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Interference of the Developing and Toxin Production of Clostridium Botulinum by Lactobacillus Paracasei Subspecies Paracasei

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Abstract
Infant botulism is an intestinal toxic-infection affecting infants younger than one year of age, and currently in some country is the most important form of human botulism by its frequency of occurrence. It is a rare neuroparalytic disease, but potentially fatal, especially if it is not early diagnosed and properly treated. It is caused by botulinum neurotoxins produced by species of *Clostridium*, principally *C. botulinum*.
C. botulinum spores are widely distributed in nature, and its most common habitat and natural reservoir is the soil, the main source of contamination for the different forms of botulism.
C. botulinum spores have been identified in some foods, such as honey, corn syrup, infant formula and in some medicinal plants. Considering the difficulty to prevent the swallowing of spores, is particularly relevant have adequate arrangements to interfere with colonization and/or toxin formation of *C. botulinum* in the intestine.
Different probiotics have been demonstrated utility against several intestinal pathogens. So, we studied and demonstrated the interference of *Lactobacillus paracasei* subsp. *paracasei* on the growth and toxin formation of one strain of *C. botulinum* type A. Therefore, administering probiotics to infants, perhaps from birth, would be effective in preventing or treating the disease.

Keywords: botulism, infant botulism, toxin formation, inhibition, probiotics, lactic acid bacteria, prevention

Introduction

Botulism is a rare neuroparalytic disease, but potentially lethal, caused by botulinum neurotoxins (BoNT) produced by different species of *Clostridium* (*C. botulinum*, *C. baratii* and *C. butyricum*).
Infant botulism (IB) affects infants between 1 and 52 weeks of life, and is currently considered the most common form of human botulism in some countries (Midura, 1996; Arnon, 1998). Argentina is the second country after the U.S. with the highest worldwide occurrence of reported infant botulism cases since first recognition of the disease in 1976 (Koepke et al., 2008). The incidence per 100,000 live birth in Argentina (2.2) is similar that in U.S. (2.1) (Lúquez et al., 2007). Between 1982 and 2012, 713 IB cases were reported in Argentina.

Spores of *C. botulinum* enter the intestine, germinate and produce BoNT that is absorbed and distributed by the blood. Its transmission is not totally clear, but certainly the environmental exposure is one of the major risk factors, and perhaps the most important form of entry. While it is important to try to prevent the access of spores to the gut through contaminated environmental dust, food, fomites, etc., it is imperative to develop concrete and accessible preventive measures for the population at risk.

Intestinal colonization is likely to be due to, among other reasons, that the infant gut microbiota does not provide effective competition to the pathogen, especially in the first half of life, with peak incidence between 2 and 3 months. In Mendoza, 90.1% of the cases reported affected infants less than six months of age, with an average age of four months.

C. botulinum is widely distributed in nature, and the soil is its main reservoir and most common habitat. Spores are also found in the dust, mud, sediments of lakes and oceans, and on the surface of vegetables, so it is not difficult their access to food and digestive tracts of humans and animals.
Currently the IB is the most important clinical epidemiological form of human botulism due to its high frequency, the difficulty of the differential diagnosis with other neurological diseases of the infant, and its possible relation to sudden infant death syndrome (SIDS) (Arnon et al., 1978, 1979b).

It is an intestinal toxi-infection with a proposed infective dose of 10 to 100 spores (Midura, 1996; Arnon, 1998). The agent colonizes and synthesizes BoNT that is absorbed and transported to cholinergic synapses where inhibits acetylcholine release, causing flaccid paralysis characteristic of botulism.

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The patient is more likely to recover fully if performed early diagnosis and proper treatment.

While intestinal colonization suggested the involvement of foods like vegetables as vehicles of clostridia (Midura, 1996), other hypotheses were also proposed, considering environmental exposure as one of the major risk factors for the entry of the spores with the environment powder and subsequent swallowing (Arnon et al. 1979b; Fernández et al., 1994; Fernández et al., 1999). In most infant botulism cases it has not been possible to identify the source of spores (Sobel, 2005).

