Vol. 15(4), pp. 191-202, April, 2021
DOI: 10.5897/AJMR2021.9484
Article Number: CD2944466708
ISSN: 1996-0808

http://www.academicjournals.org/AJMR

ACADEMIC JOURNALS
expand your knowledge

African Journal of Microbiology Research

Full Length Research Paper

Virulence and multi-resistance of gram-negative bacilli strains isolated from some artisanal fermented dairy products sold in secondary schools in Benin

Majoie Géroxie TOHOYESSOU[1], Wassiyath MOUSSE[1], Chimène NANOUKON[2], Haziz SINA[1], Akim SOCOHOU[1], Edwige AKA[3], Rodrigue TOLLO[1], Farid BABA-MOUSSA[4], Adolphe ADJANOHOUN[5] and Lamine BABA-MOUSSA[1]*

[1]Laboratory of Biology and Molecular Typing in Microbiology, Department of Biochemistry and Cell Biology, University of Abomey-Calavi, Cotonou, Benin.
[2]Laboratory of Molecular Biology and Bioinformatics Applied to Genomics, National High School of Biosciences and Applied Biotechnologies of Dassa, National University of Sciences, Technologies, Engineering and Mathematics of Abomey, Dassa-Zoumè, Benin.
[3]Microbiological Resources Management Unit (UGMI), Pastor Institute of Ivory Coast, 01 BP 490 Abidjan 01, Ivory Coast.
[4]Laboratory of Microbiology and Food Technology, Department of Plant Biology, Cotonou Benin, University of Abomey-Calavi, Benin.
[5]Benin National Institute of Agricultural Research, Cotonou, Benin.

Received 11 January, 2021; Accepted 19 April, 2021

This work aims at assessing toxin production capability and antibiotic resistance profiles of thermo-tolerant Gram-negative bacilli strains isolated from three types of fermented dairy products (yoghurt, dèguè millet and dèguè couscous). Samples collected in Abomey-Calavi and Cotonou were analyzed for microbial, biochemical and molecular parameters. Samples were contaminated with thermo-tolerant Gram-negative bacilli strains at 13.88%. The high contamination rate was recorded with the samples of dèguè couscous and the lowest contaminated samples were dèguè millet. Morning samples were more contaminated. *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Escherichia coli* and *Enterobacter cloacae* were the most identified bacteria. The most isolated species in the dry season was *E. coli.* In the rainy season, *K. pneumoniae* and *E. cloacae* were the most isolated species. *K. pneumoniae* was the most formative of biofilm (38.9%). About 12% of the isolated strains were extended-spectrum beta-lactamases (ESBL) producers. The higher resistance rate was observed with amoxicillin and doxycylin (92.3%). Imipenem was the most efficient molecule on the isolated strains with 00% of resistance. The blaTEM gene was detected in 51.43% of the isolated strains followed by blaSHV (37.14%), blaCTX-M (8.57%) and blaOXA-1 (2.86%). It is necessary to train sales people on good hygiene practices for food during their production and their distribution.

Key words: Fermented milk products, thermo-tolerant gram-negative bacilli, toxins, antibiotics resistance, Benin.

INTRODUCTION

Milk is a complex ecosystem for various microorganisms including bacteria. The widely consumed milk products are cheese, yoghourt and curdle milk (Pal and Awel, 2014). Dairy products undergo lactic fermentation to

ensure food safety through acidification and production of bacteriocins that antagonize the growth of pathogenic bacteria, and improve the final quality of dairy products by producing aromatic compounds. There is an increase demand of natural food without any artificial additive and pathogenic microorganisms. However, several germs such as yeasts and bacteria are responsible for altering the marketable and hygienic quality of these dairy products (Abdel-Aziz et al., 2016).

Food borne pathogens cause outbreak of food borne diseases through pathogenic microorganisms or their toxins (Smith and Fratamico, 2018). Thus, in developing countries, foodborne infections remain a major public health problem since they can affect a large part of population (Djogbe et al., 2019). Milk and their derived products are reported to be important sources of food borne pathogen such as bacteria (Oliver et al., 2005; Zagare et al., 2012). In Benin, traditional techniques for improving preservation and production of yoghurt and dèguè are expensive and less accessible to processors (Sessou et al., 2013; Tohoyessou et al., 2020).

Milk and its derivate products contamination by pathogenic bacteria is largely due to processing, handling, and unhygienic conditions. Garas et al. (2017) describe the presence of microorganisms that can cause food poisoning are viruses and bacteria. These include thermo-tolerant Gram-negative bacilli such as *Clostridium botulinum*, *Clostridium perfringens*, *Campylobacter*, *Salmonella* and *Escherichia coli* (Mon et al., 2015). Nowadays, food poisoning and safety is very important subject all over the world.

The Gram-negative bacilli in particular *Enterobacteriaceae* are becoming increasingly resistant to antibiotics particularly to carbapenems (Pfeifer et al., 2010; Nordmann et al., 2011). Carbapenems remain the beta-lactam with the broadest spectrum of activity. Their excellent antibacterial activity is linked to their rapid transmembrane penetration through the external wall of Gram-negative bacilli. In addition, the have stability against most natural or acquired beta-lactamases, including chromosomal and plasmid cephalosporinases and extended spectrum beta-lactamases (Nordmann and Carrer, 2010).

Considering the numerous cases of food poisoning induced by the multi-resistant bacteria, it is necessary to investigate the resistance ability of potentially pathogenic microorganism founded in foods such as fermented milk products.

Thus, the aim of this work was to identify and characterize thermo-tolerant Gram-negative bacilli isolated from three artisanal fermented dairy products (yoghurt, dèguè millet and dèguè couscous) collected from the schools of Abomey-Calavi and Cotonou (Benin).

MATERIALS AND METHODS

Sampling and samples collection

The three dairy products (yoghurt, dèguè millet and dèguè couscous) samples were randomly collected from 15 schools of Abomey-Calavi and Cotonou (Figure 1). Those samples were preferentially taken from inside schools' vendors. For each products, two samples were taken two time daily (morning and evening) and repeated twice in a week with. Thus, 180 samples (60 yoghurt, 60-dèguè couscous and 60-dèguè millet) were collected during this study. Collected samples were carried, in ice (about 4°C), to the laboratory for further analysis.

Microbiological analyses

Each collected sample (10 g) was aseptically homogenized into sterile tryptone salt water (90 ml). From this solution, a serial decimal dilution was made to obtain dilution 10^{-1}, 10^{-2}, 10^{-3}, up to 10^{-7}. For the detection and enumeration of total and fecal coliforms, dilutions 10^{-1} to 10^{-3} were used.

Total and fecal coliforms

Total and fecal coliforms were enumerated on Violet Red Bile Glucose Agar (VRBA, OXOID CM0485) medium following a previously describe method (Milani et al., 2011). Dilutions from 10^{-1} to 10^{-3} were homogenized with 15 ml of VRBA medium and after solidify, a second stratum of about 5 ml was poured. The incubation was performed at 30°C for 24 h (total coliforms) and at 44°C for 24 h (fecal coliforms).

Thermo-tolerant Gram-negative bacilli

Tryptone Bile X Glucuronide agar and Eosin Methyl Blue Agar (OXOID, CM0069) were used for the isolation of thermo-tolerant Gram-negative bacilli. The identification of such bacilli was completed with indole test and the Api20E gallery (Riegel et al., 2006).

