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Research Article

Reduction of Aflatoxin M1 Levels during Ethiopian Traditional Fermented Milk *(Ergo)* Production

Tsige Shigute and Alemayehu P. Washe [ID]

Department of Chemistry, Hawassa University, P.O. Box 05, Hawassa, Ethiopia

Correspondence should be addressed to Alemayehu P. Washe; alemayehup@yahoo.com

In this study, the reduction of aflatoxin M1 (AFM1) levels during lab-scale *ergo* production was investigated through determination of the residual levels of AFM1 using Enzyme Linked Immunosorbent Assay. The results showed gradual and incubation time dependent reduction of AFM1 level in the raw milk samples being fermented to *ergo*. The maximum reductions of 57.33 and 54.04% were recorded in AFM1 in natural and LAB inoculums initiated fermentations, respectively, in 5 days of incubation. Although a significant difference ($P = 0.05$) in the AFM1 decrease in the two types of fermentations was recorded, such findings could vary with milk samples depending on initial load of the microorganisms as determined by hygienic conditions. However, the level of AFM1 in control (sterilized) samples showed only a 5.5% decrease during the entire period of incubation. Microbiological investigation showed increasing LAB counts with incubation time. A gradual decrease in pH of the milk samples was observed during fermentation. Considering the fact that both viable and dead bacterial cells could remove AFM1 during *ergo* production, the mechanism is proposed as predominantly involving noncovalent binding of the toxin with the chemical components of the bacterial cell wall.

1. Introduction

Aflatoxins (AFs) are toxic secondary metabolites elaborated by some *Aspergillus* fungi [1]. About 20 AFs that belong to a large group of toxic compounds called difuranocoumarins have been identified. However, only four aflatoxins (AFB1, AFG1, AFB2, and AFG2) have been recognized as the main naturally occurring food contaminants [2]. AFs are among the most serious and well known naturally occurring toxins in food and feed commodities with AFB1 being the most toxic and carcinogenic [3]. Cows that consume AFB1-contaminated feed can biochemically convert the toxin into 4-hydroxy derivative, aflatoxin M1 (AFM1), which is excreted in milk. The International Agency for Research on Cancer has classified AFM1 as belonging to group 1 carcinogen to humans [4]. Although AFM1 is about ten times less toxigenic than AFB1, several studies have indicated health issues associated with AFM1 contamination of milk and milk products. This is because in many countries, every age group regularly consumes these products in their daily diet [5, 6].

Furthermore, the toxin may subsequently contaminate other dairy products including cheese and yogurt and may generate health concerns for consumers. For these reasons, different organizations have set limits on AFM1 in milk and other dairy products. The Commission Regulation of the European Union (EU) No. 165/2010 has set the maximum level of 0.05μg/L for AFM1 in milk [7]. The Food and Drug Administration in the USA (USFDA) has also set maximum level of AFM1 in milk to be 0.5μg/L [8].

Risk of human exposure to AFM1 contamination of milk is a major concern in Ethiopia where dairy farmers commonly use different mixed concentrate feeds containing traditional brewery by-product ("atela"), wheat bran, noug (*Guizotia abyssinica)* cake, maize grains, and silage to increase production. However, these feeds are susceptible to contamination with AFB1 [9, 10]. A recent survey by Gizachew et al. (2016) indicated that out of a total of 156 feed samples collected from Addis Ababa and its surrounding cities, only 16 (10.2%) contained AFB1 at a level less than or equal to 10μg/kg, and all the milk samples collected from the study

area were contaminated with AFM1 from lower (0.028 μg/L) to higher (4.98 μg/L) levels. The same authors also reported that 93% of the milk samples in the area exceeded the limit of 0.05 mg/L set by the EU [7]. The problem is exacerbated by, *inter alia,* lack of awareness about AFs and the risks associated with them in the value-chain actors including farmers, traders, and consumers. Farmers often feed left-over moldy grains to livestock. Consequently, humans are exposed since the toxins or their biotransformation products accumulate in the dairy products. That is why the Comprehensive Africa Agriculture Development Programme (CAADP) recently set AFs as a high priority research area, establishing the Partnership for Aflatoxin Control in Africa (PACA) [11].

The scientific methods reported to date on AF control have focused on three approaches: prevention of contamination of food and feed by the fungi that elaborate the toxins (mainly *Aspergillus flavus and Aspergillus parasiticus*), decontamination (removal or detoxification of the toxins), and inhibition of AF absorption in the gastrointestinal tract. Although preventing fungal contamination of food and feed commodities can be considered as the most rational approach, its implementation is difficult in tropical areas where favorable environmental and climatic conditions promote the fungal growth [12]. In addition, AFs are extremely durable and unavoidable under most conditions of storage, handling, and processing of foods or feeds [13]. In this context, decontamination of products through detoxification or reduction of the toxin is the most promising route.

