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SCREENING OF LOCALLY FERMENTED FOOD CONDIMENTS FOR PREDOMINANT MICROORGANISMS FOR SAFE UTILIZATION

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ABSTRACT

Some locally fermented foods have been associated with several microorganisms including Lactic Acid Bacteria(LAB) which have several potential health or nutritional benefits. Therefore, the aim of the study was to screen the locally fermented food condiments for predominant microorganisms for safe utilization. The fermented food condiments; fermented seeds of fluted pumpkin(ogiri) and African Oil bean seed(ukpaka) were utilized in this study. Isolation of various microorganisms were carried out using different media such as nutrient agar, mannitol salt agar, macconkey agar, salmonella shigella agar and De Man, Rogosa and Sharpe agar respectively. The developed colonies were subjected to phenotypic characterization such as macroscopic examination, Gram staining, biochemical tests and genotypic characterization. The phenotypic and genotypic characterization revealed the isolates as *Bacillus subtilis*, *Lactobacillus* sp. *Escherichia coli, Salmonella* sp. *and Staphylococcus aureus.* The total bacteria count of the fermented food condiments revealed *Bacillus subtilis* and *Lactobacillus* sp with highest colony count of $\geq 3.5 \times 10^6$ CFU/mL. The lowest count was observed in *Salmonella* sp. $\geq 2.0 \times 10^2$ CFU/mL. The obtained results proved *Bacillus subtilis* and *Lactobacillus* sp. as the predominant mivroorganisms in the assayed fermented food condiments, hence safe for utilization.

Key words : *Bacillus subtilis* FFOS, characterization, fermented food condiments, screening,

INTRODUCTION

Fermented foods are known as foods produced from the controlled growth of microbes (Dimidi *et al*.,2019) . These foods provide many health benefits such as anti-oxidant, anti-microbial, anti-inflammatory, anti-diabetic and anti-atherosclerotic activity(Şanlier *et al*.,2019). The fermented foods common in Nigeria include cassava products (garri, fufu, elubo, abacha, akara akpu), yam products (amala), maize products (ogi, agidi, soy-ogi),

millet products (ogi-baba, kwunu, tuwo, fura). The common fermented food condiments are dawadawa (African locust bean), ogiri-ugu (fluted pumpkin), ogiri-isi (castor seeds), ogiri-egusi (melon seeds), ugba (African oil bean), daddawa (soy-bean), eketeke (oil palm nut)(Tahir *et al*., 2022).

Ukpaka and ogiri are fermented condiment made from African oil bean seed and pumpkin seeds or castor bean seeds and are popularly consumed in the south-eastern part of Nigeria. Several groups of microorganisms such as *Lactobacillus, Lactococcus, Streptococcus, Saccharomyces, Corynebacterium, Bacillus* sp. and *Leuconostoc* have been isolated from these fermented foods condiments. Ogiri had been reported to have associated with *Bacillus* sp., *Staphylococcus aureus, Pseudomonas* sp.and *Lactobacillus* sp. (Ademola *et al*., 2018). Other organisms isolated from ukpaka include moulds such as *Mucor* sp., *Rhizopus* sp., *Aspergillus nidulans, A. fumigatus* and *Paecilomyces* sp. and some bacterial species, *Staphylococcus* sp., *Bacillus* and *Pseudomonas* spp and yeast;*Geotrichum* sp., *Torulopsis* sp., and *Hansenula* sp. (Okechukwu *et al*.,2011).

Most locally fermented foods/condiments are often prepared at home by uncontrolled fermentation resulting in unpredictable diversity of pathogenic microorganisms (Okafor *et al*.,2020).Some pathogens in African indigenous fermented foods or condiments such as *Staphylococcus aureus* , Gram-negative indicator bacterial strains, *Escherichia coli, Klebsiella pneumoniae* , *Proteus mirabilis* and *Pseudomonas aeruginosa* have been reported by several researchers(Ogunshe and Olasugba, 2008). Other isolated pathogens including *Klebsiella aerogenes, Citrobacter aerogenes, Enterobacter aerogenes, Shigella dysenteriae* , *Shigella flexneri* and *Shigella sonnei*have been reported (Gadaga, 2004; Enujiugha and Badejo, 2002; Ogunshe *et al*., 2006).

Therefore, the present study aimed at screening the locally fermented food condiments for predominant organisms for safe utilization.

MATERIALS AND METHODS

Sample preparation

The fermented food ogiri (fermented seeds of fluted pumpkin) and ukpaka (African Oil bean seed) utilized in this study were purchased at Mayor Market Enugu State, Nigeria. Ukpaka was homogenized using a home blender prior to use.

