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Detoxification of Zearalenone by viable and inactivated cells of *Planococcus* sp

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ABSTRACT

The capacity of *Planococcus* sp. strain S118 to remove Zearalenone (ZEN) from liquid medium in varying conditions was investigated. The results indicated that *Planococcus* sp. S118 removed ZEN by binding process. Strain S118 significantly reduced the levels of ZEN in the liquid medium; the viable and heat-inactivated bacteria could remove 21.82% and 47.82% of ZEN, respectively. Heat, acid, and Triton-100 treatment significantly enhanced the capability of removing ZEN. The detoxifying capability depended on the incubation period, concentration of bacteria, pH, and temperature. *Planococcus* sp. S118 likewise possessed the capability to remove Zearalanone (ZAN), which is one of ZEN analogues. The viable and heat-inactivated bacteria could remove 16.36% and 34.26% of ZAN, respectively. The detoxifying capability of ZEN and ZAN by heat-inactivated bacteria were significantly influenced by each other.

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1. Introduction

Zearalenone (ZEN), 6-(10-hydroxy-6-oxo-trans-1-undecenyl)-bresorcylic acid lactone, is a phenolic resorcyclic acid lactone. This mycotoxin is a non-steroidal metabolite with estrogenic-like effects which can be produced by several Fusarium species, such as *F. graminareum, F. crookwellemse, F. culmorum*, and *F. semitectum* (Elmholt & Hestbjerg, 2000; Kuiper-Godman, Scott, & Watanabe, 1987; Marasas, Nelson, & Toussoun, 1984; Thrane, 1989) after infection of corn, wheat, and other cereals (Pittet, 1998).

ZEN not only leads to economic loss by contaminating feed, but it causes serious health problems in livestock and humans as well. Owing to its structure, ZEN possesses many characteristics similar to those of steroid hormones. Biological activity of this toxin can be explained as competing with 17-β-oestradiol (Mitterbauer et al., 2003) and causing estrogenic effects and alterations in the reproductive tract of laboratory and domestic animals (D'Mello, Placinta, & Macdonald, 1999; Etienne & Jemmali, 1982; JECFA, 2000). It has likewise been reported that ZEN is characterized by carcinogenicity, genotoxicity, reproductive and developmental toxicity, and immunotoxicity (Zinedine, Soriano, Moltó, & Mañes, 2007).

Throughout the globe, ZEN has been detected in a number of cereal crops such as maize, barley, oats, wheat, rice, sorghum, and rye (CAST, 2003; Zinedine et al., 2007). Depending on climatic and storage conditions, the contents of ZEN vary within the range of 0.001–8.04 mg/kg (wheat), 0.016–0.095 mg/kg (oat), and 0.004–15 mg/kg (barley) (Placinta, D'Mello, & Macdonald, 1999). According to the United Nations Food and Agriculture Organization (FAO, 2004), ZEN was regulated in 1996 by six countries. However, the number of countries regulating the toxin rose to sixteen by 2003.

The strategies for the detoxification of mycotoxin-contaminated feedstuff remain underdeveloped on a large scale and in a cost-effective manner. One of the approaches to solving the problem is the addition of nonnutritive adsorptive materials which can bind mycotoxins. This binding process decreases the bioavailability and associated toxicities (Huwig, Freimund, Käppeli, & Dutler, 2001; Ramos, Fink-Gremmels, & Hernandez, 1996; Visconti, 1998). A large number of nonnutritive adsorptive materials were investigated *in vitro* testing, such as cholestyramine crospovidone, montmorillonite, bentonite, sepiolite, magnesium trisilicate (Ramos, Hernandez, Pla-Delfina, & Merino, 1996), and modified clinoptilolite (Döll, Dänicke, Valenta, & Flachowsky, 2004; Tomaševic-Canovic, Dakovic, Rottinghaus, Matijaševic, & Đuricic, 2003). *In vivo*, fiber or alfalfa minimizes the effects of ZEN toxicosis in rats or swine (Bursian, Aulerich, Cameron, Ames, & Steficek, 1992; Underhill, Rotter, Thompson, Prelusky, & Trenholm, 1995). The feasibility of utilizing organic adsorbents is examined as well, particularly Esterified glucomannan (Devegowda & Aravind, 2002; Swamy, Smith, MacDonald, Boermans, & Squires, 2002), which is isolated from the inner layer of yeast cell wall. It possesses a significant capability for mycotoxin adsorption.

