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Stability of the bacteria-bound zearalenone complex in ruminal fluid and in simulated gastrointestinal environment *in vitro*

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Abstract

Zearalenone (ZEA), an oestrogenic secondary metabolite of *Fusarium*, is a common feed contaminant. Lactic acid bacteria are capable to bind ZEA and this property could be used to limit its negative biological effects on farm animals. The aim of this work was to examine the stability of a lactic acid bacterium, *Streptococcus thermophilus* RAR1-ZEA complex in ruminal fluid and in conditions simulating compartments of the gastrointestinal tract. The lactic acid bacteria-ZEA complex was 70% stable in ruminal fluid incubations for up to 18 h, and about 50% of ZEA initially complexed still remained bound after incubation in pepsin, lysozyme, pancreatin and bile either alone or sequentially. However, the release of ZEA was predominantly caused by washing, except for bile which produced a partial dissociation of the complex ($P<0.05$). Addition of free ZEA to ruminal fluid resulted in instantaneous binding of about 70% of the toxin. ZEA bound to ruminal fluid was even more stable than lactic acid bacteria-ZEA in *in vitro* ruminal fermentations and in simulated gastrointestinal tract juices. It is concluded that complexation of ZEA in ruminal fluid could help protect ruminants against the toxin, and that the use of selected strains of lactic acid bacteria efficiently binding ZEA to limit its toxic effects could be more advantageous in monogastric animals.

Keywords: bacteria, binding, digestive tract

1. Introduction

Zearalenone (ZEA), a non-steroidal oestrogenic secondary metabolite produced by several *Fusarium* species such as *F. graminearum* and *F. culmorum*, is a common contaminant of cereal grains and forages. In the field, ZEA contamination is highly dependent of climatic conditions, thus its absence in the rations of farm animals cannot be fully assured. The occurrence of ZEA in animal feeds, particularly in maize-based products but also in hay and grass, was reported by several authors (reviewed by Scudamore and Livesey, 1998, and Seeling and Danicke, 2005). ZEA leads to animal reproductive disorders by activating oestrogen receptors. Pigs appear to be most susceptible to the toxic effects of ZEA, while ruminants are considered more resistant (D'Mello *et al*., 1999; Fink-Gremmels and Malekinejad, 2007). However, cases of toxic effects on ruminants have been reported (Seeling and Danicke, 2005).

There are no practical, specific and cheap methods to decontaminate affected feeds at present. For fermented feeds like silage, one possible approach is the use of selected strains of lactic acid bacteria (LAB), which can bind ZEA decreasing its absorption in the gastrointestinal tract (GIT) and hence its toxic effects. The ability of two *Lactobacillus* probiotic strains to bind ZEA and its derivative α-zearalenol (α-ZOL) *in vitro* was reported (El-Nezami *et al*., 2002). In a previous study, we screened a large number of strains for their ability to bind *Fusarium* toxins in acidified maize infusion, and showed that binding of ZEA is a common property of LAB (Niderkorn *et al*., 2007). Some strains of *Streptococcus*, among them *S. thermophilus* RAR1, were particularly efficient. In addition to the known role of

ruminal microorganisms in the partial metabolisation of ZEA (Kiessling *et al*., 1984), their high density in the rumen could also be able to bind ZEA *in situ*. However, to our knowledge, no information is available on the binding of mycotoxins in the rumen. In any case, reduction of ZEA toxicity as a consequence of binding will operate only if the complex microorganism-mycotoxin remains stable in the GIT of animals. The aim of the present work was to evaluate the stability of a bacterium, *S. thermophilus* RAR1-ZEA complex in ruminal fluid (RF) and in conditions simulating the physico-chemical environment of the GIT. Binding of ZEA in RF and stability of the complex formed was also investigated.

2. Materials and methods

The bacterium *S. thermophilus* RAR1 (LAB collection of the Research Unit for Food Process Engineering and Microbiology, INRA, Thivernal-Grignon, France), previously selected for its efficiency to bind ZEA *in vitro*, was used in this study. ZEA and derivatives α- and β-zearalenols (α- and β-ZOL), pepsin, lysozyme from chicken egg white, pancreatin and bovine bile were purchased from Sigma-Aldrich Chemie Gmbh, Steinheim, Germany.

To prepare the mycotoxin solution used for the formation of the bacterium-ZEA complex, ZEA was dissolved in methanol and concentration was determined by measuring the absorbance at 274 nm (ε = 13,909/M/cm). The methanol was evaporated with nitrogen gas, then the mycotoxin was redissolved in a small volume of ethanol, and 0.1 M citrate-phosphate buffer pH 4 was added to reach a concentration of 5 µg ZEA/ml. Ethanol represented 1.25% (v/v) in the final solution.

