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Original Article

The efficacy of yeast cell wall in the adverse effect of zearalenone on ruminal fibrolytic bacteria and *in vitro* fermentation

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Abstract

The efficacy of yeast cell wall in the adverse effects of zearalenone (ZEN) in *in vitro* gas production was determined. The experiment consisted of 4 treatments: 1) diet alone, 2) diet plus 0.5 mg/kg ZEN, 3) diet supplemented with 0.5% yeast cell wall, and 4) diet supplemented with 0.5% yeast cell wall plus 0.5 mg/kg ZEN. ZEN suppressed the *Fibrobacter succinogenes* and *Ruminococcus albus* populations ($P<0.05$), but did not interfere with the *in vitro* gas production. In contrast, yeast cell wall increased the *F. succinogenes* and *R. albus* populations and *in vitro* gas production of insoluble fractions ($P<0.05$). Moreover, ZEN and yeast cell wall did not affect digestibility, VFAs, NH_3N, or *B. fibrisolvens* population. The efficiency of yeast cell wall on ZEN decontamination was unclear. In conclusion, ZEN reduced major fibrolytic bacterial populations, while yeast cell wall could alleviate the ZEN adverse effect and enhance *in vitro* gas production.

Keywords: zearalenone, yeast cell wall, rumen fermentation, mycotoxin, fibrolytic bacteria

1. Introduction

Mycotoxins are secondary fungal metabolites and harmful to humans or animals. Among the hundreds of identified mycotoxins, aflatoxins, ochratoxins, fumonisins, and zearalenone (ZEN) are the most common mycotoxins (Omotayo, Omotayo, Mwanza, & Babalola, 2019). In particular, ZEN, a secondary metabolite of phenolic resorcylic acid lactone, is produced by several *Fusarium* species, mainly *F. graminearum* and *F. culmorum*, and is commonly known as a potent estrogenic mycotoxin causing the hyperestrogenic syndrome (Kim, Lee, Do, Nam, Li, Jang, & Lee, 2014; Rodrigues, Handl, & Binder, 2011). Owing to the estrogen-like structure, ZEN could be detrimental to reproductive organs and results in numerous reproductive problems in animals (Belli *et al.*, 2010; Mahmoud, Ghattas, Amal, & Leil, 2013; Shi *et al.*, 2018) such as reduced conception rate, inhibited oocyte maturation, ovarian atrophy, and impaired semen quality (Minervini & Dell'Aquila, 2008). Contamination by ZEN in food and feed in the field and storage had been reported worldwide (Rodrigues *et al.*, 2011; Rodrigues & Naehrer, 2012). ZEN has highly contaminated

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most compound feeds for cattle (98%) with the maximal 0.405 ppm observed in cattle fattening feeds (Kim *et al.*, 2014). Raising awareness of this issue, a European committee established the maximum limit regulations for ZEN as between 20–100 ppb in various food commodities (Alshannaq & Yu, 2017). Moreover, as anti-ZEN additives, both *S. cerevisiae* viable cells and cell walls have been introduced, with capacity to reduce the gastrointestinal absorption (Bueno, Marco, Oliver, & Bardo, 2005; Keller *et al.*, 2015) and contaminated feed (Aravind, 2003) at 1.0 g of yeast cell wall to 2.7 mg of ZEN ratio (Huwig, Freimund, Kappeli, & Dutler, 2001). D-glucan and mannoprotein portions played important roles in reducing ZEN effects (Aravind, 2003; Huwig *et al.*, 2001). While yeast cell wall is widely used in animal feed to improve animal production (Aung, Ohtsuka, & Izumi, 2019; Fowler, Kakani, Haq, Byrd, & Bailey, 2015; Hashim *et al.*, 2019), literature data clarifying the relationship of yeast cell wall with ZEN is limited. Therefore, the objective of this study was to determine the efficacy of yeast cell wall against the adverse effects of ZEN on *in vitro* gas production and in ZEN decontamination.

2. Materials and Methods

2.1 Experimental design and treatments

The experiments followed a Completely Randomized Design (CRD). The basal diet was 40 : 60 ratio of rice straw and concentrate. The chemical compositions of concentrate and rice straw are listed in Table 1. The experiment had 4 treatments: 1) diet alone (200 mg, control group, T1), 2) diet plus 0.5 mg/kg ZEN (T2), 3) diet supplemented with 0.5% yeast cell wall (*S. cerevisiae*, T3), and 4) diet supplemented with 0.5% yeast cell wall plus 0.5 mg/kg ZEN (T4), respectively, in each sample bottle.

