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Presence of *aiiA* homologue genes encoding for N-Acyl homoserine lactone-degrading enzyme in aflatoxin B_1-decontaminating *Bacillus* strains with potential use as feed additives

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ABSTRACT

Microbial degradation of aflatoxins (AFs) is an alternative to the use of mycotoxin binders. The lactone ring is a possible target for microbial enzymes and its cleavage reduces AFs toxicity. The aim of this study was to isolate and identify *Bacillus* strains able to degrade AFB_1 to less toxic metabolites and to identify *aiiA* genes encoding for N-acyl-homoserine lactone (AHL) lactonase to possibly correlate detoxification with the production of this enzyme. Eleven soilborne *Bacillus* strains were isolated and identified by MALDI-TOF MS. Ten cultures and eight cell free culture supernatants (CFCS) were able to significantly ($P < 0.05$) degrade 27.78–79.78% AFB_1. Cell lysates were also able to degrade AFB_1 ($P < 0.05$). Exposure to 70 and 80 °C did not affect enzyme activity. Aflatoxin B_1 toxicity towards *Artemia salina* was reduced after degradation by each of the *Bacillus* strains. *B. subtilis* RC1B, *B. cereus* RC1C and *B. mojavensis* RC3B, amplified a fragment of 753 pb corresponding to the *aiiA* gene encoding for AHL lactonase. AFB_1 degradation by the strains tested was due to the extracellular and intracellular enzymes. If demonstrated to be safe, these could be used to detoxify AFB_1 in contaminated food or feed.

1. Introduction

The presence of fungal toxins is inherent to numerous food and feed products throughout the world (Bhat et al., 2010). Aflatoxins (AFs), produced by *Aspergillus* section *Flavi* (mainly *A. flavus* and *A. parasiticus*) are included among the most dangerous and toxic secondary metabolites produced by fungi due to carcinogenic, mutagenic and teratogenic effects, especially aflatoxin B_1 (AFB_1) (IARC, 2002). Exposure to high levels of AFs produce acute aflatoxicosis and death in many animal species, while long-time exposure to low concentrations causes chronic toxicity and low performance in production animals (CAST, 2003). Since good agricultural, storage and transportation practices are not enough to completely prevent the entrance of mycotoxins to the food and feed chain, decontamination strategies are needed to minimize risk and economic losses (Bhat et al., 2010; Vanhoutte et al., 2016).

Diverse decontamination technologies to reduce mycotoxin levels in food and feed are offered as a last resort to salvage contaminated batches along the production chain (Vanhoutte et al., 2016). Strategies comprise physical, chemical, or biochemical principles. One popular removal strategy is the use of inorganic or organic mycotoxin binders (Kolosova and Stroka, 2011); however, some may also bind vitamins and minerals causing adverse nutritional effects (Huwig et al., 2001) or reduce the efficacy of antibiotics (De Mil et al., 2015). Microbial based decontaminating methods include mycotoxins degradation, transformation or adsorption. Microbial degradation of mycotoxins has been extensively reviewed by several authors (Adebo et al., 2017; Awad et al., 2010; Hathout and Aly, 2014; McCormick, 2013; Vanhoutte et al., 2016; Wu et al., 2009; Zhu et al., 2017 Zinedine et al., 2007). However, some of the studies reviewed wrongly consider biodegradation as a synonym of detoxification, or do not test the potential toxicity

Abbreviations: AFB_1, aflatoxin B_1; AFs, aflatoxins; AHL, N-acyl-homoserine lactone; CFCS, cell free culture supernatant; LOD, limit of detection; NA, nutrient agar; NB, nutrient broth; TLC, thin layer chromatography

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of degradation metabolites.

The toxicity of AFB_1 is mainly caused by its lactone moiety. The cleavage of AFB_1's lactone ring produces a non-fluorescent compound with 450 times reduced mutagenicity and 18 times reduced toxicity (Lee et al., 1981). The use of microbial enzymes capable to degrade AFs is an alternative to mycotoxin binders. The lactone ring is a possible target for these enzymes, since the cleavage of this structure would result in less toxic derivatives. A novel approach to microbial detoxification of mycotoxins is based on known biodegradation metabolisms of other substances that are structurally similar to mycotoxins. N-acyl homoserine lactone (AHL), which is a quorum sensing molecule associated with biofilm formation, also has a lactone ring in its structure. AHL lactonases are a family of bacterial metallo-enzymes that participate in the degradation of AHL to interrupt the Gram negative quorum sensing detection system. The *Bacillus cereus* group species (*B. cereus, B. thurigensis, B. mycoides* and *B. anthracis*), *B. subtilis, B. amyloliquefaciens* and *B. weihenstephanensi* are known to produce AHL lactonases that can hydrolyse the ester bond of the lactone ring (Huma et al., 2011; Pan et al., 2008; Sakr et al., 2013; Yin et al., 2010). These enzymes are encoded in the *aiiA* genes and its function is to auto-regulate AHL (Wang et al., 2004). The potential activity of these lactonases with respect to mycotoxins still remains elusive. New insights on microbial detoxification routes are necessary to increase the resource of new, more efficient microbial mycotoxins degraders. Screening surveys of microbial communities capable of degrading other undesirable, hazardous or recalcitrant mycotoxins-analogue molecules will lead to the finding of new mycotoxins degraders (Vanhoutte et al., 2016).

