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Research Article
Antibiotic Susceptibility Patterns of Bacteria Species Isolated from Ice-cream Vended in Ota and Lagos Metropolis

Obinna C. Nwinyi, Ebojie Obehi, Adesina Tomilola, Margaret I. Oniha and Olopade Bunmi

Department of Biological Sciences, School of Natural and Applied Sciences, College of Science and Technology, Covenant University, KM 10 Idiroko Road, Canaan Land, PMB 1023 Ota, Ogun State, Nigeria

Abstract
Background: Most milk and milk products (ice-cream) could be a veritable avenue for the transmission of multidrug resistant genes among any community. In this study, antibiotic susceptibility patterns were surveyed in some selected ice-cream sold in Ota and Lagos metropolis. **Materials and Methods:** Samples of branded ice-creams were surveyed for antibiotic susceptibility patterns, a total of 9 bacteria species were selected from the pool of isolates and characterized morphologically and biochemically. The antibiotic reactions of the isolates assigned as D^1, D^2, D^3, G^1, G^2, G^3, J^1, J^2 and J^3 to the standard Gram negative and Gram positive antibiotics was done by agar diffusion method. **Results:** Based on the comparison of the isolates with standard reference organisms, the isolates belonged to the *Salmonella*, *Pseudomonas, Yersinia, Staphylococcus* and *Micrococcus* species. Most of the organisms exhibited antibiotic reactions ranging from susceptible, intermediate to resistance to the assayed standard antibiotics. **Conclusion:** From this study, the incidence of antibiotic resistance patterns could be on the increase unless strict measure during processing and handling of ice-cream is ensured.

Key words: Ice-cream, antibiotic susceptibility patterns, Gram negative and Gram positive bacteria, characterization, standard antibiotics

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Corresponding Author: Obinna C. Nwinyi, Department of Biological Sciences, School of Natural and Applied Sciences, College of Science and Technology, Covenant University, KM 10 Idiroko Road, Canaan Land, PMB 1023 Ota, Ogun State, Nigeria Tel: +234 (0)8037027786

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INTRODUCTION

Ice-cream, a popular diary product, consists of aerated suspension of crystallized fat, hydrocolloids, casein micelles, proteins, water and highly concentrated sugar solution that are partly frozen to form rigid foam[1-4]. Ice-cream continues to receive good patronage from children and adults in most parts of world. However this, microorganisms are transmissible to human through milk and milk products[5-7]. Ice-cream being a diary product could act as a vehicle for pathogen transmission[8]. Several researchers have demonstrated that ice-creams have been responsible for outbreaks of several food borne illnesses such as cholera, typhoid and bacillary dysentery; where the high nutrient (lactose and protein), neutral pH could be an excellent medium for microbes to thrive[3]. For instance in the United States, in September, 1994 there was a recall of nationally branded ice-cream due to prevalence of *Salmonella enteritidis* infections that contaminated the tanker trailers that was used for carrying non-pasteurized liquid eggs[9].

Several organisms have been implicated in food-borne diseases from ice-cream. These organisms are classified according to their importance. These include: *Staphylococcus aureus*, a most recurrent pathogen associated with most food-borne diseases from ice-cream, it ranked about 85.5% in most outbreaks. *Salmonella* ranked about 10.1% in food-borne diseases from ice-cream outbreaks[10-12]. Among the most common spore-forming bacteria in ice-cream are the *Bacillus licheniformis*, *B. cereus*, *B. subtilis*, *B. mycoides* and *B. megaterium*[13]. Other bacteria species that cause spoilage in ice-cream include: *Eubacterium*, *Enterococcus faecalis* subsp., *liquefaciens* and *Lactobacillus fermentum*. Common coliforms species often encountered in ice-cream include: *Klebsiella*, *Enterobacter*, *Citrobacter*, *Escherichia coli* O157:H7, a pathogen of considerable importance to public health practitioners[14] and enterotoxigenic *Escherichia coli* (ETEC). *Listeria monocytogenes* has the ability to grow or survive at low temperatures, pH and water activities. This makes *L. monocytogenes* an important hazard in ice-cream[15]. Other bacteria species implicated with ice-cream contamination include *Campylobacter jejuni*, the most common cause of infectious enterocolitis and bacteria diarrhoea[16,17].

The presence of these organisms in ice-cream at certain levels may be used to assess existing quality/shelf life of the product. Aside from ice-creams harboring food borne pathogens, some of the pathogens could be resistant to different antibiotics[18]. Denyer *et al.*[19] stated that antimicrobial resistance could be acquired through horizontal transfer of genes, transposons and conjugative plasmids or by mutation in microorganisms. Thus, with the recent re-emergence of antimicrobial resistance patterns among several microorganisms, this phenomenon continues to generate serious challenges to the public health practitioners particularly in the food industry.

