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Screening for genes Encoding Virulence Factor in *Salmonella* serovar Typhimurium isolated from Tiger nut Juice

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Abstract

Background: Tiger nut juice is a popular non-alcoholic beverage in Nigeria, but it can be contaminated with bacteria that pose a risk to consumers. A study in Ogbete market, South Eastern Nigeria, aimed to identify virulence genes in *Salmonella enterica* subsp. *enterica* serovar Typhimurium found in tiger nut juice to assess the potential health threat.
Materials and Methods: Fifty tiger nut juice with codes ATC1, MECB2, BNC3, CHA4, ERT5, TYR6, VMA7, EKA8, TMM9, and ORU10 were randomly purchased from Ogbete market vendors. Standard microbiological techniques and Polymerase Chain Reaction (PCR) were used to screen the samples for *S.* Typhimurium presence and its virulence genes including *invA, spvB, pefA, csgA, orgA, msgA, spaN, spiA, spvC, sipB,* and *sefA*.
Results: Our study found that 48.0% of the samples tested positive for *S.* Typhi. *S.* Typhi positive culture rates varied by sample location: ATC1 (10%), MECB2 (4.0%), BNC3 (6.0%), CHA4 (2.0%), ERT5 (8.0%), TYR6 (0.0%), VMA7 (6.0%), EKA8 (0.0%), TMM9 (8.0%), ORU10 (6.0%). PCR amplification of the virulence gene showed the presence of *invA, spvB, pefA* and *csgA* in all isolates. *S.* Typhimurium isolates from samples TYR6, VMA7, EKA8, TMM9, and ORU10 lacked *orgA* and *msgA* genes. Other virulence genes identified include *spaN* (40-100%), *spiA* (40-100%), *spvC* (25-100%), *sipB* (20-100%), and *sefA* (100%).
Conclusion: Our findings reveal that tiger nut juice contained *Salmonella* Typhimurium harboring numerous virulence genes. Therefore, to prevent the general public's health from being harmed by tiger nut drinks contaminated with *Salmonella* Typhimurium, relevant regulatory bodies should enforce strict hygienic practices during the production, handling, and distribution of tiger nut juice.

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Introduction

Tiger nut juice is an indigenous, locally fermented, non-alcoholic beverage drink that is extensively enjoyed by urban and rural dwellers, due to its thirst-quenching and nutritional characteristics. Despite

being consumed throughout the year.[1] It demand is higher during the dry season in Nigeria.[2,3] Tiger nut juice is extracted from tiger nut (*Cyperus esculentus*), which is typically harvested in Spain, West African countries like Nigeria, Senegal, or Ghana, as well as in South America, such as Chile.[4]

In Nigeria, it is known as "Isip Okusa" in Ibibio, "Aya" in Hausa, "Ofio" in Yoruba, and "Akiausa" in Igbo, where these variants (black, brown, and yellow) are grown.[5,6] Tiger nuts are available in three varieties in Nigeria: yellow, brown, and black, with the yellow and brown variants being the most widely available. The yellow variant is more popular due to its larger size and appealing colour. The yellow type, according to Opeyemi and Obuneme,[2] yields more milk when extracted and has less fat, more protein, and fewer anti-nutritional elements, particularly polyphenol.[7]

New developments in the market demonstrate how inventive juice made from tiger nuts are emerging throughout Europe and are gaining traction in the US and other parts of the world.[4]

Because tiger nut drink is homemade, inexpensive, natural, and created using locally available raw materials, many people prefer it to industrially produced soda beverages.[7]

Since it is difficult to regulate the processing of food hawked in developing nations like Nigeria and most vendors lack sufficient knowledge of safe food processing and handling practices, the crude method and unhygienic conditions of preparation may predispose tiger nut juice to microbial contamination as the demand for this beverage increases. The occurrence of food-borne illnesses and pathogens like *Salmonella* species, *Brucella* species, *Shigella* species, *Listeria* species, and *Eschericia coli* infection can spread through local drinks.[1,2,8]

One of the common juice vendor food-borne disease is Salmonellosis. This disease is caused by *Salmonella* species which has over 2,700 known serotypes.[9,10] Consuming tiger nut juice that has been contaminated with bacteria could be linked to the development of the disease in humans because they are regarded as the primary reservoir and transport for *Salmonella.* The clinical presentation of the disease is influenced by the serotype, infectious dosage, virulence factors, and host immunity.[11,12]