The aim of the present study was to isolate and identify *Bacillus* strains with the ability to degrade AFB_1 to less toxic metabolites and to identify the *aiiA* genes in their DNA to possibly correlate detoxification with AHL-lactonase production.

2. Materials and methods

2.1. Bacillus strains isolation

Eleven *Bacillus* sp. isolates were obtained from pond mud and soil samples according to Sosa Pech et al. (2012). Observations of colony and cell morphological traits after Gram stain were recorded after 24 h incubation. At 72 h, spore formation was confirmed with malachite green stain. Isolates showing *Bacillus* genus' characteristics (Gram-positive, catalase-positive, rod-shaped, spore forming bacilli) were tested for AFB_1 degradation. Three colonies of each isolate were inoculated in microtubes containing 0.2% glycerol and stored at −80 °C for future studies.

2.2. Bacillus identification

Single colonies of 24 h cultures of each *Bacillus* strain were sent to Hospital Privado de Córdoba for species identification by MALDI-TOF MS using a Bruker Daltonics MALDI Biotyper (Bruker Daltonics, Inc., GmbH, Leipzig, Germany).

2.3. Aflatoxin B_1 degradation assays

2.3.1. Aflatoxin B_1 degradation screening by Bacillus cultures and cell free culture supernatants

The ability of the *Bacillus* cultures and CFCS to degrade AFB_1 *in vitro* was first screened using a test based on a methodology described by Zhao et al. (2010) with some modifications. Aflatoxin B_1 degradation was first tested at one temperature (30 °C) and one incubation time (48 h). Briefly, each strain was inoculated in 50 ml nutrient broth (NB) and incubated for 48 h at 30 °C and 150 rpm. Eight-hundred µl aliquots of cultures were transferred to microtubes (in triplicates), added 200 µl of a 2500 ng/ml AFB_1 solution and incubated for 48 h at 30 °C in the darkness. CFCS were obtained by centrifuging and filtering (0.22 µm pore) cultures under sterile conditions and aliquots (800 µl) were added 200 µl of the AFB_1 solution and incubated for 48 h at 30 °C in the darkness. Sterile NB medium (800 µl) supplemented with 200 µl AFB_1 solution was used as control (three replicates). After incubation, microtubes containing culture + AFB_1 were centrifuged at 5000 rpm 10 min and the supernatant was transferred to a clean glass vial. Culture supernatants, CFCSs + AFB_1 and controls were extracted three times with equal volume of chloroform (1:1, v/v) according to Teniola et al. (2005) and the combined extracts evaporated to dryness under N_2 stream. Extracts were redissolved in 500 µl mobile phase (methanol:acetonitrile:water 1:1:4, v/v) and AFB_1 content was analysed by HPLC according to Trucksess et al. (1994). For derivatization, aliquots (200 µl) were mixed with 700 µl of acetic acid:trifluoroacetic acid:water (5:10:35, v/v) solution and allowed to stand for 8,5 min at 65 °C in the dark (AOAC, 1994). An HPLC Waters Alliance 2695 system coupled to a fluorescence detector (Waters 2487) was used. Excitation and emission wavelengths were set on of 360 nm and 440 nm, respectively. Separation was carried out in a C18 Luna Phenomenex column (150 × 4.6 mm, 5 µm). The mobile phase was pumped at 1.5 ml/min flow rate. The retention time for AFB_1 was 4.7 min and the limit of detection (LOD) was 0.5 ng/ml. A calibration curve was constructed using 5; 30 and 50 ng/ml AFB_1 standards and quantification of the toxin was calculated by comparison of peak areas. The ability to reduce the amount of AFB_1 in the CFCS tubes (compared to the control) were considered to degrade the toxin by extracellular enzymes, whereas strains that reduced a higher percentage of AFB_1 in tubes containing the whole culture than in tubes containing CFCS were consider to decontaminate mainly by production of intracellular enzymes or even by adsorption of the toxin to the cell surface. All treatments were done in triplicates.

2.3.2. Aflatoxin B_1 degradation by Bacillus cell free culture supernatants at different incubation times

The progression of AFB_1 degradation was studied over a 72 h incubation period evaluating residual AFB_1 content by HPLC (as described in 2.3.1) at 24, 48 and 72 h. The degradation assay was performed according to described in 2.3.1 adding 200 µl of a 1000 ng/ml AFB_1 working solution to 800 µl of each strain's CFCS in sterile microtubes. Six replicates of each were made and two were incubated for 24 h, two for 48 h and two for 72 h at 30 °C. After each incubation period, AFB_1 was extracted as described by Iram et al. (2015). The reaction was terminated by adding equal volume (1 ml) of chloroform and the residual toxin extracted by agitating the mixture thoroughly (vortex). The chloroform fraction was separated by low speed centrifugation (2000 rpm for 5 min) and the organic phase (bottom) was withdrawn and 400 µl aliquots were evaporated to dryness under nitrogen stream. The extracts were redissolved in 100 µl acetonitrile and derivatized with 350 µl acetic acid:trifluoroacetic acid:water (5:10:35, v/v) solution and allowed to stand for 8,5 min at 65 °C in the dark (AOAC International, 1995) and analysed by HPLC according to Trucksess et al. (1994). Controls consisted of 800 µl of culture media added 200 µl of AFB_1 working solution incubated in the same conditions as samples. The visualization of new peaks corresponding to degradation products in the chromatograms was recorded along with their retention times, area and height values. All treatments were done in triplicates.

