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Screening, identification, and application of deoxynivalenol-detoxifying bacteria from mildewed alfalfa silage

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Abstract

Deoxynivalenol (DON) is very harmful to food and feed. Biological detoxification is an effective method to reduce the harmful effect and ensure safety of food and feed. In order to screen the strains that can detoxify DON, 10 samples were selected from the mouldy alfalfa silage; and the LB, NA, and MRS media with DON were used for primary screening and re-screening. The detoxification rate of DON was measured and calculated by ELISA method. The strains with high detoxification rate of DON were identified by morphology and 16S rDNA sequence analysis. The selected strains were combined and used to ferment the bran, and the detoxification efficiency of DON in the bran was then determined. Sixteen strains with the ability of detoxification were screened, and the results showed that the highest detoxification rate was 66.75%. Three strains with high detoxification rate were isolated and identified as *Bacillus subtilis*, *Pediococcus acidilactici*, and *Lactobacillus plantarum*. The combination of *B. subtilis* and *L. plantarum* yielded the highest efficiency of DON detoxification in bran, which could reach to 75.98% in 96 h.

Introduction

Deoxynivalenol (DON) is a toxic secondary metabolite primarily produced by *Fusarium graminearum* and *F. culmorum* commonly contaminating grains such as wheat, barley, corn, oat, and their by-products, and it is an important cause of animal feed poisoning (Hassan *et al.,* 2019). The pollution of DON in feed and feed products is particularly serious in China. For instance, DON is detected at 97% in feeding ingredients and complete feeds obtained from swine farms in Beijing (Li *et al.,* 2014; Ji *et al.,* 2016). In 2013, the detection rate of DON was 58.74%, and the excessive rate of the standard of DON is 4.60% of wheat flour in China (Lu and Yang, 2015); and the detection rate and excessive rate in bran is obviously higher. DON can be combined with peptide transferase active centre of ribosomal 60S subunit, and can trigger ribosomal stress response, inhibit DNA, RNA, and protein synthesis, induce cell apoptosis, damage the intestinal tract and immune system, and genotoxicity which has serious impact on human and animal health (Kang *et al.,* 2019; Lee *et al.,* 2019). Some studies have shown that pigs are very sensitive to DON in feed (Stastny *et al.,* 2019). Methylation pattern changes showing differential expression have been found in pathways of protein, nucleic acid synthesis, and ribosome biogenesis following DON exposure (Wang *et al.,* 2019c).

The chemical properties of DON are very stable and have strong resistance to heat. It is difficult to damage the toxicity of DON under the conditions of feed processing, storage, high temperature, and pressure. At present, the primary removal methods of DON are physical detoxification, chemical detoxification, and biotransformation.

Physical detoxification mainly adopts mycotoxin adsorption method. The materials used include montmorillonite, diatomite, esterified mannan, and glucomannan (Cirlini *et al.,* 2012). However, the adsorption efficiency of mycotoxin adsorbent for DON is low. Other physical methods for DON reduction often require strong oxidant, high temperature, and high pressure with more energy consumption; and the effect is not ideal as it is difficult to use in feed detoxification (Santos Alexandre *et al.,* 2018; Wang *et al.,* 2019b).

The chemical reaction method is used to transform DON into non-toxic substance under the action of alkali, acid, electrolyte, and sometimes with the help of enzymes (Lyu *et al.,* 2018; Xiong *et al.,* 2019). But strong alkali, acid, and oxidant will not only destroy the taste and nutrition of feed, but also pollute the feed. To date, there is still lack of effective and economical methods to significantly reduce the levels of trichothecene mycotoxins in food and feed, including the efforts to breed *Fusarium* pathogen-resistant crops and chemical / physical treatments to remove the mycotoxins (Yu *et al.,* 2010).

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When compared with physical and chemical detoxification methods, biotransformation method will not introduce pollution of other chemical reagents, and will not cause loss of nutrition in feed. Biological approaches, such as the use of microorganisms to convert the toxins to non- or less toxic compounds, have become a preferred choice due to their high specificity, efficacy, and environmental soundness (Wang *et al.,* 2020). Microbial detoxification will also become a promising choice, since it can be a specific, effective, irreversible, and environmentally friendly strategy of detoxification that leaves no toxic residues (Wang *et al.,* 2019b). It has been reported that some single strains or mixed cultures with the degradation ability of DON have been screened from different samples (Vanhoutte *et al.,* 2017; Zhai *et al.,* 2019).