Several efficient, safe, and reliable methods are also investigated. A number of studies on the degradation and biotransformation of ZEN by various microorganisms have been published. Significant biodegradation of ZEN has been observed by the

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mycoparasite *Gliocladium roseum* NRRL 1859, which is capable of metabolizing ZEN in 80%–90% yields (El-Sharkawy & Abul-Hajj, 1988). The strain could split the lactone ring of ZEN, and the product, which is far less oestrogenic than ZEN, consisting of a mixture of two isomeric hydroxyketones, decarboxylated spontaneously, rendering the reaction irreversible. Kakeya et al. (2002) have reported that the lactone ring of ZEN is sensitive to hydrolysis by *Clonostachys rosea*. A lactonohydrolase responsible for the detoxification is purified to homogeneity; its gene, designated as *zhd101*, is subsequently isolated from the fungus. Biological decontamination of ZEN using genetically modified organisms has also been studied since then (Higa et al., 2003; Takahashi-Ando et al., 2004).

This research aimed to search for new ZEN detoxification bacteria. It conducted a preliminary investigation on the detoxification capability, detoxification mechanisms, and factors affecting detoxification efficiency. After screening for microbial capability to detoxify ZEN in samples collected previously from various natural sources in the authors' laboratory, one of the obtained bacterial isolates exhibited detoxifying capability, which was further identified and characterized. After DNA extraction, PCR-mediated amplification of the 16S rDNA, and purification and sequencing of the PCR products, it was revealed that the isolate belonged to genus *Planococcus* sp. This research was based on this isolate of bacteria (strain S118).

2. Materials and methods

2.1. Cultivation of Planococcus sp. (strain S118)

Fresh CS medium (10 g glucose, 10 g yeast extract, 2 g $CaCO_3$, 1 L H_2O; pH = 6.5) was inoculated with isolate, agitating at 150 r/min for 48 h at 30 °C in a shaker incubator. Cells were harvested by centrifuge (Anke TGL-16G, China) at 8000× g for 5 min. The pellets were washed twice with DF medium (1.52 g KH_2PO_4, 2.44 g Na_2HPO_4, 0.2 g $MgSO_4 \bullet 7H_2O$, 0.5 g $(NH_4)_2SO_4$, 0.05 g $CaCl_2$; pH = 6.5). Meanwhile, DF medium was added to achieve the desired concentration (determined with optical density of 1.6 at 600 nm; the bacterial concentration was approximately 10^9 cfu/ml).

2.2. ZEN standard

A standard of ZEN was purchased from Sigma (Sigma–Alarich, USA). It was dissolved and diluted in methanol under sterile conditions to prepare a stock solution containing 100 mg/L of ZEN. An appropriate volume of this solution was added to the DF medium to reach the desired concentration.

2.3. Tests of detoxification

All assays were performed in Eppendorf (5 ml, safe lock) vials. Then 500 μl of the cell suspension, which was prepared as previously described, was mixed with 500 μl of ZEN solution. The final concentration of bacterial suspension was 5×10^8 cfu/ml, and ZEN was 1 μg/ml. To obtain heat-inactivated cells, bacteria were autoclaved for 20 min at 121 °C. The mixtures were incubated at 30 °C for 24 h with soft agitation (150 r/min). Subsequently, the reaction was terminated, and ZEN was determined.

2.4. Termination of detoxification and ZEN determination

All samples were terminated by centrifuge (14,000× g, 10 min), and 200 μl of supernatant was transferred to Eppendorf vials for analysis by HPLC. Controls with the same amount of DF medium, but without bacteria, were run in all the experiments as well.

