A novel strain of Cellulosimicrobium funkei can biologically detoxify aflatoxin B₁ in ducklings

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Summary

Two experiments were conducted to screen microorganisms with aflatoxin B₁ (AFB₁) removal potential from soils and to evaluate their ability in reducing the toxic effects of AFB₁ in ducklings. In experiment 1, we screened 11 isolates that showed the AFB₁ biodegradation ability, and the one exhibited the highest AFB₁ removal ability (97%) was characterized and identified as Cellulosimicrobium funkei (C. funkei). In experiment 2, 80 day-old Cherry Valley ducklings were divided into four groups with four replicates of five birds each and were used in a 2 by 2 factorial trial design, in which the main factors included administration of AFB₁ versus solvent and C. funkei versus solvent for 2 weeks. The AFB₁ treatment significantly decreased the body weight gain, feed intake and impaired feed conversion ratio. AFB₁ also decreased serum albumin and total protein concentration, while it increased activities of alanine aminotransferase and aspartate aminotransferase and liver damage in the ducklings. Supplementation of C. funkei alleviated the adverse effects of AFB₁ on growth performance, and provided protective effects on the serum biochemical indicators, and decreased hepatic injury in the ducklings. Conclusively, our results suggest that the novel isolated C. funkei strain could be used to mitigate the negative effects of aflatoxicosis in ducklings.

Introduction

Aflatoxins (AF) are secondary fungal metabolites that are largely produced by the fungi Aspergillus flavus and Aspergillus parasiticus (Diaz et al., 2002). Among the various dangerous AF and their metabolites, aflatoxin B₁ (AFB₁) is the most toxic mycotoxin, having harmful hepatotoxic, mutagenic, carcinogenic and teratogenic effects on many species of livestock. It is also classified as a group one carcinogen [International Agency for Research on Cancer (IARC), 1987]. Unfortunately, AFB₁ can easily contaminate various types of crops and is a very prevalent contaminant of maize-based food and feed all over the world (Wu and Guclu, 2012; Hamid et al., 2013). The feed contaminated by AFB₁ can pose serious problems to the health and productivity of livestock and can therefore cause significantly economic losses (Rawal et al., 2010; Wu and Guclu, 2012).

Several physical and chemical detoxification methods used to control AFB₁ have been to some extent successful, while most of them have major disadvantages including nutrients loss and high costs, which limited their practical applications (Varga et al., 2010; Jard et al., 2011). Thus, scientists have come to favor the biological method, which is utilization of microorganisms and/or their enzymatic products to remove AF through microbial binding and/or degradation of mycotoxins into less toxic compounds, giving a characterization of specific, efficient and environmentally sound detoxification (Wu et al., 2009; Guan et al., 2011).

Many studies have shown that AFB₁ can be biologically detoxified by various species of microorganisms, including fungi, such as Pleurotus ostreatus (Motomura et al., 2003), Trametes versicolor (Zalic et al., 2006), yeast such as Trichosporon mycotoxinivorans (Molnar et al., 2004) and Saccharomyces cerevisiae (Pizzolitto et al., 2013), and bacteria, such as lactic acid bacteria (Bagherzadeh Kasmani et al., 2012; Nikbakht Nasrabad et al., 2013), Stenotrophomonas maltophilia (Guan et al., 2008), Myxococcus fulvus (Guan et al., 2010) and Rhodococcus species (Cserháti et al., 2013). Unfortunately, few of these microorganisms, their metabolites and/or degradation products have been utilized in animal feed due to a lack of information on the mechanisms of detoxification, the
Table 1. Ability of AFB₁ biodegradation by screened isolates.

| Isolate<sup>b</sup> | AFB₁ biodegradation (%) |
|---------------------|-------------------------|
| T₁-1                | 84.9 ± 4.0              |
| T₁-2                | 41.7 ± 4.1              |
| T₁-3                | 86.3 ± 7.3              |
| T₁-4                | 24.9 ± 3.8              |
| T₂-1                | 75.9 ± 1.8              |
| T₃-1                | 51.5 ± 6.9              |
| T₃-2                | 81.2 ± 4.9              |
| T₃-3                | 87.1 ± 4.0              |
| T₃-4                | 20.4 ± 4.3              |
| T₃-5                | 94.2 ± 2.4              |
| T₃-6                | 70.1 ± 4.5              |

<sup>a</sup> Values are expressed as means ± SD (n = 5).  
<sup>b</sup> Isolates are screened from soil samples using coumarin as the only carbon source.