Isolation procedure

The serially diluted (10^3) fermented food condiments were inoculated into various media including nutrient agar, mannitol salt agar, macconkey agar, salmonella shigella agar and De Man, Rogosa and Sharpe agar and incubated at 37^{o}C for 24h. The developed colonies were counted using a colony counter to determine the colony forming unit per mL (CFU/mL) and the colonial colour on the various media were observed. The colonies were then subjected to further identification tests.

Identification tests on the Isolates

The developed colonies were subjected to phenotypic and genotypic characterizations.

Phenotypic characterization

The colonies were subjected to macroscopic examination, Gram staining and biochemical tests including oxidase, indole, citate, catalase, urease and voges proskauer.

Genotypic characterization

The predominant isolate, Isolate FFOS was subjected to genotypic characterization such as DNA extraction using ZR Fungal/Bacterial DNA MINIPREP (Manufactured by Zymo Research) , Electrophoresis for DNA and PCR, 16SrRNA gene amplification of the bacterial isolate and sequencing

DNA Extraction using ZR Fungal/Bacterial DNA MINIPREP (Manufactured by Zymo Research)

Bacterial cells (2mL) was added to a ZR BashingTM Lysis Tube. Thereafter 750ul lysis solution was added to the tube. It was secured in a bead fitted with 2 ml tube holder assembly and processed at maximum speed for > 5 min. The ZR BashingBeadTM Lysis Tube was centrifuged in a microcentirifuge at > 10,000 x g for 1 min. The 400 ul supernatant was transferred to a Zymo-SpinTM IV Spin Filter (orange top) in a Collection Tube and centrifuged at 7,000 x g for 1 min. Fungal/Bacterial DNA Binding Buffer (1,200 ul) was added to the filterate in the Collection Tube. The 800 ul of the mixture was transferred to a Zymo-SpinTM IIC Column in a Collection Tube and was centrifuged at 10,000 x g for 1 min. The flow through was discarded from the Collection Tube. The 800 ul of the mixture was transferred to a Zymo-SpinTM IIC Column in a collection tube and was centrifuged at 10,000 x g for 1 min. The DNA Pre-Wash Buffer (200ul) was added to the Zymo-Spin TM IIC Column in new Collection Tube and was centrifuged at 10,000 x g for 1 min. The Fungal/Bacterial DNA Wash Buffer (500 ul) was added to the Zymo-SpinTM IIC Column and centrifuged at 10,000 x g for 1 min. The Zymo-SpinTM IIC Column was transferred to a clean 1.5 ml microcentrifuge tube and 100ul (35 ul minimum) DNA Elution Buffer was added directly to the column matrix and was centrifuged at 10,000 x g for 30 seconds to elute the DNA.

Electrophoresis for DNA and PCR

The agarose powder (1g for DNA) and (2g for PCR) was dissolved in 100 mL 1xTAE in a microwavable flask and was microwave for 3 min and was allowed to cool down to 50 °C. The 10µL EZ vision DNA stain was added and agarose was poured into a gel tray with the well comb in place and was left at 4 °C for 15 min to solidify.

The loading of samples and running of an agarose gel were then carried out at 80-150 V for 1h, then the gel was carefully removed from the gel box. The DNA fragments or PCR product was visualize under UV transilluminator.

16SrRNA gene amplification of the bacterial isolate

The PCR mix was made up of 12.5µL of Taq 2X Master Mix from New England Biolabs (M0270); 1µL each of 10µM forward (27F: AGAGTTTGATCMTGGCTCAG) and reverse (1525R: AAGGAGGTGWTCCARCCGCA)primer; 2µL of DNA template and then made up with 8.5µL Nuclease free water. The cycling conditions for the amplification of the 16SrRNA gene were; initial denaturation at 94˚C for 5min, followed by 36 cycles of denaturation at 94˚C for 30sec, annealing at 56˚C for 30secs and elongation at 72˚C for 45sec. Followed by a final elongation step at 72˚C for 7 min and hold temperature at 10 ˚C forever.

Sequencing

The amplified fragments were sequenced using a Genetic Analyzer 3130xl sequencer from Applied Biosystems using manufacturers' manual while the sequencing kit used was that of BigDye terminator v3.1 cycle sequencing kit. Bio- Edit software and MEGA X.

