Oladapo *et al.*
Bulletin of the National Research Centre (2022) 46:175
https://doi.org/10.1186/s42269-022-00857-2

Bulletin of the National Research Centre

RESEARCH

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Direct detection of *iro B*, *stn* and *hil A* virulence genes in *Salmonella enterica* serovar typhimurium from non-ripened cheese

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Abstract

Background: Microorganisms' survival is based on the importance of growth factors found in the microbial environment, as well as their overwhelming appetite for survival which is controlled by their genetic material. This study was designed to investigate the virulence patterns of *Salmonella enterica* serovar *typhimurium* associated with soft cheese. Two hundred and sixty (260) soft cheese samples were collected from vendors at Akure, Ikare and Owo in Ondo State, Nigeria. Molecular characterization of six (6) *Salmonella enterica* isolates for the possession of *iro* B, *stn, hil* A and *spv* C gene were revealed through multiplex polymerase chain reaction (PCR) analysis with DNA extraction, polymerase chain reaction (PCR) amplification, sequencing and genome blasting, respectively.

Results: Four (4) possess only iro B; two (2) possess only stn gene; and another three (3) possess hil A gene as none of the isolates possess *spv* C gene. This study revealed antibiotic-resistant *Salmonella enterica* serovar *typhimurium* is present in locally made soft cheese samples. It also revealed that virulence genes comprising *hil* A, *stn* and *iro* B as well as plasmids-mediating antibiotic resistance are present in *S. enterica* serovar *typhimurium* found in locally produced cheese.

Conclusions: The presence of molecularly elucidated virulence genes comprising *Iro B*, *Stn* and *Hil A* makes the soft cheese potentially viable for pathogenicity. This study recommends food-borne salmonellosis should be constantly monitored with appropriate cleaning of preparatory paraphernalia in cheese production. Constant sensitization of nomads to improve their awareness on milk-borne zoonosis and its associated risk factors is needed.

Keywords: Antibiotic resistance, Soft cheese, *Salmonella enterica* serovar *typhimurium*, Salmonellosis, Virulence gene

Background

The presence of *Salmonella enterica* serovar *typhimurium* poses serious threat to human health and food antimicrobial sensitivity akin to drastically reducing the antimicrobial importance of food (Amiri and Moradli 2016). The bacterium is a primary enteric pathogen infecting both humans and animals. This infection begins with the ingestion of contaminated food or water by the ease passage of *Salmonellae* into intestinal epithelium of the human system to trigger gastrointestinal disease (Amiri and Moradli 2016). Also, this organism is among the most common etiological agent of cases and outbreak of food-borne diarrheal illnesses. For *Salmonella enterica* serovar *typhimurium* to cause any infection, the presence of O and H antigens is essential which indicate the somatic and the flagella strains of genes, respectively, i.e. the invasion and the spread of the pathogen (Larrisa et al. 2016). Mostly, *Salmonella enterica* serovars are found worldwide in warm-blooded animals. They are intracellular pathogens which insinuate they reside inside the body of living host (Larrisa et al. 2016).

Salmonellosis is associated with severe morbidity and even mortality in farm animals representing a major

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economic productivity loss in the food and animal industries (Leekitcharoenphon et al. 2016). Hence, it is systematic infection caused by *Salmonella* species and serotypes. The most common clinical symptoms range from headache, fever, abdominal cramps and vomiting. Young and old individuals especially those with weakened immune system, i.e. either they are immune-compromised or immune-suppressed, are bound to develop severe form of salmonellosis like reactive arthritis, irritable bowel syndrome (Leekitcharoenphon et al. 2016). *Salmonella enterica* serovars has major public health burden worldwide, causing over 1 billion human infections annually with its impact being responsible for significant morbidity and mortality (Leekitcharoenphon et al. 2016). An estimated 94% of salmonellosis is transmitted by food (Luvsansharav et al. 2020). As a result, implicated foods are often of animal origin such as beef, poultry, milk, and eggs. Any food, however, can become contaminated through cross-contamination, environmental contamination, or by the unwashed hands of food workers (Leekitcharoenphon et al. 2016).

The emergence of multidrug resistant strains limits the possibilities of effective treatment, and new antimicrobial targets are required (Akinyemi et al. 2018). *Salmonella enterica* serovar *typhimurium* is broadly adapted to the host with the respect of being the major cause of food-associated nontyphoidal gastrointestinal disease, with asymptomatic carriage in farm animals presenting a common reservoir for transmission to humans (Rivero-Calle et al. 2021). However, the prevalence of enteritis and its accompanying diarrheal and other health challenges linked to infections with *Salmonella* has continuously plagued sub-Saharan Africa (Akinyemi et al. 2018; Rivero-Calle et al. 2021). In Nigeria, typhoid fever is among the major widespread diseases affecting both young and old as a result of many interrelated factors such as inadequate sanitation, indiscriminate use of antibiotics and faecal contamination of water sources (Akinyemi et al. 2018). Morbidity associated with illness due to *Salmonella* continues to increase with untold fatal consequences, often resulting in death. An accurate figure of cases is difficult to arrive at because only large outbreaks are mostly investigated whereas sporadic cases are under-reported (Rivero-Calle et al. 2021).

In Africa, *S. enteritidis* and *S. typhimurium* were the two most common serotypes reported, occurring in 26% and 25% of the isolates, respectively (Jajere 2019). *Salmonella enterica* serovar *typhimurium* (29%) was most frequently reported in clinical isolates in North America, followed by *S. enteritidis* (21%). In spite of improvements in hygiene and sanitation, the incidence of nontyphoidal *Salmonella* (NTS) infections continue to increase, creating a burden in both industrialized and underdeveloped countries (Majowicz et al. 2010).

