

Evaluation of Lactic Acid Bacteria for the Control of Aflatoxin Contamination in Silage

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Abstract: This study evaluated the effect of lactic acid bacteria (LAB) on fermentation and aflatoxin concentrations of napier grass, whole crop green maize and whole crop dry maize plant silages, infested with aflatoxigenic mold *Aspergillus flavus*. The forage was chopped into 10kg portions in duplicates and inoculated with 100 ml of 10^{6-7} CFU/ml, B2 27 *Lb. plantarum/pentosus/paraplantarum* (LAB1); B410*Lb. plantarum/pentosus/paraplantarum* (LAB 2); LAB 1 + 100 ml of cultured spores (100 cfu/ml) of mold; LAB 2 + 100 ml of cultured spores (100 cfu/ml) of mold; control 1- no addition; control 2 – mold only; control 3 – LAB 1 only; control 4 – LAB 2 only. The inoculated silage bags were sealed for 90 days and sampling done monthly to evaluate LAB, fungal counts and aflatoxin B1. Dry matter (DM), pH, crude protein (CP), neutral detergent fiber (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) were determined using AOAC methods. Mold population decreased in month one but increased thereafter. Silage type determined LAB ($p = 6.2e-08$) and mold ($p = 3.9e-08$) proliferation. Dry maize and napier grass silages favoured LAB and mold growth, respectively. LAB inhibited mold growth and aflatoxin production ($p = 2.2e-04$). Silage pH in the third month ranged between 4.55 – 5.67 with no smell of butyric acid. Though napier and dry maize plants showed higher nutritional qualities, they favoured mold growth and aflatoxin proliferation. Aflatoxin-inhibiting LAB starters are useful in controlling aflatoxin risk in silage.

Keywords: Aflatoxin, *Aspergillus Flavus*, Lactic Acid Bacteria

1. Introduction

One of the major potential food safety risks identified in recent years in the dairy value chain of Kenya are biomolecules resulting from biological contaminants. Mycotoxin derivatives, in particular aflatoxins, carried over to milk as aflatoxin M1 (AFM1) and M2 (AFM2) from cows fed with feedstuffs contaminated with aflatoxins B1 (AFB1) and B2 (AFB2) respectively, are among such biomolecules. Aflatoxins are toxic compounds produced mainly by

Aspergillus flavus and *A. parasiticus*. The major aflatoxins are AFB1, AFB2, AFG1 and AFG2 [1]. Among them, AFB1 is the most relevant for health concern and has been classified as class 1 carcinogen by the International Agency for Research on Cancer (IARC) owing to its carcinogenic effect [2]. This mycotoxin is closely associated, in long-term exposure, to the hepatocellular carcinoma in humans, modulation of the immune system and malnutrition [3]. Aflatoxin M1 is a class 2B (possible) human carcinogen.

Dairy production is a major activity of the livestock sub-

sector in Kenya. Dairy cattle population is estimated at 4.3 million with annual milk production of 4.7 billion liters [4]. Smallholder dairy farmers based in peri-urban and rural areas own over 80% of the dairy cattle, producing over 56% of the total milk. The remaining 20% of dairy cattle is owned by large dairy farmers or belong to indigenous herd [5]. A majority of dairy cattle are kept under intensive and semi-intensive systems of livestock production and are stall-fed with planted or purchased fodder like napier grass, hay, maize stovers, weeds, grass and crop residues and occasionally supplemented with concentrate feeds, such as grain millings or compounded dairy feeds [6].

Conservation of napier grass and green maize in the form of silage has been increasing rapidly on Kenyan dairy farms alongside with the need for better performance of dairy cows, especially during dry periods [7]. Maize is susceptible to infection with fungi and aflatoxin-producing fungal species have been identified from maize varieties commonly cultivated in Kenya [8-10]. Many studies carried out in Kenya have reported the frequent presence of multiple mycotoxins, in particular AFB1, in farm-made or commercial dairy feeds and feed ingredients [11-15]. Feeding of contaminated feedstuffs to dairy cows results in transmission of mycotoxins to milk. Thus, a frequent occurrence of AFM1 has been reported recently in Kenyan raw cow milk and dairy products [14, 16-20]. Kenya has the highest per capita milk consumption, 110 liters, in sub-Saharan Africa [21]. Human exposure to aflatoxins from dairy products is, therefore, considered a public health issue [15, 22]. For this reason, maximum tolerable levels for aflatoxins in maize, dairy feeds and milk are regulated in Kenya.

For mitigation of aflatoxin risk in maize, several strategies including biological, chemical and physical methods, as well as farm practices, have been developed [23-25]. In recent field studies carried out in Kenya, promising results have been reported on application of a biocontrol method “Aflasafe”, push-pull technology; pre-harvest management of maize and good agricultural practices on farms [26-28]. Proper management practices concern also silage, which can be a

significant source of toxigenic fungi and mycotoxins as shown in many studies reviewed previously [29]. Green silage is traditionally produced by means of anaerobic fermentation generated by lactic acid bacteria (LAB), harboring naturally the ensiled forage. The potential of LAB species for prevention of the growth of aflatoxigenic fungi and binding aflatoxins has been studied extensively and a great number of prospective candidates have been identified in experimental studies [30-34]. Many microbial inoculants, including LAB strains, have been developed and are commercially available as starters or additives for forage treatment with the purpose to prevent fungal growth and mycotoxin formation, respectively, and to improve the nutritional quality of silage [35-36]. However, the efficacy of commercial preparations is subject to field trial under local conditions. Locally isolated LAB strains have been shown to reduce in vitro the growth of aflatoxigenic *A. flavus* strains isolated from maize cultivated in Kenya, and bind AFM1 in milk [37-38].

