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Cadmium-Tolerant Bacteria in Cacao Farms from Antioquia, Colombia: Isolation, Characterization and Potential Use to Mitigate Cadmium Contamination

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Abstract: Bioremediation of farm soil is a technique that merits in-depth research. There are few studies related to the use of bioremediation to reduce cadmium (Cd) availability in soils used for cacao production. This study investigates (1) field bioprospection and strain characterization using techniques including isothermal microcalorimetry to select a group of cadmium-tolerant bacteria (CdtB) for potential use as bioremediators of cacao soils and (2) the application of bacterial inoculum to compare the immobilization of Cd under field conditions. Bioprospection was carried out in four cacao farms from the Antioquia district in Colombia. Culturable CdtB strains were isolated using $CdCl_2$ as a Cd source and identified using molecular techniques. The metabolic characterization of Cd immobilization was carried out using isothermal microcalorimetry with $CdCl_2$ amendments. Five cadmium-tolerant bacteria were isolated and characterized as *Bacillus* spp. The strain CdtB14 showed better growth and Cd immobilization ability (estimated through heat ratios) than any strain isolated thus far, suggesting potential for future use in bioproduct development. Furthermore, the application of two previously characterized CdtB strains with zeolite powder was performed in the same farms where the bioprospection process was carried out. The application of the preformulated inoculum resulted in a decrease of 0.30 + 0.1 mg kg^{-1} of soil Cd in two out of the four assessed farms. The field results are preliminary and require data on the change in Cd in cacao beans to understand what this result means for Cd mitigation. This study is the first to combine bioprospecting and the performance of CdtB in laboratory and field experiments in cacao farms and shows the potential of bioremediation to mitigate Cd contamination in cacao.

Keywords: cadmium; cacao; cadmium-tolerant bacteria; isothermal microcalorimetry

Citation: Quiroga-Mateus, R.; López-Zuleta, S.; Chávez, E.; Bravo, D. Cadmium-Tolerant Bacteria in Cacao Farms from Antioquia, Colombia: Isolation, Characterization and Potential Use to Mitigate Cadmium Contamination. *Processes* **2022**, *10*, 1457. https://doi.org/10.3390/pr10081457

Academic Editor: Avelino Núñez-Delgado

Received: 11 June 2022
Accepted: 21 July 2022
Published: 26 July 2022

1. Introduction

Cadmium (Cd) is a heavy metal naturally present in farmlands and recognized for its toxicity in almost all living organisms [1]. However, a functional group of microorganisms, called cadmium-tolerant bacteria (CdtB), has been highlighted to help reduce metal uptake by plants through at least seven metabolic pathways or mechanisms in polluted soil [2]. The non-phylogenetically related group of CdtB has received little attention, and neither diversity nor the biotechnological potential for reducing Cd accumulation in cacao beans (*Theobroma cacao*, L.) have been studied systematically.

Cacao is an important crop in several South American countries, including Colombia and Ecuador. The importance of the crop dates back to ancestral cultures related to social and economic uses of the beans in sacred rituals [3]. Since 1st January 2019, the regulatory

commission of the European Union (EU) has imposed the maximum values for the permissible content of Cd in chocolates and cocoa products, affecting the commercialization of cacao beans in some areas [4,5]. This regulation is critical for producing countries, where Cd hotspots have been identified in nationwide surveys [6,7] of cacao farms.

Bioremediation-based experiments are important, since they enhance the capacity of natural populations of microorganisms and their interaction with Cd, as already demonstrated [8,9]. CdtB have the ability to transform available Cd into less harmful or geostable compounds, decreasing the availability to living organisms via biochemical speciation [9]. Among the microorganisms with bioremedial potential are rhizospheric bacteria, which are capable of interacting as metal chelators and promoters of soil pH fluctuation processes, directly influencing the bioavailability of toxic compounds [10]. Likewise, microorganisms isolated from sites with higher levels of Cd naturally show great adaptation to pressure stress conditions (i.e., high Cd concentrations) [11]. The use of bacteria isolated from contaminated soils in bioremediation processes has been previously demonstrated, as in the case of *Bacillus megaterium*, identified as a bacterium with the ability to tolerate lead, cadmium, and nickel. This bacterial strain uses its own metabolic machinery to immobilize these heavy metals through the synthesis of organic and inorganic acids and redox reactions for chemical complexation [12].

However, CdtB might not be useful when applied alone. They need some other inputs to deal with the Cd content in soils (e.g., co-application with mineral soil amendments such as zeolite). The rationale behind the use of soil amendments is to reduce the availability of Cd to plants and thus reduce its uptake [13,14]. Zeolite is a phyllosilicate mineral with a high specific surface area which increases the retention of water and cations [14], including Cd. In contrast, the application of specific Cd bioremediation agents does not result in a change in availability of all cations and only Cd, thus not resulting in the immobilization of plant nutrients. They may also be cheaper, more reactive and potentially feasible alternatives. Furthermore, the use of microorganisms has no negative environmental effects and would not be considered problematic for organic agricultural practices in soil chemical stabilization [15].

In Colombia, an important effort has been made to investigate the use of bioremediation to reduce Cd accumulation in cacao beans. This includes the bioprospection of novel autochthonous isolates and the characterization of CdtB from Cd-enriched cacao-growing soils from several districts across the country [8]. This study is part of a continuous effort to use bioprospection of CdtB in cacao-growing soils to identify produce and use the best strains to reduce Cd migration and accumulation in cacao beans.

Any bioremediation strategy should include several natural populations of CdtB that can immobilize Cd through metabolic activity under a variety of soils and environmental conditions. These bacteria can be characterized using isothermal microcalorimetry (IMC), and the choice of CdtB should consider representativeness of the Cd metabolic processes, the geological background [2], and the edaphoclimatic conditions, allowing for effective and reliable performance of CdtB throughout a wide eco-region.

In a previous bioprospecting study [8], a total of 124 CdtB were isolated from cacao farms in three districts: Arauca, Boyacá and Santander. Despite its importance in cacao production, the district of Antioquia was not included. In comparison with other districts, Antioquia has a variable (or different) geological background, mainly due to batholith enrichments, and thus different geological features related to Cd are expected in this region [16,17]. Therefore, this study is one of the first focusing on the bioprospection and utilization of CdtB in reducing the Cd content in cacao soil from Antioquia and in Colombia.

This study provides results on the bioprospection of CdtB in isolating viable cells from soils collected in cacao farms in Antioquia, Colombia. In these farms, little is known about CdtB from soils and their potential effect in Cd mitigation. As highlighted in a recent work [16], even though the Cd content in soils and cacao beans in Antioquia is lower than in

other areas, it is still important to study these areas because the fluxes of Cd in cacao farms are dynamic and might affect access to regulated markets if measures are not implemented.

Therefore, this study was conducted in three interplayed activities: (1) the identification of potentially viable CdtB populations isolated from cacao-growing soils collected at the Magdalena river basin in Antioquia, (2) a comparison of the metabolic capacities of CdtB strains when Cd is added under controlled conditions using IMC, which is a validated method to measure the immobilization activity and capacity of the isolates, and (3) the bioaugmentation of selected and preformulated CdtB and zeolite to modify the properties and Cd concentration in the soil.

2. Materials and Methods

2.1. Soil Samples and Physical-Chemical Characterization

Soil samples were collected in four farms labeled A, B, C and D. The farms are in two municipalities of the Magdalena Medio Antioqueño cacao areas: San Roque and Maceo. Farm A was located in the municipality of San Roque, while farms B, C and D were located in the municipality of Maceo. The exact locations of the farms are not disclosed in order to maintain farmers' anonymity. The total areas of the cacao in the plantations were 2, 3, 5 and 5 has for farms A, B, C and D, respectively. These areas (<5 has) are normal farm sizes in the district of Antioquia. The cacao trees were planted at a distance of 3 × 3 m. The maximum distance between farms was 30 km. Soil samples were collected in 10 × 10 m plots containing 9 cocoa trees. Samples were taken following a methodology established for geomicrobiology studies [18]. Trial pits were made at points in which mineral deposits associated with Cd were found. These deposits were identified after observing the tomograms obtained with a 2D ERT tool [19]. Samples were collected at each soil horizon using galvanized stainless steel 'Shelby' tubes previously disinfected with ethanol (70% *V/V*). Additionally, samples were taken in the plots at the extreme points (lower and upper) to obtain composite samples at a 20-cm depth. Seven composite soil samples were collected from farm A, and two composite soil samples were collected at each of the other farms. To quantify the pseudo-total cadmium in the soil, the samples were dried at 40 °C in an oven and then pulverized and sieved through a 0.5-mm sieve. One gram of dry and ground soil was mineralized using a nitric acid (HNO_3)-hydrochloric acid (HCl) solution (7:1 *V/V*) in a microwave digester (Milestone UltraWAVE, Sorisole, Bergamo, Italy) following EPA method 3051 [20]. The cadmium concentration was measured using an Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES) [21] (Thermo Scientific ICAP 6500, Waltham, MA, USA).

