Inhibitory Effect of Lactic Acid Bacteria on Aflatoxin B1 Producing *Aspergillus flavus* Isolated from Garri

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ABSTRACT

Among the potential benefits of probiotic lactic acid bacteria (LAB) is their practical use for biopreservation of foods, fungal decontamination, and novel biotherapy. A number of ready to eat foods hawked in streets of Nigeria such as garri are prone to contamination by mycotoxin-producing fungi. In this study the inhibitory effect of lactic acid bacteria (LAB) (sourced from local foods (nono and kunun- zaki)) and their bacteriocins-derivatives on aflatoxin B1 -producing *Aspergillus flavus* was conducted. Out of the five LAB isolates screened three (two strains of *Lactococcus* and one of *Pediococcus* sp) had high indices of probiotic potentials and bacteriocins extracts were obtained from them and used for the inhibition study. Forty samples of garri sold in Jos metropolis were screened for *A. flavus* and their abilities to produce Aflatoxin B1 using the Coconut Extract Agar (CEA) and Ammonia Vapour Test (AVP) methods. The three most probiotic LAB and their respective crude bacteriocin extracts were used to test for their inhibitory effect on aflatoxin producing *A. flavus* isolates. The results of the study showed that while only 17 samples (42.50%) had *A. flavus* in them, only three of the fungi ((17.65%) demonstrated capacity to produce Aflatoxin B1, making (7.5% of the total number of garri samples that had aflatoxin-producing *A. flavus*. The inhibition of fungal growth by LAB and bacteriocins was dose dependent. At an inoculum concentration of 1 ml, *Pediococcus* sp had the highest mean zone of inhibition of 43.0 mm followed by *Lactococcus* sp (Strain LAC20G with radial diameter 38.0mm and *Lactococcus* sp (LAC20G) which had a radial diameter of 29.3mm respectively. The results of the study portend that the growth of aflatoxin producing *Aspergillus flavus* in ready to eat foods such as garri could be prevented by application of appropriate dosages of bacteriocins extracts from lactic acid bacteria.

Keywords: Aflatoxin, *Aspergillus flavus*, bacteriocin, garri, lactic acid bacteria.

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I. INTRODUCTION

About 25% of the global food output is contaminated by mycotoxin causing significant economic losses (Ostry *et al.*, 2017). Aflatoxins are the most common, widespread, and most potent of all mycotoxins that contaminate food, and aflatoxin B1 (AFB1) commonly produced by *A. flavus* is responsible for about 75% of all aflatoxin contamination of foods (Zhang *et al.*, 2012) with a distribution of 43.75% in African countries as reported by (Darwish *et al.*, 2014). The World Health Organization (WHO) report indicates that over 500 million of the poorest are exposed to unsafe mycotoxins levels, most of which live in Sub-Saharan Africa, and in Nigeria, contaminations of various food commodities have been reported across all the agro-ecological zones. The world

health organization (WHO) in 2018 reported that an aflatoxin concentration of 1mg/kg or higher has been suspected to cause aflatoxicosis and aflatoxin B1 (AFB1) dose of 20-120microgram/kg BW per day is acutely toxic and potentially lethal (WHO, 2018) and according to (Wagacha & Muthomi, 2008). Due to diseases exacerbated by aflatoxin, 40% productivity loss has been incurred in developing countries. Garri is a staple, consumed by many people, and are exposed to contamination by toxigenic fungi especially those that produce aflatoxins which may be pre-harvest, post-harvest, processing phenomena, or during storage. It is hygroscopic and can absorb moisture which supports the growth of aflatoxigenic fungi.

Little or no work has been done in respect to reduction of aflatoxin in gari particularly, and not so much attention has

been paid as regards aflatoxin detection and reduction in garri bringing to bear that it is widely consumed as well as recognition of the fact that it is prone to contamination by aflatoxin B1 producing *Aspergillus flavus* and its dangers to human health. The LD50, that is, the amount or dose that is fatal to 50% of a population exposed to AFB1 is at or less than 18mg/kg BW (Kate, 2019) and more worrying of all is that normal boiling temperature cannot decontaminate foods containing aflatoxins except at sterilization temperature as decomposition temperature is at about 237-306°C (Jalili & Scotter, 2015). Although, bacteriocin has been found effective in inhibiting the growth of microorganism including fungi little have been done concerning aflatoxin B1 (AFB1) producing *A. flavus* isolated from garri.