Table 1. Ingredients and chemical compositions of the basal diet

Item	Concentrate	Rice straw
Ingredient (% of dry matter)		
Cassava chip	45.0	
Soybean meal	17.0	
Maize	10.0	
Palm kernel meal	8.0	
Rice bran	5.0	
Soybean hulls	5.0	
Molasses	6.7	
Mineral premix	0.5	
Urea	1.8	
Salt	0.5	
Sulfur	0.5	
Chemical composition		
Dry matter (%)	91.9	94.3
---------- Dry matter--------		
Organic matter	83.2	83
Ash	7.8	12.5
Crude protein	22.1	2.5
Ether extract	3.9	2.1
Neutral detergent fiber	21.1	81.4
Acid detergent fiber	11.7	50.8
Acid detergent lignin	3.6	5.9

2.2 Zearalenone preparation and determination

Commercially available ZEN (Trilogy, Catalog number: TSL-401, USA) was diluted with sterile deionized water to prepare a 1 mg/l ZEN solution. ZEN was determined by a commercial cELISA kit (R-Biopharm, Germany) according to Beg *et al.* (2006).

2.3 Rumen inoculums

Three Thai Friesian cows used as rumen fluid donors were individually penned, had free access to water, and were fed by rice straw as roughage source ad libitum for a week before rumen fluid collection through a suction pump. The rumen fluid was collected in the morning and subsequently filtered through two layers of cheesecloth. The *in vitro* fermentation was followed by the technique described by Makkar, Blummel, and Becker (1995). The rumen inoculum was transferred into each sample bottle and incubated in a hot air oven at 39°C for 72 h.

2.4 Sample collection and analysis

The basal diet was analyzed for chemical composition; dry matter (DM), ash, crude protein (CP) and ether extract (EE), using the procedures of Association of Official Analytical Chemists (AOAC, 1997), while neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined according to Van Soest, Robertson, and Lewis (1991).

During the incubation, the gas production kinetics were recorded at 1, 2, 4, 6, 8, 12, 24, 48, and 72 h. Cumulative gas production data were fitted to the model from Ørskov and McDonald (1979) as follows:

$$Y = a + b\,(1 - e^{(ct)})$$

where a = the gas production from the immediately soluble fraction, b = the gas production from the insoluble fraction, c = the gas production rate constant for the insoluble fraction (b), t = incubation time, (a+b) = the potential extent of gas production, y = gas production at time 't'. At 24 and 48 h post inoculation a set of samples were tested *in vitro* for true digestibility according to Van Soest and Robertson (1985).

2.5 Ammonia nitrogen and volatile fatty acids analysis

Thirty milliliters of each ruminal inoculum fluid were collected at 24 and 48 h post inoculation, mixed with 1 M H_2SO_4 and subsequently centrifuged at 15,000 × g for 15 min. Samples were divided into two portions, one was subjected to ammonia nitrogen (NH_3-N) analysis by the micro-Kjeldahl method (AOAC, 1990), while the other was then filtered through a nylon membrane (0.22 µm, FILtrex, Singapore) for volatile fatty acids (VFAs) analysis by high performance liquid chromatography (HPLC) (model RF-10AXmugiL; Shimadze, Japan) according to Mathew, Sagathevan, Thomas, and Mathen (1997).

2.6 Bacterial population

Total genomic DNA was extracted from 500 µl of rumen inoculum fluid after 24-h incubation by the repeated bead-beating plus column (RBB+C) method with modifications (Yu & Morrison, 2004). In brief, cell lysis was achieved by bead-beating in lysis buffer containing 4% (w/v) sodium dodecyl sulfate (SDS), 500 mM NaCl, 50 mM Tris-HCl; pH 8.0 and 50 mM EDTA using sterile zirconia/silica beads (0.1 mm; BioSpec Products, Inc., Bartlesville, USA). The mixture was homogenized in Mini-Beadbeater-1 (BioSpec Products, Inc., Bartlesville, OK, USA) for 3 min, then incubated at 70°C for 15 min with gentle shaking every 5 min, and subsequently centrifuged at 16,000 × g (Dynamica, Velocity 18R Versatile Centrifuge, Switzerland), 4°C for 5 min. Nucleic acids were removed by 10 M ammonium acetate with isopropanol. Genomic DNA was then purified via sequential digestion with RNase A and proteinase K using a commercial High Pure PCR Template Preparation Kit (Roche, Germany).