Bacterial host-pathogen interactions can modify the expression of particular genes, allowing them to adapt to their surroundings and express their potential to cause disease in the host. As a result, virulence genes promote host survival, colonization, and harm.[13] When *Salmonella* spp. encounters a harsh environment in the hosts' gastrointestinal system, virulence genes are activated. *Salmonella* pathogenicity island (SPI) refers to genomic regions that are connected to virulence functions.[14] In addition, SPI may be transmitted across bacteria by horizontal gene transfer and is associated with virulence mechanisms such as, invasiveness, toxins production, capsules, flagella, fimbriae, host colonization, serotype conversion, and secretion systems.[14,15]

According to Tung *et al*.[13] systemic infections are mostly caused by variable or factors related to bacterial virulence. As such, both the number and type of virulence genes found in the chromosomal SPIs have been linked to *Salmonella*'s pathogenicity.[16,17] For instance, genes like *spi*A and *msg*A facilitate the bacteria's evasion and internalization of macrophages while the *stn* genes are required for the serovars to display virulence in the host.[15,18] Furthermore, *Salmonella* intracellular survival genes have an important role in systemic disease in humans.[19] Meanwhile, adhesion factors like as fimbrial operons facilitate *Salmonella* serovar attachment to epithelial cell types.[20] Furthermore, the *Salmonella* virulence plasmid is critical in increasing the ability of specific serovars to grow in tissues outside the intestinal tract.[21] Other genes, such as *Cdt*B, code for the *Cdt*B component, which is considered a toxin and may play an essential role in the exceptionally long, persistent, and development of systemic disorders.[22,23]

Although *Salmonella* isolates from tiger nut juice may pose a health risk to the public, not enough research has been done on their virulence factors. In addition, the effectiveness of *Salmonella* control initiatives cannot be predicted in the absence of particular data. In order to understand the virulence determinant of *Salmonella* in commonly consumed tiger nut juice circulating the different sections of Ogbete market, it is crucial to understand the virulence peculiarity of the serotype Typhi.

Materials and methods

Study Area

The study was carried out in Ogbete market located at latitude 6.4348°N, and longitude 7.4848°E in Enugu, Enugu State. Ogbete market is one of the earliest developed markets in Igboland, grew to meet people's demands for good and services. This development was as a result of colonial infrastructures which gave rise to intra-ethnic and inter-ethnic transactions.

It is the choice market for wholesale buyers and sellers. Just like other major markets in the country, Ogbete is segmented into lines and lock-up shops. Each line is made up of various lock-up shops occupied by traders dealing on similar or related goods. In Ogbete, some of the lines are numbered alphabetically such as K-line, M-line D-line etc. However, some other major lines are not numbered alphabetically, they are railway line or electronics line, provision line, plastic line, cosmetic line, books line etc. Residents of Enugu state usually prefer going to Ogbete for major shopping, to buy things in bulk, to purchase quality and original goods, to have access to varieties, to buy new products and to buy goods at wholesale or company price. Apart from clothing and textile materials, prices of commodities in the market are moderately affordable.

Sample collection and processing

Ten vendors at Ogbete market were randomly selected to provide fifty (50) freshly produced and packed tiger nut juice with the following codes: ATC1, MECB2, BNC3, CHA4, ERT5, TYR6, VMA7, EKA8, TMM9, and ORU10. One milliliter of each sample was enriched aseptically by transferring it into 10 milliliters of Rappaport Vassiliadis (RV) selective broth enhanced with malachite green, and then incubated at 42°C for 24 hours (Sigma-Aldrich, Mumbai, India). A loopful of the turbid bacteria culture from RV broth was aseptically streaked onto Xylose-Lysine-Deoxycholate (XLD) agar (Sigma-Aldrich, Buchs, Switzerland) and incubated 35°C for 24 hours.[12] After overnight incubation red colonies on XLD agar with black colonies distinguish *S.* Typhimurium from other strains.

Gram staining, conventional biochemical tests, and the MicrogenTM Gn A+B -ID System Bioproducts Limited (Camberly, UK) were used to confirm suspected *S.* Typhimurium colonies. Further testing was done on the isolates using polyvalent *Salmonella* antisera (Oxoid, UK) in accordance with the Kauffmann-White Scheme utilizing the slide agglutination test to screen for somatic "O" and flagella "H"antigen.

