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Effect of Selected Lactic Acid Bacteria on Growth of *Aspergillus flavus* and Aflatoxin B_1 Production in *Kutukutu*

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Abstract Inhibition of *Aspergillus flavus* growth and degradation of aflatoxin B_1 (AFB_1) by six Lactic Acid Bacteria (LAB) (*Lactobacillus brevis* G11, *Lactobacillus brevis* G25, *Lactobacillus buchneri* M11, *Lactobacillus cellobiosus* M41, *Lactobacillus fermentum* N33 and *Lactobacillus fermentum* N25) were studied in *Kutukutu*, a fermented maize-based dough largely consumed in the Northern part of Cameroon. A total of twenty nine samples of *Kutukutu* were obtained from the markets in Garoua, Maroua and Ngaoundere (three towns of the Northern part of Cameroon). Then the occurrence of AFB_1 was determined using Enzyme Linked Immuno-Sorbent Assay (ELISA). Afterward, the *Kutukutu* was prepared in the Laboratory conditions following the traditional method. This *Kutukutu* was divided into several batches. The first six batches were inoculated with the spores of *Aspergillus flavus* M15 and different LAB. The second six batches were inoculated with AFB_1 along with LAB. The batches were then incubated at 25°C and 37°C for the first and second batch respectively for 120 hours. At 24, 48, 72, 96 and 120 hours, mycelia growth and AFB_1 were monitored in different *Kutukutu*. The results showed that all the *Kutukutu* samples obtained in the markets of Garoua, Maroua and Ngaoundere were contaminated by AFB_1 and those of Maroua contained the highest concentration of AFB_1 (2.3 ppb). After 24 hours of incubation, the growth of *Aspergillus flavus* M15 was totally inhibited in the presence of *L. brevis* G11, *L. buchneri* M11 and *L. cellobiosus* M41. The similar performance was observed after 120 hours with *L. fermentum* N25 and *L. fermentum* N33. The incubation of *Kutukutu* contaminated with aflatoxin showed after 120 hours that the AFB_1 was degraded by the LAB in the following order *L. buchneri* M11 (64.2%) >*L. brevis* G25 (63%) > L. *fermentum*N33 (57.2) >*L. cellobiosus* M41 (52.3%) >*L. fermentum*N25 (45.3%) >*L. brevis* G11 (43.9%).The present findings highlight the possibility of exploiting the LAB potential in the control of aflatoxinogenic strains of *A. flavus*in *Kutukutu*.

Keywords Aflatoxin B1, *Aspergillus flavus* M15, Lactic Acid Bacteria, Fermentation

1. Introduction

Kutukutu is a popular traditional fermented paste obtained from maize (*Zea mays*). It is usually consumed in Northern part of Cameroon by adults (as breakfast) and young children (as complementary food). During the Ramadan period, *Kutukutu* is frequently taken by the Muslim community before consumption of any other food [1]. Traditional process of *Kutukutu*'s production involves several steps such as: sorting: washing (optional); soaking the maize grains; grinding; filtering the paste and decanting [1]. After the production, *Kutukutu* is commonly conserved at ambient temperature in water. The renewal of this water takes place as soon as the signs of deterioration (fermentation) are perceptible. One of the most serious problems to confront the quality of *Kutukutu* is the presence of mycotoxins which are produced by different species of the genus *Aspergillus* or *Penicillium*. Aflatoxinsare mycotoxins of greatest public health concern. It is highly toxic, mutagenic, teratogenic and carcinogenic. It is also a causative agent in human hepatic and extra-hepatic carcinogenesis [2-4]. In Africa, there are ample evidences of the direct and negative effects of aflatoxin on human health through the increase of incidence of liver cancer and its potential synergistic effect on hepatitis B [5]. Moreover, recent studies have pointed out the immunosuppressive properties of aflatoxin [4-6]. A study conducted in Togo (West Africa) revealed a high frequency of kwashiorkor due to the presence of aflatoxin in the blood of 99% of the children who consumed porridge prepared with contaminated cereal. In view of health issues and economic considerations related to the presence of aflatoxin or aflatoxigenic strains in foods, the search for antifungal agents is still of today's interest [7, 8].

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Measures have been taken to reduce the level of aflatoxin in food and prevent fungal growth in stored grains. Addition of chemicals such as benzoic acid and sorbic acid are preventive methods used so far. However, the consequences such as resistance of the species, the residual level in food, remain the major problems of their use [9]. Moreover, the consumers are looking and demanding for safe products without chemical preservatives and good shelf life. Due to these strict expectations of consumers, the concept of biopreservation is gaining popularity. This concept refers to extension of shelf life and enhanced safety of foods by the growth of the natural or added microflora and their antimicrobial products [10].

Among the microorganisms which can be potentially used as biopreservatives, LAB has demonstrated their efficacy with their Generally Recognized As Safe (GRAS) status, they have traditionally been used in food and animal feed, sauerkraut and silage.

The mechanism behind the inhibition of fungi growth hand the preserving effects of LAB relate mainly to the formation of organic acids, hydrogen peroxide, competition for nutrients and production of antimicrobial substances [11, 12, 13]. However, there is reported strain variation that boosts scientists to a perpetual exploration of strains with better potential.

In view of the high level of contamination of maize sampled in north Cameroon and the awful consequences on the health of consumers, the present investigation aim to improve the safety of fermented *Kutukutu* by developing a suitable biological detoxification procedure using selected LAB strains, which may be adopted for traditional process of fermentation.

2. Materials and Methods

2.1. Concentration of Aflatoxin B_1 in *Kutukutu* sold in North Cameroon

2.1.1. Samples Collection

Twenty nine samples of *Kutukutu,* of 100g each*,* were collected at Maroua (9 samples), Ngaoundere (11 samples) and Garoua (9 samples) during the dry season in January 2013. The samples of *Kutukutu* were aseptically transferred to storage bags, maintained in ice and transported immediately to the laboratory for further analyses.

