Individual and Combined Occurrence of Mycotoxins in Feed Ingredients and Complete Feeds in China

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Abstract: The objective of this study was to investigate the individual and combined contamination of aflatoxin B1 (AFB1), zearalenone (ZEN) and deoxynivalenol (DON) in feedstuffs from different Provinces of China between 2016 and 2017. A total of 1569 samples, including 742 feed ingredients and 827 complete pig feed samples, were collected from various regions of China for mycotoxins analysis. The results showed that individual occurrence rates of AFB1, ZEN, and DON were more than 83.3%, 88%, and 74.5%, respectively, in all the tested samples. DON was the most prevalent contaminant, followed by ZEN and AFB1, with the average concentrations ranging from 450.0–4381.5 µg/kg, 2.3–729.2 µg/kg, and 1.3–10.0 µg/kg, respectively. Notable, 38.2%, 10.8%, and 0.6% of complete pig feeds were contaminated with DON, ZEN, and AFB1 over China’s regulatory limits, respectively. Moreover, over 75.0% analyzed samples were co-contaminated with two or three mycotoxins. In conclusion, the current study revealed that the feedstuffs in China were severely contaminated with DON, followed by ZEN and AFB1 during the past two years. These findings highlight the importance of monitoring mycotoxins in livestock feed and implementing feed management and bioremediation strategies to reduce mycotoxin exposure.

Keywords: aflatoxin B1; zearalenone; deoxynivalenol; feedstuffs; China

Key contribution: The current study was evaluated individual and combined occurrence of AFB1, ZEN, and DON in feedstuffs in China. These findings have important implications for the management of mycotoxins in animal breeding and feed production.

1. Introduction

Mycotoxins are a large group of fungal secondary metabolites, which are toxic to both animals and humans and that are mainly produced by five genera: Aspergillus, Fusarium, Penicillium, Claviceps and Alternaria [1]. To date, approximately 400 mycotoxins have been identified [2]. Aflatoxin B1 (AFB1), zearalenone (ZEN) and deoxynivalenol (DON) are recognized as the major mycotoxins contaminants in
agricultural products, including maize, wheat, barley, peas, peanuts, millet, oily feedstuffs, and forage, and their by-products [3,4]. As primarily produced by *Aspergillus*, AFB$_1$ is the most toxic mycotoxin, possessing hepatotoxic, mutagenic, carcinogenic, and teratogenic properties in many species of animals; it is also has been classified as a Group I carcinogen [5–7]. Both ZEN and DON are mainly produced by *Fusarium*. ZEN is an estrogenic toxin that competes with 17 β-estradiol for estrogen receptor binding, which consequently leads to fertility and reproductive problems [8,9]. In contrast, DON can induce anorexia, vomiting, and impairs immune function in various livestock species that involves inhibiting DNA, RNA, and protein synthesis [10–13].

Owing to the negative effect of mycotoxins on the health and performance of livestock, many countries have established safety standards for these toxins in feed and feed ingredients. The European Commission for example, set maximum levels of AFB$_1$, ZEN, and DON at 5–20 µg/kg, 250 µg/kg, and 900 µg/kg, respectively, in all feed ingredients and complete feed [14,15]. Recently, the Chinese government updated livestock safety standards for AFB$_1$, ZEN and DON (Table 1), which are 10 to 20 µg/kg, 100 to 250 µg/kg, and 1000 to 5000 µg/kg, respectively, for complete feeds [16].

Table 1. Chinese safety standards for AFB$_1$, ZEN and DON in feedstuffs of China.

| Feedstuff | Maximum Limit (µg/kg) |
|-----------|-----------------------|
| AFB$_1$   |                       |
| Corn by-products and peanut cake | 50 |
| Vegetable oil (except corn oil and peanut oil) | 10 |
| Corn oil and peanut oil | 20 |
| Other plant feed ingredients | 30 |
| Complete feeds for young pigs and poultry | 10 |
| Complete feeds for growing-boilers and meat-duck and laying-ducks | 15 |
| Other complete feeds | 20 |
| ZEN       |                       |
| Corn and its by-products (except Corn bran and corn steep powder) | 500 |
| Corn bran and corn steep powder | 1500 |
| Other plant feed ingredients | 1000 |
| Complete feed for young pigs | 150 |
| Complete feed for young gilts | 100 |
| Other complete feeds for pigs | 250 |
| Other complete feeds | 500 |
| DON       |                       |
| Plant feed ingredients | 5000 |
| Complete feeds for pigs | 1000 |
| Other complete feeds | 5000 |

1. AFB$_1$, aflatoxin B$_1$; DON, deoxynivalenol; ZEN, zearalenone.

Today, climate change and global warming are increasing susceptibility of staple crops, corn, wheat and soybean to fungal colonization and mycotoxin contamination, particularly AFB$_1$, ZEN and DON [17,18]. Thus, it will be important to monitor mycotoxin levels in livestock feed ingredients well into the future to maintain animal health and ensure the safety of human food products. China’s agriculture sector is contamination of mycotoxin due to, there are several climatic regions across the country, such as Yangtze and the Yellow River basins that are warm and humid with plenty of rainfall, which is favorable for mold growth and mycotoxin production in cereals [19–21]. Investigation into the prevalence of mycotoxin contamination in staple crops in China is required to help prevent exposure of livestock to mycotoxins and to ensure food and feed safety. However, such information is still very limited in China, especially regarding the co-occurrence of mycotoxins in feed ingredients. Therefore, this study was conducted to determine the contamination of individual and combined mycotoxins (AFB$_1$, ZEN and DON) in feed and feed ingredients collected from various regions of China. These results can serve as an important reference for feed manufacturers, livestock producers and Chinese regulatory authorities involved in feed and food safety.
2. Results