2.3.3. Aflatoxin B_1 degradation by cell lysates and enzyme activity at different temperatures

In order to extract intracellular enzymes (included AHL lactonases if present) from the cell, *Bacillus* strains that resulted positive to *aiiA* genes amplification (after procedures described in 2.6.) were cultivated in NB as described in 2.3. and cells were harvested by centrifugation (10 min, 3000 g, 4 °C), washed twice with 30 mM Tris/HCl 1 mM EDTA buffer [pH 7.6] and resuspended in the same buffer. The cell suspensions were stored for 15 min on ice and then ultrasonicated for pulses of

Table 1
Average residual AFB_1 (ng/ml) and AFB_1 degradation percentages after 24, 48, and 72 h incubation with *Bacillus* strains.

Strain	24 h				48 h				72 h			
	Residual AFB_1 (ng/ml)	SD*	Fisher's LSD test**	AFB_1 degradation %	Residual AFB_1 (ng/ml)	SD*	Fisher's LSD test**	AFB_1 degradation %	Residual AF_{B1} (ng/ml)	SD*	Fisher's LSD test**	AFB_1 degradation %
AFB_1 control	203.751	3.86	m	–	203.751	3.86	m	–	203.751	3.86	m	–
B. mojavensis RC1A	102.663	8.79	abcdefgh	49.62	72.029	0.05	abc	64.65	83.582	3.30	abcd	58.98
B. subtilis RC1B	170.085	10.02	klm	16.53	93.103	3.08	abcdef	54.31	103.733	10.83	abcdefgh	49.09
B. cereus RC1C	126.25	2.80	defghijk	38.04	144.889	0.56	ghijkl	28.89	118.961	9.21	cdefghij	41.62
B. mojavensis RC3A	108.37	3.75	abcdefghi	46.82	108.114	1.50	abcdefghi	46.93	69.16	1.17	ab	66.06
B. mojavensis RC3B	101.012	15.77	abcdefg	50.43	90.289	0.87	abcde	55.69	90.63	0.72	abcde	55.52
B. cereus RC3C	149.937	8.19	hijkl	24.42	172.078	9.24	klm	15.55	95.678	0.49	abcdef	53.05
B. cereus RC3E	126.475	41.37	defghijk	37.93	106.819	5.52	abcdefghi	47.58	100.278	10.13	abcdegh	50.79
B. mycoides RC4A	166.002	26.28	jklm	18.53	154.767	29.07	ijkl	24.05	137.096	0.77	efghijk	32.72
B. mycoides RC4B	95.326	0.04	abcdef	50.22	137.735	0.05	efghijk	32.41	65.547	2.24	a	67.83
B. subtilis RC6A	144.486	4.21	ghijkl	29.09	188.954	0.07	lm	7.27	83.078	5.68	abcde	59.23
B. cereus RC6B	144.889	4.67	ghijkl	28.89	138.942	0.93	fghijk	31.81	113.658	1.03	bcdefghi	44.22

*SD: Standard deviation; **Medias sharing the same letter are not significantly different (ANOVA ($P > 0.0001$) and Fisher's LSD Test ($P > 0.001$), InfoStat).

30 s each with 12 s cooling intervals (Wu et al., 2009; Thomas et al., 2005). Cell debris was removed by centrifugation (15 min, 10000 g, 4 °C) and the cell lysates were collected. Six replicates per strain were obtained. Lysates and CFCS of these strains were exposed to 25, 70 and 80 °C for 20 min (in duplicates) to study the effect of temperature on enzyme activity. After cooling cell lysates and CFCS were used to perform a AFB_1 degradation assay using the procedure described in 2.3. Concentration of the AFB_1 standard solution used fort this assay was 1000 ng/ml. All treatments were done in triplicates.

2.4. Degradation product toxicity

Toxicity of AFB_1 degradation products obtained in the CFCS degradation assay was tested using the acute toxicity test on brine shrimp (*Artemia salina*). Cysts (0.1g) were incubated in 1 l laboratory marine solution (38 g NaCl + 1000 ml commercial bottled water) for 72 h at 24–25 °C in the light and with aeration. After hatching, 2 ml of marine solution containing 30 naupilii were collected and transferred to petri dishes. Viability of naupilli was confirmed by observation of motility and larvae were transferred to glass tubes containing: i) NB + 100 ng AFB_1 extract, ii) degradation extracts of 100 ng AFB_1 obtained with each of the *Bacillus* strains (homogeneized in 1 ml water), iii) AFB_1-free NB extract or iv) marine solution controls (negative controls). All treatments were performed in triplicates. Tubes were completed to 5 ml final volume with marine solution and incubated for 24 h at 24–25 °C. Mortality (%) of naupilii was determined at 120 h and 24 h under a stereoscopic microscope according to the following equation:

$$Mortality\ \% = \frac{number\ of\ dead\ organisms}{total\ number\ of\ organisms} x\ 100$$

(Ďuračková et al., 1977).