The soil's physical-chemical parameters were measured using standardized protocols. The soil pH was determined by the potentiometric method, following a previous methodology [22], in a soil-water solution (1:2.5 *W/V*). Electrical conductivity (EC) was determined by the method proposed elsewhere [23] in a solution of soil and deionized water (relation 1:5.0 *W/V*). The organic matter content (SOM) was quantified using potassium dichromate and sulfuric acid for mineralization, where the carbon (C) content was determined using a UV-VIS spectrophotometer (Perkin Elmer Lambda 25 spectrometer, Waltham, MA, USA). Thus, the SOM was obtained by multiplying the C by 1.72 (Van Bemmelen factor). Moreover, $Ca^{2+,}$ K^+ and Mg^{2+}, were extracted using a 1M CH_3COONH_4 solution at pH 7.0 [24]. The extracted elements were quantified using atomic absorption spectrometry (AAS) (Agilent 280FS AA, Santa Clara, CA, USA). The effective cation exchange capacity (CEC) was calculated as the product of the sum of the cations. Acidity (Al+H) was determined by a standard method [25] using a T90 Automatic Titrator (Mettler Toledo, Columbus, OH, USA) in a soil extract obtained after titration of the soil in a potassium chloride solution (KCl) with 0.01 M sodium hydroxide (NaOH). The Al content was determined by AAS [26]. The content of phosphorus (P) was determined by the reduction of ascorbic acid using the Bray II method [27]. The quantification of P was carried out using UV-VIS spectrophotometry at a wavelength of 887 nm. The determination of the texture was performed using the Bouyoucos standardized method [28].

2.2. Isolation and Purification of Cadmium-Tolerant Bacteria (CdtB)

The isolation of CdtB was performed using classical microbiological methods of soil enrichment and dilutions. First, 10 g of the composite soil samples (described in Section 2.1) was inoculated with 90 mL of liquid Mergeay medium [29]. To select only CdtB, the Mergeay was adjusted to a final concentration of 6 mg L^{-1} of cadmium chloride ($CdCl_2$, Sigma Aldrich, St. Louis, MO, USA). The soil was incubated for 12 days at 28 °C and 120 rpm on an orbital shaker with a heating module (Heidolph Unimax 1010 and incubator 1000, Heidolph Instruments GmbH & Co. KG, Schwabach, Germany). At the end of this period, a 100-µL aliquot of a 10^2 dilution of each fermented soil was placed in Petri plates containing Cd-supplemented Mergeay agar. The plates were incubated at 28 °C for 5 d. Plates where a uniform growth of morphologies associated with bacteria (by their shape, size and color) was observed were replicated up to six times in fresh medium until pure cultures were obtained for characterization.

2.3. CdtB Identification, DNA Extraction and PCR Amplification of the 16S rRNA Partial Gene

Bacterial identification was carried out by sequencing the 16S ribosomal RNA (rRNA) with the aim of establishing the phylogenetic relationships and taxonomy of the isolates. Genomic DNA was extracted using the InnuPrep bacterial DNA isolation kit (Analytik Jena Kit AG, Berlin, Germany) according to a previous method [8]. The 16S rRNA partial gene was amplified by PCR with the following conditions (final volume of 50 µL): 1X Taq polymerase buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs and primers of 0.4 µM each. The primers were used according to a previous work [18]. The forward primer was 27F (5′-AGAGTTTGATCCTGGCTCAG-3′), and the reverse primer was 1492R (5′-GGTTACCTTGTTACGACTT-3′). The master mix consisted of 1.25 U of Taq DNA polymerase GoTaq® Flexi DNA Polymerase (Promega) and 10–100 ng μL^{-1} of bacterial genomic DNA. The PCR was carried out in a thermal cycler (Mastercycler Pro, Eppendorf AG) with the following program: 2 min at 95 °C with 30 cycles of 20 s at 94 °C, 20 s at 50 °C, 90 s at 72 °C and 10 min at 72 °C. The PCR products were sequenced at the Laboratory of Molecular Genetics in C.I. Tibaitatá, Mosquera-Colombia Agrosavia. Using the basic logical alignment search tool BLAST at the NCBI (National Centre for Biotechnology Information), the sequences of the isolates were compared with similar ones. Multiple alignments of different 16S rRNA sequences from related bacteria from the GenBank database were performed with the software Muscle 7 using the default configuration.

2.4. Metabolic Characterization Using Isothermal Microcalorimetry (IMC)

An isothermal microcalorimetric experiment was conducted to assess the metabolic peaks of heat flow related to the Cd immobilization capacity of the CdtB. Five CdtB strains were isolated in this study and used to test the pertinence of IMC to measure immobilization activity. *Escherichia coli* K12 DSM 6722 was used as a negative control because it has been reported as a cadmium-non-tolerant bacterium [2,9]. A strain of *Enterobacter cloacae* CdtB41 (accession code KY048271), which was isolated previously [8], was used as a positive control. This strain has been used for several studies related to heat production and Cd bacterial metabolism regionally. For growth in the microcalorimeter, except for *E. coli* K12, all strains were preincubated on solid Mergeay supplemented with a stock solution of $CdCl_2$ adjusted to a final concentration of 6 mg L^{-1}. *Escherichia coli* K12 was preinoculated on Nutrient Agar (NA). Three replicate cultures for all strains were exposed to 20-mL microcalorimetric ampoules filled with 7 mL of slanted liquid Mergeay's medium amended with 6 mg L^{-1} $CdCl_2$. The pH was adjusted to 7.0. Inoculation was performed with an electronic transferpette (Transferpette ® electronic, single Channel LH 288277, Brand, Germany) using an inoculum suspension of 1×10^7 CFU per mL, ensuring that bacteria would grow homogeneously into the ampoule. Previous studies have shown that decreasing the inoculum concentration lowered the maximum activity [30,31]. Cadmium immobilization activity was measured in an isothermal heat conduction microcalorimeter (TAM Air, Waters/TA Instruments, Delaware) equipped with 8 channels.

The temperature of the microcalorimeter thermostat was set to 28 ± 1 °C. The samples were installed into the calorimeter with Admix ampoules (TAM AIR Admix ampoule 20 mL with motor 115/320 V, TA Instrument, Lindon, UT, USA) to homogenize the mixture between Mergeay's media and the bacterial inoculum. After stable temperature conditions were obtained, each measuring channel was calibrated using a built-in electric heater of known power. The baseline was obtained from ampoules containing deionized water, only preventing any heat production due to chemical reactions.

The measurements obtained in the microcalorimeter were recorded after a required two-step thermal equilibration procedure for 1 h, recommended by the manufacturer. This methodology has also been used in previous studies [2,31–33]. During the first 15 min, samples were placed in the equilibration position to achieve preliminary thermal equilibrium. The samples were then placed in the measuring position. A final 45 additional minutes were added to achieve fine thermal equilibration conditions and start the measurements.