Although studies were conducted on different traditional and non-traditional baby foods, botulinum spores were found principally in honey (Arnon et al., 1979a; Midura et al., 1979; Kautter et al., 1982; Hauschild et al., 1988; Nakano et al., 1990; Sakaguchi et al., 1990), corn syrup (Midura, 1996), certain medicinal plants that are given as infusions (Bianco et al., 2008; Bianco et al., 2009), and powder infant formula (Brett et al., 2005). Honey has been the food linked epidemiologically as vehicle in IB cases more frequently (Arnon et al. 1979a; Midura et al., 1979; Arnon et al., 1981; Huhtanen et al., 1981; Kautter et al., 1982; Hauschild et al., 1988; Sakaguchi et al., 1990).

Public awareness about the existence of infant botulism and its transmission is critical to prevent this disease. It is very difficult to avoid the ingress of spores with environmental dust, and although infants are exposed to other vehicles (soil, herbal, food, etc.), the only current recommendation is not to feed honey to children under one year of age. Therefore, it is imperative to develop additional preventive measures, precise and accessible to the population, which could interfere with the colonization and/or toxin production of *C. botulinum* in the gut.

Therefore, and based on various studies that have described the beneficial effects of probiotics against intestinal pathogens (Saavedra et al., 1994; Suárez et al., 1994; Helander et al., 1997; Penna, 1998; Brashears et al., 1998; Brashears and Durre, 1999; FENS, 2011; De Genaro, 2012, Theodorakopouloua et al., 2013), we propose to use probiotics to provide some protection against *C. botulinum*, either by inhibiting their growth or interfering with the toxin production in the intestine.

Sullivan NM (1988), found that strains of bifidobacteria and lactobacilli isolated from infant faeces inhibited the growth of *C. botulinum* using zone inhibition procedures.

Lactic acid bacteria such as *Lactobacillus* spp., *Lactococcus* spp., *Bifidobacterium* spp., and the yeast *Saccharomyces boulardi* are among the probiotics used more frequently for human consumption. Probiotics must persist and remain viable in the gastrointestinal tract to be effective. Therefore, administration of a high number of the probiotic every day is preferable, as its residence in the gut may be no more than 72 h (Fuller, 1989).

Give the infants commercial probiotic formulations, and/or enrichment of milk formulas with this bacterium, may help prevent the occurrence of the disease or lessen its severity. However, it is necessary that the interference studies be performed *in vivo*, to evaluate the behavior of these microorganisms in the conditions provided by the infant gut.
In this study, we report the effect of *Lactobacillus paracasei* subsp. *paracasei* in the growth of *C. botulinum* and production of BoNT.

Materials And Methods

Culture media. Cooked meat medium (CMM, No.14 Middle C. Williams Hooper Foundation) (Giménez and Ciccarelli, 1970). Broth and agar Man, Rogosa and Sharpe (MRSB and MRSA) (Laboratorios Britania). MRSA supplemented with 1.9% of agar-agar (AMRS 3.2%). Spore count medium (SCM) (yeast extract 0.5%, peptone 1.0%, sodium thioglycolate 0.1%, sodium chloride 0.5%, agar 1.0%, pH 7.3±0.2), and capping medium, the same but with agar 1.5%. The media were incubated in anaerobiosis (Lúquez et al., 2005).

Buffer solutions. Citric acid 0.1 M, and dibasic sodium phosphate 0.2 M, pHs 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0 and 6.5.

Experimental animals. Swiss female mice of 20±2 g in weight. The survivors were euthanized in CO_2 chamber.
Strains. (1) *C. botulinum* type A strain BL125, isolated of an IB case from Mendoza, Argentina; (2) Probiotic: *Lactobacillus paracasei* subsp. *paracasei* (Lpp), isolated from a commercial probiotic formulation.
Growth inhibition test on solid medium (Figure 1). Two techniques were used: (1) *Streaks in cross*: On the surface of solidified MRSA 3.2% in Petri dish of 100 mm in diameter, one vertical streak of 4 cm was made with the *C. botulinum* strain. Then, two horizontal streaks with the strain of Lpp, each of them starting at both sides of streak made with the *C. botulinum* strain, without touching Lpp. Inhibition was observed by decrease or absence of growth of *C. botulinum* in the intersection of both strains. (2) *In wells*: 0.1 mL of supernatant from a 24 hours culture of *C. botulinum* in CMM was transferred into a screw

