Revista da Sociedade Brasileira de Medicina Tropical 48(4):427-431, Jul-Aug, 2015
http://dx.doi.org/10.1590/0037-8682-0092-2015

Major Article

Preliminary screening of the larvicidal effect of *Brevibacillus laterosporus* strains against the blowfly *Chrysomya megacephala* (Fabricius, 1794) (Diptera: Calliphoridae)

***Isabel Nogueira Carramaschi*[1],[2], *Lorrane de Andrade Pereira*[1], *Margareth Maria de Carvalho Queiroz*[1] *and Viviane Zahner*[1]**

[1]. Laboratório de Entomologia Médica e Forense, Pavilhão Herman Lent, Fundação Oswaldo Cruz, Rio de Janeiro, Brasil. [2]. Programa de Pós Graduação *Stricto Sensu* em Biodiversidade e Saúde, Fundação Oswaldo Cruz, Rio de Janeiro, Brasil.

ABSTRACT

Introduction: This study evaluated whether different strains of *Brevibacillus laterosporus* could be used to control larvae of the blowfly *Chrysomya megacephala*, a pest that affects both human and animal health. **Methods:** Mortality rates were recorded after 1-mL suspensions of sporulated cells of 14 different strains of *B. laterosporus* were added to 2.5g of premixed diet consisting of rotting ground beef fed to first instar larvae of *C. megacephala*. All bioassays were performed using 10 larvae per strain, with a minimum of three replicates for each bioassay. Larval mortality was recorded daily up to seven days. **Results:** Strains Bon 707, IGM 16-92, and Shi 3 showed the highest toxicity toward the larvae producing 70.5%, 64.5%, and 51.6% of larval mortality, respectively, which was significantly higher than that in the control group ($p < 0.05$). In contrast, strains NRS 1642, NRS 661, NRS 590 BL 856, NRS 342, ATCC 6457, Bon 712, and NRS 1247 showed limited or no pathogenic activity against the target larvae. **Conclusions:** Our preliminary data indicated that *B. laterosporus* could be used to develop bioinsecticides against *C. megacephala*.

Keywords: Entomopathogens. First instar larvae. Biological control.

INTRODUCTION

Brevibacillus laterosporus is known to be a potential biocontrol agent against several orders of invertebrate species, including Diptera, Coleoptera, Lepidoptera, Nematodes, and mollusks[1] [2] [3]; further, various formulations of this species as a bioinsecticide have been patented[4] [5]. In addition to its established entomopathogenic properties, *B. laterosporus* has remarkable potential in the biotechnology industry[3]. For example, some strains are known to produce antibiotics such as laterosporamine[6] that act against both gram-positive and gram-negative bacteria and chitinases that have antifungal and insecticidal activities[7]. However, unlike the entomopathogenic strains of *Bacillus thuringiensis*[8], the factors responsible for the toxicity of the different strains of *B. laterosporus* have not yet been determined.

Corresponding author: MSc. Isabel Nogueira Carramaschi. Laboratório de Entomologia Médica e Forense/FIOCRUZ. Av. Brasil 4365, Manguinhos, 21040-360 Rio de Janeiro, Brasil.
Phone: 55 21 2562-1915; **Mobile**: 55 21 99120-7153
e-mail: isabel.carramaschi@ioc.fiocruz.br
Received 18 March 2015
Accepted 22 May 2015

The blow fly *Chrysomya megacephala* (Fabricius, 1794) (Diptera: Calliphoridae) is a pest that has significant impacts on human and animal health. It is native to Australian and Oriental regions; it was subsequently introduced into Africa and South America and then rapidly dispersed to the United States of America[9]. This species was first recorded in Brazil in 1976; it was detected in the garbage from a metropolitan region of São Paulo. The presence of these flies has been associated with the risk of transmission of fecal pathogens and other disease-causing agents[10]; however, at present, there are no programs to control this species in Brazil.

