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*Full Length Research Paper*

# Toxigenic *Staphylococcus aureus* in processing of coalho and mozzarella cheese

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***Staphylococcus aureus*** **is commonly involved in food poisoning due to production of toxins responsible for causing animal and human diseases. In this study, 60 strains of presumptive *S. aureus* isolates from raw milk and cheese were biochemically identified in four dairies: 54 (90%) from refrigerated raw milk (RRM) with counts exceeding $10^6$ CFU/mL, and six (10%) from cheese with similar concentrations of CFU/mL. Out of the 60 strains of presumptive *S. aureus*, 46 (76.7%) amplified the *femA* gene and then they were investigated regarding the presence of the Toxic Shock Syndrome Toxin-1 (TSST-1) gene and the classical enterotoxin genes (SEs) types A, B, C, D and E: 31 (67.4%) carried one or more encoding toxin genes, and 13 different genotypes were identified. Twenty-one strains (61.8%) carried one gene; three (8.8%), two genes; seven (20.6%), three genes; two (5.8%), four genes; and one (3%), five genes. The *sec* gene was the most frequent one, followed by *seb* and *tst*. The *sed* gene was expressed by 10 strains (29.4%), *sea* by five (14.7%) and *see* by three (8.8%). The *S. aureus* isolates showed genetic potential for producing toxins of importance for public health that presented a danger of food poisoning.**

**Key words:** *Staphylococcus aureus*, milk, cheese, staphylococcal toxins.

## INTRODUCTION

Milk and cheese are widely consumed and appreciated foods around the world. Mozzarella cheese is widely used in Brazilian cuisine in hot dishes and sandwiches and coalho cheese is a popular dairy product consumed in the Northeast region of Brazil (Andreatta et al., 2009, Silva et al., 2012). Because cow's milk contains lipids, proteins, amino acids, vitamins and minerals (Haug et al., 2007) it is considered to be an excellent culture medium for development of a variety of microorganisms. Among the main microorganisms that contaminate milk and dairy products, staphylococci stand out. The presence of this pathogen in the mammary glands of cows, especially as the etiologic agent of bovine mastitis, makes milk and dairy products great vehicles for its dispersion in Brazil

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(Martin et al., 2016). Staphylococcal poisoning is the most frequent cause of food-borne disease (FBD) outbreaks in many countries (Kadariya et al., 2014). It occurs after ingestion of food containing preformed enterotoxins, and raw milk, pasteurized milk and cheeses can be highlighted as the dairy products most incriminated (Le Loir et al., 2003; Oliver et al., 2005).

CDC estimates that each year roughly 48 million people gets sick from a food-borne disease (FBD) (Scharff et al., 2016). Food handlers represent an unquestionable link in the epidemiological food poisoning chain. They also play the role of a hygienic-sanitary indicator in the food industry, since they represent the main source of propagation (Kadariya et al., 2014).

Several studies have reported highest prevalence of CPS in dairy products in Brazil (Rall et al., 2008; Moraes et al., 2009; Guimaraes et al., 2013; Nunes and Caldas, 2017).

Staphylococci can produce several toxins. Among them are the classical staphylococcal enterotoxins (SEs) (SEA, SEB, SEC, SED and SEE), which are responsible for most food poisoning cases presenting clinical conditions of vomiting, diarrhea, nausea and generalized debility. They can also produce Toxic Shock Syndrome Toxin-1 (TSST-1), which is responsible for multisystemic disorders and can lead to death due to lethal shock if not properly treated (Ortega et al., 2010).

The first aim of this study was to quantify coagulase-positive *Stafilococci* (CPS) in the production lines of dairies producing cheese and identify *S. aureus* isolates by biochemical and molecular methods. The second objective was to analyze the frequencies of the genes that encode for production of the classical SEs (*sea, seb, sec, sed* and *see*) and for TSST-1 (*tst*) in isolated strains of *S. aureus* isolates.

## MATERIALS AND METHODS

### Sampling procedure

One hundred and twenty samples were collected from four dairies, called A, B, C and D, in the state of Maranhão, Brazil. All factories were inspected by the state or federal inspection service, and produced cheese of mozzarella or *coalho* type from pasteurized milk.

Each dairy was visited five times, so the samples were taken from five different production batches. During each visit, two samples of refrigerated raw milk (RRM), two samples of pasteurized milk (PM) and two samples of *coalho* cheese (dairy A) or mozzarella cheese (dairies B, C and D) were taken. In total, 40 samples were of RRM, 40 of PM, and 40 of cheese (30 of mozzarella and 10 of *coalho*). The samples of refrigerated raw milk and pasteurized milk were taken from the reception tank and from the pasteurizer outlet, respectively, and kept in sterile 250 mL flasks; the cheese samples were taken after they were wrapped, in portions of 250 g. All samples were taken to the Food and Water Microbiology Laboratory in cool boxes and kept for 2 to 12 h at <4°C until microbiological analysis.

### *Quantification of coagulase-positive staphylococcus and biochemical identification of S. aureus isolates*

To quantify coagulase-positive *Staphylococcus* (CPS) and isolate *S. aureus*, serial dilutions ($10^{-1}$, $10^{-2}$, $10^{-3}$) of each sample were inoculated in dishes with Baird-Parker agar (Himedia), supplemented with egg yolk and potassium tellurite (Himedia), and were incubated at 37ºC for 48 h. After this period, typical colonies were counted and tested for Gram staining, catalase and free coagulase; the positive samples were identified as presumptive coagulase-positive *Staphylococci.* To biochemically identify *S. aureus*, all the CPS colonies were tested for Voges-Proskauer (VP) reaction and maltose and trehalose fermentation. The colonies with positive results were identified as presumptive *S. aureus* (Garcia, 2010).

### *Molecular confirmation of presumptive S. aureus isolates and toxigenic genes investigation*

The isolates of *S. aureus* were grown in BHI broth, at 37°C/24 h. After the incubation period, 1 mL of each growth was transferred to 1.5 mL microcentrifuge tubes (Axygen) and DNA extraction was performed using the Wizard Genomic DNA Purification (Promega) commercial kit according to the manufacturer's instructions for Gram-positive bacterial genomic DNA extraction. The extracted DNA was stored at -20ºC until its use.

Amplification of the 132 bp fragment of the *femA* gene (Mehrotra et al., 2000) was performed by PCR to confirm the biochemical identification of *S. aureus* isolates.

The reaction was carried out in a final volume of 25 µL, with 2.5 µL of 10x reaction buffer (100 mM Tris-HCl at pH 8.3 and 500 mM KCl), 2 mM of $MgCl_2$, 200 µM of each *dNTP*, 20 pmol of each of the oligonucleotide primers femA-1 and femA-2 (Table 1), 2.5 U of *Taq* DNA polymerase (Invitrogen, Brazil) and 5 µL of the template DNA at a concentration of approximately 200 ηg/µL.

The DNA amplification was performed in a Biocycler thermal cycler, under the following conditions: initial denaturation at 94ºC for five minutes, 35 amplification cycles (denaturation at 94ºC for two minutes, annealing at 57ºC for two minutes and extension at 72ºC for one minute) and a final extension at 72ºC for seven minutes.

In order to investigate the SE genes (*sea, seb, sec, sed* and *see*) a multiplex PCR was performed in a final volume of 50 µL was performed according Becker et al. (1998). In short, we used 5 µL of 10x reaction buffer (100 mM Tris-HCl at pH 8.3 and 500 mM KCl), 3 mM of $MgCl_2$, 160 µM of each *dNTP*, 20 pmol of each of the SEA, SEB, SEC, SED and SEE primers (Table 1), 1.2 U of *Taq* DNA polymerase (Invitrogen, Brazil) and 5 µL of the template DNA at a concentration of approximately 200 ηg/µL. The DNA of *S. aureus* ATCC 13565 (SEA), ATCC 14458 (SEB), ATCC 19095 (SEC), ATCC 23235 (SED) and ATCC 27664 (SEE), provided by Fundação Oswaldo Cruz (FIOCRUZ), were used as positive control.

To investigate the *tst* gene, PCR reaction was performed in a final volume of 25 µL according Mehrotra et al. (2000). In short 2.5 µL of 10x reaction buffer 10x (100 mM Tris-HCl was used at pH 8.3 and 500 mM KCl), 2 mM of $MgCl_2$, 200 µM of each *dNTP*, 20 pmol of each oligonucleotide primer (TSST-1 and TSST-2), 2.5 U of *Taq* DNA polymerase (Invitrogen, Brazil) and 5 µL of the template DNA at a concentration of approximately 200 ηg/µL.

From all the reactions, ten microliters of the amplified product were loaded onto 1% agarose gel with ethidium bromide (10 mg/mL) and underwent electrophoresis in TBE buffer (0.09 M Tris-HCl, 0.09 M boric acid and 2 mM EDTA, at pH 8.0), at 150 V for two hours. The amplified DNA was observed under ultraviolet light and the images were digitalized. A *100 bp ladder* (Invitrogen, Brazil)

**Table 1.** Nucleotide sequences and sizes of products from the genes investigated in the *S. aureus* isolates.

| Gene | Primer | Sequence (5'→ 3') | bp* |
|---|---|---|---|
| ***sea***** | SEA-3 | CCT TTG GAA ACG GTT AAA ACG | 127 |
| | SEA-4 | TCT GAA CCT TCC CAT CAA AAA C | |
| ***seb***** | SEB-1 | TCG CAT CAA ACT GAC AAA CG | 477 |
| | SEB-4 | GCA GGT ACT CTA TAA GTG CCT GC | |
| ***sec***** | SEC-3 | CTC AAG AAC TAG ACA TAA AAG CTA GG | 271 |
| | SEC-4 | TCA AAA TCG GAT TAA CAT TAT CC | |
| ***sed***** | SED-3 | CTA GTT TGG TAA TAT CTC CTT TAA ACG | 319 |
| | SED-4 | TTA ATG CTA TAT CTT ATA GGG TAA ACA TC | |
| ***see***** | SEE-3 | CAG TAC CTA TAG ATA AAG TTA AAA CAA GC | 178 |
| | SEE-2 | TAA CTT ACC GTG GAC CCT TC | |
| ***tst****** | TSST-1 | ACC CCT GTT CCC TTA TCA TC | 326 |
| | TSST-2 | TTT TCA GTA TTT GTA ACG CC | |
| ***femA****** | FEMA-1 | AAA AAA GCA CAT AAC AAG CG | 132 |
| | FEMA-2 | GAT AAA GAA GAA ACC AGC AG | |

*Base pairs; **Becker et al. (1998); *** Mehrotra et al. (2000).

was used as standard molecular weight.

## RESULTS

### Quantification of coagulase-positive *Staphylococcus* (CPS) and identification of *S. aureus*

CPS counts and identification of *S. aureus* are reported in Table 2. For CPS quantification, results are expressed as mean of two samples for each food. High CPS concentrations were observed in all 40 samples (100%) of RRM, with counts ranging from $1.7 \times 10^4$ to $>10^6$ CFU/mL. Twenty-six samples (65%) shown *S. aureus* contamination. From this total, 54 strains of the pathogen were biochemically identified.

In pasteurized milk *S. aureus* was not isolated but 8 (20%) out of the 40 samples analyzed presented CPS contamination with counts as high as $1.6 \times 10^4$ CFU/mL. All 40 (100%) of the mozzarella and *coalho* cheese samples also presented CPS contamination, with counts between $2.7 \times 10^3$ and $>10^6$ CFU/g. Six isolates of *S. aureus* were identified by biochemical tests, five from mozzarella cheese and one from *coalho* cheese.

Sixty presumptive *S. aureus* isolates were investigated by PCR in order to confirm the identification and search for toxins genes. The effectiveness of the molecular protocols is shown in Figure 1a and b. The individual amplification of each gene investigated was observed (*femA, sea, seb, sec, sed, see* and *tst*), as well as the specific and simultaneous amplification of the five classical SE genes through multiplex-PCR.

Among the 60 isolates, 46 (76.7%) amplified *femA*: 41 were from RRM, four from mozzarella cheese and one from *coalho* cheese.

### Identification of the Staphylococcal toxin encoding genes

Table 3 shows the results about toxin encoding genes. Among the 46 strains, 34 (74%) expressed one or more genes: 31 isolated from RRM and three from the mozzarella cheese. Twelve strains (26%) did not express any gene. All the toxin genes investigated (*sea, seb, sec, sed, see* and *tst*) were detected in the strains of *S. aureus* isolated from RRM. The three isolates from mozzarella cheese amplified only the gene responsible for the production of TSST-1; the *sea* gene was observed only in association with other genes, in contrast with the *seb, sec, sed, see* and *tst* genes, which were expressed separately.

Thirteen different genotypes were obtained. The most frequent genotype was *tst*, which was present in 10 strains of *S. aureus*, of which seven were from RRM and three from mozzarella cheese. Twenty-one strains carrying a genotype with one toxin gene, three strains with two genes (*seb* + *sec* or *seb* + *sed*) and seven strains with three genes (*sea* + *seb* + *sec*; *sea* + *sec* + *tst*; *seb* + *sec* + *sed*; or *seb* + *sec* + *tst*) were observed.

The genotype encoding four toxins (*sea* + *seb* + *sec* + *tst*) was detected in two strains, and one strain of *S*

**Table 2.** Counts of coagulase-positive *Staphylococcus* and percentage of *S. aureus* with phenotypic and genotypic identification in samples of refrigerated raw milk, pasteurized milk and cheese.

| Sample | Dairy | Coagulase-positive *staphylococcus* CFU/mL or g (mean from sampling) | | | | | Biochemical Identification of *S. aureus* | Genotypic confirmation of *S. aureus* (*femA*) |
|---|---|---|---|---|---|---|---|---|
| | | Sampling 1 | Sampling 2 | Sampling 3 | Sampling 4 | Sampling 5 | n | n |
| RRM | A | $4.9 \times 10^4$ | $2.8 \times 10^5$ | $1.7 \times 10^4$ | $7.5 \times 10^6$ | $1.2 \times 10^5$ | 54 | 41 |
| | B | $2.5 \times 10^5$ | $2.2 \times 10^6$ | $> 10^6$ | $> 10^6$ | $> 10^6$ | | |
| | C | $1.9 \times 10^5$ | $7.6 \times 10^5$ | $1.8 \times 10^6$ | $> 10^6$ | $> 10^6$ | | |
| | D | $10^5$ | $8.5 \times 10^4$ | $> 10^6$ | $> 10^6$ | $9.2 \times 10^4$ | | |
| PM | A | - | - | $1.6 \times 10^4$ | - | - | - | - |
| | B | - | $2.2 \times 10^2$ | $7.2 \times 10^3$ | - | - | | |
| | C | - | - | $3.2 \times 10^4$ | - | - | | |
| | D | - | - | - | - | - | | |
| Cheese | A | $> 10^6$ | $> 10^6$ | $> 10^6$ | $> 10^6$ | $8.9 \times 10^4$ | 01 | 01 |
| | B | $3.4 \times 10^4$ | $6.5 \times 10^3$ | $2.7 \times 10^3$ | $2.7 \times 10^4$ | $2 \times 10^4$ | | |
| | C | $6.2 \times 10^3$ | $5.8 \times 10^3$ | $8.2 \times 10^3$ | $5.4 \times 10^3$ | $2.7 \times 10^3$ | 05 | 04 |
| | D | $4.8 \times 10^3$ | $1.5 \times 10^4$ | $4.5 \times 10^4$ | $6.6 \times 10^3$ | $4 \times 10^5$ | | |
| | | **Total (%)** | | | | | **60 (100%)** | **46 (76.7%)** |

-: Absence.

