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Pyrosequencing investigation into the influence of Cu^{2+}, Zn^{2+}, Fe^{2+} and I^- mixtures on fungal diversity and toxigenic fungal growth in a fermented liquid feed

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ABSTRACT

A $L_9(3^4)$ orthogonal experiment was conducted to evaluate the influence of 9 mixtures which consisted of Cu^{2+}, Zn^{2+}, Fe^{2+} and I^- ions at different ion concentrations on fungal diversity and toxigenic fungal growth in a *Bacillus subtilis*-fermented liquid feed (FLF) using pyrosequencing. The maximal Chao estimator and Shannon index were achieved in the FLF with a mixture of Cu^{2+} (200 mg/kg), Zn^{2+} (160 mg/kg), Fe^{2+} (150 mg/kg) and I^- (2.4 mg/kg). The minimal relative abundance of *Aspergillus* was achieved when a mixture of Cu^{2+} (200 mg/kg), Zn^{2+}, Fe^{2+} and I^- was added to the FLF. Compared with Zn^{2+}, Fe^{2+} and I^-, Cu^{2+} was the most important ion in inhibiting *Aspergillus* growth. Adding Zn^{2+} (160 mg/kg), Cu^{2+}, Fe^{2+} and I^- to the FLF minimized the relative abundance of *Fusarium*. It was Zn^{2+} instead of Cu^{2+} played a critical role in suppressing the growth of *Fusarium*. The proper use of the mixture of Cu^{2+}, Zn^{2+}, Fe^{2+} and I^- in FLF contributes to inhibit the growth of mycotoxin-producing fungi during storage. The new findings of this study help farmers properly use the mixture of Cu^{2+}, Zn^{2+}, Fe^{2+} and I^- to inhibit the growth of mycotoxin-producing fungi in the production of high quality FLF and alleviate mycotoxins damages to animals and humans.

1. Introduction

The use of fermented liquid feed (FLF) is becoming more and more popular in piglets feeding, because it keeps high and regular feed and water intake of piglets (Missottena et al., 2010), alleviates the stress associated with dietary change and improves piglet health and performance (Maslowski and Mackay, 2011; Wang et al., 2011). However, how to effectively inhibit the growth of mycotoxigenic fungi during the storage of FLF has been a common concern, because mycotoxigenic fungi could grow well in high-moisture feed and produce mycotoxins.

Mycotoxins are toxic secondary metabolites produced by many species of *Aspergillus*, *Fusarium*, *Penicillium* and *Alternaria*. The most extensively investigated mycotoxins in animal feed are aflatoxin B1 (AFB1), zearalenone (ZEN) and deoxynivalenol (DON; also known as vomitoxin) (Yiannikouris and Jouany, 2002; Streit et al., 2012; Li et al., 2015). The aflatoxins (B, G and M) are natural toxins produced by the filamentous fungi *Aspergillus flavus*, *Aspergillus parasiticus*, *Aspergillus bombycis*, *Aspergillus nomius*, *Aspergillus ochraceoroseus*, *Aspergillus pseudotamarii* and *Aspergillus tamari* (Mishra and Das, 2003; Guan et al., 2011a,b). When animals continuously ingest aflatoxins through contaminated feed, gastrointestinal dysfunction, reduced feed utilization, anaemia and jaundice often occur (Streit et al., 2012). Zearalenone is mainly produced by *Fusarium graminearum* and *Fusarium culmorum* (Labuda et al., 2005). Ingesting ZEN usually results in genital problems including hyperaemia, swelling and reddening of the vulva, uterine enlargement, vaginal and rectal

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prolapse, enlargement of mammary glands, and reproductive disorders of sows (Kakeya et al., 2002). Deoxynivalenol is a trichothecene mycotoxin, which is primarily associated with *F. graminearum* and *F. culmorum* (Stroia et al., 2010). The toxicosis of DON in pigs is manifested by vomiting, feed refusal, abortion, stillbirths and weak offspring (Williams et al., 1989; Tirado et al., 2010; Xiao et al., 2013). Thus, it is a high priority to develop measures to alleviate mycotoxins contamination and toxicity in animal feed industry (Wu et al., 2013; Duan et al., 2014; Yin et al., 2014; Wu et al., 2014).