2.1.2. Determination of Aflatoxin B_1 in *Kutukutu* soldin North Cameroon

Kutukutu collected were first dried to constant weight at 60°C and AFB_1 was extracted using the method described by [14]. Quantitative estimation of AFB_1 was made using Enzyme Linked Immuno Sorbent Assay (ELISA). Immuno enzymatic kits (Celer AFB_1, Techna, Italy) were used. The Absorbance of samples was measured at 450nm using a plate reader (Metertech, 6+ Model, Version 2.01).

2.2. Preparation and Treatment of *Kutukutu*

2.2.1 Maize sample

For the preparation of *Kutukutu*, freshly harvested maize grains of CMS 8501 variety obtained from the Institute of Research and Development, Ngaoundere-Cameroon were used in the present investigation. No infestation was detected in the grains.

2.2.2. Fungal Strain and Production of Conidia

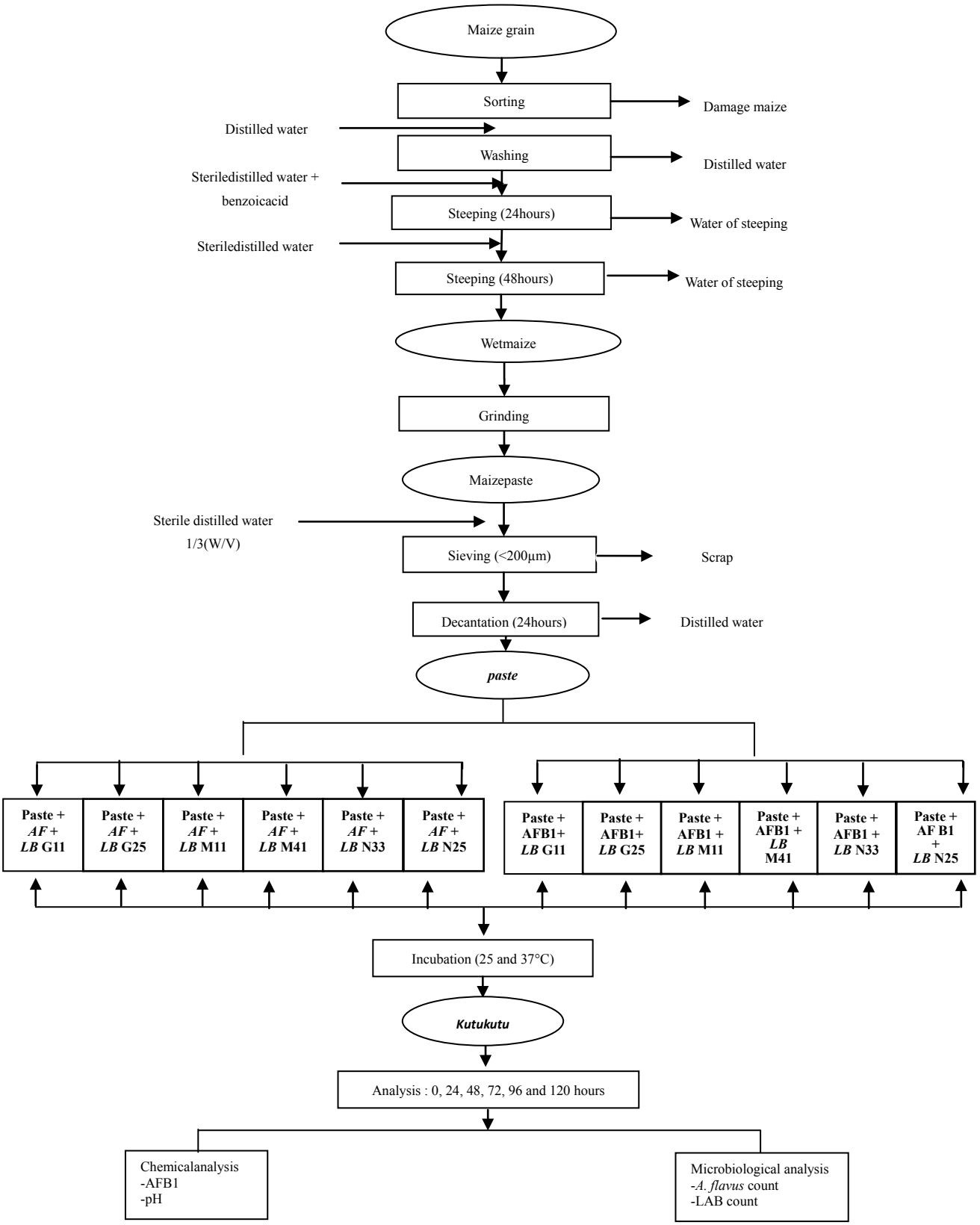
*A. flavus*M15, an aflatoxigenic strain, obtained from the Microbiology Laboratory of the National School of Agro-Industrial Sciences (University of Ngaoundere, Cameroon), was used as test microorganism. It was grown on Sabouraud dextrose agar (Difco, Detroit, MI) at 25°C for 6 days. Conidia were collected after vigorous shaking of slants with sterile peptone water (0.2% w/v). Mycelial debris were removed from conidia suspension by filtering twice through several layers of sterile damp cheese cloth [15]. The concentration of conidia was determined with a haemocytometer. The suspension was stored at 4°C until use.

2.2.3. Lactic Acid Bacteria Strains

Six LAB isolates (*L. brevis* G11, *L. brevis* G25, *L. buchneri* M11, *L. cellobiosus* M41, *L. fermentum* N33 and *L. fermentum* N25) were obtained from the Microbiology Laboratory of the National School of Agro-Industrial Sciences (University of Ngaoundere, Cameroon). Strains were subcultured from a stock culture in de Man Rogosa Sharpe broth (MRS, Difco) and incubated at 30°C for 72 hours. The perfectly insulated colonies were inoculated in test tubes containing 10mL of MRS broth and incubated at 30°C for 16 hours. The resulting preparation was centrifuged at 3000 rpm/min for 10 min and the resulting pellet was washed in 10mL of physiological peptone water (peptone 1g in saline solution (0.85% NaCl), pH 7.2) and centrifuged again at 3000 rpm/10mn. The pellet obtained was suspended in 10mL saline water. The concentration of viable cells was adjusted at 10^9 CFU/mL using McFarland Standard tube No.4.