MALDI-TOF mass spectrometry

After isolation, the MALDI-TOF mass spectrometry was used to confirm the microbial identification. Thus, the MALDI-TOF target plate (Bruker Daltonics ™) was used to receive bacterial samples. The deposited samples was covered with 1.5 µl of the matrix solution (Sigma, Lyon, France) completed with acetonitrile 50% (500 µl), trifluoroacetic acid 10% (250 µl) and water (250 µl). The mixture was then sonicated for 10 min, centrifuged (13000 g, 5 min) and transferred to a clean polypropylene tube. The target plate and matrix were then dried at room temperature before MALDI-TOF/MS (BrukerDaltonics, Germany) identification (Pfleiderer et al., 2013). A positive control was used (*E. coli* ATCC 8739).

Biofilm formation test

The thermo-tolerant Gram-negative bacilli capacity to produce

*Corresponding author. E-mail: laminesaid@yahoo.fr.

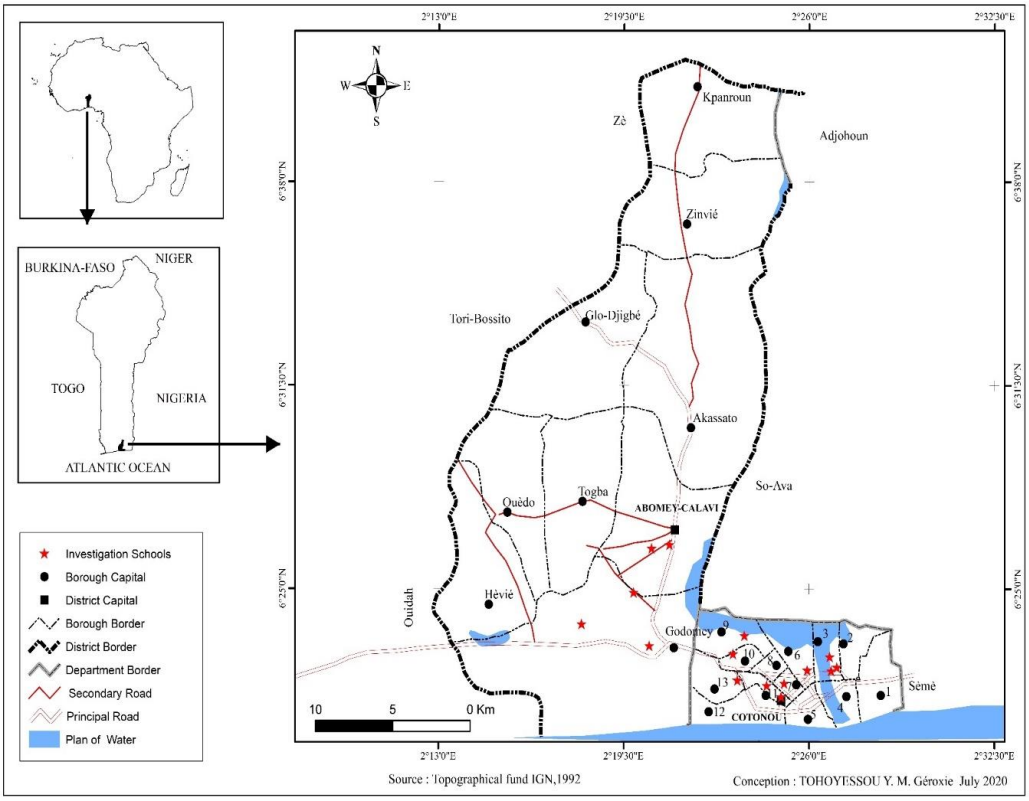

Figure 1. Localization of the investigated schools for the samples collection.

biofilm was determined according the method described by Christensen et al. (1985). Briefly, the 48 well microplate was used to assess, *in vitro*, qualitative biofilm production. Thus, on the microplates, 10 µl of 18 h old bacteria suspension was diluted with 150 µl of Brain Heath Infusion (BHI) and incubated for 24 h at 37°C. After incubation, wells were washed three times with sterile physiological water (0.2 ml) in order to eliminate the free bacteria. Biofilms formed by adhesion of sessile organisms to the microplate in each well are stained with crystal violet (0.1%) for 10 min. The excess dye was removed after thorough washing and the plates were left at room temperature for drying (Stepanović et al., 2000).

Antibiotic susceptibility of strains of thermo-tolerant Gram-negative bacilli

The susceptibility of the identified thermo-tolerant Gram-negative bacilli to 16 conventional antibiotic molecules was performed using the diffusion method (CASFM/EUCAST, 2019). The 16 antimicrobial agents (BioRad®) tested were: cefoxitin (FOX 30 µg), norfloxacin (NOR 5 µg), doxycyline (DOX 30 µg), trimethoprim sulfamethoxazole (Sxt 23.75 µg), amoxicillin-clavulamic acid (AMC 20/10 µg), amoxicillin (AMX 25 µg), ceftriaxone (CRO 30 µg), azetronam (ATM 30 µg), imipenem (IPM 10 µg), piperacilin tazoubatam (PTZ 36 µg), ticarcillin (TIC 75 µg), cefepim (FEP 30 µg), ceftazidime (CZD 10 µg), ciprofloxacin (CIP 5 µg), cefotaxime (COX 5 µg) and gentamycin (GMN 10 µg).

Penicillinase test

The penicillinase production was detected using a tube acidimetric method test on all the isolated strains as previously described (Koneman et al., 2006). Briefly, to obtain a purplish red color, 300 µl of 1% phenol red was added to the mixture. The final reaction volume is 1 ml. Two young colonies were suspended in 500 µl of physiological water (9%NaCl). The emulsion was supplemented with 150 µl of pH 8 benzyl-penicillin (600 mg of benzyl-penicillin + 400 µl of sterile distilled water). *E. coli* ATCC 25922 was used as a control. After incubation (37°C, 1 h) orange or yellow color in the tubes indicates penicillinase production.

Phenotypic detection of extended spectrum beta-lactamase

To carry out this test, a bacterial suspension (10^6 CFU/ml) was used (CASFM/EUCAST, 2019). Cefotaxim (COX 3 µg) and cefipim (FEP 30 µg) in the presence of amoxicillin + clavulanic acid (AMC) was used. AMC disc was placed in the center of Muller-Hinton agar between the two-cephalosporin discs at a distance of about 30 mm. After incubation (37°C for 18 h), the positive result was a potentiation of the corkscrew-shaped inhibition zone between the COX and AMC discs and then between the AMC and FEP discs (CASFM/EUCAST, 2019).

Hodge test

To perform this test, with *E. coli* ATCC 25922 suspension (1/10 dilution of a 0.5 McFarland suspension) and ertapenem disc (10 µg). Bacteria to be tested were seeded in line at about 20 to 25 mm of distance, from the ertapenem disc to the tip of the agar. After incubation (37°C, 24 h), the shoot of *E. coli* ATCC 25922 near the ertapenem disc around the streak of the tested strain produced a depression in the agar indicating carbapenemase activity. If the growth of *E. coli* ATCC 25922 continues to be inhibited even in the

Table 1. The PCR programs used for the amplifications.

Genes	Initial denaturation	Denaturation	Hybridization	Extension	Final extension	Number of cycles
BlaTEM	94°C for 5 min	94°C for 30 s	52°C for 30 s	72°C for 1 min	72°C for 10 min	30
BlaSHV	94°C for 10 min	94°C for 40 s	60°C for 40 s	72°C for 1 min	72°C for 7 min	30
BlaCTX-M	94°C for 10 min	94°C for 40 s	60°C for 40 s	72°C for 1 min	72°C for 7 min	35
BlaOXA-1	94°C for 10 min	94°C for 40 s	60°C for 40 s	72°C for 1 min	72°C for 7 min	30
STX-1	94°C for 2 min	94°C for 1 min	62°C for 90 s	72°C for 1 min	72°C for 5 min	30
STX-2	94°C for 2 min	94°C for 1 min	62°C for 90 s	72°C for 1 min	72°C for 5 min	30

Table 2. The sequences of primers used.