2.5. Characterization of Cd Immobilization Bacterial Capacities

The ability to immobilize cadmium by isolated bacteria was measured by fermentation in Erlenmeyer flasks. The process was divided into three parts. First, the isolated bacteria and the negative and positive control (*Cupriavidus taiwanensis* DSM 17343) were cultured in LB medium at 28 °C and 120 rpm until the exponential phase of growth was reached. A different positive control was used compared with the IMC assays, because *C. taiwanensis* is a known international reference strain for Cd immobilization. An aliquot of each culture was taken and centrifuged. The pellet was resuspended in sterile distilled water to obtain a cell suspension of 1×10^8 CFU mL^{-1} adjusted by optical density (OD) at 600 nm in the UV/Vis spectrophotometer [33]. The second step consisted of inoculating the previously prepared Erlenmeyer flasks with 20 mL Mergeay medium. The volume of inoculum added per Erlenmeyer flask was brought to a final concentration of 1×10^7 CFU mL^{-1}. This was performed in triplicate for each bacterial strain assessed in the culture medium with and without Cd. The medium was adjusted to a final concentration of 6 mg L^{-1} of $CdCl_2$ and assessed for 12 d at 28 °C in 120 rpm of constant shaking. The final step consisted of the quantification of the Cd concentration, where 12 mL of media was centrifuged for 20 min at 4600 rpm, and 10 mL of the supernatant was digested according to the standardized protocol at the analytical chemistry laboratory (C.I. Tibaitatá, in Mosquera, Colombia). The quantification was performed using the ICP-OES. The samples were analyzed at three-time intervals: 0, 6 and 12 d. The efficiency of the digestion was controlled in terms of the percentage of recovery of a reference material. The reference material was IPE 907 *Spinacia oleracea* (Wepal, Wageningen, the Netherlands). The detection limit of the samples was 0.5 µL L^{-1}, and the quantification limit was 6 µg kg^{-1}.

2.6. Biolog Gen III Metabolic Characterization

The five strains were characterized for their ability to consume different substrates using the GEN III MicroPlate test panel (Biolog Inc, Hayward, CA, USA). The plates consisted of 71 wells with carbon sources, where 23 out of the 71 substrates corresponded to C substrates and 2 corresponded to the substrate control (positive and negative) wells. A positive reaction was perceived when the OD of the well was above 1.5 units. The inoculation consisted of preparing 1000 µL of biomass (1×10^7 CFU per mL of medium) of each CdtB in Petri plates with LB culture medium. Bacterial colonies of each culture were suspended in IF-A inoculation fluid, adjusting to the recommended cell density according to the manufacturer. Each well of the microplate was inoculated with 100 µL of the cell suspension [34]. The readings were made in a plate reader (Synergy HT, Bio-Tek Instruments, Inc, Winooski, VT, USA), adjusting the absorbance to a wavelength of 600 nm with reading intervals of 30 min for 48 h at 30 °C with continuous shaking. Substrate consumption above the control well was considered a positive count. The substrate consumption profile was compared between the CdtB strains to understand their relative metabolic differences and their ability to coexist.

2.7. Bioaugmentation of CdtB and Mineral Amendment in the Selected Cacao Farms

To assess the ability of CdtB in reducing the soil Cd, two bacterial strains isolated previously, identified as 'Strain A' and 'Strain B', were applied to the farms. These strains are fully characterized, and their ability to immobilize Cd was proven previously. The strains were cultured in a preformulated media for application on the soil surface or Ap horizon (the subdivision of the plowed A horizon located near 15 cm in the soil surface) near the tree trunk. The effect of bacterial treatments was compared with a soil amendment widely applied to mitigate Cd contamination in other crops (i.e., zeolite). Due to the farms' size, this experiment could not be carried out in a single farm, and thus each treatment was applied in only one farm; farm A was the control, farm B received strain A plus zeolite, farm C received strain B plus zeolite, and farm D received only zeolite. The doses of the strains (A or B) per tree were 1×10^7 CFU g^{-1} soil and 450 g of powdered zeolite around the trunk divided between 3 holes 10 cm in depth. Treatments were applied to a block of 9 cacao trees.

It should be noted that the applications of the treatments were performed just prior to lockdown. Therefore, all the monitoring of the farms was affected. It was only possible to collect soil samples 1 year after application of the treatments. The farmers did not apply fertilizers or any other soil-based products during the course of this experiment. In the collected samples, the same parameters as those in Section 2.1 were measured.

2.8. Data Analysis

2.8.1. Phylogenetic Tree

A phylogenetic assignment of 5 CdtB strains identified in this research was verified using the GenBank database and Seqmatch version 5 of the Ribosomal Database Project (RDP). In addition, 5 referential strains found in Seqmatch, reported as CdtB, were also included in the phylogeny [8]. A 16S rRNA partial sequence of *Methanobacterium flexile* strain GH was used as an outgroup to root the tree. Phylogenetic analysis was performed using MEGA7 software. The distances were calculated using the two-parameter Kimura model. Unrooted trees were constructed by the neighbor joining method. The data set was bootstrapped 1000 times. The assigned accession codes were SAMN27387447, SAMN27387448, SAMN27387449 and SAMN27387450–SAMN27387451.

2.8.2. Kinetic Growth and Thermodynamic Parameters

The kinetic growth and thermodynamic parameters were calculated according to Richards equation [30,31,35] for the analysis of IMC data. To compare the kinetic growth parameters with heat production of each bacterium over time, a correlation was performed between the growth rate (μ) and the heat produced (Q). Moreover, the maximum growth rate (μ_{max}) calculated by calorimetry was correlated with the remaining soluble Cd (ions in a concentration of mg L^{-1}), measured at harvested cells in a parallel batch Cd immobilization test according to a previous study [8]. The adaptation phase, lambda (λ), was used to compare the differences between the CdtB to be adapted to soil enriched with Cd.

2.8.3. Soil Cadmium and Physical Chemical Properties

Descriptive statistics were applied to assess the differences in the soil cadmium, macro-elements and soil physical-chemical parameters between the starting conditions and 1 year after application of the treatments. The differences between the tested strains were verified with analysis of variance (ANOVA), performed in IMC analyses and bacterial immobilization capacities. The statistical package QtiPlot version 5.12.8 coupled to Python 3.7.2 was used. Tables and figures were constructed using Microsoft Excel and exported in QtiPlot. Due to the lack of field replicates, the statistical analysis of the field experiment is more descriptive, with no contrast among treatments.

3. Results

3.1. Soil Taxonomic Sub-Group, Cacao Varieties and CdtB Isolates

Five CdtB strains were isolated from the soils collected. Three were obtained from the surface soil layers (i.e., 0–20 cm deep), while the remaining two isolates corresponded to the samples taken in deeper layers (i.e., 20–40 cm). All the CdtB strains were isolated from farms A, C and D. It is worth mentioning that no isolates were obtained from farm B. Table 1 shows the soil type, cacao cultivar, planting year and the CdtB isolated at each farm.

Table 1. Cocoa varieties sampled in each of the farms and ages of the plantations. The ID of the cadmium-tolerant bacteria (CdtB) isolates is related to the farm origin.

Farm	Treatment	Planting Year	Cacao Cultivars	Soil Sub-Group	ID CdtB Isolates
A	Control	2016	TCS01, TCS06, TCS13, TCS19, CCN51	*Oxic Dystrudepts*	CdtB3, CdtB13
B	Strain A + zeolite	2010	FSV41, FSV155, FCH8, FTA2, CCN51	*Typic Kandiudox*	None
C	Strain B + zeolite	2015	FSV41, Hybrids	*Typic Kandiudox*	CdtB6, CdtB7.
D	Zeolite	2011	CCN51, ICS95	*Typic Kandiudox*	CdtB14.

Farm A has a topography with a slope ≥40%. The soil is well-drained with occasional waterlogging. The soil is dominated by expansive clay in water at a proportion of 2:1. The textural classes were up to 100 cm deep. Farm B presented a slightly flat topography. The soil is derived from igneous rocks with clay loam (first 10 cm of depth) and clay textures (50 cm deep) with moderate-to-low fertility. Farm C has a topography with a slope >50%. The soil has lighter colors on the surface and from red to yellowish red in the subsoil, while the texture found is predominantly clayey with low fertility up to 90 cm in depth. Farm D has a topography with slope ≥40%. The textural class was classified as sandy clay loam (first 9 cm in depth) and clay loam (85 cm deep). The soil taxonomy also indicated that three out of the four farms are oxisols (i.e., present a high degree of weathering). Additionally, there is great diversity in the cacao cultivars in all farms. Nonetheless, only the variety CCN51 is present in three out of the four farms.