Reverse-phase HPLC (system gold 125 solvent module, Beckman Coulter) was employed to quantify the residue of ZEN in the supernatant. Toxin was separated on a C18 column (250 × 4.6 mm; particle size, 5 μm; Diamonsil) with a mobile phase of water-methanol (20:80 [v/v]) at a flow rate of 1 ml/min, detected by ultraviolet (System Gold 166 Detector, Beckman Coulter) at 236 nm, and quantified by 32Karat 7.0 software (Beckman Coulter). The assay temperature was 25 °C with an injection volume of 20 μl, and the retention time was 6.0 ± 0.5 min.

The percentage of the toxin remains was calculated by using the following equation: 100×(peak area of ZEN in the supernatant/peak area of ZEN in the control).

2.5. Detoxification by intracellular cell extract and cell wall

15 ml of cell suspension was disintegrated (performed every other 5 s for 30 min) by ultrasonic cell disintegrator on ice. The disintegrated cell suspension was centrifuged at 8000× g for 10 min. The supernatant was collected, while the precipitate was suspended with 4 ml of DF medium. 500 μl, 250 μl, and 125 μl of supernatant and suspension were mixed with 500 μl, 750 μl, and 875 μl of ZEN solutions, respectively. The final concentration of ZEN was 1 μg/ml subsequently, ZEN detoxification was tested as previously described.

2.6. Impact of different treatments

Cell suspensions were treated by one of the following methods: heat treatment (autoclaved for 20 min at 121 °C), acid treatment (2 mol/L HCl), and Triton-100 (5% [v/v] Triton-100, 10 mM Tris–Cl pH = 8.0, 0.1 M NaCl, 1M EDTA, pH = 8.0). Acid-treated and Triton-100-treated suspensions were incubated at 30 °C for 1 h with soft agitation (150 r/min). After these treatments were performed, the bacterial samples were centrifuged (8000 × g, 5 min), and the supernatants were removed. The bacterial pellet was washed twice and suspended in DF medium. Subsequently, the detoxification of ZEN was tested as previously described.

2.7. Dynamics of detoxification

The previously described detoxification of ZEN was tested for 0, 2, 6, 12, 24, 48, and 72 h, respectively. Subsequently, 500 μl of mixture was centrifuged (14,000× g, 10 min), and 200 μl of the supernatant was transferred to Eppendorf vials. The bacterial pellet after centrifugation was suspended in 500 μl of water-methanol (20:80 [v/v]) for 5 min and centrifuged (14,000× g, 10 min). All the supernatants were analyzed using HPLC.

2.8. Impact of bacterial concentration, temperature, and pH value

The detoxification of ZEN by viable and heat-inactivated cell suspensions was tested under different conditions, respectively (bacterial concentration: 10^7, 5×10^7, 10^8, 5×10^8 and 10^9 cfu/ml; incubation temperature: 4, 20, 30, and 37 °C; incubation pH value: 4.5, 5.5, 6.5, 7.5, and 8.5). The final concentration of ZEN was 1 μg/ml. The mixtures were incubated for 24 h with soft agitation (150 r/min). The reactions were then terminated, and ZEN was determined.

2.9. Removal of ZEN and ZAN from a mixture of toxins

Zearalanone (ZAN) is one of ZEN analogues. The viable and heat-inactivated cell suspensions were mixed with ZEN and/or ZAN

solutions, respectively. The final incubation mixtures contained 5×10^8 cfu/ml of bacteria, 1 μg/ml ZEN and/or 1 μg/ml ZAN. Detoxification was then tested as previously described.

2.10. Statistical analyses

All results are presented as means of replicates and their standard deviations. Data were analyzed by SPSS (Statistical Product and Service Solutions) software for Windows. To determine the significant differences among means, significant tests at the 0.05 or 0.01 levels of probability were conducted according to Duncan's Multiple Range Test or Student's *t*-test. In the Duncan's Multiple Range Test, data with same letters are in the same level and there is no significant difference between them. Data with different letters are in different levels and significant differences are observed.

