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Isolation and Characterization of Potential Anti-mycotoxigenic Bacteria from Traditional Fermented Food Products in Nigeria

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ABSTRACT:
Several bacteria species secrete bioactive compounds that are antagonistic to mold and their associated toxins which lead to food spoilage. Indigenous food sources serve as a reservoir for industrially relevant bacteria. This study aimed at screening for indigenous lactic acid bacteria from nono(Fermented cow milk) and African locust beans (dawadawa) that possess inhibitory activity against *Aspergillus niger*, the causative agent of Aflatoxicosis in food. 1ml of milk and 1g of mashed dawadawa samples were serially diluted onto cyclohexamide supplemented MRS agar plates. Sugar fermentation and growth pattern analysis at different pH, NaCl, temperatures and 16S rRNA sequencing using 27F/1492R primers were used for bacteria characterization. Pure isolates obtained were assayed for their activity against aflatoxin producing *Aspergillus niger* using the dual culture method. Plates were incubated at 25^{o}C and monitored daily for percentage growth inhibition. Data obtained revealed a 16.6-53.3%, 6.6-50% inhibition of *Aspergillus niger* at 72 hours and a 41.6-71.6%, 16.6-68.5% growth inhibition of fungal growth at 120 hours for all isolates obtained from nono and dawadawa samples respectively. Growth pattern analysis revealed maximum bacteria growth after 96 hours at 33^{o}C and 37^{o}C from nono and dawadawa samples. All isolates exhibited growth in broth media containing 4.5% and 6.5% NaCl as well as in media at pH 4.5 and 9.6. Biochemical characterization revealed that all isolates were catalase negative with the ability to ferment sucrose, lactose, glucose and maltose, lactulose, trehalose and mannitol. Nucleotide sequence analysis identified these isolates as *Bacillus cereus, Lactococcus lactis, Bacillus pumilus, Bacillus valismortis, Lactobacillus plantarum, Lactobacillus fermentum, Lactobacillus helveticus, Leuconostoc citreum and Lactobacillus delbruecki*. The results of this study identified indigenous lactic acid bacteria as promising biological control agents for further testing against aflatoxicosis in food stuff.

Keyword: *Lactobacillus sp., Nono, Dawadawa, Aspergillus niger, Aflatoxicosis, growth inhibition*

INTRODUCTION:

Mycotoxins, a metabolic product churned out by filamentous fungi and yeasts, represents a major health and industrial challenge to the food industry globally as its presence in food triggers a noxious response in human referred to as mycotoxicosis [1,2,3]. Mycotoxins target both the Central and Peripheral Nervous Systems thus leading to the classification of some as neurotoxins while others generally referred to as toxins disrupt a range of normal metabolic functions [4]. The results of such include but are not limited to impaired protein synthesis, necrosis, extreme immune-deficiency, deterioration of liver and kidney function in humans, etc [5].

Researcher have discovered that the persistence of mycotoxins in processed and unprocessed foodstuffs is due to their impervious ability to withstand chemical and physical treatment options once they have established themselves irrespective of processing or storage techniques employed [6]. Aflatoxin is one of such identified toxins of mycological origin. Within the last decades, methods including pasteurization and UHT of milk products has proved ineffective towards the aflatoxin M1 reduction [7]. The inclusion of preservatives like potassium sorbate also proved ineffective when employed to thwart the growth of different mycotoxin-producing Penicillum species [8].It was also revealed that the filamentous fungi and yeast that produce these toxins are acid resistant, capable of utilizing a range or organic acids as a source of carbon, in addition to their inherent ability to grow at relatively high salt concentrations [9], [10]. In general, species of Geotrichum (51%), Aspergillus (34%), Mucor (6%) and Fusarium (3%) represent the most predominant mold strains commonly isolated in dairy products such as raw milk [11]. Controlling the surge of contamination by these pathogens and or their mycotoxins, researchers had turned towards a combination of preservatives and antimycotics such as natamycin to resolve the issue [12]. Although results from that study proved effective, toxicity studies revealed that preservatives like the previously accepted potassium sorbate was shown to be genotoxic to the human peripheral blood lymphocytes in vitro [13]. With this, the search for antimycotics, preferably of natural origins has been largely sought after. This has been bolstered by the notion that natamycin, a natural occurring antibiotic which stems from Stretomyces sp., inhibits the proliferation of filamentous fungi, molds and yeasts in relatively small quantities [12].