Various physical, chemical, and biological methods have been proposed for the decontamination of AFs in food and feed commodities through elimination, inactivation, or reduction of the levels [14, 15]. Physical methods are usually more expensive and may produce undesirable changes in foods. Sometimes it is impossible to heat foods at over 100°C to reduce AFs level [16]. Despite promising results of the use of chemicals on reduction of AFs in food, they usually produce toxic residues and cause changes in nutritional, sensory (the texture, taste, aroma, color), and functional properties of food [16, 17]. Biological control is a promising approach for reducing AF contamination in food commodities. Different strains of lactic acid bacteria inoculums were used to reduce the AFM1 level in yogurt samples [18]. This study showed the highest reduction percentage in AFM1 by certain species of LAB at the end of the storage period. Strains of probiotic bacteria were also used for the reduction of AFM1 in milk in an in vitro digestive model where up to 25.43% reduction was reported [19]. Though the use of defined starter cultures to initiate fermentation and thereby reduce AF is effective, it is difficult to implement in developing countries like Ethiopia where dairy production is dominated by traditional methods [20, 21]. One of the Ethiopian traditional dairy products commonly consumed is *ergo*.

Ergo is a naturally fermented dairy product with more or less the same characteristics to yogurt. Generally, it constitutes a sour milk product with a semisolid liquid state and has a pleasant odor, aroma, and taste. If carefully prepared, it has a smooth and thick uniform white milk appearance and can be stored for 15–20 days depending on the storage temperature. It is usually made from raw cow's milk under ambient conditions in smoked clay based containers [22]. When the ambient is cold (below ~20°C), raw milk is usually kept in a warmer place to ferment. Although, a well-smoked container is often used for milking and storage of milk, this is less effective in eliminating bacteria and some levels of LAB always remain on the porous walls of the clay based container [21]. These LAB species play key role in the fermentation process and contribute a lot to the quality of *ergo*. Due to undefined fermentation conditions, the types of bacteria and load in *ergo* could vary from sample to sample, but it is reported that lactic acid bacteria (LAB) are the predominant and ubiquitous species in *ergo* samples [21]. Therefore, it is valid to consider the effect of the LABs that are naturally developed in food products on the level of AFM1 in milk.

In this study, the reduction of AFM1 levels in a purposively collected cow's milk samples was investigated during lab-scale traditional fermentation of the milk into *ergo*. The residual levels of AFM1 were monitored using Enzyme Linked Immunosorbent Assay (ELISA). Method validation was carried out using the percentage of recovery and coefficient of variation (% CV). The findings of the current study are expected to add value to the indigenous knowledge on food preservation and preparation and aid in the development of community based aflatoxin control strategy, particularly in developing countries.

2. Materials and Methods

2.1. Apparatus and Chemicals. All the reagents and chemicals used in this study were of analytical grades and purchased from Sigma Aldrich. These reagents and chemicals include AFM1 standard, horseradish peroxidases, PBS-Tween20, tetramethylbenzidine (TMB), stop solution, and solvents (acetonitrile, anhydrous diethyl ether, methanol, and acetone). Deionized water obtained with a Milli-Q PLUS (Millipore Corporation) was used for the preparation of solutions. AFM1 standard was obtained from Sigma (St. Louis, MO, USA). Thin Layer Chromatography (TLC) was performed on a Kieselgel 60 F$_{254}$ (0.20 mm) plates (E. Merck), and the fluorescing spots were visualized under UV light (365 nm). UV-Vis spectrophotometer, coded CECIL Instrument (121–789), wavelength range 200–1100 nm, wavelength accuracy $\pm$ 0.5 nm, and wavelength precision $\pm$ 0.1 nm, supplied by Cambridge England, equipped with deuterium lamp, was used for visualizing of the TLC spots. The optical density (OD) was measured at 450 nm using a microplate reader (BioTek Instruments, Inc., USA). MRS and M17 agar (pH 6.2–6.6) and Rogosa agar (pH 5.2–5.6) were used for total LAB enumeration as well as *Lactobacilli* and *Lactococci* isolation, while YGLA (pH 7.0) was used for the isolation of *Streptococci* of LAB origin. Blood agar was used for hemolytic tests of *Streptococci*.

2.2. Study Design. Purposive sampling strategy was applied during collection of milk samples from local dairy farmers near Hawassa city (Hawassa, Ethiopia). Only AFM1 contaminated milk samples were sought for the study purpose. To this end, identification of farmers who use concentrate feeds containing wheat bran, noug cake, and moldy maize

$$\text{Residual levels of AFM1} = \text{ELISA}^{**} - \text{ELISA}^{***}$$

*ELISA carried out to determine samples that contain higher loads of AFM1 for AFM1 reduction study