Molecular Analysis

Deoxyribonuclease (DNA) Extraction: Fresh bacterial colonies were used for Genomic DNA (gDNA) extraction using the Invisorb Spin Universal Kit (Stratec Molecular, Berlin, Germany) following the protocol suggested by the fabricant and were stored at −20°C until further use. Molecular confirmation of *Salmonella* isolates was done by amplification of a designed STY0307 and STY 2021 gene with the incorporation of Internal Amplification control as which targeted the 16S rRNA gene by endpoint PCR. [24]

Virulence Genes: The molecular characterization genes involved in virulence and pathogenicity was conducted using PCR assay and the Primers used for PCR are listed in Table 1. The reactions were carried out following the manufacturer's recommendation for the GoTaq® Flexi DNA Taq polymerase (Promega, Madison, WI, United States), 1 μL of DNA, and 1 μL of each primer (10 pmol/μL). The ProFlex™ 3×32-well PCR System (Applied Biosystems, Carlsbad, CA, United States) was used to perform the amplification using an initial denaturation for 3 minutes at 95°C, 35 cycles of denaturation for 30 seconds at 95°C, 30 seconds of annealing, extension at 72°C, and final extension for 5 minutes at 72°C. The PCR products were detected by electrophoresis in agarose gel using HydraGreen (ACT Gene, Piscataway, NJ, United States) as an intercalant agent, and the visualization of the gel was conducted in the gel documentation equipment ENDURO GDS (Labnet International, Edison, NJ, United States).

Results

Of the fifty samples, other bacteria were 52.0 % while *S.* Typhi accounted for 24 (48. 0%) in tiger nut juice as shown in figure 1. ATC1 (n=5/50, 10 %), MECB2 (n=2/50, 4.0 %), BNC3 (n=3/50, 6.0 %), CHA4 (n=1/50, 2.0 %), ERT5 (n=4/50, 8.0 %), TYR6 (n=0/50, 0.0 %), VMA7 (n=2/50, 6.0 %),

EKA8 (n=0/50, 0.0 %), TMM9 (n=4/50, 8.0 %), ORU10 (n=3/50, 6.0 %).

*inv*A, *spv*B, *pef*A and *csg*A were found in all isolate. S. Typhi *org*A and *msg*A were absence from isolate found in sample TYR6, VMA7, EKA8, TMM9 and, ORU10. Virulence gene were identified as follows: *spa*N (40-100 %), *spi*A (40-100 %), *spv*C (25-100%), *sip*B (20-100%), *sef*A (100 %) as presented in Table 2

Table 1: Primer sequence of virulence gene

Virulence factor	Gene	Primer sequence (5' –3')	Ampliconsize (bp)	Reference
Structure, the Invasion-associated type III secretion system	invA	F-GTGAAATTATCGCCACGTTCGGGCAA R-TCATCGCACCGTCAAAGGAACC	55	15
	spaN	F-AAAAGCCGTGGAATCCGTTAGTGAAGT R-CAGCGCTGGGGATTACCGTTTTG	55	15
	sipB	F-GGACGCCGCCCGGGAAAAACTCTC R-ACACTCCCGTCGCCGCCTTCACAA	58	15
	orgA	F-TTTTTGGCAATGCATCAGGGAACA R-GGCGAAAGCGGGGACGGTATT	55	15
Fimbriae	csgA	F-TCCACAATGGGGCGGCGGCG R-CCTGACGCACCATTACGCTG	54	15
	sefA	R-GCGTAAATCAGCATCTGCAGTAGC F-GATACTGCTGAACGTAGAAGG	54	15
Salmonella enterotoxin	Stn	F-TTGTGTCGCTATCACTGGCAACC R-ATTCGTAACCCGCTCTCGTCC	617	25
	cdtB	F-ACAACTGTCGCATCTCGCCCCGTCATT R-CAATTTGCGTGGGTTCTGTAGGTGCGAGT	57	
Plasmid	spvC	F-TATGATGGGGCGGAAA R-AGGCTAACACGGGCTT	392	26
	spvB	F-CTATCAGCCCCGCACGGAGAGCAGTTTTTA R-GGAGGAGGCGGTGGCGGTGGCATCATA	58	
	pefA	F-TGTTTCCGGGCTTGTGCT R-CAGGGCATTTGCTGATTCTTCC	700	25
Intracellular invasion	msgA	R-GCGACCAGCCACATATCAGCCTCTTCAAAC F-CCAGGGGTCGTTAGTGTATTGCGTGAGATG	56	27
	spiA	F-CCAGGGGTCGTTAGTGTATTGCGTGAGATG R-CGCGTAACAAAGAACCCGTAGTGATGGATT	57	