Salmonella is of great concern for dairy industry. It causes morbidity and mortality throughout the world with the host immune response which can either be infection is acute (limited) or chronic (Systemic) (Pui et al. 2011; Ruszel et al. 2021). The nature of pathogenicity of an organism lies in the virulence genes or virulence factors. The majority of virulence genes of *Salmonella* are clustered in a region distributed over the chromosome, called *Salmonella* Pathogenicity Islands (SPI) (Eng et al. 2015; Dos Santos et al. 2021). *Hil* A gene, an invasive operon of salmonella, is found to trigger the internalization of deeper tissue of host systems as revealed by Yanestria et al. (2019). *Salmonella* virulence factors were also detected in virulence plasmids in certain *Salmonella* serovars like *Salmonella enterica* serovar *typhimurium* as juxtaposed by Yanestria et al. (2019).

Cheese is a nutritional versatile dairy food. A wide variety of cheese types are available to meet specific consumer requirements and allow convenience use (Aldrete-Tapia et al. 2018). The microbiota present in local soft cheese production across seasons is a crucial role as it shows the diversity in growth of microorganisms which is based on milk composition and environmental conditions like temperature, humidity, etc. (Aldrete-Tapia et al. 2018). However, bacterial species that were found at the start of production of cheese reduces, as there were subsequent processes involved in the production of cheese that can inhibit or destroy the presence of resident organisms. A preceding study has shown that these organisms are potentially antagonistic in nature (Aldrete-Tapia et al. 2018). Apart from *Salmonella enterica*, several organisms such as *Listeria monocytogenes*, *Escherichia coli*, *Enterococcus faecalis*, and *Shigella dysenteriae* are quite present in unsafe cheese production as detailed by Sangoyomi et al. (2010); Al-Gamal et al. (2019).

The production of diverse dairy products comprising soft cheese from raw milk improved its microbial quality by increasing the salinity of the manufactured dairy products (Al-Ashmawy et al. 2016). Conversely, the primordial method of manufacturing under unrestrained sanitized conditions in developing countries is a barrier to good soft cheese with sound microbiological quality (Omar et al. 2018). Satisfactory milk pasteurization application in non-ripened cheese could inactivate pathogenic derivatives during soft cheese production and storage (Fusco et al. 2020). Hence, this study set out to investigate the virulence patterns of *Salmonella enterica* serovar *typhimurium* associated with soft cheese in South-West, Nigeria.

Methods

Materials used

Colony counter (J-2 PEC MEDICAL, New Jersey, USA), Salmonella-shigella agar (SSA), sterile distilled water, 70% ethanol, sodium acetate, magnesium chloride ($MgCl_2$), BLAST-Basic local alignment search tool, soft cheese, microtube, and refrigerated universal centrifuge.

Study design

This is an experimental study conducted between January and late April 2019. It was hypothesized that there are complex genetic dynamics of mobile genetic elements that codes for the establishment of pathogens in host cells. As a rider to this, soft cheese samples were randomly collected from nomads to checkmate the hygienic practices of sellers and potential pathogenicity for susceptible consumers.

Description of Study location

The study location was accessed through experimental design. Areas of study are Ikare, Owo and Akure which are located at the Northern (Ikare and Owo), and central part (Akure) of Ondo State, respectively (Fig. 1). Ikare lies between latitudes 070.27′N; 070.31′N and longitudes 0050.43′E; 0050.47′E. Akure lies between latitudes 7.25′ N and longitude, 5.195′ E. Owo, on the other hand, is located between latitude 7.2° N and longitude, 5.59° E. These areas are moderately populated with moderate standard of living. Residents living in these areas are majorly farmers, teachers, traders, civil servants, legal and medical practitioners.

Collection of samples

Two hundred and sixty (260) cheese samples were collected from the suburbs of Ilepa at Ikare Akoko, Federal institutions and residential Estates at Akure as well as parks at Owo from January to late April 2019. The samples were stored in a cooler averagely filled with ice