In this study, we evaluated the efficacy of two LAB strains isolated from Kenya to inhibit under experimental conditions the growth of aflatoxigenic *Aspergillus flavus* strains from Kenya and AFB1 production in silage made from maize and napier grass cultivated in Kenya [9, 37].

2. Materials and Methods

2.1. Experimental Design for Microbial Treatment of Silage

The experiment was carried out between 15th August and 15th November, 2018. Forages preferred by farmers, i.e. napier grass, green maize plants and sun-dried maize plants were selected for silage making. The forage was chopped into 10 kg portions in duplicates and inoculated with single strains of *Lactobacillus* (LAB) only or with a mixture of aflatoxigenic *Aspergillus flavus* strains, wrapped in airtight, polythene silage bags and stored at room temperature (Table 1). Sampling of silage was done immediately after inoculation and thereafter monthly for three months.

Table 1. Experimental design for silage microbial treatment.

Microbial strain	Silage type and treatment		
	Napier grass	Green maize plants	Dry maize plants
B2 27 <i>Lb. plantarum/pentosus/paraplantarum</i> (isolated from Murzik) (LAB1)	LAB1 + Mold*	LAB1+ Mold	LAB1 + Mold
B2 27 <i>Lb. plantarum/pentosus/paraplantarum</i> (isolated from Murzik) (Control 3)	LAB1 only	LAB1 only	LAB1 only
B4 10 <i>Lb. plantarum/pentosus/paraplantarum</i> (isolated from Kirario) (LAB2)	LAB2 + Mold*	LAB2 + Mold	LAB2 + Mold
B4 10 <i>Lb. plantarum/pentosus/paraplantarum</i> (isolated from Kirario) (Control 4)	LAB2 only	LAB2 only	LAB2 only
Control 1	No addition	No addition	No addition
Control 2	Mold* only	Mold* only	Mold* only

**Aspergillus flavus*

2.2. Inoculation of Silage Bags

2.2.1. Source of LAB Strains

LAB strains used were (i) B2 27 *Lb. plantarum/pentosus/paraplantarum* isolated from Murzik, a spontaneously fermented cow milk and (ii) B4 10 *Lb. plantarum/pentosus/paraplantarum* isolated from Kirario, a

fermented maize gruel. These LAB strains were earlier isolated from the two traditional foods between the years 2013 and 2014 and stored in 12.5% glycerol at -80°C as described by Ahlberg et al [37]. All LAB strains were revived before use by inoculation into MRS (de Man, Rogosa and Sharpe lactobacilli medium, cooled in a water bath at 50°C) broth (500 ml each) and incubation at 30°C for 24 hours or until the cultures had grown to late logarithmic or

early stationary phase. The cells were washed with sterile saline solution and adjusted to a concentration of 10^{6-7} CFU/ml before mixing individually with the chopped feed materials at a ratio of 100 ml cell suspension to 10 kg chopped feed material to yield a cell concentration 10^{4-5} CFU/g of silage.

2.2.2. Source of Mold Strains

Three highly aflatoxigenic *A. flavus* strains (V201365, V100130, and V100095), isolated from maize kernels in Kenya were used to make inoculum [9]. The inoculum was prepared by growing the isolates on sterile maize kernels. Fifty grams of maize kernels were soaked in 25 ml sterile distilled water in 250 ml conical flasks for 6 hours or overnight, autoclaved at 120°C for 40 min and then left to cool for 6 hours or overnight in the laminar flow cabinet. The isolates were transferred onto the kernels in the conical flasks, mixed well and incubated at 30°C. To prevent clumping, the conical flasks were shaken once daily. After 7–14 days, fungal conidia were washed from the kernels with a soap solution made up of 40 µl Tween20 in 20 ml of sterile, distilled water. The suspension was sieved using sterile double cheese cloth and the conidia collected in a beaker. The filtrate was diluted further using sterile distilled water to bring it to 1×10^{10} conidia per ml for inoculation. Inoculum of each of the three isolates was raised separately and only mixed a few minutes before inoculating the silage. Equal quantities of the three *A. flavus* isolates were mixed thoroughly before silage inoculations. A five ml aliquot of the mixture was added to 100 ml of sterile phosphate buffer. This suspension was mixed with 10 kg of chopped feed material.

2.3. Ensiling Process

Napier grass (*Pennisetum purpureum*), variety Kakamegal and maize (*Zea mays* L.), variety H513 were grown at Kenya Agricultural and Livestock Research (KALRO) Muguga Centre. The grass was harvested at 1.5 meters height and left to wilt for 12 hours under shade before ensiling. The dry matter content of the grass material was 20.4%. Green maize plants were harvested at milk stage for ensiling. The dry matter content of the maize material was 21.4%. The third forage material used was dry maize stover of the variety H513. The content of dry matter of this material was 47.9%. The three forage materials were separately chopped by a chaff cutter in 2–4 cm long pieces, weighed into 10 kg batches and mixed with molasses at the rate of 5% (w/w), after which each batch was placed in a separate airtight black polythene silage bag ready for inoculation. After inoculation, the airtight silage bags were stored in a room at ambient temperature for three months. Intermittent mixing of each silage block was done every other day to allow homogenous distribution of the microbes and avoid clamping.