Trace elements (copper, zinc, iron, molybdenum, magnesium, etc.) play important roles in controlling the growth and secondary metabolism of fungi. The effect of some trace elements on the biosynthesis of aflatoxin by *A. flavus* and *A. parasiticus* has been reviewed. It was found that iron, copper, zinc, manganese, molybdenum, calcium and magnesium under certain concentrations enhanced the growth of *A. flavus* and *A. parasiticus* (Lee et al., 1966; Lillehoj et al., 1974; Aziz et al., 2000; Datsugwai et al., 2013). However, when the concentrations of trace elements are beyond optimum concentrations, trace elements could inhibit the growth of fungi progressively (Rawla, 1969).

To promote the performance of pigs and decrease the incidence of intestinal diseases, Cu^{2+}, Zn^{2+}, Fe^{2+} and I^{-} are often added to the feed of pigs at higher concentrations than the normal required concentrations, relative to other trace elements. No studies have investigated whether fungal diversity and toxigenic fungal growth are influenced by a mixture of Cu^{2+}, Zn^{2+}, Fe^{2+} and I^{-}. To find an effective method to decrease the growth of toxigenic fungi during the storage period of FLF, we designed the present study to survey the difference in fungal diversity and growth in 9 *Bacillus subtilis* FLF containing different concentrations of Cu^{2+}, Zn^{2+}, Fe^{2+} and I^{-} by pyrosequencing.

2. Materials and methods

2.1. Experimental design and sample preparation

We selected Cu^{2+}, Zn^{2+}, Fe^{2+} and I^{-} as experimental factors. Each factor had 3 concentrations which are listed in Table 1 (these ions are currently added to diets of piglets in China at concentrations more than 0 mg/kg, thus the concentration of 0 mg/kg was excluded). In addition, the selected concentrations were based on the analyzed values of 6 commercial diets for suckling piglets and 6 commercial diets for early weanling piglets. Table 2 lists 9 mineral mixtures which were prepared according to the $L_9(3^4)$ orthogonal design. Each mineral mixture was added to the basal diet to produce 9 experimental diets. The basal diet is shown in Table 3.

Each experimental diet (100 g) and tap water (300 g) were mixed into a polypropylene bag (size: 18 cm × 15 cm; thickness: 80 μm; 6 bags per experimental diet). These bags were sealed with heat sealer and heated in a steam container at 80 °C for 30 min under normal pressure to kill undesirable microorganisms to make *B. subtilis* grow well. And then, they were placed in room temperature (22.5 to 33.9 °C) for a 21-d storage. We randomly selected 4 bags of FLF from each treatment on day 22, mixed the FLF and then sampled them. All samples were stored at −80 °C before genomic DNA extraction.

Table 1
Three concentrations of trace element ions[1] (mg/kg) to be added to the basal diet.

Item	Concentration 1	Concentration 2	Concentration 3
Cu^{2+}	200	150	100
Zn^{2+}	160	110	60
Fe^{2+}	150	100	50
I^{-}	2.4	1.2	0.6

[1] Cu^{2+} of $CuSO_4 \cdot 5H_2O$, Zn^{2+} of $ZnSO_4 \cdot H_2O$, Fe^{2+} of $FeSO_4 \cdot H_2O$ and I^{-} of KI.

Table 2
Trace element mixtures to be added to the basal diet (mg/kg) in a $L_9(3^4)$ orthogonal experiment design.

Item	Cu^{2+}	Zn^{2+}	Fe^{2+}	I^{-}
Mixture 1	200	160	150	2.4
Mixture 2	200	110	100	1.2
Mixture 3	200	60	50	0.6
Mixture 4	150	160	100	0.6
Mixture 5	150	110	50	2.4
Mixture 6	150	60	150	1.2
Mixture 7	100	160	50	1.2
Mixture 8	100	110	150	0.6
Mixture 9	100	60	100	2.4

Table 3
Ingredients and nutrient levels of the basal diet (air-dry basis).