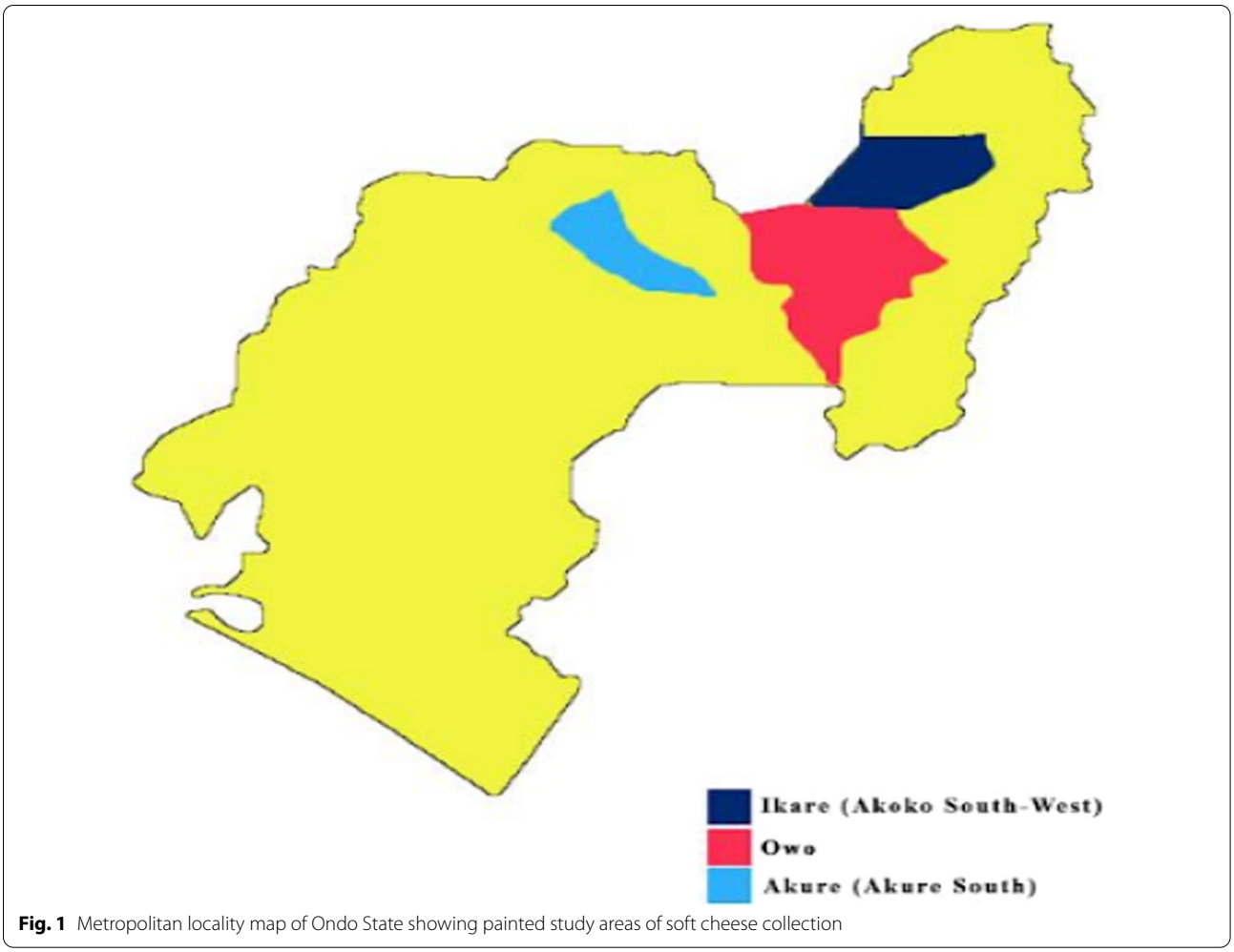

Fig. 1 Metropolitan locality map of Ondo State showing painted study areas of soft cheese collection

packs (4 °C) for three (3) hours because of the proximity between the area of collection and transfer to the laboratory. However, fresh milk of a healthy cow was the positive control experiment, while water source which was used for the cooking of cheese product was the negative control experiment.

Isolation and enumeration of bacteria

Colony counting was carried out visually by counting the number of visible colonies that appeared on the Salmonella-shigella agar (SSA) plates using rapid plating method; plates that had distinct colonies were used. Calculation of colony forming unit (CFU) per millilitre (ml) for the bacteria were recorded in standard form (Bayode et al. 2021). The number of colonies on each plate was recorded utilizing a colony counter (J-2 PEC MEDICAL, New Jersey, USA) (McHugh et al. 2017). Identification of the *Salmonella enterica* serovar *Typhimurium* was divulged via to the white–Kauffmann–Le minor protocol (Issenhuth-Jeanjean et al. 2014). Bacterial organisms were distinctively identified culturally and biochemically following the method of Onifade and Oladapo (2020). Nutrient agar and salmonella-shigella agar media were sterilized according to manufacturers' specifications. One (1) gram of each cheese sample was aspirated into 9 ml of sterile 0.1% peptone water, shaken vigorously for a minute and serially diluted up to 10^{-4}. One millilitre (1 m) of diluent 10^{-2} and 10^{-4}were aseptically transferred into two (2) sterile Petri dishes, respectively. Fifteen millimetres (15 ml) of each growth medium (NA and SSA) were aseptically poured into Petri plates containing the diluents and were homogenized gently and allowed to gel. The labelled inoculated plates were incubated at 37 °C aerobically at 24 h. Pure culture of *Salmonella enterica* were stored in sterile double strength nutrient agar slant bottles and kept at 4 °C for further studies.

Extraction of bacterial deoxyribonucleic acid (DNA)

DNA Extraction method used and DNA concentration were evaluated through the steps described by Jena bioscience Kit™ (Jena GmbH, Loebstedter, Jena, Germany). The steps were classified into three (3) which are lysis, precipitation and hybridization (Munch et al. 2019). The lysis of bacteria cells was carried out, as 1 ml of each pure isolate was transferred into a 1.5-ml microtube, centrifuged at 14,850 revolutions per minute (rpm) for 1 min using refrigerated universal centrifuge (BR Biochem Life Sciences Private Limited, New Delhi, India). The supernatant obtained was discarded, leaving the residue (pellet) in the microtube. The pellet contains peptidoglycan particles that encapsulate the chromosome (DNA or RNA). The pellets were re-suspended in cell resuspension solution. 1.5 µl of RNase A solution was added to lyse the ribonucleic acid (RNA) genome, mixed by inversion, incubated at 37 °C for 15–30 min and cooled on ice for 1 min (Vaidya et al. 2018).

Polymerase chain reaction (PCR) assay

Reconstitution of primers

Stock primers were stored in lyophilized form in order to maintain the efficacy of the primer and to avoid contamination. These were virulence primers of *S. enterica* serovar *typhimurium* which include *hil A, stn, iro B, and spv B* which were in forward and reverse (Table 1). However, to work with these primers, there is a need to reconstitute these primers to a working solution, i.e. it has to be diluted. The lyophilized stock primers were transported in microtubes and were diluted by adding some amount of PCR-grade water (nucleic water) into the microtubes containing the primer (Tassinari et al. 2020).