3. Results and discussion

The detoxification study demonstrated that after 24 h of culturing *Planococcus* sp. with ZEN, the bacteria exhibited strong detoxifying capability not only by viable bacteria (21.82%) but by inactivated bacteria (47.82%) autoclaved at 121 °C as well (Fig. 1). Heat treatment significantly enhanced the bacterial capability to remove toxin, which is approximately twice of the viable bacteria.

After disintegrating the cells using ultrasonic, the cell wall fraction was capable of detoxifying ZEN after 24 h of incubation. Meanwhile, the cell extract nearly failed to detoxify ZEN (Fig. 2). Furthermore, detoxifying capability was related to the content of cell wall fraction.

The above result indicated that the detoxifying capability of *Planococcus* sp. may depend on binding rather than metabolism. After heat treatment, the proteins were all inactive. However, inactivated bacteria also exhibited detoxification capability. Cell wall fraction could detoxify ZEN as well, but the cell extract nearly failed to do so. This indicated that ZEN was probably binding to the cell bacterial surface, causing the reduction of ZEN form incubation mixtures.

To determine if the bacterial viability affected detoxification property, or if the cell wall was involved in binding, comparative experiments were conducted with viable, heat-treated, acid-treated, and Triton-100-treated inactivated cells. Heat treatment, acid treatment, and Triton-100 treatment significantly enhanced the bacterial capability to remove ZEN (Fig. 3). Treatment of the bacteria by heat (45.81%) nearly had the same effect on detoxification as that by acid (47.43%), and better than that by Triton-100 (33.10%).

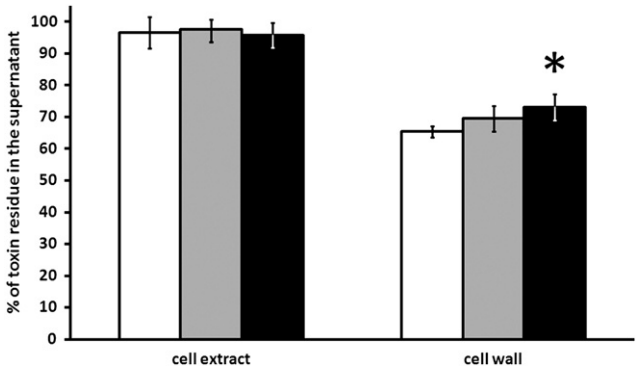

Fig. 2. ZEN detoxification by cell extract and cell wall. The incubation mixtures contained 500 μl (white bars), 250 μl (gray bars), and 125 μl (black bars) of the original suspension of the cell extract or cell wall fraction. The final concentration of ZEN was 1 μg/ml. The values were means of replicates ($n = 3$) and their standard deviations. The asterisk indicated statistical significance in comparison with the first group's (white bars) result (Student's *t*-test, $P < 0.05$).

Planococcus sp. is a Gram-positive bacterium. Cell wall polysaccharide and peptidoglycan were the two possible main elements responsible for the binding of ZEN. Both components were expected to be affected by heating and acids (Quiberoni, Stiefel, & Reinheimer, 2000). Heating might cause protein denaturation, or the formation of Maillard reaction products between polysaccharides and peptides, and proteins. Meanwhile, under acidic conditions, the glycosidic linkages in polysaccharides break down, releasing monomers that might then be further fragmented into aldehydes. Acids might also break the amide linkages in peptides and proteins, producing peptides and the component amino acids. The peptidoglycan of the cell wall was usually quite thick in these organisms, but its thickness might be reduced and/or its pore size may be increased via heat and acid treatments (Haskard, El-Nezami, Kankaanpa, Salminen, & Ahokas, 2001). This perturbation of the bacterial cell wall may allow ZEN to bind to the cell wall easily. In brief, the effective removal of ZEN suggested that binding occurred in the cell subsurface in sites exposed to heat or acid treatments (El-Nezami, Polychronaki, Salminen, & Mykkänen, 2002).