Gene	Sequences	Weight (bp)	Reference
BlaTEM	5′-TTGGGTGCACGAGTGGG TTA-3′ 5′-TAATTGTTGCCGGGAAGCTA-3′	467	Anago et al. (2015)
BlaSHV	5’ATT TGT CGC TTCTTT ACT CGC-3’ 5’ TTT ATG GCG TTACCT TTG ACC-3’	713	Dallenne et al. (2010)
BlaCTX-M	5’-ATG TGC AGYACC AGT AAR GT 3’ 5’-TGG GTRAAR TAR GTS ACC AGA 3’	688	Dallenne et al. (2010)
BlaOXA-1	5′-ATATCTCTACTGTTGCATCTCC-3′ 5′-AAACCCTTCAAACCATCC-3′	564	Dallenne et al. (2010)
Stx1	5’-TGTAACTGGAAAGGTGGAGTATACA-3’ 5’-GCTATTCTGAGTCAACGAAAAATAAC-3’	210	Meng et al. (1997)
Stx-2	5’-GTTTTTCTTCGGTATCCTATTCC-3’ 5’-GATGCATCTCTGGTCATTGTATTAC-3’	484	Meng et al. (1997)

presence of the strain tested the test result is negative (Girlich et al., 2012).

Detection of genes encoding the production of β-lactamases and of toxins for *E. coli*

All the ESBL-producer Gram-negative bacilli were used to detect genes encoding multi-resistance (TEM, CTX-M, SHV and OXA-1). In addition, the Shiga toxins genes (Stx-1 and Stx-2) were researched for E. coli isolates. The DNA template was extracted using a boiling method. Briefly, in 500 µl of sterile water a bacteria colony was incubated at 95°C for 10 min. after, the suspension was centrifuged (12000 rpm for 5 min), and 10 µl of the supernatant was used as target DNA (Rasmussen and Morrissey, 2008). DNA extracts were used for the polymerase chain reaction (PCR) reaction. The 25 µl PCR mixture containing 12.5 µl of 2xMaster Mix (BioLabs), 1.5 µl of forward primer and 1.5 µl of reverse primer, and 4 µl of DNA was used. The PCR program used for the amplifications are in the Table 1 and the primers used are in the Table 2. After 30 min electrophoresis (150 V) on 1.5% agarose gel containing ethidium bromide, 10 µl of the PCR products were visualized with a 100 bp molecular weight ladder.

Data analysis

The MS Office Excel 2010 spreadsheet was used for data

Table 3. Bacterial load of fecal coliforms and thermo-tolerant Gram-negative bacilli (TTGNB) in samples collected in Cotonou and Abomey-Calavi.

Sample	fecal coliforms (UFC/ml)	TTGNB (UFC/ml)
Yoghurt	$0.39*10^5 \pm 0.05*10^5$	$7.21*10^5 \pm 0.52*10^5$
Dèguè Millet	$11.12*10^5 \pm 0.96*10^5$	$3.09*10^5 \pm 0.17*10^5$
Dèguè Couscous	$7.75*10^5 \pm 0.16*10^5$	$10.81*10^5 \pm 0.61*10^5$

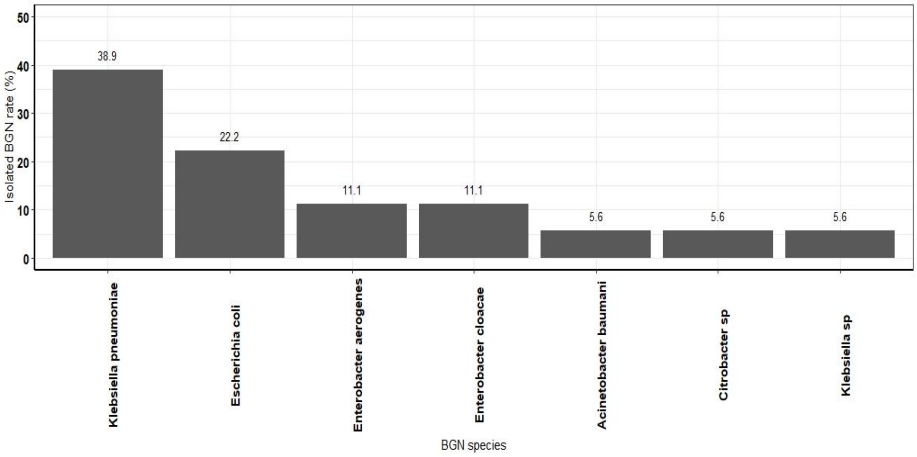

Figure 2. Rates of the different species of thermo-tolerant Gram-negative Bacilli identified.

processing. R 3.6.1 software was used for p-value test and for graphing. The test is considered statistically significant if $p < 0.05$.

RESULTS

Environment of the fermented milk products sale points

Some sales sites are unhealthy with the observation of rubbish and open gutters wastewater. Some vendors do not have adequate garbage bins for garbage collection. Dairy products are prepared at home and the rest of the activities carried out at the sale sites are done on the sale sites, using the water available at the site. Most vendors rarely change utensils rinse water. No vendors wear masks and gloves, so the hands of some vendors are in direct contact with money and the millet and couscous.

Microbiological quality of fermented dairy products

Enumeration of fecal coliforms and thermo-tolerant Gram-negative bacilli in sampled fermented milk products

The germ count results in the fermented milk products analyzed, in CFU/ml, were compiled in Table 3. Table 3 represent the microbial load of yoghurt, dèguè millet and dèguè couscous. Results indicated that the microbial loads vary according to the type of samples. For fecal coliforms, the most contaminated samples were dèguè millet samples ($11.12*10^5$ UFC/ml) and the least contaminated were yoghurt samples. For Gram-negative bacilli, couscous samples were the most contaminated and the least contaminated samples were those of dèguè millet ($3.09*10^5$ UFC/ml).

Different species of thermo-tolerant Gram-negative bacilli found in the fermented milk products

Twenty-five Gram-negative bacilli strains were isolated from the 180 samples tested. The four most represented species were strains of *Klebsiella pneumoniae* (38.9%), *E. coli* (22.2%), *Enterobacter aerogenes* (11.1%) and *E. cloacae* (11.1%). While *Acinetobacter baunami*, *Citrobacter* sp and *Klebsiella ornithinolytica* strains were the least isolated (5.6%) (Figure 2).