The tubes were spun down because pellet can often be dislodged during shipping and may be in the cap. A 100 µM master stock was prepared by adding specific amount of nucleic water to each of the lyophilized

Table 1 PCR primer pairs screened for specificity to *Salmonella enterica*

Primer pair target	Primer sequence (5′→ 3′) (*)	Annealing†	Cycles‡	Length§
SPI1 invasion gene (*hil A*)	CTG CCG CAG TGT TAA GGA TA CTG TCG CCT TAA TCG CAT GT	62	30	497
Enterotoxin gene (*stn*)	CTT TGG TCG TAA AAT AAG GCG TGC CCA AAG CAG AGA GAT TC	55	25	260
Fur-regulated gene (*iro B*)	TGC GTA TTC TGT TTG TCG GTC C TAC GTT CCC ACC ATT CTT CCC	55	30	606
Multiplex – virulence plasmid gene (*spvC*)	ACT CCT TGC ACA ACC AAA TGC GGA TGT CTT CTG CAT TTC GCC ACC ATC A			571

[*] The Primer sequence shows forward primer listed first followed by the reverse primer

[†] Annealing temperature in °C

[‡] Cycles = the number of times the dissociation, annealing and elongation steps were repeated for amplification

[§] Length of amplification product in base pairs

primer in the tube. The amount of nucleic water needed was measured according to manufacturer' specification, i.e. multiplying number of 'nmol' of primer in the tube by 10. The original primer tubes were used for this 100-µM stock. Newly suspended master stock primers were allowed to sit at room temperature for 10 min before they were used for working stock dilutions. Afterwards, the master stock primer was evenly homogenized by vortex mixer and diluted further with sterile molecular grade water in the ratio 1:10 to obtain a working stock (Shome et al. 2020).

PCR analysis of stn, hil A, iro B and spv C gene embedded in *Salmonella enterica* serovar *typhimurium*

PCR analysis was run with the working stock primers uniquely produced for the detection of *Salmonella enterica* serovar *typhimurium* virulence genes. The PCR mix comprises of 1 µl of 10X buffer, 0.4 µl of 50 mM $MgCl_{2,}$ 0.5 µl of 25 m MdNTPs, 0.5 µl of 5 mM selected working primers, 0.05 µl of 5 µl Taq polymerase with 2 µl of template DNA and 5.05 µl of sterile distilled water to make up 10 µl reaction mix (Kebede et al. 2016). The PCR profile used was initial denaturation temperature of 94 °C which lasted for 3 min, followed by 30 cycles of 60 s, 56 °C for 60 s, 72 °C for 120 s and final extension temperature of 72 °C for 5 min and the 10 °C on hold for few hours (Kebede et al. 2016).

Purification of PCR products

The amplicon was further purified before the sequencing using 2 M sodium acetate wash techniques. To about 10 µl of the PCR product, 1 µl of 2 M NaAct pH 5.2 was added. 20 µl absolute ethanol was also added, stored at -20 °C for 1 h, centrifuged at 10,000 rpm for 10 min, washed with 70% ethanol and then air-dried. The mixture was re-suspended in 5-µl sterile distilled water and kept at 4 °C for sequencing (Tassinari et al. 2020).

Preparation of samples for Gene Sequencer (ABI 3130 × 1 machine)

The cocktail mix was a combination of 9 µl of Hi Di formamide with 1 µl of purified sequence, making a total of 10 µl. The purified PCR products were loaded in the ABI 3130xl machine (genetic analyser system) CA, USA, and the nucleotide sequences were obtained (Shome et al. 2020).

PCR sequencing

The PCR mix used comprises 0.5 µl of Big Dye Terminator™ mix, 1 µl of 5X sequencing buffer, 1 µl of forward working stock primers with 6.5 µl distilled water and 1 µl of the PCR product which make up a total of 10 µl. The PCR profile for sequencing was rapid profile, the initial rapid thermal ramp to 96 °C for 1 minute, followed by 25 cycles of rapid thermal ramp to 96 °C for 10 s rapid thermal ramp to 50 °C for 5 s and rapid thermal ramp to 60 °C for 4 min. This was followed by rapid thermal ramp to 4 °C and placed on hold for few hours (Costessi et al. 2018).

Purification of PCR sequencing products

The PCR sequence product was also purified before the sequencing running using 2 M sodium acetate wash technique (Costessi et al. 2018). To 10 µl of the PCR product, 1 µl of 2 M NaAct pH 5.2 was added, followed by adding 20 µl absolute ethanol. The mixture was thereafter kept at -20 °C for 1 h, spun at 10,000 rpm for 10 min, washed with 70% ethanol, after which it was air-dried. It was re-suspended in 5-µl sterile distilled water and kept at 4 °C for sequencing running (Costessi et al. 2018). These reaction products were then re-suspended and analysed for nucleotide sequence determination using capillary electrophoresis (Applied Biosystems (ABI) prism 310 Genetic Analyser) (Shome et al. 2020). The sequence obtained was translated using gene sequential software and were compared with online genomic database using BLAST programme (http://www.ncbi.nlm.nih.gov/BLAST/blast) by submitting the sequence obtained to Gen Bank of National Centre for Biotechnology Information (NCBI) website.

Statistical analysis of data

Data obtained were subjected to one-way analysis of variance (ANOVA) and treatment means were separated using Duncan's new multiple range test (DNMRT) at $p \leq 0.05$ level of significance with the aid of Statistical Package for Social Sciences (SPSS) version 23.