Item	Content
Ingredient, %	
Corn	52.0
Wheat bran	7.0
Extruded soybean	30.0
Fishmeal (Peru)	3.0
Lactose	4.0
Premix[1]	4.0
Total	100.0
Nutrient levels,[2] %	
Digestible energy, MJ/kg	13.71
Crude protein	19.75
Calcium	1.05
Total phosphorus	0.66
Lysine	1.32
Methionine + Cystine	0.78

[1] Premix provided per kilogram diet: VA 450,000 IU, VD_3 72,000 IU, VE 2750 IU, VK_3 100 mg, VB_1 90 mg, VB_2 280 mg, VB_6 190 mg, VB_{12} 0.8 mg, Niacin 1,450 mg, Pantothenic acid 950 mg, Biotin 3 mg, Choline chloride 10,500 mg, Lysine 40,000 mg, Mn 2,000 mg, Co 38 mg, Se 10.5 mg, Ca 137,000 mg, P 40,800 mg, NaCl 80,000 mg, Wole200 (heat-resistant *Bacillus subtilis* HEWD113, effective live bacteria $\geq 2 \times 10^{10}$ CFU/g) 7,500 mg.

[2] Nutrient levels in the table were analyzed values except digestible energy.

2.2. DNA extraction and pyrosequencing

We used the E.Z.N.A Soil DNA kit (OMEGA, USA) to extract genomic DNA. The triplicate DNA extracts for each sample were pooled prior to PCR. Samples were amplified using the forward primer (A-ITS1) and reverse primer (B-ITS4). The forward primer (A-ITS1) was 5′-*CCATCTCATCCCTGCGTGTCTCCGACGACT*NNNNNNN-NNNTCCGTAGGTGAACCTGCGG-3′, where the sequence of adaptor A is shown in italics and underlined, and the Ns represent a ten-base sample specific barcode sequence. The reverse primer (B-ITS4) was 5′-*CCTATCCCCTGTGTGCCTTGGCAGTCGACT*TCCTCCGCT-TATTGATATGC-3′, where the sequence of adaptor B is shown in italics and underlined.

The polymerase chain reaction (PCR) was carried out in a 20-mL reaction volume which was containing 0.4 μL TransStart Fastpfu DNA Polymerase (Beijing TransGen Biotech Co., Ltd, China), 4 μL FastPfu buffer (5×), 2 μL dNTPs (2.5 mmol/L), 0.8 μL Forward Primer (5 μmol/L), 0.8 μL reverse primer (5 μmol/L), 0.4 μL Fastpfu Polymerase (5 μmol/L), 10 ng DNA template and de-ionized ultrapure water. The PCR protocol was performed on ABI GeneAmp 9700 Cycler in following conditions: initial denaturation for 2 min at 95 °C, followed by 30 cycles of denaturation for 30 s at 95 °C,

annealing for 30 s at 55 °C and extension for 30 s at 72 °C, then with a final extension for 5 min at 72 °C. Amplification products were visualized on 2% agarose gels, then purified using AxyPrepDNA PCR purification kit (Axygen, China), quantified using the QuantiFluor-ST system (Promega) and pooled in equimolar ratios based on concentration and subjected to emulsion PCR (Roche GS FLX Titanium emPCR Kits) to generate amplicon libraries. Amplicon pyrosequencing was performed from the A-end using a 454/Roche GS-FLX Titanium platform.

2.3. Sequences processing and bioinformatic analysis

Raw sequences that were obtained from 454/Roche GS-FLX Titanium pyrosequencer were processed with Mothur software (http://sourceforge.net/projects/seqclean/ & http://www.mothur.org/wiki/Main_Page) and the unqualified sequences were removed according to following criteria: <200 nucleotides in length (not including sample specific barcodes), contained ambiguous bases, had an imperfect match to a sample-specific barcode and a read quality score <25. The chimeric sequences were also excluded by Usearch software (version6.1, http://drive5.com/usearch/). The unique sequences were clustered into operational taxonomic units (OTUs) at 97% sequence identity by using Qiime software (http://qiime.org/scripts/assign_taxonomy.html, Naïve Bayesian Classifier). A taxonomy assignment was conducted using the ITS database (http://its2.bioapps.biozentrum.uni-wuerzburg.de/) with a confidence level of 0.7. The abundance coverage-based estimator (http://www.mothur.org/wiki/Ace) and Shannon index (http://www.mothur.org/wiki/Shannon) were calculated by Mothur, and microbial community barplot and heatmap were generated by R packages.