2.1. Concentrations of AFB$_1$ in Feed Ingredients and Complete Feeds

The occurrence of AFB$_1$ in feed ingredients and complete feeds are summarized in Table 2. A total of 1016 samples, including 522 feed ingredients and 494 complete feeds samples, were collected between 2016 and 2017 to measure the concentration of AFB$_1$. AFB$_1$ was detected in 76.9–100% of feed ingredients and complete feeds, with the mean values ranging from 1.3 to 10.0 µg/kg. The highest median level of AFB$_1$ was 11.5 µg/kg in corn bran harvested in 2016 and followed by 10.0 µg/kg in domestic DDGS from 2017. The maximum contamination of AFB$_1$ was 67.6 µg/kg in corn harvested in 2016, followed by 49.2 µg/kg in wheat flour from 2016, 36.4 µg/kg in pig complete feed (powder) from 2016, and 32.0 µg/kg in corn harvested during 2017. Only 10 samples, including 6 corn, 1 wheat flour and 3 complete pig feed (powder) from 2016, which account for 1.0% of all the analyzed feed ingredients and complete feeds, were contaminated with AFB$_1$ at levels exceeding Chinese safety standard concentrations (Table 1).

2.2. Concentrations of ZEN in Feed Ingredients and Complete Feeds

A total of 1155 samples, including 596 feed ingredients and 559 complete feeds samples, were collected between 2016 and 2017 to measure the concentration of ZEN (Table 3). ZEN was detected in 88.0–100% of feed ingredients and complete feeds, with the average concentrations from 2.3 to 729.2 µg/kg. The highest median level of ZEN was 729.2 µg/kg found in corn gluten meal from 2017, followed by 302.9 µg/kg in corn bran from 2016, and 258.7 µg/kg in domestic DDGS from 2016. The maximum contamination of ZEN was 1363.2 µg/kg in corn germ meal made in 2016, followed by 1268.6 µg/kg in corn bran from 2016, 1195.9 µg/kg in wheat middlings from 2016, 1169.2 µg/kg in imported DDGS from 2016, and 1109.7 µg/kg in pig complete feed (powder) from 2016, respectively. A total of 37 samples, which account for 3.2% of all the analyzed feed ingredients and complete feeds, were contaminated with ZEN more than the 500 µg/kg during 2016–2017. Notably, a total of 60 complete pig feeds, which account for 10.7% of all the complete pig feeds, were contaminated with ZEN at levels exceeding the Chinese safety standard concentration of 250 µg/kg (Table 1).

2.3. Concentrations of DON in Feed Ingredients and Complete Feeds

A total of 1271 samples, including 687 feed ingredients and 584 complete feeds samples, were collected between 2016 and 2017 to measure the concentration of DON (Table 4). DON was detected in 74.5–100% of feed ingredients and complete feeds, with the average concentrations ranging from 450.0 to 4381.5 µg/kg. The highest median level of DON was 4701.4 µg/kg in wheat flour from 2016, followed by 4004.3 µg/kg in wheat harvested during 2016, 3547.2 µg/kg in domestic DDGS from 2017 and 3045.2 µg/kg in corn bran from 2016. The maximum concentration of DON was 12,633.3 µg/kg in wheat middlings from 2016, followed by 11,028.9 µg/kg in barley harvested in 2016, 10,437.6 µg/kg in complete pig feed (powder) from 2017, and 9556.8 µg/kg in wheat flour from 2016, respectively. A total of the 18 samples, which account for 1.4% of all the analyzed feed ingredients and complete feeds, were contaminated with DON at levels exceeding the 5000 µg/kg during 2016–2017. Notably, a total of 223 complete pig feeds, which account for 38.2% of all the complete pig feeds samples, were contaminated with DON at levels exceeding the Chinese safety standard concentration of 1000 µg/kg (Table 1).

2.4. Co-Contamination of AFB$_1$, ZEN and DON in Feed Ingredients and Complete Feeds

AFB$_1$, ZEN and DON were present as co-contaminates in feed ingredients and complete feeds samples collected between 2016 and 2017 (Table 5). The co-occurrence of AFB$_1$ and ZEN, AFB$_1$ and DON, ZEN and DON, as well as AFB$_1$, ZEN and DON, in feed ingredients ranged from 76.9–100%, 76.9–100%, 75.0–100% and 76.9–100%, respectively. While, the co-occurrence of AFB$_1$ and ZEN, AFB$_1$ and DON, ZEN, and DON, as well as AFB$_1$, ZEN and DON in complete pig feed ranged from 96.4–100%, 96.4–100%, 97.9–100% and 96.4–100%, respectively.
Table 2. AFB$_1$ concentrations in feed ingredients and complete feeds $^1$.

