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Isolation and characterization of histamine-producing, multi-drug-resistant *Enterococcus* species in fermented oil bean seeds in Nsukka, Nigeria

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ABSTRACT

Aims: The microbiological qualities of fermented oil bean seeds depend on the indigenous microflora, personal and environmental hygiene of the handlers and the food environments. This study was aimed to evaluate the incidence of histamine-producing, multi-drug-resistant *Enterococcus* isolates from oil bean seeds during fermentation.
Methodology and results: Histamine extraction and analysis were performed on randomly sampled oil bean seeds. Histamine producing lactic acid bacteria (LAB) were isolated, from where *Enterococcus* species were further isolated. Strain-specific identification and antibiotic sensitivity tests were carried out on the identified *Enterococcus* isolates. Histamine was detected in fermented seeds. *Enterococcus* strains were identified among the histamine-producing fermenters. These include *E. faecalis* HA5, *E. faecium* VB976, *E. faecium* LMEM18, *E. gallinarum* M190262 and *E. gilvus* CR1. *Enterococcus faecalis* HA5, *E. faecium* VB976, *E. faecium* LMEM18, *E. gallinarum* M190262 and *E. gallinarum* were resistant to Ampiclox, Amoxicillin, Ceftriaxone, Ciprofloxacin and Erythromycin. *Enterococcus faecium* VB976, *E. faecium* LMEM18 and *E. gallinarum* M190262 were resistant to Streptomycin and Gentamycin. *Enterococcus faecalis* HA5 was intermediately resistant to Streptomycin and Gentamycin but sensitive to Vancomycin, while *E. gilvus* was intermediately resistant to Ampiclox, Amoxicillin and Gentamycin but sensitive to Ceftriaxone, Vancomycin, Ciprofloxacin, Streptomycin and Erythromycin.
Conclusion, significance and impact of study: The pathogenic and histamine-producing abilities of *Enterococcus* pose serious public health hazard. This is complicated by their resistance to a wide range of antibiotics. Therefore, improving the hygienic practices and regulating fermentation conditions is essential to curtailing histamine production and growth of fermenters with pathogenic potentials and ensuring the safety of the product.

Keywords: Antibiotic resistance, *Enterococcus*, fermentation, histamine production, oil bean seeds

INTRODUCTION

Oil bean (*Pentaclethra macrophylla*) (Figure 1) is a tropical plant that grows in the rainforest of the southern part of West Africa (Okwu, 2001; Asoegwu, 2006). Traditionally, the seeds are fermented between 2-6 days, depending on the individual handler, to improve its contents as well as its nutritional and organoleptic quality. In natural or unfermented form, it contains anti-nutritional contents such as undigestible oligosaccharides and phytate (Olasupo, 2016). It is rich in proteins, lipids, carbohydrates and fibres as well as bioactive components such as phenols, alkaloids, flavonoids and saponins (Okwu and Aluwuo, 2008). Fermentation of the seeds breaks down its phytochemical contents to more nutritious and flavouring delicacies. This process involves microbiological and biochemical changes influenced by raw materials and processing conditions (Steinkraus, 2018). Oil bean seed contains different varieties of amino acids. These include proline, glycine, alanine, cystine, valine, methionine, isoleucine, leucine, tyrosine phenylalanine, lysine, histidine, arginine, aspartic acid, threonine, serine and glutamic acid (Okechukwu *et al.*, 2012). Histamine formation during the processing of oil bean seeds results from the decarboxylation of the histidine component of the food. The processing in most communities is done for family consumption and as a small-scale business. The equipment used in this process

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Figure 1: *Pentaclethra macrophylla* tree.

is rudimentary and does not meet the standard processing procedures in terms of hygiene and other environmental conditions (Gadaga *et al.*, 2004). The microorganisms involved in the fermentation are not precisely known; some are the microflora of the leaves used in wrapping the fermenting seeds, while others are contaminants from the human handlers. There is no stipulated fermentation duration, as each local handler determines their own. All these factors introduce undesirable chemical components and affect the quality and safety of the product.

