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12 Carry over of aflatoxin from feed to milk in dairy cows with low or high somatic cell counts

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12.1 Abstract

Aflatoxin M1 residues in milk are regulated in many parts of the world and can cost dairy farmers significantly due to lost milk sales. Additionally, due to the carcinogenicity of this compound contaminated milk can be a mayor public health concern. Thirty four lactating dairy cows were utilized to investigate the relationship between somatic cell counts (SCC), milk yield and conversion of dietary AFB1 into milk AFM1 (carry over). The AFM1 in milk increased as soon as the first milking after animal ingestion with a pattern of increment up to the observed plateau (between 7th and 12th days of AFB1 ingestion). There was a significant ($P < 0.01$) effect of the milk yield whereas no effect could be attributed to the somatic cell count level or to the milk yield*somatic cell count interaction. Similarly, the main effect of milk yield was also observed ($P < 0.01$) on the total amount of AFM1 excreted during the ingestion period. Although plasma concentration of Gamma-glutamyl transferase was significantly affected by aflatoxin administration levels of this liver enzyme were within the normal range for lactating dairy cows. The current data suggest that milk yield is the major factor affecting the total excretion of AFM1 and that somatic cell counts as an indicator of mammary gland permeability was not related to an increase in AFM1 carry over.

Keywords: *aflatoxins, dairy cows, milk, aflatoxin M1, somatic cell count*

12.2 Introduction

The aflatoxins are secondary metabolites produced primarily by Aspergillus flavus and A. parasiticus. Aflatoxins are common crop contaminants, with contamination occurring either in the field, during harvest or during storage. The most frequently affected crops are corn (Zea mais), cotton and peanuts and their by products. The major aflatoxins are aflatoxin B1 (AFB1), B2, G1 and G2. Because of their low molecular weight, once ingested these compounds are rapidly adsorbed in the gastro-intestinal tract through a non-described passive mechanism (Yiannikouris and Jouany, 2002) and quickly appears as a metabolite in blood after just 15 minutes (Moschini et al., 2006) and milk as soon as 12 hours post feeding (Diaz et al., 2004).

Aflatoxin M1 (AFM1) is the principal oxidized metabolite of AFB1 and it can be readily found in milk and urine of most mammalians after consumption of AFB1. The aflatoxins, as a group (AFB1, AFB2, AFG1, AFG2 and AFM1), are classified as group 1 carcinogens (IARC, 2002). The European Union allowable limits for AFB1 in animal feeds and concentrates are 20 µg/kg and 5 µg/kg respectively (EC, 2003a). Furthermore, the EC limits AFM1 in milk to levels not greater than 0.05 µg/L (EC, 2006). In the US AFM1 is regulated by the US Food and Drug Administration (FDA) at 0.5 µg/L.

In dairy cows the amount of AFM1 excreted into milk can be up to 3% of the AFB1 intake (Diaz et al., 2004) and is affected by milk yield (Pettersson et al., 1989; Veldman et al., 1992) and stage of lactation (Munksgaard et al., 1987; Pettersson et al., 1989; Veldman et al., 1992). Other factors that affect carry-over into milk include species differences (Battacone et al., 2003), animal variability (Egmond, 1989; Steiner et al., 1990; Veldman et al., 1992) and mammary alveolar cell membrane health (Lafont et al., 1983).

There is limited information about the effect of udder infection on AFM1 excretion into milk. It has been suggested that an increase in AFM1 carry over (CO) occurs due to Staphylococcus udder infection (Veldman et al., 1992) whereas a previous study showed a relationship between AFM1 milk carry over and milk somatic cell counts independent of the milk yield (Lafont et al., 1983). Mastitis increases the number of Somatic Cells in milk, alters milk composition (Walstra and Jenness, 1984) and may affect AFM1 carry over rate by increasing membrane permeability.

The objective of this work was to evaluate the effect of milk yield and somatic cells count, as indicator of udder inflammatory processes, on milk AFM1 carry over in lactating dairy cows.

12.3 Material and methods

12.3.1 Animals and Treatments

An experiment was carried out utilizing 34 Holstein multiparous cows housed at the CERZOO research and experimental center (San Bonico, PC, Italy). The research protocol and animal care were in accordance with the EC council directive guidelines for animals used for experimental and other scientific purpose (EEC, 1986).