The broad range of antimicrobial compounds from organisms that are antagonistic to each other can be deployed towards the prevention of mycotoxicosis as well as the preservation of dairy products. Studies indicated that the secondary metabolites secreted by Saccharomyces cerevisiae alongside that produced by Candida krusei both avert the growth of mycotoxin-producing microorganism like Penicillum citrinum, Aspergillus flavus and Aspergillus parasiticus [13], [14]. Another report suggested that Debaryomyces hansenii secrete antimycotics with prevent the growth and proliferation of mycotoxin producers like Bysschlamys fulva, Byssochlamys nivea, Cladosporium sp., Eurotium chevalieri, Penicillium candidum and Penicillium roqueforti [15].

Lactic acid bacteria (LAB); Lactococcus, Lactobacillus, Leuconostoc and Pediococcus are industrially relevant microorganisms utilized in food and beverage fermentation practices due to a large extent to their antifungal activities which doubles with their organoleptic functions [8,16]. LAB are present in a wide range of fermented food products found globally which infers that with the different types of fermented products found around the world, there must be a pool of genetically diverse LAB. In Nigeria, the northern regions

represents the largest area in the country where milk or milk products is consumed either raw or fermented as a variety of flavoured yoghurt infused with other indigenous materials like millet. Dawdawa is a traditional food condiment used in soups and stews, produced from fermentation of Parkia boglobosa seed. Within these products, it is conceivable that certain bacterial type exist which secrete specific protein or protein complexes and compounds that inhibit the growth of mold and filamentous fungi in food sample. This paper thus focused on isolating, identifying and evaluating the anti-fungal and anti-mycotoxigenic properties of isolates obtained from Nono and dawadawa from the study area.

2. Materials and Methods

2.1. Isolation of Bacteria

Fresh samples of nono (fermented cow milk) and dawadawa were collected from Gwagwalada Area Council of Abuja (8° 56' 29" N, 7° 5' 30" E). Each was taken in a sterile container separately and placed in a polyethylene bag during transportation to the laboratory employing standard conditions for sample collection. 1ml of milk sample was immediately processed under aseptic conditions by suspending in 9ml of 0.15% Peptone water and was homogenized. 1g of dawadawa sample (mashed) was weighted aseptically and was suspended in 9ml of 0.15% peptone water under aseptic condition and vortexed for proper homogeneity. 1ml of each thoroughly mixed sample was serially diluted up to 10-6 and used to inoculate MRS agar plates supplemented with 100mg/l cycloheximide. Each plate was incubated at 37oC for 48 hours and observed for the growth of colonies. All experiments were done in triplicate.

2.2. Identification by Morphological and Biochemical Assessment

Colonies with distinct morphological characteristics were randomly selected and purified by repeat streaking on the antibiotic supplemented MRS agar. The pure strains were transferred, maintained on MRS Agar slants using 20% glycerol and stored at 4oC until needed for further studies.Distinct isolates were subjected to morphological identification aided by gram staining with the following parameters: Colour, Shape, Texture, Elevation, Margin and Opacity [17]. Wet mounts of isolated cell samples were prepared in distilled water and examined using 40X objective magnification.

Biochemical characterization was performed according to known methods [18] which included gram staining, catalase activity, production of gas from glucose, carbohydrate fermentation using phenol red broth technique [19]. Sugars used to determine the fermentation profile of the isolates were fructose, galactose, lactose, maltose, mannitol, arabinose, trehalose, sucrose and lactulose. The cultures were initially identified based on the pattern of sugar utilization.

2.3. Effect of NaCl Concentrations on Growth of Isolates

The isolates were inoculated in MRS broth having different salt concentration (4.5%, and 6.5%) and incubated at 37oC for 24–48 hours. The culture tubes were observed for the presence or absence of growth. The development of turbidity in culture tubes was recorded as the ability of isolates to grow at the given salt concentration.