Enterococci are lactic acid bacteria (LAB) comprising both pathogenic and probiotic species. They are ubiquitous in the environment as inhabitants of soil, water and plants. They are also commensals in the intestines of humans, animals, and insects (Gilmore *et al.*, 2013; Escobedo-Hinojosa and Pardo-López, 2017; Comerlato*et al.*, 2020). They are adapted to several food systems and play active roles in the organoleptic properties of foods. This is due to their ability to tolerate high salt concentrations and a wide range of temperatures and pH (Moreno *et al.*, 2006). They are noble for lactic acid and bacteriocin production. Many species, such as *E. faecium*, *E. faecalis*, *E. durans* and *E. casseliflavus* have been used as starter and non-starter cultures in the fermentation of table olives, dairy products, cheese, traditional soy paste, etc. These organisms bring about the production and scaling up of lactic acid, product maturation and enhancement of characteristic flavours and tastes (Moreno *et al.*, 2006; Hanchi *et al.*, 2014; Anagnostopoulos *et al.*, 2018; Nolasco-Hipolito *et al.*, 2019; Park *et al.*, 2020). Other foods fermented by species of *Enterococcus* include tomatoes, caper berries, sauerkraut, French beans, tea leaves, sorghum, as well as different kinds of vegetables and legumes (M'hir *et al.*, 2012; Barbieri *et al.*, 2019), thus producing various types of biogenic amines.

Histamine is the most biologically crucial biogenic amine owing to its role in the body physiological and pathological processes. They play a wide range of roles in virtually every system of the body. When histamine is produced exogenously and absorbed in large quantities by the body cells, it triggers a wide array of clinical manifestations such as respiratory allergic diseases, symptoms of the skin and soft tissue allergies and reproductive system disorders in women (Panja *et al.*, 2013; Benly, 2015). Some *Enterococcus* species, such as *E. faecium* and *E. faecalis* have been reported to possess *hdc* gene and produce histamine during the fermentation of histidine-rich foods. The quantity of histamine produced, however, depends on the amount of histidine contained in the foods (Kimura *et al.*, 2001; Gardini *et al.*, 2012).

Some species of *Enterococcus* cause invasive severe infections when there is a disruption in the commensal relationship with the host (Lengfelder *et al.*, 2019). They have been reported as the major cause of nosocomial infections, causing diseases such as urinary tract infections, bacteraemia, intra-abdominal infections and endocarditis (Kristich *et al.*, 2014). They were reported as the third most common nosocomial pathogen, causing 14% of hospital-acquired infections (HAI) in the United States between 2011 and 2014 (Weiner *et al.*, 2016). The two most important human pathogens among them are *E. faecalis* and *E. faecium.* Presently, almost all nosocomial enterococcal infections are caused by either *E. faecalis* or *E. faecium*. The pathogenic mechanism is anchored on their ability to out-compete other microbes and to evade host defenses such as gastric acid and bile to colonize the intestinal tract. Enterococci have limited susceptibility to antibiotics due to both intrinsic and extrinsic mechanisms. The intrinsic traits position them to acquire additional resistance on mobile genetic elements when they co-exist with other resistant species. Thus, many of them are multi-drug resistant (Taur *et al.*, 2012). Enterococci spread in the environment through person-to-person transmission or contamination from person to other physical objects. In hospitals, transmission occurs through contact with hospital staff, especially those in direct contact with patients and hospital equipment

