Original Article

Adsorption of Toxic Metals and Control of Mosquitos-borne Disease by *Lysinibacillus sphaericus:* Dual Benefits for Health and Environment*

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Abstract

Objective Assessment of the bacterium *L. sphaericus* as a dual-action candidate for biological control of mosquito-borne diseases and bioremediation of toxic metals.

Methods Larvae of the mosquito, *C. quinquefasciatus*, were first evaluated for metal tolerance and then exposed to 5 ppm cadmium, chromium, arsenic, and lead in assays together with seven strains of *L. sphaericus.* A probit regression analysis was used to estimate the LC_{50} of Cd, Cr, As, and Pb to *C. quinquefasciatus*. An analysis of covariance and multifactorial ANOVA examined the metal biosorption and larvicidal properties of the seven strains of *L. sphaericus.*

Results We found that *L. sphaericus* adsorbed the toxic metal ions and was toxic against mosquito larvae. The *L. sphaericus* strain III(3)7 resulted in a larvae mortality of over 80% for all the tested metals. This strain also exhibited the capacity to adsorb 76% of arsenic, 32% of lead, 25% of chromium, and 7% of cadmium.

Conclusion This study found combined metal adsorption and larval toxicity associated with three strains of *L. sphaericus* [III(3)7, OT4b.31, and CBAM5]. This suggests that a combination of these strains shows strong dual potential for biological control of mosquitos in heavy metal-contaminated areas and remediate the heavy metal contamination as well.

Key words: *Lysinibacillus sphaericus*; *Culex quinquefasciatus*; Entomopathogen; Toxic metals; Heath

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INTRODUCTION

Lysinibacillus sphaericus is a spore forming Gram-positive bacterium, which has been employed in the biological control of mosquito larvae such as *Culex quinquefasciatus* Say, 1823 (Diptera: Culicidae)[1]. Its larvicidal activity is mainly attributed to the production of a binary toxin (BinA-BinB) during its sporulating stages. The mosquitocidal toxins (Mtx1, Mtx2, and Mtx3) are produced by vegetative cells, which are degraded by proteases during the stationary phases[2-3]. In addition, the efficiency these Mtx toxic proteins has been demonstrated in synergic experiments with BinA-BinB[4]. Along with these characteristics, members of the Bacillaceae family also exhibit the potential for metal adsorption due to their additional layers and the composition of the sporing coats[5]. *L. sphaericus* shows greater persistence in polluted ponds than other Bacillaceae bacteria, such

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as *Bacillus thuringiensis*, which is known for its mosquitocidal activity[1]. Moreover, several reports have shown the ability of *L. sphaericus* to survive[6-7] and absorb metals at concentrations that are otherwise toxic[5]. The presence of the S-layer protein is correlated with these properties[4]. Furthermore, external envelops such as exopolysaccharide (EPS) and S-layer proteins can interact with metal ions. An S-layer protein is the external surface of bacteria and archaea that can form 15% of the total proteins of the cell[8,5]. The presence of these characteristics and their biotechnological potential has been reported in the Colombian strains of *L. sphaericus* CBAM5[9], OT4b.31[10], III(3)7, OT4b.26, and OT4b.49[11], which exhibit tolerance to toxic metals.

Toxic metals are normally found at low concentrations in natural aquatic ecosystems, but with the increase in the human population, and associated anthropogenic impacts, have increased the concentration of toxic metals such as cadmium, chromium, lead, arsenic, mercury, copper, and iron[12-13]. These pollutants are detrimental for the ecosystem and human health, and require appropriate remedial measures[14]. Moreover, the presence of these metals in water can influence the abundance of mosquito larvae for different species[15-17]. Mosquitoes, serving as vectors of diseases such as filariasis, malaria, dengue, Japanese encephalitis, and West Nile fever, cause millions of deaths every year. Therefore, they are the most important group of insects in terms of public health[18-20]. Most works describe the effects of individual pollutants, but unfortunately do not take into account the adverse effects that a mixture of contaminants might have[21-22] on both biological control agents and the mosquito larva. Therefore, for biological control of mosquitoes in contaminated waters, it may be necessary to inoculate metal-tolerant strains[11]. In this scenario, tools for mosquito larvae control become crucial. The different chemicals that can be used as control tools are organophosphates, insect growth regulators, chlorpyrifos, dichlorvos, cypermethrin[19,23]. To reduce transmission of mosquito-borne disease, spraying, and insecticide-treated bed nets, are some methods employed in tropical countries[23-24]. Nonetheless, these chemicals have a potential toxic effect on public health and environment, and can induce resistance in number of vector species[19,25]. For these reasons, eco-friendly and biological controls agents are the best way to avoid new mosquito resistances, while also preserving human health and the environment.

