Toxicology Research

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ARTICLE

Evaluation of the toxicity and pathogenicity of biocontrol agents in murine models, chicken embryos and dermal irritation in rabbits

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Biological control emerged as an alternative to the use of crop fungicides in fields and postharvest. It has already been demonstrated that strains of *Candida famata, Bacillus subtilis* Pla10, *Meyerozyma guilliermondii, Meyerozyma caribbica* and *Debaryomyces hansenii* are effective in controlling fungal diseases in tropical fruits. However, in order to develop applications on a field-scale, it is necessary to show that these biocontrol agents are innocuous to humans. In this study, three common toxicity studies were carried out to measure the safety of their use in food products: acute oral toxicity in adult Wistar rats, chicken embryo lethality and skin irritation studies in rabbits using concentrations of 1 and 10 mg of microbial extracts and the administration of 3 and 6 x 10^8 cel/mL of live cells for each one the tested strains used for each model. The rats showed no toxic symptoms and none died during testing. The extracts and strain cells under study did not produce a life-cycle interruption in chicken embryos. For the skin irritation studies in rabbits, the substance being studied produced no skin alteration in the animals. With these results it was concluded that the lyophilized extracts in concentrations of 1 and 10 mg, as well as the cells of the studied strains in concentrations of 3 and 6 x 10^8 cel/mL, were safe in the studied models. Therefore, their use in controlling postharvest diseases in tropical fruits is possible. Their efficiency in controlling plagues in fields and their possible effects on humans, however, require further study.

1. Introduction

Mexico is one of the most important fruit exporters[1], nonetheless, postharvest losses of up to 40% have been reported at a national level, with an estimated loss of 932,692.79 dollars in tropical fruit caused primarily by phytopathogenic microorganisms (fungi and bacteria).[2] Other sources report postharvest losses representing up to 25% of total production in industrialized nations and more than 50% in developing nations.[3] These losses generate a major economic impact due to the costs of production, harvest, transport and storage.[4] Traditionally, damage caused by microorganisms has been controlled by synthetic pesticides. However, due to the acute and chronic effects to human health, [5,7] as well as in animals, fauna, pollinators, natural enemies, terrestrial and aquatic invertebrates, [8,9] the reduction of their use has gained global importance. This has led to the search for other alternatives, namely green technologies for the control of pathogens as in the case of biological control.[10,11] Biological control is an emerging technology that consists in using microorganisms such as yeasts and bacteria that present antagonistic effects towards other microorganisms to control diseases caused by phytopathogens. Biological control, therefore, represents an alternative to the use of harmful chemical substances by being highly compatible with the environment and human health.[10,4,12,13] Bacteria that show antagonistic properties and are under widespread study, such as the genus *Bacillus,* a species that includes various strains with biocontrol activity: *Bacillus subtilis, Bacillus amyloliquefaciens, Bacillus licheniformis, Bacillus pumilus,* among others.[14-17] These microorganisms have the capacity to form spores, these act as microparasites competing for nutrients and are able to secrete metabolites such as enzymes and lipopeptides; *Bacillus* sp. UM96 inhibits the growth of phytopathogens such as *Botrytis cinerea* through production of chitinase.[18,19] In particular, the lipopeptides produced by *Bacillus* spp., such as fengicins, surfactin and iturins (iturin A), bacilomycin D and micosubtilin, have shown their effectiveness for suppressing the growth of phytopathogens. [20-30] At the same time, the utilization of certain species of yeasts have been widely studied. [4,10,31-34] Some yeasts have been reported to be effective for controlling pathogens postharvest in citrus fruit:[35] *Debaryomyces hansenii, Cryptococcus laurentii, Meyerozyma guilliermondii* and *Meyerozyma caribbica* in mango,[36,37] *Rhodotorula mucilaginosa* y *Candida famata* in papaya,[38] *Candida magnus* y *Candida sake* in grape[39] *Rhodotorula glutinis* in apple,[40] *Rhodosporidium paludigenum* in pear,[41] among others. Yeasts inhibit phytopathogenic fungi through different action mechanisms such as the production of lytic enzymes, [37,38,42] parasitism, [36] induced resistance, [43] competition for nutrients and or space, [33,36,37,43] and the formation of microbial biofilms. [37,44,45] It is important to understand the action mechanisms of the antagonist microorganisms and their metabolites for their best use and selection of new effective antagonists. [4,10,33,46] Relevant literature reports the effective use of these biocontrol agents in agriculture during harvest and postharvest, however, the introduction of these

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agents are subject to approval by regulatory authorities such as the FDA (Food and Drug Administration); to this end it is necessary to evaluate the toxic effects and thereby ensure the safety of such products. [47] Since the 1960's, toxicity studies have developed as a requisite for all new products that reach the market, said studies are conducted in animals following well established protocols laid out by the Organization for Economic Cooperation and Development (OECD). In Mexico the biocontrol agents liberation and introduction is regulated by the NOM-070-FITO-1995.[48] As studies and research on yeast and bacterial biocontrol agents have moved forward, there have also been reports, although scarce, about their toxicity as well as the toxicity of purified metabolites. The first research done on the toxicity of lipopeptides was conducted by Walton et al., (1949),[49] in which Micosubtilin isolated from *B. subtilis* is tolerated by rats up to concentrations below 20 mg/kg via subcutaneous injections. Korzybski et al., (1978)[50] demonstrated that Bacilomycin isolated from *B. subtilis*, caused toxic effects in rats in concentrations up to 75 mg/kg when delivered peritoneally. In more recent studies, it was shown that the lipopeptides from *B. subtilis* delivered to rats orally caused no toxicity. [51,52] Furthermore, studies on the toxicity of *Bacillus* extract—which contained lipopeptides—exist, and report dosis of up to 475 mg/kg not being toxic in rats. [53] Additionally, purified surfactin C delivered to rats orally, did not generate toxic effects, using up to 2500 mg/kg for 14 days[52] and in another study, a daily 500 mg/kg dosis of purified surfactin C did not cause genotoxicity nor teratogenic effects in fetuses.[54] In the particular case of purified Iturin from *Bacillus amyloliquefaciens* IUB158-03 extracts, Kim et al. (2009)[52] reported doses of up to 5000 mg/kg in rats causing no toxic effects. Additionally, Rodríguez et al., (2003)[55] assessed the dermal irritation of a commercial product that contains live bacteria from *Bacillus sphaericus* in rabbits, and found no irritation. Also, in another study with *B. thuringiensis,* Mancebo et al., (2011)[56] showed the absence of toxicity and pathogenicity measured in rats when delivered orally, intranasally and intravenously, no deaths or signs of toxicity were found. The ecotoxicity of those lipopeptides has also been studied, in this way, Daravel et al. (2014)[57] showed that pure lipopeptide compounds such as micosubtilin and surfactin in relatively high concentrations of 125 mg/L and 25 mg/L respectively, did not affect the development of lettuce plants. Even when there are no complete studies evaluating the toxicity of subproducts such as lipopeptides, it is necessary that every compound and biocontrol strain that is purporting to be used on fields or postharvest on farm products, count with formal toxicological studies that prove their safety in application and consumption. There are also no studies on the toxicity of hydrolytic enzymes produced by biocontrol yeasts. The objective of this study, therefore, is to demonstrate the low or non-existing toxicity of the extracts and cellular suspensions of five yeast cultures *Meyerozyma guilliermondii* L6D, *Meyerozyma caribbica* L6A2, *Cryptococcus laurentii* L5D*, Candida famata*, *Debaryomyces hansenii* and the bacterium *Bacillus subtilis* PLA10, through three different toxicity studies in different animal models, such as acute oral toxicity in murine, chicken embryo model and dermal irritation test in rabbits, in order to be in agreement with the NOM-070-FITO-1995.[48]