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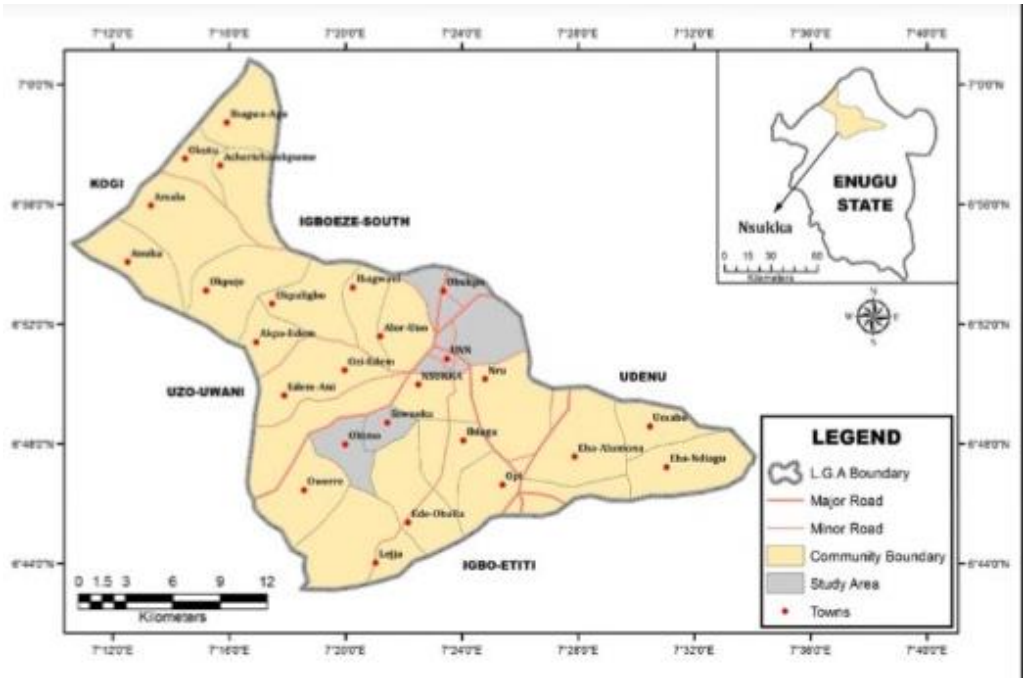

Figure 2: Map of the study area (Nsukka).

(Porwancher *et al.*, 1997). They have the ability to survive several stressful and hostile environmental conditions, such as extreme temperatures and pH, as well as high salt concentration, making them very difficult to eliminate in both hospital and non-hospital environments (Torres *et al.*, 2018). There is no information on the public health hazards associated with the presence of *Enterococcus* species in locally processed oil bean seed in Nsukka. Therefore, this study aims to investigate the incidence of histamine-producing, multi-drug-resistant *Enterococcus* isolates from oil bean seeds in the study area.

MATERIALS AND METHODS

Sample

Traditionally fermented oil bean seeds. The tree is shown in Figure 1.

Materials

Culture media and antibiotic discs were purchased from Oxoid (Cambridge UK) and they include MRS agar, Niven's agar, Bile esculin agar, Nutrient agar, peptone, Pefloxacin (10 µg), Gentamycin (65 µg), Ampiclox (30 µg), Cefuroxime (20 µg), Vancomycin (30 µg), Amoxicillin (30 µg), Ceftriaxone (25 µg), Streptomycin (250 µg), Erythromycin (10 µg) and Gentamicin (10 µg). Other materials include Bacterial/Fungal Minipres Kit (Zymo Research, Catalogue No. D6005), 2× Master Mix (NEB, Catalogue No. M0486), Gel DNA Recovery Kit (Zymo Research, Catalogue No. D4001), Terminator Cycle Sequencing Kit (V3.1, BRD3-100/1000), ZR-96 DNA Sequencing Clean up Kit (Zymo Research, Catalogue No. D4050) and ABI 3500xl Genetic Analyzer (Applied Biosystem, ThermoFisher Scientific).

Sample collection

Two hundred (200) traditionally fermented oil bean seeds were randomly collected from local producers in Ogige market, Nsukka to screen for the presence of histamine producers. The samples were obtained from market women from the adjoining rural communities and local districts within the study area as shown in Figure 2. They carry out oil bean processing as a small-scale business for livelihood. The samples, upon collection, were quickly transported to Microbiology Laboratory and stored in the refrigerator until histamine assay and microbial analysis.

Screening for the presence of histamine in the fermented oil bean seeds by high performance liquid chromatography (HPLC)

Extraction of histamine from fermented oil-been seed

Sixty samples, ten each from a group according to fermentation duration from three days to seven days of fermentation, were randomly selected for the sole purpose of ascertaining the presence of histamine in the samples. Histamine extraction and analysis were performed by modification of the previous method (Bueno-Solano*et al.*, 2012). Five g of each of the samples was ground and homogenized. About 15.00 mg of the triturated sample was placed in a 10 mL capacity volumetric flask and diluted with 5% trichloroacetic acid to

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the volumetric flask mark, mixed for one and a half min and sonicated for 6 min. From the solution, 300 mL was collected and diluted to 5 mL in a volumetric flask with methanol:water (50:50). One mL of the solution was collected and filtered with a 0.45 µL membrane.