Aflatoxins are a problem of worldwide importance in public health, agriculture, and economics (Trucksess *et al.*, 1977) with a significant impact on the health and further, the economy of a nation. The safety and quality of food are of importance if such food should be consumed especially ready to eat foods, justifying the need to identify aflatoxigenic fungi such as *Aspergillus flavus* capable of growth and contamination of street vend garri and the presence of aflatoxin B1 (AFB1) as their presence in foods could pose health challenges due to increasing cancer and immunosuppression. Lactic acid bacteria (LAB) and their bacteriocins have recently been of significant commercial interest as a natural food preservative to increase food safety and stability. Garri is a food staple in Nigeria consumed by millions of people (Tolulope & Godbless, 2015) (45-50% of the population), are prone to contamination by aflatoxin B1 (AFB1) producing *A. flavus*, and little or no work is available on it been decontaminated. The study, therefore, will go a long way as a contribution towards identifying more lactic acid bacteria isolated from local sources (e.g Nono, Kunun-zaki, etc.) capable of inhibiting the growth of aflatoxin B1 producing *A. flavus* isolated from garri. This research aims to determine the inhibitory effects of lactic acid bacteria (LAB) on aflatoxin B1 producing *Aspergillus flavus* isolated from garri sold in Jos metropolis.

II. MATERIALS AND METHODS

A. Collection of *Aspergillus flavus* Isolates

A total of 17 *A. flavus* isolated from 40 garri samples were collected from the University of Jos Microbiology Laboratory.

B. Confirmation of *A. flavus* Identity

Re-identification of *A. flavus* isolates was done by seeding pure isolate on PDA and incubated at 25 + 2 °C for 5-7 days. After incubation, macroscopic observation of colonies was done, and microscopy of distinct colonies also was carried out as well using lactophenol cotton blue. The morphological characteristics of *A. flavus* isolates were described microscopically according to (Klich, 2002).

C. Test for Aflatoxin B1 Producing *A. flavus*

1) Coconut-Based Medium – UV Test for Detection of Aflatoxin Producing *A. flavus*

Screening for aflatoxin production potentials was carried on coconut extract agar (CEA) with 40µg/ml

chloramphenicol to suppress bacterial growth, following standard methods (Davis *et al.*, 1987). All the pure fungal isolates were inoculated on CEA and incubated at 27 °C for 5 days. By observing reversed sides of plates under 365 nm ultraviolet lamb, the aflatoxin-producing potential of the isolates was determined. The emission of a characteristic blue fluorescence confirmed the presence of aflatoxin-producing potentials of the isolates with inoculated CEA as medium control.

2) Ammonia Vapor Test for Detection of Aflatoxin Producing *A. flavus*

The fungal isolates were inoculated on CEA as single colonies by cork borer (5 mm) diameter in the center of the plate and incubated in the dark at 28 °C for 7 days. The dish was inverted, and 1 or 2 drops of concentrated ammonium hydroxide solution were placed on the inside of the lid of the Petri dish. Over the lid containing ammonium hydroxide, the agar plates were inverted. A control was prepared as mentioned above.

D. Collection of Lactic Acid Bacterial Isolates and Identification

A total of thirteen (13) different Lactic Acid Bacteria isolated from Kunun-zaki and Nono were collected from the University of Jos microbiology Lab. and re-identified. Identification was done by observation of morphological characteristics, microscopic appearance, and biochemical testing.

III. RESULTS

The morphological, microscopic, as well as biochemical characteristics of the lactic acid bacteria isolated from Nono and Kunun-Zaki samples from various locations in Jos, were characterized. Morphological characteristics observed include size color, colony elevation, margin, and shape. Six of the isolates were gram-positive, catalase-negative, oxidase negative most of which appear cocci in pairs, and short rods were under the microscope, the rest were catalase-positive and gram-negative. Sugar fermentation tests done on cocci isolates that are gram-positive and catalase-negative using six different sugars which includes sucrose, glucose, fructose, mannose, lactose, maltose reveals four isolates showed different levels of fermentation of the different sugars which was observed by a change of color from purple to yellow showing acid production. Production of gas in Durham tubes was also observed. Three isolates were selected identified as *Pediococcus* sp. and *Lactococcus* sp. A and B.