2. Materials and methods

2.1 Microorganisms, media and culture conditions

The yeasts used in this study, *M. guilliermondii* L6D*, M. caribbica* L6A2,[36] *C. laurentii* L5D,[37] *C. famata*,[38] *D. hansenii*[35,58] and the bacterium *B. subtilis*,[28] phytopathogenic fungi *C. gloeosporioides* and *P. digitatum* were isolated from the surface of the Ataulfo mango and Persian lime respectively. All of the previously mentioned strains form part of the strain repository of the Instituto Tecnológico de Tepic and are preserved in 40% glycerol at -80 ºC. The *Staphylococcus aureus* isolated from infection in the urinary tract was provided by the strain repository of the Universidad de las Américas Puebla and strains of *Vibrio cholerae* was donated by the Microbiology laboratory of the Benemérita Universidad Autónoma de Puebla and were used as positive controls for pathogenicity. Both strains were preserved in a nutrient broth with 20% glycerol at -80 °C. For their certification, *S. aureus* was grown in Mannitol Salt agar (Bioxon) and the strain of *V. cholerae* was grown in agar TCBS (Bioxon) at 35 ºC. Additionally, the biochemical tests miniAPI ID32 STAPH 32500 and ID32 GN 32100 (BioMérieux®) were used for their identification. For the growth of the yeasts, a potato dextrose broth PDB (Bioxon) was used, inoculating every cryopreserved strain separately, they were incubated at 28 °C and 120 rpm for 24 h. In the particular case of *D. hansenii*, a YPD broth was used with incubation at 25 °C and 80 rpm for 24 h. For *B. subtilis* Pla10 a Luria Bertani broth was used with incubation at 30 °C and 120 rpm for 48 h. For the growth of the pathogenic strains *S. aureus* y *V. cholerae*, a Nutrient broth was used with incubation at 35 °C and 150 rpm por 12h to be in the log phase. All of the cultures were adjusted to $3x10^8$ and $6x10^8$ cel/mL, using the McFarland turbidity standards and a spectrophotometer (Spectronic 20, Bausch & Lomb) at 600nm corresponding to a DO of 0.145 and 0.275 respectively. These concentrations were assessed in the acute oral toxicity test, DRAIZE skin irritation test (cited in the Official Mexican Standard NOM-039-SSA-a-1993[59]) and chicken embryo lethality test.[60]

2.1.1 Preparation of hydrolytic yeast enzyme extracts and lipopeptides from *Bacillus subtilis* Pla10.

For stimulation of hydrolytic yeast enzyme secretion, the method described by Bautista-Rosales *al.*[57] was used*. First, C. gloeosporioides* and *P. digitatum* were cultivated separately in 50 mL of PDB. Each culture was maintained under stirred conditions (110 rpm) at 25 °C for 72 h until the appearance of mycelium. Each strain culture was sterilized at 121 °C for 20 min and then centrifuged at 1400 ×g for 10 min. Afterwards, each one of the yeasts *M. guilliermondii* L6D*, M. caribbica* L6A2*, C. laurentii* L5D*,* and *C. famata* were cultivated during 72 h at 28 °C (500 mL, PBD) in presence of 10% V/V sterile mycelium of *C. gloeosporioides*. Only *D. hansenii* grew with sterile mycelium presence of *P. digitatum.* The cultures were centrifuged at 1400 xg for 10 min and filtered through a nitrocellulose membrane with 0.20 µm pores. These extracts presented β-1, 3-glucanase, N-acetyl- β-D-glucosaminidase (Nagase) and chitinase activities.[35,36,37,38] The supernatant fluid was precipitated with ethanol (10:1 V/V) for 3 h at -20 °C and was centrifuged (5 min, 11,400×g at 4 °C). The precipitate was recovered and lyophilized. In the case of *B. subtilis*, an inoculum of 20 µl from a recent culture of 250 mL of Luria Bertani broth, was incubated in a water bath at 30 °C and stirred at 120 rpm during 18 to 22 h. The culture was centrifuged at 10,000×g for 10 min. The supernatant fluid was filtered in a 0,20 µm nitrocellulose membrane. The clarified supernatant was extracted two times with Butan-1-ol (1/10 of the broth's volume). The layer of Butan-1-ol was removed and frozen at -70 °C for its subsequent lyophilization. The butanolic fraction contained the lipopeptides iturin and surfactin.[28] All lyophilized samples were re-suspended in a PBS buffer solution (pH 7.0, 1.0 M) at a concentration of 1 mg/L and 10mg/mL for their subsequent use in the animal and chicken embryo models.

2.2 Animals

In the acute oral toxicity test, 100 *Wistar Hannover* female rats were used, with a body weight between 150 and 200 g, and 7-8 weeks old. The animals were maintained in temperature controlled rooms at 22 ± 2 ºC and dark-light cycles of 12:12 h, housing four animals per cage in polypropylene compartments. The animals' feeding consisted of a standard CMO-1000 diet for rats and water *ad libitum*. For the skin irritability test, 24 male New Zealand rabbits were used, their weights ranged between 2 and 2.5 kg and were placed in stainless steel cages. 24 h prior to experimentation, an area of the skin approximately 10 x 10 cm in size was shaven on the animals' backs. 100 SPF (Specific Pathogenic Free) chicken embryos at 11 days of embryogenesis were used for the study with chicken embryos. Applicable standards and procedures for the handling of animals established by the vivarium as well as those established internationally were taken into account. [59,61,62,63] All of the studied animals were issue from the Claude Bernard vivarium of Benemérita Universidad Autónoma de Puebla.

2.3 Acute oral toxicity test

The acute oral toxicity test was conducted according to the methods in OECD Test Guideline 401. 96 rats were used (4 rats per extract and 4 for each cell culture). The animals were submitted to an acclimatization period of 3 days. On the start day of the test, the experimental groups were each formed with 4 animals. The positive control group was delivered *V. cholerae* and the negative control group was given sterile PBS. Each of the animals in the experimental groups were administered two concentrations (1 and 10 mg/mL) of lyophilized extracts and two 3×10^8 and 6×10^8 cel/mL cellular suspensions. The body weight of each animal was registered twice a week beginning on the first day of the study for a period of 14 days. During this period, food and water consumption were also registered. The daily observations of the animals included: changes in the skin and hair, eyes, mucous membranes, occurrence of secretions and autonomic activity such as tearing, poli-erection, pupil size, respiratory pattern, changes in gait, reaction to handling as well as the presence of clonic or tonic movements, stereotypes (e.g. excessive grooming, repetitive circling) or bizarre behavior (e.g. self-mutilation, walking backwards). At the end of the study, the average gain in weight in grams per day as well as the average of solids and liquids consumed per day was obtained in grams or milliliters. At the end of this study, one animal per group was selected and a macroscopic necropsy was performed, it included exams of the outer surface of the body, all holes and cranial, thoracic and abdominal cavities. It also assessed the morphological characteristics of the heart, kidney, spleen and liver.

