischoff, and V. Molle. 2014. "A novel mode of regulation of the Staphylococcus aureus Vancomycin-resistance-associated response regulator

VraR mediated by Stk1 protein phosphorylation." *Biochem. Biophys. Res. Commun.,* 447(1): 165-71. doi: 10.1016/j.bbrc.2014.03.128.

Charlebois, A., M. Jacques, and M. Archambault. 2014. "Biofilm formation of Clostridium perfringens and its exposure to low-dose antimicrobials." *Front Microbiol.,* 5: 183. doi: 10.3389/fmicb.2014.00183.

Collie, R.E., and B.A. McClane. 1998. "Evidence that the enterotoxin gene can be episomal in Clostridium perfringens isolates associated with non-food-borne human gastrointestinal diseases." *J. Clin. Microbiol.,* 36(1): 30-6.

Corigliano, M.G., A.M. de Guzmán, and P. V. Stagnitta. 2011. "Characterization of the plasmidic or chromosomal cpe gene and metabolic activities in Clostridium perfringens isolates from food in San Luis--Argentina." *Cent. Eur. J. Public Health,* 19(1): 46-53.

Cornillot, E., B. Saint-Joanis, G. Daube, S. Katayama, P.E. Granum, B. Canard, and S.T. Cole. 1995. "The enterotoxin gene (cpe) of Clostridium perfringens can be chromosomal or plasmid-borne." *Mol. Microbiol.,* 15(4): 639-47.

Czeczulin, J.R., P.C. Hanna, and B.A. McClane. 1993. "Cloning, nucleotide sequencing, and expression of the Clostridium perfringens enterotoxin gene in Escherichia coli." *Infect. Immun.,* 61(8): 3429-39.

InfoStat, C.W. version 2012. InfoStat Group, FCA, National University of Cordoba, Argentina, http:// www.infostat.com.ar.

Donelli, G., C. Vuotto, R. Cardines, and P. Mastrantonio. 2012. "Biofilm-growing intestinal anaerobic bacteria." *FEMS Immunol. Med. Microbiol.,* 65(2): 318-25. doi: 10.1111/j.1574-695X.2012.00962.x.

Fach, P., and M.R. Popoff. 1997. "Detection of enterotoxigenic Clostridium perfringens in food and fecal samples with a duplex PCR and the slide latex agglutination test." *Appl. Environ. Microbiol.,* 63(11): 4232-6.

Fite, A., S. Macfarlane, E. Furrie, B. Bahrami, J.H. Cummings, D.T. Steinke, and G.T. Macfarlane. 2013. "Longitudinal analyses of gut mucosal microbiotas in ulcerative colitis in relation to patient age and disease severity and duration." *J. Clin. Microbiol.,* 51(3): 849-56. doi: 10.1128/JCM.02574-12.

Jay, James M., Martin J. Loessner, and David Allen Golden. 2005. *Modern food microbiol.,* 7th ed. ed. New York: Springer.

Li, J., and B.A. McClane. 2006. "Comparative effects of osmotic, sodium nitrite-induced, and pH-induced stress on growth and survival of Clostridium perfringens type A isolates carrying chromosomal or plasmid-borne enterotoxin genes." *Appl. Environ. Microbiol.,* 72(12): 7620-5. doi: 10.1128/AEM.01911-06.

Maier, S.K., S. Scherer, and M.J. Loessner. 1999. "Long-chain polyphosphate causes cell lysis and inhibits Bacillus cereus septum formation, which is dependent on divalent cations." *Appl. Environ. Microbiol.,* 65(9): 3942-9.

Man, J.C. de. 1983. "MPN Tables, Corrected " *Eur. J. Appl. Microbiol., Biotechnol.,* 17: 301-305.

Molina Ubeda, R., and Marcos Diaz. 2002. "Sanitización en la industria enológica." www.alcion.es

Monte, D.F., A.G. Tavares, A.R. Albuquerque, F.C. Sampaio, T.C. Oliveira, O.L. Franco, E.L. Souza, and M. Magnani. 2014. "Tolerance response of multidrug-resistant Salmonella enterica strains to habituation to Origanum vulgare L. essential oil." *Front Microbiol.,* 5: 721. doi: 10.3389/fmicb.2014.00721.