In toxicological studies of mosquito larvae, including *C. quinquefasciatus*, it is common to test the performance of the bacteria with a focus on larvae mortality rates[26-27]. However, in natural environments, the presence of sublethal concentrations of toxins and environmental pollutants for the larvae may have other repercussions or disrupt ecological processes[27-28]. Therefore, it is important to study the effects of the toxicants and pollutants on the control agent, i.e. the bacteria, when they appear to be sublethal for the pest, i.e. the mosquito larvae[13]. The sublethal physiological stress that generates the toxicants in polluted environments might affect the survival curves of insects in the presence of microbial pathogens[27]. Moreover, using a microorganism tolerant to particular toxicants could also provide new information about concurrent microbial activities, even under sublethal physiological stress conditions.

Previous studies have separately evaluated the larvicidal activity and the toxic metal bioremediation of *L. sphaericus.* However, the larvicidal activity of native strains of *L. sphaericus* under toxic metal-polluted conditions has not yet been described. Therefore, this study endeavors to simultaneously determine the larvicidal activity of Colombian isolates of *L. sphaericus* against *C. quinquefasciatus* under toxic metal contamination, and measure the toxic metal ion biosorption.

MATERIALS AND METHODS

Microorganisms and Culture Conditions

The *L. sphaericus* strains are shown in Table 1. All strains were cultivated in Nutrient Agar for 24 h at 30 °C[10]. A 10^8 CFU (Colony-Forming Unit) of each strain was incubated in 3 mL of Nutrient Broth for 24 h at 30 °C. One hundred µL of each liquid culture was transferred to fresh Nutrient Broth and incubated for 24 h at 30 °C. An aliquot of 100 µL was transferred to SPC Agar (Standard Plate Count) and incubated 24 h at 30 °C, for storage.

L. sphaericus Strain Synchronization

Synchronized cultures were obtained by performing cycles of cultivation in nutrient broth and sodium acetate broth: 5.00 g/L, yeast extract 3 g/L, $MgCl_2$ 1×10^{-3} mol/L, $CaCl_2$ 7×10^{-4} mol/L, and $MnCl_2$

5×10^{-5} mol/L[11]. To synchronize the metabolic states, we standardized all the strains: a 10^8 CFU aliquot of each strain was incubated on a rotary shaker for 14 h, 30 °C, 200 rpm, and inoculated on 1% v/v in sodium acetate broth with static incubation for 3 d at 30 °C, and thermally shocked at 80 °C for 12 min. These cycles were repeated until we observed 90% of cells to be fully sporulated by light microscopy.

Estimation of Metal Tolerance of C. quinquefasciatus

The tolerance for each metal was assayed in ten third-instar larvae of *C. quinquefasciatus*, Muña strain, reared at 27 °C with a relative humidity at 40%-60%, and a 12:12 h light:dark photoperiod. For each evaluated metal, analytical grade salts of $CdCl_2$, $K_2Cr_2O_7$, $Na_2HAsO_4{\cdot}7H_2O$, and $Pb(NO_3)_2$ were dissolved in chlorine-free tap water to final concentrations of 5, 25, 50, 100, and 200 ppm. The interaction of all metals was inspected using a co-ion mixture at the same concentration. The larvae were assayed in cups containing 100 mL of working solution. Larvae mortality was calculated every 12 h for 120 h of toxic metal exposure. All treatments were assayed in triplicate.

Larvicidal Activity and Toxic Metal Biosorption of L. sphaericus Strains

The concurrent larvicidal and metal sequestering activity of *L. sphaericus* vegetative cells was assayed in 5 ppm of toxic metals dissolved in chlorine-free tap water with ten third-instar larvae of *C. quinquefasciatus*. Salts of cadmium, chromium, arsenic, and lead were evaluated with the *L. sphaericus* strains CBAM5, OT4b.31, III(3)7, OT4b.49, OT4b.25, OT4b.26, 2362, and a combination of all of them, except the reference strain 2362, at the same concentration. The evaluation was done in glass cups in triplicate. Moreover, the combination treatment was tested in presence of co-ions (the mixture of all metals tested). An overnight culture of synchronized *L. sphaericus* of each strain was centrifuged at 13,000 rpm for 15 min and resuspended in tap water. The cell suspension was adjusted to an OD_{600} of 0.3 ($10e^5$ CFU) and used as 1% v/v inoculum for the 100 mL working solution. This same procedure was repeated for the pool, using the same bacterial concentration of each strain. The larvicidal activity of the bacterial strain, and the toxic metals adsorption capacity, was measured at 48 h using the median lethal concentration (LC_{50}) of the reference strain 2362[11] as a standard concentration. As a control, the metal adsorption interference of the larvae was assayed without the presence of bacteria. The determination of residual toxic metals in samples was quantified by applying the Spectroquant® Merck Millipore test for lead (test number 1.09717.0001), chromates (test number 1.14758.0001), arsenic (test number 1.01747.0001), and cadmium (test number 1.01745.0001). The assays were measured by photometry in a Photometer NOVA 60A.