RESULTS AND DISCUSSION

Phenotypic Characterization of the Isolates

Table 1 indicated phenotypic characterization of the isolates. The morphological appearance, Gram reaction and biochemical test revealed the isolates as *Lactobacillus sp., Bacillus* sp., *Salmonella* sp., *Escherichia coli* and *Staphylococcus aureus.* The presence of *Lactobacillus* and *Bacillus subtilis* in the food condiments is a welcoming result as Fijan (2014) reported these organisms as probiotics(beneficial organisms). The isolation of the pathogenic bacteria could be because the food condiments are often prepared at homeby uncontrolled fermentation resulting in unpredictable diversity of pathogenic microorganisms (Okafor *et al.*, 2020). The isolation of *Lactobacillus* and *Bacillus subtilis* from ogiri and ukpaka have been reported by Adebayo (2008) and Anyanwu *et al*. (2016). However,several pathogens including *Leuconostoc, Proteus, Klebsiella ,Staphylococcus, Streptococcus, Salmonella,Corynebacterium* and *Pseudomonas* spp.have been reported from ogiri and ukpaka (Nwachukwu *et al*., 2014; Anyanwu *et al*.,2016)

Table 1: Phenotypic characterization of the isolates

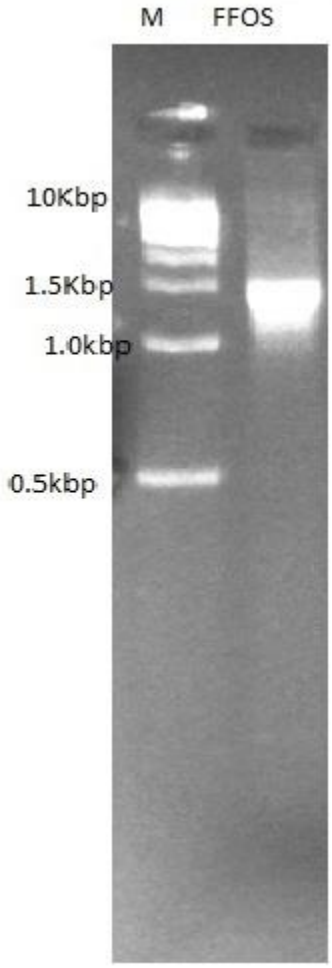
Parameters													
Isolates	**Gram reaction**	**Catalase**	**Oxidase**	**Voges Proskauer**	**Urease**	**Indole**	**Methyl red**	**Citrate**	**Growth on MSA**	**Growth on SSA**	**Growth on MAC**	**Growth on MRS**	**Inference**
1	+rod shape	-	-	-	-	-	-	-	NA	NA	NA	+	*Lactobacillus sp*
2	- Rod shape	+	-	-	-	+	+	-	NA	NA	+	NA	*Escherichia coli*
3	- rod shape	+	-	-	-	-	+	+	NA	+	NA	NA	*Salmonella* sp.
4	+spherical shape	+	-	+	+	-	+	+	+	NA	NA	NA	*Staphylococcus aureus*
5	+ rod shaped	+	-	+	-	-	-	+	NA	NA	NA	+	*Bacillus* sp.

Legend: NA= Not Applicable, MRS= Deman Rogosa Sharp, MSA= Mannitol Salt Agar, MAC= MacConkey Agar, SSA= Salmonella Shigella Agar

Genotypic Characterization

The molecular weight DNA,Amplification of 16SrRNA gene at 1500bp,Sequence of 16S rDNA and Phylogenetic tree of *Bacillus subtilis* FFOS results were shown in Figures 1-4. The genotypic characterization confirmed the predominant *Bacillus* sp. as *Bacillus subtilis* which has 92.01% pairwise similarity with *Bacillus subtilis strain C3a-FIIRO* with NCBI accession number MW577298. The obtained result revealed the fermented food a healthy condiment, considering health benefits of the isolated bacteria.

Figure 1: High Molecular weight DNA of *Bacillus subtilis* FFOS



M = 1kbp DNA ladder

Figure 2: Amplification of 16SrRNA Gene at 1500bp. by ***Bacillus subtilis* FFOS**

**TCCTCCCCAGGCGGAGTGCTTATGCGTTAGCTGCAGCACTAGGGCGGAAACCCCAA
CACTTAGCATCATCGTTTACGGTGGACTACCAGGGTATCTAATCCTGTTGCTMCCC
ACTTTCGCCTCAGCGTCAGTTACAGACCAGAAGCGCCTTCGCCACTGGTGTTCYTC
CAATMTCTACGCATTTCACCGCTACACTGGATTCCACTTCCTCTTCTGCACTCAAGT
TCCCAGTTTCCAATGACCYTCYCCGGTTGAGCCGRRGGCTTTCACATCAGACTTAA
RAAACCGCCTGCGACCTTTACGCCCAATAATTCCGGACAACGCTTGCCACCTACGT
ATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAGTACCGTCAAG
GGYCGCCTATTTGAACGGAACTTGTTCTTCCCTAACAACAAGTTTACATCCGAAAAC
CTTCATCATCAGCGGGTTGTCCGCAATTTCGTCCATTGGAAGATTCCCTACTGYTGC
CTCCCGWAGAGTCTGGGCCGGTTCAGTCCCAGGTGGCCGATCACCCTCTCAGGTC
GGTACGCATCGTGCCTGGGAGCCGTTACCTCACCAACTAGTAAGCGCCGGGRTCCA
TCTGTAAGTGAGCCGAAGCCCTTTTATGTCTGAACCATGCGTTCAAACATCCGGTA
TTAGCGGTTTCCCGGAGTATCCCGTTAAGGCAGGTTACCCACGTGTTACTCACCCG
TCCGCCGCTAAATCAGGAGCAAGCTCCATCTGTCCGCTCGACTTGCATGTATTAGC
ACCGACGGCGTCGTCCTGAGCCATGATCAAACTCTGGGSAGAG**