Hennessy *et al.*[9] and Yusuf *et al.*[20] reported of the wrong assumption by consumers that most harmful microbes in ice-creams could be controlled during pasteurization, freezing and hardening of ice-cream. Bacteria occurring in ice-creams have been uncommonly surveyed for antibiotic resistance susceptibility patterns in most communities particularly in the developing countries. Consequently, based on the severe implications and consistent failure of antibiotics due to widespread of antimicrobial resistance genes, among the developing countries, in particular Nigeria, researchers explored this all important diary product (ice-cream) to establish the common bacteria species that occur in the diary product and their antimicrobial resistance patterns. Researchers report from this study shows that Gram negative organisms were sensitive to gentamycin while all tested organisms were sensitive to ofloxacin, even at lower concentration (5 μg).

MATERIALS AND METHODS

Sample collection: Fourteen different branded packaged ice-creams were purchased randomly from different retail outlets. These include local shops and big retail malls, fast food shops around Ota and Ikeja areas of Ogun and Lagos states of Nigeria. All the purchased samples were properly packaged. In all, triplicate samples of the branded packaged ice-cream were procured. These were placed in sterile cold pack and transported to the laboratory and stored 4°C until use.

Isolation of bacterial contaminating species: De-frosted ice-cream samples (1 mL) were serially diluted from 10^{-1} to 10^{-3} in sterile distilled water. Aliquot of 1 mL of 10^{-3} of the samples were plated on Nutrient agar, Eosin Methylene Blue (EMB) agar for Total Aerobic Plate Count (TAPC) and coliform count. In addition, the same volume was inoculated by spread plate method on mannitol salt agar (Biolab, Hungary) for the isolation of *S. aureus,* while *Salmonella-Shigella* agar (Oxoid) was inoculated for the isolation of *Salmonella* species. All the samples were plated in replicates. Based on the prevalence of bacterial counts from the different samples, we selected those with very high bacteria counts more than the threshold level expected for ice-cream for further study. The selected colonies were periodically transferred to obtain the pure culture for characterization of the bacteria species and the antibacterial sensitivity study using different antibiotics. For the initial characterization of the isolates, morphological examinations of the bacterial species were used. The pure cultures incubated at 37°C for 24 h were tentatively identified as D^1, D^2, D^3, G^1, G^2, G^3, J^1, J^2 and J^3. These were further classified by cultural and biochemical techniques, thereafter compared with standard reference organisms[21,22]. The following tests were carried out: Catalase, oxidase, colony motility, urease, methyl red, voges proskauer, indole, nitrate reduction, gelatin hydrolysis, spore test, starch hydrolysis, coagulase, citrate, sugar utilization-(glucose, lactose, sucrose and galactose) and Gram stain/morphology examination.

Coliform tests: For the determination of the coliforms among the selected bacterial species, 1 mL of each bacterial species were inoculated into sterile MacConkey broth in a McCartney bottles with inverted durham tubes for coliform test. Positive results showed color change from purple to yellow and the accumulation of gas bubbles at the inverted Durham tubes. Confirmatory coliform test were carried out on the samples that showed acid and gas production. For the confirmatory tests, the positive tubes were streaked on plates of EMB incubated at 37°C for 24 h for faecal coliform organisms determination. Culture plates were examined at the end of incubation period for colony counts and cultural characteristics of formed colonies.

Antibiotic susceptibility testing: Antibiotics susceptibility testing was carried out on the isolates D^1, D^2, D^3, G^1, G^2, G^3, J^1, J^2 and J^3. The disc diffusion method according to Adeniyi *et al.*[23] and Oyetibo *et al.*[24] were adopted. Mueller Hinton (MH) agar was prepared according to the instructions of the manufacturer. The antibiotics discs were placed on MH agar plates earlier seeded with cell suspension with a turbidity of 0.5 McFarland standards. The isolates include D^1, D^2, D^3, G^1, G^2, G^3, J^1, J^2 and J^3. These were streaked on the MH agar and then antibiotics disk containing different antibiotics were placed on the MH agar and incubated at 37°C for 24 h. For the Gram positive antibiotics (Abtex Biologicals Ltd., Liverpool, UK) tested were: Cotrimoxazole (25 µg), cloxacillin (5 µg), erythromycin (5 µg), gentamycin (10 µg), augmentin (30 µg), streptomycin (10 µg), tetracycline (10 µg) and chloramphenicol (10 µg) for Gram positive organisms. The Gram negative antibiotics examined include: Ofloxacin (5 µg), augmentin (30 µg), gentamycin (10 µg), nalidixic acid (30 µg), tetracycline (25 µg), amoxicillin (25 µg), cotrimoxazole (25 µg) and nirofurantoin (20 µg). The zones of inhibition were measured and recorded. The antibiotic sensitivity was termed susceptible when the recorded zones of inhibition (mm) were ≥ 11 and resistant when the zones of inhibition to the antibiotic[25] was ≤ 9.