However, such approaches are often limited by the availability of microbial agents with the ability to detoxify the mycotoxins (Yu *et al.,* 2010). There are a lot of moulds in the mouldy alfalfa silage, which are bound to secrete some mycotoxins. Are there microorganisms that can degrade mycotoxins such as DON in the symbiotic system? In the present work, a variety of media and different culture methods were used to screen the strains with DON degradation ability. The strains were tested individually and in combination, and their detoxification capability was investigated.

Materials and methods

Samples

Ten samples were selected from different positions of mildewed alfalfa silage.

Main reagent

Standard DON was purchased from Tianjin Yifang Science and Technology Co. Ltd. with the purity of ≥ 95.0%; DON ELISA kits were purchased from Beijing Hua'an Maike Biotechnology Co. Ltd.; and other biochemical reagents and kits were purchased from Takara Company.

Main media

The main media are described as follows: (1) LB medium = yeast extract, 5 g; tryptone, 10 g; and NaCl, 10 g; added to 1,000 mL of distilled water and autoclaved at 121°C for 20 min. (2) NA medium = peptone, 10 g; NaCl, 5 g; beef extract, 3 g; added to 1,000 mL of distilled water and autoclaved at 121°C for 20 min. (3) MRS medium = glucose, 20 g; peptone, 10 g; yeast extract, 5 g; beef extract, 10 g; triamine citrate, 2 g; sodium acetate, 5 g; K_2HPO_4, 2 g; $MgSO_4 \cdot 7H_2O$, 0.58 g; $MnSO_4 \cdot 4H_2O$, 0.25 g; Tween 80, 1 g; added to 1,000 mL of distilled water and autoclaved at 121°C for 20 min. All media was added with the standard DON to a final concentration of 2 μg/mL. Later, 2% agar was added to the solid medium.

Primers

The universal primers for the amplification of 16S rDNA fragment of the strain were synthesised by Sangon Biotech (Shanghai, China). The upstream primer was 5′- AGAGTTTGATCCTGGCTCAC-3′, and the downstream primer was 5′-AAGGAGGTG ATCCAG CC-3′.

Sample treatment and primary screening of detoxification bacteria

One g of each sample was added to 10 mL of sterile water and mixed to form suspension, and 100 μL of the suspensions were coated in three kinds of modified solid culture media containing DON respectively; which were cultured aerobically and anaerobically at 37°C for 24 h. The single colonies with different morphology growing on the media were selected for pure culture, and the single colonies obtained from the preliminary screening were repeatedly sub-cultured on the same improved solid medium for three times to purify the strain.

Rescreening and detoxification rate of DON detoxification strains

The purified strain was inoculated into the modified liquid medium containing 2 μg/mL DON. Aerobic bacteria were cultured at 37°C and 200 r/min until the liquid was turbid. Anaerobic bacteria were cultured at 37°C and at constant temperature until colony precipitation appeared. The uninoculated medium was used as control. The content of DON in control and fermented broth were measured by ELISA method, and the high detoxification rate of DON detoxifying bacteria were screened and preserved. The DON detoxification rate was calculated using Eq. 1:

$$\text{DON detoxification rate} = [(\text{DON content in control} - \text{DON content in sample}) / \text{DON content in control}] \times 100\% \quad \text{(Eq. 1)}$$

Identification of DON detoxifying bacteria

The morphology, colour, and characteristics of the colonies were observed. Fresh single colonies on the plates were selected for Gram-staining, and the morphology of the bacteria was observed under the microscope. Then, 16S rDNA sequence of each strain was amplified and sequenced by Sangon Biotech (Shanghai, China). The sequencing results were compared with blast N in GenBank to determine the species of each strain.

The PCR reaction mixture contained 25 μL of: 2.5 μL 10× Taq Buffer, 2 μL dNTP, 1 μL of each primer (10 mM), 1 μL of template DNA, 0.5 μL Taq, and 17 μL of ddH_2O. PCR was performed as the following cycling conditions: 94°C for 2 min; 35 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min 30 s; 72°C for 7 min; and a 4°C soak.

Application of DON detoxifying bacteria

LB01 (*Bacillus subtilis*), NA06 (*Pediococcus acidilactici*) and MRS16 (*Lactobacillus plantarum*) were selected and cultured to 6×10^9, 2×10^9, and 3.5×10^9 CFU/mL, respectively. 10 mL of pure bacterial broth or combination bacterial broth (5 mL of each broth for two bacterial combinations, 3.3 mL of each broth for three bacterial combinations) and 40 mL of sterile water were added into 100 g of bran. The DON content in unfermented bran was used as the control and each treatment was repeated three times. All the samples were fermented at 37°C for 48, 72, and 96 h. The fermentation bran were collected, the content of DON in the fermented bran was detected by ELISA kit, and the detoxification rates were calculated using Eq. 1.