-efficiency and stability of detoxification under different oxygen, pH or bile conditions, as well as their potential side-effects needs to be further investigated.

The objective of this study was to screen novel AFB₁ biodegradation microorganisms that could be applied in feed industry. Because AFB₁ is a furanocoumarin derivative which has a similar chemical structure to polycyclic aromatic hydrocarbons (PAHs) (Yu et al., 2004; Guan et al., 2008), we therefore hypothesized that the microorganism having biodegradable activity on PAHs maybe also have the same effect towards AFB₁. We therefore chose soil samples around petroleum factories, which were contaminated with PAHs for the screening of the microorganisms that could biodegrade AFB₁ (Pampanin and Sydnes, 2013). Since coumarin is the basic chemical structure of AFB₁, along with the relatively safe and inexpensive characterization, we used medium-containing coumarin as the only carbon source to screen AFB₁ biodegrading microorganisms by replicating the well-established method previously conducted (Guan et al., 2008). Since duckling is extremely sensitive to the toxic effects of AFB₁, (Shi et al., 2013), and thus it was used to evaluate the detoxification effects of the isolate in this study. In this study, we successfully obtained the isolate Cellulosimicrobium funkei T₃-5 (C. funkei), which exhibited excellent AFB₁ biodegradation ability both in vitro and in vivo. Our findings suggested a feasible approach for a safe and efficient method to control AFB₁ levels in the animal feed industry.

Results

Experiment 1

Screening for AFB₁ biodegradation microbes. A total of 11 strains were isolated from three soil samples by coumarin medium, and all of them showed various degrees of ability to reduce concentrations of AFB₁ in the liquid medium after 72 h incubation at 37°C (Table 1), which were calculated from the high-performance liquid chromatography (HPLC) results. The chromatograms of HPLC analysed results were shown in Fig. S2. Among the 11 screened strains, seven showed the potential of reducing AFB₁ in the medium over 70%, and the isolate T₃-5 was the most effective strain with an observed 94.2% AFB₁ reduction in the medium (Table 1).

Identification of isolate T₃-5. Microscopic morphological results showed that isolate T₃-5 is a gram-positive bacterium, which appeared as circular, yellow, a smooth surface and an entire edge after 18 h of incubation at 37°C on the Luria Bertani (LB) agar (Fig. S1). Physiological and biochemical studies showed that the T₃-5 strain was able to utilize most oligosaccharides including glucose, maltose, D-xylose, galactose, D-sorbitol, D-raffinose and as well as sucrose as a sole carbon source. The T₃-5 strain could also hydrolyse cellulose, gelatin and Tween 80, but not amylum (Table 2). The 16S rDNA sequencing result of the isolate T₃-5 [National Center for Biotechnology Information (NCBI) GenBank: KM032184] showed 99% deoxyribonucleic acid (DNA) sequence homology to that of Cellulosimicrobium funkei listed by NCBI (GenBank: NR_042937.1, Fig. 1). Taken together, the morphological, physiological, biochemical characteristics of C. funkei T₃-5.

Table 2. Biochemical and physiological characteristics of C. funkei T₃-5.

| Characteristic | Result | Characteristic | Result | Characteristic | Result |
|---------------|--------|---------------|--------|---------------|--------|
| Acid fermentation of: | Phosphatidylcholine | − | 10% NaCl | − |
| Glucose | + | Tween 80 | − | Acid medium | − |
| Maltose | + | Amylum | − | Other test: | − |
| D-Xylose | + | Cellulose | + | Motility | − |
| Galactose | + | Xylan | − | Catalase test | + |
| D-Sorbitol | − | Nitrate reduction | + | Methyl red test | + |
| D-Raffinose | + | Organic acid | + | Urease test | + |
| Sucrose | + | Yeast cell | − | V-P test | − |
| Glycerol | + | Growth on: | − | Oxidase test | − |
| Hydrolysis of: | Gelatin liquefaction | + | 2%, 5% NaCl | + |
| | | | 7% NaCl | (+) |

+, Positive; −, negative; (+), weakly positive.