In addition to the vectorial capacity of adults, larvae of *C. megacephala* can cause rare but serious cases of myiasis in domestic animals and humans[11]. In Brazil, there have been reports of human myiasis caused by *C. megacephala*[12]; however, studies investigating the direct impact of large populations of this fly species on public health are lacking. Ecological studies have shown that this species is most frequently observed in open rubbish dumps in both São Paulo[13] and Rio de Janeiro, Brazil (data not published). In Thailand, where this species is considered problematic, bacterial loads carried by *C. megacephala* were found to be greater than those recorded in *Musca domestica* Linnaeus, 1758 (Diptera: Muscidae)[14], a species widely regarded as a pest of public health

significance. Furthermore, *C. megacephala* was shown to be able to carry multi-drug-resistant bacteria, including *Escherichia coli* and *Klebsiella pneumoniae*, suggesting its involvement in the potential contribution to the dissemination of antibiotic resistance genes[15,16].

Approaches for the control of populations of synanthropic Diptera species, including *C. megacephala*, included improvements in basic sanitation and application of chemical pesticides such as permethrin and deltamethrin[17], plant extracts (eucalyptol)[18], plant metabolites (neolignans)[19], microhymenopteran parasitoids[20], insect growth regulators (precocenes)[21] and a range of *Bacillus thuringiensis* strains[22]. Chemical control has been shown to be generally effective[18], but constant application might lead to the development of resistance and produce environmental contamination. Owing to the public health threat posed by *C. megacephala*, complementary control methods that can be used in urban settings need to be developed. Therefore, this study evaluated the potential toxicity of the strains of *B. laterosporus* against laboratory-reared larvae of *C. megacephala* as a preliminary step toward the development of an alternative/complementary control strategy.

METHODS

Collection of flies and colony maintenance

Adults of *C. megacephala* were collected from open dumps in the Amorim community (latitude: -22.875707, longitude: -43.250606), Rio de Janeiro, Brazil, and transported to the laboratory; they were identified using a calliphorid dichotomous key[23]. Flies were reared in net cages maintained in an acclimatized chamber at 27 ± 1°C with 70 ± 10% relative humidity (RH) and a 12-h photoperiod; they were fed a solution of 30% saccharose *ad libitum*. The first instar larvae of F1 (first) generation were placed in Petri dishes and fed 10g ground beef. The larvae were reared up to adult emergence, and the colony was maintained until the fourth generation, when the bioassays were performed.

Bacterial strains and spore suspensions

A panel of 14 strains of *B. laterosporus* was tested; these strains were selected based on their toxicity against different targets[2]. All strains were obtained from the *Coleção de Culturas do* Gênero *Bacillus e Gêneros Correlatos* (CCGB). Stock cultures were maintained at -20°C in brain heart infusion broth supplemented with glycerol (20% v/v). Working cultures were prepared on nutrient agar (NA) slants that were incubated at 30°C for 72h or until complete sporulation occurred, as assessed by light microscopy. Sporulated cultures were collected from the solid medium by scrapping the entire slope with an inoculating loop and transferred to tubes containing 9mL of autoclaved distilled water. Suspensions were heated at 80°C for 15 min to inactivate the vegetative cells and retain the spores. Serial, tenfold dilutions of spore suspensions were prepared and used to inoculate NA plates for retrospective determination of the colony forming units ($CFU \cdot mL^{-1}$) and the doses that needed to be used in the bioassays. The strategy used in this preliminary screening was to analyze the maximum number of spores to reduce the possibility of not detecting larvicidal activity of a given strain.

Feeding of larvae with experimental diet

All bioassays were conducted using 10 first instar larvae per strain; the larvae were fed 2.5g of premixed diet, consisting of rotting ground beef, along with 1mL of each bacterial suspension. Three replicates were used for each bioassay. The larval rearing diet, which was mixed with 1mL of water, served as the negative control in the bioassays. Assays were conducted in 50-mL plastic pots placed within larger 250-mL pots containing vermiculite for pupation of the larvae and were covered with a mesh; the pots were placed in an acclimatized chamber at 27 ± 1°C with RH of 70 ± 10% and photoperiod of 12h, as reported previously[19]. Pots were examined daily from on the third day of the start of the experiment, and those larvae that did not abandon the diet, or which failed to pupate, were recorded as dead. Surviving larvae were transferred to test tubes (17 × 160mm) containing vermiculite and sealed with hydrophobic cotton wool. Larval mortality was recorded daily until the end of the experiment on day 7. Since the present study aimed to conduct a preliminary screening to identify *B. laterosporus* strains that could be used for the biological control of *C. megacephala*, LC_{50} values were not calculated.