Statistical Analyses

The LC_{50} and the median lethal time (LT_{50}) of the toxic metals in the larvae were calculated using Probit regression analysis[29]. We used larvae mortality as the response variable and time or concentration as the predictors of the regressions; separate values were used for each toxic metal. An analysis of covariance (ANCOVA) explored the differences among metal treatments (arsenic, chromium, cadmium, lead, and the four metalloids) on the larvicidal toxicity over time (hours). Post-hoc Tukey tests were used to test for significant differences between metals. Univariate ANOVAs and post-hoc Tukey tests investigated the differences in

Table 1. *Lysinibacillus sphaericus* Strains and Their Larvicidal Activities

Strain	Larvicidal activity at 48 h			Origin	Reference
	S-layer	Vegetative cell	Spore		
2362	+	+	+	Reference strain	Donated by A. Delecluse
CBAM5	+	+	+	Isolated from petroleum exploration process	Villegas-Torres et al. 2011
OT4b.31	Nd	-	-	Isolated from Coleopteran larvae	Dussán et al. 2002
OT4b.49	Nd	+	+	Isolated from Coleopteran larvae	Dussán et al. 2002
III(3)7	+	+	+	Isolated from an oak forest soil	Dussán et al. 2002
OT4b.25	+	+	+	solated from Coleopteran larvae	Dussán et al. 2002
OT4b.26	+	+	+	Isolated from Coleopteran larvae	Dussán et al. 2002

Note. Nd: No data; +: Activity; -: No Activity.

larvicidal activity among *L. sphaericus* strains (seven strains plus the pool strain). In addition, a multifactorial ANOVA on larvicidal activity was conducted, including the toxic metal and strain as factors, as well as the interaction between these two influences. Similar univariate ANOVAs, Tukey tests, and the multifactorial ANOVA were run to test the differences in biosorption among strains and toxic metals. In this case, the factor strain had nine levels (seven strains, pool strain, and the control adsorption by *C. quinquefasciatus*), and the factor metal had four levels (arsenic, chromium, cadmium, and lead). The analyses were performed using the *stats* and *graphics* packages included in the R Statistical Computing Language[30].

RESULTS AND DISCUSSION

Tolerance of C. quinquefasciatus Larvae to toxic Metals

Environmental pollution generates a selective pressure on organisms, inducing different degrees of tolerance in insect larvae to toxic metals: lead, chromium, cadmium, and arsenic, even at concentrations higher than those found and reported in polluted water bodies[13]. To confirm that the concentration of toxic metals did not affect the larvae mortality rate, experiments were assayed in concentrations of 5, 25, 50, 100, and 200 ppm of arsenic, hexavalent chromium, lead, and cadmium metals. The *C. quinquefasciatus* larvae tolerated significantly higher concentrations of arsenic than the other metals (up to 150 ppm), with arsenic being the least toxic metal. According to the LC_{50} scores, the most toxic metal was cadmium (median lethality of 26 ppm), followed by chromium, lead, and then arsenic (Table 2). When comparing the metal tolerance of *C. quinquefasciatus* larvae to the toxic metals in the Muña reservoir (Cundinamarca, Colombia), the estimated concentrations are 96 times higher for arsenic, 1470 times higher for lead, 303 times higher for chromium, and 3846 times higher for cadmium, as reported by Sarmiento et al. (1999) in Colombian journal of public health. To confirm that the exposure time to the toxic metal did not affect the larvae mortality rate, an LT_{50} test was performed, measuring larval mortality every 12 h over a period of 120 h. It was found that over 55 h, cadmium and chromium produced a 50% mortality rate, whereas longer exposure was necessary to obtain the similar results for lead and arsenic (86 and 123 h respectively)(Table 2).