3.2. Identification of Bacterial Strains by 16S rRNA

The sequences obtained from 16S rRNA analysis showed enough variability to distinguish the isolated CdtB strains. The five strains isolated in our study belonged to the *Bacillus* genus, which has been previously reported as a cadmium-tolerant bacteria. The presence of *Bacillus* spp. is related to the rhizosphere of cacao trees. Figure 1 shows an exact match between *B. rugosus* and CdtB6 and *B. aryabhattai* and CdtB13. All sequences were deposited in GenBank, and the accession codes are included in Figure 1.

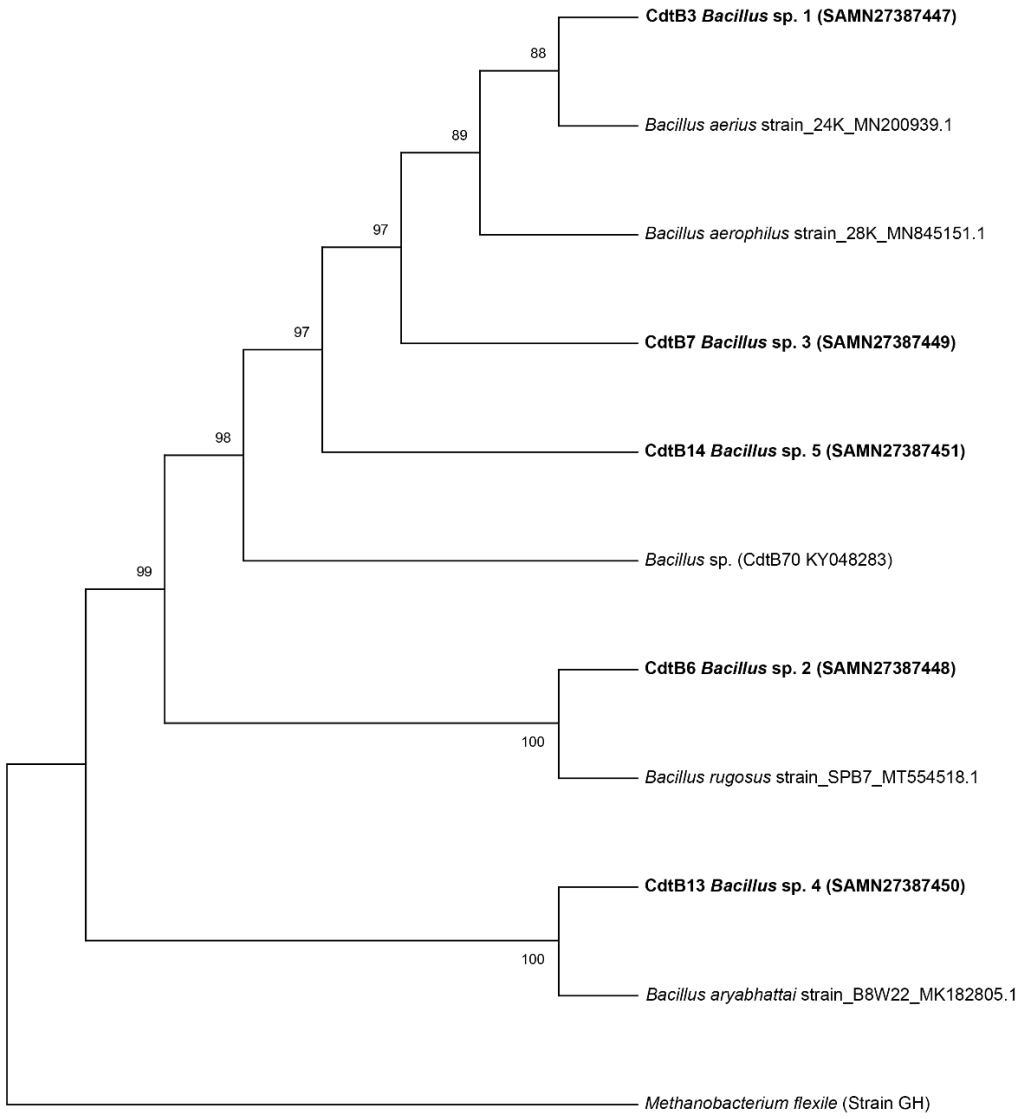

Figure 1. Phylogenetic relationship of cadmium-tolerant bacteria (CdtB) isolated in this study from cacao farms in Antioquia district. The strains were compared with reference sequences of CdtB isolated from other areas. The strains written in bold correspond to the CdtB isolated in this study.

3.3. Determining CdtB Metabolic Activity Using IMC

All the isolated CdtB strains exhibited a twofold increase in their metabolic rates when Cd was added to the media. Interestingly, as shown in Figure 2, they also showed greater immobilization activity than the control strains used for this experiment (Figure 2).