Statistical analysis

All experiments were performed in triplicate and all data was collected and recorded by Microsoft Excel. One-way analysis of variance (ANOVA) was used in bran detoxification trial for multiple comparisons with the SPSS 18.0 software package. Differences were considered to be statistically significant if $p < 0.05$.

Results

Screening of strains

Through primary and secondary screening, 16 strains with the ability of detoxification of DON were screened with three kinds of modified media containing DON in aerobic and anaerobic conditions. The strains with the ability to detoxify DON screened by the LB medium was numbered LB01, LB02, LB03, and LB04. The strains with the ability to detoxify DON screened by the NA medium was numbered NA05, NA06, NA07, NA08, and NA09. The strains with the ability to detoxify DON screened by the MRS medium was numbered MRS10, MRS11, MRS12, MRS13, MRS14, MRS15, and MRS16 (Table 1). The strains LB01, NA06, and MRS13 were selected for subsequent identification and application based on their highest detoxification rates.

Identification of strains

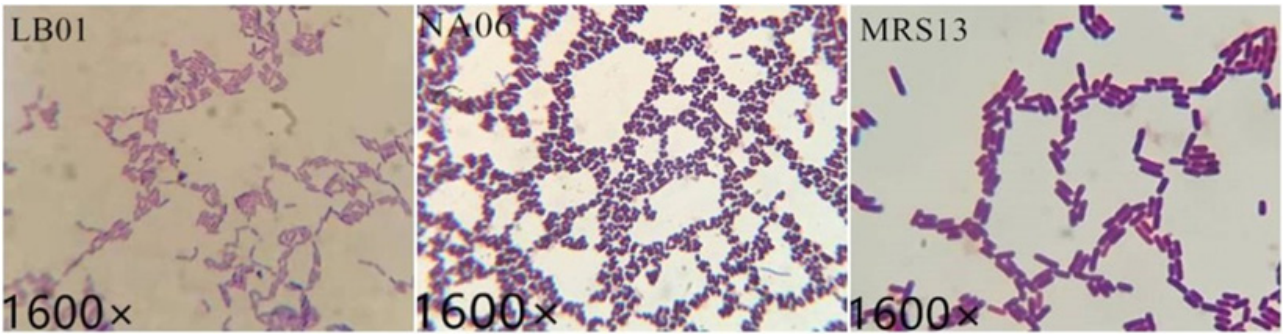
The three selected strains were cultured and stained by Gram-staining method. It was found that the colony morphology of LB01 strain was milky white, opaque, and the edge of the middle fold was irregular. The cells of LB01 were Gram-positive, long rod-shaped with blunt ends and occasional spores. The colony of strain NA06 was white and opaque, with protuberance, rough surface, and irregular edge of colony; the cells of NA06 were Gram-positive, spherical, or oval, without spore. The colony of MRS13 was milky white and translucent, the convex edge of the colony was neat, and the surface was wet and smooth; the cells of MRS13 were Gram-positive, rod-shaped, and the arrangement of the bacteria is single and in chain, without spores (Figure 1).

Table 1. The screened strains and their detoxification rate of DON.

Strain number	Average DON detoxification rate (%)
LB01	51.34
LB02	17.45
LB03	22.32
LB04	21.56
NA05	17.68
NA06	19.14
NA07	9.78
NA08	9.76
NA09	11.48
MRS10	53.16
MRS11	50.43
MRS12	54.42
MRS13	66.75
MRS14	46.83
MRS15	50.52
MRS16	48.18

The genomic DNA of strains LB01, NA06, and MRS13 were extracted and used as template to amplify the 16S rDNA. The results were shown in Figure 2. The electrophoresis showed that the amplified bands were about 1,500 bp, which was consistent with the 16S rDNA size of bacteria.

The amplified products were sequenced and compared with blast (https://www.ncbi.nlm.nih.gov/), and the results showed that the highest homology was 99% between LB01 (accession number: MT242513) and *Bacillus subtilis* (KM222187.1 and FJ393305.1), 99% between NA06 (MT242514) and *Pediococcus acidilactici* (KY550661.1, CP050079.1, and MT158636.1), and 99% between MRS13



(A) **(B)** **(C)**

Figure 1. Gram-staining of three selected strains. (A) aerobic strain on LB medium, (B) anaerobic strain on NA medium, and (C) anaerobic strain on MRS medium.