Tolerance data obtained for lead, chromium, arsenic, and cadmium allowed us to determine a working concentration of 5 ppm, in which the larvae efficiently tolerated these toxic metals within the first 24 to 48 h. At this concentration, the larvicidal effect, as a function of the exposure time (in hours), was observed (Table 2, Figure 1). Tolerance did not differ among the metal treatments (metal treatment: $F_{4,40}$=1.118, P=0.362), and increased with exposure to the metal (s) (time exposure: $F_{1,40}$=347.915, P<0.001), but this increase varied among metal treatments (metal treatment x time exposure: $F_{4,40}$=8.537, P<0.001). All metal treatments differed among each other with the exception of chromium/cadmium, chromium/co-ions, and cadmium/co-ions (Tukey tests, in all cases P<0.05). *C. quinquefasciatus* larvae tolerated significantly higher concentrations of arsenic than the other metals (up to 150 ppm), arsenic being the least toxic metal (Figure 1). The ability to tolerate different metals at a concentration of 5 ppm, as exhibited by the larvae, shows that there is a synergic effect of increased larval mortality rate when using all metals in the same treatment. However, there is a possibility of added toxicity, as shown by higher larvae mortality after the first 48 h of exposure to the metals (Figure 1). There were also clear differences in the external appearance of the larvae subjected to toxic metals as compared to the control group larvae (Figure 2).

Larvicidal Activity and Toxic Metal Adsorption of L. sphaericus

For cadmium, lead, and chromium, the larvicidal activity showed significant differences between

Table 2. Median Lethal Concentration of Toxic Metals Toward *C. quinquefasciatus* Larvae

Metals	LC_{50} (ppm)	LT_{50} at 5 ppm (hours)
Chromium	50.0	62.9
Lead	66.1	86.9
Arsenic	179.5	123.5
Cadmium	26.8	58.6

Note. The LC_{50} values were established with five concentrations for each metal 5, 25, 50, 100, 200 ppm. The control groups showed no mortality. The LT_{50} values were estimated with a metal exposure of 24, 36, 48, 60, 72, 84, 96, 108, 120 hours. The control groups showed no mortality. Significant at P<0.05.

L. sphaericus strains (cadmium: $F_{7,16}$=34.610, P<0.001; lead: $F_{7,16}$=3.929, P=0.011; chromium: $F_{7,16}$=21.810, P<0.001). For cadmium, the 2362 strain showed significantly less activity than the other strains (Tukey test, in all cases P<0.001; Figure 3). For the other assays, the strains showed larvicidal activity up to 50% for chromium and 80% and 90% for lead and arsenic, respectively (Figure 3). Similarly, the strain OT4b.49 showed significantly lower larvicidal activity than the other strains in the presence of chromium (Tukey test, in all cases P<0.030; Figure 3). The strain OT4b.31 differed from the other strains, except for the CBAM5 and 2362 strains (Tukey test, for all significant cases P<0.012; Figure 3). For lead, there was only a significant difference between the strains 2362, which exhibited the lowest performance, and the strain CBAM5, which presented the best performance for this metal (Tukey test, P=0.029; Figure 3). The data showed that the entomopathogenic activity decreases considerably in the presence of chromium for the OT4b.49 and OT4b.31 strains. However, the reference strain 2362 displayed intermediate activity in the presence of chromium, and the larvicidal activity decreases in the presence of cadmium (see Figure S1 of supporting information). In the presence of arsenic, the strain with the highest mortality percentage was OT4b.49, with 86.6%. In the cadmium and chromium treatments, the strain III(3)7 exhibited the highest mortality percentage, with 100% and 83.3%, respectively. For the other metals, this strain showed a larvicidal activity over 80%, while in the presence of

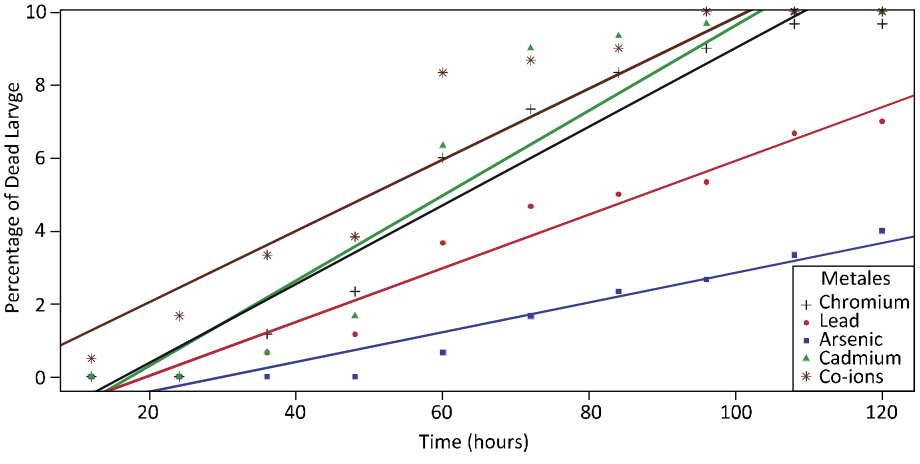

Figure 1. Effects of 5 ppm of toxic metals on larvae of *C. quinquefasciatus*. The brown line shows the effect of a combination of the four metals (co-ions). Adjusted multiple R-square=0.911.