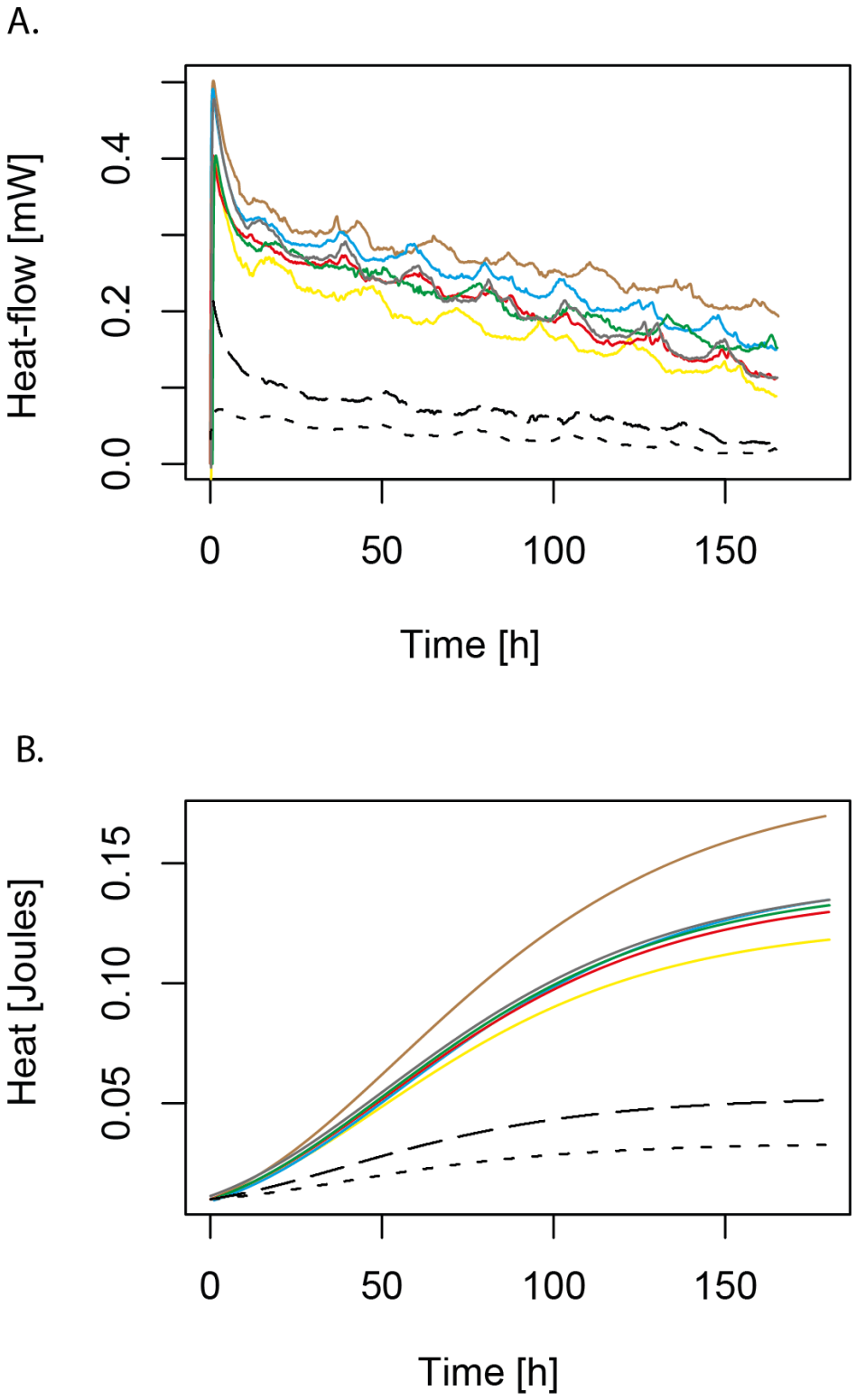

Figure 2. Calorimetric thermograms of bacterial metabolic activity. (**A**) The metabolic activity of CdtB strains by the heat flow compared to referential bacterial controls amended with cadmium in liquid culture media. (**B**) The heat produced in Joules due to the Cd immobilization activity of the assessed CdtB. Each color corresponds to the heat flow and the heat produced by one CdtB strain. Yellow = CdtB3, red = CdtB6, blue = CdtB7, green = CdtB13, brown = CdtB14, gray = CdtB41 (positive control), long dashed line = strain *E. coli* K12 DSM498 (negative control) and short dashed line = Mergeay's medium.

Table 2 shows the kinetic parameters estimated using the Richards equation [30,31,35] that fit the IMC data of the heat flow and heat to elucidate the growth rate (μ), the maximum growth rate (μ_{max}), the adaptation phase lambda (λ) and the maximum heat produced (Q_{max}). The strain CdtB14 exhibited the greatest μ (0.3 h^{-1}) and μ_{max} (0.4 h^{-1}) with statistically significant differences (Pearson coefficient $p < 0.05$). Furthermore, the same strain also exhibited the greatest Q_{max} of 0.090 Joules.

Table 2. Kinetical growth and thermodynamical parameters of cadmium-tolerant bacteria (CdtB) Cd immobilization activity. The parameters were calculated by fitting the Richards equation [30,31,35] to the heat flow data obtained by IMC. The *p*-value corresponds to the statistical significance of fitting the Richards equation into the heat flow data (for all parameters, n = 3).

Strain	μ [h^{-1}]	μ_{max} [h^{-1}]	λ [h^{-1}]	Q_{max} [J]	*p*-Value
CdtB3	0.100 ± 0.005	0.100 ± 0.006	0.191 ± 0.009	0.067 ± 0.005	0.997
CdtB6	0.200 ± 0.007	0.300 ± 0.009	2.137 ± 0.006	0.063 ± 0.004	0.997
CdtB7	0.100 ± 0.002	0.200 ± 0.003	0.572 ± 0.007	0.071 ± 0.006	0.997
CdtB13	0.100 ± 0.004	0.100 ± 0.005	2.382 ± 0.004	0.071 ± 0.007	0.997
CdtB14	0.300 ± 0.006	0.400 ± 0.007	3.757 ± 0.005	0.090 ± 0.006	0.997
CdtB41 *E. cloacae*	0.000 ± 0.000	0.001 ± 0.001	3.304 ± 0.003	0.069 ± 0.007	0.997
E. coli K12 DSM498	0.000 ± 0.000	0.000 ± 0.000	3.005 ± 0.005	0.052 ± 0.005	0.997
Mergeay + Cd	0.000 ± 0.000	0.001 ± 0.001	0.110 ± 0.003	0.005 ± 0.002	0.997

Conventions: μ = growth rate of the bacterial strain; μ_{max} = maximal growth rate estimated; λ = lambda, the adaptation phase of the assessed strains; Q_{max} = maximum heat produced by the strain in Joules.

When comparing the adaptation phase lambda (λ), the lowest parameter was found in strain CdtB3 at 0.191 h^{-1}, whereas CdtB13 and CdtB14 exhibited the greatest λ at 2.3 and 3.7 h^{-1}, respectively. This points out that strains 13 and 14 quickly adapted their metabolism and used Cd as the energy source [2].

3.4. Cd Immobilization by CdtB

Figure 3 shows the immobilization of Cd by each CdtB strain assessed and compared with the positive and negative controls and the blank. The biggest immobilization ratio occurred in strain CdtB14 with 0.6 mg of Cd L^{-1} followed by CdtB7 with 0.41 mg of Cd L^{-1}, a ratio also higher than the positive control strain *C. taiwanensis* with 0.23 mg of Cd L^{-1}, with statistically significant differences (Pearson coefficient $p < 0.05$). Other CdtB isolates in this study showed immobilization ratios lower than the positive control.

A metabolic characterization was performed using the Biolog Gen III microplate array (see Table S1). The profile shows that the strain CdtB3 was capable of mineralizing 1% NaCl, 4% NaCl, lithium chloride, Aztreonam and sodium butyrate. Moreover, the strain CdtB6 was able to mineralize the substrates: D-Meliobise, 1% NaCl, D-Mannitol, α-Hydroxy-Butiric acid, L-Aspartic acid, 1% sodium lactate, L-Glutamic acid, Guanidine HCl; Acetoacetic acid, Propionic acid, Acetic acid, Vancomycin, sodium butyrate and lithium chloride. The metabolic profile by mineralization of the C substrates from the strain CdtB7 was the highest of all the strains assessed. This substrate profile comprised 1% NaCl, 4% NaCl, 8% NaCl, 1% sodium lactate, D-Serine, D-Aspartic acid, L-Alanine, L-Aspartic acid, L-Glutamic acid, L-Serine, Lincomycin, Guanidine HCl, Quinic acid, citric acid, Bromo-Succinic acid, lithium chloride, potassium tellurite, Tween 40, Amino-Butryric acid, Acetoacetic acid, Propionic acid, Acetic acid, Aztreonam, sodium butyrate and sodium bromate. However, the higher metabolic profile to mineralize C substrates was observed in strain CdtB13. This strain degraded 45 out of 95 substrates, which shows a great versatility to adapt to several environmental stressors. The substrates degraded by Cdtb13 were D-Maltose, D-Trehalose, D-Cellobiose, Gentiobiose, sucrose, D-Turanose, Stachyose, D-Meliobise, β-Methyl-D-Glucoside, D-Salicin, N-Acetyl-D-Glucosamine, N-Acetyl-β-D-Mannosamine, 1% NaCl, 4% NaCl, 1% NaCl, D-Galactose, 1% sodium lactate, D-Mannitol, myo-Inositol, glycerol, D-Aspartic acid, L-Alanine, L-Arginine, L-Aspartic acid, L-Glutamic acid, L-Histidine, L-Pyroglutamic acid, Lincomycin, D-Gluconic acid, D-Glucoronic acid, Glucuronamine, Mucic acid, D-Saccharic acid, L-Lactic acid, citric

acid, α-Keto-Gluraric acid, L-Malic acid, Bromo-Succinic acid, lithium chloride, potassium tellurite, Amino-Butyric acid, β-Hydroxy-D,L-Butyric acid, Aztreonam, sodium butyrate and sodium bromate. CdtB14 (see Figure S1) showed a metabolic profile highlighting the mineralization of 15 substrates, including one of a pH level of 6, one antibiotic (lincomycin), three concentrations of NaCl (1, 4 and 8% *V/V*) and 10 carbon substrates, with complex substrates such as potassium tellurite and lithium chloride as well as sodium butyrate.