2.5. aiiA genes amplification

2.5.1. DNA extraction

A pure colony of each isolate grown on a NA slant was transferred to 50 ml of NA broth and incubated at 30 °C and 15 rpm for 48 h. After incubation, two ml of culture were centrifuged (12000 g, 15 min). DNA was extracted using 700 µl of extraction buffer (200 mmol l^{-1} Tris-HCl pH 8, 25 mmol l^{-1} EDTA pH 8, 25 mmol l^{-1} NaCl, 1% SDS) and incubated for 30 min at 65 °C. Deproteinization was performed twice using equal volume of chloroform:isoamyl alcohol (24:1), following the procedure proposed by Leslie and Summerell (2006).

2.5.2. Polymerase chain reaction

The full length of the *aiiA* gene was amplified using the primer pair AiiA1/AiiA2 designed based on the consensus sequences on known *aiiA* genes deposited in Genebank (Pan et al., 2008). Primer sequences were AiiA1 (forward primer), 5′-ATGACAGTAAARAARCTTTATTTC-3′ and AiiA2 (reverse primer), 5′-TCACTATATATAYTCMGGGAACTC-3′. The amplification was carried out in a 50 µl reaction volume containing 20 mM $MgCl_2$, 0.2 mM of each of the four dNTPs, 0.5 µM of each primer and 2.5 U *Taq* DNA polymerase. PCR was carried out for 30 cycles (at 94 °C for 1 min, 55 °C for 1.5 min, 72 °C for 2 min) according to Pan et al. (2008). The PCR product was analysed on a 1.5% agarose gel stained with 0.5 µg ml^{-1} ethidium bromide. Gels were photographed using a MiniBIS Pro analyser (DNR Bio Imaging Systems, Jerusalem, Israel). The fragment sizes were measured by comparison with a 100-bp DNA ladder (Invitrogen by Life Technologies, Buenos Aires, Argentina).

2.6. Lactone ring cleavage

Cleavage of AFB_1's the lactone ring was estimated by evaluating the loss or attenuation of fluorescence by thin layer chromatography (TLC) following the methodology described in the Official Methods of Analysis (AOAC International, 1995). Briefly, the extracts from the AFB_1 degradation assays were screened for AFs by spotting 10 µl of each extract together with 2, 5 and 10 µl of an AFB_1 standard solution (0.5 ng/ml) on a silica gel 60 TLC aluminium sheet (20 × 20 cm, Merck, Darmstadt, Germany) and developed with chloroform: acetone (90: 10 v / v). Chromatograms were air dried and observed under 365 and 254 nm UV light. The cleavage of AFs lactone ring was determined by visual comparison under UV light with standard solution that emitted a blue fluorescence (AFB_1). The loss of fluorescence indicated the possible cleavage of the lactone ring. The relative amounts of AFB_1 were quantitatively determined by visual comparison under UV light with the spots of the standard solution of known toxin concentration. Limit of detection of the method was 1 ng AFB_1 in a 2 µl spot of a 0.5 ng/ml standard solution.

3. Results

3.1. Bacillus identification

Three strains were identified as *B. mojavensis*, two as *B. subtilis*, four as *B. cereus*, and two as *B. mycoides,* with high score values, using the MALDI-TOF MS method (Table 1).

3.2. Aflatoxin B_1 degradation

3.2.1. Aflatoxin B_1 degradation screening

All strains were able to significantly ($P < 0.05$) degrade AFB_1 in

culture and CFCS after 48 h incubation at 30 °C showing degradation percentages that ranged from 26.56 to 79.28% and 20.14–71.56%, respectively (data not shown). *Bacillus subtilis* RC1B, *B. cereus* RC1C and *B. mojavensis* RC3B showed significantly higher ($P < 0.05$) toxin reduction percentage in culture compared to CFCS, most probably due to degradation by intracellular enzymes.

