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Genomic Studies of *Curvularia Verruculosa* and *Penicillium Citrinum* in Contaminated Commercially Processed Soymilk in Wukari: First Report

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Abstract

Soymilk has a rich nutritional profile and in its traditionally processed form, provides a favourable growth niche for fungi that cause visible and non-visible defects thereby decreasing its shelf life resulting in significant food waste that translate to huge economic losses. Fungal spoilage of food products is a constant source of worry to researchers who have sought ways of preventing and/or limiting fungal growth because they produce disease-causing mycotoxins. In this regard, twelve tainted soymilk products sold in Wukari were collected from six locations in six sample areas called Wards and identified microbiologically. Comparatively, sterilized and unsterilized soymilk samples were prepared in the laboratory as controls. The average total fungal count in the contaminated soymilk ranged from $0.7x10^7$-$1.7x10^7$cfu/ml while that of the control was $0.7x10^7$cfu/ml. The occurrence of *Penicillium* spp. was highest (66.7%) while other fungi were recovered equally (6.67%). The recovery was more in Puje (53.3%) than in Hospital (46.7%) ward. The genomic basic local alignment search tool (BLAST) confirmed the pure isolates of fungi that were presumed to be *Penicillium* spp. as *Curvularia verruculosa* and *Penicillium citrinum* with percentage identification of 100%. Using the ITS sequencing technology to genetically identify fungi isolates proves to be a faster method in characterizing food ecosystems by identifying isolates at the species level. This current study demonstrates the presence of *C. verruculosa* and *P. citrinum* in contaminated ready-to-drink soymilk. In so doing, it provides foundational information useful in developing effective management strategies that will improve soymilk quality during production, protect the health of consumers, reduce economic losses and ensure food safety in traditionally processed food products.

Keywords: Genomic, Identification, Fungi, Processed, Contaminated, Soymilk

Introduction

Soybean, a valuable source of plant protein, is a supplement source of animal protein. The bean contains all of the essential amino acids crucial for the body's healthy growth and has been employed to address numerous dietary and health-related issues globally due to its low cost and ability to improve protein deficiencies. Although the presence of anti-nutritional components such as phytates, tannins, oxalates, polyphenols and enzyme inhibitors significantly impacted its usage (Ozoh and Umeaku, 2016) [14], treatments including heat, germination, soaking and fermentation were observed to lessen these components in food products (Ogodo *et al*., 2018) [12].

Soymilk, often known as soya milk, is a type of plant milk made from dried, processed soybeans and is used as a meal for both humans and livestock. It has long been recognized as the best alternative to the substantial barriers to dairy milk intake, including lactose intolerance and milk protein allergy (Han *et al*., 2021) [7]. It presents as a white, creamy emulsion that resembles cow milk (Adegbehingbe *et al*., 2022) [1]. Sadly, soymilk is vulnerable to microbial attack if improperly processed and stored because the nutrients it contains are also necessary for the growth of a variety of microorganisms including coliforms, mesophilic aerobic bacteria, yeasts and moulds (Arekemase and Babashola, 2019) [3]. It has been previously documented that fungi like *Aspergillus* and *Penicillium* species cause soymilk to deteriorate, causing unpleasant changes in the milk (Akani and Barika, 2019) [2].

The similarities in the colonial and morphological characteristics of fungi make it difficult to isolate and classify them. The sugar assimilation and fermentation tests used to differentiate *Aspergillus* and *Penicillium* isolates are not adequate enough at speciation while newer cultivation techniques using chromogenic agar (CHROM agar), analytical profile index (API) system, Vitek 2 Identification system and molecular methods are expensive. Cost effective and easier genomic DNA extraction and sequencing technologies such as the basic local alignment search tool (BLAST) are definitive and confirmatory tools in fungal

diagnosis. Fungal isolates are characterized by sequencing and amplifying the internal transcribed spacer (ITS) target regions of their nuclear ribosomal DNA (rDNA) with ITS-1 and ITS-4 universal primers (White *et al*., 1990) [15,16].
Thus, the aim of this study therefore, was to investigate the genetic characteristics of fungi isolated from contaminated commercially processed soymilk in Wukari, Nigeria.