*aureus* expressed all five SE genes (*sea* + *seb* + *sec* + *sed* + *see*).

In Figure 2, the frequency of each of the genes investigated can be observed, independent of whether the expression was isolated or simultaneous. Out of all the genes investigated, *sec* was the most frequent one, observed in 15 strains (44.1%); followed by *seb* and *tst*, each in 14 (41.1%) strains; *sed* in 10 (29.4%), *sea* in five (14.7%) and *see* in three strains (8.8%) of *S. aureus*.

## DISCUSSION

The highest percentage of *S. aureus* strains was isolated from the RRM samples. This was an expected result because all the samples presented high CPS contamination, with counts above $10^6$ CFU/mL. This high CPS concentration could have occurred because this food is susceptible to contamination, particularly during the milking process, from the person performing the operation, from the utensils and equipment used, and even from one animal to another, especially in cases of mastitis in herds (Hait and Bennett, 2012).

Borges et al. (2008) also found that 100% of the RRM samples from a dairy in Ceará (Brazil) were contaminated by CPS with values between $10^3$ and $10^6$ CFU/mL. According to Sommerhäuser et al. (2003), the microbiological quality of milk is directly related to the hygiene of the milking process. The hygiene begins with the herd's health, since many illnesses of dairy cattle affect the original composition, flavor, smell, viscosity and microbiological quality of the milk. Another aggravating factor is inadequate storage and temperature during transportation between the farm property and the dairy, which may contribute to multiplication of the contaminating microorganisms that were present at the time of the milking.

Although the law does not set limits for the presence of pathogenic microorganisms in RRM, according to FDA, in foods with CPS counts from $10^5$ CFU/mL the presence of staphylococcal enterotoxin is likely (Hait and Bennett, 2012). Therefore, the counts measured in the present study could pose a great risk of presence of SEs

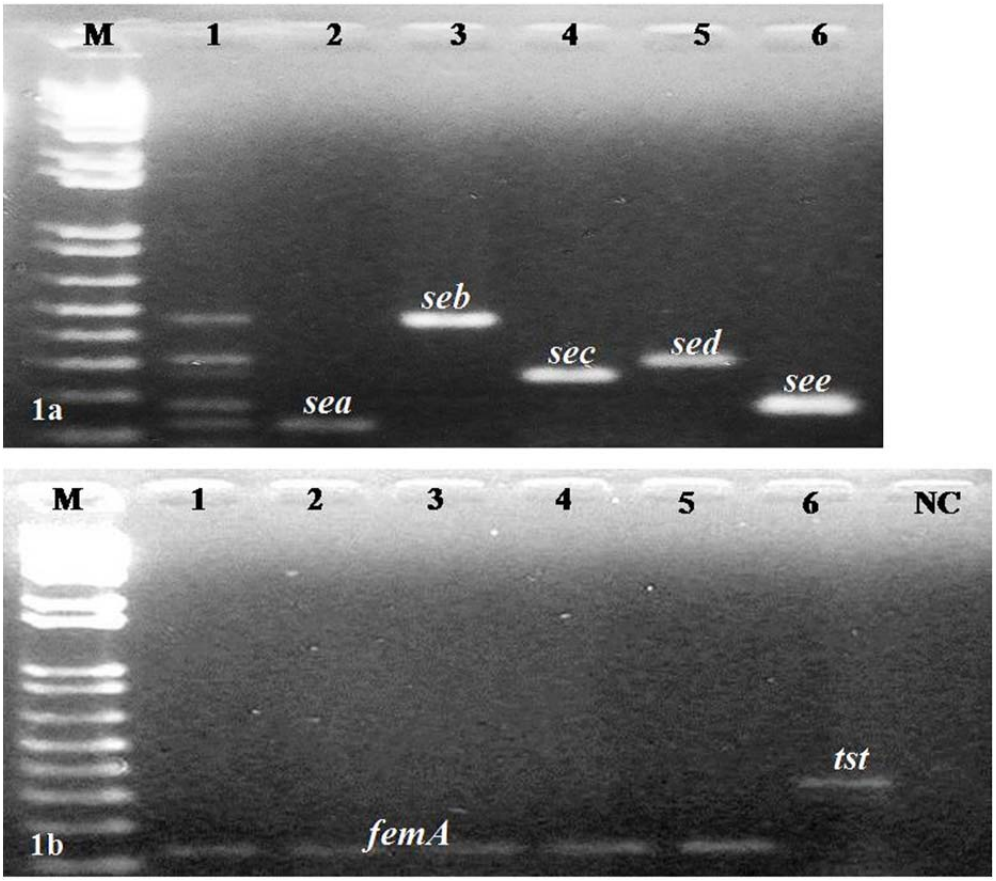


**Figure 1.** Products from simultaneous amplification of the *sea, seb, sec, sed* and *see* genes of the *S. aureus* strains through multiplex PCR and *femA* and *tst* genes in the *S. aureus* strains through uniplex PCR on 1% agarose gel. **1a**. Lane M: 100 bp molecular weight marker (Invitrogen, Brazil); Lane 1: *sea* (127 bp), *seb* (477 bp), *sec* (271 bp), *sed* (319 bp) and *see* (178 bp), simultaneously; Lane 2: *sea*; Lane 3: *seb*; Lane 4: *sec*; Lane 5: *sed*; Lane 6: *see*. **1b**. Lane M: 100 bp molecular weight marker (Invitrogen, Brazil); Lanes 1-5: *femA* (132 bp); Lane 6: *tst* (326 bp); Lane NC: negative control.

in RRM, which could reach the cheese, even after the pasteurization process, which eliminates bacteria but does not destroy the toxins produced. The thermal stability of staphylococcal toxins favors endurance of these proteins in the thermal process, with the ability to withstand temperatures as high as 100ºC for 30 min, thus remaining active in foods (Balaban and Rasooly, 2000) and causing harm to human health.

*S. aureus* was not isolated in pasteurized milk and only a small number of samples presented CPS contamination, suggesting that the pasteurization process contributed to reduce the concentration of undesirable microbiota.

Although the pasteurization process ensures destruction of the lineages of *S. aureus* that were originally present in the RRM, this bacterium may be found in PM if there is any flaw during the processing, leading to a cross-contamination and/or storage at inappropriate temperature (Corbia et al., 2000).

A study carried out in the state of São Paulo found that 38 (70.4%) out of 54 RRM samples presented CPS concentrations as high as $8.9 \times 10^5$ CFU/mL. There were eight PM samples with counts as high as $8.7 \times 10^3$ CFU/mL (Rall et al., 2008). Those values were lower than those found in the present study, which have found counts greater than $10^6$ CFU/mL and $3.2 \times 10^4$ CFU/mL for RRM and PM, respectively.

The results from the cheese samples showed that 100% did not meet the standards required by Brazilian law (Brasil, 2001), which set limits for CPS in *coalho* and mozzarella cheese of up to $5 \times 10^2$ CFU/g and $10^3$ CFU/g, respectively. Despite the low frequency of *S. aureus* isolation in cheese samples (*coalho* and mozzarella), high CPS concentrations pose a threat to public health because the production of toxins is not restricted only to the species *S. aureus*. Other CPS species can also

**Table 3.** Genotypic profile of the 46 strains of *S. aureus* with biochemical and molecular identification (*femA*), regarding the presence of the *sea, seb, sec, sed, see* and *tst* genes.

| Genotypic profile | RRM | *Coalho* cheese | Mozzarella cheese | Total (%) |
|---|---|---|---|---|
| *seb* | 2 | - | - | |
| *sec* | 3 | - | - | |
| *sed* | 4 | - | - | |
| *see* | 2 | - | - | |
| *tst* | 7 | - | 3 | |
| *seb + sec* | 2 | - | - | |
| *seb + sed* | 1 | - | - | 34 (74%) |
| *sea + seb + sec* | 1 | - | - | |
| *sea + sec + tst* | 1 | - | - | |
| *seb + sec + sed* | 4 | - | - | |
| *seb + sec + tst* | 1 | - | - | |
| *sea + seb +sec + tst* | 2 | - | - | |
| *sea + seb + sec + sed + see* | 1 | - | - | |
| Negative strains | 10 | 1 | 1 | 12 (26%) |
| **Total of *S. aureus* strains** | **41** | **1** | **4** | **46 (100%)** |

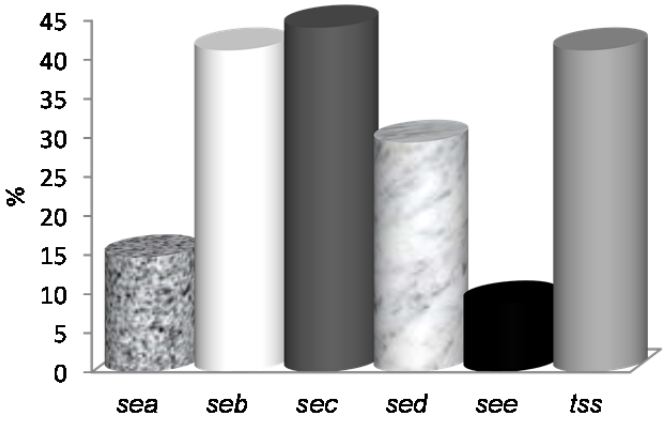


**Figure 2.** Frequencies of the classical SE genes (*sea, seb, sec, sed* and *see*) and TSST-1 (*tst*) among the 34 strains of toxigenic *S. aureus*.

produce toxins.

According to the International Commission on Microbiological Specifications for Foods (ICMSF, 1980), *S. aureus* counts between $10^3$ and $10^4$ CFU/g indicate a risk to public health. Values close to $10^5$ CFU/g signify an epidemiological threat because of the possibility that enterotoxins might be present in quantities that are enough to cause staphylococcal intoxication, if the strain of *S. aureus* is toxigenic.

Post-pasteurization contamination occurs mainly due to inappropriate handling, lack of hygiene and deficient cleaning and sanitation of the equipment and utensils used in cheese production. Pelisser et al. (2009) highlighted that one of the main sources of CPS contamination in cheese are the handlers' hands and forearms, due to deficient hygienic-sanitary control and
no use of gloves during the processing.

Molecular characterization of *Staphylococcus* isolates showed that genetic analysis is more specific than biochemical tests in identifying this microorganism.

In a study carried out on dairy farms in various municipalities in the state of Minas Gerais, 100 strains of CPS were isolated. Among these, 77 were characterized as *S. aureus* by biochemical tests but 83 strains amplified the *femA* gene (Lange et al., 2011).

Several studies have explored the *femA* gene as a specific marker for *S. aureus* genotypic identification (Mehrotra et al., 2000, Riyaz-Ul-Hassan et al., 2008, Fischer et al., 2009, Pelisser et al., 2009), given that this gene takes part in biosynthesis of the pentaglycine interpeptide bridge that is characteristic for the peptidoglycan of the cell wall of this organism (Johnson et al., 1995, Moussallem et al., 2007).

Despite the high sensitivity of biochemical identification for characterizing *S. aureus*, its specificity is not 100% satisfactory. It needs to be complemented with molecular studies on specific markers for the microorganism.

Presence of the toxin encoding genes was observed in 74% (34) of the strains of *S. aureus* with biochemical and molecular identification. A great number of genotypes were found, divided in 13 different groups, thus indicating great genetic heterogeneity between the isolates.

Considering that in the present study only six toxin genes were investigated, it can be seen that the percentage of toxigenic *S. aureus* was high. This suggests

that a great number of circulating strains of this pathogen carry toxin encoding genes. This would explain the high numbers of food poisoning cases and other infections commonly caused by this pathogen.

Studies carried out across the world have shown significant percentages of toxigenic *S. aureus*. In evaluating 78 strains of *S. aureus* isolated from milk from two farms in Tennessee with regard to the frequencies of 16 enterotoxin genes (*sea-see* and *seg-seq*) and the *tst* gene, it was observed that 73 strains (93.6%) carried one or more genes, comprising 36 different genotypic groups (Srinivasan et al., 2006).

In Italy, a study on 112 strains of *S. aureus* isolated from milk and dairy products found that 75 (67%) were positive for one or more SE genes (*sea-see* and *seg-sel*), divided into 17 genotypic profiles (Morandi et al., 2007).

Regarding the *tst* genotype, which was the one with highest frequency in the present study, Cardoso et al. (2000) and Zafalon et al. (2009) suggested that there might be a relationship between *S. aureus* strains carrying the *tst* gene and occurrences of cows with mastitis, and usually also in association with SE genes. In this, production of TSST-1 seemed to have great importance for the virulence of the samples of this microorganism, thereby influencing the severity of the cases of mastitis. In a study carried out in Brazil on 127 strains of *S. aureus* isolated from cases of clinical and subclinical mastitis, it was found that TSST-1 was one of the toxins produced with highest frequency. This was identified in 60% (475) of the samples, followed by SED (30%) and SEB (19%) (Silva et al., 2005). The presence of *S. aureus* carrying *tst* in refrigerated raw milk and in cheese could suggest that these isolates came from cows with mastitis, since TSST-1 has been associated with worsening of the inflammatory process of this illness among dairy cattle.

The strains of *S. aureus* that presented a genotypic profile with simultaneous presence of two to five toxin genes is a worrying finding because this shows the high pathogenic potential of these strains for production of different toxins, especially due to the high concentrations of the microorganism that were observed in all RRM and cheese samples.

In a study carried out in São Paulo on 132 strains of *S. aureus* isolates from raw milk, investigating the presence of the SE and TSST-1 genes, and the production of their respective toxins, 90 isolates (68.18%) were positive for one or two toxin genes, and 40 (44.44%) were capable of producing them *in vitro* (Chapaval et al., 2006).

Santana et al. (2010) reported that the risk of staphylococcal intoxication requires the presence of two factors: The food must contain staphylococci carrying the toxin genes with the ability to express this gene; and the microorganism counts should be higher than $10^5$ CFU, under the conditions that allow toxin production in the food.

The presence of strains of *S. aureus* carrying toxigenic genes does not necessarily indicate production of toxins at levels sufficient to cause food poisoning conditions, because the production could be influenced by various factors (Le Loir et al., 2003, Hennekinne et al., 2012, Bogdanovičová et al., 2017). However, the presence of these genes is required for the microorganism to be able to produce them. The PCR technique makes it possible to evaluate the genetic potential for such production and also serves as a screening test for confirming the presence of toxins in immunological assays (Zafalon et al., 2009).