2.2.4. Preparation of *Kutukutu*

Kutukutu was produced in laboratory following the process as illustrated in figure 1. After sorting, the maize grains were washed and steeped in sterile distilled water containing benzoic acid 6% (w/v) (E210) for 24 hours at room temperature. The grains were then steeped again in sterile distilled water for 48 hours at room temperature and grinded using a plate disc mill. The paste obtained was mixed (1/3 w/v) with sterile distilled water and sieved through a sieve of mesh 200µm. The paste was collected in a sterile container after decantation for 24 hours at room temperature.



(*AF*=A. *flavus* M15; LB G11 = L. *brevis* G11; LB G25 = L. *brevis* G25; LB M11 = L. *buchneri* M11; LB M41 = L. *cellobiosus*; LB N25 = L. *fermentum* N25; LB N33 = L. *fermentum* N33

Figure 1. Diagram of inoculation of *Kutukutu* with LAB, A. flavus M 15 and AFB_1

2.2.5. Inoculation of *kutukutu*

To assess the influence of LAB on fungal growth in *Kutukutu*, 700g of paste was inoculated with spores of *A. flavus* M15 and LAB strain at the concentrations 10^6 spores/mL and 10^9 CFU/mL, respectively (figure 1). The preparations were covered and incubated at 25°C for 120 hours. Aliquots were collected every 24 hours for microbiological analyses.

To evaluate the capacity of LAB to reduce AFB_1in *Kutukutu*, 300µg of paste was mixed with a pure solution of AFB_1(40 ppb) and 450µl of LAB inoculum (10^9 CFU/mL) was added, mixed and incubated at 37°C for 120 hours (Figure 1). Aliquots were collected every 24 hours for microbiological analyses.

2.3. PH Determination

The pH of the *Kutukutu* was determined using a pH-meter (Consort C863, Belgique) according to the method of [16].

2.4. Inhibition of Fungal Growth in *Kutukutu*

The mould count was carried out by the method described by [17]. One gram of *Kutukutu* was introduced in 9mL of saline water (0.85% NaCl) to provide the first dilution (10^{-1}). Serial dilutions were then performed and 0.1mL of the stock solution and all dilutions were inoculated on PDA medium. The preparations were incubated at 25°C for 48 hours.

2.5. Growth of Lactic Acid Bacteria

During fermentation of *Kutukutu*, LAB in the presence of *A. flavus*M15was enumerated using the method of [18]. For the analysis, 25g of *Kutukutu* were crushed and introduced into a conical flask containing a volume of 225ml of saline (0.85% NaCl) to provide a first dilution of 10^{-1}. Serial dilutions were performed by introducing 1ml of the mixture in 9ml of saline contained in a sterile test tube. Then, 0.1ml of the stock solution and all dilutions were placed on MRS agar and incubated at 30°C in an anaerobic jar. The enumeration of LAB was done after 48 hours of incubation in Petri dishes containing between 30 and 300 colonies.

2.6. Aflatoxin B1 Reduction

AFB_1 reduction in *Kutukutu* was performed according to the method described by [19]. Quantitative reduction of AFB1 was determined by using an ELISA KIT every 24 hours during 120 hours of fermentation.

2.7. Statistical Analysis

The results were analyzed using Statgraphics 5.0 (1998) for the analysis of variance (ANOVA), calculation of averages and standard deviations. Sigma plot 11.0 software was used to draw the curves.

3. Results and Discussion

3.1. Content of Aflatoxin B1 in *Kutukutu*

The AFB_1 content in *Kutukutu* sold in Maroua, Ngaoundere and Garoua after a spontaneous fermentation is shown in Figure 2. These results showed that, the *Kutukutu* sold in these 03 towns were contaminated with AFB_1. But, the levels of AFB_1 in *Kutukutu* vary according to the locality. The *Kutukutu* of Maroua registered the highest rate of AFB_1 (2.3±0.5 ppb) followed by the town of Ngaoundere (1.3±0.4 ppb). The rate of AFB_1 in the *Kutukutu* of Maroua was higher than the standard norm fixed by the European Union. The maximum permissible limits for AFB_1 should not exceed 2 ppb in peanuts, nuts, dried fruit, processed products as well as cereals and their derivatives [20].

3.2. PH Determination

The pH change in *Kutukutu* fermented with LAB is shown in Figure 3. After 120 hours of fermentation, the *Kutukutu* inoculated with *L. brevis* G25 had the lowest pH (2.7) follow by pH (3.1) of *Kutukutu* inoculated with *L. buchneri* M11 and *L. cellobiosus* M41.

The decrease in pH can be explained by the hydrolysis of carbohydrates in *Kutukutu* by various lactic ferments. This hydrolysis of carbohydrate is accompanied by the production of organic acid which are responsible for the reduction of the pH [21]. Wedad *et al.* [22] also showed a decrease in pH respectively in the sorghum cultivar Mugud and Karamaka after 16 hours of spontaneous fermentation at 28°C. Ejigui *et al.* [23] noted a similar reduction in pH (3.3) in the yellow corn flour after 96 hours of fermentation at 30°C. Asmahanand Muna [24] obtained a reduction of pH in the sorghum paste fermented with *Lactobacillus strains* (*L. fermentum, L. brevis and L. amylovorus*) after 6 hours of controlled fermentation at 37°C.

3.3. Inhibition of *Aspergillus flavus* M15 growth

The effect of fermentation of *Kutukutu* by various LAB on *A. flavus* M15 growth is shown in figure 3. *A. flavus* M15 was completely inhibited after 24 hours of fermentation of *Kutukutu* by *L. buchneri* M11, *L. brevis* G11 and *L. cellobiosus*M41, while control tube exhibited an increase in the number of spores during fermentation. After 48 hours of fermentation, the growth of *A. flavus*M15 was reduced to 90% in the *Kutukutu* fermented with *L. fermentum* N25. At the same time, the growth of *A. flavus* M15 was completely inhibited by *L. fermentum* N33.