2.4. Sampling

The contents of each silage bag were thoroughly mixed before sampling. About 350 g of sample was pushed to a

corner of the bag, separated from the rest of the content and the bag was tied tightly not to allow air to gain access to the bulk of the sample. The sample was emptied into a labeled plastic container. The batches were re-sealed to retain the silage in as near anaerobic conditions as possible and stored. Sampling was done as shown in Table 1. Each harvested sample was divided into two parts, 100 g transferred into labeled clean polythene zip lock bags for microbiological assays at the University of Nairobi and the remaining 250 g for chemical analysis at KALRO, Muguga. The first sampling after treatment was done at time zero, subsequent sampling was done monthly for three months.

2.5. Microbe and Aflatoxin Quantification

i. LAB count in silage

Twenty-five grams of silage was weighed and added to 225 ml of sterile physiological saline solution. Bacteria were recovered by mixing and shaking thoroughly for 15 min. Serial dilution was made from 10^1 to 10^6 with sterile normal saline. From each dilution, a sample of 100 microliters was transferred to a sterile Petri dish and 20 ml of sterilized and molten MRS added and mixed thoroughly. The mixture was allowed to cool and then the seeded Petri dishes were placed in anaerobic containers equipped with a gas generating Kit (AnaeroGen2.5L from Thermo Scientific) to generate anaerobic condition, locked and incubated at 30°C for 36 hours. Colony counting of LAB was done visually.

ii. Procedure for mold count in silage

Ten grams of silage was transferred to a sterile conical flask, 20 ml of sterile distilled water added, mixed by shaking and left to stand for 5 minutes. Nine ml of sterile distilled water was added into 3 sterile universal bottles labelled A, B and C for serial dilution. Using a micropipette with sterile tip, 1 ml of the sample solution from the conical flask was drawn and added to bottle A and mixed. From bottle A, 1 ml of the contents was transferred to B and mixed well. From bottle B, 1 ml of content was transferred to C and mixed well. From bottle C, 1 ml of the contents was drawn and discarded to ensure that all bottles contained 10 ml of mixture. Ten µl of the solution in each bottle was aseptically transferred into separate Potato Dextrose Agar (PDA) medium plate and spread evenly by using a sterile bent glass rod on the agar medium surface in triplicates and incubated at 29°C for two weeks. Colonies growing were observed visually and purified after 7 days up to 14 days and *A. flavus* identified using the taxonomic keys by Klich [39].

iii. Procedure for mold count in silage Iii Quantification of aflatoxin B1 levels in silage

Enzyme linked immunosorbent assay was used to quantify total aflatoxins. Helica Biosystems^R low matrix aflatoxin kit was applied as instructed by the manufacturer.

2.6. Chemical Analysis of Silage

2.6.1. Determination of pH

A sub-sample of 100 g collected as explained above was weighed into a 1500 ml clear plastic jug of the Ramton

RE/105 model blender. The method outlined by AOAC [40] was used.

2.6.2. Conventional or Proximate Analysis of Silage

The remaining 100 g sub-sample collected as explained above was dried overnight at 60°C in an air-circulation oven to obtain air dried samples which were thereafter ground to 1 mm particle size using a Wiley mill. All the subsequent feed analyses were conducted on the ground samples. Dry matter (DM), ash, neutral detergent fiber (NDF), acid detergent fiber (ADF), crude protein (CP) and acid detergent lignin (ADL) content of the silage were determined using the methods described by AOAC [41, 42]. Organoleptic and fermentation quality of silage was assessed using visual, smell and texture observations.

2.7. Statistical Analysis Procedure

To analyze changes in microbial growth and aflatoxin accumulation with time, the R software (R Foundation for Statistical Computing, Vienna, Austria. URL [https://www.R-](https://www.R-project.org/)

[project.org/](https://www.R-project.org/)) was used. Poisson regression was done for count data (mold count). Log of mold was obtained, and ANOVA done to compare treatments. The General linear model (GLM) in SAS was used to analyze data on quality of silage. Tukey's test was used to separate the means for the levels of each of the quality factors.

3. Results and Discussion

3.1. Effect of Substrate and LAB on *Aspergillus Flavus* Proliferation in Silage Fermentation

A. flavus population in all ensiled substrates decreased in the first month but increased and levelled off thereafter. Napier grass and green maize favoured mold growth compared with dry maize ($p=3.9\text{e-}08$), as shown in Figure 1a and Table 2. Over time, LAB2 inhibited mold growth more than LAB1, though this difference was not statistically significant (Figure 1b; Table 2).

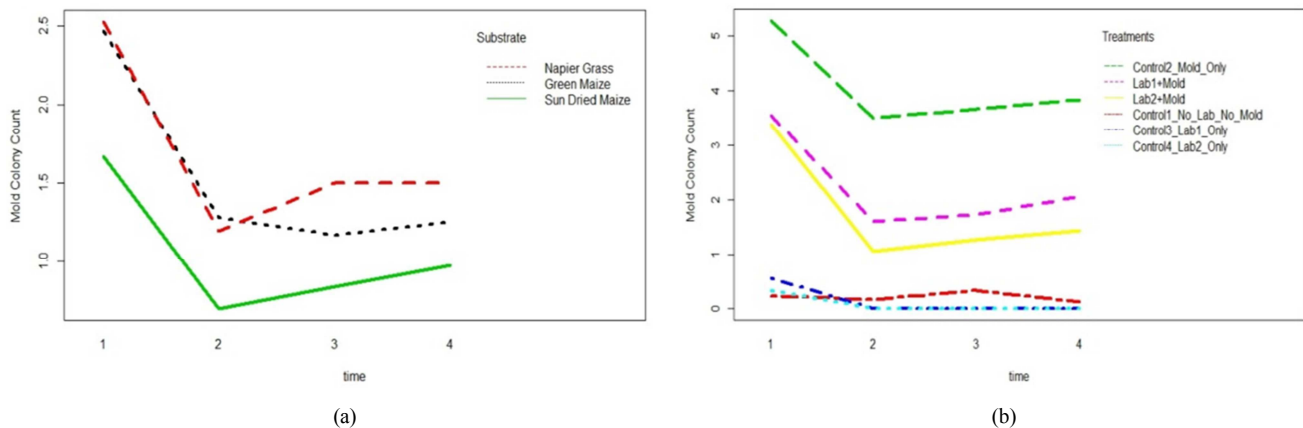


Figure 1. *Aspergillus flavus* colony counts in different silage types over time (a) *A. flavus* growth in different silage types (b) *A. flavus* growth in silage treated with *Lactobacillus*.