** and *** indicate how the residual levels of AFM1 were calculated as shown in the equation at the
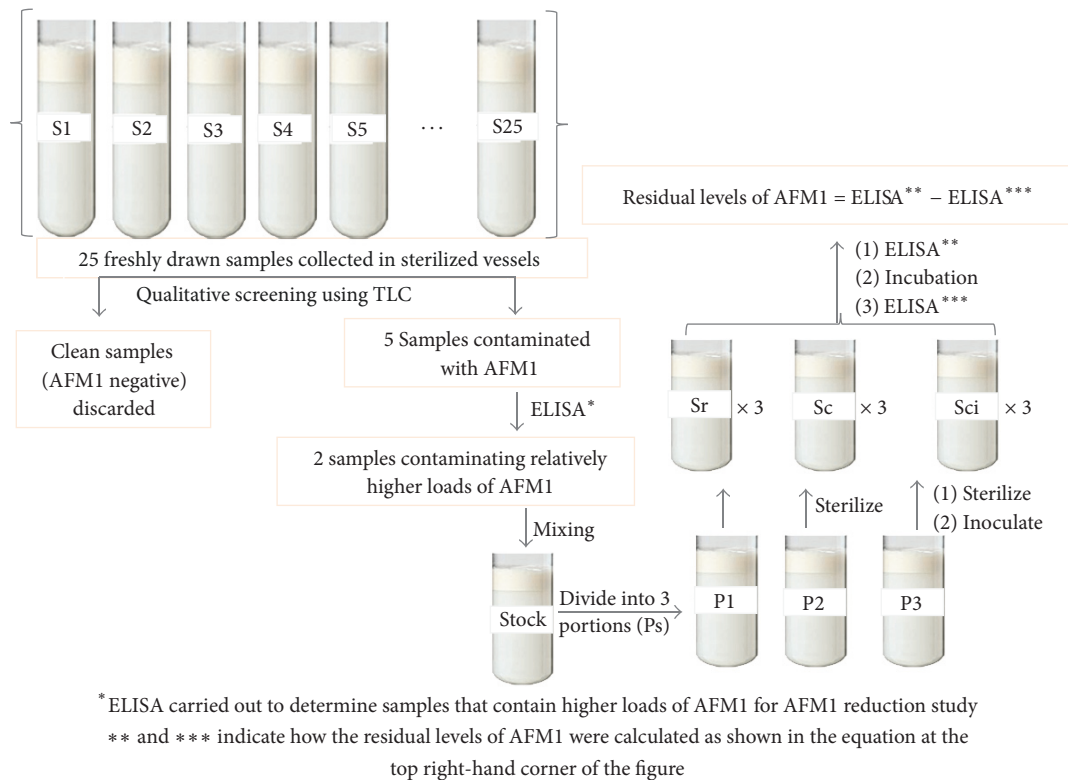
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FIGURE 1: Schematic of preparation and treatment of milk samples (Sr, Sc, and Sci) for AFM1 reduction during incubation at *ergo* fermentation condition.

grains that are susceptible to AFB1 (precursor of AFM1) contamination was made [10]. Milk samples were collected in sterilized bottles from these farmers and qualitatively screened using TLC against AFs standards for the presence or absence of AFM1. Only AFM1 contaminated milk samples were considered for further investigation. The levels of AFM1 in these samples were then quantified using sensitive ELISA. Samples containing relatively higher levels of AFM1 were mixed and homogenized to get a stock sample. The stock sample was then divided into three portions for treatment. The first portion (Sr) was directly (without sterilization) analyzed using ELISA to quantify the initial load of AFM1. The second portion (Sc) was first sterilized along with its vessel (Erlenmeyer flask), and then initial load of AFM1 was determined. The third portion was also sterilized along with its vessel (Erlenmeyer flask), analyzed for initial level of AFM1, and then inoculated with stock culture of LAB inoculums, thus considered as a positive control (Sci). Only Sr was transferred into unsterilized clay based pots (obtained from a woman in the study area) previously used for *ergo* fermentation. Finally, all the three samples (Sr, Sc, and Sci) were subjected to the traditional *ergo* fermentation conditions (ambient: 20–30°C) for five consecutive days [23]. The residual levels of AFM1 in the three samples were determined every 24 hours in triplicate using ELISA. The percentage (%) of reduction in AFM1 levels during incubation at *ergo* fermentation conditions is reported. The schematic of preparation of the three treatment groups is shown in Figure 1.

2.3. Collection and Preparation of Milk Samples.

To ensure that AFM1 levels could be detected among the aliquots taken during milk fermentation into *ergo*, a prior selection of farmers was made based on the type of feed provided to the cows. Out of 15 dairy farmers in the study area (near Arogie Gebeya, Hawassa, Ethiopia), only five farmers were purposively selected. Then a total of 25 (5 × 5) freshly harvested cow's milk samples, 1/2 litre each in sterilized bottles, was collected from each of the selected farmers. A volume of 250 ml was taken from each sample in sterilized bottles. The samples were kept in icebox and transported to Hawassa University Chemistry laboratory. In addition to the milk samples three smoked clay based containers used for *ergo* fermentation were collected from volunteer women in the study area.

2.4. Qualitative Screening Using TLC.

The extraction procedure of AFM1 from milk samples was carried out following the Horwitz [24] procedure with slight modification. Briefly, 50 ml of each of the milk samples was centrifuged at 10°C for 5 min with 4,000 rpm. After discarding the upper cream layer, the lower phases were used for qualitative testing with TLC. The sample was transferred into a 250 ml separating funnel and blended with 120 ml of methanol-water mixture of 2 : 1 proportion. Then 10.0 ml of 10% sodium chloride solution followed by 25 ml of n-hexane was added, and the mixture was shaken for 1 min. Phases were allowed to separate. The lower phase (aqueous) was drained into a second 250 ml

separating funnel, while the upper phase (organic) was discarded. The aqueous layer was then extracted three times with a total of 120 ml chloroform. The chloroform layer was collected after passing through a bedrock of anhydrous sodium sulphate to dry the mixture and consequently evaporated to the residual volume of 0.5 ml. Aliquots of aflatoxin AFM1 standard ($10 \, \mu M \cdot L^{-1}$) and sample extracts of same volume were spotted side by side on the start line of precoated TLC. The plates were developed in a development tank saturated with solvent (diethyl ether-methanol-water in the ratio 96 : 3 : 1). The plates were then removed and allowed to dry at ambient and visualized under long wave light (366 nm) to determine presence or absence of AFM1 in the samples.