Distribution of thermo-tolerant Gram-negative bacilli isolates according to the type of fermented milk products

Figure 3 presents the different thermo-tolerant Gram-negative bacilli of strains of obtained as a function of the

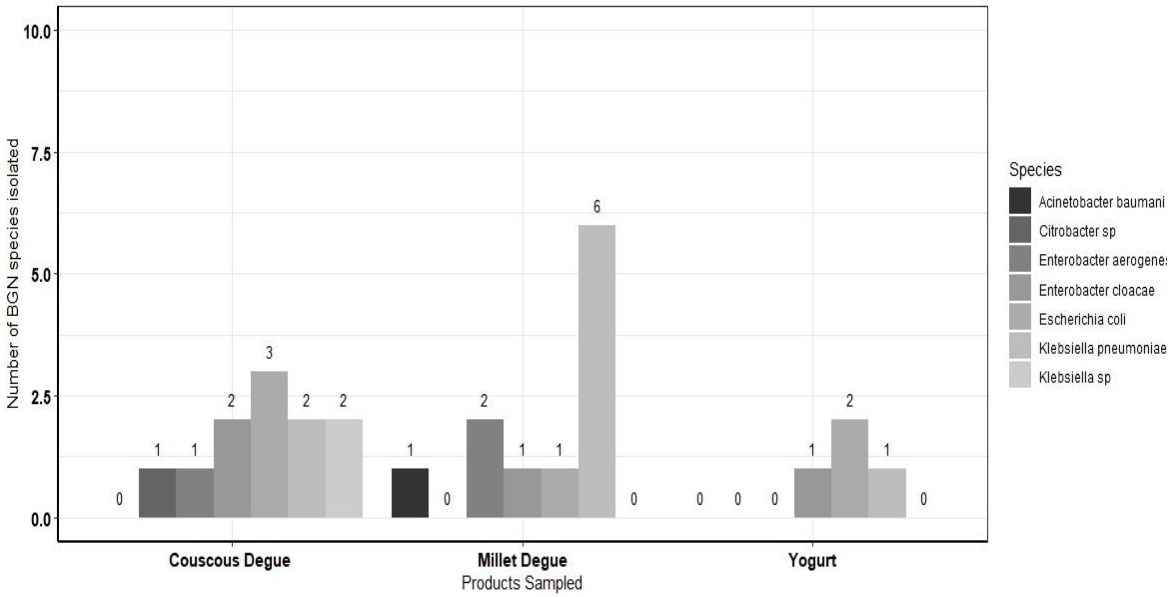

Figure 3. Distribution of thermo-tolerant Gram-negative Bacilli strains according to fermented milk products.

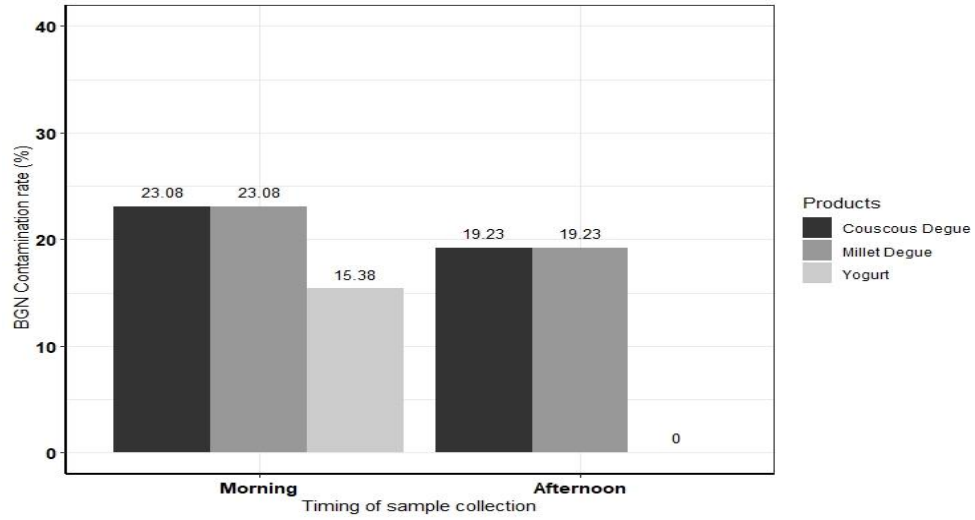

Figure 4. Distribution of thermo-tolerant Gram-negative bacilli strains according to the time of collection.

fermented milk products analyzed. A total, 7 species of thermo-tolerant Gram-negative bacilli were identified. It appears that *E. coli* strains were the most dominant in yoghourt and dèguè couscous. In the samples of dèguè millet *K. pneumoniae* was the most present. It is noted a presence of six different species in dèguè couscous, five species in dèguè millet and three species in yoghourt. *Enterobacter cloacae*, *E. coli*, and *K. pneumoniae* were present in dèguè couscous, dèguè millet and yoghourt. The distribution of species according is not significantly different (p> 0.05).

Distribution of thermo-tolerant Gram-negative bacilli according to the collection period

Samples of dèguè couscous and dèguè millet were more contaminated in the morning than in the afternoon with respective proportions of 23.08 and 19.23% (Figure 4). The yoghourt was contaminated in the morning with a proportion of 15.38% and not in the afternoon. In general, high contamination was recorded in dry season (Figure 5). The most commonly species found in the dry season was *K. pneumoniae* followed by *E. coli* and in the rainy

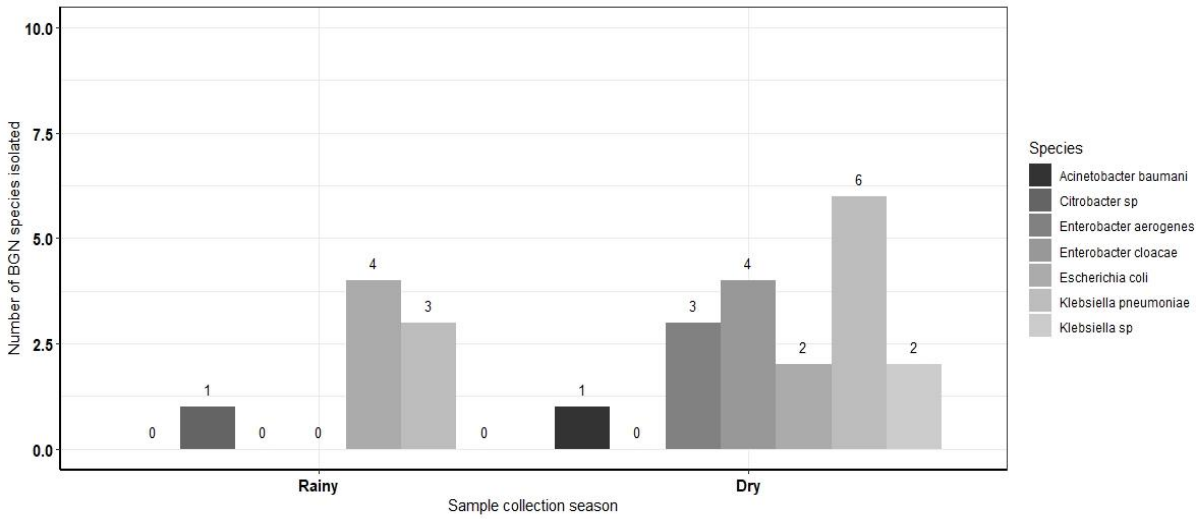

Figure 5. Distribution of thermo-tolerant Gram-negative bacilli strains according to the season of collection