Statistical analysis

Mortality values in the treated group were corrected using the formula reported by Abbott[24] as follows: Cm (%) = (%Tm - %Com)/(100 - %Com) × 100, where Cm is the corrected mortality, Tm is the mortality in the treated group, and Com is the mortality in the control group. The corrected numbers of dead larvae were subjected to a binomial test of two independent samples by using Bioestat 5.0 program. The significance level was set at 0.05. When the value of the corrected mortality of the treated group was less than that of the control group, no statistical tests were used.

RESULTS

Although standardized growth conditions were used, the number of spores per gram of diet varied between 4.8×10^6 and 2.72×10^8. The results of the larval bioassays in decreasing order of mortality are shown in **Table 1**. The strains Bon 707, IGM 16-92, and Shi 3 showed the highest larvicidal activity (70.5%, 64.5%, and 51.6% corrected mortality, respectively), with significant differences relative to the control group ($p < 0.05$). Interestingly, the Bon 707 isolate exhibited the highest toxicity, although an intermediate concentration of spores (1.46×10^7) per gram of diet was used. The mortality caused by strains Shi2 and NRS 1648 was also significantly higher than that of the control group ($p < 0.05$), but was less than 50%. In contrast, strains NRS 1642, NRS 661, NRS 590, BL 856, NRS 342, ATCC 6457, Bon 712, and NRS 1247 showed limited or no pathogenic activity against the larvae **(Table 1)**.

TABLE 1 - Toxicity of the 14 strains of *Brevibacillus laterosporus* against the larvae of *Chrysomya megacephala* (Diptera: Calliphoridae).

Strain*	Origin of the strain	Spores/g of diet	Corrected mortality[a]	Mortality in the control group	
			%	%	P value[b]
Bon 707	Institute of Hygiene, University of Aarhus, Aarhus C, Denmark	1.46×10^7	70.5	15.0	<0.0001
IGM 16-92	Institute of Genetics and Selection of Industrial Microorganisms, Moscow	3.44×10^8	64.5	22.5	<0.0001
Shi 3	Bioenvironmental Bee Laboratory (USDA), Beltsville, MD	1.36×10^8	51.6	22.5	0.0028
NRS 1648	American Type Culture Collection	6.40×10^7	43.2	26.0	0.0296
Shi 2	Bioenvironmental Bee Laboratory (USDA), Beltsville, MD	2.00×10^7	30.1	17.0	0.0481
Shi 5	Bioenvironmental Bee Laboratory (USDA), Beltsville, MD	2.49×10^7	18.0	16.7	0.4051
NRS 1642	American Type Culture Collection	1.16×10^7	13.8	27.5	-
NRS 661	Rutgers University, New Brunswick, NJ	1.88×10^8	12.0	16.7	-
NRS 590	American Type Culture Collection	7.40×10^7	10.8	26.0	-
NRS 856	Not determined	9.20×10^7	8.6	22.2	-
NRS 342	Rutgers University, New Brunswick, NJ	4.80×10^6	2.8	22.2	-
ATCC 6457	American Type Culture Collection	1.08×10^7	0.0	26.0	-
Bon 712	Institute of Higiene, University of Aarhus, Aarhus C, Denmark*	2.72×10^8	0.0	15.0	-
NRS 1247	Not determined	4.40×10^7	0.0	8.0	-

[a]Corrected mortality determined using Abbott's formula (Abbott, 1925); [b]P value was obtained after binomial test of two independent samples by using Bioestat 5.0 program. *All the strains are deposited in CCGBC. **CCGBC:** *Coleção de Culturas do Gênero Bacillus e Gêneros correlatos.*

DISCUSSION

Chrysomya megacephala densities are known to be high in urban areas that have fairs and street markets selling fresh products[9][10][13] and open dumpsters, which could serve as their breeding grounds. This situation is very concerning in Brazil owing to poor sanitary conditions in some urban areas. Hence, developing suitable control programs for *C. megacephala* is necessary in these areas. The drastic measures implemented for the eradication of the myiasis-causing fly species *Cochliomyia hominivorax* (Coquerel, 1858) in North America are extremely costly and might have caused negative ecological impacts. Therefore, they are not considered viable or practical for implementation in Brazil[25]. Thus, studies need to focus on the development of integrated pest management strategies, including biological control methods, against this pest.