Figure 3 : Sequence of 16S rDNA of *Bacillus subtilis* FFOS

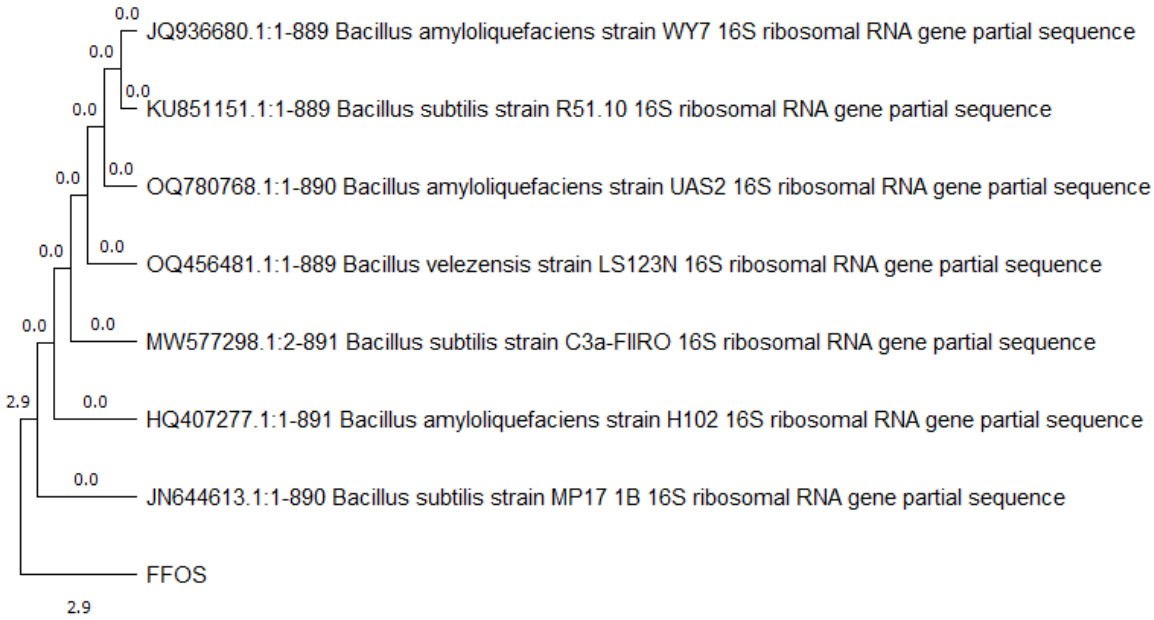


Figure 4: Phylogenetic Tree of *Bacillus subtilis* FFOS

Total bacteria count of fermented food condiments

Table 2 indicated the total bacteria count of the fermented food condiments. *Bacillus subtilis* and *Lactobacillus* sp. have the highest colony count ≥ 3.5 X 10^6 CFU/mL. The lowest bacterial count was observed with *Salmonella* sp. ≥ 2.0 X 10^2 CFU/mL. The predominant *Bacillus subtilis* and *Lactobacillus* sp. in the condiments could be that the condiments produced higher level of lactic acid making the pH value not favourable for other pathogens as most pathogens are unable to survive under these conditions . However, some pathogens such as *Escherichia coli* O157:H7 have been reported to develop acid tolerance (Gadaga *et al.*, 2004). Ozabor *et al. (*2020) and Ogbulie *et al.*(2014) also reported these Lactic Acid Bacteria as the predominant microorganism from ogiri and ukpaka sample.

Table 2: Total bacteria count (CFU/ml) of the fermented food condiments

Food condiments	*Bacillus subtilis*	*Lactobacillus* sp.	*Salmonella* sp.	*Escherichia coli*	*Staphylococcus aureus*
Ogiri	3.5×10^6	2.8×10^6	1.0×10^2	1.1×10^3	2.1×10^3
Ukpaka	2.8×10^6	2.5×10^6	2.0×10^2	1.3×10^3	3.0×10^3

Conclusion

The obtained results proved the predominant microorganisms in the assayed fermented food as *Bacillus subtilis* and *Lactobacillus* sp. hence safe for ultilization.

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