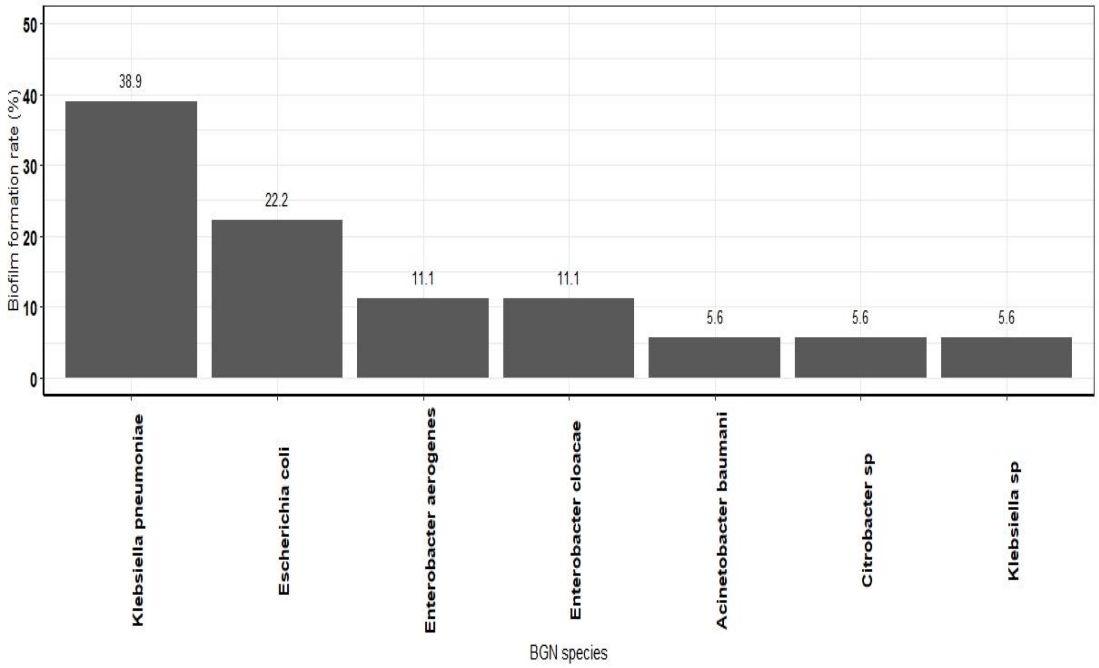

Figure 6. Biofilm production rate according to the different species of thermo-tolerant Gram-negative bacilli.

season, the species most commonly found were *E. coli* and *K. pneumoniae*.

Biofilm formation according to the isolated species of thermo-tolerant Gram-negative bacilli

The biofilm production capacity by the thermo-tolerant Gram-negative bacilli strains shows that *K. pneumoniae* was the most biofilm formative (38.9%) followed by *E. coli* (22.2%) and *Enterobacter aerogenes*, *E. cloacae* (11.1%) (Figure 6). *Acinetobacter baumani*, *Citrobacter* sp. and *Klebsiella ornithinolytica* were the lowest biofilm producers (5.6%). Meanwhile, the production of biofilm is not statistically significant different among the isolated species ($p > 0.05$). Gram-negative bacilli strains isolated from dèguè millet (34.62%) were the highest biofilm producer while those isolated from yoghourt were the

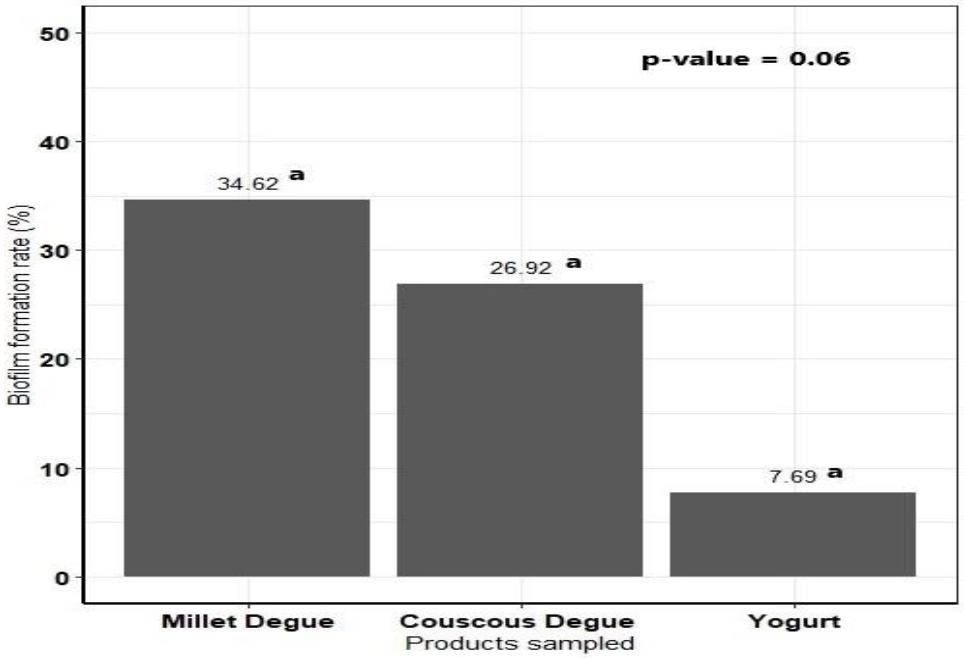

Figure 7. Biofilm production rate according to thermo-tolerant Gram-negative bacilli strains in the different samples.

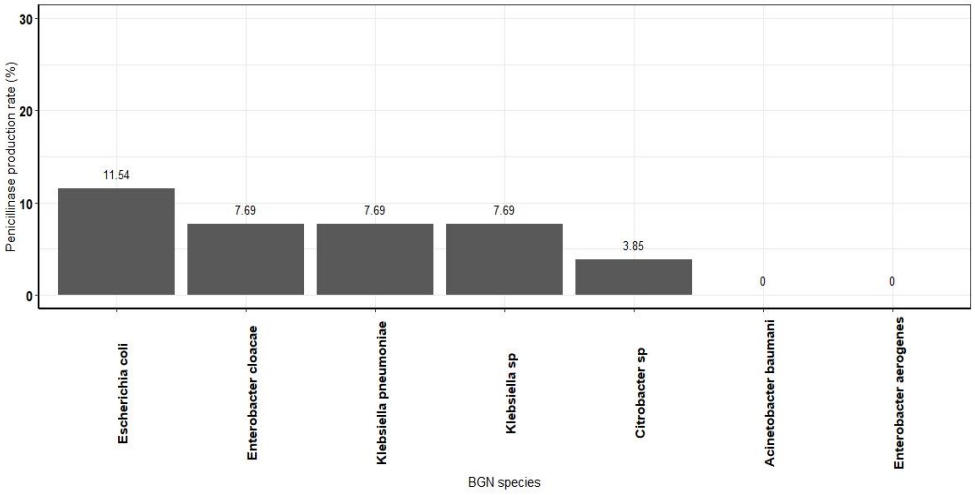

Figure 8. Penicillinase production rate according to thermo-tolerant Gram-negative bacilli strains in the different samples.

lowest (7.69%) (Figure 7). Biofilm production is statistically different from isolated species in function of samples types (p=0.06).

Penicillinase, ESBL and carbapenemase production by the thermo-tolerant Gram-negative bacilli species

The production of penicillinase by isolated thermo-tolerant Gram-negative bacilli strains was highly observed with *E. coli* (11.54%), followed by *Enterobacter cloacae, K. pneumoniae* and *Klebsiella* sp. (7.69%). *Acinetobacter baumani* and *Enterobacter aerogenes species* were not penicillinase-producers (Figure 8). Only three strains were of ESBL producers. Of the seven species isolated, only *E. coli* (7.69%) and *E. aerogenes* (3.85%) produced ESBL (Figure 9). None of the Gram-negative bacilli strains were carbapenemase-producers.