2.5. ELISA Assay Procedure. ELISA was carried out according to the protocol provided by the kit manufacturer (Helica 2000 M1 ELISA kit). The pouch of the kit was unsealed, and the required numbers of wells were taken for standards and samples to be tested. Using a fresh pipette tip for each, $200 \, \mu L$ aliquots of standards and samples or assay diluents ($20 \, \mu L$ milk + $180 \, \mu L$ of 35% methanol), kept at room temperature, were dispensed into the appropriate mixing wells in triplicate. Then, $100 \, \mu l$ solutions from the mixing wells were transferred to the assay wells precoated with AFM1 antibodies and incubated for 10 min at room temperature in the dark. Next, $100 \, \mu L$ horseradish peroxidase as a conjugate to AFM1 was added and incubation continued for further 30 min. When the incubation was complete, the wells were drained and washed with PBS-Tween 20 buffer solution three times. Residual wash buffer was removed by tapping the drained wells face down on a layer of absorbent. After addition of $100 \, \mu L$ of TMB as enzyme substrate into each well and incubation for 10 min at room temperature, $100 \, \mu L$ of the stop solution was added to the microplate wells, which changed the color from blue to yellow. The optical density (OD) was recorded at 450 nm using a microplate reader (BioTek Instruments, Inc., USA). Calibration curve was produced using AFM1 standard concentrations in the range of 0.1–2.0 μg/L. Each test sample was ten times diluted and analyzed under the conditions used to produce the calibration curve.

2.6. Microbiological Screening of Milk Samples. Microbiological examination (enumeration, isolation, and characterization) of the milk samples was carried out according to the assay procedure previously reported by Azhari (2011) [25] and Khalil and Anwar (2016) [26]. Briefly, 10 mL of each of the milk samples was homogenized with 90 mL of sterile saline (0.85% NaCl) solution to make an initial dilution (10^{-1}). Serial dilutions up to 10^{-7} were made in duplicate. After vortexing, $100 \, \mu L$ of the most dilute of each of the milk samples was spread-plated on selective media and incubated anaerobically using the Gas Pack system (Merck Anaerocult type A) at 42, 35, and 30°C for 3 days, in order to provide optimum conditions for the growth of thermophilic *Lactobacilli*, mesophilic *Lactobacilli*, and *Leuconostoc*, respectively. *Lactococci* were enumerated on M17 agar plates after aerobic incubation of the inoculated milk at 30°C for 2 days. The total counts were then performed in the highest dilution

(10^{-7}) and the counts in other samples were determined after correction for dilutions. The colony forming unit (cfu) per gm or mL was calculated by multiplying the average number of colonies with the reciprocal of dilution factor. Maintenance and activation of the bacteria were performed in respective media at 4°C and 37°C, respectively.

2.6.1. Grouping and Identification of LAB Isolates. Selection and grouping of the isolates were performed on the basis of their colony morphology in media plates and slants, Gram staining, cell morphology, catalase activity, spore formation, and motility test as described in Bergey's Manual of Determinative Bacteriology (8th edition) and reported by other researchers [25–30]. Biochemical tests such as CO_2 gas from glucose in Gibson's semisolid tomato juice medium, gas from citrate in semisolid citrated milk agar, NH_3 from arginine, and gelatin liquefaction were carried out during the differentiation [29]. Arginine dihydrolase agar and Aesculin Azide agar (Merck, Germany) were employed to perform the hydrolysis tests. Evaluation of citrate utilization was carried out using citrate and MR-VP agars (Merck, Germany). Further characterization of the isolates was carried out based on acid and gas production from glucose fermentation, growth at different conditions (temperatures, pH, NaCl concentrations), carbohydrates fermentation profiles, and hemolytic tests on blood agar [25, 26].

2.7. Determination of pH and Titratable Acid. The pH of the milk samples was measured using a digital pH meter. Percent titratable acidity, as lactic acid, was determined by titrating a volume of 1 ml of the milk sample with 0.1 M NaOH as reported by Eckles et al. 1951 [31].

2.8. Preparation of Strains Inoculums. Stock culture of LAB inoculums consisting of *Lactobacillus plantarum, Lactobacillus acidophilus, Lactobacillus brevis, Lactobacillus casei* subsp. *casei, Lactobacillus helveticus, Streptococcus faecalis, Streptococcus thermophilus,* and *Leuconostoc mesenteroides* subsp. *cremoris* previously isolated from *ergo* milk samples were kindly donated by Department of Biological Sciences (Dilla University (DU), Ethiopia). The obtained samples were thawed at room temperature and subsequently diluted to 10^4 to 10^6 with sterilized saline (0.85% NaCl). Then, 50 mL of Sci was inoculated with the stock culture of LAB (individual performance not evaluated) and incubated for the required period of fermentation at ambient as a positive control.

2.9. Statistical Analysis. All measurements and assays in this study were carried out in triplicate. Statistical analysis of the data was carried out using student t-test to test whether there is significant difference ($P = 0.05$) between the reduction in the level of AFM1 in the two treatments (Sr and Sc) vis-à-vis the presence or absence of LAB.

3. Results and Discussion

3.1. Enumeration and Characterization of LAB from Ergo. LAB species in the milk samples were identified based on cell and colony morphology, Gram and catalase reactions

TABLE 1: Physiological and biochemical characteristics of the LAB isolates from *ergo*.