| Item                        | Year | No. of Samples | Positive Samples (µg/kg) | Numbers of Samples in the Range (µg/kg) |
|-----------------------------|------|----------------|--------------------------|----------------------------------------|
|                             |      |                | % | Mean | Median | Maximum | <0.5 | 0.5–10 | 10–30 | 30–50 | 50–100 |
| Corn                        | 2016 | 175            | 94.9 | 5.8 | 4.3 | 67.6 | 9 | 156 | 5 | 1 | 4 |
|                             | 2017 | 75             | 97.3 | 4.1 | 3.3 | 32.0 | 2 | 70 | 2 | 1 | 0 |
| Domestic DDGS               | 2016 | 38             | 100 | 9.8 | 10.0 | 19.7 | 0 | 19 | 19 | 0 | 0 |
|                             | 2017 | 1              | 100 | 3.1 | 3.1 | 3.1 | 0 | 1 | 0 | 0 | 0 |
| Imported DDGS               | 2016 | 27             | 100 | 7.3 | 8.4 | 15.0 | 0 | 23 | 4 | 0 | 0 |
|                             | 2017 | 3              | 100 | 4.9 | 4.8 | 5.6 | 0 | 3 | 0 | 0 | 0 |
| Corn bran                   | 2016 | 5              | 100 | 10.0 | 11.5 | 13.5 | 0 | 2 | 3 | 0 | 0 |
|                             | 2017 | 3              | 100 | 4.6 | 4.2 | 5.8 | 0 | 3 | 0 | 0 | 0 |
| Corn germ meal              | 2016 | 9              | 100 | 8.1 | 8.3 | 13.5 | 0 | 8 | 1 | 0 | 0 |
|                             | 2017 | 5              | 100 | 5.4 | 4.2 | 10.9 | 0 | 4 | 1 | 0 | 0 |
| Corn gluten meal            | 2016 | 4              | 100 | 8.0 | 8.0 | 10.3 | 0 | 3 | 1 | 0 | 0 |
|                             | 2017 | 2              | 100 | 6.3 | 6.3 | 7.1 | 0 | 2 | 0 | 0 | 0 |
| Wheat                       | 2016 | 14             | 100 | 2.3 | 2.1 | 4.9 | 0 | 14 | 0 | 0 | 0 |
|                             | 2017 | 2              | 100 | 6.3 | 6.3 | 7.1 | 0 | 2 | 0 | 0 | 0 |
| Barley                      | 2016 | 6              | 83.3 | 1.6 | 1.7 | 2.8 | 1 | 5 | 0 | 0 | 0 |
|                             | 2016 | 45             | 100 | 2.5 | 2.4 | 6.4 | 0 | 45 | 0 | 0 | 0 |
| Wheat bran                  | 2017 | 16             | 100 | 2.7 | 2.8 | 3.8 | 0 | 16 | 0 | 0 | 0 |
| Wheat middlings             | 2016 | 22             | 86.4 | 2.7 | 2.3 | 4.5 | 3 | 19 | 0 | 0 | 0 |
|                             | 2017 | 10             | 100 | 3.1 | 3.1 | 4.3 | 0 | 2 | 0 | 0 | 0 |
| Wheat flour                 | 2016 | 9              | 88.9 | 7.5 | 2.5 | 49.2 | 1 | 7 | 0 | 1 | 0 |
|                             | 2017 | 2              | 100 | 1.6 | 1.6 | 1.9 | 0 | 2 | 0 | 0 | 0 |
| Broken rice                 | 2016 | 13             | 76.9 | 3.6 | 2.1 | 16.7 | 3 | 9 | 1 | 0 | 0 |
|                             | 2017 | 4              | 100 | 3.7 | 3.8 | 4.37 | 0 | 4 | 0 | 0 | 0 |
| Rice bran                   | 2016 | 11             | 100 | 3.4 | 3.1 | 6.6 | 0 | 11 | 0 | 0 | 0 |
|                             | 2017 | 4              | 100 | 3.7 | 3.8 | 4.37 | 0 | 4 | 0 | 0 | 0 |
| Soybean meal                | 2016 | 23             | 95.7 | 4.5 | 4.9 | 6.9 | 1 | 22 | 0 | 0 | 0 |
|                             | 2017 | 8              | 87.5 | 2.7 | 3.2 | 3.7 | 1 | 7 | 0 | 0 | 0 |
| Complete pig feed (pellet)  | 2016 | 111            | 96.4 | 3.5 | 3.3 | 26.6 | 4 | 106 | 1 | 0 | 0 |
|                             | 2017 | 9              | 100 | 2.6 | 2.8 | 3.9 | 0 | 9 | 0 | 0 | 0 |
| Complete pig feed (powder)  | 2016 | 155            | 100 | 4.0 | 3.7 | 36.4 | 0 | 151 | 3 | 1 | 0 |
|                             | 2017 | 219            | 97.7 | 3.6 | 3.5 | 25.7 | 5 | 213 | 1 | 0 | 0 |

