

## Mycoestrogen Pollution of Italian Infant Food

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**Objective** To determine the concentrations of zearalenone and its metabolites in the leading brands of infant formula milks and meat-based infant foods commonly marketed in Italy, and to assess their repercussion in the provisional tolerable daily intakes of these estrogenic mycotoxins.

**Study design** A total of 185 cow's milk-based infant formulas and 44 samples of meat-based infant foods samples were analyzed. The analysis of mycotoxins was performed by immunoaffinity column clean-up and high-pressure liquid chromatography with fluorescence detection.

**Results** Zearalenone was detected in 17 (9%) milk samples (maximum 0.76  $\mu\text{g/L}$ ). The  $\alpha$ -zearalenol was detected in 49 (26%) milk samples (maximum 12.91  $\mu\text{g/L}$ ). The  $\beta$ -zearalenol was detected in 53 (28%) milk samples (maximum 73.24  $\mu\text{g/L}$ ). The  $\alpha$ -zearalanol and  $\beta$ -zearalanol were not detected in milk samples. Although  $\alpha$ -zearalenol was detected in 12 (27%) meat samples (maximum 30.50  $\mu\text{g/kg}$ ), only one meat-based sample was contaminated by  $\alpha$ -zearalanol (950  $\mu\text{g/kg}$ ). Zearalenone,  $\beta$ -zearalenol, and  $\beta$ -zearalanol were not detected in meat samples.

**Conclusions** This study shows the presence of mycoestrogens in infant (milk-based and meat-based) food, and this is likely to have great implications for subsequent generations, suggesting the need to perform occurrence surveys in this type of food. (*J Pediatr* 2011; ■: ■-■).

Zearalenone is a nonsteroidal mycotoxin produced by *Fusarium sp* on several grains.<sup>1</sup> Despite its low acute toxicity and carcinogenicity,<sup>2</sup> zearalenone exhibits estrogenic and anabolic properties in several animal species, including human beings.<sup>2,3</sup> Zearalenone contamination of food is caused either by direct contamination of grains, fruits, and their products<sup>2</sup> or by "carry-over" of mycotoxins and their metabolites in animal tissues, milk, and eggs after intake of contaminated feedstuff.<sup>3,4</sup> The harmful effects of zearalenone may be increased through its derivatives,  $\alpha$ -zearalenol ( $\alpha$ -ZOL),  $\beta$ -zearalenol ( $\beta$ -ZOL),  $\alpha$ -zearalanol ( $\alpha$ -ZAL), and  $\beta$ -zearalanol ( $\beta$ -ZAL). Zearalenone and its derivatives have the remarkable ability to mimic estrogen, acting as an estrogen receptor agonist.<sup>5-7</sup> With in vitro assay (MCF-7 human breast cancer cell proliferation assay), the most potent estrogens were  $\alpha$ -ZAL and  $\alpha$ -ZOL, which had about the same potency as 17  $\beta$ -estradiol ( $E_2$ ), and zearalenone was about two orders of magnitude less potent than both of those metabolites.<sup>5,6</sup> The  $\beta$ -ZOL and  $\beta$ -ZAL were between one and five orders of magnitude less potent than zearalenone.<sup>5</sup> The relative potency of zearalenone compared with  $E_2$  in the uterotrophic assay was about 0.001, whereas the potency relative to that of  $E_2$  in the vaginal cornification assay was 0.001.<sup>8</sup> The  $\alpha$ -ZAL was several times more active in the uterotrophic assay than zearalenone.<sup>9</sup>

Infant formula milks constitute an important or often sole source of food for newborns and infants during their first months of life. Recently we have reported the contamination by aflatoxin M1 and ochratoxin A in infant formula milk marketed in Italy.<sup>10</sup> The introduction of complementary food is recommended to begin between the ages of 4 and 6 months for most infants. Nonetheless, only limited information exists about the presence of contaminants in commercial infant food, and none exists regarding zearalenone and their metabolites in meat-based infant foods.<sup>11,12</sup>

The aim of this study was to determine the concentrations of zearalenone and its metabolites in the leading brands of infant formulas marketed in Italy and to assess their repercussion in the provisional tolerable daily intakes of these mycotoxins. Furthermore, we determined levels of zearalenone and its metabolites contamination in meat-based infant food.

$\alpha$ -ZAL	$\alpha$ -Zearalanol
$\alpha$ -ZOL	$\alpha$ -Zearalenol
$\beta$ -ZAL	$\beta$ -zearalanol
$\beta$ -ZOL	$\beta$ -zearalenol
$E_2$	17 $\beta$ -estradiol
HPLC	High-pressure liquid chromatography
IAC	Immunoaffinity columns
LOD	Limit of determination
LOQ	Limit of quantification
PMTDI	Provisional maximum tolerable daily intake

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The authors declare no conflicts of interest.

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## Methods

A total of 185 samples from 14 brands (5 different batch numbers for each product) of infant formula powders ( $n = 17$ ) and ready-to-use preparations ( $n = 20$ ) commonly marketed in Italy were collected from 2007 to 2008 and analyzed. Different types of cow's milk-based formula were classified as preterm formula ( $n = 11$  [1 powder and 10 liquid]) and starter formula for infants from the first day to 6 months of age ( $n = 26$  [16 powder and 10 liquid]). A total of 44 samples from seven brands of meat-based infant foods commonly marketed in Italy were collected in 2008 and analyzed. Different types of meat-based infant food were classified as beef ( $n = 7$ ), chicken ( $n = 7$ ), calf ( $n = 7$ ), turkey ( $n = 5$ ), rabbit ( $n = 5$ ), pig (ham) ( $n = 5$ ), horse ( $n = 4$ ), and lamb ( $n = 4$ ).