2.4 Draize skin irritation test

The Draize skin test (described in 1944)[64] cited in the Official Mexican Standard: NOM-039-SSA1-1993[58] was used. The extracts and cell cultures were delivered on the first day of the study. For each concentration, two rabbits were used applying a single solution directly on the back with the help of a sterile cotton swab. After application, a patch of surgical gauze with 4 layers and an elastic bandage was fixated thereon in order to prevent the animal from accessing the site were the test substances were applied. The total observation period lasted 72 h and special attention was given to signs of edema and erythema at 4, 24, 48 and 72 h after removing the patches (using the value scale for skin lesions as described by Draize). The erythema values were averaged (Table 1) and added to the edema values (Equation 1) to calculate the Primary Dermal Irritation Index (PDMII).

$$\text{Primary dermal irritation index} = \bar{x} \text{ of erythema} + \bar{x} \text{ of edema} \quad \text{Eq. 1}$$

Table 1. Classification of dermal responses (Draize Scale NOM-039-SSA1-1993)

Formation of erythema and flaking	Grade
No erythema	0
Slight erythema (barely perceivable)	1
Defined erythema	2
Moderate to severe erythema	3
Grave erythema (redness) and formation of pressure sores (minor and deep lesions)	4
Formation of edema	**Grade**
No edema	0
Slight edema (barely perceivable)	1
Minor edema (presence of borders with defined concrete elevations)	2
Moderate edema (affecting an area of 1 mm)	3
Severe edema (affecting an area greater than 1mm)	4

Primary irritation index: 0.0-1Non-irritant; 1.1-2 Slightly irritant; 2.1-5 Moderately irritant; 5.1-6 Severe moderated irritant; 6.1-8 severe irritant

Based on this index for primary dermal irritation, values between 0 to 5 are considered to be within the acceptance criteria for the safe use of these extracts and cells on humans. When values range from 6 to 8, the product cannot be utilized on human skin due to it being considered as an irritant.

2.5 Viability tests in chicken embryos

The preferred inoculation route for this study is the charioallantoic membrane (MCA) utilized according to the technique described by Hitchner (1970),[65] with embryos at 11 days of embryogenesis; for the inoculation, an artificial air chamber was formed, to achieve this, two holes were made; one on the thickest pole of the egg that possesses a natural air chamber and the other on one of the sides of the egg contrary to the embryo position as observed through a candling lamp. A syringe and needle was placed at the larger pole and a bit of air was extracted favoring the formation of the artificial chamber through separation of the MCA from the shell on the inner side. It is in this artificial chamber where the extracts or cells of each test strain are inoculated using an insulin syringe and a candling lamp as a guide so as to not perforate the MCA. Any embryo whose membrane was perforated was discarded. After completing the inoculation, the holes were covered with sterile tape. Afterwards, the embryos were incubated at 35 °C and their viability examined until completing 19 days of embryogenesis using a candling lamp at all times to observe and assure that the movement of the embryo and blood stream were in good condition. A dead embryo loses the circulatory function and stops moving. For this experiment, four embryos were given doses of lyophilized extracts as well as the cultures of the strains in question, using a total of 96 embryos. An additional four embryos were used as a positive control and four were used for the negative control.

All experimentation, transportation and care of the animals were performed in compliance with the relevant laws and institutional guidelines according the Mexican norms NOM-062-ZOO-1999 (Policy for specific techniques for production, care and uses of laboratory animals),[66] NOM-003-ZOO-1994 (Policy criteria for laboratory operation for animal test approved in zoo sanitary

matter),[67] NOM-051-ZOO-1995 (Policy for humanity in animal treatment and movement),[68] NOM-033-ZOO-1995 (Policy for humanity animal treatment for domestic and wild animals sacrifice),[69] NOM-087-ECOL-1995 (Requirements for all processes related with collection, storage and final animal and all biologicals generated during animal and other biological samples).[70] All these norms are a requirement and establishment in the Bioterio Claude Bernard vivarium of Benemérita Universidad Autónoma de Puebla. At the same time this place has its own Bioethical committee who analyze each research protocol before any animal can be used.

3. Results and Discussion

3.1 Acute oral toxicity tests

Acute oral toxicity tests imply the determination of a dosage that causes the death of 50% of the treated animals. In this type of test, if the substance being tested is toxic, the death of animals after testing can span from 14 days to several months depending on the case. If only toxic effects appear, these will show in the general characteristics of the animal: like a decrease in food and water consumption and therefore weight reduction. The animal's behavior can also be affected, as well as its hair, mucous membranes, eyes, etc.[62] Many studies have been conducted on the correlation between animal and human toxicity, coming to the conclusion that no animal responds to toxins exactly the same way as a human, however, most of the effects that various lab animals experiment with different substances manifest themselves in humans and vice versa. Therefore, it is necessary to evaluate different models to avoid false negatives.[71]

3.1.1 Body weight in Wistar rats

The animals (rats) that received the extracts or the live cells orally showed no decrease in body weight during the study, in fact, they were able to gain in weight which oscillated between 1.72 ± 0.07 to 1.94±0.13 g/day and was similar to the control group that was only delivered a PBS buffer solution. This is in contrast to the control groups that received the *V. cholerae* strain, where the rats died the third day (Table 2).

3.1.2 Ingestion of food and liquids in Wistar rats

The ingestion of food and liquids during the test phase, in which each of the experimental groups had similar values to the subjects in the control group can be observed in Table 3. The inoculated rats with *V. cholerae* stopped consuming food and water and died on the third day after the experiment was started. The other animals had apparently normal behavior after 14 days, demonstrating the safety of not only the metabolites produced by the studied strains, but also the direct inoculation of the live cells of each test strain. The results in weight gain and food and liquid ingestion, indicate that the metabolites of each of the studied strains, extracts or the cells themselves, did not contain any substances with any activity that would diminish the absorption of nutrients or block the digestion of liquids and/or food in these animals. These metabolites or their cells also didn't show any inflammatory or enterotoxigenic effect that could have induced a gastroenteritis such as diarrhea. Even in the macroscopic analysis of the autopsies, no apparent change could be observed by plain sight. The possibility of an enterotoxigenic substance exists, however, its concentration is either so low that no evident effect is present or that these metabolites, if they have any activity in the gastrointestinal tract, could be sensitive to the stomach's pH level and therefore become inactive when passing through this section of the animal; this last hypothesis is left for future investigation. With regards to the live cells of each strain, three possibilities exist: a) that they are inactivated by the stomach's pH and do not reach the intestines in a viable manner, in which they have the opportunity to adhere to the intestinal epithelium and produce enterotoxigenic substances; b) that these strains do not contains adhesines that favor adhesion and colonization and therefore cannot affect the normal functions of the gastrointestinal tract or, c) even though they can survive the acidic pH of the stomach, adhere to and colonize the rats' gastrointestinal tract, these are completely safe and their presence does not negatively affect these animals. This stands in contrast to *V. cholerae*, that adheres to the intestinal epithelium upon entry, colonizes it and favors the production of choleric toxins that, as is well known, foment the hypersecretion of liquids and electrolytes, in addition to blocking nutrient absorption which ultimately cause the animal's death. [72,73]

3.1.3 Evaluation of the signs and symptoms of toxicity

The assessment of the signs and symptoms of toxicity as well as the general level of activity and reflexes showed normal parameters, at no stage did the administering of the studied substances generate anatomic-structural changes in the experimentation animals or the general level of activity and/or reflexes in the animals of each group. Similar characteristics were found with the control group that was only giving the sterile PBS solution. In summary, no toxic