The bacterium-mycotoxin complex was prepared as follows: *S. thermophilus* RAR1 was grown at 37 °C for 24 h in M17 broth (Oxoïd Ltd, Basing-stoke, UK) containing 5% v/v of a 10% w/v lactose solution. Bacterial concentration was estimated by measuring the absorbance at 600 nm as described previously (Niderkorn *et al*., 2006). Cells were harvested by centrifugation at 3,000 × g for 10 min at 5 °C, and the bacterial pellet was washed twice with 10 ml of phosphate-buffered saline (PBS 0.01 M, pH 7.4). Bacteria (5×10^8 cfu/ml) were resuspended in 5 ml of ZEA solution, and the mixture was incubated at 25 °C for 1 h with shaking (480 rpm). At the end of the incubation period, tubes were centrifuged (3,000 × g for 10 min at 5 °C). A control containing no bacteria was included to determine the fraction of ZEA initially bound to bacteria.

In a first experiment, the stability of the *S. thermophilus* RAR1-ZEA complex in ruminal fluid was tested. The complex was incubated anaerobically at 39 °C for up to 18 h in 5 ml of sheep's filtered RF-buffer solution (Goering and Van Soest, 1970) mixed in a 1 to 3 ratio. Incubations were done with and without 50 mg ground feed substrate (50% maize grain, 50% alfalfa hay). Incubations containing free (unbound) ZEA were used as controls.

In a second experiment, the stability of the *S. thermophilus* RAR1-ZEA complex was tested under simulated gastrointestinal conditions. The complex was incubated in 1 ml of solutions containing pepsin (1 g/l 0.2 M glycine-HCl buffer pH 2.5), lysozyme (3,000 U/ml 0.1 M citrate-phosphate buffer pH 6, lysozyme from chicken egg white was used as a replacement for ruminant's stomach lysozyme which is commercially unavailable), pancreatin (3 g/l 0.1 M citrate-phosphate buffer pH 7), and bovine bile (same concentration as pancreatin). Incubation conditions: enzymes and extracts concentration, pH, incubation times (1 h for pepsin and lysozyme and 2 h for pancreatin and bile), and temperature (39 °C) were chosen to mimic physico-chemical conditions and passage rate in the stomach and small intestine. The complex was also incubated sequentially in pepsin solution followed by a mixed solution of pancreatin and bovine bile. Control incubations of the complex in buffer without the addition of enzymes or bovine bile were performed concomitantly to discriminate between the biological and the washing effect of these incubations.

In a third experiment, the capacity of RF to bind ZEA and the stability of the complex formed was tested in a similar way as for the LAB-ZEA complex. Briefly, free ZEA (5 µg/ml) was allowed to bind the particulate fraction of 5 ml of filtered RF with substrate for 1 h at 39 °C. The bound ZEA (RF-ZEA complex) was then collected by centrifugation and sequentially incubated in pepsin followed by a mixture of pancreatin and bovine bile, as described above. The washing effect was also investigated for these assays.

In all three experiments, all assays and controls were carried out in triplicate. Following the corresponding incubation period, tubes were centrifuged, and ZEA and its metabolites α- and β-ZOL were quantified by reversed-phase HPLC in supernatants and bacterial pellets. ZEA was extracted from bacterial and RF pellets with methanol. The extraction recoveries were 98% and 96%, respectively. The HPLC system consisted of a P1000XR pump (SpectraSYSTEM, San Jose, CA, USA) and an automatic sampler (SpectraPhysics, San Jose, CA, USA). Separation of ZEA and derivatives was performed on a C_{18} reversed-phase column (Bischoff, 125×4.6 mm, Prontosil 120-3-C18-H 3.0 µm, Leonberg, Germany), using an isocratic mobile phase (methanol-acetic acid 1%-acetonitrile solution, 45:45:10 v/v/v) at a flow rate of 1 ml/min. Detection was carried out by photo-diode-array (scan 200-380 nm). Under these conditions, the retention times of ZEA, α-and β-ZOL were 24.2, 19.9 and 12.1 min, respectively.

Data was subjected to the analysis of variance (ANOVA). A significant difference (P<0.05) between means was determined by Duncan's multiple range test in the first experiment and Dunett's test in the second and third experiments, using the Statistical Analysis System software package (SAS Institute Inc., Cary, NC, USA).