3.2.2. Aflatoxin B_1 degradation by Bacillus cell free culture supernatants at different incubation times

All strains maintained their AFB_1 degrading ability shown in the screening. (Table 1). Average AFB_1 concentration in controls was 203.751 ng/ml, evidencing no degradation due to culture conditions. Residual AFB_1 concentrations after degradation with the different *Bacillus* strains ranged from 137.096 to 65.547 ng/ml and were significantly lower ($P < 0.001$) than the concentration in controls (Table 1). Aflatoxin B_1 degradation by strains RC3E, RC4A and RC6B increased progressively from 24 to 72 h. Strains RC1C, RC3A, RC3C, RC4B and RC6A reached their degradation maximum at 72 h, while for RC1A, RC1B and RC3B maximum degradation was achieved at 48 h. Degradation percentages ranged from 16.53 to 50.43 ng/ml at 24 h incubation time, from 7.27 to 64.64 ng/ml at 48 h and from 32.72 to 67.83 ng/ml at 72 h (Fig. 1). Strains RC1A, RC3B, RC4A, RC4B and RC3E showed significantly higher ($P < 0.0001$) degradation percentages in the first 24–48 h. All stains produced a degradation product peak that appeared in chromatograms at 11–12 min RT, most frequently at 72 h RC1A starts to produce it at 24 h and continues to produce it continuously. All strains except RC1C showed a 6 min RT peak of maximum concentration at 48 h. In addition, RC3B and RC3C showed a 5 min RT peak (Table 2).

3.2.3. Aflatoxin B_1 degradation by cell lysate extracts and enzyme activity at different temperatures

All CFCS and cell lysates were able to reduce AFB_1 concentration compared to the control. The higher degradation percentages were observed in *B. cereus* RC1C CFCS pre-exposed to 70 °C and *B. mojavensis* RC3B CFCS pre-incubated at 25 °C (77.24 and 75.33%, respectively). Degradation percentages obtained with CFCS ranged between 39.35 and 77.24% and degradation percentages obtained with cell lysates ranged between 38.18 and 53.32%. The strain *B. cereus* RC1C obtained the higher degradation percentages in each kind of extract, CFCS as well as cell lysates, when pre-exposed to 70 °C for 20 min. Exposure to high temperatures (70 and 80 °C for 20 min) did not affect enzyme activity in any of the extracts tested (Table 3).

3.3. Degradation product toxicity

All *Bacillus* strains extracellular fractions (CFCS) were able to significantly ($P < 0.05$) reduce AFB_1's toxicity according to *Artemia salina* toxicity test compared to the original toxin (AFB_1 control) after 48 h incubation at 30 °C (Table 4).

3.4. aiiA gene amplification

Three of the analysed strains, *B. subtilis* RC1B, *B. cereus* RC1C and *B. mojavensis* RC3B, amplified a fragment of ~ 800 bp corresponding to the *aiiA* gene encoding for AHL lactonase (Fig. 2). The PCR product was sequenced at Macrogen Inc. (Seoul, Korea) and the sequence of strain RC3B was submitted to NCBI Genbank database (ID 2168898). The sequences of the three strains showed 99% homology with *Bacillus* sp. B546 AHL-lactonase (aiiA) gene complete CDS (Accession FJ816104.1) after NCBI BLAST Nucleotide Sequence analysis.

3.5. Lactone ring cleavage

Cell free culture supernatants as well as cell lysates of all *Bacillus* strains were able to attenuate AFB_1 fluorescence compared to the control indicating a possible cleavage of the lactone ring. Exposure of CFCS to 70 °C and 80 °C for 20 min did not affect enzyme activity (Fig. 3).