2.4. Growth profile studies

The isolates were tested for their ability to grow in MRS broth at 10±1°C for 5 days and 28oC, 33oC and 37°C by incubating for 96 hours. For this, 10 mL of MRS broth tubes were inoculated with 1% of bacteria cultures. The development of turbidity in culture tubes was recorded as the ability of isolates to grow at the respective temperatures was noted as positive. Periodic (24 hour) measurements of growth was achieved using a UV-VIS spectrophotometer, measurements were taken at 550nm. Viability determination as well as pH measurements were analyzed [20].

2.5. Molecular Characterization

The genotypic identification of the microorganisms was performed using universal bacterial primers; 27F (51-GAGTTTGATCCTGGCTCAG-31) and 1492R (51-GGTTACCTTGTTACGACT-31). The PCR reaction was done in a 25µlcontaining 2.5µl of 10X buffer supplemented with 15mM MgCl2, 100mM Tris-HCl, 500mM KCl, 0.5µl of each oligonucleotide primer (F+R), 2µl of 0.04mM each dNTPs, 2µl of the extracted DNA, 0.25µl of 0.25 units/25µlTaq DNA polymerase and 7.25µl of autoclaved distilled water. The reaction was run for 36 cycles; denaturation at 94°C for 2 min, annealing at 52°C for 1 min as well as an elongation step at 72°C for 2 min. Amplified fragments of PCR products were purified and sequenced. The resulting 16S rRNA gene sequences were analyzed to obtain sequence similarity using the BLAST tool then compared to the corresponding neighbour sequences from GenBank-NCBI database for taxonomic identification (http://www.ncbi.nlm.nih.gov/BLAST/).

2.6. Antifungal activity screeningof the isolates

Aspergillus niger, selected as the test fungal organism, was obtained from the Specialist Teaching Hospital, University of Abuja, F.C.T., Nigeria. This fungus was collected on Potato dextrose agar slant and restored in Potato dextrose broth. Determination of aflatoxin production by this fungus was achieved by thin layer chromatography [20].The pure bacterial isolates obtained were assayed for their antagonistic activity against Aspergillus niger using the dual culture method [21]. A 40mm streak was made from 24 hour culture of bacteria, 23mm away from the center of the petri dish. Using a 6mm diameter sterilized cork borer, the growing edge of a 4 day old fungal culture was asceptically cut and placed at the center of the plate already inoculated with the test antagonist. Plates were incubated at 25oC and monitored for 5 - 7 days. Dishes inoculated with only the test pathogen (Aspergillus niger) served as control. Measurements of the percentage growth inhibition and inter-colony distance were taken daily. Percentage inhibition was calculated using the formula:

percentage of inhibition =(KR-RI)/KR X 100

Were KR = distance (measured in mm) from the point of inoculation to the colony margin on the control plate.

RI = the distance of fungal growth from the point of inoculation to the colony margin on the treated plate in the direction of the antagonist.

3. RESULTS

Twelve bacterial strains were obtained from the successful isolation of the studied samples; nono and dawadawa. Morphological assessment of the isolates revealed creamy white, flat and smooth textured appearance with a transparent opacity (Table 1). Microscopic examination was achieved during the growth phase. All the strains were gram positive (Table 1).

In the biochemical assessment, all isolates were found to be catalase negative of each individual bacterial isolate, while all tested positive in their ability to ferment tested sugars (table 2). Growth profile of the microorganisms over a four day incubation period was monitored in the broth samples for pH (pH 4.5, 9.6) and Osmo-tolerance (4.5%, 6.5% NaCl concentration) depicted in tables 3, 4. The growth profile at different temperatures (28oC, 33oC, 37oC) was also monitored (figure 2). Analysis of the data obtained indicates relatively steady growth at the two pH ranges, with stable cell integrity at the different salt concentrations, thus suggesting the reliability of the cell membranes pertaining to their robust nature as acidification occurs. Inhibitory spectrum of the bacterial isolates

All eleven pure isolates exhibited a broad antifungal activity due to their ability to impede the growth and propagation of the tested fungus (Figure 1).