Table 2. Effect of *Lactobacillus* on mold growth in different silage types: Covariate effects without time interactions, Poisson Regression.

Coefficients:	Estimate	Exp (Est.)	Std.err	Wald	Pr (> W)
(Intercept)	1.129	3.094	0.130	75.23	< 2e-16 ***
Month 2	-1.167	0.311	0.129	80.95	< 2e-16 ***
Month 3	-1.056	0.348	0.123	73.98	< 2e-16 ***
Month 4	-0.982	0.375	0.120	66.92	3.3e-16 ***
Substrate: Napier grass	0.139	1.149	0.095	2.13	0.14
Substrate: Dry maize	-0.500	0.607	0.091	30.22	3.9e-08 ***
Treatment: Control2 (Mold)	3.861	47.518	0.152	645.39	< 2e-16 ***
Treatment: Control3 (LAB1)	-0.069	0.933	0.105	0.44	0.51
Treatment: Control4 (LAB2)	-0.125	0.882	0.097	1.67	0.2
Treatment: LAB1+Mold	2.028	7.597	0.137	220.03	< 2e-16 ***
Treatment: LAB2+Mold	1.583	4.871	0.123	166.8	< 2e-16 ***

Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Mold = *Aspergillus flavus*

LAB = *Lactobacillus*

3.2. Effect of Substrate on Total Aflatoxins Production

Silage type and treatment affected aflatoxin accumulation (p

= $2.2\text{e-}04$ and $p < 2\text{e-}16$, respectively). Green maize (total aflatoxins range nil-133.4 g/ kg) and napier grass (total aflatoxins range 0.2-720 g/kg) silage, favoured aflatoxin

accumulation compared with dry maize (nil – 297 g/kg). An increase in aflatoxin production was observed in the second month and this dropped in the third month in all substrates (Table 3). The variation of aflatoxin concentration in silage with

time across the treatments was significant (Table 4). Silage treated with LAB 2 contained less aflatoxins in the third month compared with other silages treated differently.

Table 3. Variation of total aflatoxin contamination in different silage types during storage.

Silage type	Treatment	Mean total aflatoxins (g/kg)			
		Month 0	Month 1	Month 2	Month 3
Napier grass	LAB1+Mold	17.3	30	44.7	8.4
	LAB2+Mold	7.4	35.9	14.2	2.4
	Nil (control1)	3.2	16.2	28.3	10.7
	Mold (control2)	8.6	78	632	720
	LAB1 (control3)	9.6	9.3	5.2	0.2
Green maize	LAB2 (control4)	6.7	4.2	3.7	0.3
	LAB1+Mold	23.5	38.6	24.1	3.1
	LAB2+Mold	5.7	34.2	8.1	10.3
	Nil (control1)	10.7	20.8	8.8	7.7
	Mold (control2)	0.4	12.3	73.9	133.4
Dry maize	LAB1 (control3)	0.2	Nil	Nil	Nil
	LAB2 (control4)	2.8	Nil	Nil	Nil
	LAB1+Mold	7.2	23.3	14.5	5.8
	LAB2+Mold	11.9	60.7	25.7	4.5
	Nil (control1)	22.3	14.7	896.5	8.8
	Mold (control2)	23	67	311	297
	LAB1 (control3)	15.9	27	15	Nil
	LAB2 (control4)	22.2	17.8	0.2	Nil

3.3. Influence of Silage Type on LAB Growth

While mold growth decreased in the first month as explained above, LAB colony counts increased in the first month, but decreased progressively during the following two

months in all the substrates. The decrease was more in LAB1 compared with LAB2 ($p=0.0018$). Dry maize favored LAB growth followed by napier grass ($p=6.2e-08$). LAB1 + mold ($p=0.0018$) was more affected than LAB2 (Tables 4).

Table 4. Effect of silage type and LAB on aflatoxin accumulation: No interaction effect of covariates on aflatoxin concentrations.

	Df	Sum Sq	Mean Sq	F value	Pr (>F)
Treatments	5	534.8	106.96	188.7	< 2e-16 ***
Substrate	2	10.4	5.2	9.1	2.2e-04 ***
Treatments: time	15	113.04	7.536	14.38	< 2e-16 ***
Substrate: time	6	22.33	3.722	4.67	1.4e-04 ***
Residuals	100	56.7	0.57		
Effect of substrate on <i>Lactobacillus</i> Colony Counts					
Treatments	4	40.5	10.1	4.71	0.0018 **
Substrate	2	87.8	43.9	20.40	6.2e-08 ***
Residuals	83	178.5	2.2		
Effect of mold treatment on <i>Lactobacillus</i> Colony Counts					
Time	3	756	252.0	66.30	< 2e-16 ***
Treatments: time	12	182	15.2	3.99	1.1e-05 ***
Residuals	255	969	3.8		
Effect of substrate and time on <i>Lactobacillus</i> Colony Counts					
Time	3	756	252.0	76.3	< 2e-16 ***
Substrate: time	6	289	48.2	14.6	2.2e-14 ***
Residuals	261	862	3.3		