3. Results

3.1. Reads, OTUs and alpha-diversity

After processing and quality filtering of raw data, the reads (qualified sequences) and OTUs of samples from different FLF are listed in Table 4. The data of reads were deposited in the sequence read archive (SRA) database (accession number: SRP044186). Ace and Chao 1 are the indexes of microbial richness. The higher values of Ace (or, Chao 1), the more microbial communities. Thereby, the fungi richness in FLF was ranked in this order: 1 > 8 > 4 > 6 > 3 > 2 > 5 > 9 > 7, as shown in Table 4. Shannon and Simpson are the indexes of microbial diversity. The higher Shannon value (or the lower Simpson value) means the higher microbial diversity. Thus, the fungal diversity in FLF was ranked in this order: 1 > 8 > 4 > 6 > 3 > 2 > 9 > 5 > 7, as shown in Table 4.

Table 4
Reads, operational taxonomic units (OTUs), richness and diversity of fungi in fermented liquid feeds (FLF) samples.

Item	Reads	0.97 (Sequence identity)				
		OTUs	Ace	Chao 1	Shannon	Simpson
FLF 1	6748	826	1249	1258	4.86	0.0303
FLF 2	5704	317	525	445	2.88	0.2097
FLF 3	6085	397	580	574	3.32	0.1326
FLF 4	5586	609	853	860	4.24	0.0786
FLF 5	6459	309	498	424	2.64	0.2491
FLF 6	5790	560	785	784	3.98	0.0953
FLF 7	6600	283	376	395	2.50	0.2761
FLF 8	6638	704	1026	1011	4.30	0.0790
FLF 9	6740	326	418	416	2.72	0.2688

3.2. Community composition of fungi in samples of different FLF

The hierarchical heatmap was based on the top 100 abundant fungi community at genus level (as shown in Fig. 1). This heatmap disclosed that samples of FLF 1 to 9 were numerically dominated by unclassified genus. The highest similarity of fungi community was found between samples of FLF 4 and 6, FLF 5 and 7, respectively. Fig. 2 shows the fungi community compositions of the 9 FLF samples. The relative abundance of toxigenic fungi, *Aspergillus* and *Fusarium*, in the 9 FLF samples are listed as follows: for *Aspergillus*, 0.31, 0.46, 0.56, 0.36, 1.41, 1.30, 0.95, 2.21 and 4.94; for *Fusarium*, 0.07, 0.46, 0.31, 0.13, 0.09, 0.31, 0.15, 0.42 and 0.80. Samples from FLF 1 had the minimal relative abundance of *Aspergillus* and *Fusarium*, but samples from FLF 9 had the maximal relative abundance of *Aspergillus* and *Fusarium*.

3.3. Effect of trace elements on relative abundance of toxigenic fungi

The best concentration and significance order of the trace elements for the relative abundance of *Aspergillus* and *Fusarium* were different (as shown in Table 5). Compared with Zn^{2+}, Fe^{2+} and I^{-}, Cu^{2+} is the most important ion in controlling the growth of *Aspergillus*. The best concentration of Cu^{2+} in inhibiting the growth of *Aspergillus* is 200 mg/kg (Concentration 1). It was Zn^{2+} instead of Cu^{2+} plays a dominant role in promoting the growth of *Fusarium*, and the addition of Zn^{2+} at 160 mg/kg (Concentration 1) minimized *Fusarium* growth. The comprehensive analysis showed that the growth of *Aspergillus*, *Fusarium*, *Alternaria* and *Penicillium* in the FLF can be inhibited when liquid feed was fermented with a mixture of Cu^{2+}, Zn^{2+}, I^{-} and Fe^{2+} at 200, 160, 1.2 and 50 mg/kg, respectively.