In this study, 14 different strains of *B. laterosporus* were screened for their potential to be used in the biological control of *C. megacephala*. In the bioassays, first instar larvae were used since this life stage has been shown to be highly vulnerable to potential biological agents owing to the fact that first instar larvae ingest greater amounts of treated diet over a long time[3].

As reported previously[2], the toxicity of the panel of test strains used in this study varied remarkably, indicating the heterogeneity of the pathogenic mechanisms of these strains against the same target species. Only five tested strains induced significant levels of mortality compared to that of the control group. In a previous study[2], the three strains that exhibited the greatest larvicidal properties in the present study showed negligible or medium toxicity against the larvae of *Aedes aegypti* (Linnaeus, 1762) (Diptera, Culicidae), *Culex quinquefasciatus* Say (Diptera, Culicidae), *Anticarsia gemmatalis* Hübner, 1818 (Lepidoptera: Noctuidae), and *Anthonomus grandis* Boheman,

1843 (Coleoptera: Curculionidae). Interestingly, similar findings were obtained in a study[26], in which a strain of *B. laterosporus* showed elevated toxicity toward *Musca domestica*. In particular, the bacterial strain was not toxic toward *Muscidifurax raptor*: Girault & Sanders (Hymenoptera: Pteromalidae), a natural parasitoid of *M. domestica*[26]. With respect to biocontrol, the narrow spectrum of action of the larvicidal strains identified in this study is both relevant and desirable since their application might not affect non-target fauna[27].

To our knowledge, this is the first study reporting the use of *B. laterosporus* for the biological control of *Chrysomya* spp. Although the entomopathogenic activity of *Bacillus thuringiensis* against the larvae of *Chrysomya* spp. has been investigated[22] [28], the elevated mortality levels in those studies were thought to be associated with the germination of the spores of strain LFB-FIOCRUZ 907 in the hemolymph of both *C. megacephala* and *Chrysomya putoria* larvae.

The toxicity of *B. laterosporus* strains has been speculated to be associated with the activation of spore-associated proteins, or the secretion of binary toxins produced by vegetative cells, or because of the insecticidal protein crystals contained in the parasporal bodies[3]. Strains IGM 16-92 and Shi 3 are known to produce protein crystals; however, the presence of crystals should not be considered to be the only factor linked to toxicity, since strains Bon 707, NRS 1648, and Shi 2 did not produce protein crystals but were effective against *C. megacephala* larvae. Therefore, further studies are warranted to elucidate the reasons of mortality caused by *B. laterosporus* in blowflies.

In our bioassays, an initial spore suspension was fed to the larvae; however, we did not evaluate whether the spores germinated within the diet and produced vegetative cells, or determined the possible contribution of such cells, if produced, to the observed levels of larval mortality. Further, we did not determine whether the ingested spores germinated within the larvae or if the toxicity was a result of the direct interaction between the spores and the larval gut epithelium. As discussed above, the mechanisms of toxicity of *B. laterosporus* are complex and, in some cases, multi-factorial. Such analyses would have been impractical and expensive considering that this was a preliminary study. The above-mentioned factors need to be considered in future studies performed using fewer strains with confirmed toxicity toward *C. megacephala.*

Previous studies have confirmed the insecticidal potential of *B. laterosporus* strains against insects belonging to different orders[2], including the fly species *M. domestica*[29] and *Lucilia cuprina* (Wiedemann, 1830) (Diptera: Calliphoridae)[30]. This study examined the toxicity of *B. laterosporus* against other invertebrate species. The mode of action of the protein crystals of *B. laterosporus* was shown to be comparable to that of *B. thuringiensis* strains; these crystals caused the deterioration of the larval gut epithelium[31]. Similar to other invertebrate targets, the larval mortality rates caused by *B. laterosporus* treatment were variable, but significant, depending on the specific strain of the bacterium used. In particular, this study identified the strains of *B. laterosporus* that could be used for the control of *C. megacephala*. However, further studies are warranted to evaluate whether this bacterium can be used as a component of the alternative/complementary control strategy for *C. megacephala*.