The targeted fibrolytic bacteria including *F. succinogenes*, *R. albus* and *B. fibrisolvens* were quantitatively determined by real-time PCR and fluorescence detection of SYBR green mix by allowing the reactions with cycling parameters (Table 2) as described by Wongnen (2016). The species specific 16sDNA gene primers used in this experiment were confirmed by in-silico techniques with the Basic Local Alignment Search Tool (BLAST) program in the Gene-Bank Database of National Center for Biotechnology Information (NCBI) by our colleagues (Suphrap, Wachirapakorn, Thamrongyoswittayakul, & Wongnen, 2017; Wongnen, 2016).

2.7 Statistical analysis

All data were analyzed by Analysis of Variance (ANOVA) using the General Linear Model Procedure (GLM) of the SAS (1998). Duncan's New Multiple Range Test was applied to compare the treatment means (Steel & Torrie, 1980).

3. Results and Discussion

For the rice straw analysis, the result was in accordance with a previous report (Kang & Wanapat, 2013).

3.1 Gas production kinetics

Estimated parameters of gas production are presented in Figure 1 and Table 2. The results show that the gas fermentation of the soluble fraction (a) and the gas production rate constant for the insoluble fraction (c) in T2, T3, and T4 were not different (P>0.05), whereas effective gas production potential (EP) tended to be significantly lower in T2 and higher (P=0.06) with yeast cell wall supplement (T3, and T4) as compared with the control. However, gas production from the insoluble fraction (b) and the potential extent of gas production (d) were significantly higher (P<0.01) with yeast cell wall supplement (T3 and T4) than in the control group. The results suggest that rumen microbes could digest the yeast cell wall as an additional substrate

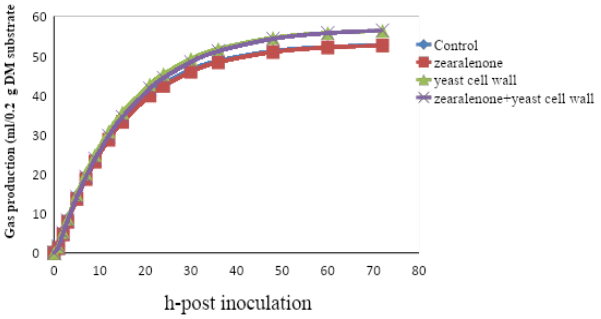

Figure 1. Effects of zearalenone and yeast cell wall on gas production during incubation
T1 = Diet alone (control group), T2 = Diet plus 0.5 mg/kg ZEN, T3 = Diet supplemented with 0.5% yeast cell wall (*S. cerevisiae)*, and T4 = Diet supplemented with 0.5% yeast cell wall plus 0.5 mg/kg ZEN

Table 2. Primer sequences used to quantify targeted fibrolytic bacteria by qPCR (Suphrap *et al.*, 2017).

Target bacteria	Primer sequences (5'–3')	Annealing Temperature (°C)	Product size (bp)
B.fibrisolvens	F-AAAGCTCTATCAGCAGGGAA R-GTAAATCCGGATAACACTTG	55.2	126
R.albus	F-GCTTACTGGGCTTTAACTGA R-CCCACACCTAGTAATCATCG	50.8	103
F. succinogenes	F-CAACCCACGTTTCCAGTT R-TGTGTAGCCCAGGATGTAA	56.9	113

(Oeztuerk, Emre, & Breves, 2016) because of the monoprotein and glucan portion of the cell wall (Lipke & Ovalle, 1988). Therefore, yeast cell wall supplement might enhance the gas production from degradation of diet (Lila *et al.*, 2004). The gas production and potential extent of gas with ZEN supplement do not differ from the control, but it is difficult to compare this matter because of limited literature data on the ZEN effects. Mycotoxins such as aflatoxin are toxic to rumen microbes and subsequently depress rumen microbial activity and gas production. In the aflatoxin B1 model, the *Streptococcus bovis* and mixed rumen bacterial growth were inhibited (Mathur *et al.*, 1976), and the asymptotic gas production declined on increasing the mycotoxin dosage (Jiang, Yang, & Lund, 2012; Mojtahedi, Danesh, Vakili, & Hayati-Ashtiani, 2013). It was suggested that microbial activities might be altered by mycotoxins, while ZEN could be metabolized to less toxic β-zearalenol (β-ZOL) by *B. fibrisolvens* (Kennedy *et al.*, 1998; Yiannikouris & Jouany, 2002).