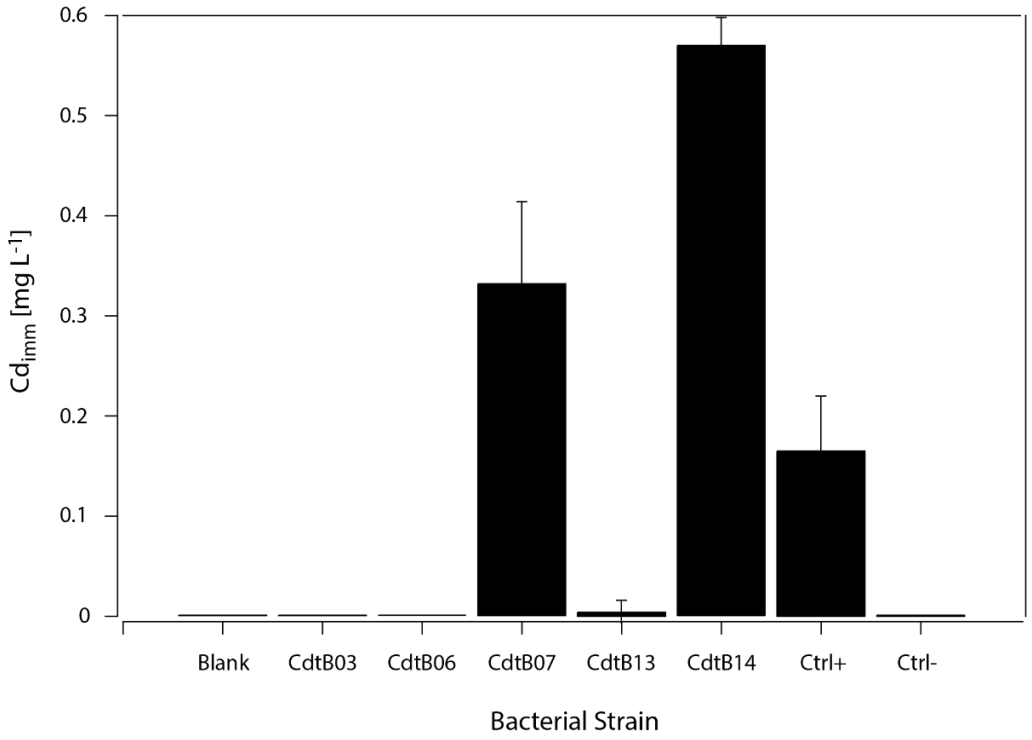

Figure 3. Results of the immobilization test using 6 mg L^{-1} cadmium chloride ($CdCl_2$). The average difference in Cd after 12 days following incubation of each strain in a solution of 6 mg L^{-1} $CdCl_2$. Cd_{imm} = Cd immobilized ratio after 12 days of incubation. The error bar indicates the standard deviation of the replicates (n = 3). Ctrl+ = positive control (*C. taiwanensis* DSM17343) and Ctrl− = negative control (*E. coli* K12 DSM 6722).

3.5. Application of Bioaugmented Inoculum of CdtB Strains and Zeolite in the Selected Cacao Farms

The effect of the application of CdtB and zeolite on the physicochemical parameters and Cd content in soils was analyzed in the four farms. More related data collected from the farms assessed here could also be found in a previous study in the Antioquias Magdalena basin area [16].

Table 3 shows the soil physicochemical properties before and after the application of treatments. The initial results showed that farms C and D contained soils with acidic pH levels, with values lower than 5.5 units, while farm A had a soil with low values of Al^{3+} and interchangeable acids. These variables showed a similar behavior for the assessed farms, except for farm C, which showed a higher Al^{3+} content and exchangeable acidity. The electrical conductivity averaged was of 0.26 $dS \cdot m^{-1}$. Regarding the Ca^{2+} content, low values were found in farms C and D, unlike farms A and B, which registered average values of the element (see Table 3). Mg^{2+} was detected at values lower than 1.5 $cmol^{+}$ kg^{-1} for the 4 farms, and the K^{+} values were highest in farm D. The phosphorus content was highest in farm B.

Table 3. Soil properties before and after treatment applications. Abbreviations: EC = electric conductivity, SOM = soil organic matter, CEC = cation exchange capacity and AlH = interchangeable acidity. Standard deviation was computed by n of each farm when replicates were obtained. Number of samples per farm (n): A before: n = 7, after: n = 2; B before: n = 2, after: n = 1; C before: n = 2, after: n = 1; D before: n = 2, after: n = 1.

Farm	Time	pH	EC	SOM	CEC	Ca	Mg	K	AlH	Al	P	Cd
			($dS \cdot m^{-1}$)	(%)	($cmol_+ \cdot kg^{-1}$)							($mg \cdot kg^{-1}$)
A	Before ‡	5.91 ± 0.34	0.34 ± 0.03	3.40 ± 0.16	7.92 ± 1.80	6.04 ± 1.57	1.43 ± 0.30	0.23 ± 0.04	0.14 ± 0.08	0.06 ± 0.05	3.87 ± 0	1.68 ± 0.03
	After	4.67	0.22-	4.07	3.49	1.34	0.39	0.13	0.83	0.46	2.66	1.68 ± 0.04 *
B	Before ‡	5.47 ± 0.47	0.24 ± 0.03	3.55 ± 0.07	7.07 ± 2.27	5.21 ± 2.14	1.05 ± 0.65	0.15 ± 0.06	0.59 ± 0.59	0.42 ± 0.42	12.41 ± 2.09	1.93 ± 0.06
	After	4.78	0.21	2.79	3.76	1.40	0.66	0.09	1.56	1.18	3.87	1.62 ± 0.01 *
C	Before ‡	4.73 ± 0.03	0.32 ± 0.05	3.57 ± 0.34	6.76 ± 0.34	2.77 ± 0.33	0.91 ± 0.04	0.14 ± 0.01	2.87 ± 0.03	2.31 ± 0.01	3.87 ± 0	1.49 ± 0.04
	After	6.06	0.34	5.52	11.41	8.64	2.57	0.15	ND	ND	6.05	1.19 ± 0 *
D	Before ‡	5.06 ± 0.04	0.17 ± 0.01	2.68 ± 0.17	3.74 ± 0.17	1.84 ± 0.15	0.80 ± 0.07	0.32 ± 0.08	0.69 ± 0.12	0.39 ± 0.12	5.30 ± 1.43	1.31 ± 0.02
	After	5.77	0.16	2.64	4.25	2.83	1.13	0.24	ND	ND	3.87	1.24 ± 0.01 *

‡ Numbers represent the average ± available standard deviation of three biological replicates. * Numbers represent the average ± standard deviation of three analytical replicates.

A year after application of the treatments, the soil chemical properties showed that the concentrations of Ca^{2+}, Mg^{2+}, K^+ and P decreased at farm A. This was also observed for the pH, which registered a decrease of 1.24 units. In farm B, the contents of Ca^{2+}, Mg^{2+}, K^+, P and SOM decreased, as well as the pH by 0.69 units. In farm C, the contents of Ca^{2+}, Mg^{2+}, K^+ and P showed an increase with respect to the initial values. Similarly, the pH had an increase of 1.33 units. In farm D, a decrease in K^+ and P was observed, but in contrast, the pH increased at this farm by 0.71 units. The exchangeable acidity and Al^{3+} contents were not detected (ND) for farm C in contrast to the 3 other farms evaluated, in which these values increased by 45%. The content of P only shows an increase in farm C and decreased in the other farms. This reduction was to a greater degree for farm B, with a percentage of 69%. Regarding the CEC, it only increased slightly in farm C, whereas in the other farms, it showed a slight decrease. Thus, it is highlighted that farm C has a greater capacity to retain and exchange nutrients. This shows a strong difference in soil parameters between the farms and sampling times.

It is worth mentioning that for the 4 farms, the available Cd content, estimated using the 0.01 M $CaCl_2$ method, was found to be below the detection limit of 0.005 mg kg^{-1} and therefore is not reported here. Regarding the initial pseudo-total Cd concentrations in the soil, farm B exhibited the highest content at 1.93 mg kg^{-1} and the farm D the lowest, with a total of 1.31 mg kg^{-1}. These values are within the same range as those reported in previous studies for soils in the studied area [16]. One year after the application of the treatments, in farm A, the content of Cd in the soil had not changed, while the greatest variation in soil Cd content was observed in farm B, which showed a decrease of 16%.

Figure 4 indicates the variation in the soil Cd content (ΔCd) between the treatments assessed in the field. The Cd concentrations in the soils showed decreases of 0.31, 0.30 and 0.07 mg kg^{-1} in farms B, C and D, respectively. The applications of CdtBs and zeolite showed the greatest decreases (Farms B and C), whereas a lower reduction was found in farm D, where only zeolite was applied. Nonetheless, we must stress that these results are preliminary and should be interpreted as such.