Zearalenone,  $\alpha$ -ZOL,  $\beta$ -ZOL,  $\alpha$ -ZAL, and  $\beta$ -ZAL reference standards, acetic acid (analytical reagent grade), and  $\beta$ -glucuronidase from *Helix pomatia* were purchased from Sigma (Milan, Italy). High-pressure liquid chromatography (HPLC)-grade water, methanol, chloroform, and acetonitrile were supplied by LABSCAN (Hasselt, Belgium). The immunoaffinity columns (IAC) ZearaStar were purchased from Tecna Srl (Trieste, Italy).

### Samples Preparation

**Milk.** Infant formula samples 5 mL were mixed with sodium acetate solution (0.2 M pH 5.5) 4 mL and hydrolyzed for 16 hours at 37 °C with glucuronidase solution 50  $\mu$ L to cleave the respective conjugates. The sample was then diluted with phosphate-buffered saline solution (pH 7.4) 40 mL. The diluted sample (pH value about 5.5 to 6.0) was filtered through a Whatman filter paper (Millipore Corporation, Maid Stone, United Kingdom). This solution was passed through the IAC at a flow rate of one to two drops  $s^{-1}$ . The column was washed with phosphate-buffered saline solution 20 mL (1-2 drops  $s^{-1}$ ). Elution was performed with methanol 3 mL. The elute was evaporated to dryness under a stream of nitrogen. The residue was redissolved in 250  $\mu$ L of the HPLC mobile phase. A 100- $\mu$ L amount of this solution was injected into the HPLC system. For samples of powdered milk, 5 g were suspended in distilled water 30 mL, heated to 50 °C, homogenized, and then processed as for liquid milk.

**Meat.** Five grams of meat-based infant food were mixed with 2 mL solution 20% acetic acid adjusted to pH 5 by addition of NaOH 1 mol/L and hydrolyzed for 16 hours at 37 °C with glucuronidase solution 50  $\mu$ L to cleave the respective conjugates. The sample was then mixed with chloroform extraction solution 7.5 mL. Extraction was done by shaking for 20 minutes on a horizontal shaker and spinning in a centrifuge at 3000 rpm for 15 minutes. The extraction step was repeated twice. The resulting organic phases were pooled, mixed with TRIS solution 3 mL (0.04 M pH 7.5), shaken for 10 minutes on a horizontal shaker, and spun in a centrifuge at 3000 rpm for 10 minutes. The organic phase was separated from TRIS solution by use of a Pasteur pipette. The

resulting organic phase was evaporated to dryness under a stream of nitrogen. The residue was redissolved in 250  $\mu$ L of the HPLC mobile phase. A 100- $\mu$ L amount of this solution was injected into the HPLC system.

### Spiked Samples

Samples spiked before extraction were used to check the performance of the extraction and clean-up procedure and to obtain validation parameters. Spiking solutions of mycotoxins were prepared daily by dilution with HPLC mobile phase. For samples of liquid milk, after thoroughly mixing for 30 minutes, the mycotoxins-fortified milks were left for at least 2 hours at room temperature to enable equilibration and used to assay the cleaning procedures before HPLC analysis. For samples of powdered milk, spiked samples were prepared by adding appropriate volume of the spiking solution of mycotoxins to the milk suspension (described above) and then processed as for liquid milk. For samples of meat-based infant foods, after being thoroughly mixed for 30 minutes, the mycotoxins-fortified samples were left for at least 2 hours at room temperature to enable equilibration and used to assay the cleaning procedures before HPLC analysis.

### HPLC

The chromatographic system consisted of a Jasco 880 pump and a Jasco 821 fluorescence detector (Jasco, Tokyo, Japan). Jasco Borwin software (Jasco UK, Essex, United Kingdom) was used for data processing. The excitation wavelength ( $\lambda_{ex}$ ) and emission wavelength ( $\lambda_{em}$ ) were set at 274 and 440 nm, respectively. The reversed-phase column was a Spherisorb Waters C<sub>18</sub> column (3  $\mu$ m, 150  $\times$  4.60 mm) connected to a Waters Guard-Pak C<sub>18</sub> pre-column (4  $\mu$ m) (Waters, Milford, Massachusetts). The column was kept at room temperature. The HPLC was operated with a mobile phase system consisting of acetonitrile-water (adjusted to pH 4 by addition of H<sub>3</sub>PO<sub>4</sub> 85%) 55/45% v/v at a flow rate of 1 mL/min.

The HPLC method was validated according to international rules<sup>13</sup>: selectivity, linearity, limits of determination (LOD) and quantification (LOQ), repeatability, and reproducibility were determined. For the linearity test calibration curves with zearalenone and  $\alpha$ -ZOL working standard solutions at 1 to 1000  $\mu$ g/L and with  $\beta$ -ZOL,  $\alpha$ -ZAL and  $\beta$ -ZOL working standard solutions at 10 to 1000  $\mu$ g/L (for each mycotoxin in acetonitrile) were obtained. Milk samples spiked with zearalenone and  $\alpha$ -ZOL at 0.05, 0.1, 0.2, 0.5 and 1  $\mu$ g/L and with  $\beta$ -ZOL,  $\alpha$ -ZAL, and  $\beta$ -ZOL at 0.5, 1, 2, 5, and 10  $\mu$ g/L were analyzed with the IAC clean-up and HPLC method. Taking into account dilution and concentration steps, these spiked samples corresponded to zearalenone and  $\alpha$ -ZOL standard concentrations of 1, 2, 4, 10 and 20  $\mu$ g/L and  $\beta$ -ZOL,  $\alpha$ -ZAL and  $\beta$ -ZOL standard concentrations of 10, 20, 40, 100 and 200  $\mu$ g/L. Meat samples spiked with zearalenone and  $\alpha$ -ZOL at 0.1, 0.5, 1, 10 and 50  $\mu$ g/kg and with  $\beta$ -ZOL,  $\alpha$ -ZAL and  $\beta$ -ZOL at 1, 5, 20, 100 and 200  $\mu$ g/kg were analyzed with the clean-up and HPLC method. Taking into account dilution and concentration steps, these spiked samples corresponded to zearalenone