Figure 2. Mosquito larvae pictured at 48 h: a) after co-ions treatment, b) untreated control.

chromium the highest mortality was caused by the strain CBAM5, with 100% mortality. Furthermore, the pool treatment (a combination of all native strains at the same time) showed the highest larvicidal activity in the presence of all metals tested (Figure 3).

The larvicidal behavior of all strains in arsenic, cadmium, chromium, and lead is shown in Figure 2. The multifactorial ANOVA showed that the entomopathogenic activity significantly differed among metals, strains, and the interaction between these two factors (metal: $F_{3,56}$=32.829, P<0.001; strain: $F_{6,56}$=12.191, P<0.001; metal x strain: $F_{18,56}$=9.562, P<0.001) (see Figure S1 of supporting information). The strains showed homologous behavior in their larvicidal activity, exhibiting higher activity for arsenic, lead, and cadmium, and lower activity for chromium at 5 ppm, except for the 2362 strain, which showed lower activity in the presence of cadmium (Figure 3). The larvicidal activity decreased considerably in the presence of chromium for the OT4b.49 and OT4b.31 strains (Figure 3). In addition, each strain exhibited a substantially different behavior for each metal tested (Figure 3). In

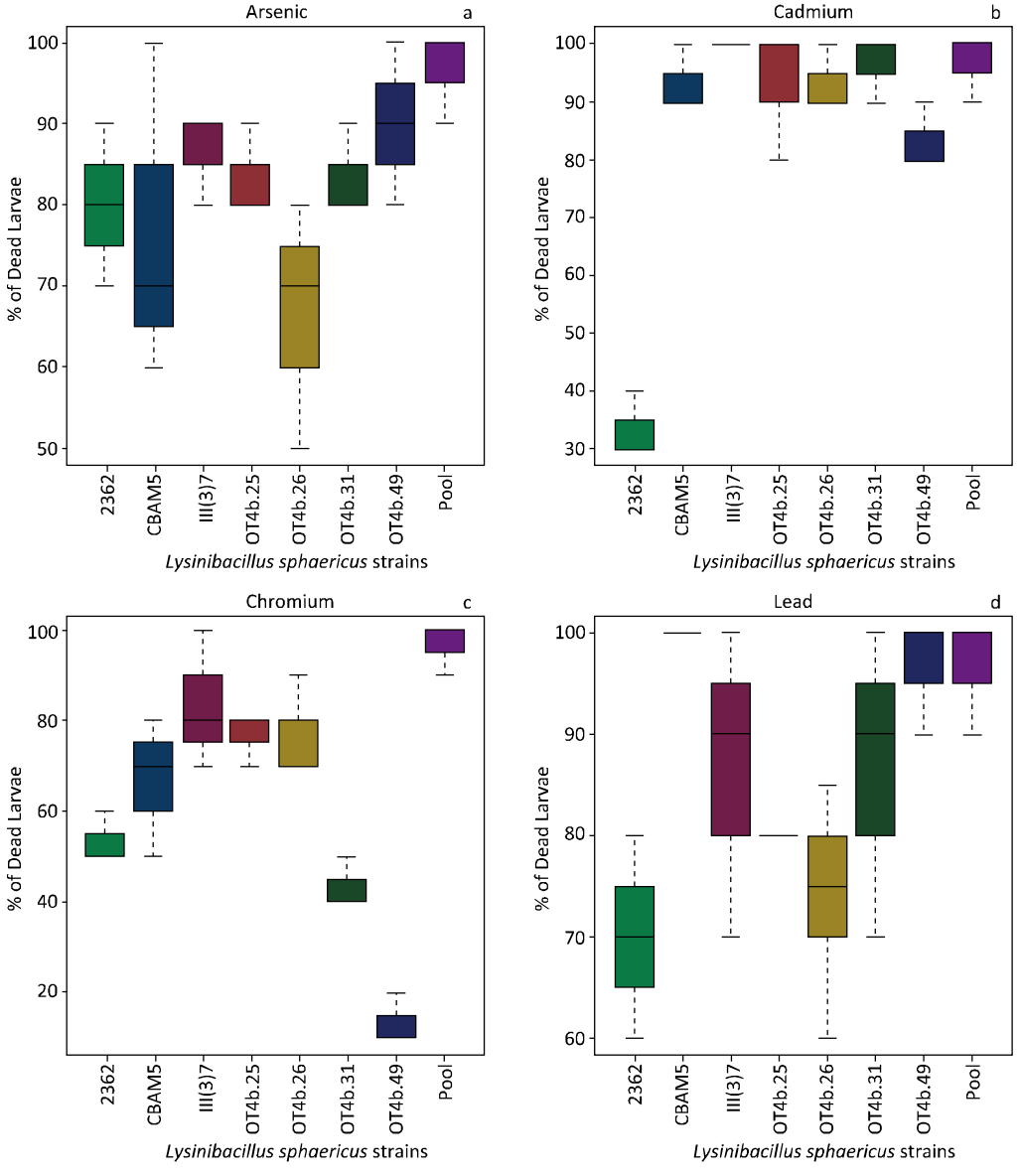

Figure 3. Mean±SE larvicidal activity at 48 h for *L. sphaericus* strains. Dead larvae in (a) arsenic. (b) cadmium. (c) chromium. (d) lead.