Milk yield and SCC data for classification of animals as high or low was obtained as the average of three measurements during a 15 d pre-experimental period. Individual milk yield for the factorial arrangement was measured daily during the last week of the pre-experimental period. Milk yield (LY: < 30 kg/head per day, HY: > 30 kg/head per day) and milk somatic cells count (SCC) (LSCC: <350000, HSCC: > 350000) were used in a 2 x 2 factorial arrangement in a completely randomized design.

Cows were housed in a free stall barn and had free access to water. The diet was formulated according to the nutrient requirements of dairy cattle (National Research Council, 2001) for an average cow weighing 600 kg, 140 days in milk and a 35 kg milk yield (3.8% fat and 3.35% protein). The bulk of the diet (table 12-1) on a dry matter basis was: corn silage (31.2%), dehydrated alfalfa hay (16.7%), grass hay (4.1%) and energy-protein supplement (48%). The diet was fed ad libitum (5% expected orts) as a total mixed ration (TMR) daily (0900 h). Cows were milked twice a day (0230 h and 1330 h) and individual milk yield was recorded at every milking (Afimilk system, Afikim, Israel).

During the experimental period (10 days) cows were given, before the morning meal, a 300 g bolus containing 1.004±0.03 g/cow per day of a naturally contaminated corn meal. This allowed for an AFB1 intake from the contaminated bolus of 98.10±0.26 μg/cow per day.

TMR samples were collected on days 0 and 10 of the experimental period, dried at 55°C in a ventilated oven until constant weight, then ground with one mm sieve (Thomas-Wiley Laboratory Mill, Arthur H. Thomas Co., Philadelphia, PA) and frozen until aflatoxin analysis.

Individual milk samples were collected at each milking for 18 consecutive days (during the last two days of the pre-experimental period, the experimental period (10 d) and for six days at the end of the AFB1 ingestion period), then a representative sample for day of milking was obtained and stored at -18°C for subsequent analysis. Milk samples collected at days 3, 7 and 10 of the treatment period were analyzed for fat, protein and lactose contents (infrared analysis, Milkoscan Model FT120 Foss Electric, Hillerod, Denmark) and for SCC (Fossomatic 360 Foss Electric, Hillerod, Denmark).

12.3.2 Sample Analysis

AFB1 assay in Feeds

Ten grams of dried feed were put in a methanol:water solution (80:20 vol/vol), shaken at 150 rpm for 45 minutes (Universal table Shaker 709) and filtered with Schleicher & Schuell 595 ½ filter paper (Dassel, Germany). Then, five mL were eluted with 45 mL of bi-distilled water through an immunoaffinity column (Aflatoxin Easi-extract, Rhône diagnostics technologies, Glasgow, UK) previously washed with 20 mL of a phosphate-buffered saline solution (pH 7.4). The column was washed with five mL water and slowly eluted with 2.5 mL of methanol. The extract was dried under nitrogen, redissolved in one mL acetonitrile:water (25:75) solution and filtered (Millipore Corporation, Bedford, Massachusetts, USA, HV 0.45 µm) before HPLC analysis.

AFM1 assay in milk samples

Extraction was done by the immunoaffinity technique according to Mortimer et al. (1987). Briefly, 50 mL of defatted milk (centrifuged at 7000 rpm for 10 minutes at 4°C) were filtered with Schleicher & Schuell 595 ½ filter paper (Dassel, Germany). Then, 20 mL were passed through an immunoaffinity column (Aflatoxin Easy-extract, Rhône diagnostics technologies, Glasgow, UK) previously washed with 20 mL of a phosphate-buffered saline solution (pH 7.4). The column was washed with five mL water, and slowly eluted with 2.5 mL of methanol. The extract was dried under nitrogen, redissolved in one mL acetonitrile:water (25:75) solution and filtered (Millipore Corporation, Bedford, Massachusetts, USA, HV 0.45 µm) before HPLC analysis.

12.3.3 Chromatography

The HPLC analysis was performed by a Perkin Elmer LC (Perkin Elmer, Norwalk, CT, USA) equipped with a LC-200 pump and a Jasco FP-1520 fluorescence detector (Jasco, Tokyo, Japan). The system and data acquisition were controlled by Jasco Borwin Chromatography PC software.