and  $\alpha$ -ZOL standard concentrations of 2, 10, 20, 200, and 100  $\mu\text{g/L}$  and  $\beta$ -ZOL,  $\alpha$ -ZAL, and  $\beta$ -ZOL standard concentrations of 20, 100, 400, 2000, and 4000  $\mu\text{g/L}$ . The experiment was repeated three times for both milk and meat samples. To test the selectivity of the procedure, samples of milk and meat spiked with the five studied mycotoxins were analyzed according to the procedure. The repeatability was tested by analyzing samples of milk and meat spiked with mycotoxins. Samples were spiked at the levels of 0.5  $\mu\text{g/L}$  or  $\mu\text{g/kg}$  (corresponding to 10  $\mu\text{g/L}$ ), 2.5  $\mu\text{g/L}$  or  $\mu\text{g/kg}$  (corresponding to 50  $\mu\text{g/L}$ ), and 5  $\mu\text{g/L}$  or  $\mu\text{g/kg}$  (corresponding to 100  $\mu\text{g/L}$ ). All samples were measured in triplicates on the same day. For the within-laboratory reproducibility test, each of the contamination level was tested in triplicates in seven days. The results of these experiments were used also for the determination of the recovery. The LOD and LOQ were calculated as threefold and tenfold the standard deviation of the intercepts of calibration curves divided by the slope of calibration curves.<sup>13</sup>

### Statistical Analysis

Values are expressed as mean  $\pm$  standard error of the mean, unless otherwise stated. Statistical analysis was performed with the Mann Whitney test, Tukey test, and one-way ANOVA where they were appropriated. Correlations between two parameters were determined by Pearson correlation ( $r$ ) coefficient analysis. Findings of  $P < .05$  were considered significant.

## Results

In **Table I** (available at [www.jpeds.com](http://www.jpeds.com)) the results regarding the method validation parameters were reported. A total of 185 cow's milk-based infant formula samples were analyzed. Zearalenone was detected in 17 (9%) samples (maximum 0.76  $\mu\text{g/L}$ ). The  $\alpha$ -ZOL was detected in 49 (26%) samples (maximum 12.91  $\mu\text{g/L}$ ) (**Table II**). The  $\beta$ -ZOL was detected in 53 (28%) samples (maximum 73.24  $\mu\text{g/L}$ ). The  $\alpha$ -ZAL and  $\beta$ -ZAL were not detected in milk samples.

Zearalenone,  $\alpha$ -ZOL, and  $\beta$ -ZOL were detected together in two starter powdered preparations (zearalenone 0.42-0.76  $\mu\text{g/L}$ ,  $\alpha$ -ZOL 0.33-0.43  $\mu\text{g/L}$  and  $\beta$ -ZOL 23.92-46.14  $\mu\text{g/L}$ ) and in a preterm ready-to-use preparation (zearalenone 0.47  $\mu\text{g/L}$ ,  $\alpha$ -ZOL 0.60  $\mu\text{g/L}$  and  $\beta$ -ZOL 1.40  $\mu\text{g/L}$ ). Nonparametric statistical analyses (Mann Whitney test) were performed with zearalenone,  $\alpha$ -ZOL, and  $\beta$ -ZOL content for comparison of the different types of formula (starter vs pre-

term, ready-to-use starter vs ready-to-use pre-term, powder starter vs powder pre-term), not establishing significant differences ( $P > .05$ ) between different groups.

Nonparametric statistical analyses (Mann Whitney test) were performed with zearalenone,  $\alpha$ -ZOL, and  $\beta$ -ZOL content for comparison of the different types of formula from the same commercial brand (preterm vs starter formula, powdered vs ready-to-use formula), not establishing significant differences ( $P > .05$ ) between different groups. Bar diagrams of zearalenone,  $\alpha$ -ZOL, and  $\beta$ -ZOL concentration distribution for all formula commercialized by each brand analyzed ( $n = 14$ ) are shown in **Figure 1**.

A statistical comparison (ANOVA) for ZEA levels between the commercial brands and starter and preterm preparations from the different commercial brands showed no significant differences ( $P > .05$ ) between the groups. By ANOVA test ( $P = .0016$ ) the  $\alpha$ -ZOL levels in samples by brand 6 ( $n = 15$ ,  $2.54 \pm 4.91 \mu\text{g/L}$ , max. 12.91  $\mu\text{g/L}$ ) and brand 13 ( $n = 5$ ,  $4.00 \pm 0.07 \mu\text{g/L}$ , range 3.95-4.05  $\mu\text{g/L}$ ) were significantly higher (Tukey test,  $P < .05$ ) than those from most of the other brands. Brands 10, 12, and 14 had no detectable levels of  $\alpha$ -ZOL, and the other brands had the lowest  $\alpha$ -ZOL concentrations. A significant difference (ANOVA,  $P < .0001$ ) between the starter preparations from the different commercial brands was detected. Among the starter preparations the  $\alpha$ -ZOL levels in samples by brand 13 ( $n = 5$ ,  $4.00 \pm 0.07 \mu\text{g/L}$ , range 3.95-4.05  $\mu\text{g/L}$ ) was significantly higher (Tukey test,  $P < .001$ ) than those from most of the other brands. An ANOVA comparison of the preterm formula from the different commercial brands showed no significant differences ( $P > .05$ ) between different groups.