Regarding the frequency of each gene investigated, the *sec* gene was the most prevalent, occurring in 15 strains (44.1%) of *S. aureus*, which was concordant with data from a study carried out in Germany, where 34 strains of *S. aureus* isolated from different dairy farms that amplified any gene (*sea-see* and *tst*) found that the *sec* gene occurred most frequently in 22 of the strains (64.7%), followed by the *tst* gene in 19 (55.8%) (Zschöck et al., 2000).

Divergent results were presented by Rall et al. (2008), who found that out of 57 strains of *S. aureus* isolated from raw and pasteurized milk, 39 (68.4%) were positive for at least one enterotoxin gene, among which the *sea* gene was the most frequent one, occurring in 16 strains (41%), followed by eight strains positive for *sec* (20.5%), five (12.8%) for *sed*, three for *seb* (7.7%) and two (5.1%) for *see*. Chapaval et al. (2006) also observed that the *sea* gene was the most frequent one in 90 strains of *S. aureus*, detected in 61 strains (67.78%), followed by *tst* in 38 (42.22%), *seb* in 30 (33.33%) and *sec* in five (5.56%), while no amplification of the *sed* and *see* genes occurred in any of the isolates.

The most frequently isolated staphylococcal enterotoxins from outbreaks of food poisoning are types A and D (Atanassova et al., 2001). In the United States, the enterotoxin A has been the type most involved, present in 77.8% of all outbreaks, followed by the enterotoxins D (37.5%) and B (10%) (Mathieu et al., 1991).

Enterotoxin types A and B have been associated with the contamination from food handlers, while types C and D have been correlated with animal-borne contamination, especially from cattle and pigs (Najera-Sanchez et al., 2003). Although outbreaks of staphylococcal intoxication have most commonly been attributed to ingestion of enterotoxin type A, and various studies have shown the prevalence of its respective gene, the data of the present study show that the *sea* gene was one of the least frequent ones, present only in five (14.7%) out of the 34 toxigenic strains of *S. aureus*.

The differences in occurrence of the toxin encoding genes between studies may be explained by the geographic distribution and ecological origin of the strains, as well as by the sensitivity of the detection methods and the

numbers and types of samples (Fagundes et al., 2010).

Based on our results, it can be concluded that all the genes of the classical enterotoxins (*sea*, *seb*, *sec*, *sed* and *see*) and the gene of the Toxic Shock Syndrome Toxin (*tst*) were identified in strains of toxigenic *S. aureus*, which presented high genetic heterogeneity and genetic potential for production of one or more toxins. All RRM and cheese samples from the dairies investigated presented high CPS counts. This suggests that the hygienic-sanitary quality was unsatisfactory and that a risk to public health could arise, due to the possible presence of toxins.

## CONFLICTS OF INTERESTS

The authors have not declared any conflict of interests.

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*Full Length Research Paper*

# A cross sectional study on *Salmonella* in apparently healthy sheep and goats slaughtered at Elfora and Luna export abattoirs, Ethiopia

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**A cross-sectional study was conducted between November 2015 and March 2016 on apparently healthy slaughtered sheep and goats, and clean knife at Luna and Elfora export abattoirs located at Modjo and Bishoftu towns to estimate the prevalence of *Salmonella* in sheep and goats, to assess the hygienic condition of flaying knife, and to isolate and identify the prevalent *Salmonella* sub-species. A total of 525 samples consisting of cecum (n=122), liver (n=122), mesenteric lymph nodes (n=122), abdominal muscle (n=122) from 44 sheep and 78 goats and 37 pooled knife samples were collected. The samples were examined for the presence of *Salmonella* following the conventional techniques of ISO standard and using OMNILOG bacterial identification system, GEN III microplate for confirmation and sub species identification. From the total of 122 animals examined, 21 (17.21%) were positive of which 12 (9.83%) were sheep and 9 (7.38%) were goats, and none of the samples from pooled knife swabs were positive for *Salmonella*. Statistically significant difference (P=0.04) in the prevalence of *Salmonella* was observed between the two species. The frequency of isolation was 10 (3.89%) and 11 (4.10%) from Luna and Elfora abattoirs, respectively. As a result, there was no significant difference (P =0.884) in the prevalence of *Salmonella* isolation between the two abattoirs. Of the total 488 tissue samples examined from apparently healthy slaughtered sheep and goat, 21 (4.3%) samples were *Salmonella* positive. *Salmonella* was isolated from 6.56% mesenteric lymph nodes, 5.73% cecum, 4.09% liver and 0.82% abdominal muscle samples. However, there was no significant difference between tissues (P=0.13). From the 21 isolated *Salmonella* species, 20 of them were confirmed to be the pathogenic *Salmonella enterica* subspp. *enterica* and 1 isolate was the non-pathogenic *Salmonella enterica* subspp. *salamae*. The results of this study showed the potential risk of sheep and goats as sources of pathogen for humans in the study area. These findings stressed the need for implementation of preventing close contact of offal and carcass during evisceration.**

**Key words:** Elfora, goats, knife, luna, prevalence, *Salmonella*, sheep, sub species.

## INTRODUCTION

Sheep and goats in Africa are noted for their ability to convent low opportunity cost feed into high value products, namely, meat, milk, fiber, manure and skin (Wilsmore, 2006). In Ethiopia their population is

estimated to be about 28.89 million sheep and 29.70 million goats (CSA, 2016).

Meat, an excellent source of protein in human diet is highly susceptible to microbial contaminations, which can cause spoilage and food borne infections in human, resulting in economic and health losses (Komba et al., 2012). A great diversity of microbes inhabit fresh meat generally, but different types may become dominant depending on pH, composition, textures, storage temperature and transportation means of raw meat (Li et al., 2006; Adu-Gyamfi et al., 2012). Specific sources that contribute microbial contamination to animal carcasses and to fresh meat during slaughter and dressing include the faeces, the skin, water, air, intestinal contents, lymph nodes, processing equipment and humans, and can be transferred to the carcass during skin removal and evisceration (Hansson et al., 2000; Reid et al., 2002).

Active surveillance data on foodborne diseases from the United States revealed that among pathogens associated with foodborne outbreaks, *Salmonella*, *Escherichia coli* O157:H7, *Campylobacter*, and *Listeria monocytogenes* are responsible for the majority of outbreaks (Chen and Jiang, 2014). *Salmonella* is among the major causes of meat contamination that can affect small ruminant as well as human being (Pepin et al., 1997; Sierra et al., 1995). The members of the genus *Salmonella* are Gram-negative, motile, facultative anaerobic, bacilli belonging to the family Enterobacteriaceae. They comprised two central species, *Salmonella enterica* and *Salmonella bongori*. Presently, six subdivisions of *S. enterica* subspecies I-VI exist with over 2500 serovars currently identified and several common serovars to human clinical infections (Dworkin et al., 2006).

Diagnosis of salmonellosis is based on the isolation of the organism either from tissues collected aseptically at the necropsy or from feacal, rectal swabs or environmental samples. It can be isolated by standard cultural techniques and various biochemical and serological tests (OIE, 2000).

Previous studies conducted in Ethiopia on salmonellosis indicated the existence of the infection in various animal species (poultry, cattle, camels, sheep and goats, fish), in retail food items (minced beef, chicken meat and offal) (Mache et al., 1997; Molla et al., 1999, 2003; Nyeleti et al., 2000; Woldemariam, 2003; Gebremedhin, 2004; Ferede et al., 2015). However, in most of the studies *Salmonella* species identification were carried out by using conventional bacteriological tests in which it is difficult to identify pathogenic from non pathogenic salmonella species, where both can show similar properties by the traditional tests. Moreover, the information on the prevalence of pathogenic species of salmonella from sheep and goat, from Elfora and Luna export abattoirs and the zoonotic importance of sheep and goat salmonellosis is not as much known. Therefore, the objectives of this study were to estimate the occurrence of *Salmonella* in different organs and flaying knife of sheep and goat and to identify the prevalent *Salmonella* subspp. Using OMNILOG bacterial identification system.

## MATERIALS AND METHODS

### Study area and population

The study was conduct at Elfora export abattoir in Bishoftu and Luna export abattoir at Modjo, Ethiopia. The study animals were apparently healthy sheep and goats slaughtered at Elfora and Luna export abattoirs and flying knifes used for slaughtering of sheep and goats by the two abattoirs that can possibly contamination the carcasses.

### Study design, sampling methods and sample size determination

A cross-sectional study was carried from November 2015 to March 2016 to isolate *Salmonella* spp. from slaughtered sheep and goats. Individual animals were sampled by using systematic random sampling depending on the number of animals slaughtered on each day. Samples were collected with interval of two weeks and each visit 10 animals and three pooled samples of knife were sampled. From each selected slaughtered sheep and goats, cecum, carcass (abdominal muscle), liver, mesenteric lymph node, and swabs from knife were collected. Sample size was calculated by considering expected prevalence of 8.7% (Teklu and Negussie, 2011), 5% desired absolute precision and 95% confidence interval using the formula recommended by Thrusfield (2007). Accordingly, the minimum sample size was 122.

$$n = Z^2 P \exp (1\text{-}Pexp) / d^2$$

Where, n is required sample size; Pexp is the expected prevalence (8.7%), d is the desired absolute precision (5%), and Z = 1.96.

### Study methodology

#### *Sample collection*

All samples were collected aseptically using sterile forceps and scalpel blades from sheep and goats during slaughtering operation. From each selected animal, sufficient amount of samples cecum, carcass (abdominal muscles), liver and mesenteric lymph node were collected separately in sterile universal bottles. As soon as the abdomen of the animal was opened, the intestine with the mesenteric lymph nodes were separated from the rest of gastrointestinal tract and kept in a separate clean container until the other tissue samples collection from the same animal have been completed. Samples from knife were collected aseptically using sterile cotton swabs and samples were taken as soon as they had slaughtered the first animal and passed to the second animal. In

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both abattoirs, the slaughter personnel did immerse their knife in hot water in every slaughtered cycle and the collected swab samples were immersed in peptone water for transportation to laboratory. Collected samples were labeled uniquely identifying name of abattoirs, species of the animal, type of sample, date of sampling and sample ID number. All the collected samples were transported by cold chain and delivered to the general microbiology laboratory of National Animal Health Diagnostic and Investigation Center/NAHDIC/ within 24 h for bacteriological processing.

#### *Bacterial isolation and identification*

The method used for the culture of *Salmonella* was according to the technique recommended by the International Organization for Standardization (Quinn et al., 1999; ISO-6579, 2002). The bacteriological media used in different stage were prepared according to the manufacturer's recommendations. The swab sample and a grinded tissue samples were transferred for pre-enrichment to buffered peptone water in the ratio of 1:9 and incubated for 24 ± 3 h at 37°C after that, 0.1 ml of the sample were selectively enriched in 10 ml of Rappaport Vassilliadis Soy Broth (RVS) and incubated aerobically at 41.5±1°C for 24 ± 3 h and then loop full of samples were platted out on xylose lysine deoxychlorate (XLD) medium incubated at 37°C for 20 to 24 h. Then *Salmonella* suspected colonies were examined for the presence of typical red colony with black center in XLD medium. Suspected colonies were cultured on nutrient agar and confirmed by biochemical tests: TSI, Urease, Indole, lysine decarboxylase and Vogues proskour tests. At this level, the genus of *Salmonella* was identified and suspected colonies were cultured on Biology Universal Growth (BUG) media for further species and subspecies confirmation.

For species and sub-species identification, OMNILOG (fully automated coated microplate based bacterial identification system) that is, GEN III microplate with protocol A method was used to test suspected colonies. A single colony grown on agar medium was selected and emulsified into 'inoculating fluid A' (IF A). According to the manufacturer's instructions, cell density of the bacterial inoculum was measured for a specified transmittance (90 to 98%) using a turbidimeter, as specified in the user guide. For each isolate, 100 µl of the cell suspension was inoculated in to each of the 96 well coated microplate, using automatic multichannel pipette and incubated aerobically at 33°C for 22 h. The OmniLog identification system automatically read each microplate and provide identification called species/sub-species ID, then the results were printed. The results were also read in the BIOLOG Micro Station reader after 22 h incubation outside GEN III incubator.

#### Data management and analysis

The Data were entered into Microsoft Office Excel spread sheets and was analyzed using STATA (version 12) statistical soft ware package. Descriptive statistics was used to determine the prevalence of salmonellosis in the study area. The association of infection with the different factors was analyzed using Chi-square test. A P-value less than 0.05 at 95% confidence interval was considered for significance.

## RESULTS

From a total of 122 animals examined, 21 (17.21%) were positive for Salmonella; 9.83% (n=12) and 7.38% (n=9) were positive sheep and goats, respectively. There was significant difference (P < 0.05) in the frequency of *Salmonella* isolation between sheep and goat (Table 1).

From Luna and Elfora abattoirs, a total of 525 samples, 488 tissues and 37 pooled knives were collected. From this out of the 257 samples collected from Luna abattoir, 10 (3.89%) were *Salmonella* positive, and out of the 268 samples collected from Elfora abattoir *Salmonella* was detected in 11 (4.10%) samples. However, there was no significant difference (P > 0.05) in the frequency of *Salmonella* isolation between these two abattoirs (Table 2).

*Salmonella* was isolated from tissue samples collected from mesenteric lymph nodes (6.56%), cecum (5.73%), liver (4.09%) and abdominal muscles (0.82%). There was no significant difference (P > 0.05) in the frequency of *Salmonella* isolation among tissue samples (Table 3).

### *Salmonella* sub species isolation

A total of 21 *Salmonella* isolates, consisting of two different subspecies were identified. Of the sub species identified during study, 20 were *S. enterica* subspp. *enterica* and 1 was *S. enterica* subspp. *salamae* (Table 4).

## DISCUSSION

In the present study, from the total of 122 animals examined, 21 (17.21%) were positive for *Salmonella* of which 12(9.83%) were sheep and 9 (7.38%) were goats. The prevalence of *Salmonella* was higher in sheep than goats. This difference was statistically significant (P=0.04). This variation in prevalence of *Salmonella* between the two species might be due to differences in feeding behavior (sheep prefer to graze while goat to browse) and rearing area as well as management differences in the two species (Wassie, 2004). The higher prevalence in sheep might be due to higher *Salmonella* carrier rate in the study population. In addition, the sheep involved in this study came from different parts of the country by different means of transport and were usually held for a day to week before slaughter. The close contact during the transport and holding time may account for the high prevalence of *Salmonella* when examined after slaughter (Hurd et al., 2002; Molla et al., 2003).