RESULTS

Table 1 shows the total viable count from the different ice-cream samples as well as the indication of presence of fecal coliforms. In some samples, high bacteria counts were enumerated. The samples I, J and K had very high bacteria count that were too numerous to count (TNTC). In all, the fecal coliform indicator *Escherichia coli* were not present in the samples, though *Salmonella* species was identified in sample D. The total viable count in all ranged between 2×10^5 CFU mL^{-1}, too numerous to count (TNTC). Based on the data obtained, samples D, G and J were selected for further studies due to the general acceptance and preference by majority of the interviewed persons on the brand ice-cream.

Table 2 indicates the morphological and biochemical reaction of organisms from samples D, G and J.

In sample D, groups D^1 and D^2 were Gram negative while, D_3 showed a Gram positive response. From the observation under the microscope, each of the organisms showed a cluster appearance in shape except for D^2 that had a bacillary shape. In all, they showed similar response to the biochemical tests. Among isolates from sample G, G^1 showed a Gram positive response to the Gram reaction whereas G^2 and G^3 showed a bacillary shape with Gram negative response to the Gram reaction. In other tests, carried out their responses were similar except for G^3 and G^1 that exhibited catalase and oxidase negative, respectively. All the organisms differed in appearance when viewed under the microscope. The G^1 showed a cluster appearance while, G^2 and G^3 showed

Table 1: Total bacterial count and determination of fecal coliforms

Sample code	TVC CFU mL^{-1} (10^3)	*Salmonella*	*Escherichia coli*
A	350	-	-
B	350	-	-
C	300	-	-
D	800	+	-
E	320	-	-
F	200	-	-
G	460	-	-
H	559	-	-
I	TNTC	-	-
J	TNTC	-	-
K	TNTC	-	-
L	455	-	-
M	TNTC	-	-
N	409	-	-

TNTC: Too numerous to count, TVC: Total viable count, -: Absence, +: Present, A: V.S, B: F.C, C: CCV, D: FS, E: BV, F: SVS, G: SV, H: IAVS, I: IUBS, J: CUVS, K: CU VS1, L: SS, M: SV and N: SRV

Table 2: Morphological and biochemical characteristics of the selected organisms

Sample code	Gram stain/ Appearance	Coagulase	Shape	Catalase	Citrate	MR	VP	Motility	Indole	Oxidase	Urease	Starch hydrolysis	H_2S	Glucose	Lactose	Galactose	Sucrose
D^1	Clusters (-)	-	Cocci	+	+	-	+	+	+	+	+	-	-	-	-	-	-
D^2	Clusters (-)	-	Bacilli	-	+	-	+	+	+	-	+	-	-	-	-	-	-
D^3	Clusters (+)	-	Cocci	-	+	-	+	+	+	-	+	-	-	-	-	-	-
G^1	Clusters (+)	-	Cocci	+	+	-	+	+	+	-	+	-	+	-	-	-	-
G^2	Chains (-/+)	-	Bacilli	+	-	-	+	+	+	+	+	-	+	-	-	-	-
G^3	Single (-/+)	-	Bacilli	-	-	-	+	+	+	+	+	-	+	-	-	-	-
J^1	Clusters (-)	-	Cocci	+	+	-	+	-	+	+	+	-	-	-	-	-	-
J^2	Clusters (-)	-	Cocci	-	+	-	+	-	+	+	+	-	-	-	-	+	-
J^3	Single (-)	-	Bacilli	+	+	-	+	+	-	+	-	-	+	-	-	-	-

MR: Methyl red, VP: Voges-proskauer, H_2S: Hydrogen sulphate, +: Positive, -: Negative and +/-: Gram variable

chain-like and single appearances, respectively. The organisms from sample J showed similar reactions to the Gram reaction which were all Gram negative. Group J^1 and J^2 were cocci in shape and appeared as cluster, J^3 emerged single in appearance with bacilli shape under the microscope. The J^2 showed a negative result to catalase test and positive response to galactose. The J^3 exhibited a motile movement and a positive response in hydrogen sulphide test. From the characterization, *Salmonella* sp. was isolated in FS ice cream under group D^1. Other suspected organisms further identified using the morphological and biochemical characteristics and comparisons with standard organisms indicate that *Pseudomonas* sp. (D^2) occurred in the coded branded ice-cream in FS, SV and CUVS. *Yersinia* (Group J^{1-3}) and *Staphylococcus* species (G^{1-3}) occurred in FS and in the CUVS, *Micrococcus* spp. was present under D^3.