$^1$ Positive samples are defined as those with AFB$_1$ $\geq$ 0.5 µg/kg (LOD). AFB$_1$, aflatoxin B$_1$; DDGS, dried distillers grains with soluble.
Table 3. ZEN concentrations in feed ingredients and complete feeds.

| Item                | Year | No. of Samples | Positive Samples (µg/kg) | Numbers of Samples in the Range (µg/kg) |
|---------------------|------|----------------|--------------------------|----------------------------------------|
|                     |      |                | % | Mean  | Median | Maximum | <10  | 10–250 | 250–500 | 500–2000 |
| Corn                | 2016 | 183            | 93.4 | 104.1 | 76.6    | 624.3    | 12   | 157    | 9       | 5       |
|                     | 2017 | 86             | 90.7 | 55.0   | 32.1    | 296.8    | 8    | 76     | 2       | 0       |
| Domestic DDGS       | 2016 | 46             | 100  | 299.4  | 258.7   | 956.7    | 0    | 20     | 22      | 4       |
|                     | 2017 | 1              | 100  | 49.3   | 49.3    | 49.3     | 0    | 1      | 0       | 0       |
| Imported DDGS       | 2016 | 33             | 100  | 274.8  | 212.7   | 1169.2   | 0    | 19     | 12      | 2       |
|                     | 2017 | 3              | 100  | 204.0  | 144.1   | 378.1    | 0    | 2      | 1       | 0       |
| Corn bran           | 2016 | 6              | 100  | 432.1  | 302.9   | 1268.6   | 0    | 3      | 1       | 2       |
|                     | 2017 | 3              | 100  | 231.5  | 109.8   | 456.6    | 0    | 2      | 1       | 0       |
| Corn germ meal      | 2016 | 10             | 100  | 316.5  | 188.7   | 1363.2   | 0    | 6      | 3       | 1       |
|                     | 2017 | 8              | 100  | 129.6  | 100.8   | 325.4    | 0    | 7      | 1       | 0       |
| Corn gluten meal    | 2016 | 5              | 100  | 494.9  | 139.8   | 1095.1   | 0    | 3      | 0       | 2       |
|                     | 2017 | 2              | 100  | 729.2  | 729.2   | 1006.3   | 0    | 0      | 1       | 1       |
| Wheat               | 2016 | 14             | 100  | 2.3    | 2.1     | 4.9      | 0    | 8      | 0       | 0       |
| Wheat               | 2017 | 14             | 100  | 154.4  | 148.4   | 393.8    | 0    | 12     | 2       | 0       |
| Barley              | 2016 | 50             | 88.0 | 94.0   | 81.8    | 439.3    | 6    | 41     | 3       | 0       |
| Wheat bran          | 2016 | 20             | 95   | 89.3   | 65.8    | 304.7    | 1    | 18     | 1       | 0       |
| Wheat middlings     | 2016 | 32             | 96.9 | 179.5  | 119.5   | 1195.9   | 1    | 24     | 6       | 1       |
|                     | 2017 | 5              | 100  | 81.4   | 57.9    | 180.8    | 0    | 5      | 0       | 0       |
| Wheat flour         | 2016 | 8              | 90   | 111.7  | 99.5    | 330.9    | 1    | 6      | 1       | 0       |
|                     | 2017 | 2              | 50   | 37.2   | 37.2    | 79.2     | 1    | 1      | 0       | 0       |
| Broken rice         | 2016 | 13             | 100  | 282.3  | 149.5   | 879.8    | 0    | 9      | 1       | 3       |
| Rice bran           | 2016 | 13             | 100  | 169.1  | 169.9   | 280.0    | 0    | 3      | 1       | 0       |
| Soybean meal        | 2016 | 27             | 96.3 | 76.9   | 69.2    | 202.4    | 1    | 26     | 0       | 0       |
|                     | 2017 | 8              | 100  | 38.8   | 32.9    | 56.9     | 0    | 9      | 0       | 0       |
| Complete pig feed   | 2016 | 123            | 100  | 210.7  | 129.5   | 916.5    | 0    | 87     | 23      | 13      |
| (pellet)            | 2017 | 9              | 100  | 55.7   | 42.5    | 138.5    | 0    | 9      | 0       | 0       |
| Complete pig feed   | 2016 | 187            | 99.5 | 129.3  | 100.8   | 1109.7   | 1    | 170    | 14      | 2       |
| (powder)            | 2017 | 240            | 99.6 | 65.1   | 43.9    | 597.8    | 1    | 231    | 7       | 1       |

1 Positive samples are defined as those with ZEN ≥ 10 µg/kg (LOD). DDGS, dried distillers grains with soluble; ZEN, zearalenone.
Table 4. DON concentrations in feed ingredients and complete feeds.