Significance codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘.’ 1

3.4. Chemical Characteristics of Silage

The pH silage dropped after the first month, indicating fermentation activity of the bacteria. As shown in Table 5, all

the three substrates were different in pH ($p<0.0001$). Dry maize stover and napier grass preserved dry matter (DM) better than green maize plants ($p<0.0001$, Table 6) Addition of LAB to silage increased the DM with time ($p=0.0490$,

Table 7). Napier grass had higher ash content than the other two substrates ($p < 0.0001$, Table 8). Napier grass also recorded the highest crude protein (CP) content ($p < 0.0001$), which varied through the storage period ($p = 0.0413$, Table 9). Silage made from napier grass produced the highest NDF (Table 10), ADF (Table 11) and ADL (Table 12).

Table 5. Effects of substrate on silage pH.

Substrate	Period (Month)				Substrate Mean pH	SEM
	0	1	2	3		
Dry maize stover	7.78	4.63	5.07	5.21	5.67 a	0.075
Green maize plants	6.67	3.23	4.20	4.10	4.55 c	0.075
Napier grass	6.95	4.23	4.86	5.10	5.31 b	0.076
Period Mean	7.13 p	4.02 r	4.71 q	4.80 q	5.17	0.043
SEM	0.086	0.088	0.086	0.086	0.043	

Means followed by similar letters (a, b, c, and p, q, r, respectively) are not significantly different ($P > 0.05$).

Table 6. Main effects of substrate and period on DM%.

Substrate	Period (Month)				Substrate Mean	SEM
	0	1	2	3		
Dry maize stover	92.98	94.61	95.29	94.51	94.34 a	14.769
Green maize plants	92.80	91.81	94.77	93.67	93.26 b	14.614
Napier grass	93.40	94.46	94.79	95.29	94.49 a	14.928
Period Mean	93.05 q	93.60 q	94.95 p	94.47 p	94.02	8.527
SEM	17.114	17.114	16.875	17.114	8.527	

Means followed by similar letters (a, b, c, and p, q, r, respectively) are not significantly different ($P > 0.05$).

Table 7. Main effects of LAB and period on DM%.

LAB	Period (Month)				LAB Mean	SEM
	0	1	2	3		
0	92.91	93.49	93.96	94.58	93.73 b	14.614
1	93.26	93.64	95.62	93.90	94.11 a	14.769
2	92.98	93.67	95.28	94.87	94.24 a	14.928
Period Mean	93.05 q	93.60 q	94.95 p	94.47 p	94.02	8.527
SEM	17.114	17.114	16.875	17.114	8.527	

Means followed by similar letters (a, b, c, and p, q, r, respectively) are not significantly different ($P > 0.05$).

Table 8. Main effect of substrate on ash content (g/kg DM).

Substrate	Substrate Mean	SEM
Dry maize stover	117.55 b	1.092
Green maize plants	96.12 b	1.080
Napier grass	160.37 a	1.104
Overall Mean	124.22	0.630

Table 9. Main effects of substrate and period on CP content (g/kg DM).

Substrate	Period (Month)				Substrate Mean	SEM
	0	1	2	3		
Dry maize stover	58.44	71.24	64.45	74.63	67.10 b	0.426
Green maize plants	87.82	86.22	83.90	92.73	87.67 a	0.422
Napier grass	61.80	62.90	54.40	65.11	60.95 c	0.431
Period Mean	69.57 qr	73.52 pq	67.59 r	77.84 p	72.10	0.246
SEM	0.494	0.494	0.487	0.494	0.246	

Means followed by similar letters (a, b, c, and p, q, r, respectively) are not significantly different ($P > 0.05$).

Table 10. Main effects of substrate and period on NDF content (g/kg DM).

Substrate	Period (Month)				Substrate Mean	SEM
	0	1	2	3		
Dry maize stover	685.66	693.45	680.02	688.38	686.74 a	0.913
Green maize plants	659.38	617.85	618.74	653.94	637.48 b	0.904
Napier grass	669.42	680.79	712.33	710.49	693.40 a	0.923
Period Mean	671.55	663.19	670.36	683.52	672.14	0.527
SEM	1.058	1.058	1.043	1.058	0.527	

Means followed by similar letters (a) are not significantly different ($P > 0.05$).

Table 11. Main effects of substrate and period on ADF content (g/kg DM).

Substrate	Period (Month)				Substrate Mean	SEM
	0	1	2	3		
Dry maize stover	429.10	423.82	397.13	441.50	422.87 b	0.673
Green maize plants	384.46	338.30	338.60	382.01	360.84 c	0.666
Napier grass	524.59	489.22	492.21	518.12	505.37 a	0.680
Period Mean	443.81 p	416.92 q	409.31 r	445.18 p	428.67	0.389
SEM	0.780	0.780	0.769	0.780	0.389	

Means followed by similar letters (b) are not significantly different ($P>0.05$).

Table 12. Main effects of substrate and period on ADL content (g/kg DM).

Substrate	Period (Month)				Substrate Mean	SEM
	0	1	2	3		
Dry maize stover	46.26	65.82	61.61	93.78	66.89 b	0.589
Green maize plants	48.95	52.80	73.43	79.32	63.62 b	0.583
Napier grass	67.48	70.17	94.69	90.80	80.86 a	0.595
Period Mean	53.85 r	62.85 r	76.58 q	87.89 p	70.34	0.340
SEM	0.682	0.682	0.673	0.682	0.340	

Means followed by similar letters (b) are not significantly different ($P>0.05$).