D'Aoust (1989) cited few studies on the prevalence of *Salmonella* on sheep and goats undertaken in different parts of the world ranging between 2 and 51.5% in sheep and 1 to 18.8% in goats. Therefore, the finding in the present study was in line with reports of D' Aoust (1989). However, the same author reported a prevalence of 14.7% in sheep which was lower than the current finding (D'Aoust, 1994). This might be due to the fact that animals had been held in the market for longer period before slaughtered where stress could contribute to the

**Table 1.** Prevalence of *Salmonella* in slaughtered sheep and goats.

| Species | No. examined | Positive | Prevalence within species (%) | Prevalence from the total (%) | $\chi^2$ | P-value |
|---|---|---|---|---|---|---|
| Ovine | 44 | 12 | 27.27 | 9.83 | | |
| Caprine | 78 | 9 | 11.54 | 7.38 | 4.88 | 0.027 |
| Total | 122 | 21 | 17.21 | - | | |

**Table 2.** Prevalence of *Salmonella* between Luna and Elfora abattoirs.

| Sample type | Number of samples from | | | | Total | |
|---|---|---|---|---|---|---|
| | Luna abattoir | | Elfora abattoir | | | |
| | Examined | Positive (%) | Examined | Positive (%) | Examined | Positive (%) |
| Cecum | 60 | 2 (3.33) | 62 | 5 (8.06) | 122 | 7 (5.73) |
| Liver | 60 | 4 (6.67) | 62 | 1 (1.61) | 122 | 5 (4.09) |
| Mesenteric lymph node | 60 | 3 (5.0) | 62 | 5 (8.06) | 122 | 8 (6.56) |
| Abdominal muscle | 60 | 1 (1.67) | 62 | 0 (0.0) | 122 | 1 (0.82) |
| Pooled knife | 17 | 0 (0.0) | 20 | 0 (0.0) | 37 | 0 (0.0) |
| Total | 257 | 10 (3.89) | 268 | 11 (4.10) | 525 | 21 (4.0) |

**Table 3.** Risk factors for isolation of salmonella from apparently healthy slaughtered sheep and goats

| Risk factors | Examined | Positive (%) | $\chi^2$ | P. value |
|---|---|---|---|---|
| **Abattoirs** | | | | |
| Elfora | 248 | 11 (4.4) | 0.021 | 0.884 |
| Luna | 240 | 10 (4.2) | | |
| **Species** | | | | |
| Goat | 312 | 9 (2.9) | 4.23 | 0.04 |
| Sheep | 176 | 12 (6.8) | | |
| **Tissues** | | | | |
| Abdominal Muscle | 122 | 1 (0.8) | 5.722 | 0.13 |
| Cecum | 122 | 7 (5.7) | | |
| Liver | 122 | 5 (4.1) | | |
| Mesenteric Lymph nodes | 122 | 8 (6.6) | | |
| Total | 488 | 21 (4.3) | | |

**Table 4.** Distributions of identified *Salmonella* subspecies by animal species and abattoir sources.

| Identified ***subspecies*** | Luna | | Elfora | | Total of the two animal species | | |
|---|---|---|---|---|---|---|---|
| | Sheep | Goat | Sheep | Goat | Sheep | Goat | Total |
| *S. enterica subspecies enterica* | 3 | 7 | 8 | 2 | 11 | 9 | 20 |
| *S. enterica subspecies salamae* | 0 | 0 | 1 | 0 | 1 | 0 | 1 |
| Total | 3 | 7 | 9 | 2 | 12 | 9 | 21 |

higher infection rate among the animals (Teklu and Negussie, 2011). This holds true for small ruminants slaughtered at Luna and Elfora export abattoir, where the animals stayed for up to a week before slaughtered, especially at Elfora export abattoir when there was scarcity of animal supply from the customer.

The overall prevalence of the current result was higher than previous findings by different researchers in different parts of the country and elsewhere; in which Sierra et al. (1995) reported 10% prevalence from freshly dressed carcasses in Spain, Woldemariam et al. (2005) reported 2.8% prevalence in Debrezeit and Wassie (2004) reported prevalence of 11.3% in Addis Ababa and Modjo abattoirs. Similarly, Teklu and Negussie (2011) had also reported prevalence of 7.7% in Modjo export abattoirs and Zubair and Ibrahim (2012) reported prevalence of 2.5% from Zakho abattoir, Kurdistan region, Iraq.

The prevalence of *Salmonella* in apparently healthy slaughtered goats in this study was 11.54%. This results fall in the range of 1 to 18.8% prevalence in goats from different countries (D'Aoust, 1989). This finding was also in line with the findings of Teklu and Negussie (2011), who reported 11.7% in Modjo, Ethiopia and Woldemariam et al. (2005), who reported 9.8% in apparently healthy slaughtered sheep and goats in Bishoftu Ethiopia. However, the prevalence in goats was higher than prevalence reported by Wassie (2004) in Addis Ababa abattoir, which was 3%, Zubair and Ibrahim (2012), 2% from Zakho abattoir, Kurdistan region, Iraq, and Bedaso et al. (2015) who reported 0.54% from apparently healthy goats and sheep at Addis Ababa abattoir enterprise, Ethiopia. Similarly Sharma et al. (2001) had also reported prevalence of 2.3% from goats samples in Zambia. Even though, the current prevalence was higher than what was discussed, it was also lower than that in Ferede et al. (2015) in which it has 17.7% was reported from apparently healthy goats at Dire Dawa municipal abattoir, and 16.7% prevalence reported from goats slaughtered at Elfora abattoir in Ethiopia (Woldemariam, 2003). This variation in reported prevalence could be associated with the sampling plan and procedures, sample type, bacteriological techniques employed in detecting *Salmonella* or difference in occurrence and distribution of *Salmonella* in the study population regardless of test samples and methods of detection and hygienic condition of the abattoir environment.

In this study, *Salmonella* was not found from 37 pooled samples of knife swab both at Luna and Elfora export abattoirs. This finding was contrary to results of Teklu and Negussie (2011), who had reported 7.4% *Salmonella* prevalence from eviscerating knife swabs and 5% prevalence report of eviscerating knives in poultry slaughter houses in Iraq Sultan and Sharif (2002). Moreover, other study on knife blades had also reported 26.7 and 10% prevalence in two Botswana abattoirs (Motsoela et al., 2002). The difference between the results of the previous and the present one could be due to improvement of the hygienic conditions of the knife. Moreover, immersion of knives in hot water is being practiced in both abattoirs after flaying each animal and this might have also resulted in the low contamination of abdominal muscle observed in both abattoirs.

From a total of 488 examined sheep and goat tissue samples, 4.3% were infected with *Salmonella*. Of the four tissue samples taken from each animal during the study period, the cecum, liver and mesenteric lymph node samples proved to be the most useful indicators of infection. Abdominal muscle samples were less infected and this result was also similar to the findings of Molla et al. (2006).

*Salmonella* isolation rate of 11.36% that was recorded from cecum samples of sheep in this study was higher than the earlier observation of 2.1% by Teklu and Negussie (2011), 4.8% by Wassie (2004) in feaces, 2.1% report of Woldemariam (2003) from feaces and 6.7% reported by Bedaso et al. (2015). The 2.56% prevalence in goat cecum in this study was comparable to that of 3.3% by Woldemariam (2003) from feaces and 2% by Wassie (2004) from feaces. Other researchers have also reported the presence of human pathogens, such as *Salmonella*, in animal feaces (Jiang et al., 2015). The higher isolation rate from cecum in this study clearly indicates that *Salmonella* is found in the cecum microflora. Usually, healthy carriers intermittently excrete only a few *Salmonella*, unless they undergo some kind of stress (example during transport or holding in the lairages prior to slaughter).

The current study revealed that the isolation rate of *Salmonella* from mesenteric lymph node in sheep was 13.6% and in goats was 2.56%. This finding was close to 8.1% finding of Pateraki et al. (1975) in Greece and 7% report of Tadesse et al. (2014) from Adama municipal abattoir, Ethiopia. High level of *Salmonella* isolation in mesenteric lymph nodes in current finding may be due to the animal stay for up to a week before slaughter. However, study conducted on apparently healthy slaughtered sheep in Australia by Moo et al. (1980) indicated a 4% prevalence of *Salmonella* in mesenteric lymph nodes that was lower than the current finding.

From this study *Salmonella* isolation rate from liver of sheep was 2.27%. This finding was low in relation to isolate found in mesenteric lymph nodes and ceacal content. Low isolation rates in liver of sheep in this study support the findings of Molla et al. (2006) 1.9%, and Wassie (2004) 1.9%. However, Bedaso et al. (2015) and Tadesse et al. (2014) reported that there was no *Salmonella* isolate found in the liver. The low detection rates in these organs indicate that localization of the organism in liver is most likely minimal. It appeared to be rare for the liver and spleen tissue to be infected with *Salmonella* before death Molla et al. (2006). In goat, the finding was higher than sheep that was 5.13%. In contrast to this result, no *Salmonella* isolate was reported by Molla et al. (2006) in goat. This variation in result may be due to cross contamination during sampling or due to different bacteriological procedures followed.

In the present study, *Salmonella* was isolated in 1.28% of the abdominal muscle of goat but not detected from the sheep' abdominal muscle. This finding is much lower

than the 10% report from freshly dressed carcasses of sheep in Spain (Sierra et al., 1995), 17.7% from goat carcass swab in Dire Dawa municipal abattoir in Ethiopia (Ferede et al., 2015). However, the present finding was in consistence with Molla et al. (2006). It is generally accepted that the carcass of healthy slaughtered animals are free of bacteria at the time of slaughter, assuming that the animals are not in a state of exhaustion (Jay, 2000). These differences in prevalence of abdominal muscle may be due to hygienic condition of abattoirs and its environment. The current study revealed that as the carcass contaminations of the study area were low and this indicates that they were found at good hygienic condition.

Out of the total 21 *Salmonella* isolates, two different *Salmonella* subspecies were identified, this were *S. enterica* subspp. *enterica* 20/21 and *S. enterica* subspp. *salamae* 1/21. The current results indicate that the pathogenic and zoonotic *S. enterica* subspp. *enterica* was highly prevalent compared to the non-pathogenic *S. enterica* subspp. *salamae.* Hence, to control and prevent *Salmonella* infection and contamination in live animals and animal products, it is critical that risk reduction strategies should be used throughout the food chain that is from farm to fork. Therefore, during evisceration the offal content and carcass should not be in contact to each other and immediate separation of the offal from the carcasses should be employed.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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*Full Length Research Paper*

# Isolation and characterization of enteropathogenic and enterotoxinogenic *Escherichia coli* from dairy products consumed in Burkina Faso

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**Food-borne diseases represent a public major health problem, and drink-water, juice, meat, and milk products are usually involved. This study aimed to evaluate the antibiotic resistance of diarrheagenic *E. coli* isolated from dairy products consumed in Burkina Faso. Five hundred and twenty-two samples were gathered. *Escherichia coli* were isolated using Standard Microbiological Methods. A 16-plex polymerase chain reaction for virulence associated genes was applied. The standard disc diffusion methods were used to assess the susceptibility to 31 antibiotics. Classes 1, 2, 3 integrons were categorized using PCR. Results showed 1.92% (10/522) of milk products was contaminated by diarrheagenic *E. coli*. Enterotoxinogenic *E. coli* was found in 4.45% (4/89) of curds, 3.4% (3/88) of pasteurized milk, and 1.15% (1/87) of "déguè". Also, "déguè" was contaminated at 2.3% (2/87) by atypical enteropathogenic *E. coli*. Antibiogram susceptibility showed that pathogenic isolated resists mainly to tetracycline, amoxicillin, ticarcillin, nalidixic acid, sulfonamide, and trimethoprim-sulfamethoxazole. Only the class 1 integrons was detected in 80% of diarrheagenic *E. coli*. Among this class 1 integrons, 4 strains contains a variable region, and the subsequent result showed a presence of *dfrA7* gene coding for trimethoprim resistance. It appears in this study that dairy products are contaminated by enteropathogenic and enterotoxinogenic *E. coli*, which are resistant to antibiotics frequently used. This study therefore recommends the training of milk products transformers.**

**Key words:** Dairy products, diarrheagenic *Escherichia coli*, antibiotics resistance, integrons, Burkina Faso.

## INTRODUCTION

Food-borne diseases represent a public major health problem, and drink-water, juice, meat, and milk products are usually involved (OMS, 2011). Abdominal cramps, vomiting, diarrhea with/without blood, fever (OMS, 2011)

are illnesses caused by foods contaminated. Diarrhea causes mortality to a fifth of all people and a third to children younger than five years old worldwide (OMS, 2014). Diarrheagenic *Escherichia coli* (DEC) remain the ones mostly associated with endemic and epidemic diarrhea, amongst all the enteropathogenic bacteria worldwide (Nataro and Kaper, 1998). In Burkina Faso, DEC is mainly responsible for diarrhea among infants younger than 5 years often associated with vomiting, fever, and dehydration (Bonkoungou et al., 2013).

Studies showed variable contaminations of milk, pasteurized milk, cheeses, and other milk products by enteropathogenic, enteroaggregative, enterohemorragic and enterotoxinogenic *E. coli* in Iran (Bonyadian et al., 2014), India (Nazir et al., 2013), Ivory Coast (Dadie et al., 2010), Brazil (Paneto et al., 2007), Nigeria (Ivbade et al., 2014), and Saudi Arabia (Al-Zogibi et al., 2015). Studies that were done on diarrheagenic *E. coli* isolated from cheeses, milk, pasteurized milk, and other milk products from Nigeria (Ivbade et al., 2014), Greece (Solomakos et al., 2009), Brazil (Paneto et al., 2007), and India (Nazir et al., 2013) revealed resistance to amoxicillin, amoxicillin-clavulanic acid, ampicillin, nalidixic acid, norfloxacin, gentamicin, streptomycin, tetracycline, doxycycline, erythromycin, cefaclor, cephradine, ceftazidime, chloramphenicol, and sulfamethoxazole-trimethoprim with variable rates. Several mechanisms are involved in antimicrobial resistance. Beyond the efflux system, reduction of the porins structure, and changing of the target of the antibiotics by methylation, plasmids, integrons and transposons also play an important role in antibiotic resistance (Stokes and Hall, 1989; Schwarz and Chaslus-Dancla, 2001; Escudero et al., 2015). These capture systems mobile elements (integrons, plasmids), are responsible for the resistance genes dissemination between the same species and the different species (Escudero et al., 2015). The studies on clinical, food and environmental DEC isolated from Nigeria, Egypt, India, and Kenya revealed that the presence of class 1, 2, and 3 integrons are the cause of the resistance of these pathogens to antibiotics. Class 1, and 2 integrons are connected to several resistance genes (cassettes) encoding antibiotic resistance such as *dfr* for trimethoprim resistance, *aac* and *aad* for aminoglycosides, *sul* for sulfonamides, *tet* for tetracycline's, *cat*, and *cmlA1* for chloramphenicol, *satA1* for streptothricin (Kiiru et al., 2013; Adelowo et al., 2014; Dureja et al., 2014; Ahmed and Shimamoto, 2015). Thus, this study aimed to examine the prevalence and mechanism of antibiotic resistance of diarrheagenic *E. coli* isolated from milk, pasteurized milk, curds, yogurts, and "déguè" (mixture of yogurt and millet lumps) consumed in Burkina Faso.