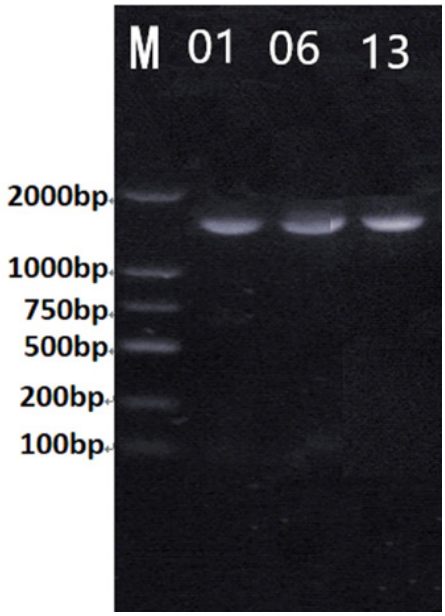


Figure 2. 16S rDNA electrophoresis of three selected strains. M is marker DL2000, 01 is strain LB01 screened on LB medium, 06 is strain NA06 screened on NA medium, and 13 is strain MRS13 screened on MRS medium.

(MT242515) and *Lactobacillus plantarum* (MG754699.1, KM485570.1, and KC887524.1).

Detoxification of DON in bran fermented by different strains

At the end of fermentation experiments, all the DON contents in bran samples were determined, and the content of DON in the control group was 701.18 μg/kg. Based on this result, the detoxification rates of DON in each experimental group were calculated and shown in Table 2. It can be seen from Table 2 that there were significant differences in the detoxification rates of different strains and different combinations of strains at different times ($p < 0.05$). The difference of detoxification rate of the same fermentation strain or combination of strains at different fermentation times was different; some differences were significant, and some differences were not. Among the three single strains, the detoxification effect of strain LB01 was the best when compared with that of single strains, and the highest detoxification rate was 58.86% after fermented for 96 h. The detoxification rate increased significantly with the extension of fermentation time ($p < 0.05$). The detoxification effect of strain NA06

Table 2. Detoxification rate of DON in bran fermented with selected strains.

Strains	Detoxification rate (%)		
	48 h	72 h	96 h
LB01	51.45 ± 0.23^{aB}	54.49 ± 0.37^{bB}	58.86 ± 0.77^{cB}
NA06	39.36 ± 0.49^{aE}	40.08 ± 1.04^{aE}	41.70 ± 0.57^{aF}
MRS13	40.03 ± 0.28^{aE}	43.38 ± 0.64^{bD}	44.65 ± 0.67^{bE}
LB01+NA06	49.33 ± 0.66^{aC}	48.46 ± 0.38^{aC}	49.44 ± 0.71^{aC}
LB01+MRS13	71.24 ± 0.86^{aA}	75.46 ± 0.63^{bA}	75.98 ± 0.79^{bA}
NA06+MRS13	44.34 ± 0.48^{aD}	45.37 ± 0.69^{aD}	47.06 ± 0.45^{bD}
LB01+NA06+MRS13	39.23 ± 0.47^{aE}	39.65 ± 0.44^{aE}	40.82 ± 0.38^{aF}

Different lowercase letters within the same row, and different capital letters within the same column indicate significant difference ($p < 0.05$).

was the least, and there was no significant difference in the detoxification rate of extended fermentation time ($p > 0.05$). Among the combined strains, the combination of LB01 and MRS13 had the best detoxification effect on DON in bran (75.98%) after fermented for 96 h, and the detoxification rate did not increase significantly after fermented for 72 h ($p > 0.05$).

It was shown that *B. subtilis* had a strong ability to degrade DON, and there was a certain synergistic effect on the detoxification of DON when combined with *L. plantarum*. The detoxification rate of *P. acidilactici* was not high, and there was no synergistic effect with other strain in each group of fermentation, thus was not considered suitable for the detoxification of DON.