3.5. Organoleptic Characteristics of Fermented Silage

Visual and texture assessments indicated that all the silage bags were well preserved. No silage bag had the characteristic smell of butyric acid, which is an indicator of poor fermentation.

Non-LAB treated silage (control) produced more aflatoxin B1 compared with the LAB treated samples, confirming the suggested influence of LAB to mitigate aflatoxin risk in silage. Aflatoxin level of non-LAB treated Napier silage was the highest and lowest on dry maize-based silage. This shows the importance of silage substrate on the fermentation process. Further, during the experimental period, pH of green maize silage dropped to 3. This development could have caused the decline in observed LAB counts. Fungal growth was inversely proportional to LAB growth, suggesting a fungistatic activity of the employed bacterial strains. *A. flavus* grows optimally at pH 5, aw 0.99 and temperatures between 30 and 35°C, whereas the greatest production of aflatoxins occurs at 25°C [43]. To optimize the silage production technique, temperature and water activity should be measured in further experiments.

The results of this study support many earlier studies about the benefits of using LAB strains as starters in silage making for dairy cattle [29, 36]. We can conclude that LAB strains originating from the same ecosystem as fungal strains and forage substrates can be useful as additive for reduction of aflatoxin contamination in silage, and at the same time improve the nutritional quality of silage. Cultivated green maize plants, napier grass and sun-dried maize are commonly used for cattle feeding in Kenya in addition to natural grass and hay. They are also preserved as silage to supplement feeding, especially during dry periods when there is a shortage of adequate feed supplies. However, this practice is challenged by frequent contamination of feeding materials, especially maize, with mycotoxin producing molds and subsequently, occurrence of mycotoxins in milk when contaminated silage is fed to dairy cattle. Therefore,

improved technologies are needed to prevent the growth of potentially toxigenic fungi and concurrent production of mycotoxins during the ensiling process and storage of ensiled forage before feeding to livestock.

In the current study, all the silage bags were well preserved within the period of three months' experiment. No silage bag had the characteristic smell of butyric acid which is an indicator of poor fermentation [44]. Silage pH is a key criterion to evaluate fermentation quality; the lower the pH, the better preserved and more stable the silage is [45]. Over the experimental period, silage pH decreased from 7.13 to 4.80, which is within the maximum growth pH for LAB, *A. flavus* and aflatoxin production [46-48]. Lower pH (lower and 4.8) has been reported to deactivate or lyse LAB. This could have caused the low counts of LAB observed in green maize silage and the general decrease in LAB counts with time. Aflatoxin levels decreased with increasing acidity of silage with time, which may be explained by potential mycotoxin binding activity of the employed LAB strains. Acidity of the silage may have damaged the cell integrity of LAB, thus allowing further binding or degradation of aflatoxins by intercellular components [31]. In some studies, non-viable LAB cells have been reported to be more effective at binding aflatoxins than viable forms [49, 50]. On the other hand, the same LAB strains used in this study proved in previous study, more effective in aflatoxin binding when in viable form compared to heat-treated cells [38].

The results point to an improved nutritional quality of silage in the presence of added LAB starters. However, further research using genomic fingerprinting techniques, such as DGGE or RAPD-PCR technique, to investigate the microbial population dynamics during ensiling process is needed [51]. This would confirm the response of temperature and pH changes on both *A. flavus* and LAB and further confirm the genetic diversity of both LAB and *A. flavus* during ensiling, since apart from the inoculants, these microbes are found inherently on the surface of plant materials [51]. Decrease in counts of *A. flavus* was more

pronounced in silage inoculated with LAB1 than with LAB2, though the level of aflatoxin contamination was the same in both silage types.

4. Conclusion

Napier grass rated the best substrate for silage with high DM and ash content, moderate NDF and ADF, and low CP. Dry maize followed napier grass as substrate for silage making; these forages were also the most suitable for controlling fungal growth with addition of LAB starters. Thus, for mitigation of the aflatoxin risk in feed-dairy ecosystems, application of aflatoxin-inhibiting LAB starters in silage appears as a recommendable strategy in addition to other good agricultural practices.