| Item                | Year | No. of Samples | Positive Samples (µg/kg) | Numbers of Samples in the Range (µg/kg) |
|---------------------|------|----------------|--------------------------|----------------------------------------|
|                     |      |                | % | Mean       | Median      | Maximum  | <100 | 100–1000 | 1000–5000 | >5000 |
| Corn                | 2016 | 187            | 98.4 | 857.4       | 718.4       | 4590.8   | 3   | 130      | 54        | 0     |
|                     | 2017 | 97             | 97.9 | 750.3       | 639.6       | 2250.9   | 2   | 72       | 23        | 0     |
| Domestic DDGS       | 2016 | 48             | 100  | 2599.7      | 2458.21     | 6044.7   | 0   | 2        | 45        | 1     |
|                     | 2017 | 2              | 100  | 3547.2      | 3547.2      | 5406.8   | 0   | 0        | 1         | 1     |
| Imported DDGS       | 2016 | 34             | 100  | 1855.4      | 1717.1      | 4044.7   | 0   | 3        | 31        | 0     |
|                     | 2017 | 55             | 74.5 | 872.8       | 523.6       | 7297.8   | 14  | 16       | 24        | 1     |
| Corn bran           | 2016 | 6              | 100  | 2943.2      | 3045.2      | 4710.7   | 0   | 1        | 5         | 0     |
|                     | 2017 | 3              | 100  | 1295.3      | 1086.4      | 1916.7   | 0   | 1        | 2         | 0     |
| Corn germ meal      | 2016 | 10             | 100  | 1426.5      | 1229.2      | 2900.6   | 0   | 4        | 6         | 0     |
|                     | 2017 | 7              | 100  | 1206.9      | 1001.4      | 2374.6   | 0   | 3        | 4         | 0     |
| Corn gluten meal    | 2016 | 4              | 100  | 1688.1      | 1665.2      | 2229.1   | 0   | 0        | 4         | 0     |
|                     | 2017 | 2              | 100  | 559.9       | 559.9       | 620.3    | 0   | 2        | 0         | 0     |
| Wheat               | 2016 | 14             | 100  | 3613.8      | 4004.3      | 6595.6   | 0   | 2        | 10        | 2     |
|                     | 2017 | 15             | 100  | 2635.8      | 492.6       | 11028.9  | 0   | 9        | 3         | 3     |
| Barley              | 2016 | 53             | 100  | 2304.2      | 2405.6      | 6054.4   | 0   | 8        | 44        | 1     |
|                     | 2017 | 23             | 100  | 1394.6      | 1014.3      | 5642.1   | 0   | 9        | 13        | 1     |
| Wheat bran          | 2016 | 34             | 100  | 2961.2      | 2647.6      | 12633.3  | 0   | 2        | 30        | 2     |
|                     | 2017 | 7              | 100  | 1543.0      | 1017.7      | 3563.0   | 0   | 3        | 4         | 0     |
| Wheat middlings     | 2016 | 9              | 100  | 4381.5      | 4701.4      | 9568     | 0   | 1        | 4         | 4     |
|                     | 2017 | 3              | 100  | 450.0       | 456.4       | 736.9    | 0   | 3        | 0         | 0     |
| Wheat flour         | 2016 | 13             | 100  | 1607.3      | 1038.7      | 4075.4   | 0   | 6        | 7         | 0     |
|                     | 2017 | 16             | 100  | 1532.7      | 1505.1      | 3148.5   | 0   | 6        | 10        | 0     |
| Broken rice         | 2016 | 4              | 100  | 1271.6      | 1117.3      | 1900.0   | 0   | 1        | 3         | 0     |
|                     | 2017 | 29             | 96.6 | 451.6       | 377.9       | 1171.4   | 1   | 26       | 2         | 0     |
| Soybean meal        | 2016 | 12             | 100  | 610.7       | 498.3       | 1478.5   | 0   | 11       | 1         | 0     |
|                     | 2017 | 128            | 99.2 | 1194.0      | 1089.6      | 4279.3   | 1   | 53       | 74        | 0     |
| Complete pig feed   | 2016 | 195            | 100  | 1018.1      | 936.8       | 3400.9   | 0   | 108      | 87        | 0     |
| (pellet)            | 2017 | 252            | 98   | 876.3       | 706.2       | 10437.6  | 5   | 187      | 58        | 2     |
| Complete pig feed   | 2016 | 13             | 100  | 1532.7      | 1505.1      | 3148.5   | 0   | 6        | 10        | 0     |
| (powder)            | 2017 | 16             | 100  | 1271.6      | 1117.3      | 1900.0   | 0   | 1        | 3         | 0     |

1 Positive samples are defined as those with DON \( \geq 100 \) µg/kg (LOD). DDGS, dried distillers grains with soluble; DON, deoxynivalenol.
Table 5. Percentage of AFB$_1$, ZEN and DON co-contaminants in feed ingredients and complete feeds.