The antifungal properties of LAB have already been reported by a few authors. Most of them belong to the genera *Lactococcus* and *Lactobacillus*. Zuo *et al.* [25] showed the antifungal activity of *L. casei*CGMCC1 against *A. flavus* in liquid medium. Omemu [26] showed that the growth of fungal populationwas significantly ($P<0.05$) reduced from 6.8 to 3.7 Log_{10}CFU/g after 12 hours of fermentation of *Ogi* (fermented corn paste). Similarly, Roy *et al.* [27] showed antifungal activity of six LAB against *A. flavus* IARI. The interest for LAB in biopreservation of food is growing. Their efficacy lays on the production of wide range of antifungal and antimicrobial compounds such as lactic acid, acetic acid,

and bacteriocin. Lavermicocca *et al.* [28] demonstrated that the antifungal compounds such as phenyllactic acid and 4-hydrophenyllactic acid were produced by *Lactobacillus plantarum*.

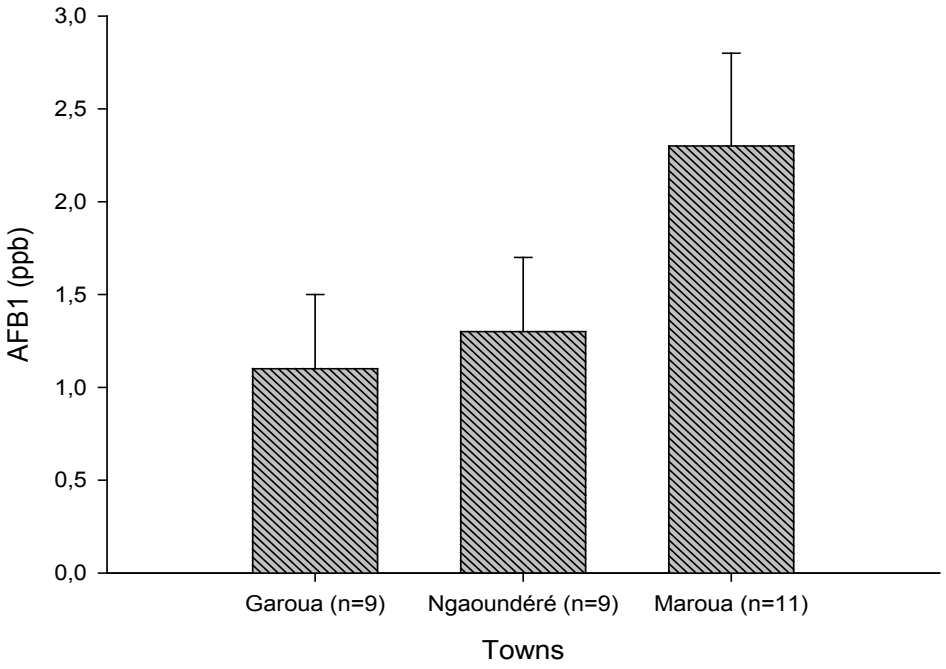
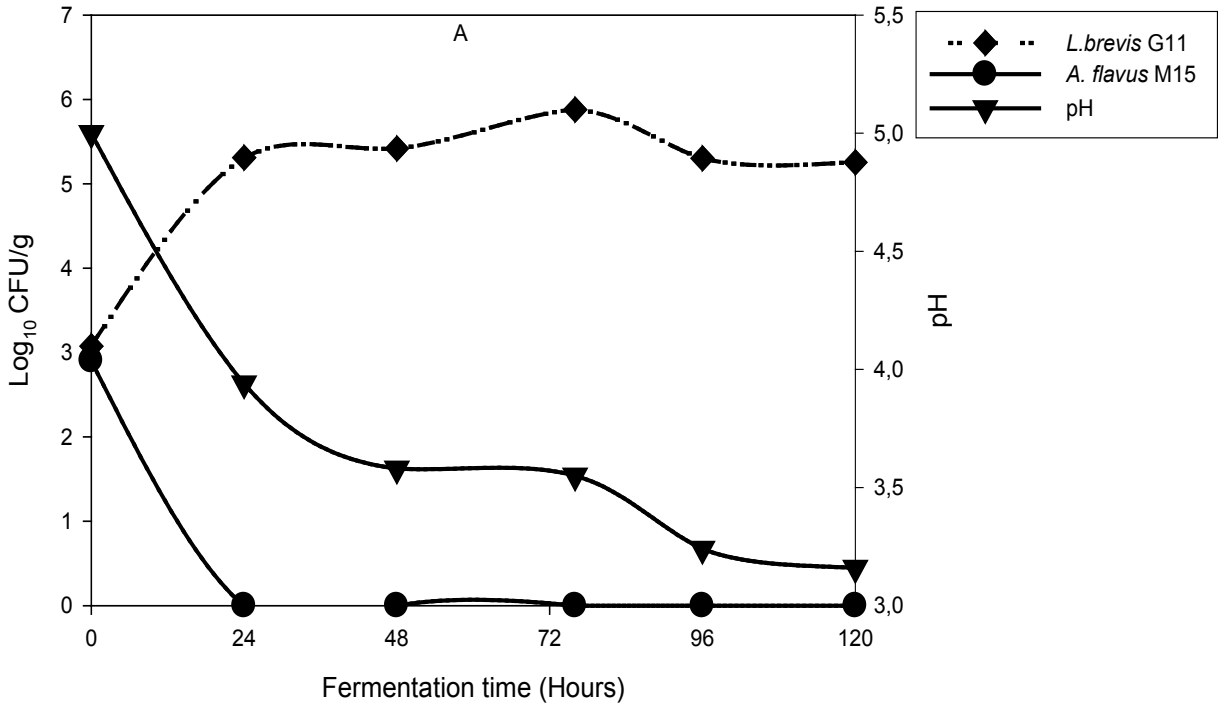
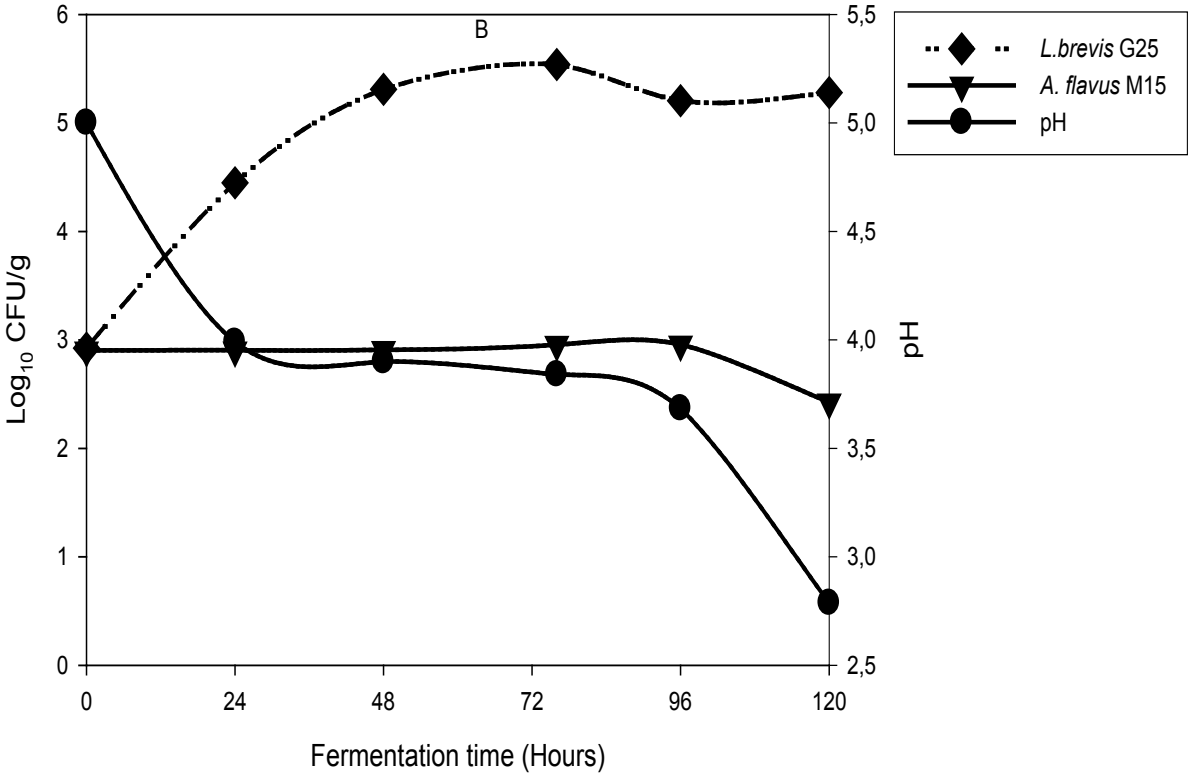