the presence of arsenic and lead, all the strains exhibited effective larvicidal activity; whereas, in the presence of cadmium, the larval mortality caused by strain 2362 was less than 50%. Similarly, in the presence of chromium, the mortality due to strains T4b.49, OT4b.31 was less than 50% of the larvae tested (Figure 3). *L. sphaericus* III(3)7 was the only strain that maintained its entomopathogenic activity with a mortality rate of 80% of the population in the presence of all metals tested. The difference in larvicidal activity in chromium for the OT4b.49 and OT4b.31 strains might be explained by a lack of, or low larvicidal activity of, S-layer proteins (Table 1) that provide a larvicidal mechanism[3]. This is in accordance with Allievi et al.[4], where the S-layer protein of the strain C7 was found to be more active against mosquitos than the S-layer protein from the 2362 strain, particularly against *Aedes aegypti.* Furthermore, certain metals such as chromium could affect the larvicidal activity of the *L. sphaericus* strains. Moreover, it has been previously shown that exposure to metals increases susceptibility to pathogens[27].

The adsorption of the toxic metals at the time that the *L. sphaericus* strain exhibited its entomopathogenic activity in *C. quinquefasciatus* was calculated by subtracting the residual concentration in ppm of metal in the media from its initial concentration in ppm. It was observed that the different strains displayed the ability to adsorb the evaluated toxic metals from the medium. The biosorption significantly differed among strains for the four metals (arsenic: $F_{8,18}$=22.280, P<0.001; cadmium: $F_{8,18}$=28.020, P<0.001; chromium: $F_{8,18}$=31.930, P<0.001; lead: $F_{8,18}$=8.178, P<0.001). This assay showed that the biosorption of arsenic by all strains is higher with respect to the adsorption of the other metals, followed by lead, chromium, and cadmium (Figure 4). These last three metals were adsorbed in very similar concentrations by the strain 2362: between 27%-30% of the initial concentration of the metal. Regarding the other strains, they exhibited different adsorption concentrations for each metal, between 5%-38%, with the exception of the strain OT4b.26, which was only able to adsorb 1% of chromium. For chromium treatment, the OT4b.31 strain achieved the highest biosorption percentages: 30.3%. For lead, the OT4b.25 reached the highest adsorption performance with 38.6%, followed by the OT4b.31 strain with 35.7%. For cadmium, the strain 2362 reached the highest adsorption: 27.4%. For arsenic, the III(3)7 strain reached the highest biosorption value: 76% of the initial concentration (Figure 4). Furthermore, the strains OT4b.31 and OT4b.26 also exhibited favorable cadmium and arsenic adsorption over other strains of *L. sphaericus*. The only significant differences in the adsorption of cadmium were found between the III(3)7 strain and the strains CBAM5, OT4b.31, 2362, OT4b.26, and the pool (Tukey test, in all these cases, P<0.012). Similarly, all strains exhibited significant differences against the control treatment (*C. quinquefasciatus* alone in the different metals), except the III(3)7 strain in cadmium, and both the OT4.b25 and OT4.b26 in chromium (Tukey test, in all these cases, P<0.05). Significant differences in the cadmium treatment with the OT4b.31, 2362, OT4b.26, and CBAM5 strains, with respect to OT4b.49, were also observed (P<0.05). The 2362 and OT4b.31 strains were better at adsorbing cadmium, with an average of 1.373 and 1.293 ppm respectively (Figure 4). Cadmium was the metal with the lowest adsorbed concentration for all strains tested, except for the OT4b.25 and OT4b.26 strains, which adsorbed less in the presence of chromium (Figure 4). In addition, the differences of metal adsorption between the strains can be attributed to differences in the mechanisms of metal adsorption of each strain, such as efflux bombs, biomass accumulation, and S-layer adsorption, among others[5,9-10,31]. The percentage of adsorption for the *C. quinquefasciatus* control treatments in the tested metals might be explained by the intrinsic capacity of the larvae to accumulate metals such as arsenic[27].