4. Discussion

At the turn of the century, increasing number of research efforts have been made pertaining to the composition and various natural resources indigenous to the study locale. In the West African region, traditional dairy products like cow milk and fermented African locust beans, known as dawadawa are important dietary components owing to their socio-economic and nutritional value. As part of both the fermentative measures employed as well as the quality of the dairy products, a plethora or microbial species can be found in such products, thus making traditional food products a suitable target for the isolation of diverse strains of bacteria with desired industrial applications [22]. Frontiers in biotechnology has led to bolstering yields and improvement of targeted products using naturally evolved microorganisms which serve to lower production process costs is an economically viable attempt to deliver useful products to mankind [23]. Morphological examination of the isolates obtained from the study samples revealed twelve distinctive colonies, assumed to be lactic acid producers owing to their source of origin. Microscopic evaluation suggested that all the obtained microbial isolates were Gram positive with further characteristics revealing that the colour range for all isolates were variants of cream, white and yellow, smooth textured with a transparent opacity (Table 1). Further analysis of the morphological features maintained the same consistency for shape, colour, elevation, margin and surface for biological replicates when cultured on MRS agar plates. Disparities in colony colours was observed between each sample, most notable of which was the yellow colonies isolated only from the milk sample and the dark cream colonies obtained from dawadawa samples (Table 1). Of the 12 isolates, six colonies were irregularly shaped while the other six were circular in shape.

In this investigation, all isolates exhibited negative biochemical characteristics when tested for catalase while fermentation pattern analysis revealed positive consumption of glucose, lactose and maltose (Table 2). With the exception of isolate designated MM, all other isolates were able to metabolize trehalose, all but MC were able to metabolize sucrose and all DJ were able to utilize dextrose in Durham's tube. These results appear similar to studies performed in northern Africa [24-26].

Studies suggest that the physiology of several lactic acid bacteria possess adequate metabolic machinery to utilize a range of sugars as sources of carbon for growth [27-30].All bacterial isolates obtained from the milk and dawadawa samples withstood broth environments with 4.5% and 6.5% NaCl concentrations which is in tandem with data obtained from previous studies, incubated and monitored daily over 96 hours [24]. Data from this study suggested that the isolates obtained possessed a degree of osmo-tolerance hence can be applied to a range of industrial and environmental applications since high levels of salt in the environment would otherwise elicit cell death [24].

Table 1: Morphological assessment of bacteria

Isolate	Colour	Shape	Elevation	Texture	Margin	Opacity	Surface	Gram Reaction
MS	Creamy	Irregular	Convex	Smooth	Undulate	Transparent	Shinny	Positive
MF	Yellowish	Irregular	Flat	Smooth	Filamentous	Transparent	Dry	Positive
MU	Cream	Circular	Convex	Smooth	Entire	Transparent	Smooth	Positive
ME	White	Circular	Flat	Smooth	Entire	Transparent	Smooth	Positive
MC	Light Cream	Circular	Flat	Smooth	Entire	Transparent	Smooth	Positive
MM	Light Cream	Circular	Convex	Smooth	Entire	Transparent	Smooth	Positive
DN	White	Irregular	Flat	Smooth	Entire	Transparent	Smooth	Positive
DO	Cream	Circular	Convex	Smooth	Entire	Transparent	Smooth	Positive
DJ	Light Cream	Irregular	Flat	Smooth	Entire	Transparent	Dry	Positive
DT	Dark Cream	Irregular	Flat	Smooth	Entire	Transparent	Dry	Positive
DG	Creamy	Circular	Flat	Smooth	Entire	Transparent	Smooth	Positive

Table 2: Phenotypic assessment of bacteria

Characteristics	DJ	MM	DN	DO	MC	MF	DT	MS	ME	MU	DG
CO_2 Production	-	+	-	+	+	+	+	+	-	-	+
Catalase	-	-	-	-	-	-	-	-	-	-	-
Fermentation pattern											
Sucrose	+	+	+	+	-	±	+	+	+	+	+
Lactose	±	+	+	+	+	±	+	+	+	+	+
Trehalose	+	-	+	+	±	+	+	+	+	+	+
Dextrose	-	+	+	+	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+	+	+
Mannitol	+	-	+	+	-	+	+	+	+	+	+
Lactulose	+	-	+	+	±	±	+	+	+	+	+
Grow at pH:											
4.5	+	+	+	+	+	+	+	+	+	+	+
9.6	+	+	+	+	+	+	+	+	+	+	+