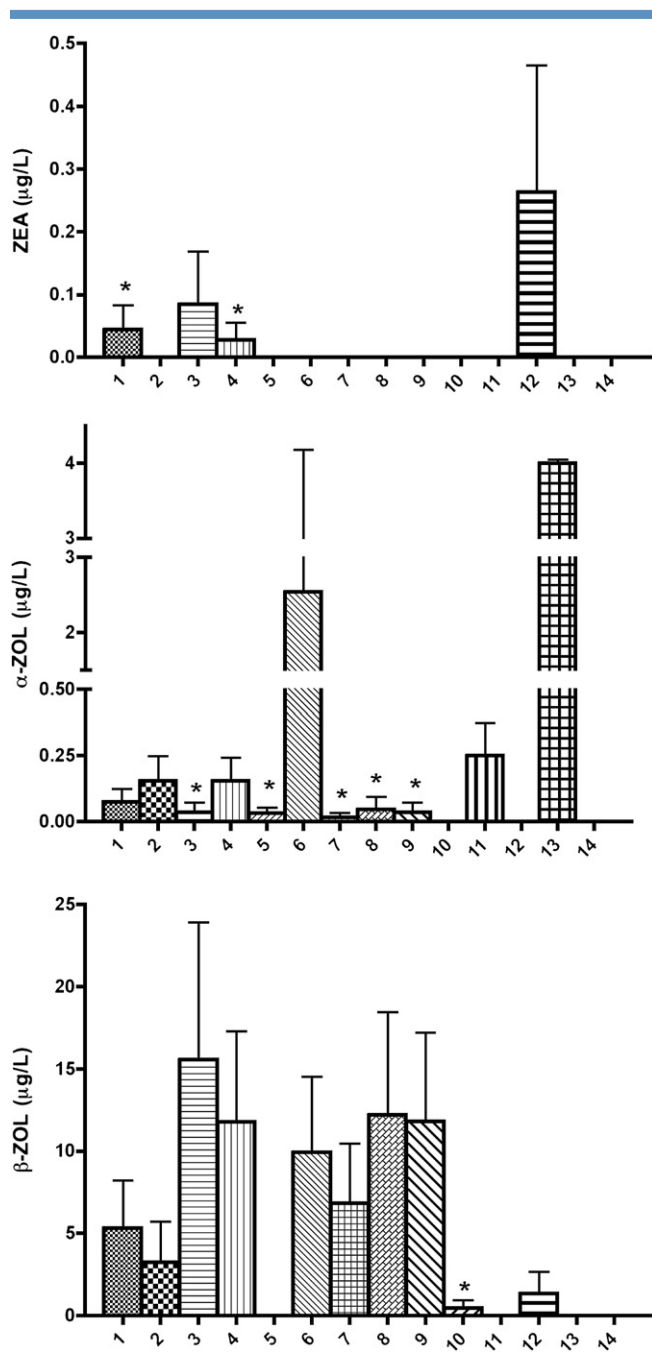
For  $\beta$ -ZOL no significant difference was established by ANOVA ( $P > .05$ ) between the commercial brands. A comparison of statistical differences (ANOVA,  $P = .035$ ) between the starter preparations from the different commercial brands was performed. Among the starter preparations the  $\beta$ -ZOL levels in samples by brands 2 ( $n = 15$ ,  $24.44 \pm 6.36 \mu\text{g/L}$ , range 13.41-35.46  $\mu\text{g/L}$ ) and 3 ( $n = 10$ ,  $28.00 \pm 12.85 \mu\text{g/L}$ , range 3.29-69.41  $\mu\text{g/L}$ ) were higher than those from most of the other brands but not significantly (Tukey test,  $P > .05$ ). No significant difference ( $P > .05$ ) was detected between the preterm formulas from the different commercial brands.

The mycotoxin values determined in the different types of formula were used to calculate mean daily dietary mycotoxins intakes by infants on the basis of feeding tables provided by brands, under the assumption that they receive only formula.

**Table II.** Zearalenone,  $\alpha$  ZOL, and  $\beta$ -ZOL concentrations in different types of infant formula investigated

Infant formula	Number of samples	Zearalenone		$\alpha$ -ZOL		$\beta$ -ZOL	
		Number of positives (%)	Mean $\pm$ SEM	Number of positives (%)	Mean $\pm$ SEM	Number of positives (%)	Mean $\pm$ SEM
Preterm	55	6 (11%)	0.014 $\pm$ 0.010 $\mu\text{g/L}$	7 (12%)	0.69 $\pm$ 0.47 $\mu\text{g/L}$	16 (29%)	7.80 $\pm$ 2.90 $\mu\text{g/L}$
Liquid	50	6 (12%)	0.020 $\pm$ 0.010 $\mu\text{g/L}$	7 (14%)	0.76 $\pm$ 0.52 $\mu\text{g/L}$	16 (32%)	8.60 $\pm$ 3.13 $\mu\text{g/L}$
Powder	5	0 (0%)	—	0 (0%)	—	0 (0%)	—
Starter	130	11 (8%)	0.026 $\pm$ 0.014 $\mu\text{g/L}$	42 (32%)	0.20 $\pm$ 0.07 $\mu\text{g/L}$	37 (28%)	6.13 $\pm$ 1.49 $\mu\text{g/L}$
Liquid	50	0 (0%)	—	12 (24%)	0.15 $\pm$ 0.07 $\mu\text{g/L}$	16 (32%)	10.85 $\pm$ 3.10 $\mu\text{g/L}$
Powder	80	11 (13%)	0.042 $\pm$ 0.022 $\mu\text{g/L}$	30 (37%)	0.23 $\pm$ 0.11 $\mu\text{g/L}$	21 (26%)	3.48 $\pm$ 1.31 $\mu\text{g/L}$

SEM, standard error of the mean.



**Figure 1.** Zearalenone,  $\alpha$ -ZOL, and  $\beta$ -ZOL levels ( $\mu\text{g/L}$ ) in the infant milk-based formulas provided by different manufacturers ( $n = 14$ ). Bars marked with asterisks had mean concentrations below LOD; brands 10, 12, and 14 were found negative for  $\alpha$ -ZOL; brands 5, 11, 13, and 14 were found negative for  $\beta$ -ZOL.