Table 2. Average values and standard deviation in weight gain expressed in grams/day in the Wistar rat groups treated with lyophilized extracts and live cells of biocontrol agents

Strain/ Treatment	*C. laurentii* L5D g/day^{X} (SE*)	*M. guilliermondii* L6D g/day (SE)	*M. caribbica* L6A2 g/day (SE)	*C. famata* g/day (SE)	D. *hansenii* g/day (SE)	*B. subtilis* PLA10 g/day (SE)
1 mg	1.79±0.08	1.79±0.07	1.84±0.13	1.74±0.03	1.84±0.13	1.94±0.13
10 mg	1.79±0.14	1.81±0.17	1.81±0.17	1.91±0.39	1.72±0.07	1.88±0.17
3x10^{8} cel/ml	1.86±0.11	1.74±0.03	1.77±0.06	1.79±0.06	1.80±0.15	1.88±0.15
6x10^{8} cel/ml	1.88±0.15	1.74±0.03	1.72±0.07	1.80±0.18	1.91±0.39	1.87±0.17
Control (+): Died on the third day						
Control (-): X=1.77; DE: 0.07						

Control (+): *V. cholerae*; Control (-): PBS sterile X: Arithmetic Average; SE: Standard Deviation

Table 3. Ingestion of foods and liquids in Wistar rats.

Strain/ Treatment		*C. laurentii* L5D	*M. guilliermondii* L6D	*M. caribbica* L6A2	*C. famata*	D. *hansenii*	*B. subtilis* PLA10
1 mg	SI g/day	54	48	60	57	55	60
	LI mL/day	60	50	70	50	50	65
10 mg	SI g/day	61	54	67	57	66	58
	LI mL/day	65	55	60	60	50	50
3x10^8 cel/ml	SI g/day	59	58	63	54	50	60
	LI mL/day	60	55	50	55	50	50
6x10^8 cel/ml	SI g/day	63	53	55	57	66	68
	LI mL/day	65	55	60	60	50	50
Control (+): Died on the third day Control (-): IS:62 g/day; IL: 55 ml/day							

IS: Solid intake; IL: Liquid intake; Control (+): *V. cholerae*; Control (-): Solution de PBS sterile

symptoms were observed in the experimentation groups with the delivered dosages, neither with the extracts nor the cells of the cell cultures of the strains in question. These results indicate that the live cells as well as their metabolites had no negative effects, whether over the nervous system or over organs and tissue that could affect the animals' normal behavior.

3.1.4 Mortality in Wistar Rats

The main parameter measured in the acute oral toxicity studies is death. During the test study, and with the administering of lyophilized extracts in concentrations of 1 and 10 mg as well as live cells of the cultures of each one of the studied strains with concentrations of 3x10^8 y 6x10^8 cel/mL, death did not occur in the treated animals; contrary to what happened with the positive control group that was inoculated with *V. cholerae*, where these died on the third day. Furthermore, these animals stopped consuming food and drink after 8 h of having delivered the bacterial control. In the macroscopic autopsies, no evidence of pathological alterations in the organs analyzed could be found in each of the groups treated with lyophilized extract or live cells of the studied strains, showing similar characteristics to the negative control group. Therefore, the need to carry out histopathological studies was not discarded. Reports about the toxicity of biocontrol agents and of the metabolites that exercise a biocontrol function, such as hydrolytic enzymes, are scarce. Nonetheless, hydrolytic enzymes, produced by biocontrol yeasts have the capacity to hydrolyze essential components of the cellular wall of fungi and bacteria, β-1, 3-glucanase hydrolyzes β-1-3-glucan, chitinase hydrolyzes chitin and nagase hydrolyzes N – acetyl – β – D – glucosamine,[36] considering that these substrates are not from cells or human tissue, it is not possible that these cause lysis or structural damage. Fuentes-Silva (2006)[74] reports that enzymes such as plant β-1-3-glucanase, have manifested the capacity to induce allergic reaction in humans, in comparison with this study, no symptoms of gastric allergies in the animals related to the delivered dosages were registered. In regard to reports of lipopeptide toxicity produced by *Bacillus*, similar experiments have shown a close relationship with the results of our work. Sahnoun et al., (2014)[53] assessed the toxicity of crude lipopeptide extracts produced by *Bacillus subtilis* SPB1 through determination of LD50 in rats, no abnormal behavior, nor toxic symptoms in any of the treated animals was observed, clearly demonstrating that the extracts are not toxic. Ayed et al., (2015)[75] assessed the acute oral and subchronic toxicity in rats of a mix of lipopeptides produced by *Bacillus majovensis* A21. The results showed that the delivery of 400 mg/kg, did not cause any bodily changes in the rats as well as no toxic alterations. This represents a great advantage, since the lipopeptides from the strain B. subtilis PLA10 would be applied in the form of extracts, not purified, in such a way, that even when in this study pure compounds were assessed, the purified extracts contain smaller quantities then the amount that they reported, thus making the extracts safer. There are cases in which the extracts are less toxic then the pure compounds, as is the case with acetogenins of *Annona muricata*, where the anticancer compound is more potent, but also more toxic, supporting the safe use of the complete extract from this plant.[76]

3.2 Viability in chicken embryos

Chicken embryos are a biological model utilized in a wide range of toxicological studies due to their easy use, rapid development, early embryogenesis and sensitivity to substances and pathogenic microorganisms so that the virulence factors of a microorganism are directly reflected in the development of embriogenesis.[77] These grow under controlled conditions and small variations of these result in abnormal development or even death; that characteristic makes them highly appreciated in toxicity studies since any change in their environment offers immediate answers of the toxic effects of the substances under study.[78] For our experiments, the lyophilized extracts in concentrations of 1 and 10 mg as well as the live cells of the cultures of each one of the studied strains in concentrations of 3x10^8 y 6x10^8 cel/mL, inoculated in the chicken embryos, did not cause interruption of embryogenesis; these stayed alive similar to those embryos inoculated with a sterile PBS solution for 19 days, even with a dosage of 6 x10^8 cel/mL. The embryos inoculated with *Staphylococcus aureus* a 3x10^8 cel/mL did not survive more than 36 h after being inoculated. In this manner, it can be affirmed that the strains analyzed in this study lacked the capacity to invade tissue and therefore could not cause damage in embryo development.

3.3 Skin irritation tests in rabbits with extracts and live cells of biocontrol agents

Table 4. Dermal irritation index of lyophilized extracts of biocontrol agents in rabbit skin according to the Draize Scale

Strain	Evaluation (H)	IID*	Classification
C. laurentii L5D	4, 24, 48, y 72	0	No irritante
M. guilliermondii L6D	4, 24, 48, y 72	0	No irritante
M. caribbica L6A2	4, 24, 48, y 72	0	No irritante
C. famata	4, 24, 48, y 72	0	No irritante
D. *hansenii*	4, 24, 48, y 72	0	No irritante
B. subtilis PLA10	4, 24, 48, y 72	0	No irritante
Control (+)	4, 24, 48, y 72	6	Irritante
Control (-)	4, 24, 48, y 72	0	No irritante

Control (+): *S. aureus*; Control (-): Solution sterile isotonic saline; Dose: (10mg).