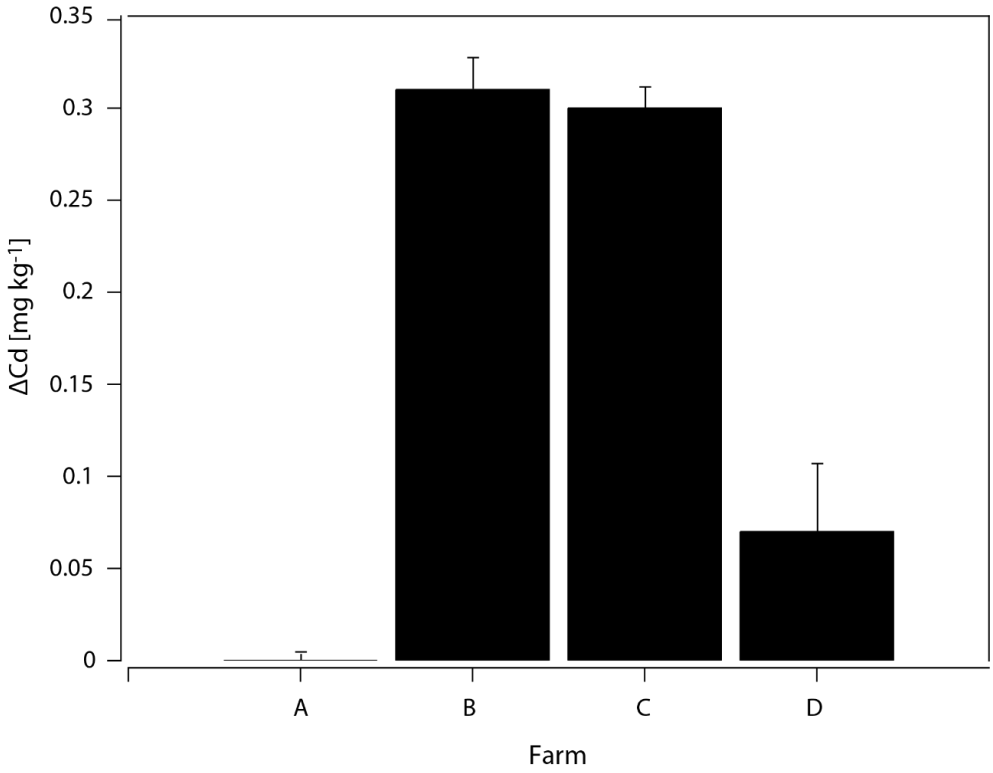

Figure 4. Variation of Cd content in soil (ΔCd) observed in the 4 farms assessed 1 year after applying the treatments. Cadmium content in soil by the closed digestion method by Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES) [21]. Farm A showed a minimal variation in Cd soil content (less than 0.004 mg kg^{-1} Cd).

4. Discussion

4.1. Soil Properties and Cd Availability

The soil Cd observed in this study is likely to be related to the weathering of the parent material in each of the soils and the potential input from chemically synthesized fertilizers used at each of the farms. However, the latter was not assessed in this research.

Several soil chemical factors have an impact on the availability of Cd for absorption by cacao trees. The most relevant are the soil pH, SOM and salinity. These factors are considered important in determining the most suitable agronomic practices for mitigation, such as by liming, where an alkaline amendment composed of calcium carbonate is added to the soil. This practice has been considered a remediation method under the premise that soils with alkaline pH decrease the plant-available soil Cd [36].

The soils found in this research were acidic, suggesting a high availability of Cd. In contrast, these soils contain higher concentrations of P, Al and Fe than other nutrients [37]. The concentration of P, for instance, varied in a range from 3.87 to 5.30 mg kg^{-1}. This P content is important to take into account when selecting CdtB to ensure a P solubilization capacity [8,9] in the context of the farms assessed here. Moreover, the low content of SOM found at the four farms (<4%) is probably unrelated to the availability of Cd in the soils. While this fraction has the capacity to retain Cd on its surface, this low percentage is unlikely to play an important role. This has been observed in a major database analysis in another study about the drivers of cadmium accumulation in cacao beans [38]. The study highlights that both the available soil Cd and soil organic carbon (SOC) content had negligible effects on the bean Cd.

4.2. Identification of Potentially Highly Effective CdtB Strains for Bioremediation

Calorimetry is a technique *in stato nascendi* and can be very useful for CdtB research [8,39,40]. In situ studies have demonstrated that the changes in heat flow in

soils with high Cd contents (natural or spiked) detected by IMC thermograms are due to the bacterial CdtB community [40]. Fungal activity can be distinguished from bacterial activity, as the metabolic activity is much higher in the former, being greater than 2000 μW [31], compared with 400–1000 μW, respectively [18,41]. In this study, the heat flow was found to be <1300 μW.

Interestingly, all the bacterial strains isolated in this study correspond to the genus *Bacillus.* This genus has been recognized in the cacao rhizosphere as nitrogen fixing and with an ability to solubilize and mineralize phosphorus and other nutrients, thus facilitating their absorption by the plant [42]. Moreover, this genus has previously been proposed as part of a practical strategy for the bioremediation of soils contaminated with Cd [15].

In terms of the IMC assessment in this study, it is worth mentioning that the heat-flow produced by CdtB14 is twice the maximum heat flow detected by the positive control, CdtB41, which has already been reported as a good bioindicator for cadmium immobilization due to the highest 'total heat' (Q) and maximum heat (Q_{max}) observed in CdtB isolated in cacao farms in Colombia prior to this study [8]. In spite of that, the strain CdtB14, found here, showed a greater Cd immobilization capacity than any of the strains previously reported. The strain *Bacillus* sp. CdtB14 showed a higher Cd immobilization capacity and ratio compared with the positive control (*Enterobacter cloacae* CdtB41 and other CdtB isolates found in a previous bioprospection study, such as *Achromobacter* sp. CdtBDB30, *Burkholderia* sp. CdtBDB36 and *Cupriavidus* sp. CdtBDB37).

Other important parameters assessed in this study were the maximum growth rate (μ_{max}) and the adaptation phase (λ). Again, comparing the strain CdtB14 with the positive control CdtB41, both parameters were greater for the former (0.400 h^{-1} and 3.757 h^{-1}, respectively). This means that this strain grows faster and, at the same time, adapts easily to higher Cd environments, both of which are key parameters in successful bioformulation.

4.3. Metabolic Plasticity of CdtB for a Challenging Environment

The metabolic plasticity of the CdtB isolated here was demonstrated by the Biolog Gen III microplate assay, where a wide variety of substrates were mineralized, specifically by the strain CdtB13 (45 out of 95 C substrates). Interestingly, the substrates lithium chloride, potassium tellurite, Amino-Butyric acid, β-Hydroxy-D, L-Butyric acid, Aztreonam and sodium butyrate were used for this strain, which might be related to the metabolic pathway of biochelation and bioweathering, also related to P solubilization with nine amino acids [2]. The strain CdtB14 showed a metabolic profile with high mineralization of 10 carbon substrates, where complex substrates such as potassium tellurite and lithium chloride, as well as sodium butyrate, were related to bioweathering, and the amino acids were more related to biochelation by the activation of the ATP-dependant P-type enzymes in the membrane of bacterial cells [2,43]. The adaptation to an acidic pH is also highlighted, since the strain was grown in pH 6. This adaptation could also be explained by the fast λ observed during the calorimetric experiment. Moreover, this strain was one of the few (CdtB7 also has the ability) that grows in the presence of an antibiotic such as lincomycin. This is important because the resistance to antibiotics is also related to heavy metal tolerance, as has been documented previously [2,44].

The response of CdtB to a high-Cd environment is related to several parameters, such as the temperature, pH, soil type, Cd enrichment and soil function. For instance, Cd-tolerant microorganisms have demonstrated a great metabolic plasticity by calorimetric assays, with a heat production between 350 and 500 μW in agricultural soils related to higher concentrations of Cd in the range of 1000–6000 μg g^{-1} [39].