Materials and Methods

Study Area and Sampling Technique

The investigation was conducted in Wukari, a town located in Southern Taraba State of Nigeria. Two (2) study locations namely Puje and Hospital Wards were chosen. Sample collection points located in the Puje Ward were Takum Junction, Keystone Bank, and New Market while those located in the Hospital Ward were Federal University Wukari, Old BB and Wapan-Nghaku Primary School.
A total of twelve (12) soymilk samples were variously purchased from six (6) soymilk vendors in the wards with two (2) samples from each collection point. The samples were transferred into clean sterile bottles under aseptic conditions and transported to the Microbiology Laboratory at Federal University Wukari for microbiological assay. As controls, two (2) soymilk samples were aseptically prepared in the laboratory. One of these samples underwent sterilization whereas the other did not.

Serial Dilution, Inoculation and Incubation

After thoroughly shaking each of the soymilk sample bottle, 1ml of the sample was transferred, using a sterile pipette, into a number of sterile test tubes. Thereafter, a tenfold dilution of each sample was performed. All the samples were plated utilizing the pour plate technique (Dhawale and LaMaster, 2003) [5]. In triplicates, 0.1ml of dilution 10^{-6} was placed into sterile petri dishes that were filled with molten sabouraud dextrose agar (SDA) and incubated at 25^0C for 7 days. The fungal isolates were identified macroscopically, microscopically and genetically (Cheesbrough, 2006) [4].

Genetic Analyses

The fungal isolates were characterized by sequencing the Internal Transcribed Spacer (ITS) region of the nuclear DNA (rDNA). The universal primers ITS 1 and ITS 4 were used to amplify the ITS target region (White *et al*., 1990; Altschul *et al*., 1997) [15,16]. The target and 5' to 3' sequence of ITS-1 primer was ITS rDNA sequence and TCCGTAGGTGAACCTGCGG respectively while the target and 5' to 3' sequence of ITS-4 primer was ITS rDNA sequence and TCCTCCGCTTATTGATATGC respectively.
The genomic DNA of each sample was extracted using the Quick-DNA Fungal/Bacterial kit (Zymo Research, Catalogue No. D6005). One Tag "Quick-Load"2X Master Mix (NEB, Catalogue No. MO486) was used to amplify the ITS target area with the primers. The EXOSAP method was used to enzymatically clean up the PCR products after they had been run on a gel. The Nimagen and BrilliantDye™Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000, was used sequence to the extracted fragments in the forward and reverse directions and purified (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™, Catalogue No. D4050). Purified fragments were analyzed on the ABI 3500xl Genetic Analyzer (Applied Biosystems, ThermoFisher Scientific) for each reaction for every fungal isolate, BLAST results were obtained when DNASTAR was used to analyze the ab1 files created by the ABI 3500XL Genetic Analyzer (NCBI).

Results

Tables 1 and 2 contain information on the colony and microscopic (cellular) morphologies of sixteen (16) fungal isolates comprising of six (6) genera from soymilk collected from all the wards and sample location points. Strikingly, all were of filamentous form and opaque but with varying degrees of colour, morphological hyphae and branches.

Table 1: Colonial morphology of fungal isolates

Collection points	Form	Elevation	Margin	Surface	Opacity	Colour	Reverse	Probable fungi
OB	Filamentous Filamentous	Raised Convex	Filiform Undulate	Wooly Velvet	Opaque Opaque	W GG	White Dark tan	*Apophysomyces* *Penicillium*
NM	Filamentous Irregular	Umbonate Convex	Filiform Entire	Cottony Smooth	Opaque Opaque	W CW	White-black White	*Rhizopus* *Saccharamyces*
KB	Filamentous Filamentous	Umbonate Flat	Undulate Undulate	Dull Powdery	Opaque Opaque	G BG	Brown Dark tan	*Penicillium* *Penicillium*
TJ	Filamentous Filamentous	Convex Convex	Filiform Undulate	Fluffy Velvet	Opaque Opaque	GB GG	Dark tan Dark tan	*Penicillium* *Penicillium*
FUW	Filamentous Filamentous Filamentous	Flat Umbonate Convex	Filiform Filiform Undulate	Dull Fluffy Velvet	Opaque Opaque Opaque	GW GG	Brown White Dark tan	*Penicillium* *Sclerotinia* *Penicillium*
WPS	Filamentous Filamentous	Crated-form Umbonate	Undulate Undulate	Rough Rough	Opaque Opaque	G W	Brown White	*Penicillium* *Sclerotinia*
USC	Filamentous Filamentous	Convex Convex	Undulate Undulate	Opaque Opaque	Opaque Opaque	GG W	Dark tan White	*Penicillium* *Penicillium*
SC	Filamentous	Crated-form	Lobate	Opaque	Opaque	YG	Cream white	*Aspergillus flavus*