Probiotic potentials testing was carried out on the isolates. Antibacterial susceptibility tests of the isolates to three different test organisms which include *S. aureus*, *E. coli*, *Bacillus* sp. and level of inhibition is represented by a zone of inhibition as shown in Table II below *S. aureus* was susceptible to all five lactic acid bacteria with a zone of inhibition of 23, 22, 28, 20, 21 mm, *E. coli* was susceptible to 2 and *Bacillus* sp. to (3) three of lactic acid bacteria. All lactic acid bacteria isolates showed different tolerance to NaCl at a different concentration as represented in the chart (Fig. 1) below. Determination of milk coagulation prototype 1% (v/v) of the probiotic culture bacteria was done. Determination of tolerance of the isolates to phenol at different concentrations

carried out showed all isolate tested showed a different level of tolerance to phenol as represented in Table III.

TABLE I: ANTIMICROBIAL SENSITIVITY TESTING OF LACTIC ACID BACTERIA ISOLATES FROM KUNUN-ZAKI AND NONO

Isolate Name	Isolate Code	Zone of Inhibition (mm)		
		<i>S. aureus</i>	<i>E. coli</i>	<i>Bacillus sp</i>
<i>Pediococcus sp.</i>	LCG20A	23	15	21
<i>Lactococcus sp.</i>	LCG20F	22	R	R
<i>Lactococcus sp.</i>	LCG20G	28	21	25
<i>Pediococcus sp.</i>	LCG20J	20	R	R
<i>Lactococcus sp.</i>	LCG20M	21	R	5

TABLE II: PROBIOTIC POTENTIALS OF LACTIC ACID BACTERIA USING PHENOL TOLERANCE TEST AS AN INDEX

Conc. Of Phenol:	0.10%	0.30%	0.60%
LAB Isolate	Absorbance (nm)		
LCG20A	0.4	0.42	0.39
(<i>Pediococcus sp.</i>)			
LCG20G	0.48	0.42	0.25
(<i>Lactococcus sp.</i>)			
LCG20M	0.36	0.38	0.27
(<i>Lactococcus sp.</i>)

Fig. 1. Probiotic Potential Measured by NaCl Tolerance of *Lactococcus sp* and *Pediococcus sp* Isolated from kunun-zaki and Nono.

Key: Column; A = sample LCG20A, B = sample LCG20F, C = sample LCG20G, D = samples LCG20M.

Seventeen *A. flavus* isolated from forty gari samples from four markets in Jos metropolis were considered. Detection of aflatoxin B1 and non-aflatoxin B1 isolated using Coconut based - U.V and ammonium vapor test revealed that out of the seventeen (17) *A. flavus* isolates that were isolated from the 40 garri samples representing a presence frequency of

42.5%, 3(17.6%) isolates coded as Af05-Gby1, Af09-Ty1 and Af10-Ty2 were aflatoxin B1 positive in both methods (Table IV). The detection by UV light at 365nm recognized aflatoxigenic by the production of blue-green fluorescent colonies in the center of glass Petri dish of CEA in the reverse, from non-fluorescence non inoculated CEA plate.

The inhibitory effect of the bacteriocin producer isolates identified as *Pediococcus sp.* and *Lactococcus sp* A and B tested using a modified overlay method of Magnusson and Schnurer (2001), showed a different level of inhibition. The LAB isolates assayed for possible inhibition of the *Aspergillus flavus* isolates result is as shown in Table IV. All the LAB isolates inhibited at least one aflatoxin B1 producing *A. flavus* isolate to varying extents. The highest inhibition (32 mm) was observed with *Pediococcus sp* against all three *A. flavus* isolates. Least inhibition was observed with *Lactococcus sp* (B) inhibiting only *A. flavus* (Af09-Ty1) with a zone of inhibition of 14 mm. The inhibitory effect of the crude lactic acid bacteria bacteriocin extracts was also tested by inoculating 2 ml, 1 ml of stock *A. flavus* mycelia solution as well as 1ml of dilution 101, 102, 103, and 104 on PDA by pour plate method. *Pediococcus sp* has the highest zone of inhibition of 35 mm at the lowest mycelia dilutions of 2 ml of stock mycelia solution on *A. flavus* (Af05-Gby1), *Lactococcus sp* (A) has the highest zone of 25 mm on *A. flavus* (Af05-Gby1) and *Lactococcus sp.* (B) has the highest zone of inhibition of 20 mm on *A. flavus* Af09-Ty1 and Af10-Ty2. Maximum Zone of inhibition observed by *Pediococcus sp*, *Lactococcus sp* (A), and *Lactococcus sp.* B at highest mycelia dilution of 0.0001/ml are 58,56 and 48 mm, respectively on *A. flavus* isolate Af10-Ty2, Af09-Ty1, and Af05-Ty1 respectively, measured in millimeters is shown in Table V.