Table 5. Primary dermal irritation index of live cells of biocontrol agents in rabbit skin according to the Draize Scale

Strain	Evaluation (H)	IID*	Classification
C. laurentii L5D	4, 24, 48, y 72	0	No irritante
M. guilliermondii L6D	4, 24, 48, y 72	0	No irritante
M. caribbica L6A2	4, 24, 48, y 72	0	No irritante
C. famata	4, 24, 48, y 72	0	No irritante
D. *hansenii*	4, 24, 48, y 72	0	No irritante
B. subtilis PLA10	4, 24, 48, y 72	0	No irritante
Control (+)	4, 24, 48, y 72	3	Irritante
Control (-)	4, 24, 48, y 72	0	No irritante

Control (+): *S. aureus*; Control (-): Solution sterile isotonic saline; Dose: $6x10^8$ cel/mL

In the case of studies determining skin irritation and skin corrosion, the traditional test method is the Draize test, (1944),[69] which consists of qualitative values of the degree of inflammation or redness caused on the animal's skin after applying a chemical or biosynthetic product. Skin irritation tests in rabbits are currently readily utilized due to them being models in which the skin irritation of pharmaceuticals—that can be used on sensitive skin such as that of babies—is tested. Cosmetics that are applied to sensitive tissues of the face and that could produce some irritation of the skin or dermatitis or even hypersensitivity (allergies) are also assessed. For our experiments, the lyophilized extracts in concentrations of 1 and 10 mg, as well as the live cell cultures of each of the studied strains with a concentration of $3x10^8$ and $6x10^8$ cel/mL did not generate changes in any of the assessed times of the rabbit skin, measured during 72 h (Table 4) and (Table 5). In general, the areas of the skin maintained their normal base condition and showed no change. The measurements of skin irritation in skin of experimental rabbits for each of the assessed concentrations had a value of zero, also, the value for the Primary Skin Irritation Index was also zero. Therefore, it is considered that the extracts and cells of the biocontrol agents subject to study in this investigation cause no irritation in skin. These results were compared with those of the positive control where the lowest concentration was used (*S. aureus* $3x10^8$ cel/mL). This strain showed irritation 24 h after its application and the substance according to the Draize scale was considered to be an irritant after obtaining a value of 6 on the Primary Irritation Index. In figure 1, the positive effect of *S. aureus*, the negative control (PBS) and the effect of an extract and a strain of *C. laurenti*, that is similar to the effects observed for the other extracts and tested strains, is shown. Due to the dramatic increase in the number of people affected by this pathology, the phenomenon of skin allergies has gained importance globally. Genetic predisposition, environmental conditions, and the frequency of exposure to allergens are the main characteristics observed that appear to participate in an important way to sensitization. In the case of allergies caused by hydrolytic enzymes, a few were reported to be produced by plants, a reduced group being responsible for triggering type I immediate hypersensitive reactions. A representative of this class of allergens is β-1,3-glucanase of *H. brasiliensis* of plant origin, related to the latex allergy.[79] Even when scant information about the allergenic properties of hydrolytic enzymes produced by plants is available, there are no reports of studies that relate microbial enzymes with allergies in humans. On the other hand, among the few studies on lipopeptide skin irritation, there is one conducted by Hwang et al. (2005),[80] where the dermal skin irritation index in rabbits of surfactin produced by *Bacillus subtillis* complex BC2121 was studied. This studied placed surfactin on the Draize scale as a non-irritant with a scale value of 0.125. In our study, the lyophilized extracts used that contained lipopeptides such as surfactin and

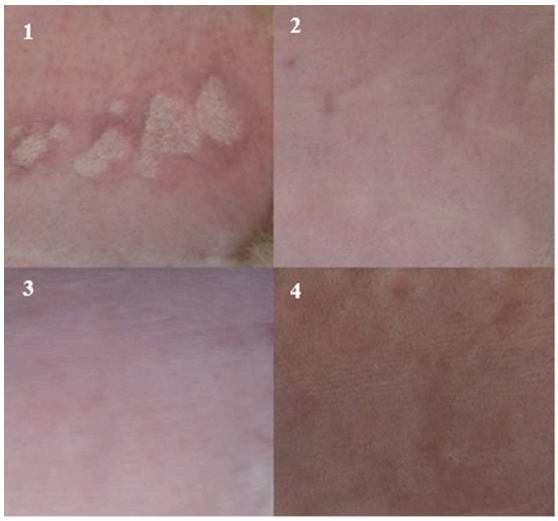

Fig. 1 Analysis of the irritation index in rabbit skin: 1. Positive control: *S. aureus* (irritant). 2. Negative control: Sterile PBS Solution (non-irritant). *3.* Lyophilized extract of *C. laurenti* L5D (10mg/ mL: Non-irritant). 4. Culture of *C. laurenti* ($6x10^8$ cel/mL: Non- irritant).

Iturin[28] derived from *B. subtilis* PLA10 did not cause inflammatory or erythemateous reactions in the skin of treated rabbits, therefore the dermal irritation index acquired a value of zero, placing it according to the Draize scale as a non-irritatant substance. It is rare for individuals of the general population that are exposed to these substances to become sensitized. In fact, within the general population, the prevalence of sensitization to fungal enzymes, for example, has been reported to be as low as 1% and as high as 15%.[81]

4. Conclusions

According to the results found in this study, we can conclude that the different delivered doses of lyophilized extracts (1 and 10 mg/mL) and live cells of *M. guilliermondii* L6D*, M. caribbica* L6A2*, C. laurenti* L5D*, C. famata, D.* hansenii, and *B. subtilis*, did not show any toxicity after the delivery of high doses of more than 600 million cells. This number is more than a 100 times higher than the amount of cells that a fruit treated with these microorganisms could have or 100 times more than the metabolites that could be applied to them for phytopathogenic biocontrol. This is supported by the lack of deaths in both models (rat and chicken embryo), toxic symptoms, or any type of change in the rest of the studied parameters, such as body weight, microscopic analyses of organs and tissues in the studied rats. These same products in the studied concentrations had no effect on rabbit skin. These type of products, due to their low toxicity and high biodegradability in the environment, low toxicity to plants and animals, are considered green products that will reduce the use of synthetic chemical products, providing a more sustainable form of agriculture and with less toxic effects to humans and the environment.