| Item                  | Year | AFB$_1$ & ZEN (%) | AFB$_1$ & DON (%) | ZEN & DON (%) | AFB$_1$ & ZEN & DON (%) |
|-----------------------|------|--------------------|-------------------|---------------|--------------------------|
| Corn                  | 2016 | 92.4               | 94.7              | 93.4          | 92.4                     |
|                       | 2017 | 92.0               | 97.3              | 90.7          | 92.1                     |
| Domestic DDGS         | 2016 | 100                | 100               | 100           | 100                      |
|                       | 2017 | 100                | 100               | 100           | 100                      |
| Imported DDGS         | 2016 | 100                | 100               | 100           | 100                      |
|                       | 2017 | 100                | 100               | 100           | 100                      |
| Corn bran             | 2016 | 100                | 100               | 100           | 100                      |
|                       | 2017 | 100                | 100               | 100           | 100                      |
| Corn germ meal        | 2016 | 100                | 100               | 100           | 100                      |
|                       | 2017 | 100                | 100               | 100           | 100                      |
| Corn gluten meal      | 2016 | 100                | 100               | 100           | 100                      |
|                       | 2017 | 100                | 100               | 100           | 100                      |
| Wheat                 | 2016 | 83.3               | 83.3              | 75.0          | 83.3                     |
|                       | 2017 | 86.7               | 93.8              | 95.0          | 93.8                     |
| Wheat bran            | 2016 | 86.4               | 86.4              | 96.9          | 86.4                     |
|                       | 2017 | 100                | 100               | 100           | 100                      |
| Wheat middlings       | 2016 | 87.5               | 87.5              | 87.5          | 87.5                     |
|                       | 2017 | 100                | 100               | 100           | 100                      |
| Wheat flour           | 2016 | 76.9               | 76.9              | 100           | 76.9                     |
|                       | 2017 | 100                | 100               | 100           | 100                      |
| Broken rice           | 2016 | 100                | 100               | 100           | 100                      |
|                       | 2017 | 100                | 100               | 100           | 100                      |
| Rice bran             | 2016 | 91.3               | 95.7              | 96.3          | 91.3                     |
|                       | 2017 | 87.5               | 87.5              | 97.9          | 87.5                     |
| Soybean meal          | 2016 | 96.4               | 96.4              | 99.2          | 96.4                     |
|                       | 2017 | 100                | 100               | 100           | 100                      |
| Complete pig feed     | 2016 | 99.3               | 100               | 99.5          | 99.3                     |
| (pellet)              | 2017 | 97.7               | 97.6              | 97.9          | 97.6                     |
| Complete pig feed     | 2016 | 96.4               | 96.4              | 99.2          | 96.4                     |
| (powder)              | 2017 | 100                | 100               | 100           | 100                      |

1 Co-occurrence of mycotoxins samples is defined as those simultaneously contain AFB$_1$, ZEN, and/or DON $\geq$ 0.5 µg/kg, 10 µg/kg, and 100 µg/kg (LOD), respectively. AFB$_1$, aflatoxin B$_1$; DON, deoxynivalenol; ZEN, zearalenone; AFB$_1$ & ZEN, feedstuffs co-contaminated with AFB$_1$ and ZEN; AFB$_1$ & DON, feedstuffs co-contaminated with AFB$_1$ and DON; ZEN&DON, feedstuffs co-contaminated with ZEN and DON; AFB$_1$ & ZEN & DON, feedstuffs co-contaminated with AFB$_1$, ZEN and DON; DDGS, dried distillers grains with soluble.
3. Discussion

The current study was conducted to determine the individual and combined occurrence of three of the most prevalent and toxic mycotoxins (AFB₁, ZEN and DON) in feed ingredients and complete pig feeds from different regions of China from 2016 to 2017. Generally, all three mycotoxins showed a quite high prevalence in the analyzed samples during the past two years, ranging from 74.5% to 100% in total. The mean level of AFB₁ (1.6–10.0 µg/kg) measured in the current study was relatively lower than previously reported levels (0.4–627 µg/kg) in China during the period of 2012–2015 [21,22], while the percentage of samples containing AFB₁ (76.9–100%) was very high, and 1.0% of the 1016 samples exceeded China’s safety standards. Notably, the relatively lower mean level of AFB₁ in the current study can explain that only 1.2% of the whole tested complete pig feeds were contaminated with AFB₁ over 10 µg/kg, which was much lower than those of the previously reported 7.7–7.8% [21,22]. This discrepancy may be because the analyzed feedstuffs were randomly collected from various areas, and weather varies in these regions during the collection period. A recently summarized report showed that the range values of AFB₁ in feed materials and feedstuffs were 8 µg/kg, 2–3 µg/kg, 0–3 µg/kg, 8–90 µg/kg, 1 µg/kg, and 42 µg/kg in North America, Central South America, Europe, Asia, Oceania, and Africa, respectively [23]. The range value of AFB₁ in feedstuffs in the current study was 0–67.6 µg/kg, which is similar to the value from Asia, which was the highest compared with other areas [23]. Differences in the occurrence of AFB₁ among various geographical areas may be due to the differences of the seasonal and local weather conditions during critical plant growing stages. In addition, since feedstuffs are major goods for import and export between countries, 2.1% feed ingredients samples (>20.0 µg/kg) and 14.6% complete pig feed samples (>5.0 µg/kg) exceeded the European Commission regulation (250 µg/kg) should not be ignored [21]. Because AFB₁ is the most carcinogenic mycotoxin, it will be necessary to continue monitoring AFB₁ levels in feed and feed ingredients well into the future.