No degradation products of ZEN were observed on the HPLC chromatogram after 72 h of incubation (data not shown), indicating that the strain was not capable of metabolizing ZEN under these experiment conditions. According to the recovery rates of ZEN

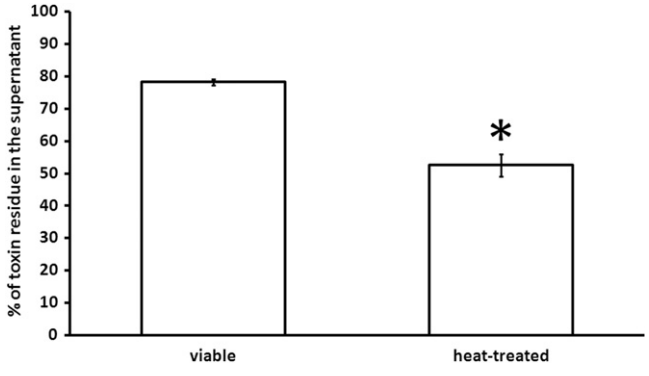

Fig. 1. The detoxifying capability of viable and heat-inactivated bacteria. The incubation mixtures contained 5×10^8 cfu/ml of bacteria and 1 μg/ml of ZEN. The values were means of replicates ($n = 3$) and their standard deviations. Stars indicated statistical significance in comparison with the viable group result (Student's *t*-test, $P < 0.01$).

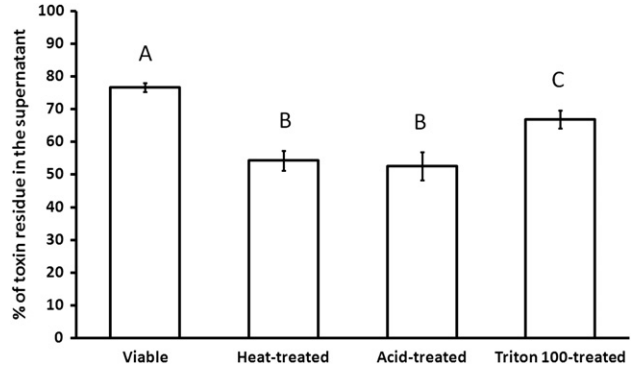

Fig. 3. ZEN detoxification by viable, heat-treated, acid-treated, and Triton-100-treated bacteria. The incubation mixtures contained 5×10^8 cfu/ml of bacteria and 1 μg/ml of ZEN. The values were means of replicates ($n = 5$) and their standard deviations. Means with different letters were significant different according to Duncan's Multiple Range Test ($P < 0.05$).

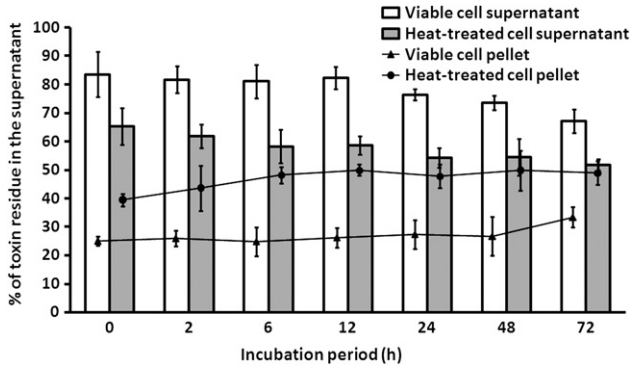

Fig. 4. Dynamics of ZEN detoxification by strain S118. The viable and heat-inactivated bacteria were shown in white and gray, respectively. The incubation mixtures contained 5×10^8 cfu/ml of bacteria and 1 μg/ml of ZEN, and were incubated at 30 °C for 0, 2, 6, 12, 24, 48, and 72 h, respectively. The values were means of replicates ($n = 5$) and their standard deviations.