Results

Frequency of bacterial occurrence in cheese samples from selected cities

Table 2 shows that Akure metropolis had the highest plate count of enteric pathogens with $18.50 \pm 0.71 \times 10^5$ CFU/ml on nutrient agar as well as $5.79 \pm 0.45 \times 10^4$ CFU/ml on Salmonella-shigella agar. The frequency of bacterial occurrence isolated from cheese samples obtained from three (3) senatorial districts of Ondo State (Akure, Ikare and Owo) is shown in Table 3. A total of two hundred and sixty (260) cheese samples were studied for the presence and the virulence patterns of *Salmonella enterica* serovar *typhimurium* as illustrated in Table 3. Fifty (50) cheese samples were vended in Ikare province with varying locations ranging from transit garages, and market square. At Akure, one hundred and thirty (130) vended cheese samples were obtained from bus stops, car parks, suburbs, as well as market square. The remaining 80 cheese samples

Table 2 Mean count of enteric pathogens in cheese samples on Nutrient and *Salmonella-Shigella* (SS) agar (CFU/ml)

Town/City	Period (week)	Samples (Cheese)	Bacterial colonies on Nutrient agar (CFU/ml) (10^{-2}) diluent	Bacterial colonies on S.S. agar (CFU/ml)
Ikare	1	10	8.00 ± 0.14^{e}	1.95 ± 0.07^{a}
	2	10	4.99 ± 0.02^{c}	4.10 ± 0.14^{a}
	3	10	5.75 ± 0.07^{d}	3.30 ± 0.00^{e}
	4	20	8.50 ± 0.14^{ef}	3.85 ± 0.07^{f}
Akure	5	20	18.50 ± 0.71^{l}	0.50 ± 0.71^{c}
	6	20	13.20 ± 0.21^{i}	5.79 ± 0.45^{f}
	7	20	15.30 ± 0.57^{j}	2.40 ± 0.14^{cd}
	8	20	17.35 ± 0.49^{k}	4.15 ± 0.07^{f}
	9	10	18.15 ± 0.21^{l}	2.10 ± 0.14^{d}
	10	15	12.35 ± 0.49^{h}	3.60 ± 0.00^{e}
	11	15	8.93 ± 0.12^{f}	2.10 ± 0.00
	12	10	10.85 ± 0.07^{g}	1.65 ± 0.07^{a}
Owo	13	21	1.65 ± 0.07^{a}	2.25 ± 0.07^{c}
	14	20	2.40 ± 0.14^{b}	0.00 ± 0.00
	15	19	2.40 ± 0.00^{b}	0.00 ± 0.00
	16	20	1.45 ± 0.07^{a}	1.55 ± 0.07^{a}

Data are presented as mean ± Standard error (n = 2). Values of the same superscript in the same column are not significantly different at $p \leq 0.05$. S.S—Salmonella-shigella; CFU—Colony forming unit

Table 3 Percentage frequency of occurrence of bacteria isolated from cheese samples across towns

Town/City	Number of cheese tested	Number of positive cheese sample (%)
Akure	130	110 (84.6)
Ikare	50	50 (100)
Owo	80	41 (51.3)
Total	260	

Percentage frequency = (Number of positive cheese samples/Number of cheese tested) × 100

were vended across towns in Owo province. The highest percentage frequency of bacterial occurrence in local soft cheese obtained was accounted from Ikare Akoko, (100%) while the lowest frequency of bacterial occurrence was detected in samples obtained from vendors in Owo town (51.3%).

Presumptive identification of bacterial isolates obtained from cheese samples

Twenty-nine (29) bacterial isolates were obtained from cheese samples and analysed for morphological and biochemical characteristics (Table 4). These isolates were presumptively identified as *Salmonella enterica*, *Shigella* spp, *Escherichia coli*, *Listeria monocytogenes*, *Yersinia enterocolitica*, and *Enterococcus faecalis* all of which are gram negative.

Molecular identity of virulence genes in *Salmonella enterica* serovar *typhimurium*

Six (6) *Salmonella enterica* isolates were screened for the possessions of four (4) virulence genes, namely *iro* B, *stn, hil* A and *spv* C. Figure 2 shows the gel electrophoretic image of *iro* gene fragments of *Salmonella enterica* isolates with 1.1 kilo base pairs. Isolate code, A6, IK2, IK3, W2 were found to possess *iro* B genes which were molecularly identified as glycosol transferase *iro* B with accession numbers, CQ108275.1, CQA90090.1, AFD59678.1, and AVU70088.1, respectively, as shown in Table 5.

The electrophoretic gel image of *stn* fragments shown in Fig. 3 reveals that *Salmonella isolates,* A8 and IK3, possess *stn* genes which are molecularly identified as domain containing protein and accession numbers, ECY4622154.1, BTY1142154.1, respectively as shown in Table 6. Isolates, A8, IK2, IK3 were molecularly confirmed to have hyper-invasion *hil* A (Fig. 4) with accession numbers, AOY22154.1, CT_33212.5, and ECN5074153.1 as shown in Table 7. spvC virulence gene was found to be negative in all six *Salmonella enterica* isolates as no visible bands were observed as shown in Fig. 5.

Discussion

Nutrient agar (NA) was used to quantify the total viable bacterial count of the cheese samples collected, while salmonella-shigella agar (SSA) was used to identify the

Table 4 Biochemical characteristics of bacteria isolated from cheese samples

Isolate code	Catalase	Motility	Indole	Triple sugar iron test				Mannose	Citrate	Urease			Probable Organisms
				Slant	Butt	H_2S	Gas				Methyl Red	V. P	
IK1-5, A1, A2, A4, A6-8, W1, W2, W3	+	+	−	k	a	+	+	+	+	−	+	−	*Salmonella enterica*
A3, A5, A11, A14, A15, W5, IK6, IK7,W7	+	+	−	a	a	−	−	+	+	−	+	−	*Shigella, Escherichia coli*
A9, A12, A16, IK8, W4	+	+	−	a	a	−	−	−	+	−	+	+	*Listeria monocytogenes*
W8	+	+	−	a	a	−	+	−	+	+	+	−	*Enterococcus faecalis*
A13	+	−	−	a	a	−	−	+	−	−	+	+	*Yersinia enterocolitica*