ACKNOWLEDGMENTS

We thank Dr. Douglas McIntosh for the English revision.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

FINANCIAL SUPPORT

This study was supported by grants from *Centro Integrado Empresa Escola* (CIEE), *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES), *Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro* (FAPERJ) and *Conselho Nacional de Pesquisa e Desenvolvimento Tecnológico* (CNPq) process number 485303/2013-2.

REFERENCES

1. Huang X, Tian B, Niu Q, Yang J, Zhang L, Zhang K. An extracellular protease from *Brevibacillus laterosporus* G4 without parasporal crystals can serve as a pathogenic factor in infection of nematodes. Res Microbiol 2005; 156:719-727.
2. Oliveira EJ, Rabinovitch L, Monnerat RG, Passos LK, Zahner V. Molecular characterization of *Brevibacillus laterosporus* and its potential use in biological control. Appl Environ Microbiol 2004; 70:6657-6664.
3. Ruiu L. *Brevibacillus laterosporus*, a Pathogen of Invertebrates and a Broad-Spectrum Antimicrobial Species. Insects 2013; 4:476-492.
4. Arnaut G, Boets A, Damme N, Van Rie J. Toxins. U.S. Patent 7,919,609, 5 April 2011.
5. Delrio G, Ellar DJ, Floris I, Paglietti B, Pantaleoni RA, Rubino S, et al. *Brevibacillus laterosporus* strain compositions containing the same and method for the biological control of dipters. U.S. Patent 8,076,119, 13 December 2011.
6. Shoji J, Sakazaki R, Wakisaka Y, Koizumi K, Mayama M. Isolation of a new antibiotic, laterosporamine (Studies on antibiotics from the genus *Bacillus*. XIII). J Antibiot 1976; 29:390-393.
7. Prasanna L, Eijsink VGH, Meadow R, Gaseidnes S. A novel strain of *Brevibacillus laterosporus* produces chitinases that contribute to its biocontrol potential. Appl Microbiol Biotechnol 2013; 97:1601-1611.
8. Palma L, Muñoz D, Berry C, Murillo J, Caballero P. *Bacillus thuringiensis* toxins: an overview of their biocidal activity. Toxins 2014; 6: 3296-3325.
9. Braack LE. Spread in South Africa of the Oriental latrine fly *Chrysomya megacephala* (Fabricius) (Diptera: Calliphoridae), an introduced species closely resembling *Chrysomya bezziana* Villeneuve. Onderstepoort J Vet Res 1991; 58:311-312.
10. Guimarães JH, Prado AP, Linhares AX. Three newly introduced blowfly species in southern Brazil (Diptera: Calliphoridae). Rev Bras Entomol 1978; 22:53-60.