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data as well as the NCBI blast results suggest that the isolate T3-5 belonged to C. funkei, which is an aerobic and facultatively anaerobic gram-positive bacterium. The above strain is deposited at China Center for Type Culture Collection (CCTCC) in Wuhan University, and has a preservation number of CCTCC NO: M 2013564.

Characterization of AFB1 biodegradation by C. funkei T3-5. The culture supernatant of C. funkei T3-5 showed the strongest ($P < 0.05$) AFB1 biodegradation ability compared with viable cell and cell extract, which removed 97%, 20% and 16% AFB1 after 72 h incubation respectively (Fig. 2A). These results indicated that the activity of AFB1 biodegradation occurred primarily within the culture supernatant of C. funkei. The AFB1 biodegradation ability of the culture supernatant of C. funkei was decreased ($P < 0.05$) 54% and 56% after treated with proteinase K with or without SDS, respectively, while it was only slightly decreased by heat treatment (Fig. 2B).

Experiment 2

Performance. Non-significant differences in initial body weight were observed among the four groups (Table 3). After 2 weeks of experimental treatments, the growth performance was significantly affected by oral administration of AFB1 and C. funkei or their interactions (Table 3). Compared with the control, the final body weight (BW), overall daily BW gain and overall daily feed intake of ducklings were decreased ($P < 0.05$) 42%, 49% and 38%, along with increased ($P < 0.05$) 23% overall feed/gain ratio by AFB1 administration respectively. Although the AFB1 + C. funkei group showed the similar trend to AFB1

![Neighbor-joining phylogenetic tree based on 16S rDNA gene sequences showing the relationships among the species of the genus Cellulomonas and related species.](image)

**Fig. 1.** Neighbour-joining phylogenetic tree based on 16S rDNA gene sequences showing the relationships among the species of the genus Cellulomonas and related species. Bootstrap values calculated for 1000 replications are indicated. Bar, 2 substitutions per 100 nucleotides. Accession numbers from Genbank are given in brackets.

![Ability of AFB1 biodegradation by culture supernatant, cell and cell extract of C. funkei after 72 h fermentation (A); and culture supernatant of C. funkei was determined by pretreating the supernatant by proteinase K with or without SDS or heat respectively (B).](image)

**Fig. 2.** Ability of AFB1 biodegradation by culture supernatant, cell and cell extract of C. funkei after 72 h fermentation (A); and culture supernatant of C. funkei was determined by pretreating the supernatant by proteinase K with or without SDS or heat respectively (B). Values are means ± SD, n = 5. Bars without a common letter differ, $P < 0.05$. 

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group that decreased (P < 0.05) the final BW (33%), overall daily BW gain (39%) and overall daily feed intake (39%) of ducklings respectively, administration of _C. funkei_ prevented (P < 0.05) the loss in final feed conversion and BW (9%) at the first week. In addition, administration of _C. funkei_ alone reduced (P < 0.05) the overall feed/gain ratio (9%), and did not affect the other growth performance parameters, when compared with the control. No mortality due to AFB1 administration was found in this study.

**Serum biochemistry and liver histology.** The results showed that the serum biochemical and histological parameters were significantly affected by administration of AFB1 and _C. funkei_ or their interactions (Table 4). The AFB1 administration led to increased (P < 0.05) activity of aspartate aminotransferase (AST; 404% and 867%) and alanine aminotransferase (ALT; 82% and 282%), along with decreased (P < 0.05) concentration of total protein (TP; 34% and –) and albumin (ALB; 44% and –) in the serum of ducklings at the first and second week respectively. Strikingly, AFB1 + _C. funkei_ group decreased (P < 0.05) the activity of AST (43 and 44%) and ALT (28 and 20%), along with increased (P < 0.05) concentration of TP (22% and –) and ALB (42% and –) in serum of ducklings at the first and second week, respectively, compared with the AFB1 group (Table 4). Furthermore, the histological analysis results showed AFB1 administration-induced hepatic injury, such as vacuolar degeneration, necrosis and bile duct hyperplasia at the first week and increased liver damage on the second week. Notably, AFB1 + _C. funkei_ group alleviated the liver damage was observed in the AFB1 group (Fig. 3).