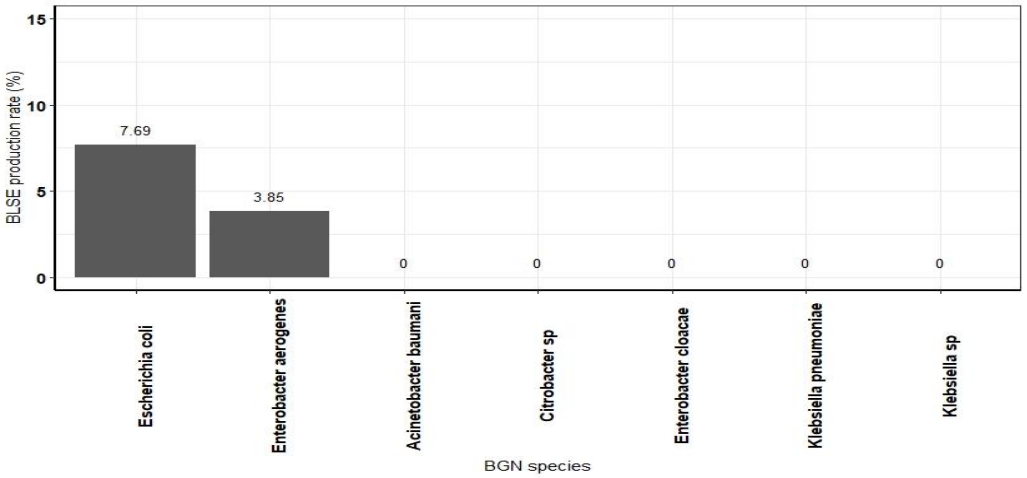

Figure 9. BLSE production by the rate according to thermo-tolerant Gram-negative bacilli strains in the different samples.

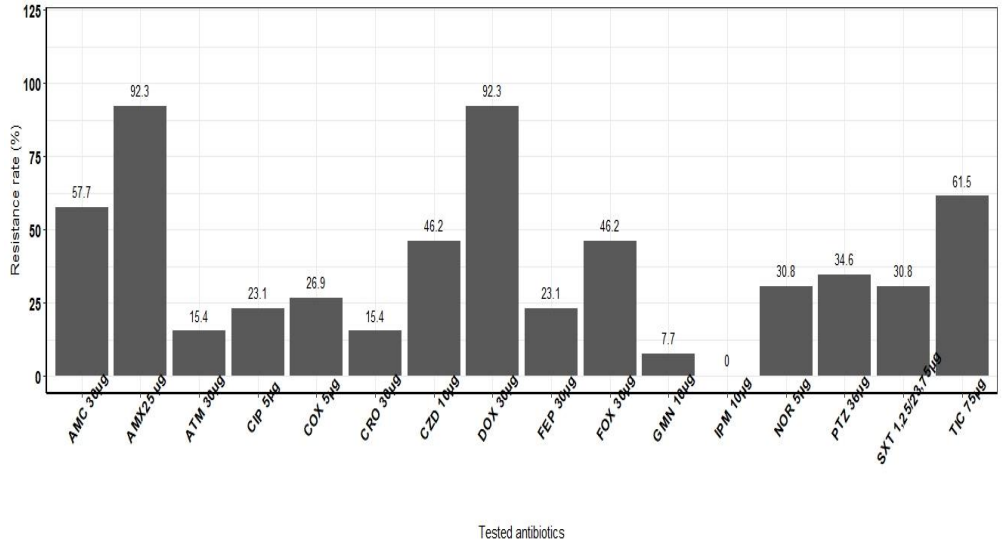

Figure 10. Resistance rate of isolated thermo-tolerant Gram-negative bacilli strains to antibiotics. Legend: Amoxicillin-clavulamic acid (AMC 20/10 µg), Amoxicillin (AMX 25 µg), Azetronam (ATM 30 µg), Ciprofloxacin (CIP 5 µg), Cefotaxim (COX 5 µg), Ceftriaxone (CRO 30 µg), Ceftazidim (CZD 10 µg), Doxycylin (DOX 30 µg), Cefepim (FEP 30 µg), Cefoxitin (FOX 30 µg), Gentamycin (GMN 10 µg), Imipénèm (IPM 10µg), Norfloxacin (NOR 5 µg), Piperacilin tazoubatam (PTZ 36 µg), trimethoprime sulfamethoxazole (Sxt 23.75 µg), Ticarcillin (TIC 75 µg).

Susceptibility to antibiotics of thermo-tolerant Gram-negative bacilli strains

Most of thermo-tolerant Gram-negative bacilli were resistant to β-lactam antibiotics. The highest resistance rate (92.3%) was recorded with amoxicillin and doxycylin followed by ticarcillin (61.5%) and amoxicillin+ clavulanic Acid (57.7%). The only sensitivity was observed with imipenem which showed no resistance (Figure 10).

Presence of genes encoding the production of β-lactamases and toxins genes among identified E. coli

The blaTEM gene was the most observed (51.43%) followed by blaSHV gene (37.14%). Genes encoding blaTEM, blaSHV and blaCTX-M were observed in the *E. coli* isolates at respective proportions of 17.14, 8.57 and 8.57%. Only one strain of *K. pneumoniae* carried the blaOXA-1 gene. blaCTX-M was only present in *E. coli*

Table 4. proportions of presence of genes coding for the production of β-lactamases.

	BlaTEM	BlaSHV	BlaCTX-M	BlaOXA-1
Enterobacter cloacae	3 (8.58%)	2 (5.71%)	0 (0%)	0 (0%)
Enterobacter aerogenes	2 (5.71%)	1 (2.86%)	0 (0%)	0 (0%)
Acinetobacter baumani	0 (0%)	1 (2.86%)	0 (0%)	0 (0%)
Citrobacter sp	1 (2.86%)	0 (0%)	0 (0%)	0 (0%)
K. ornithinolytica	1 (2.86%)	1 (2.86%)	0 (0%)	0 (0%)
K. pneumoniae	5 (14.28%)	5 (14.28%)	0 (0%)	1 (2.86%)
Escherichia coli	6 (17.14%)	3(8.57%)	3 (8.57%)	0 (0%)
Total	**18 (51.43%)**	**13 (37.14%)**	**3 (8.57%)**	**1 (2.86%)**

strains. The only isolated *Acinetobacter baumani* carried only blaSHV gene (Table 4). No strain of *E. coli* isolated carried genes coding for the production of shiga-toxins.

DISCUSSION

The observation of the sites investigated during our study, indicate insalubrity of certain sites with garbage and open gutters wastewater. Some sellers do not have adequate garbage collection bins. Fermented dairy products were displayed on makeshift tables. Inadequate garbage bins were observed with sellers for garbage collection. This increase the attraction of flies, which are not only indicators of poor hygiene, but also vectors of fecal contamination germs (Samapundo et al., 2016). Dairy products are prepared at home and the rest of the activities carried out in the context of the sale is done at the point of sale, using the water available at that site. Most female vendors rarely change rinsing utensils water. No sellers wear masks and gloves, so the hands of some vendors are in direct contact with the money and sold food. These practices increase the possibility of cross-contamination among the vended products. All this is detrimental to good practices in the preparation and sale of these desserts. Previous studies on street foods in Benin made similar observations (Moussé et al., 2016). The microbiological quality of fermented dairy products reveals the presence of fecal coliforms and thermo-tolerant Gram-negative bacilli isolates including *E. coli.* This found illustrates a failure of hygiene and implementation of Good Manufacturing Practices (GMP) recorded during the survey. Kouame-Sina et al. (2010) did similar report on the contamination occurs from hands, sanitation of vendors as well as the environment, the water used in the production. Also, according to Zelalem and Bernard (2006), high level of contamination of milk products might be explain by initial contamination originating from the udder surface, washed water, milking materials and utensils used for filtering the milk.

