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Determination of the Biodetoxification Potentials of *Lactobacillus Brevis* on Aflatoxin M1 from Fresh Raw Cow Milk within Zaria Metropolis

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Abstract

Background: The negative impacts of Aflatoxins on the economy and health led to investigations for strategies to prevent their contamination in food and feed. Biological methods gained popularity due to its friendliness to both environment and body health.

Aim: The aim of this research work is to determine the bio-detoxification potentials of Lactobacillus brevis on Aflatoxin M1 (AFM1) from fresh raw cow milk.

Methodology: L. brevis was isolated from locally fermented cow milk, the isolates were identified using cultural, morphological, physiological, biochemical and sugar fermentation test. Nine (9) samples were collected from three (3) different sampling sites within Zaria metropolis namely; Dan-Magaji, Kufena and Gabari, three (3) samples each from the sampling sites. The samples were screened for AFM1 contamination using a rapid test kit specific for milk samples (Ring Biotechnology Co., Ltd. Art no.:100004-96T). AFM1 contaminated samples were subjected to HPLC analysis to determine the extent of contamination. Surface binding assay and HPLC analysis were used to quantify the amount of unbound AFM1 in the samples, LAB-AFM1 complex stability testing and recovery of bound AFM1 were also conducted. The result were analysed using 'ANOVA' single factor, 2 way ANOVA and Duncan's multiple range test.

Results: L. brevis was isolated from locally fermented cow milk. All the samples collected were contaminated with aflatoxin M1 at varying concentrations, Lactobacillus brevis isolates were able to bind AFM1 at a range of 36-69%, LAB-AFM1 complex stability was about 63% and 68% of the bound AFM1 was recovered.

Conclusion: It can be concluded from this research that lactic acid bacteria can be a good option for reducing the level of AFM1 contamination from milk samples as they are harmless and generally recognized as safe for human consumption.

Keywords: Bio-detoxification; Aflatoxin M1; Milk; Lactobacillus brevis; High Performance Liquid Chromatography(HPLC)

Abbreviations: PEM: Protein Energy Malnutrition; LAB: Lactic Acid Bacteria; GRAS: Generally Recognized as Safe; EU: European Union; AFB1: Aflatoxin B1; AFM1: Aflatoxin M1; NAFDAC: National Agency for Food and Drug Administration and Control.

Introduction

According to the Food and Agriculture Organization of the United Nations [1], a quarter of the world's food crop is spoiled by filamentous fungi and thus should be rejected for food safety reasons at the expense of the food supply of a steadily rising world population. More than 250 mold types that produce mycotoxins are particularly problematic. Among the known mycotoxins, aflatoxins are the most important [2]. Milk is considered a staple food for humans of all age groups due to its high nutritional value [3]. It plays a central role in human diet and therefore holds a great economic significance on the global nutritional level [4]. However, it may also be a source of natural food contaminants that may cause disease.

Lactobacillus Brevis

L. brevis is a Gram-positive, non-spore forming rod shaped obligate heterofermentative bacteria that ferment glucose primarily to lactic acid, CO_2 and ethanol. They generally are non-respiratory and lack catalase with a DNA base composition of about 44-47 (mol %) G+C, they grow anaerobically, but unlike most anaerobes, they can grow in the presence of O2 as "aerotolerant anaerobes". Although they lack catalase, they possess superoxide dismutase and have alternative means to detoxify peroxide radicals, generally through peroxidase enzymes [5]. *L. brevis* is normally isolated from milk, cheese, plants, sewage, fermented vegetables, cow manure and the intestinal tract of humans and rats [6]. Mycotoxins including aflatoxin have affected most crops grown worldwide; however, the extent of aflatoxin toxicity varies according to commodities [7]. It has been reported that approximately 25-50 % of world's agricultural crops are contaminated with mycotoxins, among which aflatoxin is the most significant [8]. It has been estimated that more than 5 billion people in developing countries worldwide are at risk of chronic exposure to aflatoxins through contaminated foods [9].