3. Results and discussion

Fractions of ZEA bound to *S. thermophilus* RAR1 before incubation in RF and solutions simulating GIT were 59±2% and 51±3% for the first and second experiment, respectively. These binding values are within normal ranges (Niderkorn *et al*., 2006). The final concentration of ZEA was 2.95 µg/ml and 2.55 µg/ml in the first and second experiment, respectively.

In the first experiment, immediately after mixing free ZEA and RF followed by centrifugation, 73% of ZEA added was found in pellet, indicating that ZEA was instantaneously bound to the particulate matter present in RF even without added probiotic bacteria (Table 1). This observation may be explained by the high density of microorganisms including LAB in RF, although it has to be noted that the role of small feed particles could not be quantified in this work. The instantaneous complexation is consistent with previous studies on binding of ZEA and other compounds by LAB (El-Nezami *et al*., 2002; Morotomi and Mutai, 1986). Considering the bound fractions, the RF-ZEA complex remained stable in rumen fluid up to 18 h since this fraction did not change at the end of the incubation period (P>0.05). When the *S. thermophilus* RAR1-ZEA complex was added to rumen incubations in presence of feed, more than 90% remained in the complex at 0 h and at 18 h, the bound mycotoxin fraction was similar to that obtained with free ZEA (P>0.05) (Table 1). In contrast, in the absence of substrate, the bound fraction drastically decreased following incubation (P<0.05). Substrate can play a positive role in complexation either by increasing the bacterial biomass or directly by binding a fraction of ZEA. In the absence of substrate, bacterial lysis and competition among ruminal microorganisms could also result in a partial release of ZEA. Independently of treatments, a fraction of ZEA was metabolised to α- and β-ZOL, and these metabolites were found in both the free and bound fractions. The metabolisation of ZEA to α- and β-ZOL by ruminal microflora is well-known (Kiessling *et al*., 1984) and our results are consistent with this observation. In addition, we showed that a fraction of metabolites was also bound by RF.

In the second experiment, the *S. thermophilus* RAR1-ZEA complex stability was tested in conditions simulating the post-ruminal GIT environment. The complex was stable in solutions of pepsin, lysozyme or pancreatin, between 65% and 77% of ZEA remained bound after incubation (Table 2). The abomasum of ruminants has the special ability to produce lysozyme. This enzyme was tested because its hydrolytic action against gram positive bacteria could have an effect on the LAB-ZEA complex stability.

Table 1. Stability of *S. thermophilus* RAR1-ZEA complex in ruminal fluid.

	RF + feed + ZEA[c]		RF + feed + RAR1-ZEA complex[c]		RF without feed + RAR1-ZEA complex[c]	
Incubation time (h)[a]	**0**	**18**	**0**	**18**	**0**	**18**
Free fraction (%)[b]						
ZEA	24 (2)	24 (3)	5 (0)	22 (6)	10 (1)	45 (4)
α-ZOL	nd	5 (1)	nd	5 (2)	nd	7 (2)
β-ZOL	nd	4 (2)	nd	nd	nd	nd
Total	24 (2)	33 (3) *,z	5 (0)	27 (4) *,z	10 (1)	52 (3) *,y
Bound fraction (%)[b]						
ZEA	73 (2)	52 (7)	91 (4)	48 (4)	83 (5)	36 (1)
α-ZOL	nd	10 (0)	nd	9 (0)	nd	6 (0)
β-ZOL	nd	7 (2)	nd	10 (0)	nd	4 (1)
Total	73 (2)	69 (6) y	91 (4)	67 (4) *,y	83 (5)	46 (1) *,z
Recovery (%)	97	102	96	94	93	98

[a] For incubation time = 0, samples were homogenised and immediately centrifuged.

[b] Free and bound fractions of ZEA and metabolites α- and β-ZOL were percentages found in supernatants and pellets, respectively, from the amount of ZEA initially added. Data are means (standard deviations) of triplicate tubes.

[c] RF = ruminal fluid; nd = not detected. Data are means (standard deviation) of triplicate tubes. Within a row, for a same treatment, total values at 18 h incubation followed by * are significantly different from total values at 0 h incubation (P<0.05). Within a row, total values at 18 h incubation followed by a different letter (y, z) are significantly different (P<0.05).

Table 2. Stability of *S. thermophilus* RAR1-ZEA complex in solutions simulating post-ruminal compartments of ruminant's gastrointestinal tract.