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References

1. Maya-Ambía, K. Sakamoto and L. A. R. Camacho, Diversificación de los mercados frutícolas externos de México ante los desafíos de la globalización: el caso de las exportaciones de mango a Japón, México y la Cuenca del Pacífico, 2011, **14**(42): 67-96.
2. (SAGARPA, 2015)
3. C. A. Nunes, Biological control of postharvest diseases of fruit. Eur J Plant Pathol, 2012, **133**(1), 181-196.
4. R. R. Sharma, D Singh, and R. Singh, Biological control of postharvest diseases of fruits and vegetables by microbial antagonists: A review, Biol Control, 2009, **50**(3), 205-221.
5. J. E. Kester, Endocrine-Disrupting, Clinical Environmental Health and Toxic Exposures, 2001, 362.
6. D. M. Soderlund, J. M. Clark, L. P. Sheets, L. S. Mullin, V. J. Piccirillo, D. Sargent, and M. L,Weiner, Mechanisms of pyrethroid neurotoxicity: implications for cumulative risk assessment, Toxicology, 2002,**171**(1), 3-59.
7. A. Ferrer, Pesticide poisoning. Annales Sis, San Navarra, 2003, **26**: 155-171.
8. B. Weiss, S. Amler, and R. W. Amler, Pesticides Pediatrics, 2004, **113**: 1030–1036.
9. M. Watts and S. Williamson. Replacing Chemicals with Biology: Phasing out highly hazardous pesticides with agroecology. Copyright © Pesticide Action Network Asia and the Pacific, 2015. All rights reserved ISBN 978-983-9381-70-2
10. S. Droby, M. Wisniewski, D. Macarisin, and C. Wilson, Twenty years of postharvest biocontrol research: is it time for a new paradigm? Postharvest Biol Tec, 2009, **52**(2), 137-145.
11. M. Jamalizadeh, H. R. Etebarian, H. Aminian, and A. Alizadeh, A review of mechanisms of action of biological control organisms against post-harvest fruit spoilage, EPPO Bulletin, 2011, **41**(1), 65-71.
12. V. Y. Zhimo, D. D. Bhutia, J. Saha, and B. Panja, Exploitation of Yeasts as an Alternative Strategy to Control Post Harvest Diseases of Fruits-A Review. World Appl Sci J, 2014, **31**(5), 785-793.
13. F.Liu, R. L. Zhan, Y. B.He, Y.L. Zhao,S.J. Yang and J. M. Chang,Mechanism of biological control to anthracnoses in mango by antimicrobial cinnamaldehyde. Guoshu Xuebao, Journal of Fruit Science, 2011, ***28***(4), 651-656.
14. B. J. Jacobsen, N. K Zidack, and B. J Larson, The role of Bacillus-based biological control agents in integrated pest management systems: plant diseases. Phytopathology, 2004, **94**, 1272–1275

15. M. Ongena, and P. Jacques, Bacillus lipopeptides: versatile weapons for plant disease biocontrol, Trends Microbiol, 2007, **16**, 115–125.
16. C. H. Jiang, F.Wu, Z. Y. Yu, P. Xie, H. J. Ke, H. W Li, and J. H Guo, Study on screening and antagonistic mechanisms of *Bacillus amyloliquefaciens* 54 against bacterial fruit blotch (BFB) caused by Acidovorax avenae subsp. citrulli. Microbiol Res**,** 2015, **170**, 95-104.
17. C. Pane, and M. Zaccardelli, Evaluation of Bacillus strains isolated from solanaceous phylloplane for biocontrol of Alternaria early blight of tomato, Biol Control, 2015, **84**, 11-18.
18. S. C. Martinez-Absalon, M. D. C. Orozco-Mosqueda, M. M. Martínez-Pacheco, R. Farias-Rodriguez, M. Govindappa and G. Santoyo, Isolation and molecular characterization of a novel strain of Bacillus with antifungal activity from the sorghum rhizosphere, Genet Mol Res, 2012, 1-9.
19. S. Martínez-Absalón, D. Rojas-Solís, R. Hernández-León, C. Prieto-Barajas, M. D. C. Orozco-Mosqueda, J. J. Peña-Cabriales and G. Santoyo, Potential use and mode of action of the new strain *Bacillus thuringiensis* UM96 for the biological control of the grey mould phytopathogen Botrytis cinerea, Biocontrol Sci Techn, 2014, **24**(12), 1349-1362
20. L. Volpon, F. Besson, and J. M Lancelin, NMR structure of active and inactive forms of the sterol-dependent antifungal antibiotic bacillomycin L, Eur J Biochem, 1999, **264**(1), 200-210.
21. R. S. Bongers, J. W. Veening, M. Van Wieringen, O. P., Kuipers, and M. Kleerebezem, Development and characterization of a subtilin-regulated expression system in *Bacillus subtilis*: strict control of gene expression by addition of subtilin, Appl Environ Microb, 2005,**71**(12), 8818-8824
22. X.H. Chen, A. Koumoutsi, R. Scholz, K. Schneider, J. Vater, R. Süssmuth, J. Piel, R.J. Borriss, Genome analysis of *Bacillus amyloliquefaciens* FZB42 reveals its potential for biocontrol of plant pathogens. Biotechnol. J, 2009,**140**:27-37.
23. V. Leclère, M. Béchet, A. Adam, J. S. Guez, B. Wathelet, M. Ongena, P. Thonart, F. Gancel, M. Chollet-Imbert and P. Jacques, Mycosubtilin overproduction by *Bacillus subtilis* BBG100 enhances the organism's antagonistic and biocontrol activities, Appl Environ Microb, 2005, **71**:4577–4584.
24. M. Ongena, P. Jaques, Y. Touré, J. Destain, A. Jabrane, and P. Thonart, Involvement of Fengycin-type Lipopetides in the Multifaceted Biocontrol Potential of *Bacillus subtilis*, Appl Microbiol Biotechnol, 2005, **69**: 29-38
25. M. León, P. M. Yaryura, M. S. Montecchia, A. I. Hernández, O. S. Correa, N. L. Pucheu, N. L. Kerber and A.F. García, Antifungal activity of selected indigenous pseudomonas and bacillus from the soybean rhizosphere, Int J Microbiol 2009, **57**:20-49
26. H. Cawoy, W. Bettiol, P. Fickers, and M. Ongena, Bacillus-based biological control of plant diseases. In: Stoytcheva, M. (Ed.), Pesticides in the Modern World Pesticides Use and Management. InTech, 2011, 273–302.
27. H. J. Lee, and H. Y. Kim, Review: Lantibiotics, Class I bacteriocins from the genus Bacillus, J Microbiol Biotechn, 2011, **21**(3), 229-235.
28. J. A. Ragazzo-Sánchez, A. Robles-Cabrera, L. Lomelí-González, G. Luna-Solano, and M. Calderón-Santoyo, Selección de cepas de Bacillus spp., productoras de antiobióticos aisladas de frutos tropicales, Rev Chapingo Ser Hortic, 2011,**17**(SPE. 1), 5-11.
29. C. Pane, and M. Zaccardelli, Evaluation of Bacillus strains isolated from solanaceous phylloplane for biocontrol of Alternaria early blight of tomato, Biol Control, 2015, **84**, 11-18.
30. S. B. Khedher, H. Boukedi, O. Kilani-Feki, I Chaib, A Laarif, L. Abdelkefi-Mesrati, and S. Tounsi, Bacillus amyloliquefaciens AG1 biosurfactant: Putative receptor diversity and histopathological effects on Tuta absoluta midgut, J Invertebr Pathol, 2015, **132**, 42-47.
31. D. Spadaro, R. Vola, S. Piano, and M. L. Gullino, Mechanisms of action and efficacy of four isolates of the yeast *Metschnikowia pulcherrima* active against postharvest pathogens on apples, Postharvest Biol Technol, 2002, **24**:123–134.
32. D. Spadaro, and M. L. Gullino, State of the art and future prospects of the biological control of postharvest fruit diseases, **Int J Food Microbiol**, 2004, **91**(2), 185-194.
33. W. J. Janisiewicz, and L. Korsten, Biological control of postharvest diseases of fruits, Annu Rev Phytopathol, 2002, **40**(1), 411-441.
34. J. Liu, Y. Sui, M. Wisniewski, S Droby, and Y. Liu, Review: utilization of antagonistic yeasts to manage postharvest fungal diseases of fruit, Int J Food Microbiol, 2013, **167**(2), 153-160.
35. L. G. Hernández-Montiel, C. P. Larralde-Corona, S. Vero, M. G. López-Aburto, J. L. Ochoa, and F. Ascencio-Valle. Caracterizacion de levaduras *Debaryomyces hansenii* para el control biologico de la podredumbre azul del limon mexicano. Characterization of yeast *Debaryomyces hansenii* for the biological control of blue mold decay of Mexican lemon, CyTA J. Food, 2010, **8**(1), 49-56.
36. P. U. Bautista-Rosales, M. Calderon-Santoyo, R. Servín-Villegas, N. A. Ochoa-Álvarez, and J. A. Ragazzo-Sánchez, Action mechanisms of the yeast *Meyerozyma caribbica* for the control of the phytopathogen *Colletotrichum gloeosporioides* in mangoes, Biol Control, 2013, **65**(3), 293-301.
37. P. U. Bautista-Rosales, M. Calderón-Santoyo, R. Servín-Villegas, N. A. Ochoa-Álvarez, R. Vázquez-Juárez, and J. A. Ragazzo-Sánchez, Biocontrol action mechanisms of *Cryptococcus laurentii* on *Colletotrichum gloeosporioides* of mango, Crop Prot., 2014, **65**:194-201.