4. Discussion

Trace elements (e.g., Cu, Zn, Fe and I) are crucial for the proper growth and metabolism of fungi. A lack or overly concentrated addition of trace elements is adverse to the growth of fungi. No information is available for the influence of Cu^{2+}, Zn^{2+}, Fe^{2+} and I^{-} mixtures on the growth of fungi in FLF. Results in the current study showed that the mixture of Cu^{2+}, Zn^{2+}, Fe^{2+} and I^{-} added at different concentrations affected the growth and diversity of fungi in FLF during storage, and the growth and diversity of fungi in FLF decreased with decreasing single Cu^{2+}, Zn^{2+} or Fe^{2+} concentration.

Toxigenic fungi grow well in liquid feeds with abundant moistures and other nutrients and produce mycotoxins at optimal temperatures. Thus, how to suppress the growth of toxigenic fungi in FLF is highly concerned by the producers of animal husbandry industry. Toxigenic fungi that are most extensively investigated in animal feed are *Aspergillus* and *Fusarium* (Streit et al., 2012; Stroia et al., 2010; Guan et al., 2011a,b). Previous results found that trace elements affect the growth of *Aspergillus* and *Fusarium* (Adiga et al., 1962; Jackson et al., 1989; Cuero et al., 2003; Cuero and Ouellet, 2005). The influence of trace elements is different depending on the type of trace element ions and whether they were used alone or in a mixture (Cuero and Ouellet, 2005). Some trace elements such as Cu^{2+} and Zn^{2+} are essential micronutrients. However, when they exceed certain threshold levels, they are toxic to fungi; the optimal concentrations of Fe^{2+}, Cu^{2+} and Zn^{2+} vary much widely with different strains (Hartikainen et al., 2012).

Data of the present study indicated that the relative abundance of *Aspergillus* in FLF varied with different mixtures of Cu^{2+}, Zn^{2+}, Fe^{2+} and I^{-}. *Aspergillus* showed the lowest relative abundance in the FLF with a mixture of Cu^{2+} (200 mg/kg) and Zn^{2+}, Fe^{2+}, I^{-}. The ability of mixtures in inhibiting *Aspergillus* growth was ranked as 1 > 4 > 2 > 3 > 7 > 6 > 5 > 8 > 9. This showed that addition of Cu^{2+}