## MATERIALS AND METHODS

### Study design and sampling

The study was conducted between October 2011 and June 2015, in ten major cities producing and consuming bovine milk products in Burkina Faso (Figure 1a). Sampling was carried out regularly within three steps of milk production: firstly, 69 farms' milk had been collected in eight cities: "Bobo-Dioulasso" in the Southwest (19), "Dori" in the North (5), "Fada N'Gourma" in the East (12), "Kongoussi" and "Sabcè" in the North Central with respectively 4 and 3 farms, "Koudougou" in the West Central (6), "Léo" in the South (12) and "Ouahigouya" in the North (8). All the farms in an area were connected to dairy transformation units in the same city. Secondly, four yogurts and 13 pasteurized milk products samples were collected from the dairy transformation units associated with the above-cited farms. Thirdly, 436 milk products of consumption from distribution chain were gathered in "Ouagadougou" (Figure 1b) and "Ziniaré". These consisted of 84 sets of milk and 89 curds samples (a traditional production) from open markets, 88 pasteurized milks, 88 yogurts and 87 "dégué" samples from food shop and supermarkets. A total of 125 to 500 ml samples of milk products were gathered and transported at 4°C to the "Laboratoire de Biologie Moléculaire, d'Epidémiologie et de Surveillance des Bactéries et Virus Transmissibles par les Aliments (LaBESTA)/ Université Ouaga I Pr Joseph KI-ZERBO", and examined immediately.

### *Escherichia coli* isolation and identification

The ISO 4832 (ISO, 1991) modified method was used for isolating and identifying *E. coli*. Twenty-five milliliter samples of milk were homogenized into 225 ml of buffered peptone water (Liofilchem, Italy) and incubated at 37°C. Then, after 24 h of incubation, two loopfuls of enriched broth were streaked into violet red bile lactose (VRBL) agar (Liofilchem, Italy) and ChromoCult coliform agar (Merck, Germany), which were incubated at 44.0 ± 0.1°C for 24 h. Suspicious *E. coli* colonies appear small, purple with purple cloud and blue at violet respectively on VRBL and ChromoCult coliform agar. Three to five presumptive colonies were carefully chosen and tested for lactose and glucose metabolism, indole, urea, citrate, and fermentative gas production. *E. coli* colonies were confirmed by API 20E system (BioMerieux, France).

### Diarrheagenic *Escherichia coli* characterization

A boiling process was used to obtain the DNA of each strain. This was carried out by homogenizing two loopfuls of each strain into an Eppendorf Tube comprising 250 µl of sterile water. The mixture was boiled afterwards for 10 min and centrifuged for 10 min. The supernatant was collected and used for the PCR reactions.

A multiplex polymerase chain reaction was used for the detection of five major diarrheagenic *E. coli* (DEC). This characterization was carried out for the intensifying of 16 virulence genes of DEC with specific primers (Table 1). The virulence genes that follow were categorized according to Antikainen et al. (2009): For Entero-pathogenic *E. coli* (EPEC), the presence of *eaeA*, *escv* and/or *ent* and *bfpB*. The absence of *bfpB* indicated atypical EPEC; for Shiga Toxin producing *E. coli* (STEC), the presence of $stx_1$, and/or $stx_2$ with a possible additional genes as *eaeA*, *escv*, *ent*, and EHEC-*hly*;

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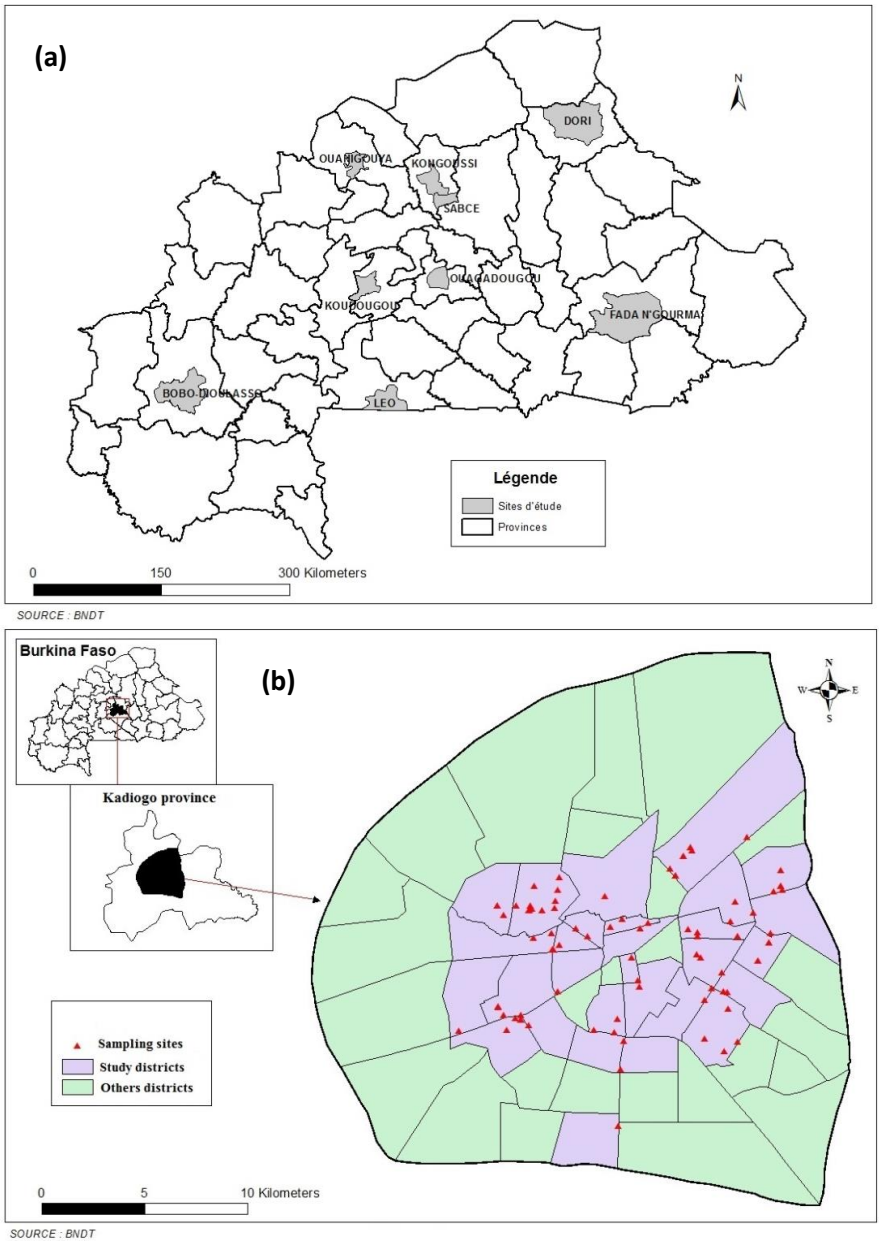


**Figure 1.** (a): Map of Burkina Faso with sampling sites in the nine (9) majors' cities producing and/or consuming of milk products. (b): Sampling sites in Ouagadougou, Burkina Faso.

for Enteroinvasive *E. coli* (EIEC), the presence of *ipaH*, and *invE* genes*;* for Enteroaggregative *E. coli* (EAEC), the presence of *virF*, and/or *aggR*, and/or *pic*, and/or *astA* genes; for Enterotoxinogenic *E. coli* (ETEC), the presence of *elt*, and/or *sta*, and/or *stb* genes.

A 25 µl reactional mixture was employed to perform the Multiplex. A volume of 2.5 µl of DNA samples were added into 22.5 µl of PCR mixture comprising 5.0 µl of buffer GC, 0.6 µl of $MgCl_2$, 1.0 µl of dNTPs, 0.4 µl of Taq polymerase, 10.5 µl of $H_2O$, 2.5 µl of Muller mix (*escV, bfpB, stx1, stx2, lt, sta, stb, invE, astA*, *aggR, pic, uidA*), and 2.5 µl of Jenni mix (*eaeA, ent,* EHEC-*hly, ipaH*). Amplification programmes were 98°C for 30 s following to 30 cycles of 98°C for 30 s, 62,5°C for 60 s, 72°C for 90 s, and final extension of 72°C for 10 min. Amplified DNA fragments were divided by agarose gel electrophoresis (1% weight/volume), added ethidium bromide, and visualized under UV light. DNAs of following reference strains were used for positive control: 17.2 for EAEC, E2348-69 for EPEC, EDL 933 for EHEC, M90T for EIEC and EDL 1493 for ETEC. The strain HB101 DNA was also used for negative control.

### Antimicrobial susceptibility testing

The agar disc diffusion method (CASFM, 2014) was used to carry out Antimicrobial susceptibility of diarrheagenic *E. coli* isolated. Diameters of inhibition were determined according to « Comité de l'Antibiogramme de la Société Française de Microbiologie » instructions (CASFM, 2014). Thirty-one (31) common antibiotic (BioRad, France) discs used were: amoxicillin 25 µg (AMX),

**Table 1.** Diarrheagenic *E. coli* primers and the virulence genes detected.

| Pathotypes | Target gene | Primer sequence (5'-3') | Product size (bp) | [C] (µM) |
|---|---|---|---|---|
| STEC, EPEC | *eaeA* | F: TCAATGCAGTTCCGTTATCAGTT<br>R: GTAAAGTCCGTTACCCCAACCTG | 482 | 0.1 |
| | *escV* | F: ATTCTGGCTCTCTTCTTCTTTATGGCTG<br>R: CGTCCCCTTTTACAAACTTCATCGC | 544 | 0.4 |
| | *ent* | F: TGGGCTAAAAGAAGACACACTG<br>R: CAAGCATCCTGATTATCTCACC | 629 | 0.4 |
| EPEC typique | *bfp B* | F: GACACCTCATTGCTGAAGTCG<br>R:CCAGAACACCTCCGTTATGC | 910 | 0.1 |
| STEC | EHEC-*hly* | F: TTCTGGGAAACAGTGACGCACATA<br>R: TCACCGATCTTCTCATCCCAATG | 688 | 0.1 |
| | *stx1* | F: CGATGTTACGGTTTGTTACTGTGACAGC<br>R: AATGCCACGCTTCCCAGAATTG | 244 | 0.2 |
| | *stx2* | F:GTTTTGACCATCTTCGTCTGATTATTGAG<br>R: AGCGTAAGGCTTCTGCTGTGAC | 324 | 0.4 |
| EIEC | *ipaH* | F: GAAAACCCTCCTGGTCCATCAGG<br>R: GCCGGTCAGCCACCCTCTGAGAGTAC | 437 | 0.1 |
| | *invE* | F: CGATAGATGGCGAGAAATTATATCCCG<br>R:CGATCAAGAATCCCTAACAGAAGAATCAC | 766 | 0.2 |
| EAEC | *aggR* | F: ACGCAGAGTTGCCTGATAAAG<br>R: AATACAGAATCGTCAGCATCAGC | 400 | 0.2 |
| | *pic* | F: AGCCGTTTCCGCAGAAGCC<br>R: AAATGTCAGTGAACCGACGATTGG | 1111 | 0.2 |
| | *astA* | F: TGCCATCAACACAGTATATCCG<br>R: ACGGCTTTGTAGTCCTTCCAT | 102 | 0.4 |
| ETEC | *elt* | F: GAACAGGAGGTTTCTGCGTTAGGTG<br>R: CTTTCAATGGCTTTTTTTTGGGAGTC | 655 | 0.1 |
| | *estIa* | F:CCTCTTTTAGYCAGACARCTGAATCASTTG<br>R: CAGGCAGGATTACAACAAAGTTCACAG | 157 | 0.4 |
| | *estIb* | F: TGTCTTTTTCACCTTTCGCTC<br>R: CGGTACAAGCAGGATTACAACAC | 171 | 0.2 |
| *E. coli* | *uidA* | F: ATGCCAGTCCAGCGTTTTTGC<br>R:AAAGTGTGGGTCAATAATCAGGAAGTG | 1487 | 0.2 |

STEC : Shiga toxin producing *E coli* ; EPEC: Enteropathogenic *E. coli*; EIEC: Enteroinvensive *E. coli*; EAEC: Enteroaggregative *E. coli;* ETEC: Enterotoxigenic *E. coli ;* [C] : Concentration.

ticarcillin 75 µg (TIC), piperacillin 75 µg (PIP), piperacillin + tazobactam 85 µg (PPT), amoxicillin + clavulanic acid 30 µg (AMC), ticarcillin + clavulanic acid 85 µg (TCC), cefotaxim 30 µg (CTX), ceftazidim 30 µg (CAZ), cefolatin 30 µg (CEF), cefepim 30 µg (FEP), aztreonam 30 µg (ATM), imipenem 10 µg (IPM), cefuroxim 30 µg (CXM), cefoxitin 30 µg (FOX), imipenem + EDTA 10 µg (EIP), nalidixic acid 30 µg (NAL), norfloxacin 5 µg, ofloxacin 5 µg (OFX), ciprofloxacin 5 µg (CIP), tobramycin 10 µg (TMN), gentamicin 10 µg (GMN), amikacin 30 µg (AKN), chloramphenicol 30 µg (CHL), tetracycline 30 µg (TET), minocyclin 30 µg (MNO), tigecyclin 15 µg (TGC), fosfomycin 50 µg (FSF), sulfonamide 200 µg (SUL), trimethoprim + sulfamethoxazol 25 µg (SXT), nitrofuran 300 µg (FTN), nitroxolin 20 µg (NIT). A multi-drug resistance of strains was examined and defined as being a resistance to at least three families of antibiotics (Dureja et al., 2014). The inhibition zones were evaluated as "resistant", "intermediate sensitive", or "sensitive" according to CASFM (2014) criteria with Antimicrobial susceptibility testing system version 3.0.0.