Table 3. Effects of zearalenone and yeast cell wall on *in vitro* gas production in dietary treatments
a = the gas production from the immediately soluble fraction, b = the gas production from the insoluble fraction, c = the gas production rate constant for the insoluble fraction, d = the potential extent of gas production, EP = a + [bc/(k + c)] where k = 0.05 (Ørskov and McDonald, 1979)
T1 = Diet alone (control group), T2 = Diet plus 0.5 mg/kg ZEN, T3 = Diet supplemented with 0.5% yeast cell wall *(S. cerevisiae)*, and T4 = Diet supplemented with 0.5% yeast cell wall plus 0.5 mg/kg ZEN

Item	Treatment				SEM	P-value
	T1	T2	T3	T4		
Gas production						
a	-1.91	-2.45	-2.31	-2.15	0.14	0.59
b	54.9^{B}	55.4^{B}	59.1^{A}	59.1^{A}	0.52	<0.01
c	0.071	0.069	0.069	0.064	0.001	0.12
d	54.5^{B}	58.0^{A}	56.8^{A}	58.0^{A}	0.42	<0.01
Effective gas production potential (EP)						
	30.3	29.6	31.8	31.8	0.32	0.06

$^{A, B}$ Means in the same row with different superscripts differ (P<0.05)

3.2 *In vitro* degradability and rumen metabolites

The *in vitro* dry matter digestibility (IVDMD) and organic matter digestibility (IVOMD) were not influenced by ZEN and yeast cell wall supplementation (P>0.05) (Table 4). Moreover, volatile fatty acids (Acetic acid, Propionic acid, & Butyric acid) and ammonia nitrogen (NH_3-N) did not differ among the treatments (Table 5, P>0.05). Possibly ZEN could be metabolized to β-ZOL (Keller *et al.*, 2015), and therefore ZEN had no effect on the degradability (Khatoon & Abidin, 2018; Yiannikouris & Jouany, 2002).

3.3 Rumen bacterial populations

F. succinogenes, *R. albus*, and *R. flavefaciens* were selected for the trial because they are mainly involved the fibrolytic activities in the rumen (Krause *et al.*, 2003; Morgavi, Kelly, Janssen, & Attwood, 2013). Effects of ZEN and yeast cell wall on the fibrolytic bacterial populations from *in vitro* incubation are shown in Table 6. The findings reveal a significant increase in the *F. succinogenes* and *R. albus* population means (P<0.01) by 11.1% and 2.8%, respectively, elevated with the yeast cell wall supplement (T3) from than in the control. In contrast, the population means of *F. succinogenes* and *R. albus* in ZEN group (T2) were by 6.5% and 8.5% lower than in the control. The yeast cell wall could stimulate the growth of several ruminal bacteria, especially the lactate utilizing and the cellulolytic bacteria (Alugongo *et al.*, 2017), and might synergistically alleviate the negative ZEN effects. Moreover, it has been suggested that increased bacterial population in rumen involve the action of yeast cell wall, thus elevating the ruminal digestibility. However, the population of *B. fibrisolvens* did not differ across the treatments. Correspondingly, ZEN did not affect the *B. fibrisolvens* specific growth (Westlake, Mackie, & Dutton, 1987).

Table 4. Effects of zearalenone and yeast cell wall on *in vitro* digestibility
T1 = Diet alone (control group), T2 = Diet plus 0.5 mg/kg ZEN, T3 = Diet supplemented with 0.5% yeast cell wall (*S. cerevisiae*), and T4 = Diet supplemented with 0.5% yeast cell wall plus 0.5 mg/kg ZEN

Item	Treatment				SEM	P-value
	T1	T2	T3	T4		
In vitro dry matter digestibility (IVDMD), %						
24h	66.3	67.6	64.9	65.3	0.51	0.21
48h	69.5	70.3	66.7	58.3	0.58	0.08
In vitro organic matter digestibility (IVOMD), %						
24h	74.4	73.3	73.0	75.3	0.41	0.16
48h	77.6	77.3	78.1	78.0	0.44	0.23