Key: F, forward; R, reverse

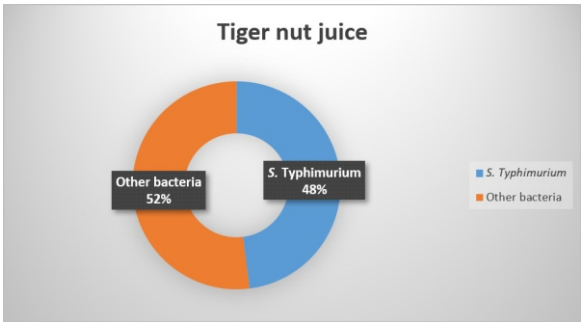

Figure 1: Overall distribution of *S*. Typhi in tiger nut juice

Discussion

Salmonella serovars Typhi 48. 0% was identified in tiger nut juice. Previous research has outlined a similar report.[28] There is evidence of fecal contamination in these samples, which could be the consequence of either a subpar sewage drainage system or the unhygienic personal habits of the beverage's makers. Every strain of Salmonella Typhi poses a risk to food safety and has the potential to spread salmonellosis to humans. *Salmonella* Typhi exhibit pathogenic mechanisms mostly due to the expression of virulence genes, which are triggered by physiological and environmental cues.[29]

The fact that every isolate tested positive for invasion protein A (*inv*A) 100% indicate that *inv*A genes in *Salmonella* Typhi is necessary for the invasion of epithelial cells. The Pathogenicity island-1 of *Salmonella* Typhi contains the Type Three Secretion System (TTSS) apparatus gene *inv*A, which secretes invasion effectors such as invasion factor A, suggests that they can all invade, induce gastroenteritis,[30,31,32] and internalize antigen presenting cells such as macrophages in human.

The curli-specific gene (*csg*A) were completely amplified in all isolates. Previous research has showed that the *csg*A gene is highly detected in *Salmonella* serotypes.[15,33]

The development of biofilm and the upkeep of bacteria in the environment, including on inert or abiotic surfaces, are associated with the *csg*A gene.[34] Similar to how *Salmonella*'s *csg* genes are linked to the bacterium's capacity to form biofilms, which increases drug resistance, the existence of the *csg*A gene is important for public health due to severity and persistent nature of biofilm forming bacterial infection.[35-37] The fact that all of the strains of *Salmonella* typhi have the *csg*A gene suggests that the bacteria may be maintained on abiotic surfaces,

Table 2: Distribution of *S*. Typhi virulence genes in different sample location

Sample location (*n*)	*invA*	*spaN*	*sipB*	*orgA*	*csgA*	*sefA*	*Stn*	*cdtB*	*spvC*	*spvB*	*pefA*	*msgA*	*spiA*
ATC1 (5)	5(100)	2(40)	1(20)	3(60)	5(100)	5(100)	4(80)	5(100)	5(100)	5(100)	5(100)	3(60)	2(40)
MECB2 (2)	2(100)	0(0.0)	1(50)	2(100)	2(100)	2(100)	2(100)	2(100)	0(0.0)	2(100)	2(100)	1(50)	2(100)
BNC3 (3)	3(100)	3(100)	3(100)	1(33.3)	3(100)	3(100)	3(100)	3(100)	0(0.0)	3(100)	3(100)	3(100)	3(100)
CHA4 (1)	1(100)	1(100)	1(100)	0(0.0)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)
ERT5 (4)	4(100)	2 (50)	4(100)	3(75.0)	4(100)	4(100)	4(100)	4(100)	(0.0)	4(100)	4(100)	0(0.0)	4(100)
TYR6 (0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
VMA7 (2)	5(100)	5(100)	3(60)	0(0.0)	5(100)	5(100)	0(0.0)	5(100)	0(0.0)	5(100)	5(100)	0(0.0)	2(40)
EKA8 (0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
TMM9 (4)	4(100)	4(100)	1(25)	0(0.0)	4(100)	4(100)	1(25)	4(100)	1(25)	4(100)	4(100)	0(0.0)	4(100)
ORU10 (3)	3(100)	3(100)	0(0.0)	0(0.0)	3(100)	3(100)	2(66.7)	3(100)	2(66.7)	3(100)	3(100)	0(0.0)	3(100)

like those used in food processing, which is relevant to public health.