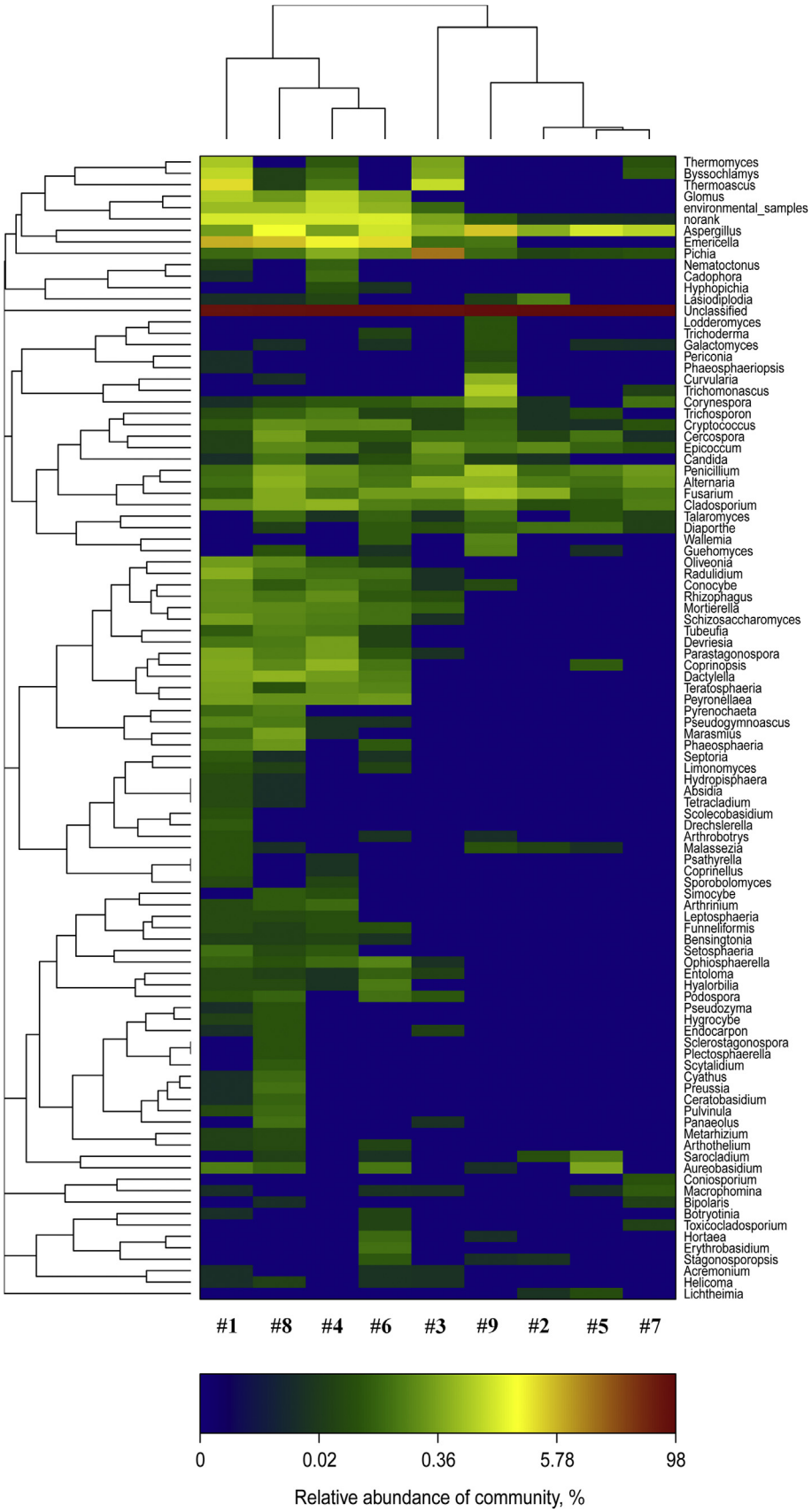

Fig. 1. Hierarchical dendrogram of the top 100 fungi in different fermented liquid feed (FLF). Distribution of the top 100 abundant fungi genus in different FLF. The phylogenetic tree was calculated using the neighbour-joining method and the relationship among samples was determined by Bray–Curtis distance. The heatmap plot depicted the relative percentage of each fungal genus within each sample. The relative values for fungal genus were indicated by colour intensity.