Figure 2. AFB_1 content in *Kutukutu* sold in Maroua, Garoua and Ngaoundere




B
Log10 CFU/g
pH
Fermentation time (Hours)
L.brevis G25
A. flavus M15
pH
0
1
2
3
4
5
6
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3,0
3,5
4,0
4,5
5,0
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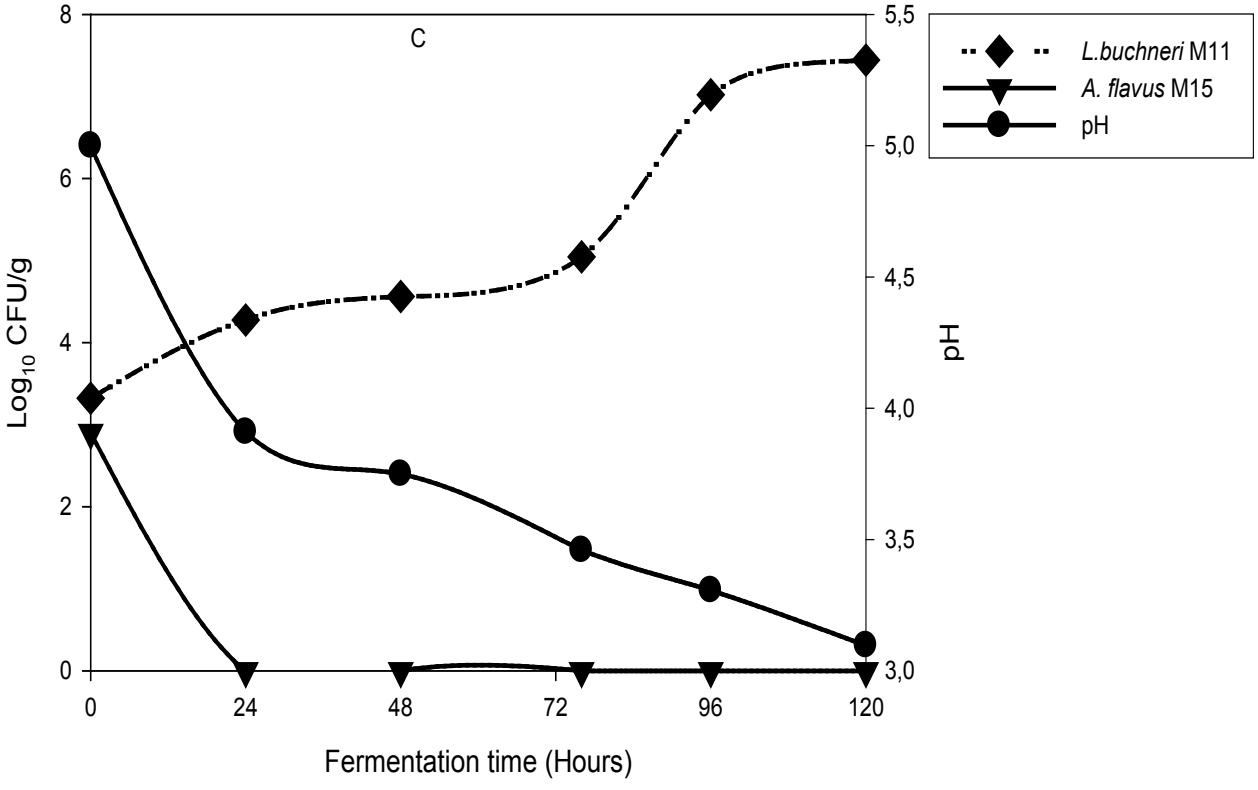
24
48
72
96
120