The AFB1 was separated with a reverse-phase C18 Superspher column (four µm particle size, 125 x 4 mm i.d.; Merck, Darmstadt, Germany) at room temperature and isocratic conditions, with a mobile phase of water and acetonitrile:methanol solution (17:29, vol/vol) with a 64:36 (vol/vol) ratio. The flow rate was 1 mL/min. Then, the AFB1 was detected by fluorescence, after post-column dramatization (Jasco 2080 Plus HPLC pump) with pyridinium hydrobromide

perbromide (PBPB) at flow 0.1 mL/min. The fluorescence detector was set at 365nm excitation and 440nm emission wavelengths. The standard stock solution was checked for AFB1 concentration according to A.O.A.C. method 970.44 (AOAC, 1995) and stored at -20°C when not in use.

The AFM1 was separated with a reverse-phase C18 LiChospher 100 column (Merck, Darmstadt, Germany, five μm particle size, 125 x 4 mm I.D.) at room temperature, with a water and acetonitrile (75:25 vol/vol) mobile phase, and the flow rate set at one mL/min. The fluorescence detector was set at 365nm excitation and 440nm emission wavelengths. The standard stock solution was checked for AFM1 concentration according to A.O.A.C. method 970.44 (AOAC, 1995) and stored at -20°C when not in use.

12.3.4 Blood samples

Blood samples were taken before morning meal via jugular venipuncture on day 0 and 10 of the experimental period. The blood was collected into Li-Heparinized (17 U of heparin/mL of blood) Vacutainer (Vacutainer systems, Belliver industrial estate, Plymounth, UK). Then, plasma was obtained by centrifugation (3000 rpm for 15 minutes). The plasma fraction was isolated and stored at -20ºC until analyzed for albumin, globulin, aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT) and bilirubin and Beta-hydroxybutyrate concentrations (Bertoni, 1999).

12.3.5 Carry over calculation

The carry over of AFM1 in milk was calculated as the percentage of the AFB1 consumed that was excreted as AFM1 in milk at the time when the toxin output in milk reached a steady-state.

12.3.6 Statistical Analyses

The AFB1 intake, AFM1 milk concentration and total excretion and the CO in milk were analyzed using the mixed procedure of SAS® (Statistical Analysis System Institute, 2001). A factorial arrangement was used and fixed effect in the model included the milk yield and the milk SCC. The animal, within milk yield and SSC interactions were included as random effects. The day of collection was a repeated measure (compound symmetry covariance structure).

The CO (%) was regressed on milk yield (Kg) over time of collection (day) and the linear equation was calculated.

Plasma parameters (pre-experimental and after AFB1 ingestion period) were compared using the paired t-test (Statistical Analysis System Institute, 2001).

12.4 Results

As expected, the low AFB1 concentration fed to animals did not cause negative health problems during the experimental period. The initial milk yield and the milk SCC for groups were (mean ± SD): 21.2 ± 3.8 and 127600 ± 161203, 21.7 ± 3.9 and 1171889 ± 676859, 41.8 ± 8.4 and 240000 ± 20075, 34.8 ± 4.6 and 2030667 ± 2451213; respectively for LY-LSCC, LY-HSCC, HY-LSCC, HY-HSCC.

The TMR had a base AFB1 content of 3.70 ± 0.21 μg/kg contributing to a bulk milk AFM1 content of 4.80±1.80 and 3.90±1.72 ng/L, respectively before and after the ingestion period.

The AFM1 in milk increased as soon as the first milking after animal ingestion with a pattern of increment up to the observed plateau (between 7th and 12th days of ingestion) as reported in figure 12-1. At day one of AFB1 ingestion the AFM1 contents in milk were 52.9 ng/L vs. 24.9 ng/L and 44.0 ng/L vs. 39.3 ng/L, respectively for the HY-HSCC, HY-LSCC, LY-HSCC and LY-LSCC groups. Closer values among groups were observed from the third day until the end of the ingestion period with average AFM1 contents at the plateau condition of 65.8, 61.9, 66.7 and 59.2 ng/L respectively for the HY-HSCC, HY-LSCC, LY-HSCC and LY-LSCC groups (table 12-2).

The AFB1 CO into milk calculated at the plateau was 2.32, 2.70, 1.48 and 1.29 % of the AFB1 consumed, respectively for the HY-HSCC, HY-LSCC, LY-HSCC and LY-LSCC groups. There was a significant ($P < 0.01$) effect of the milk yield whereas no effect could be attributed to the somatic cell count level or to the milk yield x somatic cell count interaction. Similarly, the main effect of milk was also observed ($P < 0.01$) on the total amount of AFM1 excreted during the AFB1 ingestion period.