Thus, during our study, among the thermo-tolerant Gram-negative bacilli isolates, 38.9% were *K. pneumoniae* and 22.2% were *E. coli*. For *E. coli*, this rate is lower than the 38% reported by Bagré et al. (2014) on raw milk. The presence of different strain of *E. coli* gives a good indication of fecal pollution and contamination of milk products which lead to gastroenteritis and food poisoning in human (Galal et al., 2013). Presence of *E. coli* in milk products constitute a public health hazard. This contamination by *E. coli* could be explained also by the low hygiene level including handlers' hand, quality of water used and the used utensils during the processing and dairy products sale. The exposure of dairy products for sale in bowls and packet can be a source of contamination (Zagare et al., 2012). Similar results were early reported on fermented milks in Burkina Faso (Barro et al., 2002; Koussou et al., 2007; Savadogo et al., 2010). In our study dèguè, couscous and dèguè millet samples were more contaminated in the morning (23.08%) than in the afternoon (19.23%) with respective proportions of 23.08 and 19.23%. The yoghourt samples were only contaminated at 15.38% in the morning. This could be explained by the fact that the initial contamination will be affected by the lowering of pH and the antimicrobial activities of lactic acid bacteria present by the production of bacteriocines (Savadogo et al., 2004; Labioui et al., 2005). Moreover, their decrease may be due to inappropriate conditions for their development such as temperature (Le Conte and Navajas, 2008). The formation of biofilm by strains of thermo-tolerant Gram-negative bacilli, *K. pneumoniae* displays highest rate of 38.9% followed by the strains *E. coli* with a proportion of 22.2%. Samples of dèguè millet contained more biofilm-forming thermo-tolerant Gram-negative bacilli (34.62%) strains. The formation of biofilm by food-borne thermo-tolerant Gram-negative bacilli strains (especially dairy products) is very serious for human, especially for children. Biofilms are reported to be involved in both device associated infections and tissue infections such as pneumonia and osteomyelitis (Costerton et al., 1999). The study of the sensitivity to antibiotics of the strains of isolated thermo-tolerant Gram-negative bacilli showed the existence of variable resistance proportions according to the antibiotics families. Indeed, against the 16 antibiotics tested, the strains of thermo-tolerant Gram-negative Bacilli show a resistance rate of 92.3% to

amoxicillin and to doxycillin, a resistance rate of 61.5% to ticarcillin and 57.7% resistance to amoxicillin+ clavulanic acid. These founded are similar to those of Bagré et al. (2014) on raw and curdled milk sold in Ouagadougou who found a high rate of resistance of *E. coli* strains to amoxixcillin (78.26%) and the highest rate of resistance to amoxicillin+ clavulanic acid (100%). Our data is similar to those of Virpari et al. (2013) which observed the highest resistance rate to Ampicillin (43.75%). This high resistance level could be explained by the abusive and uncontrolled use of antibiotics both in medicine and agriculture, especially in developing countries. This abusive use could induce the acquisition of antibiotic resistance factors by microorganisms (Okeke et al., 1999; Lesch et al., 2001; Trivedi et al., 2011), only *E. coli* (7.69%) and *Enterobacter aerogenes* (3.85%) strains produced ESBL. These rates are lower than the 16% observed by Lonchel et al. (2012) in Cameroon on clinical strains. The difference observed can be explained by the origin of the strains involved. Indeed, the strains of clinical origin acquired resistance to antibiotics because they are currently in contact with molecule other than food strains. The observed resistance is due to the abusive and uncontrolled antibiotics use. On the other hand, our strains come from food materials that are supposed to not yet been confronted with the abusive antibiotics use. The only sensitivity was observed with imipenem, which showed a resistance rate of 00%. This suggests that imipenem is still a treatment of choice against the Gram-negative bacilli. During our research, *E. coli* strains carriers' blaCTX-M gene and had the highest level of blaTEM. Studies in the food sector have observed the presence of blaSHV, blaTEM and blaCTXM-M genes in *E coli* strains at high levels. These are the studies conducted by Saad et al. (2019) on chickens in Egypt and that carried out in Iran by Dallal et al. (2018). These studies have shown that the more the strains develop a multidrug resistance to antibiotics the more they are carriers of resistance genes. In our research, we observed the presence of two multi-resistant *E. coli* strains that are both biofilm-forming, ESBL-producing and carriers of blaCTX-M, blaTEM and blaSHV genes. This state of affairs is very bad for the health of consuming populations.

Conclusion

It appears from our study that artisanal fermented dairy products sold in some secondary schools of Abomey-Calavi and Cotonou are contaminated with thermo-tolerant Gram-negative bacilli. They have capabilities for biofilm formation, penicillinase production and ESBL production. Resistance strains observed in food are a reality. Awareness raising and training of sellers on good hygiene and manufacturing practices for fermented milk products are necessary to reduce bacterial contamination in order to increase the safety of these products and thus preserve the health of young consumers.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGMENT

Grant I-3-E-5393-2 of International Foundation of Science (IFS) supported this work.

REFERENCES

Abdel-Aziz SM, Asker MM, Keera AA, Mahmoud MG (2016). Microbial food spoilage: control strategies for shelf life extension. Microbes in Food and Health pp. 239-264.

Anago E, Ayi-Fanou L, Akpovi CD, Hounkpe WB, Tchibozo MAD, Bankole HS, Sanni A (2015). Antibiotic resistance and genotype of beta-lactamase producing *Escherichia coli* in nosocomial infections in Cotonou, Benin. Annals of Clinical Microbiology and Antimicrobials 14(5):1-6.

Bagré TS, Kagambèga A, Bawa IH, Bsadjo TG, Dembélé RE, Zongo C, Barro N (2014). Antibiotic susceptibility of *Escherichia coli* and *Salmonella* strains isolated from raw and curds milk consumed in Ouagadougou and Ziniaré, Burkina Faso. African Journal of Microbiology Research 8(10):1012-1016.

Barro N, Outtara CAT, Nikiema AP, Outtara AS, Traore SA (2002). Microbiological quality assessment of some street food widely consumed in the city of Ouagadougou, Burkina Faso. Santé 12(4):369-374.

CASFM/EUCAST (2019). Available online: https://www.sfm-microbiologie.org/2019/05/06/casfm-eucast-2019-v2/ (accessed on 3 April 2019).

Christensen GD, Simpson WA, Bisno AL, Beachy EH (1985). Adherence of biofilm producing strains of *Staphylococci epidermidis* to smooth surfaces. Infections and Immunity 37(1):318-326.

Costerton JW, Stewart PS, Greenberg EP (1999). Bacterial biofilms: a common cause of persistent infections. Science 284(5418):1318-1322.

Dallal MMS, Fani F, Rajabi Z, Karami-Talab M (2018). Prevalence of blaCTX-M, blaSHV and blaTEM β-Lactamase Genes among *Escherichia coli* isolates in Foodborne Outbreak in Iran. International Journal of Enteric Pathogens 6(2):48-52.

Dallenne C, Da Costa A, Decré D, Favier C, Arlet G (2010). Development of a set of multiplex PCR assays for the detection of genes encoding important β-lactamases in *Enterobacteriaceae*. Journal of Antimicrobial and Chemotherapy 65(3):490-495.

Djogbe AAM, Tchekessi CKC, Sachi P, Degbey C, Bleoussi R, Banon J, Yaou IB (2019). Microbiological Characterization of Salty Bread, Soy Cheese and Three Yogurt Varieties Sold in the Streets of Benin. International Journal of Current Microbiology Applied Sciences 8(8):2201-2216.

Galal HM, Hakim AS, Dorgham SM (2013). Phenotypic and virulence genes screening of *Escherichia coli* strains isolated from different sources in delta Egypt. Life Sciences Journal 10(2):352-361.

Garas LC, Cooper CA, Dawson MW, Wang JL, Murray JD, Maga EA (2017). Young pigs consuming lysozyme transgenic goat milk are protected from clinical symptoms of enterotoxigenic *Escherichia coli* infection. Journal of Nutrition 147(11):2050-2059.

Girlich D, Poirel L, Nordmann P (2012). Value of the modified Hodge test for detection of emerging carbapenemases in *Enterobacteriaceae*. Journal of Clinical Microbiology 50(2):477-479.