Characteristics	*Lactobacillus*				*Streptococcus*		*Lactococcus*
	Lb1	Lb2	Lb3	Lb4	St1	St2	Lc1
Cell morphology	Rods	Rods	Rods	Rods	Cocci	Cocci	Cocci
Gram stain reaction	+	+	+	+	+	+	+
Catalase activity	−	−	−	−	−	−	−
Spore formation	−	−	−	−	−	−	−
Glucose fermentation	+	+	+	−	+	+	+
NH_3 from arginine	−	−	+	−	−	+	−
CO_2 in Gibson medium	−	+	−	+	−	+	−
Citrate hydrolysis	−	+	−	−	−	+	−
Growth at temperature °C							
4	−	−	−	−	−	−	−
10	−	−	−	−	−	+	+
37	+	+	+	+	+	+	−
45	+	+	+	+	+	−	−
Heat tolerance							
55–60	−	−	+	+	+	−	−
Growth at pH							
4.5	−	+	−	−	+	+	−
6.5	+	+	+	+	+	+	+
Growth at NaCl (%)							
2	+	+	+	+	+	−	+
4	+	+	+	−	+	+	−
6	−	+	+	−	+	+	−
Carbohydrate fermentation:							
Fructose	+	+	+	+	+	+	−
Galactose	+	+	−	−	+	+	−
Glucose	+	+	+	−	+	+	+
Lactose	+	+	−	+	+	+	+
Mannose	+	+	−	−	+	+	−
Sucrose	+	+	+	−	+	+	−
Sorbitol	+	−	−	−	−	+	−
Xylose	+	−	−	−	+	+	−

Lactobacillus: Lactobacillus plantarum (Lb1)*, Lactobacillus acidophilus* (Lb2)*, Lactobacillus leichmannii* (Lb3)*, Lactobacillus delbrueckii ssp. bulgaricus* (Lb4)*,
Streptococcus: Streptococcus thermophilus* (st1)*, Streptococcus lactis* (st2)*, and Lactococcus: Lactococcus lactic ssp. cremoris* (Lc1).

when grown on MRS growth media. Gram positive, catalase negative, cocci, coccobacilli, or rod shaped nonspore forming, nonmotile isolates with characteristic cell arrangements were considered as lactic acid bacteria [32]. Though the LAB species in the inoculums culture were known as per the information provided by the supplier, it was important to carry out the total LAB expressed as cfu per mL of the inoculated milk samples (Sci) so that the AFM1 reduction in the two samples (Sr and Sci) can be compared. The total LAB counts in Sr and Sci were 8.57 and 9.75 log(cfu·mL^{-1}), respectively. No viable LAB species were detected in the sterilized sample (Sc). Isolation, grouping, and characterization of the LAB isolates from Sr were carried out based on morphological, biochemical, and physiochemical characteristics [25–30]. The result is summarized in Table 1. Isolates observed as short to long rods in pairs or chains, homo/heterofermentative, grown at 5 to 45°C and with NaCl were grouped as *Lactobacillus*.

Gram positive, catalase negative cocci occurring in pairs or chains were grouped as *Streptococcus*. Cocci observed in pairs or chains displaying homofermentative characteristics, grown at 5–10°C but not at 45°C, and not grown in presence of NaCl, were identified as *Lactococci*. Seven LAB species belonging to three genera (*Lactobacilli*, *Streptococci*, and *Lactococci*) were isolated from Sr. These LAB species included 4 (57%) *Lactobacilli* (*Lactobacillus leichmannii, Lactobacillus acidophilus, Lactobacillus plantarum*, and *Lactobacillus delbrueckii* ssp. *bulgaricus*), 2 (29%) *Streptococci* (*Streptococcus thermophilus*, and *Streptococcus lactis*), and 1 (14 %) *Lactococci* (*Lactococcus lactic* ssp. *cremoris*). The results are in agreement with the previous reports and the fact that LAB is among the most predominantly encountered microorganisms in dairy products causing milk to sour naturally. It is also reported that *ergo* fermented in presmoked traditional vessels contained LABs belonging to the genera *Lactobacillus, Pediococcus,*

Enterococcus, *Streptococcus*, *Leuconostoc*, and *Lactococcus* as predominant microbes [21]. The dominance of *Lactobacilli* species in the current sample is also consistent with the observations reported by other researchers who worked on traditionally fermented milk [21–23, 33]. For instance, Eyassu et al. (2012) [33] reported that *Lactobacillus* species isolated from ititu (fermented camel milk) was the dominant genus which comprised about 58% of the total LAB isolates followed by *Lactococcus* species which accounted for 25%. The variation in the type and number of the lactic acid bacteria isolated from current milk sample and other milk samples previously reported could be attributed to the traditional practices (like smoking), the handling (hygienic quality), and organoleptic property of the fermented milk.

3.2. Calibration Curve and Method Validation.

Standard concentrations of AFM1 in the range of 0.1–2.0 $\mu g \cdot L^{-1}$ were used to construct the calibration curve. Linearity of OD percentage with a linear regression equation of $y = -0.266x + 1.6$ and R^2 value of 1 is observed in the suggested concentration range. The validation of ELISA data was then carried with determination of percentage of recoveries and coefficient of variation (CV percentage). The recoveries were recorded by spiking milk samples with known amounts of AFM1 standard and determining the recovered amount. Simultaneously, CV percentage corresponding to triplicate measurements were tabulated. The recorded recovery percentage ranged between 72 and 89.1% with CV percentage of 5.2%. The recovered values were found to increase with increasing homogenization by vortexing the samples before measurement. The improvement in sensitivity of the measurement with homogenization indicated possible matrix effect that could interfere with the binding event. Nevertheless, the obtained recoveries and CV percentage were consistent with the guidelines for analysis of aflatoxins (Commission of the European Communities, 2006). According to the regulation, the recommended value of recovery for concentrations of aflatoxins from 0.01–0.05 $\mu g/kg$ must be of 60 to 120% and for higher concentrations (>0.05 $\mu g/kg$) must be of 70 to 110%.