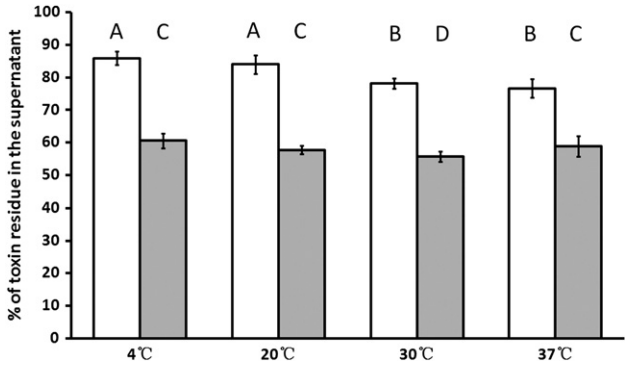

Fig. 6. Impact of ZEN removal by temperature. The viable and heat-inactivated bacteria were shown in white and gray, respectively. The incubation mixtures contained 1 μg/ml of ZEN and 5×10^8 cfu/ml of bacteria. The mixtures were incubated at 4, 20, 30 and 37 °C, respectively. The values were means of replicates ($n = 5$) and their standard deviations. Means with different letters were significant different according to Duncan's Multiple Range Test ($P < 0.05$).

through water-methanol extraction, ZEN was all recovered from the bacterial cells and supernatant. This indicated that ZEN was chemically stable under these incubation conditions, and it was associated with the bacterial surface, as previously mentioned.

The detoxification of ZEN was a relatively rapid and continued process since approximately 16.43% and 34.59% were removed after mixing with either viable bacteria or heat-inactivated bacteria (Fig. 4). When incubation was continued, the percentage of ZEN in the supernatant was reduced. At the same time, the recovery rate from the bacterial cell was increased. After 24 h, the percentage of ZEN in the supernatant in the heat-inactivated bacteria group was basically stable. However, the percentage of ZEN in the supernatant in viable bacteria was nonetheless reduced even after 72 h.

Several experiments were conducted to screen the optimal conditions for detoxification. Fig. 5 demonstrated that the detoxifying capability of ZEN relied strongly on the concentration of bacteria in the incubation mixtures. Significant different effects of detoxification between the viable bacteria and heat-inactivated bacteria were significantly displayed when cfu/ml was $\geq 5 \times 10^7$/ml. The higher the concentration of bacteria, the greater the difference between viable bacteria and heat-inactivated bacteria.

ZEN detoxification by viable and heat-inactivated bacteria varied under different temperatures (Fig. 6). The detoxification was lower ($P < 0.05$) at 4 °C (14.10%) and 20 °C (16.04%) as compared to that at 30 °C (21.93%) and 37 °C (23.39%) by viable bacteria. In the heat-inactivated bacterial case, detoxification at 30 °C (44.34%) was significantly higher as compared to that at 4 °C, 20 °C, and 30 °C.

ZEN detoxification was pH sensitive (Fig. 7). The highest removal by viable bacteria (31.75%) was observed at pH 4.5, decreasing gradually as the pH value rose, with the lowest recorded at pH = 8.5 (12.04%). However, no significant differences were observed between the pH 7.5 and 8.5 groups. Detoxification by heat-inactivated bacteria exhibited a similar trend: the lower the pH value, the higher the detoxification capability. Detoxification at pH 4.5 was highest, but there were no significant differences among the pH 4.5, 5.5, and 6.5 groups. The correlation of ZEN detoxification with pH values was probably a result of the cell surface's electric charge distribution. Further study on this is required.

To investigate whether strain S118 could detoxify ZAN, one of the ZEN analogues, or if ZAN could affect ZEN detoxification, we incubated ZEN with or without ZAN, as well as ZAN with or without ZEN. Data indicated that strain S118 could also remove ZAN by viable bacteria (16.36%) and heat-inactivated bacteria (34.26%) (Table 1). Incubation of a mixture of toxins (1:1) significantly decreased ($P < 0.01$) the detoxifying capability of a single toxin by heat-inactivated bacteria. Detoxification of ZEN was significantly affected by ZAN, dropping from 49.59% to 38.90%. The bacteria possibly contained a number of binding sites for this type of toxins, and ZAN was a competitive inhibition in ZEN binding.