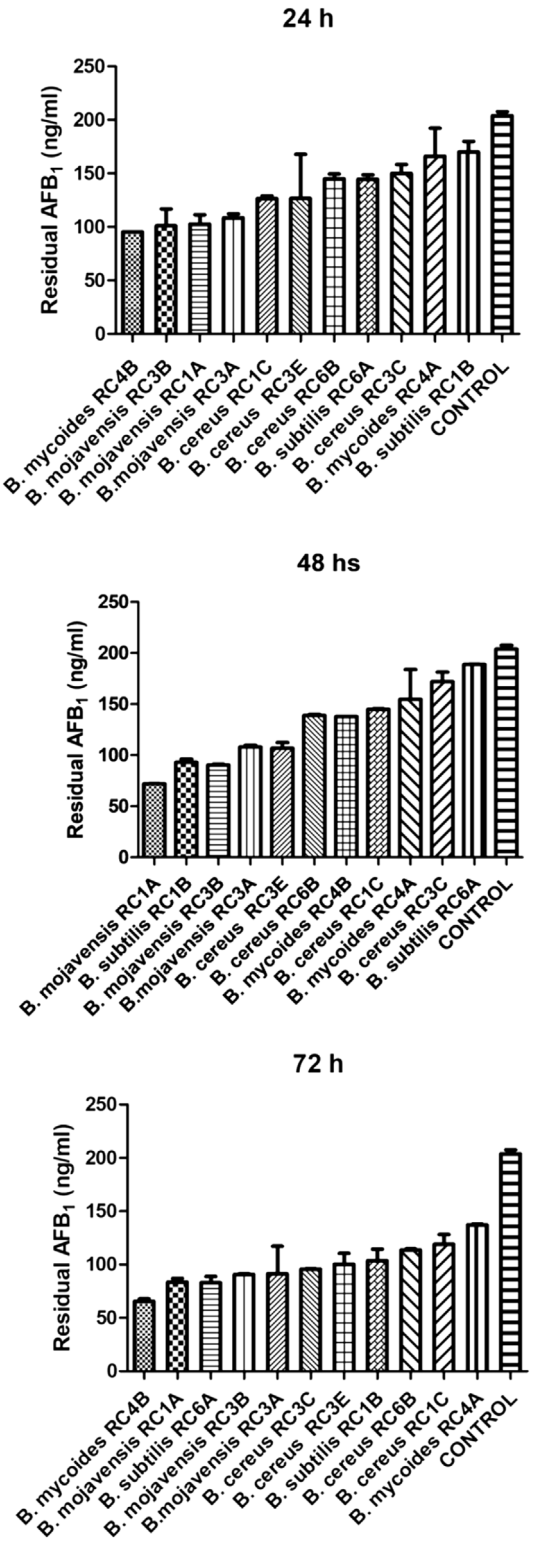

Fig. 1. Residual AFB_1 concentration (ng/ml) after 24, 48 and 72 h and 30 °C incubation with *Bacillus* strains cell free culture supernatants (CFCS) and nutrient broth used as control.

Table 2
Retention time and area (media ± standard deviation) of AFB_1 degradation products peaks after 24, 48 and 72 h incubation with *Bacillus* cell free culture supernatants.

Treatment	24 h		48 h		72 h	
	TR*	Area (media ± SD**)	TR*	Area (media ± SD**)	TR*	Area (media ± SD**)
Control	ND***	ND***	ND***	ND***	ND***	ND***
B. mojavensis RC1A	–	–	6.054	1.4 ± 0	–	–
	11.865	19 ± 13.57	11.553	7.8 ± 2.68	12.459	21.05 ± 12.09
B. subtilis RC1B	–	–	6.153	2.4 ± 0	6.53	2.4 ± 0
	12	1.3 ± 0			12.789	16.9 ± 9.47
B. cereus RC1C			11.565	2.2 ± 0	12.749	13.6 ± 0
B. mojavensis RC3A	6.074	10.1 ± 0	6.079	8.9 ± 0	6.765	2 ± 0
	–	–	–	–	12.512	10.2 ± 4.80
B. mojavensis RC3B	5.079	7.1 ± 0	–	–	5.664	25 ± 0
	–	–	6.313	5.15 ± 0.07	6.525	6.8 ± 3.81
	–	–	–	–	12.447	15.15 ± 5.72
B. cereus RC3C	5.384	35.8 ± 2.26	5.192	50.55 ± 2.89	5.696	25.2 ± 10.32
	6.105	7 ± 3.39	5.79	18.05 ± 2.05	6.482	4.85 ± 0.35
	–	–	–	–	12.423	16.05 ± 4.31
B. cereus RC3E	5.693	3.6 ± 1.83	5.519	11.7 ± 2.12	6.389	6.7 ± 4.52
	–	–	–	–	12.134	15.2 ± 3.53
B. mycoides RC4A			6.622	14.7 ± 0		
			12.753	9.8 ± 0	12.121	24.45 ± 5.72
B. mycoides RC4B	6.457	2.4 ± 0	–	–		
	–	–	–	–	12.16	10.55 ± 0.91
B. subtilis RC6A	–	–	6.208	12.55 ± 1.34		
	–	–			11.833	15.65 ± 3.46
B. cereus RC6B	6.277	8.75 ± 0.63	6.247	9.6 ± 0.84	6.115	6.7 ± 2.96
	–	–	–	–	11.724	20.35 ± 0.63

* RT: Retention time; **SD: Standard deviation; ***ND: non detectable.

Table 3
Degradation of AFB_1 by cell free culture supernatants (CFCS) and cell lysates of *Bacillus* strains isolated from soil and pond mud incubated for 72 h at 30 °C with a previous exposure of the extracts (20 min) to different temperatures.

Strain	CFCS			Cell lysate		
	AFB_1 (ng/g)			AFB_1 (ng/g)		
	Average	SD*	Reduction %	Average	SD*	Reduction %
AFB_1 control	179.38^{d}	0				
B. subtilis RC1B						
25 °C	80.61^{bc}	1.15	51.72	110.86^{c}	18.32	38.20
70 °C	71.58^{ab}	1.20	60.10	84.56^{bc}	2.82	52.86
80 °C	82.04^{bc}	1.50	54.26	96.81^{bc}	2.43	46.03
B. cereus RC1C						
25 °C	97.35^{bc}	0.90	45.73	110.90^{c}	27.15	38.18
70 °C	40.82^{a}	0.48	77.24	83.73^{bc}	0.90	53.32
80 °C	85.66^{bc}	0.30	52.25	89.13^{bc}	0.5	50.31
B. mojavensis RC3B						
25 °C	44.25^{a}	0.02	75.33	103.87^{bc}	31.60	42.09
70 °C	108.79^{e}	0.05	39.35	108.29^{c}	32.09	39.63
80 °C	70.86^{ab}	1.37	60.50	91.81^{bc}	2.42	48.82

*SD: Standard deviation.