C
Log10 CFU/g
pH
Fermentation time (Hours)
L.buchneri M11
A. flavus M15
pH
0
2
4
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8
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3,5
4,0
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5,0
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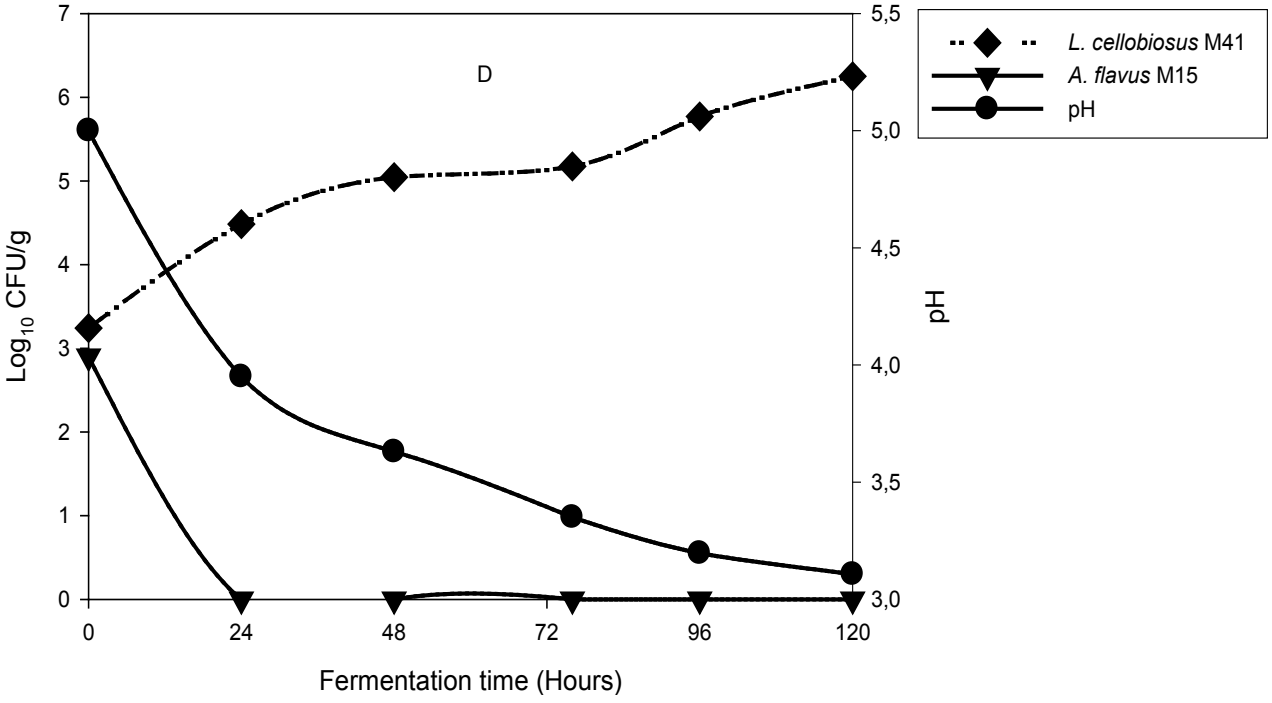
96
120