Growth in NaCl											
4.5%	+	+	+	+	+	+	+	+	+	+	+
6.5%	+	+	+	+	+	+	+	+	+	+	+

Key: + = Positive, - + Negative, ± = Partial

Table 3: Growth Profile at different temperatures @ 550nm

Isolate	28°C				33°C				37°C			
	24Hrs	48Hrs	72Hrs	96Hrs	24Hrs	48Hrs	72Hrs	96Hrs	24Hrs	48Hrs	72Hrs	96Hrs
ME	0.241	1.939	2.126	2.177	0.24	1.788	2.693	3.011	0.294	1.532	1.73	2.046
MU	1.871	2.890	2.991	3.213	1.806	2.521	2.943	3.333	2.193	2.33	2.695	2.981
MF	1.061	1.729	2.926	3.222	1.233	1.296	2.497	3.248	1.919	1.936	2.013	2.947
MC	0.629	2.129	2.801	3.145	0.52	1.790	1.896	1.984	0.589	1.574	1.374	2.468
MS	0.488	2.296	2.649	2.918	0.624	2.258	3.025	3.321	1.296	1.823	2.076	2.111
DO	1.837	2.88	2.967	3.298	1.645	2.429	2.969	3.348	2.313	2.789	3.209	3.465
DT	1.042	0.964	1.172	1.539	0.933	0.398	1.509	1.714	1.088	0.807	1.294	1.529
DN	1.461	1.965	1.908	1.912	1.524	2.061	2.088	1.986	1.775	1.986	2.008	2.772
DG	0.562	2.071	2.328	2.441	0.746	1.986	2.174	0.717	0.515	2.348	2.578	2.545
MM	0.194	2.14	2.86	3.251	0.205	1.184	1.456	1.721	0.254	1.687	1.372	2.892
DJ	2.082	2.28	2.814	3.131	2.113	2.834	3.211	3.142	2.482	2.995	3.326	3.488

Table 4: Identification of yeast isolate based on sequence alignment (BLAST)

Culture code	Nearest Phylogenetic relative	Strain	Accession Number	Sequence Similarity (%)
1	*Bacillus anthracis*	HA528	KJ535337.1	95
2	*Lactococcus lactis*	200703	KU899037.1	96
3	*Bacillus pumilus*	CE4	JQ435701.1	96
4	*Bacillus cereus*	Vivi8	MF629786.1	95
5	*Bacillus valismortis*	MJ12	KX7888661.1	93
6	*Lactobacillus plantarum*	Z135A	MH392792.1	90
7	*Lactobacillus fermentum*	NW11	HQ026756.1	96
8	*Lactobacillus helveticus*	H1	MF942366.1	100
9	*Leuconostoc cittreum*	CAU9855	MF429692.1	100
10	*Lactobacillus delbrueckii*	TS1-06	EU346727.1	93

Key: 1=DG , 2=ME , 3=DJ , 4=MC , 5=DN , 6= MS, 7=DT , 8=MM , 9=DO , 10=MU. (MF sequence not identified)

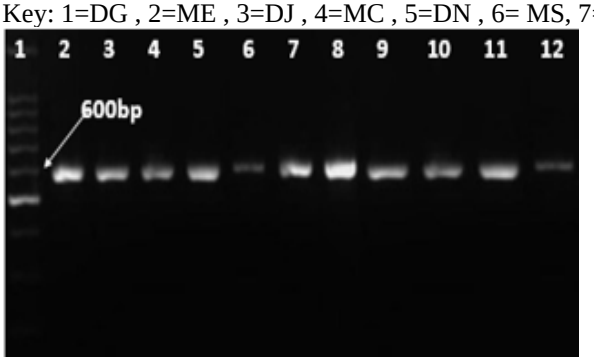

Figure 1: Gel electrophoresis micrograph of amplified products

Most of the antifungal isolates were identified by 16S rDNA sequencing as *Bacillus cereus, Lactococcus lactis, Bacillus pumilus, Bacillus valismortis, Lactobacillus plantarum, Lactobacillus fermentum, Lactobacillus helveticus, Leuconostoc citreum and Lactobacillus delbrueckii.*