One of the applications of IMC to characterize Cd immobilization is the use of data to calculate the inhibitory ratio (I) [40,45,46]. This will be used in forthcoming studies, applying the CdtB selected in this study, to understand how CdtB interact in the presence of Cd non-tolerant bacteria, fungi or both in soils enriched with different forms and concentrations of Cd.

The thermograms showed here pointed out that using IMC to select the Cd tolerance is an accurate tool to compare heat production related to the Cd immobilization capacity as well as Cd immobilization rates. However, this is just the first step, and more studies are needed to obtain a final well-designed bioproduct.

For example, a metagenomic analysis would be a great complimenting tool once the bioprospection has been carried out, especially for collecting subsamples on top of heat production peaks, using the thermodynamic parameter of the time to reach the maximum metabolic peak of heat (TTP) to take subsamples at that particular peak and assigning the corresponding CdtB populations tuned to Cd immobilization (Jaramillo et al. In Prep.). Future work with a subset of soil samples assessed in this study to follow this new approach will increase our knowledge on the metabolic plasticity of the CdtB selected in this initial bioprospection. Once a bioproduct has been developed, field experiments will be essential in determining how the selected CdtB reacts to field conditions.

4.4. Preliminary Results of the Co-Application of CdtB and Zeolite in the Selected Cacao Farms

While research carried out by the authors indicates that the strains CdtB13 and 14 have great Cd immobilization capacities in laboratory experiments, they are in the initial stage of characterization for their potential to be used as a bioproduct. However, these strains have not yet been produced massively throughout a pre-formulation stage designed for field trials. In contrast, strains A and B, used here in the field experiment, are in an advanced stage of bioproduct development and are currently available as a pre-formulated product. This is important, because a single bacterial inoculum that has not yet been conditioned to the environmental stresses it will experience in the field may not work due to bad formulation, rather than an inability per se to immobilize Cd.

Therefore, the preliminary results of the two CdtB strains applied here suggest a potential applicability to reduce the available Cd in the soil. While it has been pointed out in previous field studies [14] that the capacity of zeolite to retain Cd is less successful compared with other amendments, the application of a CdtB strain in combination with zeolite appears to be more effective than its application alone. This could be the result of the effect on soil parameters such as the CEC, Ca, Mg, K and P. Nonetheless, it remains unclear how it works, given that these parameters did not change in a consistent direction in farms B and C (both of which received zeolite and a CdtB strain). Additionally, the CdtB was not applied alone or in consortium to each farm, so the cause of these changes is unclear. It is also important to understand if these changes in Cd content in the soil are sufficiently significant to lead to a decrease in Cd in the cacao bean. Due to the P and K solubilization capacities of CdtB, the metabolic pathways of bioweathering and biochelation [2] might explain the variation of these parameters in farms B and C, but this needs to be confirmed with meta-transcriptomic assays.

The retention of Cd in this study could be related to zeolite and the increasing CEC in farm D. Several studies have reported the effect of amendments on the immobilization of Cd due to increase in CEC [14,47,48]. Here, the decrease in Cd in soils occurred in farms where the CdtB treatments were applied with zeolite. Even though we did not find any direct correlation between the CdtB and the soil parameters before and after applications, the use of CdtB could be very useful due to its ability to help to reduce the available Cd in a well-designed amendment. This reduction occurs because the bioavailable Cd in soils is affected by CdtB through various immobilization, translocation and mineralization mechanisms used by these microorganisms [2].

Although our soil Cd reduction in the assessed cacao farms could be considered promising results, our findings should be considered as preliminary because of the lack of replication and long-term monitoring (i.e., number of samples taken in consecutive years) as well as the lack of data on the accumulation of Cd by the plant (considering the Igeo or the Geoaccumulation indexes and translocation factor [40]). It is important to determine the effect of CdtB on the bean Cd concentration. Nevertheless, this section intends to show that the application of the bioprospection processes to solve a real field problem is currently

under study. Despite that, this is the first time that the selected CdtB have been applied in a field experiment on cacao plantations in Colombia. We highlight this statement because even if the beneficial application of CdtB has also been observed in other crops such as rice [10,46–49], Indian mustard [35], tomato [50] with similar reductions (3–20% reductions on average) and in other pollutants such as Pb [49,50] in other regions, the use of CdtB for the Cd content in cacao soils is innovative in neotropical regions in America as well as worldwide.

This research is still ongoing in the four farms mentioned in this study. Thus, more conclusive and solid data will be presented in the near future. Additionally, the effect of both CdtB strains on soil parameters should be studied in a systematic manner with more replicates using single- and mixed-strain applications. Considering that cacao is a perennial crop, it is expected that the results will display significant changes after some years of treatment.

5. Conclusions

In this study, five CdtB from four cacao farms in the district of Antioquia, Colombia were isolated, and their Cd immobilization capacities were tested. The use of calorimetry in combination with a Cd immobilization test resulted in an understanding of both the Cd immobilization capacity and Cd immobilization ratios. The strain *Bacillus* sp. CdtB14 showed a higher Cd immobilization capacity and ratio compared with the positive control (*Enterobacter cloacae* CdtB41 and other CdtB isolates found in a previous bioprospection study, such as *Achromobacter* sp. CdtBDB30, *Burkholderia* sp. CdtBDB36 and *Cupriavidus* sp. CdtBDB37. On top of that, the metabolic plasticity was demonstrated by the strain CdtB14 with potassium tellurite and lithium chloride, two complex carbon sources linked to the biochelation of heavy metals.

Furthermore, two bacterial strains used in the field bioaugmentation experiments (A and B) in addition to zeolite displayed a potential ability to immobilize the soil Cd, reducing the initial concentrations by 16% and 20%, respectively. Thus, we suggest that the use of a bioremediation technique may be a novel alternative to Cd mitigation in cacao, in addition to the use of soil amendments. Despite the fact that these results are preliminary, and the change in accumulation of Cd in the cacao bean has yet to be validated, the results analyzed here are promising and have merit to be 'spread out'. This ongoing study will continue for several years to understand better the utility of Cd bioremediation.

In forthcoming studies, metagenomic studies should be included to assess the effect of CdtB bioaugmentation in cadmium non-tolerant microbial populations and in key soil parameters that might influence plant nutrition in cacao. Moreover, the trade-offs of the co-application of the different CdtB strains in tandem should also be a target in forthcoming bioproduct development for future field trials under different environmental conditions.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/pr10081457/s1. Figure S1: Plot of metabolic profile of the strain CdtB14 obtained by the Biolog Gen III Microplate assay. Table S1: Raw data of the metabolic profiles of the assessed CdtB strains by Biolog Gen III Microplate assay.

Author Contributions: Conceptualization, D.B. and E.C.; methodology, R.Q.-M. and S.L.-Z.; software, D.B. and E.C.; validation, D.B., E.C. and R.Q.-M.; formal analysis, D.B., R.Q.-M., S.L.-Z. and E.C.; investigation, E.C. and D.B.; resources, E.C.; data curation, E.C.; writing—original draft preparation, R.Q.-M., S.L.-Z. and D.B.; writing—review and editing, R.Q.-M., D.B. and E.C.; visualization, R.Q.-M.; supervision, D.B. and E.C.; project administration, E.C. and D.B.; funding acquisition, E.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research and the APC fees were funded by FONTAGRO through the project 'multi-agency cocoa platform for Latin America and the Caribbean Cocoa 2030-2050' with grant ATN/RF-17235-RG. The opinions expressed in this publication are exclusively those of the authors and do not necessarily reflect the point of view of FONTAGRO, its Board of Directors, or the countries it represents. This manuscript was also financed by Agrosavia under grant number 1001351.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data obtained during this study could be partially shared under reserve of the correspondence author.

Acknowledgments: We thank to the senior researcher Rachel Atkinson from Bioversity/CIAT Alliance in Lima, Peru for the proofreading and technical suggestions of this manuscript. We would also like to thank Yeni Rodriguez, head leader of the Laboratory of Soil Chemistry, C.I. Tibaitatá in Mosquera, Colombia, for timely provision of the soil data during the edition of this manuscript.

Conflicts of Interest: The authors declare no conflict of interest. Aside from that, the funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript or in the decision to publish the results.

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