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References

- [1] Okoth S, Improving the Evidence Base on Aflatoxin Contamination and Exposure in Africa: Strengthening the Agriculture-Nutrition Nexus; Partnership for Aflatoxin Control in Africa: Addis Ababa, Ethiopia, p. 128 (2016).
- [2] International Agency for Research on Cancer, A Review of Human Carcinogens. F. Chemical Agents and Related Occupations: IARC Monographs on the Evaluation of Carcinogenic Risks to Humans (2012).
- [3] Thanushree MP, Sailendri D, Yoha KS, Moses JA, Anandharamakrishnan C, Mycotoxin contamination in food: An exposition on spices. *Trends Food Sci Technol* 93: 69–80 (2019).
- [4] KDB, Kenya Dairy Board, Dairy information. <https://www.kdb.go.ke/dairy-data/>. Accessed 3, January 2022 (2022).
- [5] FAO OECD-FAO Agricultural Outlook 2019-2028 © OECD/FAO 2019. https://www.fao.org/3/CA4076EN/CA4076EN_Chapter7_Dairy.pdf
- [6] Njarui DM, Gatheru M, Wambua JM, Nguluu SN, Mwangi DM, Keya GA, Feeding management for dairy cattle in smallholder farming systems of semi-arid tropical Kenya. *Livest Res Rural Dev* 23: 5 (2011).
- [7] Muia JM, Tamminga S, Mbugua PN, Kariuki JN, The nutritive value of Napier grass (*Pennisetum purpureum*) and its potential for milk production with or without supplementation: a review. *Trop Sci* 40: 109–131 (2000).
- [8] Muthomi JW, Mureithi BK, Chemining'wa GN, Gathumbi JK, Mutit EW, *Aspergillus* species and Aflatoxin b1 in soil, maize grain and flour samples from semi-arid and humid regions of Kenya. *International Journal of AgriScience* 2: 22–34 (2012).
- [9] Okoth S, Nyongesa B, Ayugi V, Kang'ethe E, Korhonen H, Joutsjoki V, Toxigenic potential of *Aspergillus* species occurring on maize kernels from two agro-ecological zones in Kenya. *Toxins* 4: 991–1007 (2012).
- [10] Probst C, Njapau H, and Cotty, PJ, Outbreak of an acute aflatoxicosis in Kenya in 2004: identification of the causal agent. *Appl Environ Microbiol* 73: 2762–2764 (2007).
- [11] Kang'ethe EK, Lang'a K, Aflatoxin B1 and M1 contamination of animal feeds and milk from urban centers in Kenya. *Afr Health Sci* 9: 218–226 (2009).
- [12] Senerwa DM, Sirma AJ, Mtimet N, Kang'ethe EK, Grace D, Lindahl JF, Prevalence of aflatoxin in feeds and cow milk from five counties in Kenya. *African J Food Agric Nutr Dev* 16: 11004–11021 (2016).
- [13] Walte HG, Schwake-Anduschus C, Geisen R, Fritsche J, Aflatoxin: Food chain transfer from feed to milk. *J Verbrauch Lebensm* 11: 295–297 (2016).
- [14] Kang'ethe EK, Sirma AJ, Murithi G, Mburugu-Mosoti CK, Ouko EO, Korhonen HJ, Nduhiu GJ, Mungatu JK, Joutsjoki V, Lindfors E, Ramo S, Occurrence of mycotoxins in food, feed, and milk in two counties from different agro-ecological zones and with historical outbreak of aflatoxins and fumonisins poisonings in Kenya. *Food Qual Saf* 1: 161–170 (2017).
- [15] Kemboi DC, Ochieng PE, Antonissen G, Croubels S, Scippo ML, Okoth S, Kangethe EK, Faas J, Doupovec B, Lindahl JF, Gathumbi JK, Multi-mycotoxin occurrence in dairy cattle and poultry feeds and feed ingredients from Machakos Town, Kenya. *Toxins* 12: 762 (2020).
- [16] Langat G, Tetsuhiro M, Gonoi T, Matiru V, Bii C, Aflatoxin M1 contamination of milk and its products in Bomet County, Kenya. *Adv Microbiol* 6: 528 (2016).
- [17] Anyango G, Mutua F, Kagera I, Andang'O P, Grace D, Lindahl JF, A survey of aflatoxin M1 contamination in raw milk produced in urban and peri-urban areas of Kisumu County, Kenya. *Infect Ecol Epidemiology* 8: 1547094 (2018).
- [18] Kagera I, Kahenya P, Mutua F, Anyango G, Kyallo F, Grace D, Lindahl J, Status of aflatoxin contamination in cow milk produced in smallholder dairy farms in urban and peri-urban areas of Nairobi County: a case study of Kasarani sub county, Kenya. *Infect Ecol Epidemiology* 9: 1547095 (2019).
- [19] Lindahl JF, Kagera IN, Grace D, Aflatoxin M1 levels in different marketed milk products in Nairobi, Kenya. *Mycotoxin Res* 34: 289–295 (2018).
- [20] Kuboka MM, Imungi JK, Njue L, Mutua F, Grace D, Lindahl JF, Occurrence of aflatoxin M1 in raw milk traded in peri-urban Nairobi, and the effect of boiling and fermentation. *Infect Ecol Epidemiology* 9: 1625703 (2019).
- [21] Ahlberg S, Grace D, Kiarie G, Kirino Y, Lindahl J. A risk assessment of aflatoxin M1 exposure in low and mid-income dairy consumers in Kenya. *Toxins* 10: 348 (2018).
- [22] Monda EO, Alakonya AE, A review of agricultural aflatoxin management strategies and emerging innovations in sub-Saharan Africa. *African J Food Agric Nutr Dev* 16: 11126–11138 (2016).
- [23] ICIPE 3R Kenya Project, Dairy. African Centre for Technology Studies. <https://www.3r-kenya.org/dairy/> (2020).