Plasma biochemistry for samples collected before and after the aflatoxin ingestion period are reported in table 12-3.

12.5 Discussion

The presence of AFM1 in milk was detectable from the first milking after the animal AFB1 ingestion (figure 12-1) which is in accordance with previous work (Allcroft et al., 1968; Diaz et al., 2004; Trucksess et al., 1983). In particular, in the early stage of increase of the AFM1 plateau (between 7th and 12th days of ingestion) there was an effect ($P < 0.01$) of the SCC. However, the

SCC effect was confined to the high yield groups ($P < 0.01$ and $P < 0.05$, respectively for the 1st and 2nd d of ingestion) due to the milk yield and SCC interaction being separated from the level of production (figure 12-1). Results suggest that high milk yield could intensify the effect of SCC on AFM1 CO. A previous report suggested a positive correlation between SCC and milk AFM1 content in dairy cows fed an AFB1 contaminated diet (Lafont et al., 1983). However, these authors did not report the effect of milk yield in their study.

The observed plateau developed later than in previous studies, in which the steady state for AFM1 was established at 24 (Frobish et al., 1986) and 76 hours (Polan et al., 1974, Diaz et al., 2004) from the initial AFB1 ingestion, but is in agreement with results reported by Battaccone et al. (2003) on sheep, in which the observed plateau condition was between day 9 and 13 from the first AFB1 ingestion. However, milk AFM1 levels in the present study were lower than previously reported maximum concentrations. Previous studies with dairy cows report a plateau at maximum AFM1 concentrations in milk (Frobish et al., 1986; Pettersson et al., 1989; Polan et al., 1974).

The AFB1 is promptly absorbed within the gastro intestinal tract of dairy cows and rapidly transferred as AFM1 into milk (Polan et al., 1974). Milking cows fed a 5 mg AFB1 bolus had detectable blood plasma AFM1 and AFB1 concentrations as soon as 15 minutes from treatment, indicating both a rapid absorption of AFB1 through the rumen wall and metabolism into AFM1 (Moschini et al., 2006). The authors in this study used the retinol palmitate plasma level as a marker for the intestinal adsorption (Bertoni et al., 2001) which indicated a probable AFB1 absorption at rumen level and an intestinal contribution to the AFM1 plasma level 120 minutes after drenching.

The adsorption and consequent transfer to blood and biological fluids is by passive diffusion of the polar component into the liquid phase and by diffusion or active transport of the non polar component into the lipid phase. Because of their low molecular weight (AFB1 = 312.27 and AFM1 = 328.27 formula weight), the toxins are rapidly adsorbed through membranes by a passive mechanism (Yiannikouris and Jouany, 2002). Upon adsorption, the body's ability for AFB1 detoxification is associated with the action of the liver microsomal cytochrome P-450 enzyme family and the enzyme S-glutation-transferase (Galtier, 1999). This system is effective within 7 and 351 µg/head/day (Munksgaard et al., 1987). The level of AFB1 being used in our trial was lower than 80 µg/kg body weight, a threshold value after which a decrease of feed intake was observed in calves (Lynch et al., 1971).

Milk yield was decreased when feeding 100 µg/kg AFB1 (Patterson and Anderson, 1982), and a considerable milk yield reduction was observed in cows fed 100 and 300 µg AFB1/kg body weight (B.W.) (Mertens and Wyatt, 1977). Similar results were obtained by (Applebaum et al., 1982). The level of AFB1 contamination used in our trial (0.16 µg/kg B.W.) was lower than the

indicated threshold value of 100 μg/kg B.W. for milk yield depression and no changes in milk yield pattern were observed during the AFB1 ingestion period, as would be expected (figure 12-2).

Several factors could affect aflatoxin CO. The variability observed among animals could be related to differences in rumen degradation activity (Westlake et al., 1989), difference in rumen biotransformation to aflatoxicol and other metabolites other than AFM1 (Auerbach et al., 1998), differences in term of induction of the enzymatic AFB1 oxidation system (Steiner et al., 1990) and differences in mammary gland permeability (Lafont et al., 1983).

Milk AFM1 content has been previously related to SCC (Lafont et al., 1983). However, more recent work suggests that milk yield is the main factor contributing to the total AFM1 excretion (Pettersson et al., 1989; Veldman et al., 1992). In our trial, the total AFM1 excretion and the CO in milk were affected by the milk yield and not by the SCC during the plateau period (table 12-2). This data suggest a higher AFM1 excretion in high milk yield cows compared to low milk yield cows.