3.3. Quantification of Aflatoxin M1 in Milk Samples.

Determination of the levels of AFM1 in the milk samples was carried out for two purposes: to identify samples with higher loads of AFM1 so that the levels could be detected in the aliquots taken during the reduction study and to determine initial levels of the toxin at the onset of fermentation so that the percentage reduction can be calculated. Therefore, the samples were first qualitatively screened for the presence of AFM1 using TLC. TLC results showed that 5 out of 25 samples were contaminated with AFM1. Quantification of AFM1 in the contaminated samples was then carried out using ELISA. AFM1 levels ranging from 1.47 to 5.27 $\mu g \cdot L^{-1}$ were detected in the five contaminated milk samples (Figure 2). All of the contaminated samples contained AFM1 above the limit level. This could be due to the purposive sampling strategy implemented as described in Section 2.3. To ensure that aflatoxin M1 levels could be detected among the aliquots taken during milk fermentation, only two of highly aflatoxin-contaminated
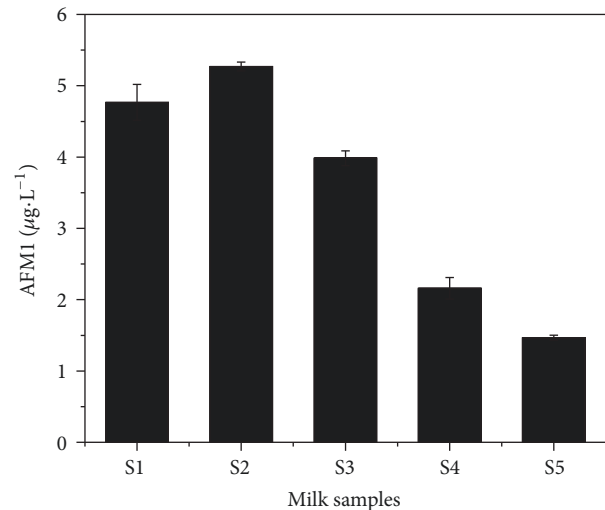

Figure 2: ELISA results of the level of AFM1 in the contaminated milk samples.

samples (S1 and S2) were considered to determine the fate of AFM1 during fermentation.

3.4. Reduction of AFM1 during Ergo Production.

The AFM1 reduction study was carried out on the three treatment groups (Sr, Sc, and Sci) prepared using two of the five AFM1 contaminated samples as described in Section 2.2. The mean initial levels of AFM1 in triplicate samples of Sr, Sc, and Sci were found to be 5.0, 5.1, and 5.1 $\mu g \cdot L^{-1}$, respectively. The levels of AFM1 in Sc and Sci were the same, as expected. This is because these samples were subjected to the same treatment (sterilization) except the inoculation of Sci with LAB cultures, which is carried out after the initial level of AFM1 was determined. A slight difference in the values of Sr and other samples (Sc and Sci) could be due to concentration of sample aliquots during sterilization. Quantification of the residual levels of AFM1 every 24 hours during incubation at *ergo* fermentation condition gave the results indicated in Figure 3. As shown in the figure, a gradual decrease of AFM1 levels in the fermenting samples (Sr and Sci) was recorded during incubation. The mean percent decrease of 57.33 and 54.04 was recorded in Sr and Sci, respectively, after the 5 days of incubation. Although the number of bacteria in Sci is slightly higher that in Sr, a comparable or even greater reduction in the level of AFM1 was recorded in Sr than Sci. This could be attributed to higher number of more efficient strains in Sr than Sci, regardless of the total count. However, this should be verified through further investigation on the individual performances of the LAB strains. Only 5.53% decrease was recorded with the control (Sc) samples. This slight decrease in the level of AFM1 in Sc samples could be attributed to binding of the AFM1 with the trace levels of dead bacterial cells as previously reported by different researchers [34, 35]. It is trace because the milk sample was immediately sterilized after collection, and no time was allowed for the proliferation of the bacteria that might probably present. The bacteria in Sr and Sci continued growth during the fermentation. The

TABLE 2: Effect of pH on the fate of AFM1 in naturally fermenting milk *(ergo)* at 25°C.