**Discussion**

The two most novel findings from the present study were: (i) we successfully screened a novel AFB1 biodegradation microorganism _C. funkei_ T3-5, and (ii) oral administration of _C. funkei_ effectively alleviated the adverse effects induced by AFB1, in the ducklings. The _C. funkei_ is a gram-positive, aerobic and facultatively anaerobic, non-sporing rod or coccus-shaped bacterium of the genus *Cellulosimicrobium*, consistent with previously reported (Brown et al., 2006). In vivo, _C. funkei_ demonstrated effective AFB1 biodegradation ability that 97% of AFB1 can be removed after 72 h incubation. Interestingly, these reported values were much higher than those of previously reported various microorganisms, such as *Rhodococcus erythropolis* (67%, Alberts et al., 2006), *Flavobacterium aurantiacum* (74.5%, Smiley and Draughon, 2000), *Mycobacterium strain* (80%, Hormisch et al., 2004), _M. fulvus_ (81%, Guan et al., 2010) and _S. maltophilia_ (83%, Guan et al., 2008). Strikingly, Cserháti and colleagues (2013) found that several *Rhodococcus* species displayed more than 97% AFB1-degrading ability, along with effective degrading ability to other common mycotoxins also, which also offered a promising strategy to control mycotoxins. Moreover, our results implied that the compounds biodegrading AFB1 were mainly within the fermentation supernatant of _C. funkei_ rather than in its viable cell and cell extract. Notably, the AFB1 biodegradation activity of fermentation supernatant of _C. funkei_ was decreased more than 50% after treated with proteinase K or plus SDS, which is similar to the AFB1 biodegradation by the culture supernatant of _F. aurantiacum_ and

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**Table 3. Effects of administration of AFB1, and _C. funkei_ on growth performance in ducklings.**

|                | Control          | AFB1a            | _C. funkei_ F  | AFB1 + _C. funkei_  |
|----------------|------------------|------------------|---------------|---------------------|
| week 0 BW, g   | 119.3 ± 1.3      | 118.8 ± 1.0      | 118.5 ± 0.6   | 118.8 ± 0.5         |
| week 1 BW, g   | 372.5 ± 30.0a    | 309.4 ± 7.0a     | 364.7 ± 16.2a | 336.6 ± 16.4a       |
| week 2 BW, g   | 786.9 ± 61.2a    | 458.5 ± 61.7a    | 858.8 ± 42.6a | 527.8 ± 63.3a       |
| week 2 BW gain, g/day | 47.7 ± 4.3a | 24.3 ± 4.4a | 52.9 ± 3.0a | 29.2 ± 4.5a |
| week 2 feed intake, g/day | 89.7 ± 9.0a | 55.5 ± 5.7a | 90.3 ± 3.1a | 55.1 ± 7.8a |
| week 2 feed/gain, g/g | 1.88 ± 0.04a | 2.32 ± 0.25a | 1.71 ± 0.03a | 1.89 ± 0.16a |

a. Values are expressed as means ± SD (n = 5), and means with different superscript letters differ (P < 0.05).

**Table 4. Effects of administration of AFB1 and _C. funkei_ on serum biochemical parameters in ducklings.**

|                | Control          | AFB1b            | _C. funkei_ F  | AFB1 + _C. funkei_  |
|----------------|------------------|------------------|---------------|---------------------|
| week 1 ALT, μg/l | 46.5 ± 5.8a      | 234.8 ± 48.7a    | 48.3 ± 7.8a   | 132.8 ± 26.7a       |
| week 1 AST, μg/l | 68.3 ± 8.4a      | 124.5 ± 5.4a     | 68.8 ± 11.3a  | 89.8 ± 8.1a         |
| week 1 TP, μg/l  | 29.4 ± 1.2a      | 19.4 ± 1.8a      | 28.9 ± 0.8a   | 23.6 ± 1.0a         |
| week 1 ALB, μg/l | 13.5 ± 0.4a      | 7.6 ± 0.8a       | 13.4 ± 0.7a   | 10.8 ± 0.1a         |
| week 2 ALT, μg/l | 35.3 ± 10.2a     | 341.0 ± 52.0a    | 39.00 ± 11.0a | 190.0 ± 43.3a       |
| week 2 AST, μg/l | 53.5 ± 8.7a      | 204.5 ± 44.7a    | 54.3 ± 8.3a   | 162.8 ± 30.9a       |
| week 2 TP, μg/l  | 29.9 ± 1.7 –     | 30.0 ± 0.3 –     | 30.0 ± 0.3 –  | 30.0 ± 0.3 –        |
| week 2 ALB, μg/l | 14.0 ± 0.9 –     | 13.9 ± 0.2 –     | 13.9 ± 0.2 –  | 13.9 ± 0.2 –        |

a. Values are expressed as means ± SD (n = 5), and means with different superscript letters differ (P < 0.05).