The percentage and concentration of the analyzed Fusarium mycotoxin ZEN in the present study were quite high. The percentage and range of mean ZEN concentrations in feedstuffs were 88.0–100% and 2.3–729.2 µg/kg, respectively, which is similar with previous Chinese reports showing 50.0–100% and 0–630.2 µg/kg, respectively, in feedstuffs between 2012 and 2015 [21,22]. ZEN was mainly contaminated in corn gluten meal, corn germ meal, corn bran, and DDGS, barley, wheat middlings and rice bran, which is similar to previous reports that showed that ZEN primarily occurred in corn, wheat and barley and their by-products in China, South Korean, Europe, Middle East, and Africa [21,22,24–26]. Also, 10.7% of 559 complete pig feed samples exceeded the regulatory limits in China, which is relatively lower than a previous investigation conducted in China [21]. This discrepancy may also be attributed to the different sampling areas and different weather conditions during the collection periods. Notably, although no feed ingredients samples exceeded the European Commission regulation (2000–3000 µg/kg), similar regulatory limits in European and China for complete pig feeds indicated that exporting these feeds should be strictly monitored [26]. Notably, the incidence rate and the mean range of ZEN concentrations in the complete pig feeds in China were higher than those of in South Korean which were 95% and 31.7 µg/kg, respectively [26]. Meanwhile, the recently summarized report showed that the range values of ZEN in feed materials and feedstuffs were 217 µg/kg, 0–111 µg/kg, 3–37 µg/kg, 32–219 µg/kg, 50 µg/kg, and 25 µg/kg in North America, Central South America, Europe, Asia, Oceania, and Africa, respectively [23]. The maximum value of ZEN in feedstuffs in the current study is 1363.2 µg/kg, which is higher than the values in all the reported areas [23]. Differences in the occurrence of ZEN among various geographical areas can be a result of the differences in the seasonal and local weather conditions during critical plant growing stages.

The percentage and concentration of the analyzed Fusarium mycotoxin DON in the present study were also high. The percentage of samples and average concentration of DON were 74.5–100% and 450.0–4381.5 µg/kg, respectively, which are similar to previous reports of 50.0–100% and 364.5–3931.7 µg/kg, respectively, in feedstuffs collected in China during the period of 2012–2015 [21,22].
The results indicate that wheat, barley, wheat flour, wheat middlings, wheat brain, DDGS and corn bran were seriously contaminated with DON, and 5.6% of the 306 feed ingredients exceeded the 5000 µg/kg safety concentration set by China. Surprisingly, 38.2% of the 584 complete pig feed samples were contaminated with DON at concentrations that exceeded the 1000 µg/kg regulatory limit set by China; this degree of contamination was much higher than the previously reported percent ages (14.0–23.5%) [20–22], which could be due again to the different regions and their weather conditions. Only four feed ingredients (barley, wheat middlings and wheat flour) over the relatively loose regulatory limits in Europe (8000–12,000 µg/kg), similar safety standards in Europe and China for the complete pig feeds indicate that export these feeds should be rigorously supervised [21].

The recently summarized report showed that the range values of DON in feed material and feedstuffs were 1947 µg/kg, 51–237 µg/kg, 88–968 µg/kg, 61–691 µg/kg, 94 µg/kg, and 745 µg/kg in North America, Central South America, Europe, Asia, Oceania, and Africa, respectively [23]. The maximum value of DON in feedstuffs in the current study is 12,633.3 µg/kg, which is at least 6 times higher than the values in all the reported areas [23]. Differences in the occurrence of DON among various geographical areas may be due to the differences in the seasonal and local weather conditions during critical plant growing stages. Taken together, this study indicates that the investigated feed ingredients and complete pig feeds in China during the past 2 years were severely contaminated with Fusarium mycotoxins, especially DON; and the government, feed company and farmers need to be aware of this.

Co-occurring mycotoxins may exhibit addictive or synergetic toxic effects, and this has been well documented by many studies [4,27–30]. Unfortunately, co-occurrence of mycotoxins was quite common in the present study, with more than 75% of samples contaminated with two or three mycotoxins. Notably, the DDGS, corn bran, corn germ meal, corn gluten meal, wheat, and rice bran samples were 100% co-contaminated with AFB1, ZEN and DON in all the tested samples, and more than 96.4% complete pig feeds were co-contaminated with these three mycotoxins. These outcomes were consistent with previous investigations that revealed that mycotoxin co-occurrence is an extremely common problem in feed industry world widely [23,25,31–35]. Because current safety regulations do not consider the toxic potential of co-occurring mycotoxins, the combined effects on animal and human health are probably under estimated; these combined toxic effects need to be investigated further and should be taken into consideration when new regulatory limits are set in the future.

It is also worth noting that the mean levels of the three analyzed mycotoxins in most of the feed ingredients and complete feeds were lower in 2017 compared to those of in 2016. The domestic DDGS had similar AFB1 concentrations compared to the imported DDGS in both 2016 and 2017, lower mean ZEN concentrations than imported DDGS in 2017, and much higher DON concentrations than the imported DDGS in both 2016 and 2017; the three analyzed mycotoxins concentrations appeared similar in both pellet and powder pig complete feeds.