In this study, antibiotic-resistant bacteria are those bacteria not inhibited or killed by the tested antibiotics. They survive and even increase in the presence of antibiotics. Bacteria that are resistant to many antibiotics are known as multi-resistant organisms.

DISCUSSION

Food safety is an essential issue globally. It is one of the cardinal objectives of WHO strategic goals between the periods[26] of 2008-2013. In addition, food borne diseases which originates due to presence of microorganisms or its metabolites in food products is one of the most common challenges in the food industry with considerable threat to the public health.

Raw milk has been noted to be one of the easily contaminated/polluted foods. As a result, it could have direct impact on everyone most especially the immunocompromised people, children and the aged. Based on the significance of this, researchers surveyed a milk product (ice-cream) for the presence of microorganisms or its genes that can severely hamper the health of the wide populace that consume this milk product. In the present study, the prevalence of *Salmonella*, *Pseudomonas, Yersinia, Staphylococcus* and *Micrococcus* species were recorded among the branded ice-cream. *Staphylococcus* species among which include the *S. aureus* has been noted for food poisoning[27]. In a survey done in Japan, between the periods of 1993-1998, the *Staphylococcus* species account for 32.5% of food contamination with raw milk having about 76.3% contamination rate[28]. In the developing countries, due to constant power outage, the right condition required for the preservation of the ice-cream cannot be maintained thus the occurrence of *Staphylococcus* species recorded from this study cannot be doubted. In addition, *Staphylococcus* and *Pseudomonas* species have potential of producing biofilms which is a factor that contributes to their tolerance to the pasteurization processes, survival of environmental factors, entrance via faults in storage tanks and cracks in the plant and packaging materials[29]. Several researchers[5,30] have reported, that the use of unclean equipment in the manufacturing of ice-cream products, contaminated raw material and inadequate aseptic measures during handling, inappropriate storage temperatures, direct transfer of pathogens by vectors/human increase the likelihood of ice-cream acting as a vehicle for pathogen transmission. In Table 1, the high bacterial count which represents the hygiene quality of the branded ice-cream ranged between 2×10^5 CFU mL^{-1}, too numerous to count (TNTC). This high bacteria count were also recorded by Clarence *et al.*[31], Nonga *et al.*[6] and Okonko *et al.*[32] who isolated almost similar organisms from sausages, meat pie and seafood processors that were vended publicly. According to Ambily and Beena[33], bacteriological quality of ice-cream estimates the hygiene and food safety state of the product. High estimation of bacteria counts and presence of *Salmonella* indicate that the ice-creams may have been contaminated during production. This therefore suggests that there is a possible risk of food infection on consumers. Among the organisms *Staphylococcus* sp. (an enterotoxin producer) and *Pseudomonas* sp. have been implicated in food spoilage[34,35]. The presence of *Micrococcus* and *Pseudomonas* species has been attributed to poor handling, unhygienic conditions during preparation and packaging of ice creams. It is reported that *Staphylococcus* species can survive and elaborate on the starch and proteins contained in the ice-cream thus leading to production of enterotoxins. The *Yersinia* species could be transmitted by contaminated water and food, it has been isolated in several food products such as meat, meat products, fish, vegetables, milk and ice-cream[36-39]. *Yersinia* species has been known to cause fever, diarrhea, abdominal pain and vomiting. It has potentials of growing at 4°C and below[40].