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S. maltophilia reported earlier, and indicated the active ingredient could therefore be protein or perhaps an enzyme (Alberts et al., 2006; Guan et al., 2008). Furthermore, the AFB₁ biodegradation activity was positively correlated with the protein content from the fermentation supernatant of C. funkei by ammonium sulfate precipitation (Table S1) which provides further evidence that the active ingredient could be protein (Callejón et al., 2014; Duong-Ly and Gabelli, 2014). However, heating may cause denaturation of proteins, strikingly, the AFB₁ biodegradation activity of fermentation supernatant of C. funkei was only slightly affected by heating. It may be interpreted by (i) the protein involved in the AFB₁ biodegradation is heat resistant (Wang et al., 2007), and (ii) the fermentation supernatant may contain cell wall, and heating may increase permeability of their external layer and lead to the increasing availability of the otherwise hidden binding sites for AFB₁ (Shetty et al., 2007). Taken together, our results revealed that the active ingredient of AFB₁ biodegradation in the fermentation supernatant of C. funkei could be enzyme and other active ingredients such as, cell wall. However, systematic identification of the active ingredients in the fermentation supernatant of C. funkei and its detoxification mechanism is still needed to be explored in the future.

Administration of AFB₁ reduced the growth rate and efficiency of feed utilization of ducklings, which were in accordance with those in previous studies (Pasha et al., 2007). The negative effects of AFB₁ on feed intake, BW gain and the feed conversion have been associated with anorexia, reluctance and inhibition of protein synthesis and lipogenesis (Bagherzadeh Kasmani et al., 2012). The present study showed that supplementation of C. funkei at dose of 10⁸ cfu/day prevented the loss in feed conversion throughout. Meanwhile, although supplementation of C. funkei had no significant effect on the final BW, while improved body weight was observed during the first week. The beneficial effects of supplementation of C. funkei could be due to (i) toxin biodegraded by C. funkei in vivo was evidenced by a 97% removal of AFB₁, and (ii) the C. funkei could hydrolyse cellulose and improved the cellulose utilization. In addition, no adverse effects in productivity parameters were found between ducklings in control group and the experimental group administered C. funkei alone, indicating that C. funkei was non-toxic and safe. Similar results were obtained from recent studies that Nocardia corynebacteroides and S. cerevisiae can partly detoxify chicken feed contaminated with AFB₁ (Tejada-Castañeda et al., 2008; Pizzolitto et al., 2013).

Activities of serum enzymes such as ALT and AST, and concentrations of serum TP and ALB have been described as valuable parameters of hepatic injury and function (Bagherzadeh Kasmani et al., 2012; Lv et al., 2014). Administration of AFB₁ alone increased ALT and AST activity, along with decreased TP and ALB concentrations compared with the control diet. These outcomes were consistent with previous studies, which provided evidence that liver injury was induced by AFB₁ (Bagherzadeh Kasmani et al., 2012; Shi et al., 2013). Results obtained from the present study showed that serum biochemical changes could be ameliorated by C. funkei administration. Moreover, histopathological changes in the livers of ducklings exposed to AFB₁ were similar to those reported on avian aflatoxicosis (Denli et al., 2009). Administration of C. funkei showed stronger

Fig. 3. Photomicrographs of hepatic sections stained with haematoxylin and eosin (40× magnification) of ducklings from different treatment groups on (A) week 1 and (B) week 2 respectively.
C. funkei may therefore exert better protective effects on aflatoxicosis in practice. Although C. funkei has showed potent AFB1 biodegradation capability and safety in vivo study, directly using this microbe as a feed additive seems challenged by the fact that C. funkei is an opportunistic pathogen (Petkar et al., 2011). Therefore, our ongoing research was focus in two directions: (i) exploring the mechanism of AFB1 biodegradation by C. funkei, which try to separate the enzyme and/or other active ingredients such as cell wall that could biodegradation the AFB1; and (ii) using C. funkei alone or with other microbial to do the solid-state fermentation on rapeseed meal and cottonseed meal to improve crude fiber digestibility, reduce AFB1 contents and produce AFB1 biodegradation active ingredients in these feedstuffs.