$^{A, B}$Means in the same row with different superscripts differ (P<0.05)

Table 5. Effects of zearalenone and yeast cell wall on volatile fatty acids and ammonia nitrogen
T1 = Diet alone (control group), T2 = Diet plus 0.5 mg/kg ZEN, T3 = Diet supplemented with 0.5% yeast cell wall (*S. cerevisiae*), and T4 = Diet supplemented with 0.5% yeast cell wall plus 0.5 mg/kg ZEN

Item	Treatment				SEM	P-value
	T1	T2	T3	T4		
Acetic acid (C_2), %						
24h	63.1	62.4	62.9	61.3	0.33	0.21
48h	64.4	63.0	62.1	62.2	0.18	0.39
Propionic acid (C_3), %						
24h	23.8	24.6	23.1	24.6	0.36	0.51
48h	24.7	23.7	24.1	24.3	0.17	0.15
Butyric acid (C_4), %						
24h	13.2	13.1	14.1	14.2	0.22	0.10
48h	12.9	13.5	13.8	13.5	0.17	0.35
C_2:C_3 ratio						
24h	2.7	2.6	2.7	2.5	0.05	0.58
48h	2.6	2.7	2.6	2.6	0.02	0.38
NH_3N, mg%						
24h	13.4	13.4	13.2	13.4	0.20	0.17
48h	16.4	15.8	17.6	17.0	0.30	0.14

Table 6. Effects of zearalenone and yeast cell wall on the fibrolytic bacterial population
T1 = Diet alone (control group), T2 = Diet plus 0.5 mg/kg ZEN, T3 = Diet supplemented with 0.5% yeast cell wall (*S. cerevisiae*), and T4 = Diet supplemented with 0.5% yeast cell wall plus 0.5 mg/kg ZEN

Item	Treatment				SEM	P-value
	T1	T2	T3	T4		
F. succinogenes ($\times 10^6$ copies/ml)	1.08^{B}	1.01^{C}	1.20^{A}	1.11^{B}	0.02	<0.01
R. albus ($\times 10^6$ copies/ml)	3.52^{A}	3.22^{B}	3.62^{A}	3.57^{A}	0.15	<0.01
B. fibrisolvens ($\times 10^8$ copies/ml)	1.46	1.41	1.48	1.58	0.16	0.56

$^{A, B, C}$Means in the same row with different superscripts differ (P<0.05)

Generally, decontamination activity of *S. cerevisiae* against ZEN and its derivatives is well documented, although viable *S. cerevisiae* cells seem to have the most potential for ZEN detoxification (Keller *et al.*, 2015). As a probiotic, *S. cerevisiae* yeast has been approved as a potential feed additive against ZEN detoxification with 52.0% adsorption rate within a 24-h incubation (Chlebicz & Śliżewska, 2019). Likewise, the detoxification ability of yeast cell wall against ZEN is in accordance with the viable yeast models (Chlebicz & Śliżewska, 2019; Keller *et al.*, 2015). Although the literature data on yeast cell wall are limited, the detoxification properties could be demonstrated in this study. After a 24-h incubation, about 92.0% decrease in average ZEN level (from 0.521 to 0.004 μg/kg) was also observed with yeast cell wall (T3); nevertheless, the adsorption efficiency by the yeast cell of ZEN varied among the treatments and is unclear.

Microbial-based methods are the most common detoxification or decontamination approaches for ZEN, not only using adsorption but also conversion processes (Wang, Wu, Pan, & Long, 2019). The latter theoretically involves biotransformations of ZEN either in animals or in feed, and results in α- and β-ZOL metabolites. However, this might not fully detoxify the ZEN, leaving behind residual metabolites and favoring formation of less estrogenic β-ZOL (Keller *et al.*, 2015).

However, the ZEN-derived interference could be alleviated by supplemented yeast cell wall. The yeast cell wall may provide some beneficial effects on rumen fermentation *in vitro* and could be alternatively implemented in animal models or in field practice. However, the issue regarding the efficiency of the yeast cell wall on the effect of ZEN has not yet been completely settled. Further studies are warranted to understand these relationships.

4. Conclusions

The current *in vitro* model demonstrated that *S. cerevisiae* yeast cell wall had potential and beneficial properties to enhance *in vitro* gas production from insoluble fractions and alleviate the adverse effects of ZEN on major fibrolytic bacterial populations.

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