**Integrons detection**

For all DEC, the single polymerase chain reaction (Bissonette and Roy, 1992; Ploy et al., 2000; Mazel, 2004) was used to detected classes 1, 2 and 3 integrons. Integrase genes *IntI1*, *IntI2*, and *IntI3*

**Table 2.** Integrons, and resistance genes primers.

| Target gene | Primer sequence (5'-3') | Product size (bp) | Concentration (µM) |
|---|---|---|---|
| *IntI1* | F: ATTTCTGTCCTGGCTGGCGA<br>R: ACATGTGATGGCGACGCACGA | 600 | 10 |
| *IntI2* | F: CACGGATATGCGACAAAAAGG T<br>R: GTAGCAAACGACTGACGAAATG | 806 | 10 |
| *IntI3* | F: GCCCCGGCAGCGACTTTCAG<br>R: ACGGCTCTGCCAAACCTGACT | 600 | 10 |
| *36854*<br>*36855* | GGCATGCAAGCAGCAAGCGCGTTA<br>AACCGAACTTGACCTGATAGTTTG | | 10 |
| *Sul1*<br>*Orf4* | GTCCGACATCCACGACGTCTGATC<br>CAAACTATCAGGTCAAGTCTGCTT | | 10 |
| *Sul3*<br>*Orf6* | CCTGGAGATCTGCGAAGCGCAATC<br>GTCGCTGCAACTCGCGACT | | 10 |

**Table 3.** Prevalence of *E. coli* in milk products consumed in Burkina.

| Types of dairy products | *Escherichia coli* | |
|---|---|---|
| | Number | % |
| Farm milk (n=69) | 68 | 98.55 |
| Milk (n=84) | 29 | 34.52 |
| Curd (n=89) | 29 | 32.58 |
| Pasteurized milk (n=101) | 29 | 28.71 |
| Yoghourt (n=92) | 04 | 04.35 |
| Déguè (=87) | 14 | 16.09 |
| **Total (n=522)** | **174** | **33.33** |

were carried out with specific primers (Genecust, Luxembourg) (Table 2). Primers 36854 and 36855 were used to categorize the variables regions (VR) of class 1 integrons. Sizes of variables regions were measured, purified, and sequenced to determine resistance genes. For the 3' conserved segment (3'CS), primers sul1-Orf4, and sul3-Orf6 were used by single PCR. Thermocycling conditions were 94°C for 5 min, following to 35 cycles at 94°C for 30 s, 60 s to 60 and 62°C respectively for *IntI1*, VR, 3'CS and *IntI2*/*IntI3*, and 72°C for 60 s. The ultimate extension was 72°C for 10 min. The amplicons were visualized by electrophoresis on 1% (weight / volume) gel agarose in the TAE buffer.

#### Data analysis

MS Excel 2010 was used to analyze the data. The obtained sequences were compared in GenBank database with the use of BLAST software in NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to determined resistance genes with a similarity of 98.5%.

## RESULTS

### Prevalence of *E. coli* in dairy products

The results show that 33.33% of dairy products are contaminated by *E. coli* with 315 strains. High rates were observed in traditional dairy products with 98.55% of farm milk, followed by 34.52% of milk sold in Ouagadougou's open markets, and curds (32.58%). This study also showed that pasteurized milk and "déguè" are contaminated by *E. coli* respectively at 28.71 and 16.09% (Table 3).

### Prevalence of diarrheagenic *E. coli*

The study revealed that the milk products consumed in study sites are contaminated by some diarrheagenic *E. coli* with variables rates (Table 4). Atypical enteropathogenic *E. coli* (presence of *eaeA* and *ent* genes) and enterotoxinogenic *E. coli* (presence of *stb* gene) was detected with 0.38 and 1.53% of total milk products respectively. Atypical enteropathogenic *E. coli* (aEPEC) was found in 2.3% of "déguè" while 4.45% of curds, 2.97% of pasteurized milk, and 1.15% of "déguè" were contaminated by enterotoxinogenic *E. coli*.

### Antimicrobial resistance of diarrheagenic *E. coli*

Antibiogram results showed diarrheagenic *E. coli* isolated from milk products resists at least to 11 antibiotics used. Atypical enteropathogenic and enterotoxinogenic *E. coli* were resistant to amoxicillin, ticarcillin, piperacillin, nalidixic acid, norfloxacin, ofloxacin, tobramycin, nitroxolin, nitrofuran, tetracycline, sulfonamide, and trimethoprim-sulfamethoxazole (Table 5).

### Class 1 integrons, and resistance genes

Class 1 integrons were detected in eight out of the 10 pathogenic *E. coli* isolates (80%). Class 2 or 3 integrons

**Table 4.** Prevalence of diarrheagenic *Escherichia coli*.

| Types of dairy products | Diarrheagenic *Escherichia coli* (DEC) N (%) | | | | | |
|---|---|---|---|---|---|---|
| | EPEC | STEC | EHEC | EIEC | EAEC | ETEC |
| Farm milk (n=69) | - | - | - | - | - | - |
| Milk (n=84) | - | - | - | - | - | - |
| Curd (n=89) | - | - | - | - | - | 4 (4.45) |
| Pasteurized milk (n=101) | - | - | - | - | - | 3 (2.97) |
| Yoghourt (n=92) | - | - | - | - | - | - |
| Déguè (=87) | 2 (2.3) | - | - | - | - | 1 (1.15) |
| Total (n=522) | 2 (0.38) | - | - | - | - | 8 (1.53) |

-: None; EPEC: Enteropathogenic *E. coli*; STEC: Shiga toxin producing *E. coli*; EHEC: Enterohemorrhagic *E. coli;* EIEC: Enteroinvasive *E. coli;* EAEC: Enteroaggregative *E. coli;* ETEC: Enterotoxinogenic *E. coli*.

**Table 5.** Resistance of diarrheagenic *Escherichia coli* to antibiotics used.

| Strains | Milk products | Pathovars | Antibiotic resistance | Intermediary resistance |
|---|---|---|---|---|
| Ld5.4 | Déguè | EPEC | TET, SUL | NIT |
| Ld5.1 | | EPEC | AMX, TIC, NAL, TET | NIT, PIP |
| Ld5.2 | | ETEC | AMX, TIC, NAL, TET | NIT, PIP |
| Lc37 | Curds | ETEC | - | NIT |
| Lc2.2 | | ETEC | | NIT, PIP, TIC |
| Lc7 | | ETEC | FTN | NIT |
| Lc51.2 | | ETEC | - | NIT |
| Lp2.2 | Pasteurized milk | ETEC | AMX, TIC, PIP, NAL, NOR, OFX, TMN | NIT |
| Lp56.4 | | ETEC | TET | NIT, TIC, PIP |
| Lp70.2 | | ETEC | AMX, TIC, PIP, TET, SUL, SXT | NIT |

EPEC: Enteropathogenic *E. coli*; ETEC: Enterotoxinogenic *E. coli*, TET: Tetracyclin 30 µg; AMX: Amoxicillin 25 µg; SUL: Sulphonamid 200 µg; TIC: Ticarcillin 75 µg; NAL: Nalidixic acid 30 µg, FTN: Nitrofuran 300 µg; PIP: Piperacillin 75 µg; NOR: Norfloxacin 5 µg; OFX: Ofloxacin 5 µg; TMN: Tobramycin 10 µg; SXT: Trimethoprim-sulfamethoxazol 25 µg; NIT: Nitroxolin 20 µg.

were not detected. Four class 1 integron-containing EPEC, and ETEC strains contained an identical integron harboring a single cassette, *dfrA7*, encoding resistance to trimethoprim. No classes 2 and 3 integrons were detected in this study (Table 6).

## DISCUSSION

### Prevalence of *E. coli* in dairy products

Investigation showed that the milk products consumed in Burkina Faso are widely contaminated by *E. coli* with variable rates. Traditional milk products, such as farm milk (98.55%), milk (34.5%), and curds (32.58%), mostly sold in open markets, are highly contaminated by *E. coli*. Comparable outcomes have been reported in Ivory Coast and Burkina Faso with slim differences (Katinan et al., 2012; Bagré et al., 2014). These results could be explained by milking conditions in farms, and handling conditions during selling of milk. In fact, Bagré et al. (2015) showed that a majority of farms in Burkina Faso are mainly traditional with unhygienic practices. In these farms, 43.9% do not clean udders before milking, with a calabash being the main collection utensil. These practices, due to poor hygienic training, could explain the traditional milks' contamination during its production. In addition, in this study, fermented and pasteurized milks are contaminated by *E. coli*. The pasteurized milks consumed in Burkina Faso are contaminated by *E. coli* (28.71%). These results are lower than those found in Iran (93.75%) (Nazir et al., 2013). The contamination by *E. coli* could be also explained by a post-contamination during packaging. In unit, packaging is a manual that could cause contamination by workers. The results have shown that the yogurts, which are consumed in Burkina Faso, are less contaminated. In fact, this low contamination could be explained by the acidity of yogurt. Investigations have revealed lactic bacteria produce bacteriocins, which inhibit pathogens as *E. coli, Listeria*

**Table 6.** Resistance genes associated to class 1 integrons.

| Stains | Milk product | Pathovars | Class integrons | Size of cassettes (bp) | Resistance genes |
|---|---|---|---|---|---|
| Ld5.4 | Déguè | EPEC | *IntI1* | 800 | *dfrA7* |
| Ld5.1 | | EPEC | *IntI1* | 800 | *dfrA7* |
| Ld5.2 | | ETEC | - | - | - |
| Lc37 | Curds | ETEC | *IntI1* | 800 | *dfrA7* |
| Lc2.2 | | ETEC | - | - | - |
| Lc7 | | ETEC | *IntI1* | - | - |
| Lc51.2 | | ETEC | *IntI1* | 800 | *dfrA7* |
| Lp2.2 | Pasteurized milk | ETEC | *IntI1* | - | - |
| Lp56.4 | | ETEC | *IntI1* | - | - |
| Lp70.2 | | ETEC | *IntI1* | - | - |

EPEC: Enteropathogenic *E. coli*; ETEC: Enterotoxinogenic *E. coli.*

*innocua* and reduce bacterial flora (Khay et al., 2011; Yang et al., 2012); but high contamination of milk products by *E. coli* before transformation can still contain these bacteria. About “déguè”, this contamination could be explained by the supplies used to fermented milk with lumps of millet. Lumps, which are not pasteurized and are often exposed to sun, could bring enteropathogens bacteria particularly for the period of the mixture.

## Prevalence of diarrheagenic *E. coli*

Our study reveals a contamination of milk products by some pathotypes of *E. coli*. In this study, 522 milk products consumed in Burkina Faso were contaminated characteristically enteropathogenic and enterotoxinogenic *E. coli*. None of enterohemorrhagic, enteroinvasive and enteroaggregative *E. coli* was found in this study. Indeed, “déguè” (semi-modern milk product) is contaminated by atypical enteropathogenic *E. coli* (aEPEC) with the presence of *eaeA* and *ent* genes. A number of studies in some countries revealed that milk products are contaminated by atypical EPEC. For example, several authors noted that milk are contaminated at 1.2 to 1.6% in Ivory Coast (Dadie et al., 2010); 1.56% and 8.25% in Iran (Rahimi et al., 2012; Mohammadi and Abiri, 2013), and 7.03% in Saudi Arabia (Al-Zogibi et al., 2015). Correspondingly, pasteurized milks are contaminated at 22.1 and 28.12% in Brazil (Da Silva et al., 2001) and India (Nazir et al., 2013); milk cheeses in Brazil (2, 4, and 6% respectively by EPEC O125, O111, and O55), and in Iran (19.48% with the serotype O127) (Najand and Ghanbarpour, 2006; Paneto et al., 2007). About these pathovars in “déguè”, none of the studies was carried out in Africa. The contamination of milk products consumed in Burkina Faso may constitute a public health concern particularly for children younger than five years. Studies on diarrhea etiologies in Burkina Faso revealed that atypical EPEC are one of the most typical bacterial causes (Nitiema et al., 2011; Bonkoungou et al., 2013; Dembélé et al., 2015). In addition, Bonkoungou et al. (2013) reported that aEPEC is more predominant than classical EPEC in diarrhea infections from Burkina Faso. In recent times, studies displayed lower prevalence of EPEC in children younger than five years in Burkina Faso (4%) (Bonkoungou et al., 2013) and Senegal (1.16%) (Sambe-Ba et al., 2013). Among DEC found, enterotoxinogenic *E. coli* (ETEC) became predominant in traditional (curd) and semi-modern (“déguè”, and pasteurized milk) milk products. Studies in Germany (Franke et al., 1984), India (Nazir et al., 2013) and Iran (Bonyadian et al., 2014) revealed that milk products (3.2%), pasteurized milk (3.13%), and cheeses (1.66%) are contaminated by ETEC. It gives the impression that ETEC is associated with travelers’ and infantile diarrhea (Nataro and Kaper, 1998). Enterotoxinogenic *E. coli* can create heat-stable toxin (ST) and heat-labile toxin (LT), which are responsible to profuse water diarrhea and others symptoms such as fever, vomiting, abdominal cramps. In this study, heat-stable toxin (*stb*) gene was detected in all strains. In Burkina Faso, ETEC is responsible for infantile watery diarrhea, often associated with dehydration. The consumption of water, foods, unpasteurized milk, raw juice, fruits, vegetables and unheated meals are commonly implicated with ETEC infection (CDC, 2005).

## Antimicrobial resistance of diarrheagenic *E. coli*

Antibiogram patterns revealed multidrug resistance of enteropathogenic and enterotoxinogenic *E. coli*. A small number of studies have been carried out on pathogenic *E. coli* isolated from a number of dairy products in the world. Resistance of diarrheagenic *E. coli* to nalidixic acid is comparable to that observed with enterotoxinogenic *E. coli* (ETEC) in Brazil (40%) (Paneto et al., 2007), and STEC in Nigeria (20%) isolated from milk products (Ivbade et al., 2014). However, the tetracycline resistance

is lower than that found in Nigeria (90%) (Ivbade et al., 2014) and Greece (100%) (Solomakos et al., 2009), and higher than that found in Brazil (31%) (Paneto et al., 2007) concerning STEC and ETEC strains. Resistances to norfloxacin and trimethoprim-sulfamethoxazole were observed. Higher results were observed mainly in Nigeria (20%) (Ivbade et al., 2014) and Greece (100%) (Solomakos et al., 2009) for the trimethoprim-sulfamethoxazole on *E. coli* producing Shiga toxin. Additionally, resistances to penicillins (amoxicillin, ticarcillin, and piperacillin), aminoglycosides (tobramycin), sulfonamides and others fluoroquinolones such as ofloxacin were observed. Diarrheagenic *E. coli* isolated from curds, pasteurized milks and "déguè" resist five antibiotics families, such as tetracycline, penicillin, aminoglycoside, sulfonamide, and fluoroquinolone. Such resistance could be clarified by the presence of genes encoding resistance to these antibiotics. Integrons characterization revealed mostly the presence of *dfrA7* genes encoding resistance to trimethoprim. This classification displays resistances to other antibiotics may be encoded by other mechanisms or unwanted genes in this study. Studies carried out on *E. coli* producing shiga toxin in Egypt showed 3' conserved regions of integrons contains *qnrB*, *qnrS*, and *floR* genes encoding resistance to quinolones (Ahmed and Shimamoto, 2015). In this study, 80% (8/10) of diarrheagenic *E. coli* harboured class 1 integrons, with *dfrA7* gene encoding trimethoprim resistance. Previous studies carried out in China on *E. coli* isolated from dairy products revealed the presence of *dfrA17* and *dfrA1* genes encoding trimethoprim resistance (Zhao et al., 2014). In addition, resistance to antibiotics belonging to other families of tetracycline, penicillin, aminoglycoside and quinolone could be due to a selection of resistant strains in dairy products by antibiotic residues. Our earlier data (Bagré et al., 2015) on the same dairy products revealed antibiotics residues belonging to beta-lactam and/or sulfonamides and/or tetracycline and aminoglycosides and/or quinolones and/or macrolides in several magnitudes. For that reason, antibiotic residues could exert selection pressure on pathogenic strains in these dairy products.