Figure 3 illustrates the behavior of metal biosorption for the *L. sphaericus* 2362, CBAM5, III(3)7, OT4b.25, OT4b.26, OT4b.31, and OT4b.49 strains at 48 h. The multifactorial ANOVA showed that the adsorption of the metals significantly differed among metals, strains, and the interaction between these two factors (metal: $F_{3,54}$=305.232, P<0.001; strain: $F_{6,54}$=12.725, P<0.001; metal x strain: $F_{18,54}$=6.751, P<0.001) (see Figure S2 of supporting information). By comparing Figures 3 and 4, it is possible to suggest that each toxic metal can influence the larvicidal activity of the *L. sphaericus* strains, but this effect is not necessarily dependent on the metal biosorption capacities. The various mechanisms for removing toxic metals utilized by indigenous bacteria to overcome toxicity, and the mechanisms by which they spread in the environment[9], may affect the ability to kill larvae. The metals can inhibit the growth of some mosquitocidal strains and

can thereby increase larvae survival[11]. Similarly, metal exposure can affect the larvae by inducing sublethal physiological stress, as found in previous studies with arsenic[27,32].

CONCLUDING REMARKS

This study demonstrated that *C. quinquefasciatus* larvae are resistant to certain concentrations of toxic metals (shown above as LC_{50}), stressing the need to seek new entomopathogenic bacterial strains with additional dual characteristics. The concurrent activity found in the different strains of *L. sphaericus* suggests that each metal could affect the LC_{50} of the bacteria strain in polluted waters. Moreover, all the strains tested here exhibited a dual activity, but not toward all the metals. Furthermore, to effectively remove different metals, it is necessary to establish a combination of *L. sphaericus* strains, as demonstrated in this study. In addition, the activity of the vegetative cultures of *L. sphaericus* in contaminated waters suggests that the strains III(3)7,