The mean daily intakes of zearalenone,  $\alpha$ -ZOL, and  $\beta$ -ZOL for ready-to-use pre-term formula were  $0.078 \pm 0.01$ ,  $0.098 \pm 0.07$  and  $2.90 \pm 0.43 \mu\text{g/kg}$  body weight, respectively. The mean daily intakes of  $\alpha$ -ZOL and  $\beta$ -ZOL for ready-to-use starter formula were  $0.17 \pm 0.03$  and  $2.89 \pm 0.19 \mu\text{g/kg}$  body weight, respectively. The mean daily intakes of zearale-

none,  $\alpha$ -ZOL, and  $\beta$ -ZOL for powdered starter formula were  $0.021 \pm 0.02$ ,  $0.087 \pm 0.04$ , and  $0.76 \pm 0.20 \mu\text{g/kg}$  body weight, respectively. Correlations analysis between the contamination levels of different types of infant formula and their main characteristics (lipid, protein and carbohydrates contents) showed no significant ( $P > .05$ ) correlation between measures for all the mycotoxins analyzed.

A total of 44 samples of meat-based infant foods were analyzed. The  $\alpha$ -ZOL was detected in 12 (27%) samples (max.  $30.50 \mu\text{g/kg}$ ) (Table III). The  $\alpha$ -ZAL was detected in one sample ( $950 \mu\text{g/kg}$ ). Zearalenone,  $\beta$ -ZOL, and  $\beta$ -ZAL were not detected in meat samples.

Bar diagrams of  $\alpha$ -ZOL amount distribution for all meat-based infant food commercialized by each brand analyzed ( $n = 7$ ) and for all meat types analyzed are shown in Figure 2. By ANOVA, no significant difference ( $P > .05$ ) was detected between the commercial brands. A comparison of statistically significant differences (ANOVA,  $P = .0107$ ) between all meat types analyzed was performed. Globally, the  $\alpha$ -ZOL levels in samples of calf meat ( $n = 7$ ,  $8.51 \pm 4.49 \mu\text{g/kg}$ , max.  $30.50 \mu\text{g/kg}$ ) were significantly higher (Tukey test,  $P < .05$ ) than those from most of the other brands. Turkey and chicken meat samples had no detectable levels of  $\alpha$ -ZOL.

## Discussion

Several studies agree with a low transfer rate of zearalenone and its metabolites to milk.<sup>14,15</sup> However, livestock fed with feeds containing zearalenone may produce milk and milk products that contain high doses of these mycotoxins.<sup>15-18</sup> According to Prelusky et al,<sup>15</sup> the maximum concentrations ( $6.1 \mu\text{g/L}$  zearalenone,  $4 \mu\text{g/L}$   $\alpha$ -ZOL, and  $6.6 \mu\text{g/L}$   $\beta$ -ZOL) were found in the milk of one cow given an oral dose of  $6000 \text{ mg}$  zearalenone (equivalent to  $12 \text{ mg/kg}$  body weight), but neither zearalenone nor its metabolites were found in the milk ( $<0.5 \mu\text{g/L}$ ) of three lactating cows fed  $50$  or  $165 \text{ mg}$  zearalenone (equivalent to  $0.1$  and  $0.33 \text{ mg/kg}$  body weight) for 21 days. Moreover, recently it was reported<sup>19</sup> that rumen metabolic capacity can be saturated depending on varying feeding regimes.

These results agree with studies regarding zearalenone metabolism in cows. In the cow, free and conjugated (glucuronic and sulphate) zearalenone (29%),  $\alpha$ -ZOL (20%), and  $\beta$ -ZOL (51%) were found;  $\beta$ -ZOL was the predominant species.<sup>20</sup> An exception to this trend was represented from samples of brand

**Table III.** The  $\alpha$ -ZOL amounts in different types of meat-based infant foods

Type of meat	Number of samples	Number of positives (%)	Mean $\pm$ SEM	Maximum
Calf	7	4	$8.51 \pm 4.48 \mu\text{g/kg}$	$30.50 \mu\text{g/kg}$
Beef	7	2	$1.10 \pm 0.74 \mu\text{g/kg}$	$4.80 \mu\text{g/kg}$
Rabbit	5	2	$1.32 \pm 0.85 \mu\text{g/kg}$	$4.10 \mu\text{g/kg}$
Ham	5	2	$1.64 \pm 1.04 \mu\text{g/kg}$	$5.00 \mu\text{g/kg}$
Horse	4	1	$1.37 \pm 1.37 \mu\text{g/kg}$	$5.50 \mu\text{g/kg}$
Lamb	4	1	$1.80 \pm 1.80 \mu\text{g/kg}$	$7.20 \mu\text{g/kg}$
Turkey	5	0	—	—
Chicken	7	0	—	—

SEM, standard error of the mean.

13, which had the highest concentrations of  $\alpha$ -ZOL and no  $\beta$ -ZOL. However, from some brands (13 and 14) we had only one type of product (5 samples), so the results of samples by brand 13 could be explained by few number of samples analyzed with respect to others brands. From a research standpoint, we have shown that there are significant variations of zearalenone metabolites levels across the infant formula brands. This could be attributed to different manufacturing practices and probably to variation in the quality of raw material used by the infant formula manufacturer.