Koneman EW, Allen SD, Janda WM, Schreckenberger PC, Winn WCJr. (2006). Koneman's color atlas and textbook of diagnostic microbiology. Lippincott Williams & Wilkins 6:930-940.

Kouame-Sina SM, Dadie A, Makita K, Grace D, Dje M, Taminiau B,

Bonfoh B (2010). Biodiversity, phylogenetic relationship and antibacterial potential of Bifidobacterium species isolated from raw milk production chain in Abidjan, Côte d'Ivoire. Presented at the 11th CSRS Scientific Seminar, Abidjan, Côte d'Ivoire. 4 November 2010. Centre Suisse de Recherches Scientifiques (CSRS).

Koussou MO, Grimaud P, Mopaté LY (2007). Evaluation de la qualité physico-chimique et hygiénique du lait de brousse et des produits laitiers locaux commercialisés dans les bars laitiers de N'Djamena au Tchad. Revue d'Elevage et de Médecine Vétérinaire des Pays Tropicaux 60(1-4):45-49.

Labioui H, Elmoualdi L, El Yachioui M, Ouhssine M (2005). Sélection de souches de bactéries lactiques antibactériennes. Bulletin de la Société de Pharmacie de Bordeaux 144(3/4):237.

Le Conte Y, Navajas M (2008). Climate change: impact on honeybee populations and diseases. Revue des Sciences et Techniques OIE 27(2):499-510.

Lesch CA, Itokazu GS, Danziger LH, Weinstein RA (2001). Multi-hospital analysis of antimicrobial usage and resistance trends. Diagnostic Microbiology and Infectious Diseases 41(3):149-154.

Lonchel CM, Meex C, Gangoué-Piéboji J, Boreux R, Assoumou MCO, Melin P, De Mol P (2012). Proportion of extended-spectrum ß-lactamase-producing Enterobacteriaceae in community setting in Ngaoundere, Cameroon. BMC Infectious Diseases 12(1):53.

Meng J, Zhao S, Doyle MP, Mitchell SE, Kresovich S (1997). A multiplex PCR for identifying Shiga-like toxin-producing *Escherichia coli* O157: H7. Letters in Applied Microbiology 24(3):172-176.

Milani E, Koocheki A, Moeenfard M, Mortazavi SA (2011). The distribution of some pathogenic micro-organisms in Kurdish cheese during ripening. In IDF International Symposium on Sheep, Goat and other non-Cow Milk.

Mon KK, Saelao P, Halstead MM, Chanthavixay G, Chang HC, Garas L, Zhou H (2015). *Salmonella enterica* serovars *Enteritidis* infection alters the indigenous microbiota diversity in young layer chicks. Frontier in Veterinary Sciences 2:61. https://doi.org/10.3389/fvets.2015.00061

Moussé W, Baba-Moussa F, Adjanohoun A, Noumavo PA, Sina H, Assogba S, Baba-Moussa L (2016). Virulence profiles of pathogenic *Escherichia coli* strains isolated from street foods in Benin. International Journal of Biotechnology and Food Science 4(3):52-64.

Nordmann P, Carrer A (2010). Les carbapénèmases des entérobactéries. Archives de Pédiatrie 17:S154-S162.

Nordmann P, Naas T, Poirel L (2011). Global spread of carbapenemase-producing *Enterobacteriaceae*. Emerging Infectious Diseases 17(10):1791-1798.

Okeke IN, Lamikanra A, Edelman R (1999). Socioeconomic and behavioral factors leading to acquired bacterial resistance to antibiotics in developing countries. Emerging Infectious Diseases 5(1):18-27.

Oliver SP, Jayarao BM, Almeida RA (2005). Foodborne pathogens in milk and the dairy farm environment: food safety and public health implications. Foodborne Pathogens Disease 2(2):115-129.

Pal M, Awel H (2014). Public health significance of Listeria monocytogenes in milk and milk products: An overview. Journal of Veterinary Public Health 12(1):01-05.

Pfeifer Y, Cullik A, Witte W (2010). Resistance to cephalosporins and carbapenems in Gram-negative bacterial pathogens. International Journal of Medical Microbiology 300(6):371-379.

Pfleiderer A, Lagier JC, Armougom F, Robert C, Vialettes B, Raoult D (2013). Culturomics identified 11 new bacterial species from a single anorexia nervosa stool sample. European Journal of Clinical Microbiology and Infectious Diseases 32(11):1471-1481.

Rasmussen RS, Morrissey MT (2008). DNA based methods for the identification of commercial fish and seafood species. Comprehensive Reviews *in* Food Science *and* Food Safety 7(3):280-295.

Riegel P, Archambaud M, Clavé D, Vergnaud M (2006). Bacteria difficult to culture and identify. Ed. Biomerous. France pp. 93-112.

Saad DN, Sultan S, Abdelhalem MA, Al-Azeem MWA (2019). Molecular Detection of blaTEM, blaSHV and blaOXA from *Escherichia coli* Isolated from Chickens. Journal of Veterinary and Animal Research 2:102-110.

Samapundo S, Thanh TC, Xhaferi R, Devlieghere F (2016). Food safety knowledge, attitudes and practices of street food vendors and consumers in Ho Chi Minh city, Vietnam. Food Control 70:79-89.

Savadogo A, Ouattara CAT, Ilboudo AJ, Karou D, Traore AS (2010). Isolation and susceptibility to antibiotics of bacterial strains from Burkina Faso fermented milk samples. Advance Journal of Food Science and Technology 2(2):91-95.

Savadogo A, Ouattara CA, Bassole IH, Traore AS (2004). Antimicrobial activities of lactic acid bacteria strains isolated from Burkina Faso fermented milk. Pakistan Journal of Nutrition 3(3):174-179.

Sessou P, Farougou S, Azokpota P, Youssao I, Yehouenou B, Ahounou S, Sohounhloue DCK (2013). Inventory and analysis of endogenous conservation practices for wagashi, a traditional cheese produced in Benin. International Journal of Biology and Chemical Sciences 7(3):938-952.

Smith JL, Fratamico PM (2018). Emerging and re-emerging foodborne pathogens. Foodborne Pathogens Diseases 15(12):737-757.

Stepanović S, Vuković D, Dakić I, Savić B, Švabić-Vlahović M (2000). A modified microtiter-plate test for quantification of staphylococcal biofilm formation. Journal of Microbiology Methods 40(2):175-179.

Tohoyessou MGE, Mousse W, Sina H, Bade F, Boton CEA, Ahouissou OR, Baba-Moussa L (2020). Microbial quality of artisanal yoghurt and Dèguè products collected in schools of Cotonou and Abomey-Calavi (Benin). African Journal of Food Science 14(5):112-118.

Trivedi K, Cupakova S, Karpiskova R (2011). Virulence factors and antibiotic resistance in enterococci isolated from food-stuffs. Veterinary Medicine Journal (Praha) 56(7):352-357.

Virpari PK, Nayak JB, Brahmbhatt MN, Thaker HC (2013). Study on isolation, molecular detection of virulence gene and antibiotic sensitivity pattern of *Escherichia coli* isolated from milk and milk products. Veterinary World 6(8):541-545.

Zagare MS, Deshmukh AM, Patil SS (2012). Analysis of dairy pack food for presence of bacterial pathogens. DAV International Journal of Science 1(1):2277-5641.

Zelalem Y, Bernard F (2006). Handling and microbial load of cow's milk and irgo-fermented milk collected from different shops and producers in central highlands of Ethiopia. Ethiopian Journal of Animal Production 6(2):67-82.