Food contamination is often by yeasts and spoilage molds owing to the unsterilized processing conditions as well as inadequate storage, thus serving as an avenue for transmission of food borne diseases [38,39,40]. The most predominantly isolated species of mold and fungi isolated from food in developing countries include but are not limited to *Aspergillus niger, Aspergillus fumigatus, Penicillium chrysogenum, Rhizopus spp, Fusarium moniliforme,* and *Trichoderma reesii* [38,41].

The effects of consuming food products tainted with such pathogenic fungi stretch from acutely toxic to immunosuppressive orcarcinogenic, owing to the release of mycotoxins like aflatoxins, fumonisins, patulin, etc [42,43].

In this investigation, the antagonistic behavior of all isolated bacteria assayed against *Aspergillus niger* displayed increased growth inhibition on increasing duration of incubation (figure 2). Data obtained revealed that isolate designated MC (*Bacillus cereus*) displayed the highest % inhibition; 71.6% at 120 hours, quickly followed by DG-*Bacillus cereus* (68.5%), DT-*Lactobacillus fermentum* (68.3%) and MM-*Lactobacillus helveticus* (68.2%).Lactic acid bacteria is known for their ability to synthesize bioactive compounds that are capable of mediating the growth and subsequent spoilage of food by mold especially during fermentation [32]. Using the dual-culture method, 9 of the 11 isolates exhibited strong antifungal spectrum against *A. niger*, similar to observations by Coloretti and colleagues [44] for which isolates DJ and MF displayed less effective activity (figure 2).

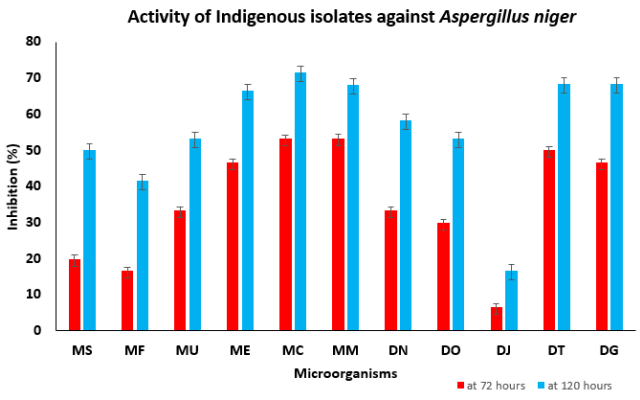

Figure 2. Antagonistic activity of bacteria isolates against fungi

The present study has demonstrated the availability of indigenous lactic acid bacterial species that could be

manipulated to serve as food preservatives and deter food spoilage by pathogenic molds and yeasts, thus reaffirming data obtained in another study [44,45].

5. CONCLUSION

Data obtained in this study revealed that Bacillus cereus, Lactococcus lactis, Bacillus pumilus, Bacillus valismortis, Lactobacillus plantarum, Lactobacillus fermentum, Lactobacillus helveticus, Leuconostoc citreum and Lactobacillus delbruecki were the predominant lactic acid strains of bacteria isolated from fermented dawadawa seeds and nono using MRS media. These microorganisms are similar to the bacterial isolates obtained from fermented cow, goat and lamb milk in other studies in which the similarities extend towards acid production, Gram's reaction, catalase test and fermentation patterns under microaerophylic conditions [26,46]. All isolates obtained were characterized as lactic acid fermenters (Table 2) as the major feature of bacteria obtained from the studied samples. These findings validate the notion that lactic acid bacteria are the main microorganisms that trigger the fermentation of nono and dawadawa (Parkia biglobosa) seeds [24]. All the isolates obtained exhibited antagonistic activity towards Aspergillus niger, an aflatoxin producing fungi and causative agent of aflatoxicosis in food.

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