Derivatization

One hundred microlitres of the derivatization agent, orthorphthalaldehyde, was collected from the reagent vial and injected into the samples. The mixture was vortexed, and the reaction was allowed to be completed in 5 min. A thoroughly washed micro syringe was used to pipette 5.0 µL of the derivatized samples into the HPLC column.

Chromatographic separation

The HPLC system (Agilent 1200 Series) was set up and allowed to stabilize in 30 min. Five µm of the derivatized samples were injected into the system with a flow rate of 1.0 mL/min. The HPLC conditions were maintained as follows: Column (Chromsep SS C18 with the dimension of 150 mm × 4.6 mm × 5 µm), Mobile phase (Mobile phase (A), Tetrahydrofuran:methanol:phosphate-buffer (100 mmol/L) (1:8:9) and Mobile phase (B), Methanol:phosphate-buffer (100 mmol/L) (80:20), with gradient of Min/A%B%, (8/75/25). (12/67/33). (25/50/50). (30/0/100). (35/67/33). The mobile phase was filtered through 0.4 µm membrane and degassed. Histamine was quantified using detector (AGILENT 1260) at a wavelength of 254 nm.

Isolation of lactic acid bacteria

Isolation of LAB was done by modification of the previously used method (Ngene *et al.*, 2019). Three g of each of the 200 samples of fermented oil bean seeds were ground and suspended in 10 mL of sterile 0.1% peptone solution. 1 mL of each of the supernatant was further diluted to obtain 10^{-3} diluents. 0.5 mL of each of the final diluents were smeared on duplicate plates of MRS (DeMan, Rogosa, and Sharpe) agar and incubated at 37 °C for 48 h to isolate lactic acid bacteria (LAB). These were done under aerobic conditions. Growth in MRS is presumed to be the presence of LAB. Discrete colonies on the MRS were sub-cultured on MRS agar to obtain a pure culture. Morphologically different colonies from each sample were preserved separately.

Isolation of histamine producers

The pure isolates of LAB were inoculated onto duplicate plates of Niven's agar and incubated under aerobic conditions for 72 h at 37 °C. Purple colonies were indicative of histamine production (Niven *et al.*, 1981). The purple colonies were then streaked on MRS agar to obtain pure isolates. The isolates were incubated in MRS agar slant at 37 °C for 72 h and then preserved in the refrigerator for future use.

Isolation of *Enterococcus* species

The histamine-positive LAB were subjected to Bile Esculin Test according to the method described by Albert and Anicet (1999). Overnight cultures of the pure isolates were inoculated in a slant of Bile Esculin agar and incubated for 48 h at 37 °C to rule out non group D viridians streptococci. Agar slant with growths which darkened with time (positive test) were selected for salt tolerance test to isolate *Enterococcus*. Bile esculin-positive isolates were inoculated into tubes of Trypticase Soy Broth containing 6.5% NaCl to isolate *Enterococcus* species (Manero and Anicet-Blanch, 1999). The cultures were incubated for 48 h at 37 °C and monitored for turbidity. Salt-tolerant isolates were selected for molecular identification.

Strain-specific identification of the isolates

All the presumptive *Enterococcus* isolates were subjected to molecular test. The genomic DNA of the isolates was extracted using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, Catalogue No. D6005). The 16S target region was amplified using OneTaq® Quick-Load® 2× Master Mix (NEB, Catalogue No. M0486), with AGAGTTTGATCMTGGCTCAG and CGGTTACCTTGTTACGACTT as primer sequences. The PCR products were run in a gel and gel-extracted with Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Catalogue No. D4001). The extracted fragments were sequenced in the forward and reverse direction (Nimagen, BrilliantDye™ Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000) and purified (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™, Catalogue No. D4050). The purified fragments were analyzed on the ABI 3500xl Genetic Analyzer (Applied Biosystem ThermoFisher Scientific) for each reaction for every sample, as listed below. CLC Bio Main Workbench v7.6 was used to analyze the abi files generated by the ABI 3500XL Genetic Analyzer and results were obtained by a blast search (NCBI).