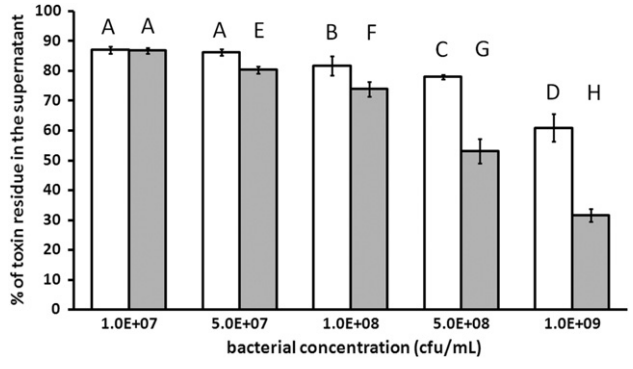

Fig. 5. Impact of ZEN removal by cell density. The viable and heat-inactivated bacteria were shown in white and gray, respectively. The incubation mixtures contained 1 μg/ml of ZEN and 10^7, 5×10^7, 10^8, 5×10^8, and 10^9 cfu/ml bacteria, respectively. The values were means of replicates ($n = 5$) and their standard deviations. Means with different letters were significant different according to Duncan's Multiple Range Test ($P < 0.05$).

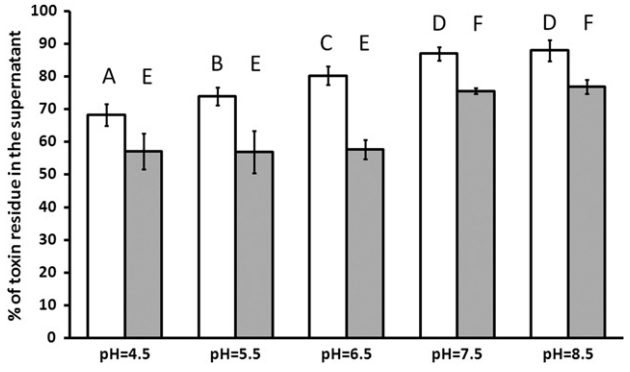

Fig. 7. Impact of ZEN removal by pH value. The viable and heat-inactivated bacteria were shown in white and gray, respectively. The incubation mixtures contained 1 μg/ml of ZEN and 5×10^8 cfu/ml of bacteria. The mixtures were incubated in pH 4.5, 5.5, 6.5, 7.5 and 8.5, respectively. The values were means of replicates ($n = 5$) and their standard deviations. Means with different letters were significant different according to Duncan's Multiple Range Test ($P < 0.05$).

Table 1
Detoxification of ZEN and ZAN from a mixture of toxins[a].

	% Residue of toxin in solution			
	ZEN		ZAN	
	Alone	With ZAN	Alone	With ZEN
Viable	77.44 ± 0.83	80.30 ± 2.27	83.64 ± 1.51	84.02 ± 3.33
Heat-treated	50.41 ± 1.20	61.10 ± 6.22*	65.74 ± 2.62	71.53 ± 2.55*

[a] The final incubation mixtures contained 5×10^8 cfu/ml of bacteria, 1 μg/ml ZEN and/or 1 μg/ml ZAN. The asterisk indicated statistical significance different in comparison with the incubation with single toxin group results (Student's *t*-test, $P < 0.01$) ($n = 5$).

4. Conclusion

The results of this study indicated that *Planococcus* sp. strain S118 significantly reduced the levels of ZEN in the incubation mixtures by viable and inactivated bacteria. Binding rather than metabolism could possibly explain the interaction of *Planococcus* sp. strain S118 with ZEN. Heat, acid, and Triton-100 treatment significantly enhanced the bacterial capability to remove ZEN. Incubation period, concentration of bacteria, pH, and temperature were strong factors affecting the detoxifying capability of ZEN.

Planococcus sp. strain S118 could likewise remove ZAN from the incubation mixtures by viable and heat-inactivated bacteria. A complex interaction existed between ZEN and ZAN detoxification. As a potential mycotoxin binder, more research is needed on detoxifying application in real food and feed samples.

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