11. Hall MJR, Wall R. Myiasis of humans and domestic animals. Adv Parasitol 1995; 35:257-334.
12. Fernandes LF, Pimenta FC, Fernandes FF. First report of human myiasis in Goiás State, Brazil: frequency of different types of myiasis, their various etiological agents, and associated factors. J Parasitol 2009; 95:32-38.
13. Dias LS, Santarém VA, Almeida MSR, Medina AO, da Silva AV. Biodiversidade de moscas *Calliphoridae* no lixão urbano de Presidente Prudente, São Paulo, Brasil. Arq Inst Biol 2009; 76:659-663.
14. Sukontason KL, Bunchoo M, Khantawa B, Piangjai S, Rongsriyam Y, Sukontason K. Comparison between *Musca domestica* and *Chrysomya megacephala* as carriers of bacteria in Northern Thailand. Southeast Asian J Trop Med Public Health 2007; 38: 38-44.
15. Liu Y, Yang Y, Zhao F, Fan X, Zhong W, Qiao D, et al. Multi-drug resistant Gram-negative enteric bacteria isolated from flies at Chengdu Airport, China. Southeast Asian J Trop Med Public Health 2013; 44:988-996.
16. Zurek L, Ghosh A. Insects represent a link between food animal farms and the urban environment for antibiotic resistance traits. Appl Environ Microbiol 2014; 80:3562-3567.
17. Sukontason K, Chaiwong T, Tayutivutikul J, Somboon P, Choochote W, Piangjai S, et al. Susceptibility of *Musca domestica* and *Chrysomya megacephala* to Permethrin and deltamethrin in Thailand. J Med Entomol 2005; 5:812-814.
18. Sukontason KL, Boonchu N, Sukontason K, Choochote W. Effects of eucalyptol on house fly (diptera: muscidae) and blow fly (diptera: calliphoridae). Rev Inst Med Trop Sao Paulo 2004; 46:97-101.
19. Cabral MMO, Mendonça PM, Barbosa-Filho JM, Queiroz MMC, Mello RP. Biological activity of neolignans on the post-embryonic development of *Chrysomya megacephala* (Fabricius, 1974) (Diptera: Calliphoridae). Fitoterapia 2007; 78:20-24.
20. Carvalho AR, de D'almeida JM, de Mello RP. Mortalidade de larvas e pupas de *Chrysomya megacephala* (Fabricius) (Diptera: Calliphoridae) e seu parasitismo por microhimenópteros na cidade do Rio de Janeiro, RJ. Neotrop Entomol 2004; 33:505-509.
21. Singh S, Kumar K. Sensitivity of last larval stadium of *Chrysomya megacephala* (Fabricius) to anti-allatin ageratochromene Precocene II. Indian J Exp Biol 2011; 49:600-608.
22. Cavados CFG, Chaves JQ, Queiroz MMC, Serra-Freire NM, Rabinovitch L. An assessment of the biological activity of *Bacillus thuringiensis* LFB-FIOCRUZ 907 in *Chrysomya megacephala* (Diptera: Calliphoridae). Israel J Entomol 1998; 32:117-123.
23. Mello RP. Chave para identificação das formas adultas das espécies da família Calliphoridae (Diptera, Brachycera, Cyclorrapha) encontradas no Brasil. Entomol Vect 2003; 10:255-268.
24. Abbott WS. A method of computing the effectiveness of an insecticide. J Econ Entomol 1925; 18:265-267.
25. Moya Borja GE. Erradicação ou manejo integrado das miíases neotropicais das Américas? Pesq Vet Bras 2003; 23:131-138.
26. Ruiu L, Satta A, Floris I. Susceptibility of the house fly pupal parasitoid *Muscidifurax raptor* (Hymenoptera: Pteromalidae) to the entomopathogenic bacteria *Bacillus thuringiensis* and *Brevibacillus laterosporus*. Bio Control 2007; 4:188-194.
27. Brodeur J. Host specificity in biological control: insights from opportunistic pathogens. Evol Appl 2012; 5:470-480.
28. Oliveira MS, Nascimento MA, Cavados CF, Chaves JQ, Rabinovitch L, Lima MM, Queiroz MMC. Biological activity of *Bacillus thuringiensis* strains against larvae of the blowfly *Chrysomya putoria* (Wiedemann) (Diptera: Calliphoridae). Neotrop Entomol 2006; 3:849-852.
29. Zimmer CR, De Castro LLD, Pires SM, Menezes AMD, Ribeiro PB, Leite FPL. Efficacy of entomopathogenic bacteria for control of *Musca domestica*. J Invertebr Pathol 2013; 114:241-244.
30. Pessanha RR, Carramaschi IN, Mallet JRS, Queiroz MMC, Zahner V. Evaluation of larvicidal activity and effects on post embrionary development of laboratory reared *Lucilia cuprina* (Wiedemann, 1830) (Diptera: Calliphoridae), treated with *Brevibacillus laterosporus*. J Invertebr Pathol 2015; 128:44-46.
31. Ruiu L, Satta A, Floris I. Observations on house fly larvae midgut ultrastructure after *Brevibacillus laterosporus* ingestion. J Invertebr Pathol 2012; 111:211-216.