Antibiotic sensitivity testing

Genetically confirmed species of *Enterococcus* isolates were cultured for 16 h at 37 °C on nutrient agar to obtain fresh cultures. The colonies were then standardized and subjected to agar diffusion test using Gram-positive disc (Oxoid UK), containing Pefloxacin (10 µg), Gentamycin (65 µg), Ampiclox (30 µg), Cefuroxime (20 µg), Vancomycin (30 µg), Amoxicillin (30 µg), Ceftriaxone (25 µg), Streptomycin (250 µg), Erythromycin (10 µg) and Gentamicin (10 µg). The plates were incubated for 16 h and zones of inhibition diameter were determined. The sensitivities of the isolates were determined using Clinical Laboratory Standard Institute (CLSI) version 2020 guideline.

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Table 1: Results of HPLC enhanced integrator analysis of histamine contents of the fermented oil bean seeds.

Sample group according to fermentation duration	No of samples per group	Average quantity per group in µg/kg	Average quantity in all fermented samples
Control (unfermented)	10	0.00000 e^{-1}	0.00000 e^{-1}
Group 1 (3 days)	10	1.08027 e^{-1}	6.70793 e^{-1}
Group 2 (4 days)	10	7.63971 e^{-1}	
Group 3 (5 days)	10	7.93773 e^{-1}	
Group 4 (6 days)	10	8.22040 e^{-1}	
Group 5 (7 days)	10	8.66155 e^{-1}	
Total	60		

Table 2: Antibiotic resistance profiles of histamine-producing *Enterococcus* strains isolated from fermented oil-been seeds.

Organism	APX	AM	C	V	CPX	S	E	GN
E. faecalis HA5	R	R	R	S	R	I	R	I
E. faecium VB976	R	R	R	R	R	R	R	R
E. faecium LMEM18	R	R	R	R	R	R	R	R
E. gallinarum M190262	R	R	R	I	R	R	R	R
E. gallinarum ELU0020-T93-S-NI_000167	R	R	R	I	R	R	R	R
E. gilvus CR1	I	I	S	S	S	S	S	I

Key: R = Resistant, I = Intermediate, S = Sensitive; APX = Ampiclox, AM = Amoxicillin, C = Cetriaxone, V = Vancomycin, CPX = Ciprofloxacin, S = Streptomycin, E = Erythromycin, GN = Gentamycin.

RESULTS

Average quantity of histamine in 15.00 mg of the triturated sample of fermented oil bean seeds.

The oil-bean extracts were subjected to HPLC and the result showed the presence of histamine in all the sixty samples used. The quantity of histamine varies across the samples, with an average quantity of 6.70793^{e-1} per sample (Table 1).

Prevalence of histamine-producing *Enterococcus* in the fermented oil bean seeds

Out of two hundred samples screened, 121(60.5%) were lactic acid bacteria. Out of these, a total of 32 distinct colonies were identified as *Enterococcus* species, representing 26.4% of the total LAB. The *Enterococcus* isolates were later confirmed genetically as *E. faecium* LMEM18, *E. faecium* VB976, *E. gallinarium* 190262, *E. gallinarium* ELu0020-T93-S-NI_000167, *E. faecalis* HA5 and *E. gilvus* CR1.

Antibiotic sensitivity profile and resistance indices of the genetically identified strains

The antibiogram of the isolates shows that *E. faecium* and *E. gallinarum* were totally resistant to Ampiclox, Cefuroxime, Amoxicillin, Ceftriaxone, Ciprofloxacin, Streptomycin, Cotrimoxazole, Erythromycin, Pefloxacin and Gentamicin (10 µg). *Enterococcus faecalis* was intermediately resistant to Streptomycin and Gentamicin and totally resistant to all others. *Enterococcus gilvus*, however, was intermediately resistant to Ampiclox, Cefuroxime, Amoxicillin and Gentamicin but sensitive to Cefriaxone, Ciprofloxacin, Streptomycin, Cotrimoxacin, Erythromycin and Pefloxacin (Table 2).