Table 4
Evaluation of aflatoxin B_1 (AFB_1) breakup products toxicity on brine shrimp (*Artemia salina*): Naupilii mortality (%) was evaluated after 2 and 24 h and no difference in number of dead larvae was observed. Extracts of the AFB_1 degradation assay with cell free culture supernatant (CFCS) were used for the toxicity test. Nutrient broth added 100 ng/ml AFB_1 and nutrient broth without AFB_1 were extracted in same way as the samples and used as positive and negative controls, respectively. Laboratory marine solution was used as negative control as well.

Extract	Mortality (%)	Fisher's LSD test
AFB_1 positive control	30.56	a
NB without AFB_1	4.89	c
Marine solution control	2.18	c
B. mojavensis RC1A	10,00	c
B. subtilis RC1B	7.5	c
B. cereus RC1C	8,71	c
B. mojavensis RC3A	10.42	c
B. mojavensis RC3B	7.92	bc
B. cereus RC3E	12,50	c
B. cereus RC3F	6.73	c
B. mycoides RC4A	10.8	c
B. subtilis RC6A	11.25	c
B. cereus RC6B	7.09	c
B. mycoides RC6C	6.52	c

*Different letters indicate statistically significant differences (ANOVA ($P < 0.05$) and Fisher's LSD test ($P < 0.05$), InfoStat).

4. Discussion

In the present study, the ability to degrade AFB_1 of 11 *Bacillus* strains isolated from soil and pond mud was evaluated. In addition, the presence of the *aiiA* genes encoding for AHL lactonase was analysed since it was considered a possible responsible for AFB_1 degradation. Degradation of AFs by *Bacillus* species has been documented in recent studies (Farzaneh et al., 2012; Petchkongkaew et al., 2008; Xia et al., 2017; Xu et al., 2017). However, most authors do not test the toxicity of AFB_1 breakdown products, which is essential if we intend to use the strain or enzyme in agriculture or the food industry as an alternative to the classical chemical and physical decontamination methods (Zhu et al., 2017).

Bacillus strains used for this study were isolated from soil and pond mud, usual habitats for other mycotoxin degrading or AHL-producing *Bacillus* species according to other authors (Chen et al., 2010; Gao et al., 2011; Xia et al., 2017). The MALDI-TOF MS method used for species identification was effective to differentiate between closely related species in *B. cereus* and *B. subtilis* groups.

In the AFB_1 degradation screening, all strains were able to degrade AFB_1 in culture after 72 h incubation at 30 °C showing degradation percentages that ranged from 26.56 to 79.28%. Similarly, Xia et al. (2017) obtained 67.2% AFB_1 degradation by *B. subtilis* JSW-1 and Xu et al. (2017) obtained 97% AFB_1 degradation by *B. shackletonii* in 72 h cultures. Farzaneh et al. (2012) also observed a decrease in AFB_1

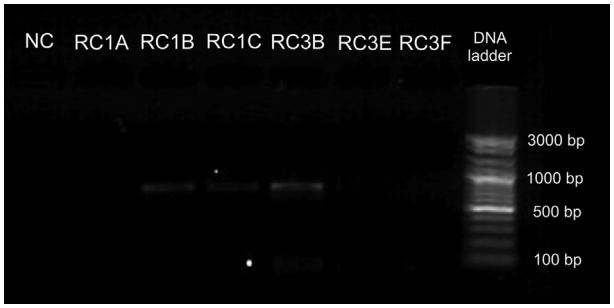

Fig. 2. Presence of *aiiA* gene amplicons of *S. subtilis* RC1B (lane 3), *B. cereus* RC1C (lane 4) and *B. mojavensis* RC3B (lane 5). *B. mojavensis* RC1A (lane 2), *B. cereus* RC3E (lane 6) *and B. cereus* RC3F (lane 7) showed no amplification. NC: negative control (lane 1) DNA Ladder: DNA Ladder Plus 100bp (Genbiotech S.R.L., Buenos Aires, Argentina).

concentration of 52.67% and 80.53% after 24 and 48 h incubation with *B. subtilis* UTBSP1, respectively.

All strains were able to significantly degrade AFB_1 by extracellular compounds present in the CFCS over 24, 48 and 72 h incubation. This result validates the claim that microbial detoxification of AFB_1 is most probably due to degradation by extracellular proteins, perhaps enzymes present in CFCSs, and not by a physical adsorption to the cell wall (Adebo et al., 2017; Farzaneh et al., 2012; Teniola et al., 2005; Xia et al., 2017). However, intracellular enzymes could also play a part on the detoxification process as observed for *B. subtilis* RC1B, *B. cereus* RC1C and *B. mojavensis* RC3B which cell lysates were able to degrade 38.20–53.32% AFB_1.