cap tube containing 15 mL of MRSA 3.2%, melted and maintained at 48°C. It was homogenized by gentle inversion of the tube and poured into Petri dishes of 100 mm diameter, letting solidify for 45 min at room temperature, replacing the glass cover by filter paper to permit the surface of the medium to dry properly. Two holes of 6 mm in diameter were made, one was filled with 100 µL of active culture of Lpp of 24 hours in MRSB, and the other with 100 µL of sterile broth (without Lpp). After incubation (48 to 72 hours at 34ºC), the growth inhibition halos of colonies of *C. botulinum* were measured.

To verify that inhibition was not due to only the reduction in pH by the development of Lpp in MRSB, the test was repeated by filling the holes with MRSB buffered at different pHs, from 2.5 to 6.5 using increments of 0.5 units. The final pH values were monitored with Beckman digital pH meter model PHI 720.

Inhibition of BoNT production assays in co-cultures of *C. botulinum* and Lpp in liquid medium (Figure 3). The co-cultures were performed in 10 mL of MRSB, with different proportions of individual previous active cultures of 24 hours at 34ºC of *C. botulinum* and Lpp in CMRS, mixing in all possible combinations, pure broths and diluted 1:10 and 1:100 (Figure 3). After incubation at 34ºC for 48 hours, the levels of toxin in the co-cultures were compared with the corresponding *C. botulinum* monocultures (controls of toxin production). In supernatants (centrifuged 20 min at 12,000xg) the levels of toxin (LD_{50}) were performed according to method described by Reed and Muench (1938), using Crisley diluent for the dilutions (Harrell et al., 1964).

At the same time, it was consigned the average time of death of two mice inoculated intraperitoneally with 0.5 mL each (Table1.B.).

To determine the number of organisms inoculated into each co-culture, the count of colony forming units (cfu) of *C. botulinum* was performed in tubes in SCM, and the count of cfu of Lpp in MRSA plates incubated anaerobically 72 h at 34ºC. Counts were considered for the tubes containing between 10 and 30 cfu, and the plates with cfu between 30 and 80.

Results

Growth inhibition test on solid medium

Streaks in cross: Figure 1 shows the results of the test by cross-striations. Inhibition of growth of *C. botulinum* is observed at the intersection of both horizontal and vertical streaks.

Figure 2. Growth inhibition assay by cross-striations.

In wells: Figure 3 shows the results of the test by the method in wells. The well on the left was filled with culture of Lpp in MRSB, and on the right -as negative- MRSB without Lpp, regulated to pH 3.5 with phosphate buffer. To the left is observed the halo of growth inhibition of the *C. botulinum*.

Figure3. Inhibition with Lpp in wells, and growth of Cb BL125 in mass.

Inhibition of BoNT production assays in co-cultures of *C. botulinum* and Lpp in liquid medium

Table 1 (co-cultures of BL125 and Lpp) contains, in Part A the results of the counts of cfu/0.1 mL used in the tests, and in Part B the results of interference consigning times of death and levels of toxin.

Tubes 1, 2 and 3 correspond to three different concentrations of BL125 (10^6, 10^5 and 10^4 cfu/0.1 mL) in monoculture as controls. Toxin levels in LD_{50}/mL were $2.26x10^6$, $2.33x10^6$ and $2.56x10^6$ respectively. In the following three groups of tubes have been faced the same concentrations than in monoculture of BL125, against different concentrations of Lpp in each group (tubes 4, 5 and 6 with 10^8, 10^7 and 10^6 cfu/0.1 mL respectively, and the same in tubes 7, 8 and 9, and 10, 11 and 12) (Table 1.A).

For all three concentrations of BL125 (1:1, 1:10 and 1:100), the reducing level toxin was similar regardless of the concentration of Lpp, 3 to 6 $\log_{10}$ (Table 1.B and Table 2).