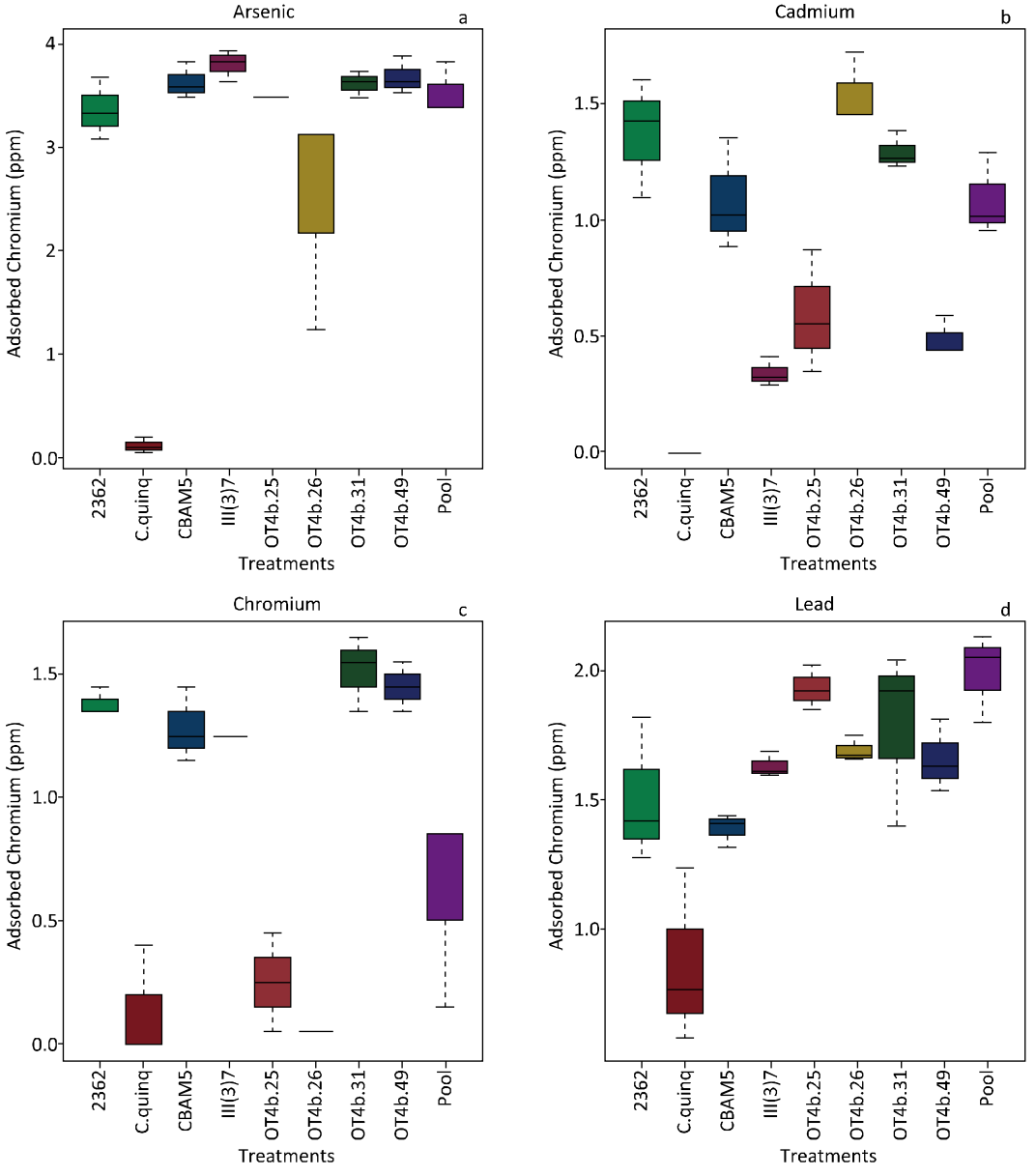

Figure 4. Mean±SE toxic metal accumulation at 48 h for *L. sphaericus*: Adsorbed (a) arsenic. (b) cadmium. (c) chromium. (d) lead.

OT4b.31, and CBAM5 could be successful candidates for biological control in environments polluted with arsenic, lead, hexavalent chromium, and cadmium. This is demonstrated by the effective larvicidal performance of these three bacterial strains in different metals and co-ions. It might be possible that the *L. sphaericus* strains could exhibit different mechanisms to counteract metal toxicity, such as efflux bombs, although this requires further research. More work at the molecular level is also required to discover the mechanisms behind the interactions of environmental contaminants and bacterial entomopathogenicity, such as toxins or S-layer proteins.

Therefore *Lysinibacillus sphaericus* is a microorganism with dual benefits for the environment and human health, with applications toward mitigating mosquito-borne tropical diseases and the adsorption of toxic metals.

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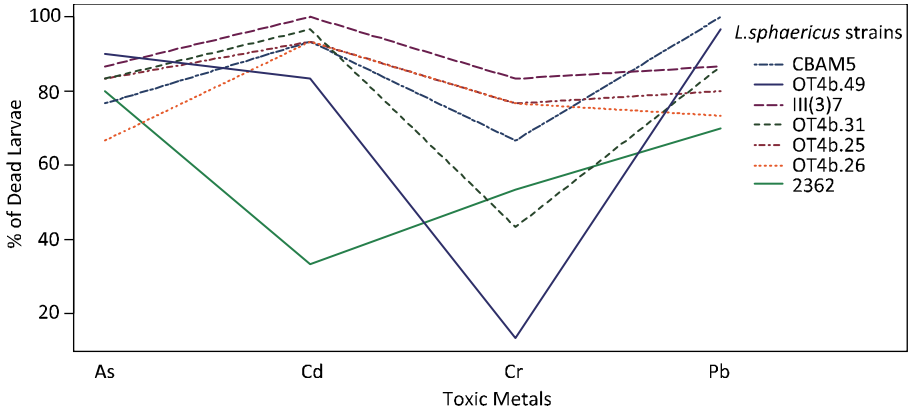

Figure S1. Larvicidal activity of *L. sphaericus* strains with each toxic metal at 48 h.

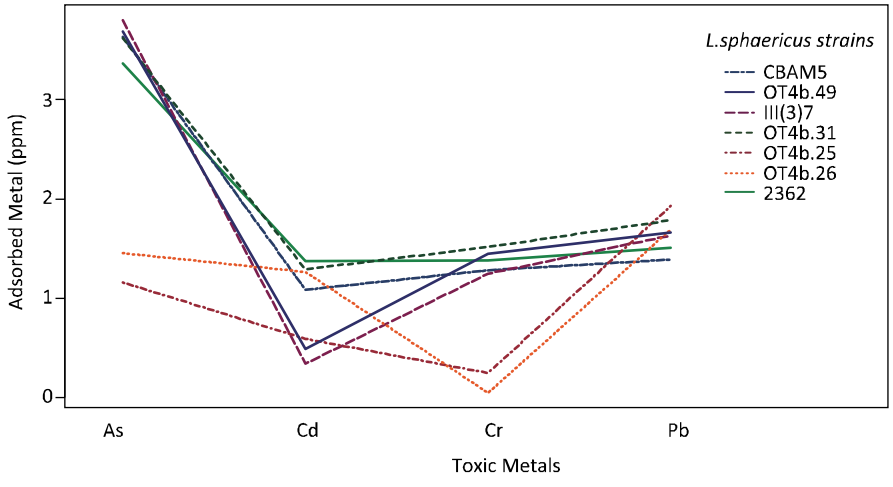

Figure S2. Metal adsorption of *L. sphaericus* strains with each toxic metal at 48 h.