On the international scale, mycotoxins have also cost Nigeria huge losses as they cause reduced yield and food shortage in the country. This affects the quantity of produce available for export, another major problem lies in the perception and policies of the buyer nations. The culminative effect of fungal infestation of the farmland and stored produce summarily gives a bad international reputation of all agricultural products emanating from the country. This leads to reduced demand of agricultural produce from the country, or a total ban on the goods leading to economic loses [10]. The International Agency for Research on Cancer has classified AFB1 and AFM1 as class 1 human carcinogen [11]. Aflatoxin M1 has been well known to have cytotoxic, genotoxic, and carcinogenic effects [12]. Human exposure to AFM1 is due to the consumption of contaminated milk and dairy products of which daily intake could be highly variable in the world. Infants represent the most susceptibly exposed population due to their high consumption of dairy products either as cow's milk and related by-products in their diet or from breast milk where the mycotoxin can be excreted [13]. Dietary exposure to aflatoxin M1 is one of the major causes of hepatocellular carcinoma, the fifth most common cancer in humans worldwide and suppress the immune system particularly for population that test positive for the hepatitis B virus [14]. Aflatoxin M1 have been implicated in the pathogenesis of protein energy malnutrition (PEM), a condition affecting more than 118 million (32% of) children in the developing world and hence the development of kwashiorkor, marasmus, immunosuppression and underweight in infants. In dairy cattle, consumption of feeds contaminated with aflatoxins causes death due to hepatic damage, decreased milk production, immunity suppression and reduced oxygen supply to tissues due to anemia, which reduces appetite and growth [15].

Harmful effects caused by aflatoxin have directed researchers towards finding new strategies for prevention and detoxification in order to preserve the safety of products intended for human and animal consumption [16]. several approaches have been applied to detoxify AFs in crops and during postharvest such as the physical, chemical and biological methods [17], but there is a common drawback of physical and chemical treatments, since they may cause a significant decline in the quality of food products, losses of nutritional value, high cost and cause undesirable health effects [18]. Over the past decades. The use of selected microorganism to control aflatoxins and postharvest disease has greatly increased, providing an attractive alternative tool for removing toxins and safeguarding the value of food and feed. Biological control provides safe methods to remove aflatoxins from foods [19]. Several studies have reported the capability of many microorganisms, including bacteria, yeast, fungi, actinomycetes and algae in removing or degradation of aflatoxins from food and feed [20]. Among all types of available microorganisms that may be utilized to remove aflatoxin from contaminated medium, lactic acid bacteria (LAB) would be a suitable choice for reducing the bioavailability of aflatoxins because of their unique characteristics, they are Generally Recognized as Safe (GRAS) by USFDA, and also some of them have beneficial effects on health which are termed probiotics [19]. The European Union (EU) has set the limit of aflatoxin B1 (AFB1) in feed for dairy cattle to be 5 ng/kg, while the limit for aflatoxin M1 (AFM1) in milk is 50 ng/kg which was adopted in Nigeria by the National Agency for Food and Drug Administration and Control (NAFDAC) as 50 ng/L and 25 ng/L in milk intended as food for adults and infants, respectively. The Codex Alimentarius limit is 500 ng/kg [21].

Materials and Methods

The study was conducted in Zaria local government of Kaduna State. *L. brevis* was isolated from locally fermented cow milk, the isolates were identified using cultural, morphological, physiological, biochemical and sugar fermentation test using carbohydrate free MRS medium.

Sugar Fermentation Tests

Nine (9) ml carbohydrate free MRS broth medium was used containing 1% of the desired sugar, (Arabinose, Cellobiose, Melibiose, Raffinose, Ribose, and Sucrose). Phenol red indicator was added to each tube and 1ml (10^7) CFU/ml of the fresh test culture was inoculated, the tubes were incubated at 37°c for 48hours. Colour change from red to yellow indicates the utilization of the sugars by the isolates. The results were compared with the standard table of Bergey's Manual of Systematic Bacteriology after the experiment (Bergey's Manual of Systematic Bacteriology 8th edition).

Sample Collection and Screening for AFM1

One (1) litre each of Nine (9) fresh raw cow milk samples were collected and labelled from three (3) different Fulani settlements namely; Dan-magaji, Gabari and Kufena within Zaria metropolis for AFM1 screening. The samples were transported in ice packs to Multiuser Science Research Laboratory ABU Zaria for analysis. Rapid test kit specific for milk samples was used to screen samples based on manufacturer's instructions (Ring Biotechnology Co., Ltd. Art no.:100004-96T). The kit utilizes high affinity of monoclonal antibody against AFM1 which can easily identify its contamination in milk. Its AFMI detection limit can meet both EU and USA acceptable limits.