This study show that curds, pasteurized milk and “dégué” consumed in Burkina Faso are lowly contaminated by enteropathogenic and enterotoxinogenic *E. coli*. In addition, the results of the Integrons showed that resistance is carried out by plamsids, with risks of a transmission of inter/intra pathogenic species. These mechanisms could help to understand the genetic materials of the DEC resistance isolated from dairy products. Nevertheless, the risk appears low in prevalence terms; special attention should be giving to the dairy products process. Training and awareness should be done with dairy products transformers and farmers, with the view of protecting the health of consumers and avoid the emergence of resistant pathogens.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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# Riboflavin enriched iru: A fermented vegetable protein

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**African locust bean (*Parkia biglobosa*) cotyledon is fermented in most countries of West Africa to produce a soup condiment, known as 'iru' in Yoruba language, or 'dawadawa' in the predominant Hausa language. Iru is rich in minerals and serves as a source of protein supplement in the diet of poor families. Riboflavin (Vitamin $B_2$) is an essential component of basic cellular metabolism but its daily requirement is not met in Nigeria particularly among the rural dwellers. Therefore, the provision of a riboflavin enriched iru will help to eradicate problems encountered from riboflavin deficient diet. Iru was purchased from three different markets in Ibadan, Oyo State, Nigeria. From the iru, microorganisms were isolated, characterised, screened for riboflavin production and co-cultured for the production of riboflavin enriched iru. Sixty-three bacteria were isolated and identified as *Micrococcus varians* (9), *Staphylococcus* species (27), *Bacillus* species (24) and *Micrococcus luteus* (3). *Bacillus subtilis* IR50 produced highest riboflavin 25.77 mg/L, followed by *Staphylococcus* spp. strain IR26 23.37 mg/L, while *M. varians* IR49 had the least riboflavin production 6.35 mg/L. Mixed culture of *B. subtilis* IR50 and *Bacillus licheniformis* IR28 produced the highest riboflavin of 4.5 mg/L, *Staphylococcus aureus* IR06 and *B. subtilis* IR50 produced 2.3 mg/L, while *B. subtilis* IR50 produced 1.5 mg/L when used singly. The result shows that *B. subtilis* IR50 have the potential to increase the riboflavin content of iru and therefore will contribute to bioenrichment technology.**

**Key words:** African locust bean, iru, riboflavin, *Bacillus subtilis*, bioenrichment.

## INTRODUCTION

African locust bean (*Parkia biglobosa*) cotyledon is fermented in most West African belt countries to produce a soup condiment, known as 'iru', or 'dawadawa', depending on the ethnic group. Till date, the production process is a traditional art; and the fermentation is carried out by indigenous microflora derived from the immediate environment. Iru which is the Yoruba name is a product of alkaline fermentation of African locust bean (*P. biglobosa)* which is rich in protein and usually fermented to a tasty food condiment used as a flavour intensifier for soups and stews and also adds proteins to a protein-poor diet. Apart from imparting flavour, it serves as a source of protein supplement in the diet of poor families (Odunfa, 1985).

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The pods are flat, large, irregular clusters from which the locust bean seeds are obtained (Omafuvbe et al., 2004). It contains about 40.4% protein, 31.5% fat, 3.1% fibre and 15% carbohydrate (Fetuga et al., 1974). The African locust bean is consumed mainly because of the flavouring attributes. Locust beans are not usually used for food in their natural state, because of the presence of non-digestible carbohydrates which may include arabinogalactan, stachyose, and raffinose (Odunfa, 1983) and the presence of anti-nutritional factors which are a diverse range of naturally occurring compounds in many tropical plants (Esenwah and Ikenebomeh, 2008). Fermentation makes the food to be more nutritious, digestible and safer with better flavour. The cooked African locust beans are unpalatable but when fermented into condiment, Iru, the physical, chemical and nutritional characteristics of the seeds change (Amoa-Awu et al., 2005).

The fermentation is brought about by strains of *Bacillus subtilis* (Odunfa, 1981). Many strains of the *B. subtilis* group have been isolated from iru samples obtained from different sources in southwestern. Quantity of iru consumed varies with the country and within the country. The average per capita per day consumption of iru in Togo and Ghana is 4 and 2 g, respectively, the Yorubas of Southwestern Nigeria consume 10 g per day per person (Dema, 1965), while the overall consumption estimated for parts of Nigeria range from 1 to 17 g per person per day (Nicol, 1959).

Riboflavin (vitamin $B_2$) is a water-soluble vitamin derived from plants and many micro-organisms. Because this biosynthetic capability is lacking in higher animals, they must therefore obtain this essential nutrient from their diet. Riboflavin is the precursor of the enzyme cofactors flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), which are vital in many of the body's enzymatic functions for the transfer of electrons in oxidation-reduction reactions (Burgess et al., 2006). Riboflavin deficiency is most commonly seen in developing countries (Blanck et al., 2002), among the elderly (McKinley et al., 2002), and in chronic alcoholics (Langohr et al., 1981). Riboflavin deficiency mainly manifests itself clinically in the mucocutaneous surfaces of the mouth, through the occurrence of cracks at the corners, and inflammation of the lips and tongue (Baker and Dickerson, 1996), but deficiency is also associated with vision deterioration and growth failure. In recent years the vitamin has been found to be effective in the treatment of migraine (Boehnke et al., 2004), malaria (Akompong et al., 2000) and Parkinson's disease (Coimbra and Junqueera, 2003). The recommended daily requirement of riboflavin is set at 1.3 mg (Food and Nutrition Board, 1999). The aim of this research work is to bioenrich iru using microorganisms that have the ability to produce riboflavin naturally and also ferment the locust beans.

## MATERIALS AND METHODS

### Sample collection

African locust bean seeds (*P. biglobosa*) and iru used for this study were purchased from retail markets in Ibadan, Oyo State, South-west Nigeria. They were transported to the Food and Industrial Microbiology Laboratory of the Department of Microbiology, University of Ibadan in clean polythene bags until further use.

### Isolation of microorganisms from locust beans

The isolation of bacteria associated with the fermentation of locust beans to produce iru was done on Plate count agar, Mannitol salt agar, and tryptone soy agar using pour plate method according to Harrigan and McCance, (1966) and the viable populations were determined by plating out 1 ml of the $10^{-3}$, $10^{-5}$, $10^{-6}$ and $10^{-7}$ dilutions. The plates were incubated at 37° for 24 to 48 h. After incubation, pure isolates were obtained, and stored on Nutrient agar slants until further investigations. The cultural characteristics of each isolates were observed. Microscopic examination and several biochemical tests were also carried out for the purpose of identification.

### Screening of isolates for riboflavin production

The standard medium used in screening the isolates for riboflavin production was composed of 25 g/L sucrose, $KH_2PO_4$, $MgSO_4.7H_2O$, $ZnSO_4$ (0.2% solution) and 10% sodium phosphate buffer 0.1 M, pH 7.0, according to the method of Suzuki et al. (2009). A volume of 15 ml of the medium was dispensed into McCartney bottles and autoclaved for 20 min at 121°C and 1 atm. An aliquot of 0.3 ml of each isolates were inoculated into the 15 ml culture medium, respectively. The incubation was carried out for 48 h at 30°C and 100 rpm on a rotary shaker, in aerobic condition. Riboflavin production was assayed in the culture broth after 48 h. The culture broth for each isolates was dispensed into 15 ml centrifuge tubes and was centrifuged at 1°250 × g for 15 min at 5°C. The experiment was carried out in the dark to avoid riboflavin oxidation. The supernatant were separated for riboflavin analysis.

### Riboflavin assay

A 0.8 ml of the supernatant obtained from the culture broth was added to 0.2 ml of 1 M NaOH. A 0.4-ml volume of the resulting solution was neutralized with 1 ml of 0.1 M potassium phosphate buffer (pH 6.0). Using a Spectrum lab 752S UV VIS Spectrophotometer, the absorbance of the mixture for each isolates in the culture broth at 444 nm was measured (Ming et al., 2003). The riboflavin concentration was calculated using the extinction coefficient of $1.04 \times 10^{-2}\ M^{-1}cm^{-1}$ (Sauer et al., 1996).

### Laboratory production of iru and inoculation with screened isolates

#### *Boiling*

Locust beans seeds were boiled in a pressure pot in order to enhance good dehulling characteristics and high quality of final product. After 2 h of boiling, the locust bean seed was allowed to cool for 5 min after boiling before dehulling.

***Dehulling***

This was achieved by rubbing seeds in between palms to remove the seed coats.

***Separation***

The cotyledons were washed thoroughly in clean, potable water and the testae were removed using a sieve. The dehulled seeds were then cooked further for 30 min in the pressure pot to soften the cotyledons after which they are drained and cooled.
For the fermentation, 20 g of the locust bean seeds were placed aseptically into 6 sterile petridishes that were lined with aluminium foil which creates a warm environment for the fermentation. Using plating method, $1.7\times10^{7}$cfu/ml of *Bacillus* species and *Staphylococcus* species that had the highest riboflavin concentrations, obtained from fermented iru were inoculated into the locust bean plates and incubated at 37°C for 4 days.

**Estimation of riboflavin content in the inoculated locust beans**

The riboflavin concentration of the locust bean samples that were inoculated with *Bacillus* spp. and *Staphylococcus* spp. was estimated at different time intervals of 12 to 96 h. The fermenting locust bean samples were washed in sterile distilled water and sodium borate buffer at pH 7.52; this was done to regulate the pH of the samples. The mixture was then filtered using Whatman filter paper no 1. The absorbance of the filtrate was determined and estimated using a JENWAY, 6405 UV/ Vis spectrophotometer at 444 nm. The riboflavin concentration of the fermented locust bean was calculated using the extinction coefficient $1.04\times10^{-2}$ $M^{-1}$ $cm^{-1}$ (Sauer et al., 1996).

**Effects of different concentrations of NaCl on riboflavin concentration of iru**

Twenty grams of the laboratory prepared iru was transferred into five sterile containers that contain different concentration of NaCl ranging from 0 to 10%. A volume of 1 ml from the 18 to 24 h old harvested cells of selected isolates which produced high concentration of riboflavin (inoculum size was determined to be $1.7 \times 10^{7}$ cfu/ml) was inoculated into the iru samples. The inoculated samples were covered properly and kept at room temperature for 4 days. At 24 h interval, 1 g of each sample at different concentration was transferred into 10 ml of already sterilized distilled water to serve as stock, and then it was serially diluted in subsequent 9 ml of sterile distilled water. Appropriate dilution factors were plated on sterile Petri dishes and incubated at 37°C for 18 to 48 h. After 24 to 48 h, the colonies that showed the characteristics of the organisms on the plates were counted and recorded. The riboflavin concentration of the locust bean sample was read by taking the absorbance of the samples using JENWAY, 6405 UV visible spectrophotometer at 444 nm and the estimated concentration was read from the standard curve.

**Effect of different temperatures on the growth of isolates used as starters in enriching iru**

Nutrient broth were inoculated with the appropriate starter and incubated at 30, 35, 40, 45 and 50°C for 24 h. After 24 h, the turbidity of each isolates was read as absorbance at 540 nm using JENWAY, 6405 UV visible spectrophotometer.

**Effect of pH on the growth of isolates used in fermenting iru**

Nutrient broth was dissolved in potassium phosphate buffer at different pH 3, 4, 5, 6, 7 and 8, after which it was dispensed in properly labelled test tubes for each isolate. Each test tube was sterilized at 121°C for 20 min and inoculated with appropriate starter. The turbidity of each isolate was read as absorbance at 540 nm using JENWAY, 6405 UV visible spectrophotometer after 24 h.

**Statistical analysis**

The data obtained in this research work were subjected to analysis of variance (ANOVA) and the Duncan's multiple range tests were used to separate the means while significant difference was obtained for sample treatments ($P\leq0.05$).

## RESULTS

Sixty-three bacteria strains were isolated from the fermenting iru. They were identified as *Staphylococcus aureus* (19), *Micrococcus varians* (9), *Staphylococcus* spp. (5), *Bacillus licheniformis* (4), *Bacillus alvei* (4), *Micrococcus luteus* (3), *Bufo marinus* (3), *Bacillus brevis* (2), *Bacillus megaterium* (2), *Bacillus pumilus* (2), *Bacillus sphaericus* (2), *Bacillus subtilis* (2), *Staphylococcus intermedius* (2), *Bacillus badius* (1), *Bacillus maequariensis* (1), *Bacillus polymxa* (1), and *Staphylococcus horminis* (1).

### Screening of isolates for riboflavin production

The 63 isolates were screened for riboflavin production; it was observed that *Bacillus subtilis IR50* produced the highest riboflavin of 25.8 mg/L, followed by *Staphylococcus* spp. IR26, while *M. varians* IR49 have the least of 6.4 mg/L.

### Riboflavin concentration (mg/L) in locust bean fermented with selected starters that are capable of high riboflavin production

Figure 1 shows the mean riboflavin concentrations (mg/L) under laboratory fermentation of locust bean using *Bacillus* and *Staphylococcus* spp. for 12 to 96 h of fermentation. *B. subtilis* IR50 and *B. licheniformis* IR28 when used together in locust bean fermentation produced the highest riboflavin of 4.5 mg/L, combination of *S. aureus* IR06 and *B. subtilis* IR50 produced 2.3 mg/L riboflavin concentration, while *B. subtilis* IR50 alone produced the least riboflavin concentration of 1.5 mg/L. Figure 2 shows the growth response of selected isolate to temperature at 30, 35, 40, 45 and 50°C, respectively. At 35 and 50°C all the isolates had minimum growth. The optimum temperature for growth of all isolates is at 40°C.