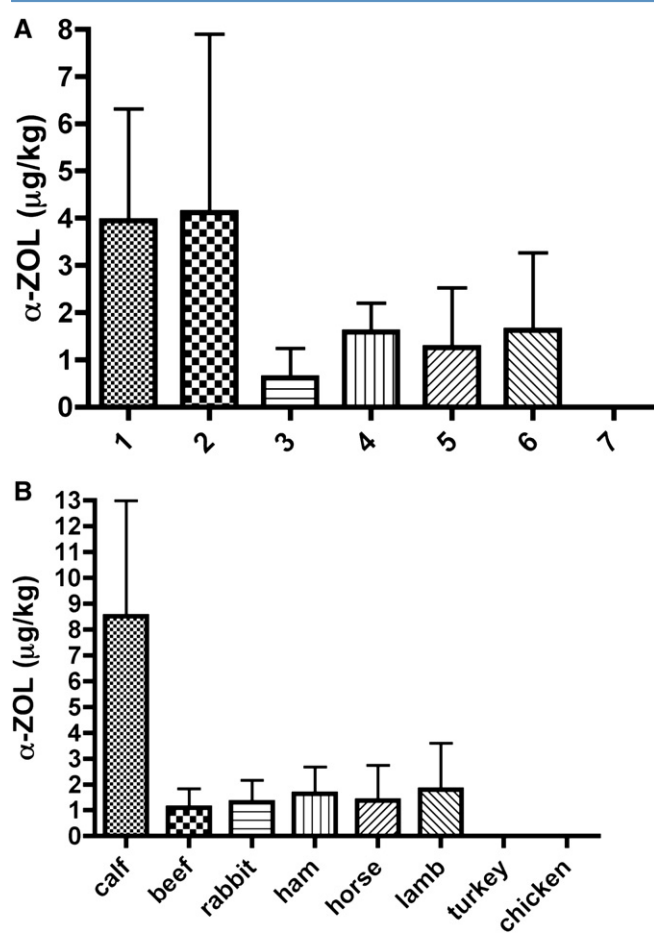
Our data could be also explained considering a high contamination of feedstuffs in Italy or the existence of other possible routes of zearalenone exposure than the original milk used for manufacturing infant formulas. Different ingredients have been added to infant formula not only to simulate better the composition of breast milk, but also to impart health benefits. Raw material (cow's milk or isolated protein), processing methods, additives, mineral supplements, and storage will in part determine the levels of minor and trace constituents present in the final product. In this study, correlations analysis between the zearalenone and its metabolite contamination levels of different types of infant formula and their

main characteristics (lipid, protein and carbohydrates contents) showed no significant correlation between parameters.

Diet is obviously an important pathway of exposure to all sorts of chemical agents and naturally occurring compounds. Zearalenone and its derivatives are naturally occurring, together with phytoestrogens, and can enter the food chain with consequences for human reproduction and health. Considering the mean levels of zearalenone in the principal foods and their consumption, the average daily intakes of zearalenone ranged among adults from 0.8 to 29 ng/kg body weight. Small children have the highest average daily intakes ranging from 6 to 55 ng/kg body weight.<sup>21</sup> A provisional maximum tolerable daily intake (PMTDI) for zearalenone of 0.5  $\mu$ g/kg of body weight is now established by the Joint Committee Food and Agriculture Organization of the United Nations and the World Health Organization, on the basis of the no-observed-effect level of 40  $\mu$ g/kg body weight/day obtained in a 15-day study in pigs. The committee recommended that the total intake for zearalenone and its metabolites (including  $\beta$ -ZOL) should not exceed this value.<sup>22</sup> The average zearalenone and  $\alpha$ -ZOL levels in the analyzed samples were much lower than the PMTDI. However, the average  $\beta$ -ZOL levels in both preterm and starter ready-to-use infant formula were sufficient to cause a higher intake of  $\beta$ -ZOL (fourfold higher) than the suggested PMTDI of 0.5  $\mu$ g/kg/d. Infants and children are more susceptible to different toxins than adults because of their lower body weight, higher metabolic rate, lower ability to detoxify, and because of incomplete development of some organs and tissues such as the central nervous system. Therefore it would be of great importance to reduce the risk of children's exposure to zearalenone and its metabolites.

The dietary intake of zearalenone and its metabolites from meat and products seems to be of little significance because of the rapid biotransformation and excretion of zearalenone in animals.<sup>23</sup> Zearalenone and its metabolite concentrations in liver and bile increases with administered dose.<sup>14,24</sup> Neither zearalenone nor its metabolites are detected in muscles, kidneys, liver, bladder, dorsal fat of male bovine ingesting 0.1 mg/d/kg feed.<sup>20</sup> However, zearalenone has been detected in 20% of 25 fresh meat samples at levels of  $8.7 \pm 1.6$   $\mu$ g/kg.<sup>18</sup>

These results, concerning meat-based infant food samples, agree with studies regarding zearalenone metabolism in different animal species and showing  $\alpha$ -ZOL as one of the most important metabolite in tissue samples.<sup>25</sup> This is the first report regarding the presence of zearalenone metabolites in commercial meat-based infant foods and it points out the need to perform occurrence surveys in this type of food. The  $\alpha$ -ZAL was detected in one sample of lamb meat-based infant food at a very high concentration (950  $\mu$ g/kg). Since 1969  $\alpha$ -ZAL has been widely adopted as a growth stimulant in the United States to improve fattening rates of cattle.<sup>26,27</sup> Its use has been banned in the European Union since 1985 together with a variety of other growth hormones, such as 17  $\beta$ -estradiol, progesterone, testosterone, trenbolone acetate, and melenigestrol acetate. This also includes a ban on imported meat and meat products derived from cattle given these hormones other than for veterinary reasons.<sup>28,29</sup> The Joint Food and



**Figure 2.** The  $\alpha$ -ZOL levels ( $\mu$ g/kg) in the meat-based infant foods provided by **A**, seven manufacturers and **B**, by different type of meat.