38. Magallon-Andalon, C., G., (2008), Determinación de los mecanismos de acción en el control de *Colletotrichum gloeosporioides* en papaya (*Carica papaya L.*), Maestría en Ciencias en alimentos. Instituto Tecnológico de Tepic.
39. I. S. Pantelides, O. Christou, M. D. Tsolakidou, D. Tsaltas, and N. Ioannou, Isolation, identification aned in vitro screening of grapevine yeasts for the control of black aspergilli on grapes, Biol Control, 2015, **88**, 46-53.
40. H. Zhang, L. Wang, L. Ma, Y. Dong, S. Jiang, B. Xu, and X Zheng, Biocontrol of major postharvest pathogens on apple using *Rhodotorula glutinis* and its effects on postharvest quality parameters, Bio Control, 2009, **48**(1), 79-83.
41. C.Yu, T. Zhou, K. Sheng, L. Zeng, C. Ye, T. Yu, and X, Zheng, Effect of pyrimethanil on *Cryptococcus laurentii*, *Rhodosporidium paludigenum*, and *Rhodotorula glutinis* biocontrol of *Penicillium expansum* infection in pear fruit, Int J Food Microbiol, 2013,**164**(2), 155-160.
42. H.Yehuda, S. Droby, M. Bar-Shimon, M. Wisniewski, and M. Goldway, The effect of under-and overexpressed CoEXG1-encoded exoglucanase secreted by *Candida oleophila* on the biocontrol of *Penicillium digitatum*. Yeast, 2003, **20**(9), 771-780
43. S. Droby, V. Vinokur, B. Weiss, L. Cohen, A. Daus, E. E. Goldschmidt, and R. Porat, Induction of resistance to *Penicillium digitatum* in grapefruit by the yeast biocontrol agent *Candida oleophila*, Phytopathology, 2002, **92**(4), 393-399
44. M. Chi, G. Li, Y. Liu, G. Liu, M. Li, X. Zhang, and J. Liu, Increase in antioxidant enzyme activity, stress tolerance and biocontrol efficacy of *Pichia kudriavzevii* with the transition from a yeast-like to biofilm morphology, Biol Control, 2015,**90**, 113-119.Chi et al., 2015;
45. D. Spadaro, and S. Droby, Development of biocontrol products for postharvest diseases of fruit: The importance of elucidating the mechanisms of action of yeast antagonists, *Trends Food* Sci Technol, 2016, **47**, 39-49
46. J. D'aes, K. De Maeyer, E. Pauwelyn, and M. Höfte, Biosurfactants in plant–Pseudomonas interactions and their importance to biocontrol, Environ. Microbiol. Rep., 2009, **2**: 359-372.
47. A. Skrobek, D. Boss, G. Défago, T. M. Butt and M. Maurhofer, Evaluation of different biological test systems to assess the toxicity of metabolites from fungal biocontrol agents, Toxicol Lett, 2006, **161**(1), 43-52.
48. Norma Oficial Mexicana NOM-070-FITO-1995, Requisitos y Especificaciones Fitosanitarios para la Importación, Introducción, Movilización y Liberación de Agentes de Control Biológico (http://www.economia-noms.gob.mx/noms/consultaXNormaAction.do; 17/Octubre/2016)
49. R. B. Walton and H B. Woodruff, A crystalline antifungal agent, mycosubtilin, isolated from subtilin broth, J Clin Invest, 1949, **28**(5 Pt 1), 924
50. T. Korzybski, Z. Kowszyk-Gindifer, and W. Kurylowicz. Antibiotics isolated from the genus Bacillus (Bacillaceae), 1978, 1529-1661. In: Antibiotics - Origin, Nature and Properties, Vol. III. American Society of Microbiology, Washington, DC.
51. B. K. Park, J. H. Lim, Y. H. Hwang, M. S. Kim, I. B. Song, H. G. Lee, and S. C. Park, Acute oral toxicity of surfactin C in mice, Toxicol Res, 2006, **22**(4), 453-458.
52. H. K. Kim, and T. S. Lee, Toxicity and characteristics of antifungal substances produced by Bacillus amyloliquefaciens IUB158-03, Life Sci, 2009, **19**(11), 1672-1678.
53. R. Sahnoun, I. Mnif, H. Fetoui, R. Gdoura, K. Chaabouni, F. Makni-Ayadi, and D. Ghribi, Evaluation of *Bacillus subtilis* SPB1 lipopeptide biosurfactant toxicity towards mice, Int J Pept Res Ther, 2014, **20**(3), 333-340.
54. Y. H Hwang, B. K Park, J. H.Lim, M. S Kim, I. B. Song, S. C. Park and H. I. Yun, Evaluation of genetic and developmental toxicity of surfactin C from *Bacillus subtilis* BC1212, J Health Sci, 2008, **54**(1), 101-106.
55. A. M. Rodríguez, B. G. Navarro, L. Riera, S. Lugo, Y. G. Torres, M. E. Arteaga, and D. Fuentes, Ausencia de toxicidad/patogenicidad de una formulación de *Bacillus sphaericus* 2362, J Toxicol, 2003, **20**(3), 210-215.
56. A. Mancebo, T. Molier, B. González, S. Lugo, L. Riera, M. E. Arteaga, and C. González. Acute oral, pulmonary and intravenous toxicity/pathogenicity testing of a new formulation of *Bacillus thuringiensis* var israelensis SH-14 in rats, Regul Toxicol Pharmacol, 2011, **59**(1), 184-190.
57. J. Deravel, S. Lemière, F.Coutte, F. Krier, N. Van Hese, M. Béchet, and P. Jacques, Mycosubtilin and surfactin are efficient, low ecotoxicity molecules for the biocontrol of lettuce downy mildew, App.. Microbiol. Biotechnol., 2014, **98**(14), 6255-626l.
58. R. González-Estrada, M. Calderón-Santoyo, E. Carvajal-Millan, F. D. A. J. Valle, J. A., Ragazzo-Sánchez, F. Brown-Bojorquez, and A. Rascón-Chu, Covalently Cross-Linked Arabinoxylans Films for *Debaryomyces hansenii* Entrapment, Molecules, 2015, **20**(6), 11373-11386.
59. Norma Oficial Mexicana NOM-039-SSA-1-1993, Bienes y Servicios. Productos de perfumería y belleza. Determinación de los índices de irritación ocular, primaria dérmica y sensibilización. (http://www.salud.gob.mx/unidades/cdi/nom/039ssa13.html; 10/Febrero /2016).
60. I. D. Jacobsen, K. Große, S. Slesiona, , B. Hube, A. Berndt, , & M. Brock, Embryonated eggs as an alternative infection model to investigate *Aspergillus fumigatus* virulence, Infect. Immun, 2010, **78**(7), 2995-3006.