The greatest reductions occurred with BL125 concentration of 10^4 cfu per 0.1 mL, surpassed by Lpp concentration in 2, 3 and 4 log (10^6, 10^7 and 10^8 cfu/0.1 mL). With a reduction of $2.56x10^6$ DL_{50}/mL to $4.00x10^0$, $4.60x10^1$ and, $4.00x10^0$ DL_{50}/mL respectively (Table 1.B). And, times of death of the mice decreased dramatically, from an average of 1 h 56 min to >12 h (ANOVA, $p<0.0001$).

Statistical analysis. The comparison of means was done using analysis of variance (ANOVA), resulting average time of death of both experiments (BL125 and BL125+Lpp) with significant differences (p-value <0.0001).

For the coculture BL125+Lpp the death time at any of the dilutions was greater than 720 minutes, therefore, in this case it was not necessary the comparison of the means because the values of the time of death for this experiments were constants.

Discussion And Conclusions

Many studies have described the beneficial effects of probiotics in infectious processes versus intestinal pathogens. For example, adhesion of probiotics to

intestinal epithelium would prevent the binding of pathogens such as enteropathogenic and enterotoxigenic *E. coli*, *Salmonella* spp., *Yersinia* spp., etc. (Helander et al., 1997; Brashears et al., 1998; Brashears and Durre, 1999).

On the other hand, as some authors had referred that probiotic strains have showed beneficial effects in treatment and prevention of *C. difficile* infections (Theodorakopoulou et al., 2013), and although there have not been reports of the benefits of probiotic use in IB, Radsel A. et al. (2013) considered that probiotic could potentially alleviate symptoms of IB. And they refer the administration of a probiotic strain of *Lactobacillus reuteri* DSM17938 at therapeutic dose of $1x10^9$ bacteria daily in the treatment of two cases of IB in Slovenia.

There are publications in reference to growth inhibition of *C. botulinum* by probiotics (Sullivan et al., 1988; Uymaz B et al., 2011), but as far as we know, there are no publications regarding interference on BoNT production.

Since probiotics have shown protection against some intestinal pathogens, we studied *in vitro* interference by *Lactobacillus paracasei* subspecie *paracasei* on growth and BoNT production of *C. botulinum* strain BL125 isolated of an IB case. The results of both tests were positive.

The tests of growth inhibition in a solid medium were began with the method of cross-striations, but because of the difficulties by the drag of microorganisms at the junction of the two striations, the tests were continued with the method with BL125 in all the mass of solid medium in Petri dish, and broth with Lpp in wells.

Growth inhibition was not due to changes in pH, neither could not be verified the presence of soluble inhibitory substances (bacteriocins?), investigations that could be addressed in future works.

Regarding the interference of toxin production in liquid media, depending upon the relative proportions in the co-cultures, we obtained significant reductions of 3 to 6 $\log_{10}$ in toxin levels.

Since in IB main entry of the *C. botulinum* spores is by ingestion of contaminated environmental dust, very hard to avoid, it should be consider the probiotic administration as prophylactic measure, to interfere with the development and/or toxin formation when spores enter the intestine. And why not, use probiotic as therapeutic measure too.
While the results of toxin production, and growth inhibition confirmed our hypothesis, it is necessary to extend the research to higher number of probiotic and *C. botulinum* strains.

We consider that this finding is very relevant from the point of view of prevention, and treatment perhaps, in infant botulism. And, it should be assessed the amount and frequency of administration to infants, perhaps from birth.

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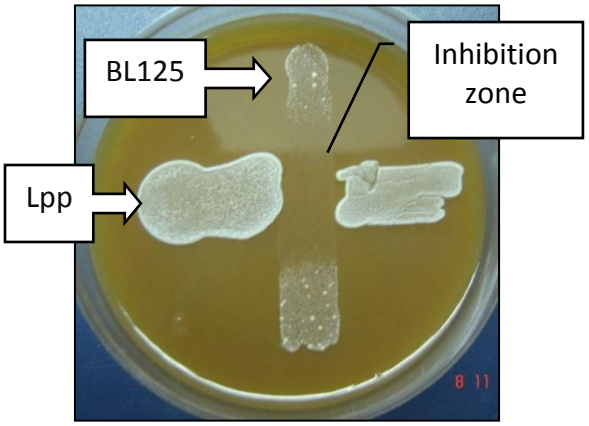

Figure 1. Growth inhibition assay by cross-striations.