Antibiotics are natural, semi-synthetic or synthetic drugs with antibacterial or antiparasitic or antifungal activities. Conversely the constant administration of antibiotics to man and animals poses a selective pressure on the resistance of the bacteria species giving rise to resistant strains that affect negatively the public health. For the purposes of keeping track of medically important antibiotics for the treatment of food poisoning and infection, antimicrobial susceptibility testing among organisms isolated from food products is very necessary. In addition, it is always paramount among the food safety practitioners to avoid the dissemination of bacteria or it's with mobile elements in foods. Several researchers[41,42] reported that extensive data on antimicrobial susceptibility on clinical and subclinical samples exist, however very scanty report on antimicrobial susceptibility patterns from isolates from food samples exist. Owing to the direct implication of the antibiotic resistance burden on the health any community, it is proper to assess the extent of antibiotic resistance of the isolates from ice-creams because antibiotic treatment is the main method for combating bacterial infections. From Table 3 and 4, *Salmonella*, *Pseudomonas*, *Yersinia*, *Staphylococcus* and *Micrococcus* species showed resistance to the assayed antibiotics. All the organisms showed resistance to cotrimazole, cloxacillin, erythromycin, Augmentin, amoxicillin, streptomycin and tetracycline. This result compares well with previous studies of Hu *et al.*[43] that isolated 32 organisms from food product with most of the showing resistance to tetracycline, erythromycin, chloramphenicol . In a study conducted by Teuber *et al.*[44] most of the organisms isolated from food samples were resistant to one or more antibiotics including streptomycin, erythromycin, penicillin, tetracycline, rifampicin, gentamicin and chloramphenicol. The *Salmonella*, *Pseudomonas* and *Yersinia* species were all susceptible to ofloxacin while, *Salmonella* species showed an intermediate resistance to nalidixic antibiotics.

In this study, most of the organisms have shown multidrug resistance patterns since there were resistant to more than three antibiotics. Thus, the development of antibiotic resistance by the isolates may be due to horizontal gene transfer over the species by the mobile genetic elements such as plasmids transposons[45].

According to Davies[46] several methods have been evolved by bacteria for enzymatic inactivation of antibiotics. These include but not limited to the use of lactamases, aminoglycoside acetyl-, nucleotidyl- and phosphoryltransferases against the antibiotics or through restricted import of antibiotics by use of penicillin binding proteins, active export of antibiotics by membrane inserted ATP-dependent efflux systems or target modification via methylation of 23S rRNA, mutation of amino acid sequence of topoisomerase.

Now-a-days, the increase in the spreading of antibiotic resistance genes in food products has continued to generate interests especially where the potential of transferring

Table 3: Antibiotic susceptbility activities of isolates test with Gram positive antibiotics

Sample code	Cotrimazole (25 µg)	Cloxacillin (5 µg)	Erythromycin (5 µg)	Gentamycin (10 µg)	Augmentin (30 µg)	Streptomycin (10 µg)	Tetracycline (10 µg)	Chloramphenicol (10 µg)
D3	R	R	R	S (20)	R	R	R	I
G1	R	R	R	S (20)	R	R	R	S (15)

Table 4: Antibiotic susceptibility activities of isolates tested with Gram negative antibiotics

Sample code	Augmentin (30 µg)	Ofloxacin (5 µg)	Gentamycin (10 µg)	Nalidixic acid (30 µg)	Nitrofurantoin (20 µg)	Cotrimazole (25 µg)	Amoxicillin (25 µg)	Tetracycline (25 µg)
D1	R	S (27)	S (21)	I	R	R	R	R
D2	R	S (14)	I	I	I	R	R	R
G2	R	S (22)	S (15)	R	R	R	R	R
G3	R	S (21)	I	R	R	R	R	R
J1	R	S (27)	S (20)	I	R	R	R	R
J2	R	S (20)	S (16)	I	R	R	R	R
J3	R	S (24)	S (21)	R	R	I	R	R

antibiotics resistance could occur between opportunistic pathogens and normal flora in the gut. This has a potential of becoming virulent in immunocompromised individuals and children that consume such products like ice-cream. Also it has been noted that some *Staphylococcus* species produce biofilms which aids the organism to produce chronic debilitated conditions in human due to reduced susceptibility to antibiotics because of the decreased diffusion of antimicrobials through the biofilm matrix[29].

Thus, this upsurge in resistance among these organisms has continued to bear upon government policies on sustainable ways of eliminating such organisms in food products. Thus, the presence of these bacteria species poses serious public health risks. The perceived notion that freezing ice-cream and other frozen dairy products could slow bacterial growth or kill the organisms has been perceived as wrong assertion. Most bacteria under cold condition can produce cold-shock proteins to withstand the cold environment.

CONCLUSION

The detection of multidrug resistant Gram negative organisms from the branded ice-creams is a threat to the growing antibiotics resistance challenge. Many of these antibiotics resistance are borne on plasmids and can easily confer resistance to other Gram negative organisms. Their presence in ice-cream can also lead to high incidence of untreatable community acquired infections. In this study, the Gram negative and Gram positive organisms showed susceptible to intermediate resistance to gentamycin, while all the tested bacteria species were sensitive to ofloxacin, even at lower concentration (5 μg). Thus, the class of antibiotics aminoglycosides and fluoroquinolones still remain the choice of drug for treating food infections. To forestall, the spread of antimicrobial resistance, it is advocated that there should be periodic monitoring and risk assessment of street vended ice-creams notwithstanding the brand.

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