Key: W=White; GG = Gray-green; CW = Creamy-White; G = Gray; BG = Black-gray; GB = Gray-Black; YG = Yellow-green; OB = Old BB; NM = New market; KB = Keystone Bank; TJ = Takum junction; FUW = Federal University Wukari; WPS = Wapan-Nghaku Primary School; USC = Unsterilized control; PAS = Sterilized control

Table 2: Microscopic characteristics of fungal isolates

Cellular Morphology	Probable fungal species
Septate hyphae, branched conidiophore with chains of conidia produced by phialides with bluish peniciliale	*Penicillium*
Branching mycelia with aseptate hyphae and unbranching sporangiophores	*Rhizopus*
Broad, aseptate hyphae and unbranched, straight or curved sporangiophores	*Apophysomyces*
Composed of septate hyphae with branched conidiophores	*Sclerotinia*
Creamy, oval shape budding cell with rounded shape at the end	*Saccharomyces*
Single and double cover, entire vesicle point out in all directions	*A. flavus*

The total fungal count in the commercially processed contaminated soymilk in both wards was $6.7x10^7$ while the fungal count at sample collection points ranged from $0.7x10^7$-$1.7x10^7$cfu/ml. The fungal count in each of the controls was $0.7x10^7$cfu/ml (Table 3).

Table 4 shows the percentage occurrence and overall distribution of fungi at different study location wards and sample collection points. The occurrence of *Penicillium* species was highest (66.7%) while the other fungal species were equally least recovered (6.67%). The recovery was more in Puje (53.3%) than in Hospital (46.7%) ward.

Table 3: Distribution and average total fungal load in soymilk samples

Study location wards	Sample collection points	Fungal count (cfu/ml)
Puje	Takum junction	1.3×10^7
Puje	Keystone Bank	1.7×10^7
Puje	New market	1.0×10^7
Hospital	Federal University Wukari	1.3×10^7
Hospital	Old BB	0.7×10^7
Hospital	Wapan-Nghaku Primary School	0.7×10^7
Total		6.7×10^7
Laboratory unsterilized control		0.7×10^7
Laboratory sterilized control		0.7×10^7

Table 4: Frequency and percentage occurrence of fungi at different locations, wards and collection points

Fungi species	Sample locations									Percentage Occurrence (%)		
	Puje ward				Hospital ward							
	TJ	KB	NM	Total	FUW	OB	WPS	Total	Total		USC	SC
Penicillium	3	3	0	6	2	1	1	4	10	66.7	2	0
Rhizopus	0	0	1	1	0	0	0	0	1	6.67	0	0
Aspergillus flavus	0	0	0	0	0	0	0	0	0	0	0	1
Apophysomyces	0	0	0	0	0	1	0	1	1	6.67	0	0
Sclerotinia	0	0	0	0	1	0	1	2	2	13.3	0	0
S. cerevisiae	0	0	1	1	0	0	0	0	1	6.67	0	0
Total	3	3	2	8(53.3)	3	2	2	7(46.7)	15	100	2	1

Figures in parentheses represent percentages

Key: TJ = Takum junction; KB = Keystone Bank; NM = New market; FUW = Federal University Wukari; OB = Old BB; WPS = Wapan-Nghaku Primary School; USC = Unsterilized control; SC = Sterilized control

The subsequent genomic basic local alignment search tool (BLAST) of the two (2) culturally identified pure isolates that were presumed to be *Penicillium* species, confirmed them as *Curvularia verruculosa* and *Penicillium citrinum* with percentage identification of 100% (Table 5).