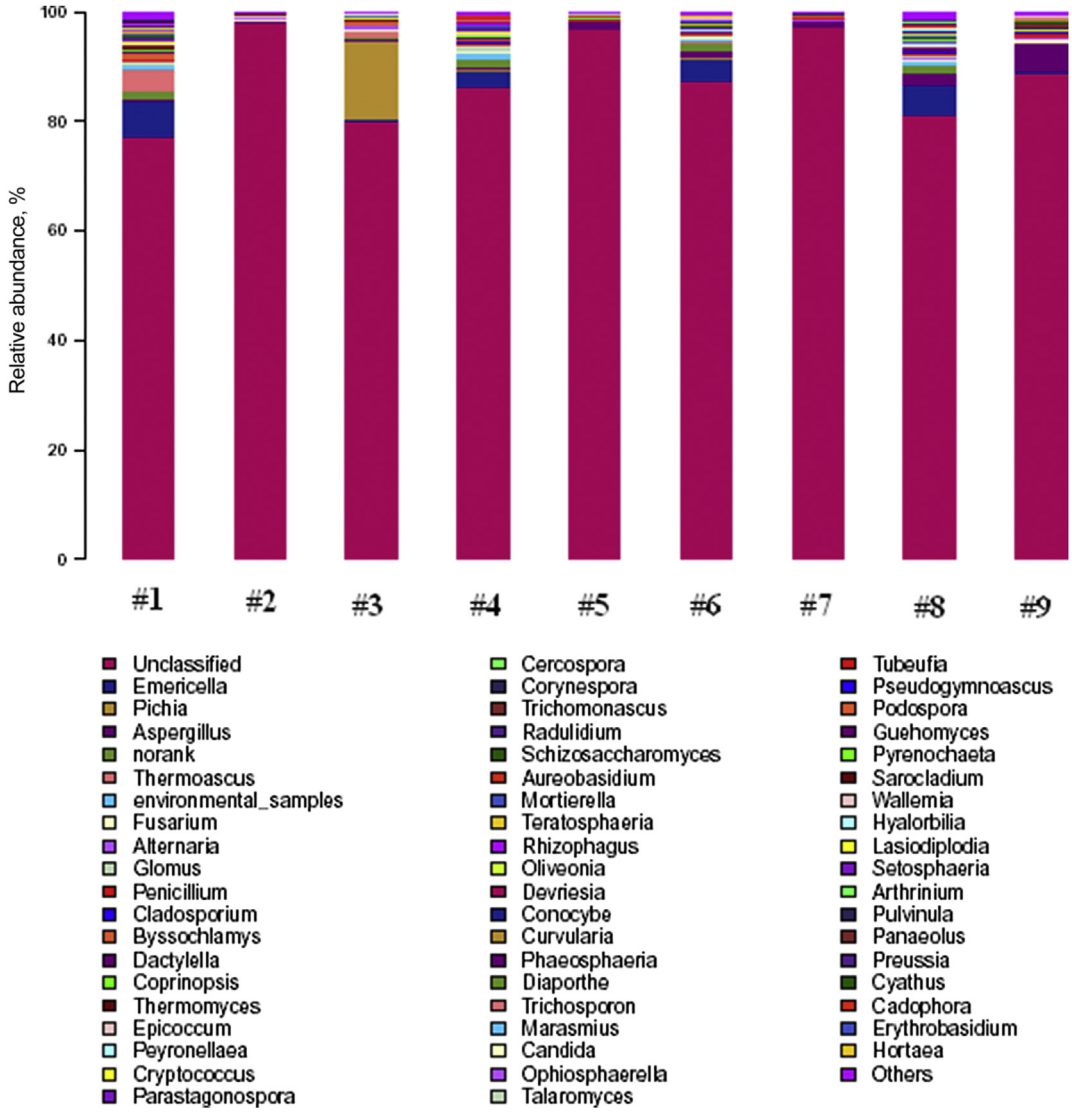

Fig. 2. Genus-level taxonomic compositions of fungi in different FLF. Genus-level taxonomic compositions of fungi in FLF. Sequences that could not be classified into any known group were named as No-rank. Sequences that could not be matched to any known sequences were designated as unclassified. Sequences that had relative abundance of less than 1% were grouped into others.

Table 5
Effect of trace elements on the relative abundance of toxigenic fungi in FLF.

Item	Factor	Mean			Range[1]	Best concen.[2]	Significance order
		Concen. 1	Concen. 2	Concen. 3			
Aspergillus	Cu^{2+}	0.44	1.02	2.70	2.26	Concen. 1	$Cu^{2+} > Zn^{2+} > I^{-} > Fe^{2+}$
	Zn^{2+}	0.54	1.36	2.26	1.72	Concen. 1	
	Fe^{2+}	1.27	1.92	0.97	0.94	Concen. 3	
	I^{-}	2.22	0.90	1.04	1.32	Concen. 2	
Fusarium	Cu^{2+}	0.28	0.18	0.46	0.28	Concen. 2	$Zn^{2+} > Fe^{2+} = Cu^{2+} > I^{-}$
	Zn^{2+}	0.12	0.32	0.47	0.36	Concen. 1	
	Fe^{2+}	0.27	0.46	0.19	0.28	Concen. 3	
	I^{-}	0.32	0.31	0.29	0.04	Concen. 3	
Alternaria	Cu^{2+}	0.26	0.17	0.42	0.25	Concen. 2	$Cu^{2+} > I^{-} > Zn^{2+} > Fe^{2+}$
	Zn^{2+}	0.21	0.25	0.38	0.17	Concen. 1	
	Fe^{2+}	0.25	0.30	0.30	0.05	Concen. 1	
	I^{-}	0.25	0.19	0.41	0.22	Concen. 2	
Penicillium	Cu^{2+}	0.12	0.19	0.48	0.36	Concen. 1	$Cu^{2+} > Fe^{2+} = I^{-} > Zn^{2+}$
	Zn^{2+}	0.23	0.23	0.33	0.10	Concen. 2	
	Fe^{2+}	0.22	0.36	0.21	0.15	Concen. 3	
	I^{-}	0.33	0.18	0.28	0.15	Concen. 2	

[1] Range = maximal relative abundance minus minimal relative abundance.
[2] Concen. = concentration. The best concentration is the concentration to achieve the minimal relative abundance.

at 200 mg/kg combined with Zn^{2+}, Fe^{2+} and I^- to the FLF could minimize the growth of *Aspergillus*.