A, Akure; IK, Ikare; W, Owo; a, acid production; k, alkaline production; V.P., Voges Proskauer; +, positive; −, negative

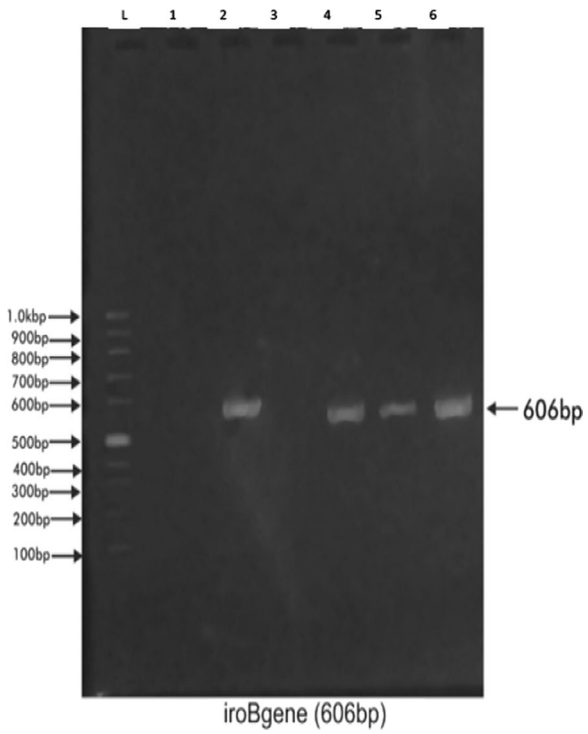

Fig. 2 PCR amplification of genomic DNA targeted to amplify the *iro* B virulence factor embedded in *Salmonella enterica* serovar *typhimurium* on 1.0% agarose gel electrophoresis. L = Molecular Marker. 1 = A1, 2 = A6, 3 = A8, 4 = IK2, 5 = IK3, 6 = W2

specific *Salmonella enterica* and other species of Salmonella from the collected cheese samples. The high total bacterial count, $18.50 \pm 0.71 \times 10^5$ CFU/ml, recorded in this study (Table 2) corroborates with the work of Omemu et al. (2014) who observed the bacterial count of soft white cheese at 20.0×10^7 CFU/ml vended within Abeokuta metropolis in Ogun State, Nigeria. This high count might be attributed to the use of low milk quality. According to the outcome of their study, the microbial quality of raw milk was crucial for the production of any high quality dairy food. It could also be as a result of unsanitary conditions by the vendors during processing and handling of the cheese. Mishandling and disregard of hygienic measures on the part of the food vendors may enable pathogens to come into contact with foods and in some cases, survive and multiply in sufficient numbers to cause gastrointestinal illness in the consumers after ingestion (Birke and Zawide 2019).

The bacterial occurrence revolves around the water used in the process of production. This claim was supported by the research carried out by Saka et al. (2013) whose team worked on the morphological and chemical assessment of water from different rural settings in Ikare Akoko. The team quantified that the mean count of faecal coliforms to be 1.04×10^6 CFU/ml. The highest frequency of bacterial occurrence obtained in Table 3 was accounted for soft cheese samples vended at the rural settings of Ikare, Ondo State, Nigeria. Ikare town is known to be a recreational centre for tourist and in yesteryears has friendly people whose hospitality knows no bound for all tribes of Nigeria. The Fulani are the main vendors of soft cheese in Ondo State and 90% are young girls with piece of personal hygiene.

Hence, the highest frequency of occurrence is a rider to the behavioural pattern of the vendors and the water used at the final stage of production, i.e. cleaning of utensils, washing of hands and packaging baskets. Also, the source of water supply from polluted wells and streams whose locations are at sewer channels and tanks utilized for cheese processing could be considered as a rationale for contamination.

The virulence patterns of plasmid-mediated multidrug resistant *Salmonella enterica* serovar *typhimurium* was detected in a similar study (Onifade and Oladapo 2020) associated with soft cheese in South-West, Nigeria. Apart from *Salmonella enterica* identified in Table 4, the centre of the study, five (5) other enteric organisms were identified in cheese samples. These are *E. coli, Shigella dysenteriae, Enterococcus faecalis* and *Listeria monocytogenes*. The biochemistry of *S. enterica* serovar *typhimurium* observed in this study is in line with the outcome of Sangoyomi et al. (2010) who performed biochemical characterization on bacteria isolated from cheese, a claim also supported by Al-Gamal et al. (2019).

The presence of *Salmonella enterica* in soft cheese samples recorded in this study (Table 4) is in line with the

Table 5 Molecular identity of *iro* B gene in *Salmonella enterica*

Salmonella isolate codes	Molecular identity	Percentage similarity (%)	Strain	Accession number
A6	Glycosol transferase *iro* B	93.10	–	CQ108275.1
IK2	Glycosol transferase *iro* B	90.10	–	CQA90090.1
IK3	Putative glycosol transferase *iro* B	90.11	798	AFD59678.1
W2	Glycosol transferase *iro* B	90.40	–	AVU70088.1