Agriculture Organization of the United Nations and the World Health Organization Expert Committee on Food Additives proposed in 1987 a maximum acceptable intake of 0.5  $\mu\text{g}/\text{kg}$  body weight corresponding to maximum residue levels in liver of 10  $\mu\text{g}/\text{kg}$  and in muscle of 2  $\mu\text{g}/\text{kg}$ , which are based on the no hormonal effect level of  $\alpha$ -ZAL. The Food and Drug Administration established safe concentration levels for total  $\alpha$ -ZAL residues in uncooked edible tissues of cattle as high as 150  $\mu\text{g}/\text{kg}$  in muscle, 300  $\mu\text{g}/\text{kg}$  in liver, 450  $\mu\text{g}/\text{kg}$  in kidney, and 600  $\mu\text{g}/\text{kg}$  in fat. The European Food Safety Authority report for 2008 on the monitoring of veterinary medicinal product residues and other substances in food of animal origin in the member states showed there were 69 noncompliant results for  $\alpha$ -ZAL and  $\beta$ -ZAL in bovines, but the feed contamination was suspected as the origin of all noncompliant results for bovine samples.<sup>30</sup> The high level of  $\alpha$ -ZAL found in this investigation did not fit with feed contamination; however, more studies need to verify and address these points.

Our study shows the presence of mycoestrogens in food. This is likely to have greater implications for infants and young children than for adults having a more varied diet. These data clearly suggest the need to identify risk markers of the infant feed quality. Analysis of infant milk-based formula, hence, may serve as a marker of exposure to zearalenone and their metabolites. At the same time, zearalenone metabolites levels in milk indicate the exposure of newborns or infants, which needs to be considered in the overall risk characterization.

In addition, specific studies should be initiated that allow the establishment of safe levels of zearalenone metabolites in feed materials and compounded feeds, particularly for infants and children of different ages, as they are considered to be the most sensitive to environmental chemicals. Finally the finding of  $\alpha$ -ZOL and  $\alpha$ -ZAL in meat-based commercial infant foods points out the need for further studies in this type of sample. ■

Submitted for publication Aug 7, 2010; last revision received Dec 19, 2010; accepted Jan 18, 2011.

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## References

- Miller JD. Aspects of the ecology of Fusarium toxins in cereals. *Adv Exp Med Biol* 2002;504:19-27.
- Joint FAO/WHO Expert Committee on Food Additive (JECFA). 53<sup>rd</sup> Report: Safety Evaluation of Certain Food Additives; WHO Food Additives Series; WHO: Geneva, Switzerland, 2000; vol. 44.
- Danicke S, Swiech E, Buraczewska L, Ueberschar KH. Kinetics and metabolism of zearalenone in young female pigs. *J Anim Physiol Anim Nutr* 2005;89:268-76.
- Kleinova M, Zollner P, Kahlbacher H, Hochsteiner W, Lindner W. Metabolic profiles of the mycotoxin zearalenone and of the growth promoter zeranol in urine, liver, and muscle of heifers. *J Agric Food Chem* 2002;50:4769-76.
- Leffers H, Naesby M, Vendelbo B, Skakkebaek NE, Jorgensen M. Oestrogenic potencies of Zeranol, oestradiol, diethylstilboestrol, Bisphenol-A and genistein: implications for exposure assessment of potential endocrine disrupters. *Hum Reprod* 2001;16:1037-45.
- Shier WT, Shier AC, Xie W, Mirocha CJ. Structure-activity relationships for human estrogenic activity in zearalenone mycotoxins. *Toxicol* 2001; 39:1435-8.
- Tiemann U, Tomek W, Schneider F, Vancelow J. Effects of the mycotoxins  $\alpha$ - and  $\beta$ -zearalenol on regulation of progesterone synthesis in cultured granulosa cells from porcine ovaries. *Reprod Toxicol* 2003;17: 673-81.
- Kuiper-Goodman T, Scott PM, Watanabe H. Risk assessment of the mycotoxin zearalenone. *Reg Toxicol Pharmacol* 1987;7:253-306.
- Takemura H, Shim JY, Sayama K, Tsubura A, Zhu BT, Kayoko S. Characterization of the estrogenic activities of zearalenone and zeranol in vivo and in vitro. *J Steroid Biochem Mol Biol* 2007;103:170-7.
- Meucci V, Razzuoli E, Soldani G, Massart F. Mycotoxin detection in infant formula milks in Italy. *Food Addit Contam* 2010;27:64-71.
- Rey-Salgueiro L, Martínez-Carballo E, García-Falcón MS, González-Barreiro C, Simal-Gándara J. Occurrence of polycyclic aromatic hydrocarbons and their hydroxylated metabolites in infant foods. *Food Chem* 2009;115:814-9.
- Schecter A, Wallace D, Pavuk M, Piskac A, Pápe O. Dioxins in commercial United States baby food. *J Toxicol Environ Health* 2002;65:1937-43.
- Q2(R1): Validation of Analytical Procedures: Text and Methodology, CPMP/ICH/381/95.
- Shreeve BJ, Patterson DS, Roberts BA. The carry-over of aflatoxin, ochratoxin and zearalenone from naturally contaminated feed to tissues, urine and milk of dairy cows. *Food Cosmet Toxicol* 1979;17:151-2.
- Prelusky B, Scott PM, Trenholm H, Lawrence GA. Minimal transmission of earalenone to milk of dairy cows. *J Environ Sci Health B* 1990;25:87-103.
- Mirocha CJ, Pathre SV, Robison TS. Comparative metabolism of zearalenone and transmission into bovine milk. *Food Cosmet Toxicol* 1981;19:25-30.
- Coffey R, Cummins E, Ward S. Exposure assessment of mycotoxins in dairy milk. *Food Control* 2009;20:239-49.
- El-Hoshy SM. Occurrence of zearalenone in milk, meat and their products with emphasis on influence of heat treatments on its level. *Arch Leb-ensmittelhyg* 1999;50:140-3.
- Seeling K, Dänicke S, Ueberschar KH, Lebzien P, Flachowsky G. On the effects of Fusarium toxin-contaminated wheat and the feed intake level on the metabolism and carry over of zearalenone in dairy cows. *Food Addit Contam* 2005;22:847-55.
- Danicke S, Ueberschar KH, Halle I, Matthes S, Valenta H, Flachowsky G. Effect of a detoxifying agent to laying hen diets containing uncontaminated or Fusarium toxin-contaminated maize on performance of hens and carryover of zearalenone. *Poultry Sci* 2002;81:1671-80.
- Minervini F, Giannoccaro A, Cavallini A, Visconti A. Investigations on cellular proliferation induced by zearalenone and its derivatives in relation to the estrogenic parameters. *Toxicol Lett* 2005;159:272-83.
- Codex Committee on Food Additives and Contaminants (CCFAC) Posting Date. Joint FAO/WHO Expert Committee on Food Additives: Position Paper on Zearalenone. Publication CCFAC 00/19. Codex Alimentarius Commission, Rome, Italy. 2000.
- Creppy EE. Update of survey, regulation and toxic effects of mycotoxins in Europe. *Toxicol Lett* 2002;127:19-28.
- Doll S, Danicke S, Ueberschar K, Valenta H, Schnurrbusch U, Klobasa F, Flachowsky G. Effects of graded levels of Fusarium toxin contaminated maize in diets female weaned piglets. *Arch Anim Nutr* 2003;57:311-34.
- Minervini F, Dell'Aquila ME. Zearalenone and reproductive function in farm animals. *Int J Mol Sci* 2008;9:2570-84.
- Moran C, Quirke JF, Prendiville DJ, Bourke S, Roche JF. The effect of estradiol, trenbolone acetate, or zeranol on growth rate, mammary development, carcass traits, and plasma estradiol concentrations of beef heifers. *J Anim Sci* 1991;69:4249-58.
- Williams JE, Ireland SJ, Mollett TA, Hancock DL, Beaver EE, Hannah S. Influence of zeranol and breed on growth, composition of gain, and plasma hormone concentrations. *J Anim Sci* 1991;69:1688-96.
- Council Directive 85/649/EEC, 1985.
- Directive of The European Parliament and of the Council 96/22/EC, 1996.
- EFSA. Report for 2008 on the results from the monitoring of veterinary medicinal product residues and other substances in food of animal origin in the Member States. *EFSA J* 2010;8:1-55.