Fimbria-associated genes such as *Salmonella* encoding fimbriae A (*sef*A) and plasmid encoding fimbriae A (*pef*A) were detected with 100% accuracy. Previous reports have found *pef*A in isolates from human gastroenteritis patients.[15] *Salmonella typhi* can adapt and proliferate in variety of hosts through the sef operon, which is promoted by the *sef*A gene,[20,33] which makes its presence in the isolated bacteria significant. Additionally, the *sef*A gene has been linked to the serotypes Moscow and Enteritidis; however, other serotypes were able to acquire different genes than those found in this study.[15,38] *Salmonella* plasmid virulence (*Spv*) gene expression requires both *spv*C and *spv*B. When *spv*B and *spv*B concomitantly expressed, they enhances or elevate *Salmonella*'s pathogenicity and may result in systemic infection.[39] *spv*C was detected in 25–100% of the isolates in the current investigation. Generally speaking, a high detection rate of virulence genes suggests a relatively high potential for pathogenicity in *Salmonella typhi.*

In every isolate, the *spv*B gene was discovered. The existence of the *spv*B gene in these isolates is significant, though, as spv genes are strongly linked to strains that infect humans widely and cause non-typhoid bacteremia.[21,40] Although our study unable to determine the expression and carrying status of these gene in cases of non-typhoid bacteremia among the juice consumers but in addition to identify the reservoir of salmonellosis outbreaks, our research into virulence genes has revealed important information into the pathogenicity of *Salmonella* in tiger nut juice drink.

The presence of Oxygen-regulated invasion protein A (*org*A), *Salmonella* Typhimurium surface presentation of antigens N (*Spa*N), and *Salmonella* invasion protein B (*sip*B) were found in the studied isolates. The *spa*N gene is one of the 12 genes that form a cluster associated with host invasion properties while *sip*B is one of the genes encoding *Salmonella* type 3 secretion system transport effector proteins. The expression of these gene via the type III secretion system (TTSS) structure is linked to *org*A, spaN, and *sip*B, allowing *Salmonella Typhi* to infiltrate both phagocytic and nonphagocytic cells.[41]

According to Borah *et al*.[42] the *sip*B gene might be essential to *Salmonella* pathogenicity. In the event that the *inv*A gene's detection rates are as anticipated, this gene is acknowledged as a quick identification tool for the *Salmonella* genus and signifies that every strain is capable of invading cells and causing gastroenteritis.[30] Similar to this, earlier studies have found the presence of the genes *sip*B, *org*A, and *spa*N in *Salmonella* isolates from human and poultry sources.[15,43]

The *Salmonella* enterotoxin (*stn*) genes vary from 25.0 to 100%. Several researchers explored *stn* from food, faeces, or clinical samples using PCR.[25,44] These genes are dominant virulence genes required for the serovars of S. enterica to display virulence in the host.

Other genes, such as Cytolethal distending toxin B (*cdt*B), code for the *Cdt*B subunit, which is regarded a toxin with a potentially key function in the abnormally long, persistent, and development of systemic disorders.[22,43]

In our investigation, we discovered that *Salmonella* pathogenicity island A (*spi*A) and (macrophage survival gene A (*msg*A) contribute to macrophage survival or intracellular survival. The high frequency of the *spi*A gene in juice samples is regarded essential due to the gene's function, which is related to the capacity of *Salmonella* serotypes to form biofilms.[33] According to Abdullahi et al.[45] and Steenackers et al.,[46] biofilm is a significant public health concern because it strengthens resistance to physical forces, the host immune system, and antibiotics.[47] Since the *spi*A gene is connected with *Salmonella* strains linked to poultry,[15] using chicken droppings as manure for tiger nut cultivation may help this gene converge during the harvesting phase to the juice processing stage.

Salmonella strains carrying the *spi*A gene have been shown to be more resilient in chicken farms and to potentially contaminate meat and eggs. Contaminated food is a major source of *Salmonella* infection in humans.[15] Given the reported occurrence of the genes from food-producing animals similar with those genes found in tiger nut juice in this study, there is a greater chance that humans would contract food-borne diseases driven by these bacteria from zoonotic origin.

Conclusion

The results of our analysis show that tiger nut juice had a high occurrence of *Salmonella* virulence genes, suggesting that *Salmonella* could present significant obstacles to maintaining local public health. To avoid *Salmonella* infection outbreaks, we thus advise active surveillance in order to obtain up-to-date knowledge on the pathogenicity of circulating strains of *Salmonella* Typhimurium and the source of contamination.

To protect the public's health from consuming tiger nut juice tainted with pathogenic microorganisms, relevant regulatory bodies should enforce strict hygienic practices during the production, handling, and distribution of tiger nut juice.

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