A, Akure; IK, Ikare; W, Owo

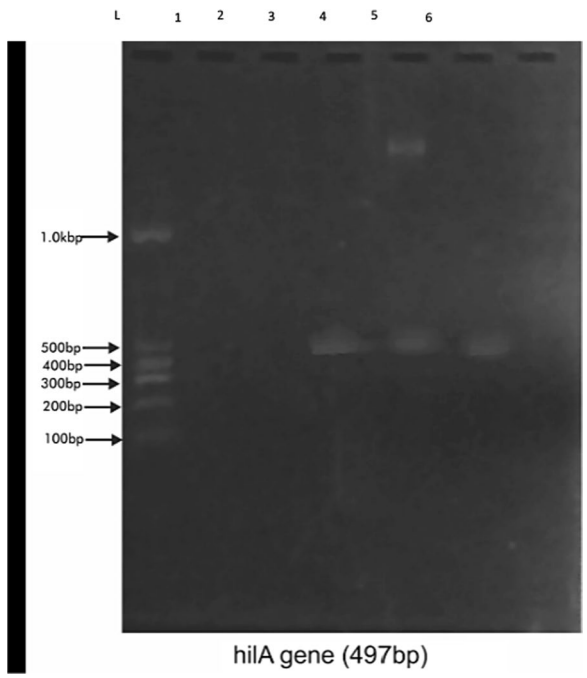

Fig. 3 PCR amplification of genomic DNA targeted to amplify the *Hil* A virulence factor embedded in *Salmonella enterica* serovar *typhimurium* on 1.0% agarose gel electrophoresis. L = Molecular Marker. 1 = A1, 2 = A6, 3 = A8, 4 = IK2, 5 = IK3, 6 = W2

observation made by Frasson et al. (2016) who accounted for the presence of *Salmonella enterica* serovars in dairy foods. However, the findings from this study have shown that *Salmonella enterica* isolated from cheese is relatively small. This is due to the in-processes involved in the cheese-making which affects the growth of the bacterium. Nonetheless, they were susceptible pathogens associated with cheese. In fact, the earlier observations made by Frasson et al. (2016) showed that the prevalence of food-borne *Salmonella enterica* was reported to be 81%, indicating the ease of contamination involved in cheese production if not carefully monitored.

Findings from the study also revealed the virulence genes of *Salmonella enterica* serovar *typhimurium* through the PCR amplification of *iro* B, *stn,* and *hil* A. The primer pairs targeting the enterotoxin, stn and the histidine transport operon met all of our criteria for application to cheese samples which is in agreement with the evaluation of primers as demonstrated by Ziemer and Steadham (2003), Heymans et al. (2018). The criteria for accepting a primer set was based on PCR amplification of all *Salmonella* strains tested (Heymans et al. 2018).

The molecular determination of ferric uptake regulator in *Salmonella enterica* serovar *typhimurium* has revealed the presence of *iro* B gene in cheese samples in Fig. 2. This is in agreement with the study carried out by Zishiri et al. (2016) who worked on the prevalence of virulence genes of *Salmonella* in South Africa. They revealed that out of the overall 146 *Salmonella*-positive samples that were screened for the *iro* B gene, most of them were confirmed to be *Salmonella enterica* with high prevalence rates of *iro* B ranging between 69–85%, a gene responsible for the shortage of blood in host. In fact, findings from Bearson et al. (2008) demonstrated the influence of *iro* B gene in *S. enterica* serovar *typhimurium*. They claimed that there is notable competition of iron acquisition between the host and *S. enterica* serovar *typhimurium*. This confirmed the travail of *Salmonella enterica* serovar *typhimurium* in host system, once consumed.

The *hil* A (hyper-invasion locus A) confirmed positive in this study (Fig. 3) is the grand orator of invasion in *Salmonella enterica* serovar *typhimurium* which were distributed in all sources of cheese samples. This corroborates with the research conducted by Yanestria et al. (2019). They opined that 12.5% of *hil A* gene exhibited by *Salmonella* were distributed in Milkfish sources. This clearly questions the potential location and the routes of *Salmonella* transmission in cheese making. The First step in the intracellular pathogenicity cycle of *Salmonella* is the invasion of intestinal epithelial cells, and this step is controlled by the *hil A* gene, in a review confirmed by Sharma (2016). Therefore, intake of cheese samples contaminated with *Salmonella enterica* serovar *typhimurium* may result to inflammation, liver cirrhosis and some other chronic gastro intestinal infections.

However, *stn* gene operon with molecular size of 260 bp was found in 33.3% of the isolates analysed for virulence (Fig. 4). The two (2) pure isolates of *S. enterica* serovar *typhimurium* had enterotoxic and cytotoxic effects on host systems. The photographic plates of *Salmonella enterica* serovar *typhimurium* showed the differences in the bands, as a band appears blurred and the other, distinct, which is a clear indication that there is difference

Table 6 Molecular identity of *stn* gene in *Salmonella enterica*

Salmonella isolate codes	Molecular identity	Percentage similarity (%)	Strain	Accession number
A8	Domain containing protein enterotoxin *stn*	100	DUF1205	ECY4622154.1
IK3	Enterotoxin *stn*	97.50	–	BTY1142154.1

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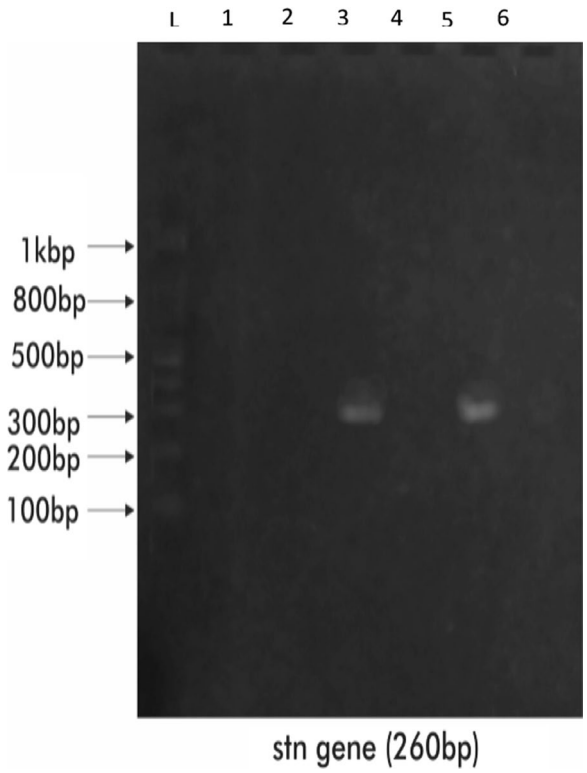