In summary, the C. funkei isolated in the present study, exhibited significant improvements in the capabilities of biodegradation of AFB1, in vitro. Moreover, an in vivo study verified its AFB1 biodegradation activity in ducklings with respect to partial improvement growth performance, serum biochemistry, hepatotoxicity and histopathology of livers. Additionally, the in vivo study showed that administration of C. funkei at 10^5 CFU/day was non-toxic and safe to administer to ducklings. Overall, these findings suggest that the use of C. funkei in AFB1-contaminated feed offers a new strategy to reduce the adverse effects of aflatoxicosis in ducklings.

Experimental procedures

**Experiment 1**

**Soil samples and AFB1 biodegradation microorganism isolation.** Three soil samples designated as T1, T2 and T3 were collected around the factories of SINOPEC Wuhan Company, Shandong Jinjiao Coal Mine Company and Shandong Yankuang International Coking Company. All these samples were air-dried at room temperature. Microorganisms that could use coumarin as the only carbon source were then isolated from soil samples by using a standard procedure with minor modifications (Guan et al., 2008). Single colonies that were able to grow on the coumarin (Sigma Chemical Co., Bellefonte, USA) plate were selected for AFB1 biodegradation activity analysis according to the protocol described by Guan and colleagues (2008) with minor modifications. Initially, candidate isolates were cultured at 37°C in LB medium for 72 h, and then 950 μl fermented supernatant was taken and mixed with 50 μl 10 μg/ml AFB1 solution (Sigma Chemical Co., Bellefonte, USA) in a sterilized centrifuge tube, and then biodegradation tests were conducted at 37°C for 72 h. Finally, the reaction solution was centrifuged at 10 000 g at 4°C for 10 min to remove cells and the supernatant, and then it was collected for AFB1 quantification (Guan et al., 2008). The AFB1 concentration was determined by HPLC (Teniola et al., 2005) with a minor modification. AFB1 was extracted three times with chloroform from liquid cultures and cell-free extracts. The chloroform was evaporated under nitrogen gas, and the samples were dissolved in methanol, filtered by 0.22 μm filters for HPLC analysis. HPLC analysis was performed on a Shimadzu LC-20A binary gradient liquid chromatography equipped with a 5 μm × 4.6 mm × 250 mm C-18 reverse-phase column (ZORBAX Eclipse XDB-C18, Agilent). The mobile phase was acetonitrile/methanol/water (1:1.2, v/v/v) at a flow rate of 1 ml/min, and the sample temperature was set at 30°C. AFB1 was measured by UV (365 nm.) detector (Shimadzu SPD-20A). The stabilized LB medium alone substituted fermentation supernatant incubated with AFB1 solution was used as the negative control.

**Identification of the AFB1 biodegradation isolate.** Total DNA was extracted from the AFB1 biodegradation isolate using TIANamp Bacterial DNA Kit (Tiangen Biotech, Beijing, China) according to the manufacturer’s instructions. The forward primer (27f: 5'-GAGATTTGATCCTGGCTCAG-3') and the reverse primer (1492r: 5'-CTACGGCTACCTTGT TACGA-3') were used to amplify the 16S ribosomal (r)DNA (Minerdi et al., 2012). After the amplified 16S rDNA fragment was purified using the Gel Extraction Kit, it was ligated into the pMD18-T vector, and transformed into the Escherichia coli JM109 strain by calcium chloride activation (Dagert and Ehrlich, 1979). The positive colonies were selected for DNA sequencing (Tsingke, Wuhan, China). The obtained DNA sequence and NCBI GenBank-derived sequences were aligned using the CLUSTALX program (Thompson et al., 1997). Neighbour-joining phylogenetic tree and bootstrap values were analysed by the MEGA program (Tamura et al., 2013). Physiological and biochemical tests were carried out following the method described by Holt and colleagues (1994).