- [24] Udomkun P, Wiredu AN, Nagle M, Müller J, Vanlauwe B, Bandyopadhyay R, Innovative technologies to manage aflatoxins in foods and feeds and the profitability of application—A review. *Food Control* 76: 127–138 (2017).
- [25] Gnonlonfin BG, Traore SY, Samake S. Effective control of aflatoxin contamination in staple maize food crop in Sub-Saharan Africa: A review of current pre-and postharvest low-cost technologies and perspectives. *J Agric Sci Technol* 9: 135–151 (2019).
- [26] Mutegi CK, Cotty PJ, Bandyopadhyay R, Prevalence and mitigation of aflatoxins in Kenya (1960-to date). *World Mycotoxin J* 11: 341 (2018).
- [27] Njeru NK, Midega CA, Muthomi JW, Wagacha JM, Khan ZR, Impact of push–pull cropping system on pest management and occurrence of ear rots and mycotoxin contamination of maize in western Kenya. *Plant Pathol* 69: 1644–1654 (2020).
- [28] Mahuku G, Nzioki HS, Mutegi C, Kanampiu F, Narrod C, Makumbi D, Pre-harvest management is a critical practice for minimizing aflatoxin contamination of maize. *Food Control* 96: 219–226 (2019).
- [29] Ogunade IM, Martinez-Tupia C, Queiroz OC, Jiang Y, Drouin P, Wu F, Vyas D, Adesogan AT, Silage review: Mycotoxins in silage: Occurrence, effects, prevention, and mitigation. *J Dairy Sci* 101: 4034–4059 (2018).
- [30] Ahlberg SH, Joutsjoki V, Korhonen HJ. Potential of lactic acid bacteria in aflatoxin risk mitigation. *Int J Food Microbiol* 207: 87–102 (2015).
- [31] Ma ZX, Amaro FX, Romero JJ, Pereira OG, Jeong KC, Adesogan AT, The capacity of silage inoculant bacteria to bind aflatoxin B1 in vitro and in artificially contaminated corn silage. *J Dairy Sci* 100: 7198–7210 (2017).
- [32] Sadiq FA, Yan B, Tian F, Zhao J, Zhang H, Chen W, Lactic acid bacteria as antifungal and anti-mycotoxigenic agents: a comprehensive review. *Compr Rev Food Sci Food Saf* 18: 1403–1436 (2019).
- [33] Ren H, Feng Y, Pei J, Li J, Wang Z, Fu S, Zheng Y, Li Z, Peng Z, Effects of *Lactobacillus plantarum* additive and temperature on the ensiling quality and microbial community dynamics of cauliflower leaf silages. *Bioresour Technol* 307: 123238 (2020).
- [34] Li J, Wang W, Chen S, Shao T, Tao X, Yuan X, Effect of lactic acid bacteria on the fermentation quality and mycotoxins concentrations of corn silage infested with mycotoxigenic Fungi. *Toxins* 13: 699 (2021).
- [35] Zielińska KJ and Fabiszewska AU, Improvement of the quality of maize grain silage by a synergistic action of selected lactobacilli strains. *World J Microbiol Biotechnol* 34: 1–8 (2018).
- [36] Muck RE, Nadeau EM, McAllister TA, Contreras-Govea FE, Santos MC, Kung Jr L, Silage review: Recent advances and future uses of silage additives. *J Dairy Sci* 101: 3980–4000 (2018).
- [37] Ahlberg S, Joutsjoki V, Laurikkala S, Varmanen P, Korhonen H, *Aspergillus flavus* growth inhibition by *Lactobacillus* strains isolated from traditional fermented Kenyan milk and maize products. *Arch Microbiol* 199: 457–464 (2017).
- [38] Ahlberg S, Kärki P, Kolmonen M, Korhonen H, Joutsjoki V. Aflatoxin M1 binding by lactic acid bacteria in milk. *World Mycotoxin J* 12: 379–386 (2019).
- [39] Klich MA, Identification of common *Aspergillus* species. Utrecht: Centraalbureau voor Schimmelcultures (2002).
- [40] Procedure AO. Official methods of analysis of AOAC. International 17th ed. Gaithersburg, MD, USA: Association of Analytical Communities (2000).
- [41] AOAC, Official methods of analysis, 16th edition. AOAC International, Arlington, Virginia, USA (1995).
- [42] AOAC, Official methods of analysis, 17th edition. AOAC International, Gaithersburg, MD, USA (2000).
- [43] Kosegarten CE, Ramírez-Corona N, Mani-López E, Palou E, López-Malo A, Description of *Aspergillus flavus* growth under the influence of different factors (water activity, incubation temperature, protein and fat concentration, pH, and cinnamon essential oil concentration) by kinetic, probability of growth, and time-to-detection models. *Int J Food Microbiol* 240: 115–123 (2017).
- [44] Wang S, Yuan X, Dong Z, Li J, Shao T, Effect of ensiling corn stover with legume herbages in different proportions on fermentation characteristics, nutritive quality and in vitro digestibility on the Tibetan Plateau. *Grassl Sci* 63: 236–244 (2017).
- [45] Alonso VA, Pereyra CM, Keller LA, Dalcero AM, Rosa CA, Chiacchiera SM, Cavaglieri LR, Fungi and mycotoxins in silage: an overview. *J Appl Microbiol* 115: 637–643 (2013).
- [46] Hamed MA, Abdel Ghany TM, Elhussieny NI, Nabih MA, Exploration of fungal infection in agricultural grains, aflatoxin and zearalenone synthesis under pH stress. *Int J Curr Microbiol App Sci* 5: 1007–1017 (2016).
- [47] Tai B, Chang J, Liu Y, Xing F, Recent progress of the effect of environmental factors on *Aspergillus flavus* growth and aflatoxins production on foods. *Food Qual Saf* 4: 21–28 (2020).
- [48] Elsanhoty RM, Salam SA, Ramadan MF, Badr FH, Detoxification of aflatoxin M1 in yoghurt using probiotics and lactic acid bacteria. *Food Control* 43: 129–134 (2014).
- [49] Sarlak Z, Rouhi M, Mohammadi R, Khaksar R, Mortazavian AM, Sohrabvandi S, Garavand F. Probiotic biological strategies to decontaminate aflatoxin M1 in a traditional Iranian fermented milk drink (Doogh). *Food Control* 71: 152–159 (2017).
- [50] McAllister TA, Dunière L, Drouin P, Xu S, Wang Y, Munns K, Zaheer R, Silage review: Using molecular approaches to define the microbial ecology of silage, *J Dairy Sci* 101: 4060–4074 (2018).
- [51] Fabiszewska AU, Zielińska KJ, Wróbel B, Trends in designing microbial silage quality by biotechnological methods using lactic acid bacteria inoculants: a minireview. *World J Microbiol Biotechnol* 35: 1–8 (2019).