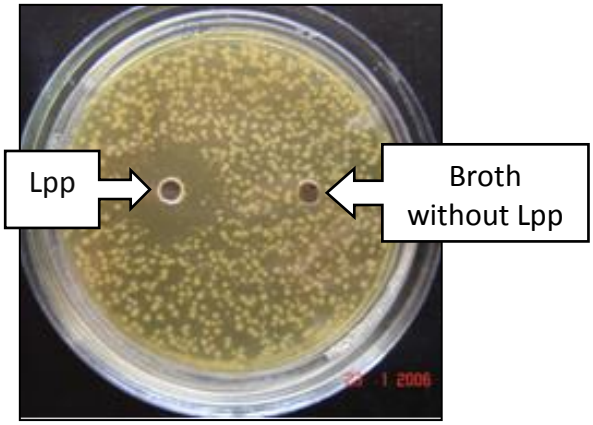

Figure 2. Inhibition with Lpp in wells, and growth of Cb BL125 in mass

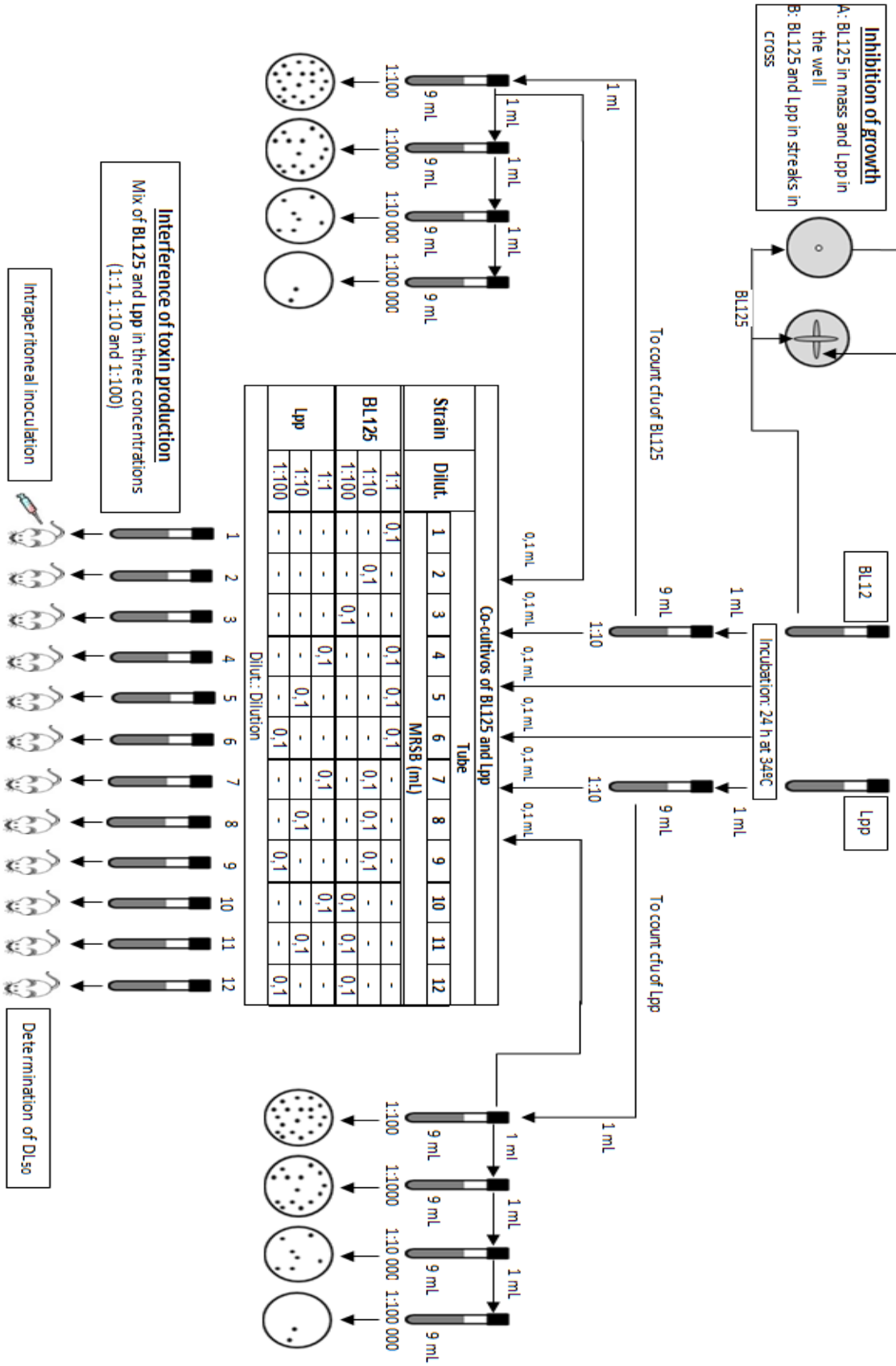

Figure 3. Interference of growth and toxin production of BL125 by Lpp

Table 1. Co-cultures of BL125 and Lpp

A: cfu of BL125 and Lpp used for the tests. B: Results of interference of toxin production in the co-cultures.													
A: cfu/0,1 mL de BL125 and Lpp													
Strain	**Dil.**	**Count**	**Tube**										
			1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
BL125	1:1	ufc/ 0,1 mL	$1.8x10^6$	-	-	$1.8x10^6$	$1.8x10^6$	$1.8x10^6$	-	-	-	-	-	-
	1:10		-	$1.8x10^5$	-	-	-	-	$1.8x10^5$	$1.8x10^5$	$1.8x10^5$	-	-	-
	1:100		-	-	$1.8x10^4$	-	-	-	-	-	-	$1.8x10^4$	$1.8x10^4$	$1.8x10^4$
Lpp	1:1		-	-	-	$2.3x10^8$	-	-	$2.3x10^8$	-	-	$2.3x10^8$	-	-
	1:10		-	-	-	-	$2.3x10^7$	-	-	$2.3x10^7$	-	-	$2.3x10^7$	-
	1:100		-	-	-	-	-	$2.3x10^6$	-	-	$2.3x10^6$	-	-	$2.3x10^6$