**Characterization of AFB1 biodegradation activity of C. funkei T3-5.** After C. funkei T3-5 grew at 37°C in LB medium for 72 h, then the cell, cell extract and fermentation supernatant were prepared as previously described (Guan et al., 2008), and their AFB1 biodegradation ability was tested as described before. Specifically, supernatant was obtained by centrifuging fermentation supernatant at 12 000 g at 4°C for 20 min; cell was collected after being centrifuged at 12 000 g at 4°C for 20 min and washed twice with phosphate buffer (50 mM; pH 7.0); cell extract was produced by using ultrasonic cell disintegrator on ice, and the suspension was centrifuged at 12 000 g for 20 min at 4°C, and then it was...
filtered by 0.22 μm pore size sterile cellulose pyrogen free filters. Since the main active ingredients for AFB1, biodegradation were found within the fermentation supernatant, further assessment was conducted through in vivo experiments. The AFB1 biodegradation stability of fermentation supernatant of C. funkei was determined by the residual activity after the supernatant was treated by proteinase K (0.5 mg/ml) with or without SDS (5.0%) at 37°C for 6 h, or boiled at 100 °C for 15 min respectively. The untreated fermentation supernatant of C. funkei was used as the positive control.

Experiment 2

Ducklings, treatments and samples collection. Our animal protocol was approved by the Institutional Animal Care and Use Committee of Huazhong Agricultural University, China. A total of 80 day-old Cherry Valley commercial ducklings were randomly divided into four treatment groups with four replicates of five birds each. The trial was arranged in a 2 by 2 factorial design that included oral administration of AFB1, solvent and C. funkei or solvent respectively. All birds were allowed free access to a similar corn-soybean meal diet (Shi et al., 2008) and distilled water ad libitum. The LD50 of AFB1, in duckling is 2.8 mg/kg BW (Yunus et al., 2011), and we chose 100 μg/kg BW since the dose of subchronic toxicity test was chosen between 1/10-1/50 LD50 (Jin et al., 2008). After 3 days of acclimation, each group was administered an oral dose of AFB1 [dissolved in 1.0% dimethylsulphoxide (DMSO)] at 100 μg/kg BW or an equivalent amount of sterile DMSO, along with an administration of 1 ml 106 cfu/ml C. funkei or an equivalent amount of sterile LB medium per day respectively. The administration continued for 2 week. Birds were monitored mortality daily, along with body weight and feed intake measured weekly. Meanwhile, four birds from each treatment group were slaughtered weekly to collect blood and liver for the preparation of serum, and liver histological tissue samples were prepared as previously described (Shi et al., 2013; Sun et al., 2013).

Serum biochemical and histological analysis. The serum activities of ALT and AST, along with concentrations of TP and ALB were determined in serum samples. Analyses of the serum samples were measured by an automatic biochemistry analyser (Beckman Synchron CX4 PRO, CA, USA). The liver tissues were fixed in 10% neutral buffered formalin and processed for paraffin embedding, sectioned at 5 μm and stained with haematoxylin and eosin, by standard procedure (Pizzolitto et al., 2013). Liver sections from all birds were microscopically examined.

Statistical analysis. Data generated from experiment 1 were analysed by one-way ANOVA to test the main effects of AFB1, biodegradation activity of C. funkei. Data generated from experiment 2 were analysed by two-way ANOVA to test the main effects of administration AFB1, and C. funkei. The Bonferroni t-test was followed for multiple mean comparisons if there was a main effect. All analyses were conducted using SAS 8.2 (SAS Institute). Data were presented as means ± SD, and significance level was set at P < 0.05.

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Conflict of interest

Lv-Hui Sun, Ni-Ya Zhang, Ran-Ran Sun, Xin Gao, Changqin Gu, Christopher Steven Krumm and De-Sheng Qi have no conflicts of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

Fig. S1. Morphology of (A) colony and (B) gram staining of C. funkei T3-5.

Fig. S2. The selected chromatogram of HPLC, (A) 25 ug/kg AFB1 standard; (B) negative control; (C) positive control; (D) after AFB1 biodegradation by C. funkei. AFB1 biodegradation (%) = (Cpeak area − Dpeak area)/ Cpeak area × 100%.

Table S1. Ability of AFB1 biodegradation by the protein from the culture supernatant of C. funkei by ammonium sulfate precipitation1.