The significance of single Cu^{2+}, Zn^{2+}, Fe^{2+} and I^- for the relative abundance of *Aspergillus* was different and ranked as $Cu^{2+} > Zn^{2+} > I^- > Fe^{2+}$ in the FLF according to the value of range. The best concentrations of single Cu^{2+}, Zn^{2+}, Fe^{2+} and I^- ions in inhibiting the growth of *Aspergillus* were 200, 160, 50 and 1.2 mg/kg, respectively. Elevated concentration (from 100 to 200 mg/kg) of Cu^{2+} decreased the growth of *Aspergillus*, which is consistent with the previous result (Al Abboud and Alawlaqi, 2011). Elevated concentration (from 60 to 160 mg/kg) of Zn^{2+} suppressed the relative abundance of *Aspergillus*. Aziz et al. (2000) also found that elevated Zn^{2+} (from 100 to 300 mg/kg) decreased the growth of *A. flavus* in a 14-d incubation, but increased the growth of *A. flavus* in a 28-d incubation, and the increase in Cu^{2+} or Fe^{2+} concentration numerically decreased the growth of *A. flavus* (Aziz et al., 2000). Our results also showed high Fe^{2+} concentration inhibited the growth of *Aspergillus*, and high I^- concentration stimulated the growth of *Aspergillus* during a 21-d storage.

Fusarium showed minimal relative abundance in the FLF with a combination of Zn^{2+} (110 mg/kg) and Cu^{2+}, Fe^{2+}, I^-. The ability of Cu^{2+}, Zn^{2+}, Fe^{2+} and I^- mixtures in suppressing the growth of *Fusarium* was ranked as $1 > 5 > 4 > 7 > 3 = 6 > 8 > 2 > 9$. This indicated that high concentration of Zn^{2+} combined with Cu^{2+}, Fe^{2+} and I^- decreased the growth of *Fusarium* in FLF.

Compared with Cu^{2+}, Fe^{2+} and I^-, Zn^{2+} was the most important ion in inhibiting the growth of *Fusarium* in the FLF. Elevated Zn^{2+} concentration suppressed *Fusarium* growth. Thind and Madan (1977) found that Zn^{2+} was necessary for the growth and sporulation of *Fusarium moniliforme*, and Zn^{2+} from 0.0001 to 10 mg/kg supplementation increased the growth of the fungus, but from 10 to 400 mg/kg decreased it progressively, and the optimal concentration of Zn^{2+} for *F. moniliforme* is 1.0 mg/kg. Cuero and Ouellet (2005) reported that the supplementation of Zn^{2+}, Cu^{2+} or Fe^{2+} to *F. graminearum* liquid cultures stimulated the growth of *F. graminearum*, and single Fe^{2+} ion had the largest effect, but single Cu^{2+} ion had the smallest effect. The triple mixture of Zn^{2+}, Cu^{2+} and Fe^{2+} also stimulated the growth of *F. graminearum* (Cuero and Ouellet, 2005). Our results validated that Zn^{2+} instead of Cu^{2+} played a dominant role in the growth of *Fusarium*, and the best concentration for Zn^{2+} to decrease the growth of *Fusarium* was 110 mg/kg.

5. Conclusions

The diversity and relative abundance of fungi in FLF are influenced by a mixture of Cu^{2+}, Zn^{2+}, Fe^{2+} and I^- at different concentrations. The most important ion in inhibiting *Aspergillus* growth is Cu^{2+}, and Zn^{2+} plays a critical role in suppressing the growth of *Fusarium*. The supplementation of Cu^{2+} at 200 mg/kg, Zn^{2+} at 160 mg/kg, Fe^{2+} at 150 mg/kg and I^- at 2.4 mg/kg to a *B. subtilis* FLF could minimize the relative abundance of *Aspergillus* and *Fusarium*.

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