**Table I.** Validation parameters for HPLC methods of mycotoxins

Parameters	Zearalenone	$\alpha$ -ZOL	$\beta$ -ZOL	$\alpha$ -ZAL	$\beta$ -ZAL
<b>Liquid milk</b>					
CV% Intra-day	3	3	2	2	2
CV% Inter-day	8	7	7	5	3
LOD ( $\mu\text{g/L}$ )	0.02	0.02	0.2	0.2	0.2
LOQ ( $\mu\text{g/L}$ )	0.05	0.05	0.5	0.5	0.5
R <sup>2</sup>	0.9985	0.9986	0.9952	0.9923	0.9952
R <sup>2</sup> matrix	0.9910	0.9920	0.9888	0.9889	0.9875
<b>Recovery</b>					
0.5 $\mu\text{g/L}$	89.9 $\pm$ 1.8	79.9 $\pm$ 5.0	78.2 $\pm$ 5.5	75.5 $\pm$ 7.6	69.9 $\pm$ 6.7
2.5 $\mu\text{g/L}$	91.2 $\pm$ 3.5	89.4 $\pm$ 0.8	88.4 $\pm$ 1.8	85.0 $\pm$ 9.0	79.5 $\pm$ 4.8
5 $\mu\text{g/L}$	95.0 $\pm$ 1.2	85.9 $\pm$ 6.8	85.9 $\pm$ 7.1	88.0 $\pm$ 5.0	88.9 $\pm$ 4.5
<b>Powdered milk</b>					
CV% Intra-day	3	3	2	2	2
CV% Inter-day	8	7	7	5	3
LOD ( $\mu\text{g/L}$ )	0.02	0.02	0.2	0.2	0.2
LOQ ( $\mu\text{g/L}$ )	0.05	0.05	0.5	0.5	0.5
R <sup>2</sup>	0.9985	0.9986	0.9952	0.9923	0.9952
R <sup>2</sup> matrix	0.9910	0.9920	0.9888	0.9889	0.9875
<b>Recovery</b>					
0.5 $\mu\text{g/L}$	85.0 $\pm$ 0.3	88.1 $\pm$ 0.8	89.0 $\pm$ 8.0	79.8 $\pm$ 3.0	75.0 $\pm$ 1.8
2.5 $\mu\text{g/L}$	79.9 $\pm$ 2.8	78.0 $\pm$ 3.5	93.2 $\pm$ 4.9	84.6 $\pm$ 0.9	79.8 $\pm$ 3.5
5 $\mu\text{g/L}$	96.0 $\pm$ 3.0	86.0 $\pm$ 3.5	85.0 $\pm$ 6.8	89.0 $\pm$ 8.0	85.0 $\pm$ 2.0
<b>Meat</b>					
CV% Intra-day	2	2	3	3	2
CV% Inter-day	7	6	7	8	6
LOD ( $\mu\text{g/kg}$ )	0.5	0.5	2	2	2
LOQ ( $\mu\text{g/kg}$ )	1.0	1.0	5	5	5
R <sup>2</sup>	0.9993	0.9989	0.9987	0.9950	0.9992
R <sup>2</sup> matrix	0.9980	0.9952	0.9888	0.9870	0.9850
<b>Recovery</b>					
0.5 $\mu\text{g/kg}$	87.0 $\pm$ 0.8	91.0 $\pm$ 1.2	85.9 $\pm$ 2.1	85.4 $\pm$ 3.7	75.0 $\pm$ 7.8
2.5 $\mu\text{g/kg}$	93.2 $\pm$ 0.4	90.5 $\pm$ 0.5	87.0 $\pm$ 1.4	86.8 $\pm$ 0.9	79.2 $\pm$ 3.1
5 $\mu\text{g/kg}$	96.5 $\pm$ 0.5	89.7 $\pm$ 1.3	89.4 $\pm$ 3.0	90.2 $\pm$ 0.5	85.5 $\pm$ 0.5

CV, coefficient of variation.