The presence of *aiiA* homologue genes has been described in different *Bacillus* species (Chen et al., 2010; Dong et al., 2002; Easwaran et al., 2015; Lee et al., 2002; Iram et al., 2015; Pan et al., 2008). In the present study, only three of the analysed strains amplified with the AiiA1/AiiA2 primer pair designed by Pan et al. (2008). These strains were selected for the subsequent studies to possibly correlate the production of AHL lactonase with AFB_1 degradation. In the AFB_1 degradation screening, the strains that carried the gene encoding for AHL lactonase, showed significantly higher degradation percentages in culture than in CFCS. An AHL lactonase was thought the as the probable responsible for the degradation since they are unlikely to be secretary enzymes (Dong et al., 2000). Based on this result, lysates of *B. subtilis* RC1B, *B. cereus* RC1C and *B. mojavensis* RC 3B were tested obtaining up to53% AFB_1 degradation. The activity of the enzymes was not affected by high temperatures. Similarly, Xu et al. (2017) and Xia et al. (2017) described AFB_1-degrading enzymes from *B. shackletonii* and *B. subtilis* resisted exposure to boiling water for 10 min. AHL lactonase has also demonstrated to be resistant to high temperatures. Cao et al. (2012) informed $AiiA_{AI96}$ from *Bacillus* sp. AI96 retained 60–90% of its activity after incubation 80 and 70 °C, respectively. The stability of enzymes to wide ranges of temperatures facilitates the production, commercialization, application and conservation of enzyme based products.

The significant reduction of naupilii mortality demonstrated that the AFB_1 breakup products were less toxic than the parent toxin. Xu et al. (2017) reported AFB_1degradation products obtained with *B. sackletonii* LMG 18435 (L7) supernatant showed no genotoxicity according to results of SOS Chromotest.

Homologes to the *aiiA* gene have been described to be widespread among *Bacillus* species and subspecies, especially from *B. cereus* and *B. subtilis* groups (Dong et al., 2002; Easwaran et al., 2015; Pan et al., 2008). They encode for AiiA proteins with lactonase activity responsible of the cleavage of the ester bond of the lactone ring of the AHL molecule. For this reason, our first hypothesis was that these could be the enzymes responsible for AFB_1 degradation if they could cleave the toxin's lactone moiety. However, only three of the isolates amplified the *aiiA* gene demonstrating to carry the genes encoding for these enzyme. It is clear that AHL lactonases are not the only enzymes involved in AFB_1 degradation in *Bacillus* strains since not all strains are able to produce them and moreover, a high degradation percentage was obtained with CFCS. However their participation in AFB_1 degradation is not excluded. There must be a combination of secretary and non-secretary enzymes responsible for AFB_1 decontamination. Two cleavage products were detected in most of the samples and the elucidation of their molecular structures is the next step to follow to ensure the absence of toxicity. The loss of fluorescence of degradation products indicated the decontamination process most probably involves the cleavage of the lactone ring moiety. This is going to be confirmed in a future study by LC-MS/MS method as described by Iram et al. (2015).

Microbial detoxification of mycotoxins is an attractive choice for decontamination since it can be a specific, effective, irreversible and environmental-friendly (Ji et al., 2016; Vanhoutte et al., 2016; Zhu et al., 2017). Bacteria can degrade toxins at high rate and wide reaction conditions, favouring their application in the food/feed chain. *Bacillus*

Fig. 3. Attenuation of aflatoxin B_1 fluorescence under UV light (365 nm). Controls: A) 2 µl; B) 5 µl and C) 10 µl of 500 ng/ml AFB_1 solution. Spots D to M: 10 µl of degradation assays extracts of initial 500 ng/ml AFB_1 concentration. Spot J: Degradation of AFB_1 by *B. cereus* RC3F produced a fluorescent compound with a RF value different from AFB_1.

spp. with mycotoxin biotransformation activities have been more sought after, due to their perceived probiotic properties and the possibility of being applied directly on feeds (Bernardeau et al., 2017; Das et al., 2013; Ji et al., 2016); Permpoonpattana et al. (2012); Rhayat et al. (2017). The use of active enzymes for mycotoxins degradation is also being used in commercial feed additives. Many other exogenous enzymes such as phytases, amylases, proteases and many others have been purified and successfully applied in feed industries. Further studies aimed to purify and identify the enzymes that take part in the AFB_1 degradation process will be carried out in the near future. New degradation assays will be performed in order to continue the study of degradation metabolites using LC-MS/MS, Q-TOF-MS methodologies in the search of different AFB_1 breakup products and confirmation of the lactone ring cleavage. *In vivo* assays will be performed on Wistar rats and broilers to ensure the reduction of toxicity. Production parameters, genotxicity, cytotoxicity and toxicity signs in different organs (especially liver, lung, intestine and kidney) will be assessed to evaluate the absence of toxicity related to the strains or their products.

5. Conclusion

In conclusion, 11 *Bacillus* strains capable of degrading AFB_1 to less toxic metabolites were isolated, and their degradation activity was extracellular as well as intracellular. Therefore, if demonstrated to be safe when applied to animals, these strains have potential to be used to detoxify AFB_1 in contaminated feed. In addition, *B. subtilis* RC1B, *B. cereus* RC1C and *B. mojavensis* RC3B were demonstrated to carry de genes encoding for an AiiA AHL lactonase, an enzyme able to cleave the lactone ring and reduce toxicity of mycotoxins such as aflatoxins, ochratoxin A and zearalenone.Declarations of interest

The authors declare there are no conflicts of interest regarding the publication of the present work.

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