Quantification of Aflatoxin M1 Level from Positive Samples Using HPLC Analysis

Aflatoxin M1 standard was obtained from R-Biopharm (Darmstadt, Germany). HPLC grade methanol, acetonitrile and water were obtained from Fisher Scientific Company, UK. The liquid chromatographic system (1260 Infinity Agilent Technologies, USA) consisted of a HPLC pump, an auto injector, a column oven, and a fluorescence detector. The HPLC conditions for analysis of AFM1 were as follows: column, Hypersil 5AA-ODS 200 x 2.1mm (Agilent Technologies, USA); column temperature, 25°C; mobile phase, water: acetonitrile: methanol (60:30:10); flow rate, 0.7ml/min, retention time 2m, injection volume 5µl and detector, fluorescence spectrophotometer (excitation 360 nm; emission 440 nm).

Extraction and Purification of Aflatoxin M1 (AFM1)

The extraction procedure was performed as previously described by Ruangwises and Ruangwises [22]. One hundred (100) ml of raw cow milk sample was measured into a 100ml glass beaker and were placed in a freezer to attain a temperature of 4°C. The sample was pipetted into a 50ml plastic centrifuge tubes. The milk samples were defatted by centrifugation at 4,000 rpm for 10 min. Fatty layer was separated and filtered using whattman filter size 4, the resulting skimmed milk was transferred into a 50ml plastic syringe with a Luer tip which was attached to an immuno affinity column. The skimmed milk was allowed to flow into the column by gravity at a flow rate of approximately 2 ml/min. After the skimmed milk had run through, 20 ml of PBS was used to wash the column at a flow rate of 5ml/min. Air was passed through the column to remove residual liquid.

AFM_1 was eluted from the column at a flow rate of 1 drop/second with 1.25 ml of acetonitrile: methanol (60:40v/v) and 1.25 ml of HPLC water giving a total volume of 2.5 ml. One hundred (100) µl was injected into the HPLC system and AFM_1 in the final solution was determined using HPLC analysis. Equation for the amount of aflatoxin is made according to the following;

$$Wm = Wa \times \left(\frac{Vf}{Vi}\right) \times \left(\frac{1}{VS}\right)$$

Where: Wm = Amount of aflatoxin M1 in the test sample in µg/ L
Wa = Absorbance corresponding to area of aflatoxin M1 peak of the test extract (ng) Vf = Final volume of re-dissolved eluate (µL)
Vi = Volume of injected eluate (µL)
Vs = Volume of test portion (milk) passing through the column (mL) [23]

Quantified levels of AFM1 from the samples were analysed using ANOVA single factor and mean AFM1 were separated using Duncan's Multiple range test

Determination of Aflatoxin M1 Binding Capacity of LAB Using Surface Binding Assay

Preparation of Bacterial Inoculum: The Lactic acid bacteria used was cultivated in 100ml MRS Broth at 37°C for 48hrs anearobically. The bacterial inoculum was adjusted and maintained at 0.5 and 1.0 McFarland standard which is equivalent to $1.5x10^8$ and $3.0x10^8$cfu/ml using a U.V visible spectrophotometer (Agilent technologies) at a wavelength of 600nm and absorbance range of 0.08-0.1 and 0.225-0.257 for 0.5 and 1.0 McFarland respectively.

Surface Binding Assay: Two (2) ml of the bacterial inoculum equivalent to 0.5 McFarland ($1.5x10^8$ cells) and 1.0 McFarland ($3.0x10^8$ cells) were inoculated in 8ml aflatoxin M1 contaminated milk, the inoculated milk was incubated at 4°c and 37°c for 30min and 60min respectively, after 30min 5ml of the inoculated milk was taken and centrifuge at 3500g for 10min and same after 60min. Unbound AFM1 in the supernatant was quantified using HPLC analysis. All assays were performed in triplicate and both positive control (known concentration of aflatoxin M1) and negative control (milk free from aflatoxin M1 with LAB strain cultures) were included. Detection was done by fluorescence with excitation and emission wavelengths of 360 and 440 nm, respectively. The retention time was 1-2 min using a very sensitive hypersil 5AA-ODS 200 x 2.1mm column. The percentage of AFM1 removed was calculated using the equation 100 x [1 - (peak area of AFM1 in the test sample)/(peak area of AFM1 in the positive control)] [24].