4. Conclusions

In summary, the current study showed that AFB1, ZEN and DON were highly prevalent in all the analyzed feedstuffs from various regions of China between January 2016 and December 2017. DON exhibited the most serious contamination in feedstuffs, followed by ZEN and AFB1. Moreover, the co-occurrence of AFB1, ZEN and DON were extremely common in the tested feedstuffs. Particularly, the percentage of these co-occurring mycotoxins was 100% in DDGS, corn bran, corn germ meal, corn gluten meal, wheat, and rice bran samples, and over 96.4% in complete pig feeds. Overall, these findings warn us that (1) mycotoxin contamination in feedstuffs should be routinely monitored; (2) appropriate detoxification strategies for mycotoxins should be used in the feed industry; and (3) new regulatory limits for mycotoxins need to consider their co-occurrence in feedstuffs.
5. Materials and Methods

5.1. Samples Collection and Preparation

A total of 1569 samples were collected at livestock farms, or feed companies from various locations in China between January 2016 and December 2017. There were 742 feed ingredient samples including 287 corn, 86 domestic DDGS, 88 imported DDGS, 8 corn bran, 17 corn germ meal, 6 corn gluten meal, 13 wheat, 15 barley, 76 wheat bran, 40 wheat middlings, 22 wheat flour, 24 broken rice, 20 rice bran and 40 soybean meal, as well as 827 complete pig feed samples, including 461 pelleted and 366 powdered forms. These samples were mainly collected from Guangxi, Guangdong, Shandong, Jiangsu, Shanghai, Zhejiang, Fujian, Jiangxi, Hunan, Hubei, Hebei, Shanxi, Ningxia, Gansu, Heilongjiang, Jilin, Liaoning, and Inner Mongolia provinces. Most of the feed and feed ingredient samples were analyzed for AFB$_1$, ZEN and DON, while few of them have been analyzed for only 1 or 2 mycotoxins due to the lack of available quantity. All the samples were stored in bags at $-20^\circ$C until analysis. All the samples were ground to a fine powder according to the method described by the European Commission Regulation EC 152/2009 for the analysis [36].

5.2. Extraction of Mycotoxins from Samples

Extractions of mycotoxins from samples were prepared as described in previous reports [21,22]. Briefly, 25 g of the ground samples were mixed with 100 mL of methanol:water (80:20, v/v) for AFB$_1$ measurement, acetonitrile:water solution (84:16, v/v) for ZEN measurement or methanol:water (60:40, v/v) for DON measurement. The samples were blended at high speed for 3 min, and then filtered through Mycosep® #226 (Romer Labs. Inc., Singapore). The solvent extracts were diluted with phosphate-buffered saline solution (PBS, pH 7.4), then filtered through immunoaffinity columns; ZearaStar (Romer Labs, Tulln, Austria) for ZEN, AokinImmunoClean CF AFLA and CF DON (Aokin AG, Berlin, Germany) for AFB$_1$ and DON, respectively. After column washing with PBS and a methanol-water solution, the mycotoxins were eluted from the columns with methanol, and mycotoxins concentrated to dryness under nitrogen steam. The mycotoxin residues were then immediately re-dissolved in a mobile phase described below, filtered through a Millex PTFE 0.22 µm filter (Merck, Tianjin, China), and analyzed by high-performance liquid chromatography (HPLC).

5.3. HPLC Analysis

The mycotoxins were quantified as previously described [21,22]. Briefly, AFB$_1$ was analyzed with a reversed-phase HPLC/fluorescence detection system (Agilent 1260, Agilent Technologies, Waldbronn, Germany) with a 360 nm excitation and 440 nm emission fluorescence detector. A C$_{18}$ column (4.6 mm × 250 mm, 5 µm, Dikma, Shanghai, China) was employed with the limits of detection (LOD) and quantification (LOQ) set at 0.5 µg/kg and 1.5 µg/kg, respectively. The analysis was performed using a mobile phase of methanol:water:acetonitrile (30:60:10, v/v/v) at a flow rate of 1 mL/min. The temperature of the column was set at 30 ºC. ZEN and DON were analyzed with a Shimadzu LC-20A binary gradient liquid chromatograph (Shimadzu Europa GmbH, Duisburg, Germany) equipped with a C$_{18}$ (4.6 mm × 150 mm, 5 µm) reversed-phase column (ZORBAX Eclipse XDB-C18, Agilent Technologies, Waldbronn, Germany). ZEN analysis was conducted using a mobile phase of methanol:water:acetonitrile (8:46:46, v/v/v) at a flow rate of 1 mL/min and detected under 274 nm excitation and 440 nm emission wavelengths [37]; the LOD and LOQ for ZEN were 10 µg/kg and 24 µg/kg, respectively. DON was analyzed using a mobile phase of methanol:water solution (20:80, v/v) at a flow rate of 0.8 mL/min under UV light at a wavelength of 218 nm [38] and the LOD and LOQ for DON were 100 µg/kg and 260 µg/kg, respectively. The validity of mycotoxin peaks in HPLC chromatograms have been shown in Figure S1. The blank samples are the solvents that were used to dissolve standard samples before HPLC detection. LOD and LOQ correspond to the analyte amount for which the signal-to-noise ratio is equal to 3 and 10 [39,40], respectively, with a minor adjustment according to our previous study [22].
5.4. Statistical Analysis

All the data were calculated by Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA, USA) and expressed as percentages or means, median and maximum.

Supplementary Materials: The following are available online at www.mdpi.com/2072-6651/10/3/113/s1, Figure S1: The HPLC chromatogram of AFB1 (A), ZEN (B), and DON (C).

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