Incubation period (days)	Raw milk fermenting to ergo		Sterilized milk sample		Starter culture inoculated milk sample	
	pH	% AFM1 reduction	pH	% AFM1 reduction	PH	% AFM1 reduction
0	4.45	-	4.50	0	4.50	-
1	4.40	13.53 ± 1.136	4.48	0.36 ± 0.10	4.40	10.7 ± 1.70
2	4.30	25.13 ± 2.10	4.41	1.24 ± 0.39	4.35	14.41 ± 2.13
3	4.10	35.67 ± 0.10	4.40	3.25 ± 0.21	4.15	22.30 ± 3.13
4	3.70	44.80 ± 1.17	3.93	4.90 ± 1.10	3.67	44.14 ± 1.50
5	3.50	57.33 ± 3.10	3.89	5.50 ± 2.40	3.53	54.04 ± 2.94

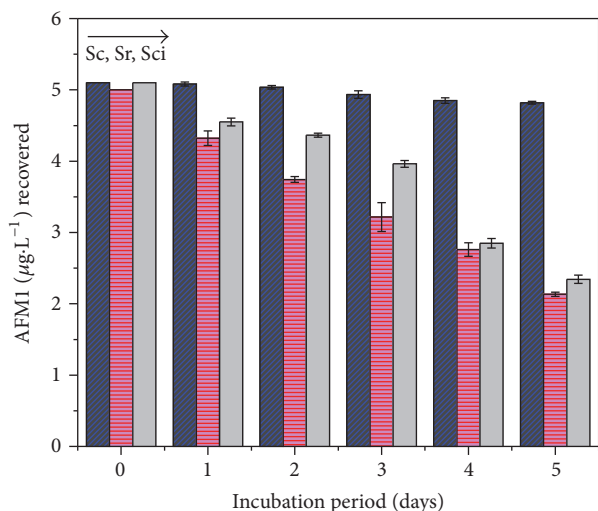Results are the average ± SD for triplicate samples.

FIGURE 3: ELISA results of the fate of AFM1 in, left to right, sterilized (Sc), raw (Sr), and sterilized and starter culture inoculated (Sci) milk samples during incubation under traditional milk fermentation to Ethiopian naturally sour dairy product "*ergo.*"

FIGURE 4: Effect of incubation temperature on AFM1 reduction during *ergo* fermentation.

observed higher reduction of AFM1 is the expected one. The current result also reenforced the fact that *ergo* fermentation is induced by the natural presence of LAB in milk samples [21]. In general, the study revealed the nonobvious benefits of consuming *ergo* rather than freshly harvested raw milk and suggested the possibility of using fermentation of milk into *ergo* as a strategy to reduce the effect of milk contamination by aflatoxin.

3.5. Effect of pH on the Fate of AFM1 in Ergo.

The pH of the fermenting milk samples was monitored every 24 hours during incubation at 25°C. The result is indicated in Table 2. The pH showed a slight and gradual decrease with incubation time in fermenting samples (Sr and Sci). The decrease in the pH was accompanied by the souring and fermentation of the milk samples. It also suggests proliferation of the LAB species because the pH of the control samples (sterilized) remained relatively unchanged during the incubation period, 5 days. The current findings are also in agreement with a relatively low pH of *ergo*, ranging from 4.3 to 4.5, reported elsewhere [21].
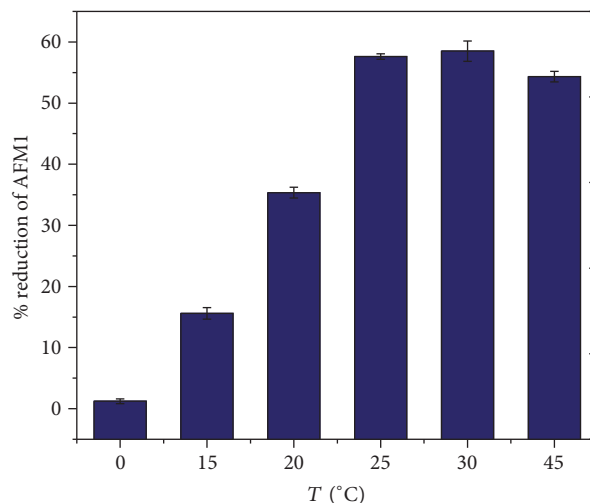
3.6. Effect of Temperature on Fate of AFM1.

The effect of temperature on the AFM1 reduction during *ergo* fermentation was studied at 0, 15, 25, 37, and 45°C. The temperature range was decided based on the possible climatic conditions of the country where *ergo* is produced. The result is indicated in Figure 4. A gradual increase in reduction percentage with increasing incubation temperature can be observed. However, the highest reduction was observed near ambient temperatures whereas the lowest at temperatures near zero degrees Celsius. This is in agreement with the fact that fermentation and growth of LAB, key players in AFM1 removal, are slow at low temperatures [36]. Thus, the highest reduction of AFM1 in fermenting *ergo* near ambient temperature (25–30°C) might be attributed to the proliferation of LABs under this condition.