Determination of Lactic Acid bacteria-aflatoxin Complex Stability: The stabilities of the bacteria-AFM1 complexes were evaluated by determining the amount of AFM1 remaining bound following three washes. Bacterial pellets (sedimented portion that accumulates after centrifugation) were washed with PBS then, suspended in Milli-Q water (1.5 ml) and incubated at room temperature for 10 min. After centrifugation, the supernatant was not removed so as not lost some part of the released AFM1, the tube was shooked well and the released AFM1 was quantified by HPLC, and the percent AFM1 bound was calculated as % Bound = sum of AFM1 released after three washes/bound AFM1 X 100. This washing procedure was repeated another two times.

Recovery of the Bound Aflatoxin: The aflatoxin bound to lactic acid bacteria was recovered by suspending the washed bacterial pellets in chloroform (5 ml). The chloroform was evaporated using Nitrogen stream evapouration, and the residue was dissolved in methanol (1ml) and was quantified using HPLC analysis.

The % recovered AFM1 was calculated as recovered AFM1/bound AFM1 X 100. Results were analysed using excel and SPSS version 20.

Results and Discussion

Isolate	Colony morphology	Gram reaction and morphology	Spore staining	Temperature survivability test			Salt tolerance test	Catalase test	Indole	Citrate	motility
				15°	30°	45°					
LAB A	Large cream colored colonies with rough edges	Gram +ve short rods in chains	-	-	+	+	+	-	-	-	-
LAB B	Convex small rough flat colonies	Gram +ve rods in chains	-	+	+	-	+	-	-	-	-
LAB C	Large cream colored colonies with rough edges	Gram +ve cocci in clusters	-	-	+	-	+	-	-	-	-
LAB D	Small, dull, flat opaque colonies with rough edges	Gram +ve coccabacilli in chains	-	-	+	-	-	-	-	-	-
LAB E	Round cream colored colonies	Gram +ve cocci in pairs	-	-	+	+	+	-	-	-	-

Table 1: Colonial morphology, Physiological and some Biochemical tests for determination of *Lactobacillus brevis*.

Table 1 shows result for some morphological, microscopic, physiological and biochemical test for the identification of *Lactobacillus brevis* from locally fermented cow milk samples. Five (5) different species of LAB were isolated and were further characterized using sugar fermentation tests.

Isolates	Arabinose	Cellobiose	Raffinose	Ribose	Sucrose	Melibiose
LAB A	-	+	+	-	+	+
LAB B	-	+	-	+	+	-
LAB C	+	+	-	-	+	-
LAB D	+	-	+	+	-	+
LAB E	+	-	+	+	+	-

Table 2: Sugar fermentation tests.

Table 2 shows result for the sugar fermentation tests, and based on the results obtained from Table 1 and 2, LAB. B was selected as *L. brevis* according to Bergey's Manual of Systematic Bacteriology.

Table 3 shows result for screened milk samples aflatoxin M1 contamination, all the samples were contaminated with AFM1 after testing with a rapid test kit specific for milk samples, the kit had a limit of detection of 0.05ppb which is equivalent to 0.05ng/ml/g. This result is the same with the reports of Maureen, et al. [25] in Kenya, the authors collected 96 raw milk samples and all the samples were contaminated with AFM1.

S/N	Location	No. of samples tested	No. positive
1	Dan-magaji	3	3
2	Gabari	3	3
3	Kufena	3	3
	Total	9	9

Table 3: Screening of raw cow milk samples for AFM1 using rapid test strip from three (3) collection sites.

S/N	Location	No. of samples tested	AFM1 in each sample(µg/L)	Mean AFM 1 concentrations/location (µg/L)	No. below EU limit(<0.05µg/L)	No. above EU limit(>0.05µg/L)
1	Dan-magaji	3	119.99	101.02[a]	0	3
			102.84			
			80.22			
2	Gabari	3	71.15	60.71[b]	0	3
			58.62			
			80.22			
3	Kufena	3	79.28	62.96[b]	0	3
			62.34			
			47.26			

Table 4: Quantified AFM1 from raw cow milk samples using HPLC analysis.