DISCUSSION

The accumulation of exogenous histamine in fermented foods is a serious public health concern. This study focused on the activities of histamine-producing *Enterococcus* species that are involved in the fermentation of oil bean seeds. The presence of histamine in all the samples of fermented oil bean seeds screened is a strong indication of microbial decarboxylation of the histidine content of the Oil bean seed which is one of the amino acid contents of the food (Okechukwu *et al.*, 2012). Fermentation duration played a significant role in histamine accumulation. The longer the fermentation duration, the more the quantity of histamine accumulated. This is because the microbial counts and decarboxylation of histidine in the food samples increased with time. Though the histidine quantity in the sample may be relatively little, its decarboxylation is of great importance to the health of the consumers owing to the pathological roles of histamine. The average histamine concentration was 6.7 µg/kg of the samples screened (Table 1). Histamine accumulation of more than 40 mg/meal or 0.75 mg/kg body weight is considered hazardous (Douen *et al.*, 2017). Oil beans, being a popular delicacy and flavouring agent in the study area, could spike the chances of consumption of large quantities of histamine by the locales.

The presence of histamine-producing *Enterococcus* in the fermented oil bean seeds calls for serious public health concerns. Some species are highly invasive pathogens implicated in many nosocomial infections, especially in immunocompromised individuals (Kimura *et*

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Table 3: Multi-drug resistant indices of the isolates.

Organism	Resistance index
E. faecalis HA5	0.88
E. faecium VB976	1.00
E. faecium LMEM18	1.00
E. gallinarum M190262	1.00
E. gallinarum ELU0020-T93-S-NI_000167	1.00
E. gilvus CR1	0.38

al., 2001; Gardini *et al.*, 2012; Lengfelder *et al.*, 2019). The bacteria may have gained entry into the food as green leaf microflora or as environmental and human contaminants. Recall that the genera are found in plants, animals, including human GIT as well as soil (Gilmore *et al.*, 2013; Escobedo-Hinojosa and Pardo-López, 2017; Comerlato *et al.*, 2020). The fermentation conditions of oil bean seeds are suitable for the growth of *Enterococcus*. The bacteria grow in a wide range of pH, temperature, and salt concentrations (Moreno *et al.*, 2006), which are typical of oil bean seed fermentation. The production of lactic acid during the fermentation process lowers the food's acidity, making it possible for the *hdc* gene to be activated for histamine production.

Five of the six identified strains of *Enterococcus* belong to the species *E. faecalis*, *E. faecium* and *E. gallinarium* which are implicated as human pathogens (Lengfelder *et al.*, 2019). This further buttress the danger they pose in food when consumed raw, as is sometimes the case in the study area. The high antibiotic resistance indices are possibly because of several exposures to various antibiotics during treatments (Table 3). Recall that these organisms have been implicated in several nosocomial and community acquired infections, especially *E. faecalis* and *E. faecium*, which are considered the most important human pathogenic *Enterococcus* species (Dubin and Pamer, 2014). Their regular exposures to the drugs give room for selection of adapted strains and subsequent multiplications. Therefore, it is not suprising to observe that the two most implicated species (*E. faecalis* and *E. faecium*) are totally resistant to most antibiotics. *Enterococcus gallinarum*, though not acknowledged as a leading human pathogen, inhabits the gut and has been exposed to antibiotics. This may have accounted for its high resistance index. Again, gene exchange is possible as it co-inhabits with other species. *E. gilvus* was susceptible to most antibiotics and only showed intermediate resistance to a few others. The reason for this pattern is not clear but may be connected with the source of the isolates, either from the environment or human handler.

CONCLUSION

Enterococcus isolates are potential pathogens. Their ability to produce histamine in foods poses additional food poisoning hazards, while their resistance to antibiotics poses a treatment challenge. Therefore, the involvement of *Enterococcus* species in the fermentation constitutes serious public health hazards as histamine producers and as resistant pathogens, thus making traditionally fermented Oil bean seeds in rural communities subtly unsafe for human consumption. With high levels of antibiotic resistance, treatment of food poisoning caused by these strains using antibiotics may be defeated. Therefore, adequate hygiene and proper regulation of all fermentation conditions have to be improved to ensure the safety of this product.

CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest whatsoever.

AUTHOR'S CONTRIBUTION

All authors listed have made significant intellectual contributions to the work and approved it for publication.

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