Simulated compartment	ZEA released (%) [a,b]	ZEA remaining bound (%) [a,b]	Recovery (%)
Abomasum			
Pepsin (pH 2.5; 1 h)	16 (0) *	77 (7)	93
Lysozyme (pH 6; 1 h)	32 (2)	77 (5)	109
Buffer (pH 4; 1 h)	29 (3)	69 (5)	98
Small intestine			
Pancreatin (pH 7; 2 h)	27 (4)	65 (5)	92
Bovine bile (pH 7; 2 h)	52 (7) *	51 (2) *	103
Buffer (pH 4; 2 h)	28 (5)	73 (5)	101
Abomasum + small intestine [c]			
Pepsin followed by pancreatin/bovine bile	37 (6) *	48 (5)	101
Buffer followed by buffer	24 (5)	45 (7)	98

[a] Fractions of ZEA released and remaining bound were percentages found in supernatants and pellets, respectively, from the amount of mycotoxin initially bound to bacteria.
[b] Data are means (standard deviations) of triplicate tubes. Within a column, for a same treatment, means followed by * are significantly different from incubation in buffer (P<0.05).
[c] Data are fractions of ZEA released and remaining bound after the second incubation period; recovery is the sum of both values and the fraction released after the first incubation period.

These percentages were similar to those observed in buffer (P>0.05), indicating that the proportion of ZEA dissociated was due to washing rather than to a chemical effect of these digestive substances. In contrast, ZEA release was more significant in bovine bile than in other solutions including buffer (P<0.05), indicating that bile can partially dissociate the complex. If this observation also occurs *in vivo*, a fraction of ZEA bound to bacteria could be released in the small intestine. The effect of bile was also shown when the complex was sequentially incubated in pepsin followed by the mixture of pancreatin and bile. However, the percentage of bound ZEA remaining after the pancreatin/bile treatment was not different from that observed with sequential incubations in buffer (P>0.05), indicating that washing remained the main cause of release. The washing effect observed is consistent with results of a previous study indicating a limited release after repeated washes of viable *Lactobacillus*-ZEA complex with water (El-Nezami *et al*., 2004).

The high proportion of ZEA that binds naturally to the RF prompted us to test the stability of the RF-ZEA complex in simulated gastric and intestinal environments. The fraction of ZEA bound in this third experiment was 71±4% (3.55 µg/ml) and the complex was stable after incubating in pepsin alone and after a sequential incubation in pepsin followed by the mixture of pancreatin and bovine bile (Table 3). Few data are available on the kinetics of ZEA in ruminants, some studies reported that only negligible levels of ZEA and its metabolites were found in plasma and tissues of dairy cows fed high oral doses of this mycotoxin (Kleinova *et al*., 2002; Prelusky *et al*., 1990). According to the results of the present work, binding of ZEA by RF could contribute to this low absorption. The tolerance of ruminants to ZEA has been explained by a high efficiency in the biotransformation and elimination of ZEA by the liver as compared to pigs and also by ZEA metabolisation by rumen microbes (Fink-Gremmels and Malekinejad, 2007). This work also suggests that a decrease in mycotoxin bioavailability due to binding could be a contributing factor in ruminants' tolerance to ZEA.

The use of selected LAB capable to bind ZEA seems not to give any particular benefit to ruminants. However, this property could be more advantageous in monogastric animals such as pigs, which can be fed fermented cereal grains. In these *in vitro* experiments, about 50% of ZEA initially complexed to bacteria remained stable under conditions simulating the GIT of animals. *In vivo* experiments are needed to evaluate the biological importance of ZEA binding by selected strains of LAB.

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Table 3. Stability of RF-ZEA complex in solutions simulating post-ruminal compartments of ruminant's gastrointestinal tract.

Simulated compartment	ZEA released (%) [a,b]	ZEA remaining bound (%) [a,b]	Recovery (%)
Abomasum			
Pepsin (pH 2.5; 1 h)	5 (0) *	94 (3)	99
Buffer (pH 4; 1 h)	8 (1)	95 (4)	103
Abomasum + small intestine [c]			
Pepsin followed by pancreatin/bovine bile	9 (2) *	90 (6)	104
Buffer followed by buffer	5 (0)	86 (5)	99

[a] Fractions of ZEA released and remaining bound were percentages found in supernatants and pellets, respectively, from the amount of mycotoxin initially bound to bacteria.

[b.] Data are means (standard deviation) of triplicate tubes. Within a column, for the same treatment, means followed by * are significantly different from incubation in buffer ($P<0.05$).

[c] Data are fractions of ZEA released and remaining bound after the second incubation period; recovery is the sum of both values and the fraction released after the first incubation period.

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