The results were analyzed using Annova single factor, statistically there is significant differences between the amounts of quantified AFM1 and sampling locations. Calculated p-value is 0.000785 which is less than 0.05 at 95% confidence interval. Means were separated using Duncan's multiple range test, mean obtained from location Dan-Magaji is significantly different from other sampling sites and the samples collected from the location had the highest level of AFM1 contamination (101.02 µg/L), followed by location Kufena (62.96 µg/L) and location Gabari (60.71 µg/L). This variation may be due to differences in the type of feed given to the animals (as animals in location Dan-Magaji are being fed with cotton seed meal. Cotton is among the products highly vulnerable to AF attack while the animals in the other two locations are being fed with Maize fiber and grasses), metabolic activities and the degree of AFB1 contamination in the feed.

Odeda and Atanda, et al. [26], they reported the AFM1 level in the range of 2.04-4.00 µg/L in processed milk and ice cream in Abeokuta, Nigeria. This variation in level of AFM1 may be due to differences in samples considered as fresh raw milk samples were used in this study while in their study processed products were considered (milk and ice cream).

Markaki and Melissari [27] investigated the levels of AFM1 in commercial pasteurized milk in Greece and reported AFM1 ranging from 0.5 to 5ng/L. In Portugal, Martins and Martins [28] studied the levels of AFM1 in 31 samples of raw and 70 samples of heat-processed milk, and found that 80.6% of raw milk and 84.2% of heat-processed milk samples were contaminated with AFM1. Among raw milk samples, 54.8% contained levels of AFM1 between 5 and 10mg/L and 19.3% had levels between 21 and 50mg/L. However, the occurrence of AFM1 in milk and milk products has been reported in the Mediterranean region including Egypt [29].

Pittet [30] reported that concentrations of AFM1 in raw milk are usually less than 0.1ng/L in Europe, but might be greater than 1.0ng/L in other parts of the world. Varying levels of AFM1 in milk have been reported in surveys carried out in various parts of the world, although other factors might contribute to production of fungal toxins in food and feedstuffs. Fardos, et al. [31] reported AFM1 contamination of cow, sheep and goat milk as 95%, 62% and 40% in Jeddah, Saudi Arabia. The authors reported that all the samples collected were contaminated with AFM1 but in levels below the US recommended limit (0.5ppm) thus, the milk is safe for human consumption.

Dan-magaji	**Inoculum Density**	**1.5×10^8(CFU/mL)**				**3.0×10^8(CFU/mL)**			
	Temp.(°C)	4°	4°	37°	37°	4°	4°	37°	37°
	Time(m)	30	60	30	60	30	60	30	60
	Bound AFM1(µg/L)	8.8	14.1	23.3	32.9	28.4	29.8	36.4	43.2
Gabari	**Inoculum Density**	1.5×10(CFU/mL)				3.0×10(CFU/mL)			
	Temp.(°C)	4°	4°	37°	37°	4°	4°	37°	37°
	Time(m)	30	60	30	60	30	60	30	60
	Bound AFM1(µg/L)	3.6	4.7	5.6	16.8	18.7	27.2	32.7	48.9
Kufena	**Inoculum Density**	**1.5×10^8(CFU/mL)**				3.0×10^8(CFU/mL)			
	Temp.(°C)	4°	4°	37°	37°	4°	4°	37°	37°
	Time(m)	30	60	30	60	30	60	30	60
	Bound AFM1(µg/L)	3.8	5.0	9.1	6.9	6.4	14.1	22.6	29.1

Table 5: AFM1 binding assay using *L. brevis* isolates at 0.5 and 1.0 McFarland standard, 4° and 37°C temperature and circulation time of 30 and 60minutes.

Table 5 shows result for AFM1 binding assay, *L. brevis* removes about 69% of AFM1 from raw cow milk and the highest binding occurred at a temperature of 37°c, cell density of 1.0 McFarland ($3.0x10^8$cfu/ml) and circulation time of 60minutes.