Discussion

DON is a food and feed safety issue, which greatly affects grain and feed production worldwide. Exposure to DON for a long time may increase toxicity in animals due to the synergistic with other contents in feed (Ahad *et al.,* 2017). Biological detoxification is regarded as the preferred method, and many microorganisms have been screened from different sources and used to degrade DON. Several studies have demonstrated that DON could be metabolised to lower toxicity compounds by different microorganisms under different culture conditions, either individually or in combination (Islam *et al.,* 2012). For instance, a bioassay was implemented to screen microbial enrichment cultures, originating from rumen fluid, soil, digestate, and activated sludge on their biotransformation and detoxification capability of DON (Vanhoutte *et al.,* 2017). The enrichment cultures originating from soil and activated sludge were capable of detoxifying and degrading 5 and 50 mg/L DON. Ten isolates were identified and isolated from chicken intestines using conventional microbiological selection strategies guided by PCR-DGGE (denaturing gradient gel electrophoresis). They were all able to transform DON to DOM-1. Most isolates were potent in transforming DON and the activity was stable during sub-culturing (Yu *et al.,* 2010). In the process of long-term symbiosis with DON, living organisms will produce specific enzymes to degrade and utilise it. For instance, yellow mealworms (*Tenebrio molitor* L.) can be grown with diets containing a large number of DON; and then they could digest, decompose, and excrete DON (Van Broekhoven *et al.,* 2017). There was a significant increase of DON observed during ensiling (Jensen *et al.,* 2019); so we assume that there will be a certain amount of DON in the mouldy alfalfa silage, and then we screened the 16 DON detoxification strains from the mouldy alfalfa silage.

There are many bacteria that can detoxify DON such as *Clostridiales*, *Anaerofilum*, *Collinsella*, and *Bacillus* (Yu *et al.,* 2010); *Serratia*, *Clostridium*, *Citrobacter*, *Enterococcus*, *Stenotrophomonas*, and *Streptomyces* (Islam *et al.,* 2012). Zhai *et al.* (2019) reported that high throughput sequencing was used to characterise the composition of the involved degradation microflora, and the analysis of 16S rRNA sequences indicated that the mixed culture was composed of at least 11 bacterial genera, with *Pseudomonas* accounting for nearly half of the relative abundance (Zhai *et al.,* 2019). Also, Wang *et al.* (2019b) isolated a Gram-negative bacterial strain with effective biodegrading abilities on DON. The strain was identified as *Devosia insulae* on the basis of morphological and physiological characteristics, and 16S rRNA-based phylogenetic analysis (Wang *et al.,* 2019a). In the present work, three different strains with the ability of DON degradation were identified and preliminarily determined as *B. subtilis*, *P. acidilactici* and *L. plantarum*. Obtaining strains for the detoxification of DON from probiotic strains and ascertaining the mechanisms of detoxification will be helpful for assuring feed safety in feeding. Fortunately, the strains we isolated were animal probiotics. These strains have the ability of DON detoxification, which is consistent with the characteristics of other reported strains. For instance, two *Bacillus* strains possessing the capability of detoxifying DON were selected from 59 probiotics by using a two-run screening, and the *Bacillus* strains presented stronger detoxification ability of DON (Cheng *et al.,* 2010). Also, *L. sakei* and *P. acidilactici* were reported to have the ability to detoxify DON (Juodeikiene *et al.,* 2018).

Different strains have different detoxification efficiency on DON. It was reported that the highest degradation rate of DON is by a *Paradevosia* strain DDB001, which could grow well and thoroughly eliminate 200 mg/L of DON in a complete growth medium (Wang *et al.,* 2017). Also, DON treatment with *P. acidilactici* ICTU05 resulted in 34% reduction of DON in malting grains (Juodeikiene *et al.,* 2018). In addition to different strains, the detoxification ability of DON may be closely related to the form of DON. In the present work, the strains with the highest detoxification ability of DON standard showed great difference in the detoxification of DON in bran.

When compared with single bacterium, bacterial consortia have a higher ability of DON detoxification. Wang *et al.* (2020) screened the bacterial consortium C20, which efficiently degraded

almost 70 µg/mL DON within 5 d, and the bacterial consortium C20 was able to degrade DON under a wide range of pH and temperature conditions (Wang *et al.,* 2020). This may be due to the synergism between different strains. In the present work, the combination of *Lactobacillus* and *Bacillus* yielded the highest detoxification rate of DON in bran. These two strains could have a great development potential and values since the results obtained agree with those demonstrated by Hahn *et al.* (2015) who also aerobically and anaerobically tested the detoxification of DON.

Conclusion

In the present work, 16 bacterial strains with the ability to detoxify DON were screened, and the best three were further identified. The highest detoxification rate was by *B. subtilis* (66.75%). It was demonstrated that *B. subtilis* had a strong ability to detoxify DON, and there was a certain synergistic effect on the detoxification of DON when it was combined with *L. plantarum*.

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