Under the conditions of this experiment, in which all cows received AFB1, and the levels of AFB1 exposure, the relationship between CO (%) and milk yield (kg) can be described by the following (figure 12-3):

CO = - 0.326 + 0.077 x milk yield
RSD = 0.692
R2=0.58

The distribution of the residuals outlines the absence of any bias related to the milk yield on CO estimate. On average, the high milk yielding cows had a 1.81 fold increase in the CO to milk, which is in agreement with previous reported data in early and late lactating dairy cows (Lafont et al., 1983; Veldman et al., 1992). The inappropriate implementation of equations relating CO and milk yield could lead to erroneous conclusions in terms of maximum AFB1 daily intake to comply with the EU limit of AFM1 in milk. For instance, the plotting of the estimated CO obtained when applying the Veldman et al. (1992) equation to our milk yield against values calculated with our equation clearly indicate the presence of factors not accounted for (source of contamination, animal variability, etc..) in both equations which limits the equations to their respective trials for CO estimates (figure 12-4). A simple steady-state model has been proposed for the disposition of AFB1 and AFM1 in the lactating cow (Van Eijkeren et al., 2006). The model attempts to better define kinetics of carry over of AFB1 in feed to AFM1 in milk. However, the application of the model to our data did not fit: while daily intake of AFB1 was similar among different milk yielding groups

(LY, HY), the calculated AFM1 concentrations were 1.3 and 0.9-folds the corresponding observed mean levels, respectively for the LY and HY groups.

Mastitis as measured by high SCC could cause disruption of the tight junction of alveolar cell membranes in the mammary gland. Because of this reduction in the integrity of the blood-udder barrier, an influx of pro-inflammatory factors might further disrupt the tight junction and increased blood-udder permeability (Davis et al., 1999). Similar results were observed in sheep where distended udders were related to the pro-inflammatory factors have been found in the milk of sheep under similar circumstances (Colditz, 1988). Furthermore, anti-inflammatory factors from hyper-immunised cows reduced the cell membrane tight junction permeability (Stelwagen et al., 1997).

The animal arrangement for the SCC content in our trial obtained groups (LY-LSCC, HY-LSCC) with average SCC below or slightly over 100000/mL, which is considered a threshold value for a healthy udder (Steiner et al., 1990; Walstra and Jenness, 1984), and groups considerably higher in SCC (LY-HSCC, HY-HSCC) in which the integrity of tight junction were probably damaged allowing leaking of blood and milk components (Bruckmaier et al., 2004).

Thus, factors affecting the permeability of the blood-udder barrier, together with the low AFB1 molecular weight (312.27 formula weight), could regulate the excretion of AFM1 into milk, particularly in high producing dairy cows. From our data the increased mammary gland permeability as a consequence of inflammatory processes alone does not seem to explain the increase of the CO (table 12-2).

As previously reported (Diaz et al., 2004; Frobish et al., 1986), the AFM1 clearance at the end of the AFB1 ingestion period was fast, bringing the AFM1 below the legislative limit (50 ng/L) within 24 hours (all groups) and lower than 15ng/L (low somatic cells groups) within 48 hours from last day of ingestion.

The AFB1 ingestion period did induce changes in some of the evaluated plasma parameters (table 12-3). Even though the change in the Gamma-glutamyl transferase might suggest a damage of the liver at cellular level, the values for cows in this study were within the upper limit for cows at their stage of lactation (Bertoni et al., 2000).

12.6 Conclusions

The current data suggest that milk yield is the major factor affecting the total excretion of AFM1. In this study the CO calculated from a predictive equation was lower than previously reported for similar levels of AFB1 intake, although not outside the range. Previously reported differences in CO associated to membrane permeability due to inflammatory factors was only

detectable during the first days of AFB1 ingestion and only occurred in the high yielding cows on experiment. It is possible that the low molecular weight of aflatoxins could account for the absence of the SCC effect at plateau conditions.