Fig. 4 PCR amplification of genomic DNA targeted to amplify the Stn virulence factor embedded in *Salmonella enterica* serovar *typhimurium* on 1.0% agarose gel electrophoresis. L = Molecular Marker. 1 = A1, 2 = A6, 3 = A8, 4 = IK2, 5 = IK3, 6 = W2

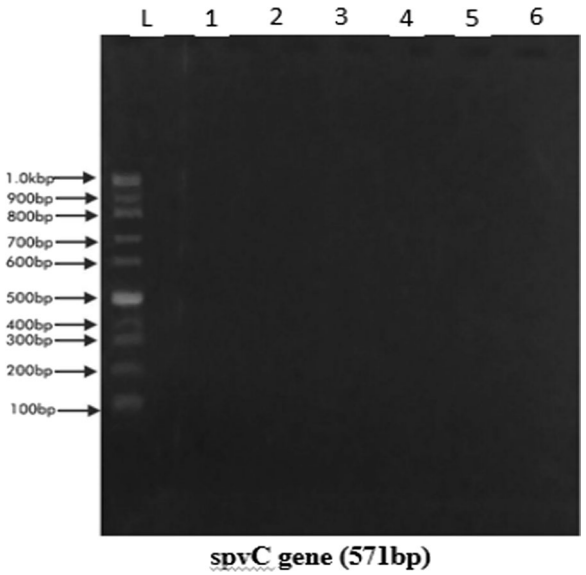

Fig. 5 PCR amplification of genomic DNA targeted to amplify the spvC virulence factor embedded in *Salmonella enterica* serovar *typhimurium* on 1.0% agarose gel electrophoresis. L = Molecular Marker. 1 = A1, 2 = A6, 3 = A8, 4 = IK2, 5 = IK3, 6 = W2

of phenotypes of gene and regulation in the membrane composition and integrity. Moreover, Masayuki et al. (2012); ElSheikh et al. (2019) analysed the importance of Stn gene and how it regulates membrane composition and *Salmonella*' integrity. These were done through *in vitro* and *in vivo* using wild-type and *Δstn* mutant *Salmonella*. From their research, the team opined that differences of virulence phenotypes between the wild type and *Δstn* led to the production of R-S-EXE triad motif, which is essential for the ADP-ribosyltransferase of CT and LT, a virulence factor of *Salmonella* as reported by Masayuki et al. (2012) and ElSheikh et al. (2019).

From this, it is possible that *stn* might play a role in unique function, a known operon capable of causing severe acute gastroenteritis. Spv operon, on the other hand, has not been detected (Fig. 5), as the *Salmonella* plasmid gene is noted for growth regulation which is being activated during the interaction with the host cell framework. However, the deprivation of nutrient by the phagosome present in host phagocytic cells can be the environmental signal activating *Salmonella* plasmid virulence gene expression. In fact, a study confirmed that analyses of reporter gene expression, mRNA synthesis, deletion mutagenesis, and gene complementation have demonstrated a novel 'regulatory system'. This is a consequence of the selective transcription of *spv* during the stationary phase of the bacterial growth (Guiney and Fierer 2011; Lv et al. 2019).

Conclusions

Therefore, locally produced soft cheese used for this study was found to be unsafe for human consumption due to the low microbiological quality made evident in

Table 7 Molecular identity of *hil* A gene in *Salmonella enterica*

Salmonella isolate codes	Molecular identity	Percentage similarity (%)	Strain	Accession number
A8	Biosynthetic hyper-invasion loci	89.20	–	AOY22154.1
IK2	Ostensive invasion	91.32	–	CT_33212.5
IK3	Biosynthetic hyper-invasion loci	95.30	–	ECN5074153.1

A, Akure; IK, Ikare

the microbiological profile of the vended soft cheese. The presence of molecularly elucidated virulence genes comprising *Iro B*, *Stn* and *Hil A* makes the soft cheese potentially viable for pathogenicity. This study recommends that food-borne salmonellosis should be constantly monitored with appropriate cleaning of preparatory paraphernalia in cheese production encouraged. Constant sensitization of nomads to improve their awareness on milk-borne zoonosis and its associated risk factors is needed.

Abbreviations

DNA: Deoxyribose nucleic acid; RNA: Ribose nucleic acid; $MgCl_2$: Magnesium chloride; MdNTPs: Modified dynamic needle tip positioning; Mm: Millimolar; BLAST: Basic local alignment search tool; ADP: Adenosine di-phosphate; CT: Cholera toxin; LT: Heat-labile toxin; µL: Microlitre; µM: Micromolar.

Acknowledgements

Authors are grateful to Erevna laboratories, Adebajo Street, New Bodija, Ibadan, Oyo State, Nigeria, for their technical input in the virulence analysis performed in this study. Authors also acknowledge the efforts of Inqaba Biotech West Africa Ltd, located at the Bioscience Unit, International Institute for Tropical Agriculture (IITA), Ibadan, Nigeria, for the virulence primer stocks utilized in this study.

Author contributions

AKO designed and supervised the study. ODO developed the methodology, literature, conducted the study, acquired, analysed, and interpreted the data obtained. ODO and MTB wrote the first draft, previewed, and fine-tuned the draft before submission. Authors ODO and MTB both read and approved the final manuscript draft.

Funding

None was received for this study.

Availability of data and materials

There is availability of data and materials in supplementary data files.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

Authors declare that no competing financial interests or personal relationship exist.

Received: 2 March 2022 Accepted: 2 June 2022
Published online: 18 June 2022

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