B: Death time (DT) and LD_{50}/mL														
Strain	**Dil.**	**Toxin**	**BL125**			**Lpp**								
			Without Lpp			**Dilution**								
						1:1	**1:10**	**1:100**	**1:1**	**1:10**	**1:100**	**1:1**	**1:10**	**1:100**
			Tube											
			1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
BL125	**1:1**	DT	1:30	-	-	>12	>12	>12	-	-	-	-	-	-
		LD_{50}/mL	$2.3x10^6$	-	-	$4.7x10^2$	$1.1x10^3$	$2.4x10^2$	-	-	-	-	-	-
	1:10	DT	-	2:00	-	-	-	-	>12	>12	>12	-	-	-
		LD_{50}/mL	-	$2.3x10^6$	-	-	-	-	$1.4x10^2$	$1.4x10^2$	$5.8x10^1$	-	-	-
	1:100	DT	-	-	2:20	-	-	-	-	-	-	>12	>12	>12
		LD_{50}/mL	-	-	$2.6x10^6$	-	-	-	-	-	-	$4.0x10^0$	$4,6x10^1$	$4.0x10^0$
Dil.: Dilution														

Tabla 2. Comparison of DL_{50} and cfu for BL125 and Lpp

Cfu		**DL_{50}**		**Reduction log**
BL125	**Lpp**	**BL125**	**BL125+Lpp**	
10^6	10^8 10^7 10^6	10^6	10^2 10^3 10^2	3 - 4
10^5	10^8 10^7 10^6	10^6	10^2 10^2 10^1	4 - 5
10^4	10^8 10^7 10^6	10^6	10^0 10^1 10^0	5 - 6