AflatoxinM1 binding by LAB and incubation temperature could be strain dependent, some strains could bind AFM1 best at low temperatures while others may prefer moderate temperature range. This finding was in accordance with Rayes [32], who found that the optimum temperature for AFM1 removal from milk was 37°C and the lowest removal at 5°C and Diaa, et al. [33] reported that *L. acidophilus* bind AFM1 best at a temperature of 37°C but in contrary, *L. plantarum* bind AFM1 best at 4°C in whole milk. Incubation time may differ hugely from strain to strain, however, as a very quick procedure, increasing in the incubation time does not expand LAB strains to bind more mycotoxins. In this research binding of significant amount of AFM1 was achieved at 60minutes of incubation, El- Nezami, et al. [24] found that by varying the incubation time, there was no significant difference in the amount of the removed AFB1 by LAB strain, the process was fast and binding was observed since the 1st minute. While, Kasmani, et al. [34] reported that the amount of the adsorbed AFB1 in PBS buffer by LAB strains was time dependent recording the best adsorbing after 12 hours. Khanafari, et al. [35] showed that *L. plantarum* bound AFB1 at the rate of 45% in 1 hour and total binding after 90 hours was observed. Likewise, Sezer, et al. [36] found that AFB1 binding by *L. plantarum* and *L. lactis* was almost complete in the first 6 hours. Binding process is dose-dependent, and the quantity of mycotoxins binding will rise dramatically due to increasing in cell concentration [37].

S/N	Location	Bound AFM1(µg/L)	Released AFM1 after 1st wash(µg/L)	Released AFM1 after 2nd wash(µg/L)	Released AFM1 after 3rd wash(µg/L)	%Released	%Bound
1	Dan Magaji	43.2	14.02	2.07	0.42	38	62
2	Gabari Kufena	48.9	11.26	2.89	0.1	29	71
3	Kufena	29.1	7.52	5.03	0.08	43	57

% Released = Sum of released AFM1 after 3 washes/Bound AFM1 X 100
% Bound = 100 - %Released
Table 6: Results for *L. brevis*-AFM1 complex stability test.

Table 6 shows result for *L. brevis*-AFM1 complex stability testing, the complex formed was relatively stable (63%). The calculated p-value is 0.2015 which is > α- at 95% confidence interval. (0.05), statistically there is no significant differences between the amount of AFM1 released after a series of three (3) washes, therefore H_o is accepted.

S/N	Location	Bound AFM1(µg/L)	Recovered AFM1(µg/L)	%Recovered AFM1 (µg/L)
1	L.D	43.2	36.83	85
2	L.G	48.9	29.65	61
3	L.K	29.1	17.22	59

%Recovered = RecoveredAFM1/Bound AFM1 X 100
Table 7: Result for AFM1 recovery using *L. brevis* in the three (3) locations.

Table 7 shows result for AFM1 recovery, about 68% of the AFM1 bound by *L. brevis* was recovered.

Paired sample t-test was used to analyze the result for AFM1 recovery, calculated p- value is 0.001 which is less than α at 95% confidence interval, since p- value is < 0.05, H_o is rejected. There is a strong correlation between bound AFM1 and recovered AFM1 (as the amount of bound AFM1 increases, the amount of recovered AFM1 also increases), the value obtained is 0.933. Statistically, there is significant difference between the amount of bound and recovered AFM1, p-value is 0.001. This also showed that AFM1 binding by LAB is reversible, if the process of solvent extraction continued, probably more of the bound AFM1 will be recovered. Bovo, et al. [38] reported that the LAB/AFM1 complex was unstable and the amount of toxin released varied widely from strain to strain. Corroborating with this Serrano-Niño, et al. [39] reported that AFM1 binding capacity was reversible process since all strain tested released a small portion of bound AFM1 after a single wash with PBS.

Conclusion

All the samples were found to contain AFM1 at a level above EU set limit which was adopted by National Agency for Food and Drug Administration and Control (NAFDAC) in Nigeria. *Lactobacillus brevis* was able to reduce the level of AFM1 contamination 69% from the samples and forms a relatively stable complex with AFM1, the binding is also reversible since about 68% of the bound AFM1 was recovered, and this further showed that LAB bind AF to their cell components and binding site may differ from strain to strain.

Recommendations

- Regulatory agencies should employ adequate monitoring to ensure that AFM1 levels are below the set limit in milk and milk products.
- The use of LAB could be a good option for reducing the levels of AFM1 in milk and milk products as they are generally recognized as safe for human consumption by USFDA.

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