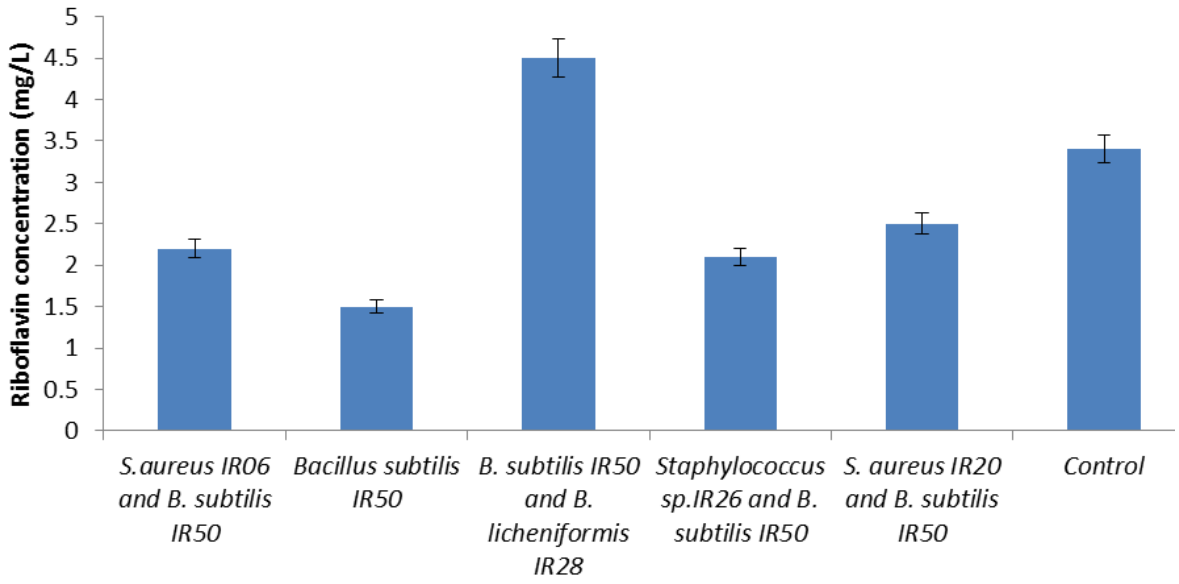


**Figure 1.** Mean riboflavin concentrations (mg/L) in locust bean fermented with *Bacillus* and *Staphylococcus* spp.

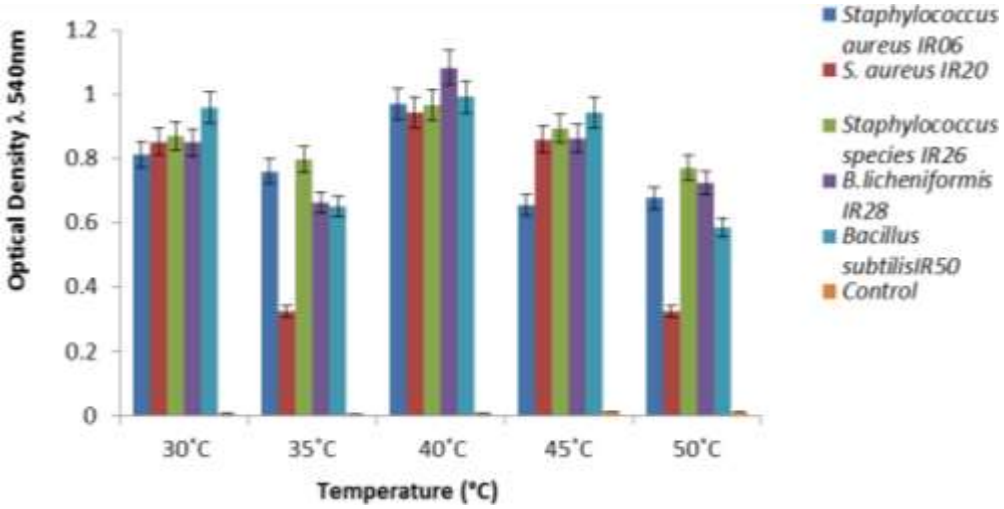


**Figure 2.** Growth response of selected isolates capable of high riboflavin production at different temperatures.

Also, the growth response of selected starters to different pH is shown in Figure 3. *S. aureus* IR06 and IR20 had optimum pH for growth at pH 6 while the optimum pH for *Staphylococcus* spp. IR26, *B. licheniformis* IR28 and *B. subtilis* IR50 is 7 and the least growth is obtained at pH 3 for all isolates. Isolates' growth response to different concentration of NaCl is as shown in Figure 4. All the isolates grew better at 0% NaCl and they had scant growth at 2.5% NaCl. *B. subtilis* IR50 in combination with *Staphylococcus* spp. IR26 had minimal growth at 7.5% NaCl, while *B. licheniformis* IR28 in combination with *B. subtilis* IR50 were able to grow well at the different concentrations of NaCl compared to other isolates but had their least growth at 10% salt concentration. *B. subtilis* IR50 in combination with *S. aureus* IR06 survived at the different concentrations of NaCl but had their best growth at 0% NaCl and least growth at 5%. *B. subtilis* IR50 used singly was able to grow at different concentration of NaCl but had least growth at 7.5% NaCl and thrived best at 0% NaCl.

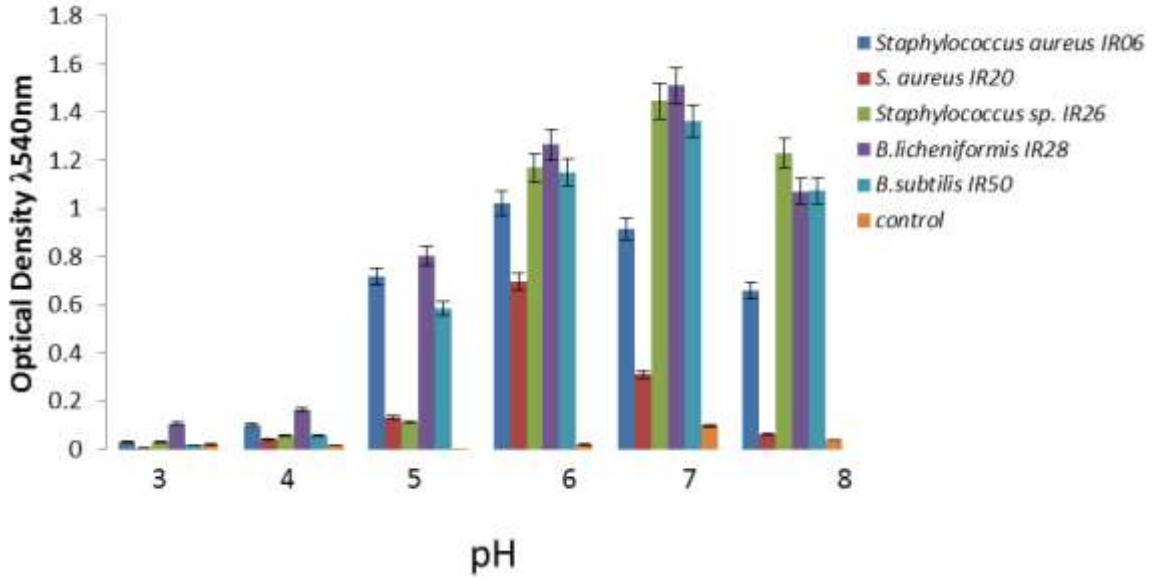


**Figure 3.** Growth response of selected starters capable of high riboflavin production at different pH.

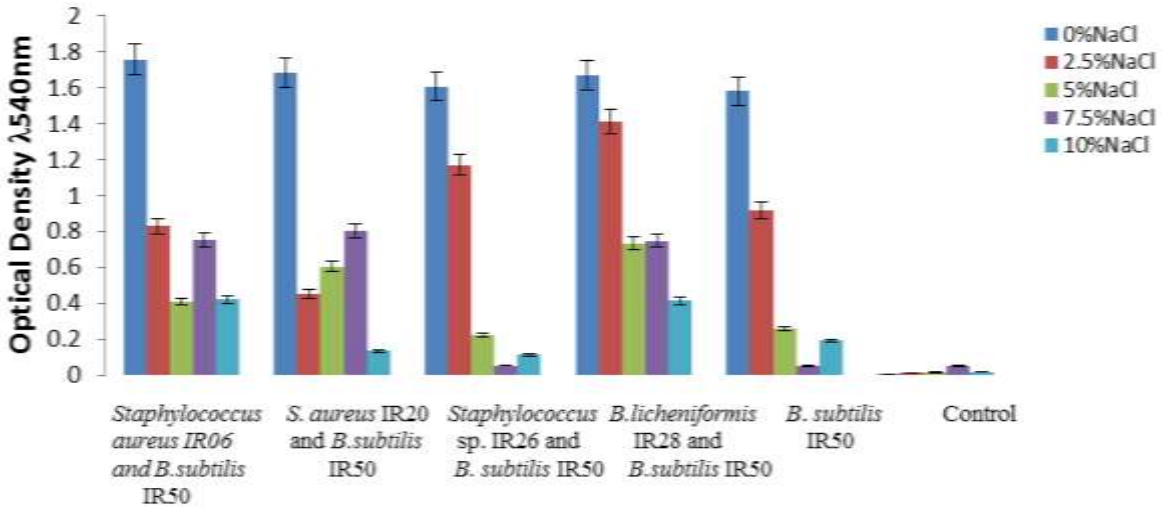


**Figure 4.** Growth response of selected starters capable of high riboflavin production to different concentrations of NaCl.

### Effects of different concentrations of NaCl on riboflavin produced in locust bean fermented with *Bacillus* and *Staphylococcus* spp., respectively

The mean riboflavin concentrations of locust bean fermented with *Bacillus* and *Staphylococcus* spp. at different concentrations of NaCl is shown in Table 1. All the selected starters produced high concentration of riboflavin at 0% NaCl. There was a gradual decrease in riboflavin produced by the selected isolates at 2.5 to 10% NaCl concentrations. *Staphylococcus* spp. IR26 combined with *B. subtilis* IR50 for the fermentation of locust beans at 0% NaCl produced highest riboflavin of 11.430 mg/L, while *B. subtilis* IR50 alone gave 3.890 mg/L .

## DISCUSSION

The dynamics of fermentation in food or condiment such

**Table 1.** Mean riboflavin concentration of iru fermented with *Bacillus* and *Staphylococcus* species at different concentrations of NaCl.

| Isolates code | Isolate | 0% NaCl | 2.5% NaCl | 5% NaCl | 7.5% NaCl | 10% NaCl |
|---|---|---|---|---|---|---|
| IR06+IR50 | *S. aureus* and *B. subtilis* | 8.850 | 4.560 | 5.220 | 3.320 | 3.560 |
| IR20+IR50 | *S. aureus* and *B. subtilis* | 8.580 | 8.800 | 4.820 | 4.280 | 3.520 |
| IR26+IR50 | *S. species* and *B. subtilis* | 11.430 | 6.140 | 3.760 | 4.480 | 5.980 |
| IR28+IR50 | *B. licheniformis* and *B. subtilis* | 10.310 | 5.070 | 4.720 | 4.550 | 4.40 |
| IR50 | *B. subtilis* | 3.890 | 4.70 | 4.480 | 4.50 | 3.040 |
| No isolate | Control | 3.090 | 3.320 | 3.670 | 3.870 | 4.050 |

as iru is a complex microbiological process involving interactions among different microorganisms (Omafuvbe et al., 2003). The contribution of the accompanying flora of fermenting substrate is determined by the substrate composition and hygiene during production. The microorganisms isolated from iru samples at species level include different species of *Staphylococcus*, *Micrococcu*s, and *Bacillus*. *Bacillus* is the most predominant because of its proteolytic ability and also their ability to break down oils. *B. subtilis* has been associated with iru fermentation (Enujiugha, 2009). *Staphylococcus* and *Micrococcus* were present in low numbers compared to *Bacillus*. These organisms do not appear to be important in the fermentation process. *Bacillus* spp. have been implicated in the fermentation of most vegetable oil protein seeds (Odunfa, 1981; Ogueke and Aririatu, 2004). *Staphylococcus* spp. and *Micrococcus* spp. have also been isolated from fermenting African oil bean seeds but from the health point of view, the presence and isolation of *S. aureus* indicated poor hygienic practices during production (Ibeabuchi et al., 2014).

*B. subtilis* was screened to produce the highest concentration of riboflavin among other isolates. Several studies have been established on the ability of this organism to produce riboflavin for feed and food fortification purposes (Stahmann et al., 2000; Burgess et al., 2006). Species of *B. subtilis* group have been reported to be generally regarded as safe (GRAS) by the U. S. Food and Drug Administration, and their role in the fermentation of locust bean to produce different condiments has been thoroughly investigated (Beaumont, 2002).

Riboflavin has been traditionally synthesised for food and feed fortification by chemical means but recently biotechnological processes which involve the use of various bacteria, yeast and fungi have been put in place (Stahmann et al., 2000). One of these biotechnological processes employs the use of *B. subtilis* and much work has been carried out in characterising the vitamins biosynthetic pathway (Perkins and Pero, 2002). Bacillus subtilis IR50 isolated in this study was screened to produce highest concentration of riboflavin.

Food fermentation processes mostly depend on co-culturing of microbes which act in conjunction to produce desired product characteristics. This study highlights the microbial diversity of riboflavin producing strains isolated from iru. Mixed culture of *B. licheniformis* IR28 and *B. subtilis* IR50 produced highest concentration of riboflavin; there could be a form of protocooperation interaction between the organisms which may means that both interacting species gain fitness with each other thereby leading to an increase in riboflavin production (Sieuwerts et al., 2008).

The use of high riboflavin producer as starters in locust beans fermentation contributes significantly to the functional value of iru produced and also will bring about enrichment of poor riboflavin diets; this will eventually lead to a decrease in riboflavin deficiencies among the populace of rural dwellers. Iru fermentation is solid substrate fermentation with an exothermic process, where the temperature of the fermenting seed increases gradually from ambient temperature to about 30˚C to 45˚C (Odunfa and Oyewole, 1986). The bacteria isolated from iru that are, *Staphylococcus* spp. and *Bacillus* spp. grew best at 35 to 40°C so this is evidence to their ability to ferment iru substrate.

One of the most common local/natural food preservatives is salt, which tends to improve the shelf life of processed African locust bean seeds by reducing the number of microbial load on the samples, which could have been agent(s) of deterioration or spoilage to the sample and reduce the shelf life (Ademola et al., 2013). There have been reports on methods of improving the shelf life of fermented locust beans (Omabuvfe, 2007; Kolapo et al., 2007), but little is known about its effect on the riboflavin content of the fermented product. In this study 0 to 10% concentration of NaCl was used. It has been reported in the findings of Odunfa et al., (1981) that a gradual decrease in growth rate of *Staphylococcus* spp. was observed with increasing concentration of sodium chloride. Ikenebomeh (1989) also states that variations in salt content influence the microbial development leading to lower microbial counts at salt addition above 3%. In this study, all the isolates used experienced a significant decrease in their microbial growth after exposure to 0%

NaCl. Furthermore, addition of NaCl to iru brought about a decrease in the riboflavin production due to inhibitory effect of NaCl on the selected isolates. This is in agreement with Ratnakar and Rai (2013) who observed reduction in riboflavin content of leaves of *Amaranthus polygamous* with increase in concentration of NaCl. This signifies that using salt as a means of preserving iru which is a common practice among the rural dwellers have a significant impact on the riboflavin content of the condiment and the practice should be discouraged.

## Conclusion

*B. subtilis* IR50 and *B. licheniformis* IR28 isolated from fermented locust beans were able to increase the riboflavin content of iru *in vivo* significantly. However, the use of salt as a means of preservative has effect on both the growth of the isolates and the stability of the riboflavin content of the produced condiment (iru).

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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