3.7. Effect of LAB and Mechanistic Insights.

The effect of LAB on AF removal was studied by recording LAB count changes during incubation of the milk samples (Sr) to verify whether it is correlated with the observed reduction in the level of AFM1. The result is indicated in Figure 5. Interestingly, LAB count (Figure 5(a)) and titratable acid percentage
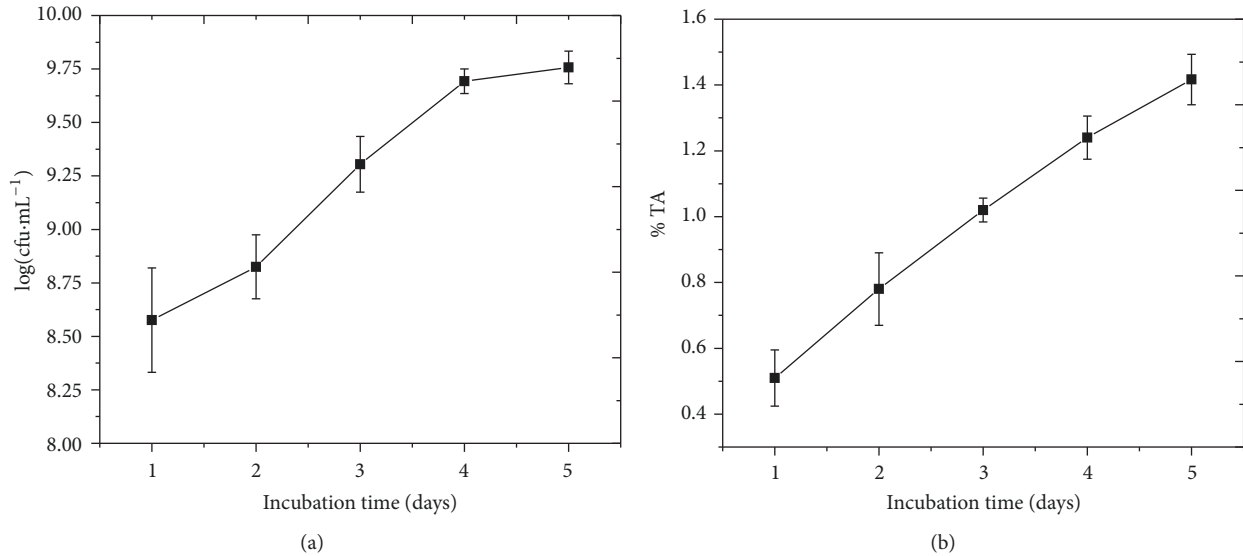
FIGURE 5: (a) Changes in LAB count; (b) changes in titratable acid percentage (% TA) in Ethiopian traditionally fermenting milk (*ergo*) incubated at ambient (20 to 25°C).

(Figure 5(b)) increased with increasing duration of incubation. The increase and total LAB counts and titratable acid percentage correlated with the corresponding decrease in the level of AFM1 (Table 2). The observed correlation between the increase in total LAB and the reduction in the level of AFM1 in Sr and lack of this observation in the control sample (Sc) during incubation at *ergo* fermentation conditions were considered as a direct evidence of the effect of LAB on the reduction of AFM1 in *ergo*. Although, a universal consensus regarding the mechanism of AF reduction by LAB has not been reached, two possible mechanisms can be considered. The first is removal of the toxin by noncovalent binding with the bacterial cells. The second is the degradation through hydrolysis of the lactone ring of the toxin by the developing acid during the fermentation [37]. However, numerous recent investigations on the possible mechanisms of AFM1 removal by treatment with dairy strains of LAB and bifidobacteria suggested a physical union, adhesion to bacterial cell wall components (polysaccharides and peptidoglycans), instead of degradation by hydrolysis or covalent binding [35, 37–41]. For instance, Bovo et al. 2013 [35] demonstrated AFM1 binding by *Saccharomyces cerevisiae* strain and a pool of three LAB strains (*Lactobacillus rhamnosus*, *Lactobacillus delbrueckii* spp. *bulgaricus*, and *Bifidobacterium lactis*), alone or in combination, in UHT (ultrahigh temperature) skim milk. On the other hand, other researchers showed that the binding ability of AFB1 to LAB is strain specific [18, 34, 41]. Regarding the effect of pH change during *ergo* fermentation, Elsanhoty et al. (2014) [18] considered the pH change during fermentation as a factor affecting the binding interaction and showed an increase in the level of AFM1 reduction by the decrease in pH during bacterial treatment. By contrast, Haskard et al. (2001) [40] reported that the AF binding with bacterial cells is independent of pH. It seems that AF removal by treatment with dairy strains of LAB could

occur regardless of pH change and that the toxin interacts with certain chemical components of the bacterial cell wall whose composition may vary with LAB strains. Besides, the observation that heat-killed cells show AFM1 removal higher than viable cells [18, 35] favors the binding mechanism and attenuates the importance of the hydrolysis mechanism. This evidence strongly supports the first mechanism that the toxin is removed predominantly by noncovalent binding with the chemical components of the bacterial cell wall. This mechanism is further evidenced by the reports of Hernandez-Mendoza et al. (2009) [41] who demonstrated that cell components such as teichoic acids are the possible binding sites for AFB1.

4. Conclusions

The reduction of aflatoxin M1 levels during Ethiopian traditional fermented dairy product *(ergo)* production was successfully demonstrated through the determination of residual levels of the toxin in raw and sterilized (control) milk samples using Enzyme Linked Immunosorbent Assay. The study showed that traditional fermentation of milk into *ergo* can significantly reduce AFM1. Microbiological investigations of the milk samples fermenting into *ergo* showed the presence of LAB species. Though no viable bacterial counts were recorded in sterilized samples, a small decrease in the level of AFM1 was observed which could be attributed to the trace levels of dead bacterial cells possibly present in the sample before the sterilization. The reduction in the level of AFM1 in raw milk samples during fermentation to *ergo* was, therefore, proposed to be attributed to the natural presence and proliferation of lactic acid bacteria, which is also responsible for the fermentation. The current findings could assist in the scientific development of indigenous knowledge as an effective food safety control strategy against

aflatoxin, particularly in tropical and subtropical areas where cow's feeds are susceptible to mold growth and aflatoxin contamination. Further studies are needed to evaluate the individual efficiency of the LAB strains commonly found in milk samples and whether any degradation product is formed during the treatment.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

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