TABLE III: INHIBITORY EFFECT OF LACTIC ACID BACTERIA ON AFLATOXIN B1 PRODUCING *A. FLAVUS* ISOLATES OBTAIN FROM GARI SAMPLES

LAB Isolates	Aflatoxin Producing <i>A. flavus</i> Isolates		
	Af05-Gby1	Af09-Ty1	Af10-Ty2
LCG20A	++	++	++
<i>Pediococcus sp.</i>			
LCG20G	+	-	+
<i>Lactococcus sp.</i>			
LCG20M	-	+	-
<i>Lactococcus sp.</i>			
Control (-)	-	-	-

Key: - = No inhibition, + = Inhibition < 30 mm, ++ = Inhibition > 30 mm, +++ = Inhibition.

TABLE IV: FREQUENCY OF OCCURRENCE OF AFLATOXIN B1 PRODUCING *A. FLAVUS* ISOLATED FROM GARI SOLD IN 4 LOCATIONS

Location	Total no of sample	Occurrence of AfB1 producing <i>A. flavus</i>			
		No. of <i>A. flavus</i>	%	No. of AfB1 positive <i>A. flavus</i> in both Coconut bases medium and Ammonia vapor test	%
Bukuru	10	2	20.00	0	0.00
Gada-biu	10	5	50.00	1	20.00
Taminus	10	5	50.00	2	20.00
Chobe	10	5	50.00	0	20.00
Total	40	17		3	17.60

TABLE V: INHIBITORY EFFECT OF EXTRACTS OF BACTERIOCIN FROM LAB ON VARYING CONCENTRATION OF MYCELIA OF *ASPERGILLUS FLAVUS* MEASURED BY ZONE OF INHIBITION

AFB1-producing isolate	Conc. of <i>A. flavus</i> mycelia (ml/Conc.)	Lactic acid bacteria Crude extract (0.1ml)			Mean zone of inhibition mm & STDEV
		<i>Pediococcus</i> sp.(LCG20A) (mm)	<i>Lactococcus</i> sp.(LCG20G) (mm)	<i>Lactococcus</i> sp.(LCG20M) (mm)	
Af05-Gby1	2	35	25	15	25.00 $\pm$ 10.00
	1	38	40	30	36.00 $\pm$ 5.29
	0.1	40	45	40	41.60 $\pm$ 2.89
	0.01	45	48	42	45.00 $\pm$ 3.00
	0.001	50	55	44	49.70 $\pm$ 5.50
	0.0001	56	55	48	53.00 $\pm$ 4.36
Af09-Ty1	2	20	15	20	18.30 $\pm$ 2.89
	1	45	45	28	39.30 $\pm$ 9.81
	0.1	55	55	35	48.30 $\pm$ 11.55
	0.01	50	58	40	49.30 $\pm$ 9.01
	0.001	55	55	42	50.76 $\pm$ 7.51
	0.0001	56	56	40	50.76 $\pm$ 9.24
Af10-Ty2	2	24	20	20	21.30 $\pm$ 2.31
	1	40	30	30	33.30 $\pm$ 5.77
	0.1	46	35	32	37.70 $\pm$ 2.08
	0.01	50	48	38	45.30 $\pm$ 6.43
	0.001	54	55	40	49.70 $\pm$ 8.39
	0.0001	58	55	45	52.70 $\pm$ 6.81

P-values: Af05-Gby1= 0.2323, Af09-Ty1= 0.0004, Af10-Ty2 = 0.1034 (Alpha value = 0.05).
P-value for mean zone of inhibition = 0.6576

Fig. 2 Mean Zone of Inhibition Curve of Bacteriocins against Varying *Aspergillus flavus* Mycelia Concentration. (Series 1, 2 and 3 represented by color blue, red and grey curve is the Mean Zone of inhibition of bacteriocins extract in mm against a concentration of *A. flavus* Af05-Gby1, Af09- Ty1 and Af10- Ty2 respectively in ml.