61. Norma Oficial Mexicana NOM-062-ZOO-1999, Especificaciones Tecnicas para la Producción, Cuidado y Uso de los animals de Laboratorio. NOM-062-ZOO-1999
62. OECD (1996) Guidelines for Testing of Chemicals No 401 Acute Oral Toxicity. Principles and Methos of Toxicology. Ginebra. https://ntp.niehs.nih.gov/iccvam/docs/acutetox_docs/udpproc/udpfin01/append/appi.pdf; 10/Febrero /2016
63. OECD (1987) Guidelines for the Testing of Chemicals No 402: Acute Dermal Toxicity. Fixed Dose Procedure, Paris: Organisation for Economic Co-operation and Development. (http://www.oecd.org/chemicalsafety/risk-assessment/1948333.pdf; 10/Febrero /2016)
64. J. H. Draize, G. Woodward and H. O. Calvery, Methods to the study of the irritation and toxicity of substances applied topically to the skin and mucous membranes, J Pharmacol Exper Ther, 1944, **82**: 377-390
65. S. B. Hitchner, Infectivity of infectious bursal disease virus for embryonating eggs, Poult Sci, 1970, 49(2), 511-516.
66. Norma Oficial Mexicana NOM-062-ZOO-1999, Especificaciones técnicas para la producción, cuidado y uso de los animales de laboratorio. (http://www.economia-noms.gob.mx/noms/consultaXNormaAction.do; 17/Octubre/2016)
67. Norma Oficial Mexicana NOM-003-ZOO-1994, Criterios para la operación de laboratorios de pruebas aprobados en materia zoosanitaria. (http://www.economia-noms.gob.mx/noms/consultaXNormaAction.do; 17/Octubre/2016).
68. Norma Oficial Mexicana NOM-051-ZOO-1995, Trato humanitario en la movilización de animales. (http://www.economia-noms.gob.mx/noms/consultaXNormaAction.do; 17/Octubre/2016)
69. Norma Oficial Mexicana NOM-033-ZOO-1995, Sacrificio humanitario de los animales domésticos y silvestres. (http://www.dof.gob.mx/nota_detalle.php?codigo=5376424&fecha=18/12/2014; 17/Octubre/2016).
70. Norma Oficial Mexicana NOM-087-ECOL-1995, Requisitos para la separación, envasado, almacenamiento, recolección, transporte, tratamiento y disposición final de los residuos peligrosos biológico-infecciosos que se generan en establecimientos que presten atención médica. (http://www.dof.gob.mx/nota_detalle.php?codigo=4884397&fecha=07/11/1995; 17/Octubre/2016).
71. H. Olson, G. Betton, D. Robinson, K. Thomas, A. Monro, G. Kolaja and M. Dorato, M. Concordance of the toxicity of pharmaceuticals in humans and in animals. Regul Toxicol Pharmacol, 2000, **32**(1), 56-67.
72. E. G.Vázquez, A. H. Torres, J. H. Martínez, J. G. Gómez, Cholera and other Vibrio infections, Medicine, 2014, **11**(56), 3317-3321.
73. M. Maheshwari, K. Nelapati and B. Kiranmayi, Vibrio cholerae—a review. *Vet World*, 2011, **4**(9), 423-428.
74. D. Fuentes-Silva and A. Rodríguez-Romero, ENDO-β-1, 3-glucanasas reconocidas por anticuerpos tipo IgE de sueros de pacientes alérgicos. Alergia e Inmunol Pediatr, 2006,**15**, 35-42.
75. H. B. Ayed, R. Nasri, N. Jemil, I. B. Amor, J. Gargouri, N. Hmidet and M. Nasri, Acute and sub-chronic oral toxicity profiles of lipopeptides from *Bacillus mojavensis* A21 and evaluation of their in vitro anticoagulant activity, Chem. Biol. Interact, 2015, **236**, 1-6.
76. C. Yang, S. R.Gundala, R.Mukkavilli, S.Vangala, M. D. Reid and R Aneja, Synergistic interactions among flavonoids and acetogenins in Graviola (*Annona muricata*) leaves confer protection against prostate cáncer. Carcinogenesis, 2015, 046.
77. D. Sequera, J. M. Ercolino, and M. Álvarez, Embrión de Pollo: Un modelo con cardiovideograma para el estudio de la toxicidad experimental inducida por Thevetia peruviana, Retel, 2010, **28**, 37-48.
78. M. Giannaccini, A. Cuschieri, L. Dente, and V. Raffa, Non-mammalian vertebrate embryos as models in nanomedicine. Nanomedicine, NANOMED, 2014, **10**(4), 703-719.
79. C. A. S. R. López, and A. R. Romero, Características bioquímicas y moleculares de alergenos del látex de *Hevea brasiliensis*, Alergia, Asma e inmunología pediátrica, 2002,**11**(3) 92-100.
80. M. H. Hwang, H. I. Yun, J. H. Lim, K. S. Kim, M. H. Rhee, N. W. Kim and S. C. Park, Antibacterial activity in vitro and primary dermal irritation test in rabbits of surfactin produced *Bacillus subtilis* complex BC2121, **Toxicol Res**, 2005, **21**(1), 39-43.
81. B. J. Green, and D. H. Beezhold, Industrial fungal enzymes: an occupational allergen perspective. *J Allergy*, 2011, http://dx.doi.org/10.1155/2011/682574.

Evaluation of the toxicity and pathogenicity of biocontrol agents

1. Substance Preparation

Microorganisms

Extracts

Cell cultures

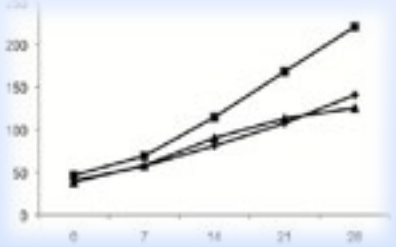
2. Substances administration

a) Acute oral toxicity test

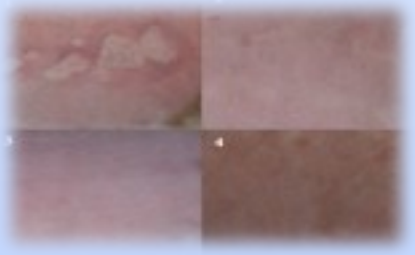
b) Draize skin irritation test

c) Chicken embryo lethality test

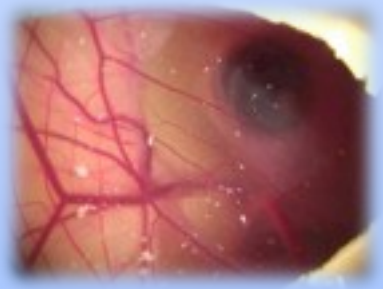
3. Toxicity evaluation



Weight gain



Irritation index



Viability