Table 12-1. Ingredients and chemical composition of the basal diet

	Base diet
Ingredients (g/kg dry matter)	
Corn silage	312
Alfalfa hay, dehydrate	167
Grass hay	41
Cotton seed, whole with lint	85
Corn meal	183
Barley meal	66
Protein supplement[1]	103
Calcium soap[2]	9
Soybean meal	34
Chemical composition (g/kg dry matter)	
Crude Protein	162
Crude lipids	48.6
ADF[3]	204
NDF[4]	340
Calculated	
PeNDF[5]	266.8
NSC[6]	411.8
Forage (%)	52
Net energy lactation (MJ[7]/kg DM)	7.08

[1]Contains per kg of premix: Soybean meal 600 g, sunflower meal 300 g, mineral and vitamin supplement 100 g.; 120000 IU of Vitamin A; 9000 IU of Vitamin D3; 90 mg of Vitamin E; 3.6 mg of Co; 19.2 mg of I; 1.44 mg of Se; 600 mg of Mn; 62.4 mg of Cu; 2240 mg of Zn; 1.92 mg of Mo; 360 mg of Fe.

[2]Megalac;

[3]expressed without residual ash.

[4]according to Van Soest et al. (1991) without sodium sulfite and with alpha-amylase; expressed without residual ash.

[5]PeNDF: Physical effective neutral detergent fiber (Mertens, 1997), calculated according to the contribution of the single feed present into the diet (concentrates were considered with PeNDF=0; whole cotton seeds PeNDF=70)

[6] Calculate as NSC= 100-(NDF+ASH+Crude protein+Crude Lipids)

[7]according to NRC, 2001

Table 12-2. AFB1 intake (µg), AFM1 milk concentration (ng/L) and total excretion (µg/cow/day) and carry-over (%) at plateau (7th – 12th day on AFB1 ingestion)

Item	Groups				s.e.	Main effects ($P <$)		
	HY-HSCC	HY-LSCC	LY-HSCC	LY-LSCC		Milk Yield	SCC	Milk Yield x SCC
AFB1 intake (µg)	98.0	98.3	98.0	98.1	0.064	0.455	0.007	0.141
AFM1 (ng/L)	65.8	61.9	66.7	59.2	4.908	0.915	0.500	0.832
Total AFM1 excreted (µg/cow/day)	2.27	2.66	1.45	1.27	0.177	0.001	0.756	0.360
Carry over (%)	2.32	2.70	1.48	1.29	0.180	0.001	0.767	0.366

Table 12-3. Blood parameters for cows with low somatic cell counts (LY-LSCC & HY-LSCC) before and after AFB1 ingestion

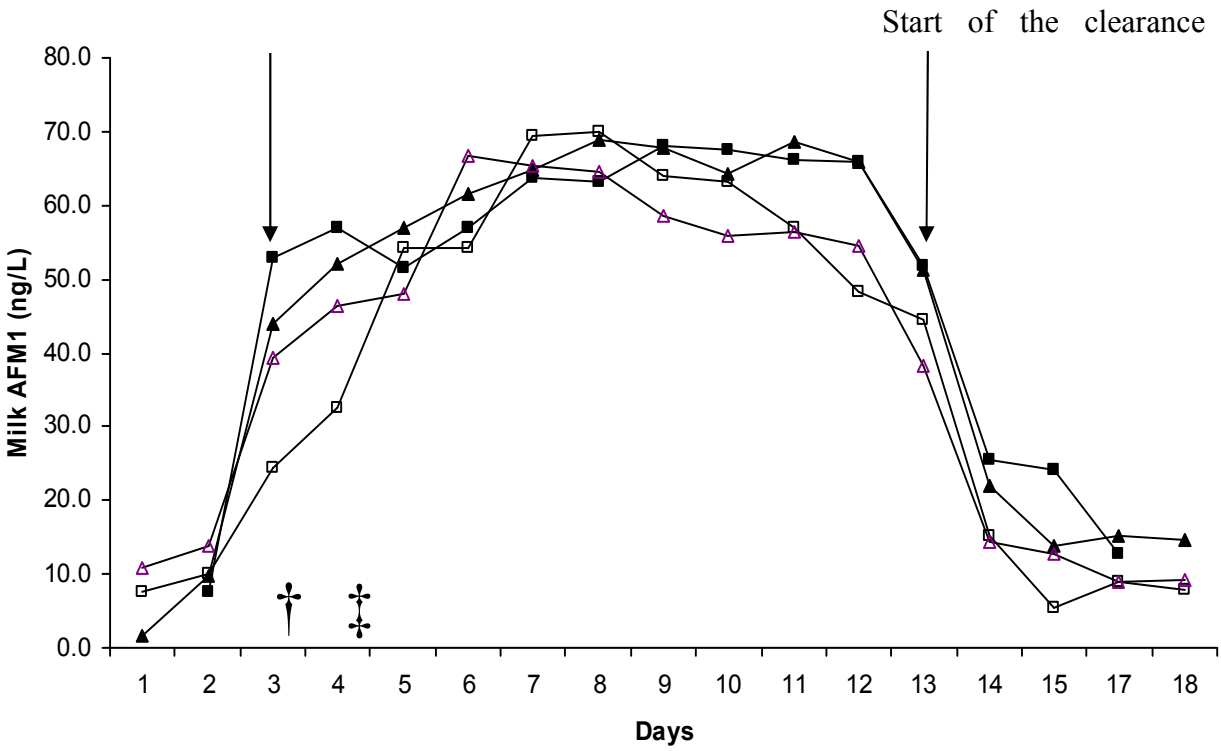
Parameter	Before ingestion	After ingestion	s.e.	P[1]	Range[2]
Albumin (g/L)	33.75	37.50	0.871	0.0012	32.3-35.9
Globulin (g/L)	33.83	38.50	1.469	0.0088	38.3-57.6
Aspartate aminotrasnferase (U/L)	63.17	68.25	3.903	0.2194	61.1-103
Gamma-glutamyl transferase (U/L)	26.58	29.58	0.739	0.0019	20.8-45.1
Bilirubin (µmol/L)	2.78	2.56	0.272	0.4261	1.63-4.58
Beta-hydroxybutyrate (mmol/L)	0.51	0.46	0.082	0.5357	0.16-0.75