Fig. 3. Inhibition of *Aspergillus flavus* by crude extract of bacteriocin from *Pediococcus* sp, *Lactococcus* sp A and B respectively with distilled water (H₂O) used as control on opposite wells.

IV. DISCUSSION

The study indicated that out of the total of thirteen (13) lactic acid bacteria isolated from kunun-zaki and Nono, five (5) cocci were tested for their antimicrobial effect out of which three (3) demonstrated antimicrobial effects. Organism identified includes *Pediococcus* sp, *Lactococcus* sp A and B with isolate code of LCG20A, LCG20G, and LCG20M respectively which conforms with the research findings of (Peyer, 2017) and that of (Okekeaji, 2017). They were the lactic acid bacteria (LAB) from which the bacteriocins were isolated and used for the test of their inhibitory effect on aflatoxin-producing *Aspergillus flavus*. *Pediococcus* sp and *Lactococcus* sp, which showed a higher degree of antimicrobial effect among all LAB tested, have been known for their bacteriocins producing potentials which agree with a report by (El-Nezami, 1998) on the ability of dairy strains of LAB to bind a common food carcinogen, aflatoxin B1 and (Savadogo *et al.*, 2006) on Bacteriocins and lactic acid bacteria.

The research showed that three (3) (17.6 %) out of the 17 species of *A. flavus* produced aflatoxin B1. This result compares with those reported by (Klich, 2002). The difference between the aflatoxin-producing potential of these three could be due to several factors among which may include environmental conditions such as relative humidity, temperature, water activities medium type, etc. as reported by (Davis *et al.*, 1987) and (O'Riordan & Wilkinson, 2009).

It was shown that *Pediococcus* sp. had the highest inhibitory effect on aflatoxin-producing *A. flavus* followed by the *Lactococcus* sp. (coded as LCG20G). This study showed, however, that the effect of *Pediococcus* sp. on aflatoxin producing *A. flavus* is much higher than that of *Lactococcus* sp. which is slightly in variance with a study reported by (Onilude *et al.*, 2005).

This study clearly demonstrated that the inhibitory effect of crude bacteriocins extracts was significantly higher on aflatoxin-producing *A. flavus* than those of the live organisms

used. This is expected since the bacteriocins extract are indeed the major antagonistic principle behind the probiotic function of LAB (Aljewicz, & Cichosz, 2017; Aljewicz *et al.*, 2014; Aljewicz *et al.*, 2014b).

V. STATISTICAL ANALYSIS

The inhibitory effects of the bacteriocins were dose-dependent, this is proved by the significance in the P-value (< 0.05) of the mean zone of inhibition as clearly shown on the graph in Fig. 2.

VI. CONCLUSION

The inhibitory effects of bacteriocins on aflatoxin-producing *A. flavus* increase with the increase in dose level of bacteriocin and vice versa. Identification of LAB with high ability as well as low dose level of bacteriocins capable of inhibiting aflatoxin producing *Aspergillus flavus* from ready to eat food will improve food safety and ensure sustainable quality of foods such as garri.

VII. CONCLUSION

Lactic acid bacteria and their bacteriocin thus can be used as probiotics (as food supplements especially in widely consumed readily to eat foods such as garri) to inhibit the growth of aflatoxin producing *Aspergillus flavus* which may produce these toxins that have a deleterious health effect on humans. More studies should be done in this regard, by identifying more lactic acid bacteria isolated from different local sources, and their inhibitory effect is tested in comparison against toxigenic fungi especially aflatoxin B1 producing fungi as well as identifying factors that can influence the inhibitory effect of the lactic acid bacteria.

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