Table 5: The blast identification of fungal isolates from soymilk

S.No.	Fungi isolate	Genbank accession (sequence ID)	Percentage ID (%)	Predicted fungi
1	*Penicillium* spp.	MH859788.1	100	*Curvularia verruculosa*
2	*Penicillium* spp.	MT558921.1	100	*Penicillium citrinum*

Discussion

Storage fungi like *Aspergillus* and *Penicillium* have been reported to cause soymilk to spoil and cause unfavorable alterations to the milk (Momoh *et al*., 2011) [10]. The identification of *C. verruculosa* and *P. citrinum* in this current study, to the best of our knowledge, is the first report of such. The occurrence of other fungi with the exception of *Apophysomyces* and *Sclerotinia* species is consistent with studies that found similar fungi in soymilk (Oladele and Ofure, 2020; Adegbehingbe *et al*., 2022) [13,1]. The twelve soymilk samples that were analyzed differed greatly in microbiological quality and had fungal loads higher than the laboratory control samples due to improved processing and storage conditions. It is not impossible that these samples could have been contaminated by biotic or abiotic factors, which seem to be the main drivers of fungal growth in food products. Additionally, the use of the same storage containers by market vendors to store the soybean used to produce the soymilk might have aided in the propagation of the fungi possibly due to the rise in the crop product's humidity and temperature, which ultimately favour the growth of fungi (Drisu, 2018) [6]. The places where the soybeans were purchased and processed after harvest are all additional elements that could have led to the contamination of the soymilk.

The recovery rate of *Penicillium* was highest because of its highly evolved physiology and ability to adapt and grow in a diverse range of habitats, from soil to vegetation to air as well as indoor environments and various food products especially meals high in protein and other nutrients like

soymilk (Obi, 2014) [11]. More so, the soymilk samples used in this study were not refrigerated and, when left to stand, were good niches for fungi that are acidotolerant, psychrotolerant and can even tolerate to some extent, chemical preservatives sometimes added to prolong its shelf life causing changes in their organoleptic qualities due to their metabolic activities.

The isolation of *Rhizopus*, *Aspergillus*, *Apophysomyces* and *Sclerotinia* species may indicate environmental rather than substrate contamination given that their spores are typical pollutants (Oladele and Ofure, 2020) [13]. *Saccharomyces* species might have also occurred as opportunistic pathogens (Maduka *et al*., 2021) [9]. Generally, they can spread through the air, bodily fluids, contaminated food, or through polluted water infecting both plants and animals. Therefore, every major aspect of food supply including agricultural practice, postharvest, handling, transport, storage and processing must be monitored to prevent infection.

The use of the ITS sequencing technology to genetically identify isolates of fungi that were initially morphologically identified as *Penicillium*, showed that it is a faster method to characterize food ecosystems by identifying isolates at the species level. Utilizing multi-locus sequencing of DNA regions with taxonomical interest has changed fungal taxonomy by reclassifying numerous species and at the same time continuously recognizing new phylogenetic species within the "species complexes" in which members possess hardly any differences in their morphological characters or even more so, are morphologically indistinguishable from each other (Litoriya and Modi, 2021) [8].

This present outcome as far as could be ascertained, is the first report of isolation of *C. verruculosa* and *P. citrinum* in commercially processed contaminated readymade soymilk. This implies that effective management strategies must be industrially instituted in order to reduce economic losses and ensure food safety in traditionally processed food products.

Conclusion

The presence of fungi especially *Curvularia verruculosa* and *Penicillium citrinum* in soymilk poses a serious risk for some fungi-related mycotoxicoses to animals and unwary customers, especially the elderly and young children for whom soymilk obtained from soybean food crop, is used as a weaning diet. Therefore, the preparation, storage and distribution environments should be kept clean to avoid contamination in order to maintain the intended shelf life.

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