Oh, E.T., and J.S. So. 2003. "A rapid method for RNA preparation from Gram-positive bacteria." *J. Microbiol. Methods,* 52(3): 395-8.

Pantaléon, V., S. Bouttier, A.P. Soavelomandroso, C. Janoir, and T. Candela. 2014. "Biofilms of Clostridium species." *Anaerobe,* 30: 193-8. doi: 10.1016/j.anaerobe.2014.09.010.

Radhika, B., N.V. Kumar, and D. Sreenivasulu. 2016. "Detection of Clostridium perfringens alpha toxin gene in lambs by loop mediated isothermal amplification." *Vet. World,* 9(1): 60-4. doi: 10.14202/vetworld.2016.60-64.

Rood, J.I., and S.T. Cole. 1991. "Molecular genetics and pathogenesis of Clostridium perfringens." *Microbiol. Rev.,* 55(4): 621-48.

Sarker, M.R., R.P. Shivers, S.G. Sparks, V.K. Juneja, and B.A. McClane. 2000. "Comparative experiments to examine the effects of heating on vegetative cells and spores of Clostridium perfringens isolates carrying plasmid genes versus chromosomal enterotoxin genes." *Appl. Environ. Microbiol.,* 66(8): 3234-40.

Shandera, W.X., C.O. Tacket, and P.A. Blake. 1983. "Food poisoning due to Clostridium perfringens in the United States." *J. Infect. Dis.,* 147(1): 167-70.

Smedley, J.G., D.J. Fisher, S. Sayeed, G. Chakrabarti, and B.A. McClane. 2004. "The enteric toxins of Clostridium perfringens." *Rev. Physiol. Biochem. Pharmacol.,* 152: 183-204. doi: 10.1007/s10254-004-0036-2.

Sparks, S.G., R.J. Carman, M.R. Sarker, and B.A. McClane. 2001. "Genotyping of enterotoxigenic Clostridium perfringens fecal isolates associated with antibiotic-associated diarrhea and food poisoning in North America." *J. Clin. Microbiol.,* 39(3): 883-8. doi: 10.1128/JCM.39.3.883-888.2001.

Stagnitta, P.V., B. Micalizzi, and A.M. de Guzmán. 2006. "Prevalence of some bacteria yeasts and molds in meat foods in San Luis, Argentina." *Cent Eur. J. Public Health*, 14(3): 141-4.

Tórtora, J.C. 1984. "Alternative medium for Clostridium perfringens sporulation." *Appl. Environ. Microbiol.,* 47(5): 1172-4.

Tórtora, J.C., M.S. Pinheiro, and M.C. Pitard. 1986. "Biochemical differentiation between entero-toxigenic heat-sensitive and heat-resistant Clostridium perfringens strains." *Microbiol. Immunol.,* 30(2): 89-95.

Varga, J.J., B. Therit, S.B. Melville. 2008. "Type IV pili and the CcpA protein are needed for maximal biofilm formation by the gram-positive anaerobic pathogen Clostridium perfringens." *Infect. Immun.,* 76(11): 4944-51. doi: 10.1128/IAI.00692-08.

Wang, R., N. Kalchayanand, D.A. King, B.E. Luedtke, J.M. Bosilevac, and T.M. Arthur. 2014. "Biofilm formation and sanitizer resistance of Escherichia coli O157:H7 strains isolated from "high event period" meat contamination." *J. Food Prot.,* 77(11): 1982-7. doi: 10.4315/0362-028X.JFP-14-253.

Yantorno, O., A. Bosch, D. Serra, L. Arnal, and S.M Ehling. 2010. "Biofilm en la interfase entre microbiología y biofísica." II TALler Interdisciplianrio Sobre Biofilms Resumenes Fundación Instituto Leloir.