D
Log10 CFU/g
pH
Fermentation time (Hours)
L. cellobiosus M41
A. flavus M15
pH
0
1
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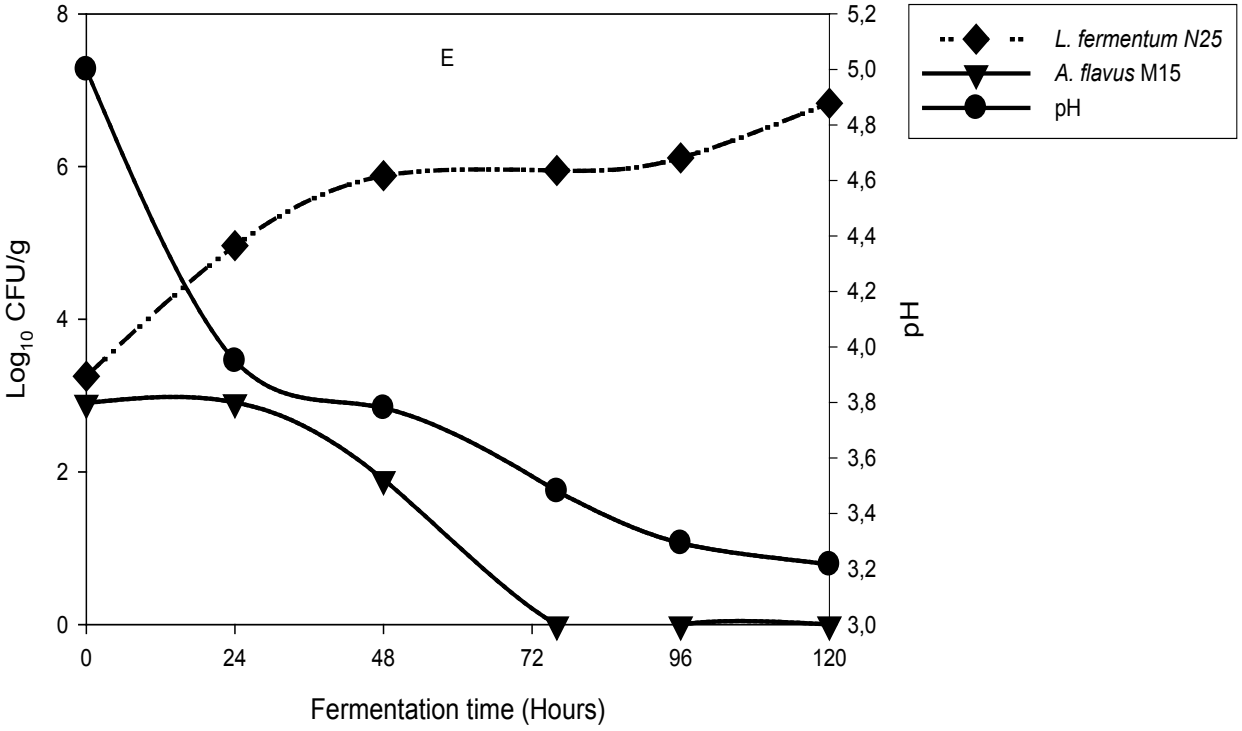
E
Log10 CFU/g
pH
Fermentation time (Hours)
L. fermentum N25
A. flavus M15
pH
0
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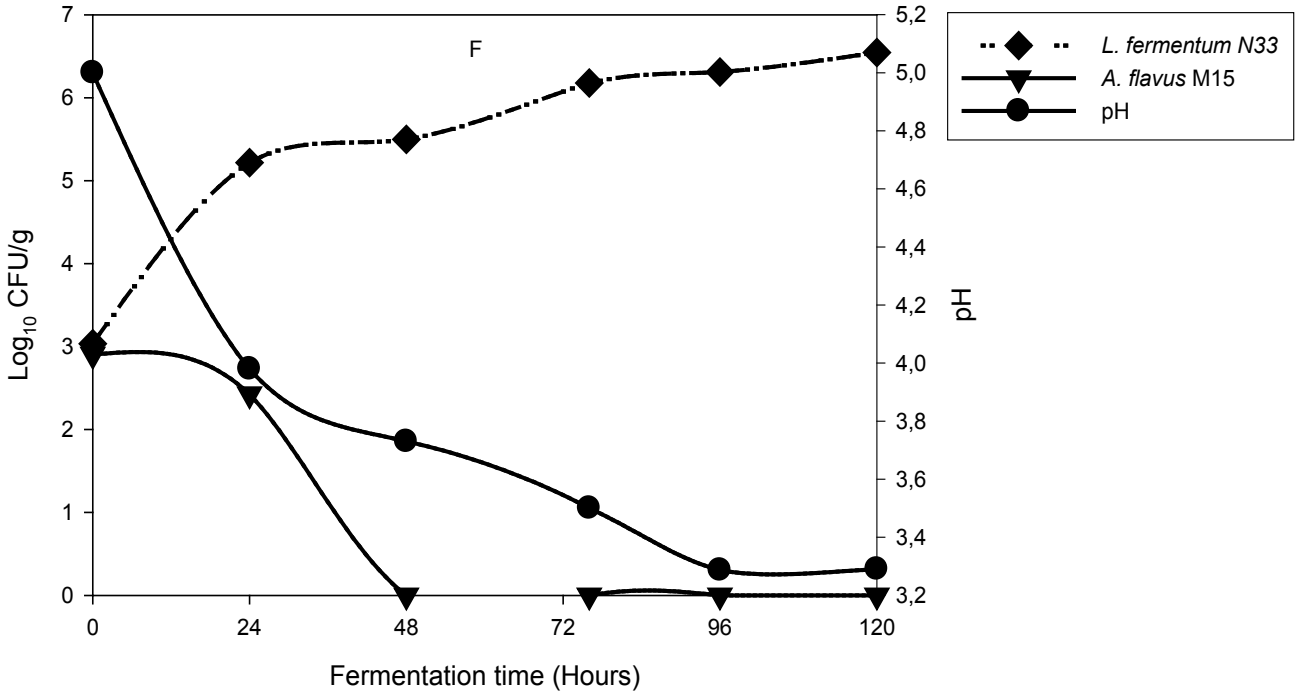

Figure 3. Inhibition of *A. flavus* M15 growth in *Kutukutu* during fermentation by various LAB (A = *L. brevis* G11; B = *L. brevis* G25; C = *L. buchneri* M11; D = *L. cellobiosus*; E = *L. fermentum* N25 and F = *L. fermentum* N33) at 25°C

3.4. Reduction of Aflatoxin B_1 in *Kutukutu*

The figure 4 (A and B) give the removal percentage of AFB_1 in *Kutukutu* fermented with LAB. It was observed soon after 24 hours of incubation, a reduction of AFB_1 in *Kutukutu* fermented with all the LAB. A significant decrease of AFB_1 was observed in *Kutukutu* fermented with *L. fermentum* N33 (21.8%) and *L. brevis* G25 (19.1%). However, the highest AFB_1 reduction ranging from 6.5 to 2.3 ppb (64.2%) and 6.5 to 2.4 ppb (63%) was observed respectively in *Kutukutu* fermented with *L. buchneri* M11 and *L. brevis* G25 after 120 hours. Studies conducted in fermented foods also showed the reduced of AFB_1 by LAB [29].

The mechanism of removal of AFB_1 by LAB is still unclear, but it was hypothesized to depend on the LAB cell wall structure [30]. Other theory suggested that removal of AFB_1 is through noncovalent binding of mutagens by fractions of the cell wall skeleton of LAB [31]. Alternative mechanism of aflatoxin B_1 removal has been reported. LAB fermentation opens up the aflatoxin B1 lactone ring resulting in its complete detoxification [32]. In addition to this, the lower pH could also contribute to the removal of AFB_1 in *Kutukutu*. Studies have shown that treatment of LAB pellets with hydrochloric acid significantly enhanced the binding ability of the bacteria [33]. This hypothesis is supported by the weak pH found in the *Kutukutu* fermented with *L. buchneri* M11 (3.1) and *L. brevis* G25 (2.7) (figure 3). This is an agreement with the finding of [34] who concluded that the pH may contribute to the transformation of AFB1 to the non-toxic AFB_{2a} in acidogenous yogurt. Similar results were also reported elsewhere [29, 35]. Rasic *et al.* [36] found that the fermentation of yogurt and the acidification of milk containing AFB_1 greatly reduced the amount of the toxin.