[1]Cows consuming a 300g bolus containing 1.004±0.03 g/cow per day of a naturally contaminated corn meal. This allowed for an AFB1 intake from the contaminated bolus of 98.10±0.26 µg/cow per day

[2]For difference being different than zero

[3] Bertoni, G., 1999

Figure 12-1 AFM1 concentration in the milk of cows from different factorial arrangement: (■) HYHSCC, (□) HYLSCC, (▲) LYHSCC, (Δ) LYLSCC



†Somatic cells count effect ($P < 0.01$). Production and somatic cells count interaction effect sliced for the level of production significant ($P < 0.01$) in the HY group

‡Somatic cells count effect ($P < 0.05$). Production and somatic cells count interaction effect sliced for the level of production significant ($P < 0.05$) in the HY group

Figure 12-2. Milk yield of cows from different factorial arrangement: (■) HYHSCC, (□) HYLSCC, (▲) LYHSCC, (Δ) LYLSCC

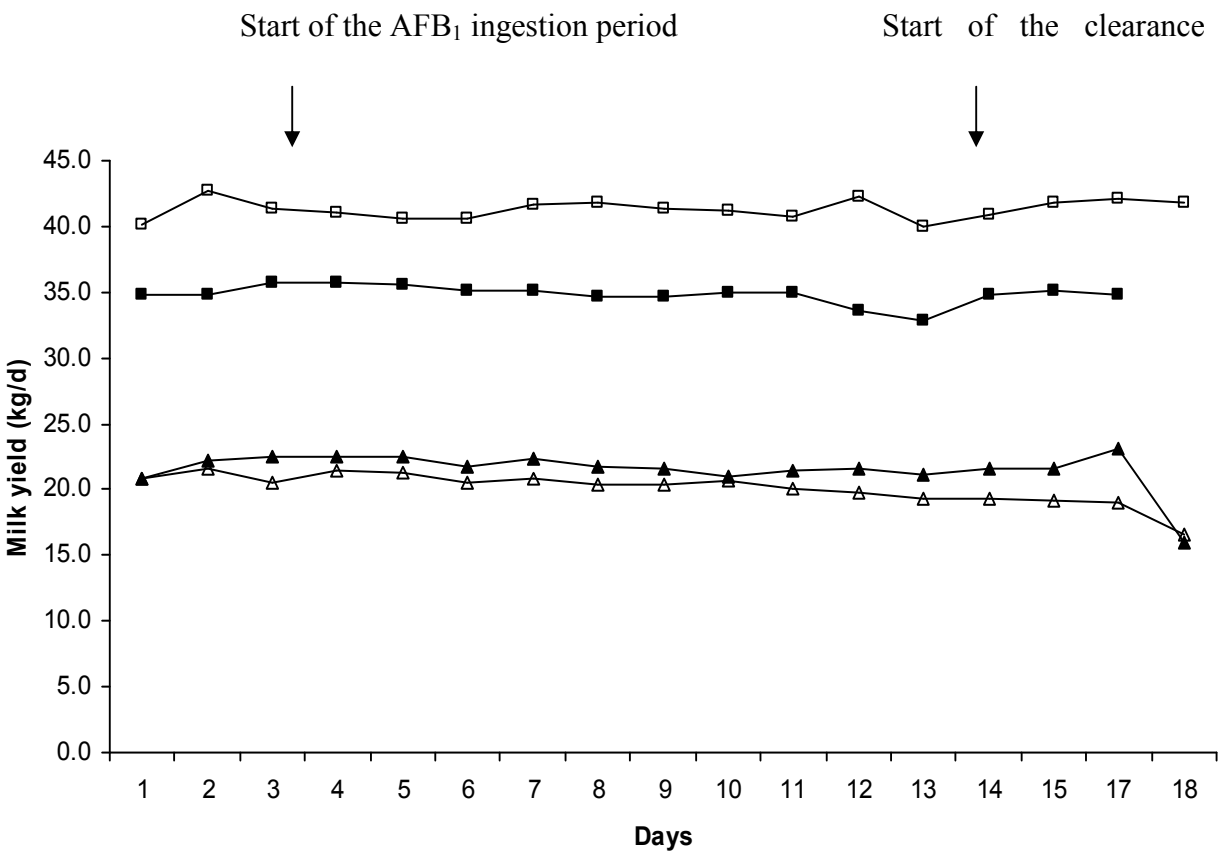


Figure 12-3. Plot of observed (■) and residuals (□) for carry-over versus milk yield (kg)

carry-over = - 0.3255 + 0.0769 * milk yield (RSD = 0.692, r2=0.58)

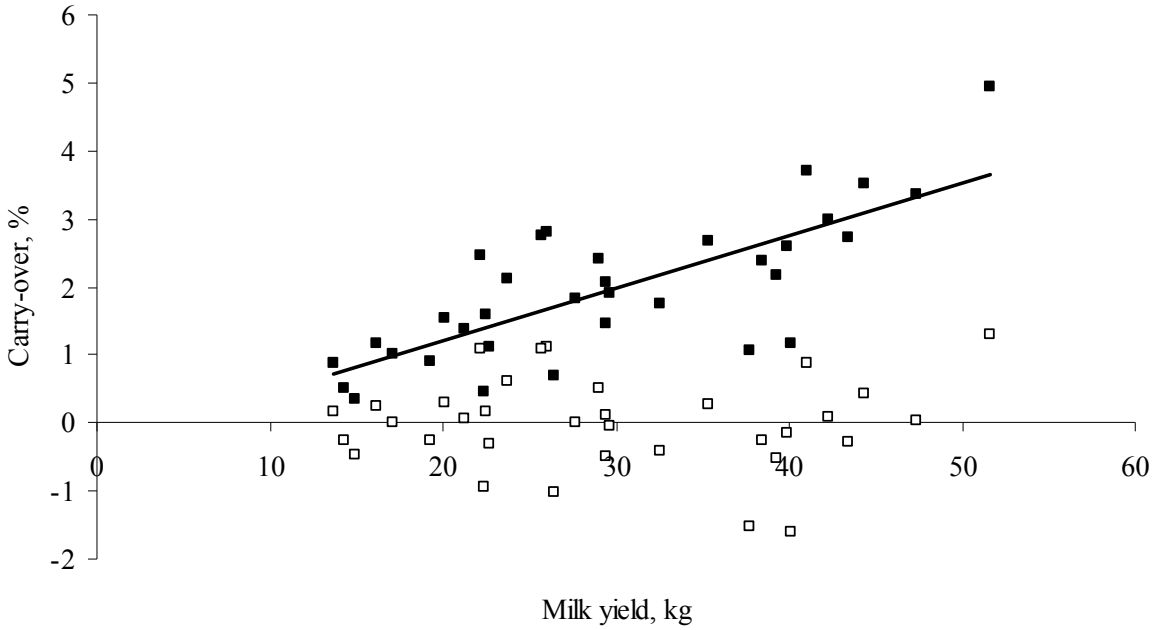


Figure 12-4. Plot of the predicted carry-over according to the obtained equation versus the predicted carry-over as proposed by Veldman et al. (1992)