3.5. Growth of Lactic Acid Bacteria

The growth of LAB in *Kutukutu* in the presence of *A. flavus*M15 is presented in figure 3. The results show that, even in the presence of the moulds, we observed the growth of LAB during the fermentation. This increase of LAB was ranging from 2.9 to 7.4 Log10CFU/g. Conversely, the moulds were inhibited in the presence of low pH it was observed that the LAB can multiply with weak pH (2.7-3.1) during fermentation (Figure 3). This could be explained by their acidophilic character. Ogunbanwo *et al.* [37] reported that the genus *Lactobacillus* commonly predominates during food fermentation are the most aciduric of all LAB.

After 72 hours of fermentation, there was growth reduction from 5.8 to 5.2 Log_{10}CFU/g and 5.5 to 5.2 Log_{10}CFU/g for *L. brevis* G11 and *L. brevis* G25 respectively. *L. buchneri*M11, *L. cellobiosus*M41, *L. fermentum* N25 and *L. fermentum*N33 showed an exponential growth until 120 hours of fermentation. For *L. buchneri* M11 and *L. fermentum* N25, growth ranged from 3.3 to 7.4 Log_{10}CFU/g and 3.3 to 6.3 Log_{10}CFU/g after 120 hours of fermentation.These results are in agreement with few reports showing that the population of LAB is increasing with the time of fermentation [24, 38, 26].

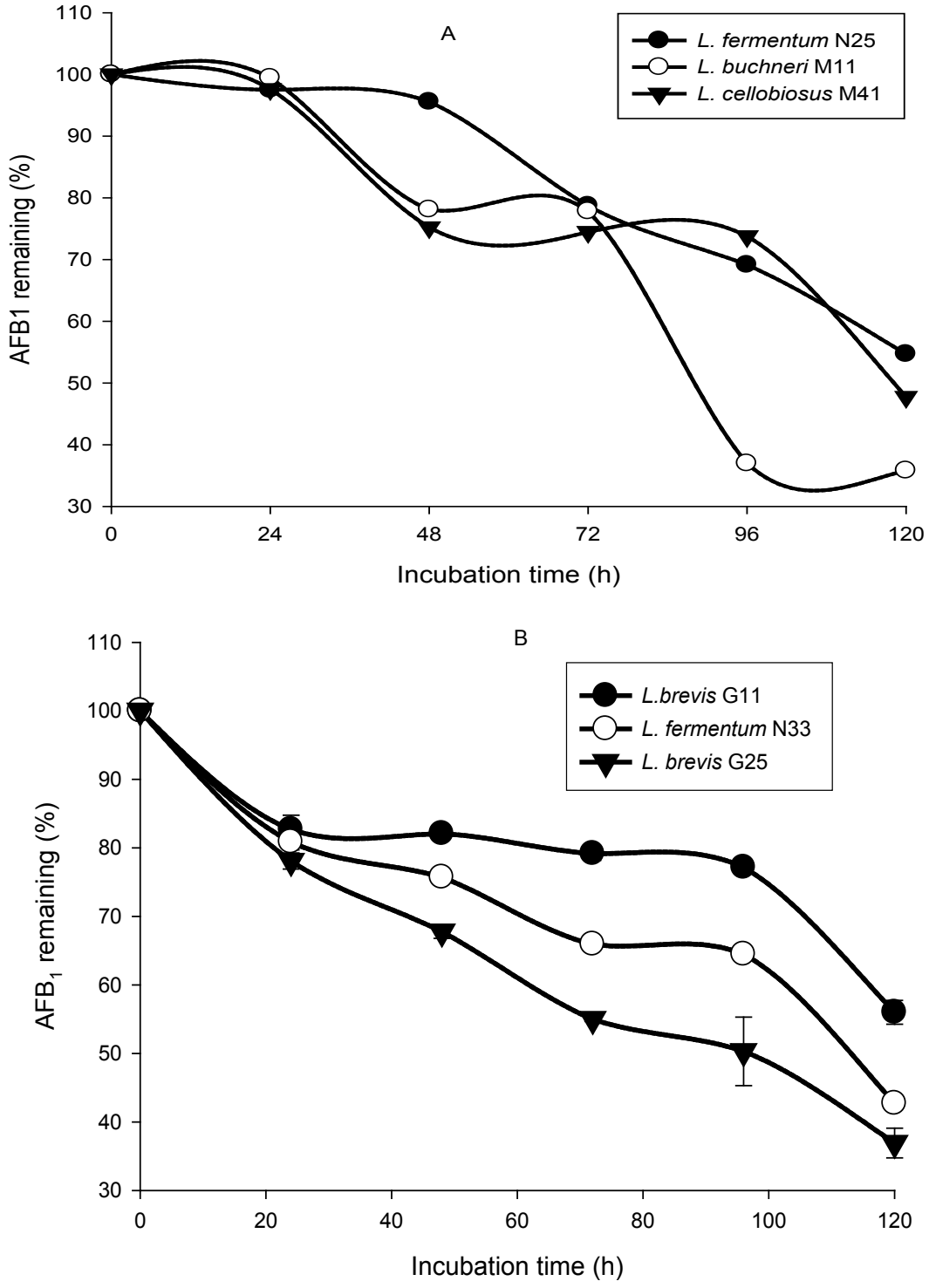

Figure 4. Remaining percentage of AFB_1 in *Kutukutu* fermented with LAB (A = *L. fermentum* N25, *L. cellobiosus* M41 and *L. buchneri* M11; B = *L. brevis G11*, *L. fermentum*N33 and *L. brevis G25*)

4. Conclusions

LAB used in this work have shown their ability to reduce the pH during fermentation. This certainly helped to significantly reduce fungal growth and AFB_1 content in *Kutukutu* during fermentation. With the increasing interest in food safety throughout the world, this LAB cultures with high antifungal and antimycotoxigenic potential could be of immense value in limiting AFB_1 in the food.

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