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Antibiotics Resistance among Bacteria Isolated from Evaporated Milk

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ABSTRACT

This study was aimed at examination of cans of evaporated milk samples collected at different location in Ogbomoso, Nigeria for evidence of spoilage, isolation and characterization of bacterial pathogen from the evaporated milk samples, determination of the antibiotic susceptibility profiles and evaluation of the proteolytic activity of the isolates. Twelve bacterial pathogens were isolated from some evaporated milk, the isolate were characterized and were identified to be *Bacillus coagulans, Bacillus licheniformis, Bacillus cereus, Bacillus circulans, Paracoccos denitrificans, Micrococcus luteus, Micrococcus varians, Proteus mirabilis, Enterobacter aerogenes, Staphylococcus saprophyticus, Klebsiella, pneumoniae* and *Pseudomonas fluorescens*. The cans of evaporated milk were examined and it was discovered that two of the samples had flipper swell, one had springer while the others were flat. Two of the evaporated milk samples were positive when tested for the presence of hydrogen and carbondioxide gases. All the sample cans were negative when tested for the presence of hydrogen sulphide. Antibiotics susceptibility profile of the isolates was determined; it was found that 16.67% of the organisms were sensitive to the antibiotics while 83.33% were resistant. The result of proteolytic activity test shows that six of the isolates were highly proteolytic, five were weakly proteolytic while only *Enterobacter aerogenes* was non proteolytic.

Key words: Antibiotics, proteolytic, casein, carbondioxide, hydrogen, hydrogen sulphide

INTRODUCTION

Milk and milk products constitute important nutritional components for all groups. Good quality milk meets the nutritional needs of the body better than any single food as it contains all essential food constituents (Sharm and Joshi, 1992). As a result of the presence of these nutrients, milk is an excellent culture medium for many kinds of micro-organisms (Henry and Newlander, 1997). The presence and multiplication of these microorganisms in milk brings about changes in the properties of milk thus reducing it quality (Frazier and Westhoff, 1986). In order to extend the shelf life of milk for human consumption by preventing the growth of spoilage organisms as well as preventing the transmission of diseases via milk, this highly nutritious, versatile food is usually pasteurized for a short time (Edema and Akingbade, 2007).

Pathogenic bacteria in milk have been a major factor for public health concern since the early days of the diary industry (Altug and Bayrak, 2003). Many diseases are transmissible via milk products; traditionally raw or unpasteurized milk has been a major vehicle for transmission of

pathogens (Vasavada, 1988). Another source of contamination by microorganism is unclean teats (Altug and Bayrak, 2003). The use of unclean milking and transporting equipment contributed also to the poor hygienic quality (Bonfoh *et al.*, 2003).

Contamination of milk and dairy products by pathogenic micro-organisms can be of endogenous origin, following excretion from the udder of an infected animal and/or exogenous origin, through direct contact with infected herds or through the environment e.g., water, personnel (Farzana *et al.*, 2009). Treatment and processing of milk can inhibit or encourage the multiplication of microorganisms (Brisabois *et al.*, 1997). Food borne pathogens can survive and thrive in post-pasteurization processing environments, thus leading to recontamination of dairy products. These pathways pose a risk to the consumers from direct exposure to food borne pathogens present in unpasteurized dairy products as well as dairy products that become re-contaminated after pasteurization (Oliver *et al.*, 2005).

Staphylococcus aureus by far is the most frequent pathogen associated with outbreaks (85.5% of the outbreaks), followed by *Salmonella* (10.1%) (De Buyser *et al.*, 2001). Cooked food products and raw milk were most commonly contaminated with food borne pathogens and many of them were resistant to different antibiotics. Milk products are often contaminated with enterotoxigenic strains of *S. aureus* (Chao *et al.*, 2007). It is currently not possible to effectively and consistently exclude such multi antibiotic resistant strains from the human food chain, which means that they continue to pose a significant clinical threat to consumers and concomitant economic threats to the food production and processing industry (Walsh *et al.*, 2005). Presence of enterotoxigenic and antimicrobial resistant strains of *S. aureus* have become remarkably widespread in foods. This requires a better control of food contamination sources and distribution of antimicrobial-resistance organisms (Normanno *et al.*, 2007). Contamination of dairy foods with virulent pathogens render them to be a source of public health hazard. The possible contamination sources are either mastitis in dairy cow or the milk itself (Carter, 1995). Growing concerns over food safety among the consumers call for the manufacturing and processing of foods under extremely hygienic conditions to avoid possible health challenges (Farzana *et al.*, 2009).

Bearing this in mind, this study was therefore aimed at; examination of cans of evaporated milk for physical changes, detection of some gases produced in the cans by microbial activity, isolation and characterization of bacterial pathogen from some evaporated milk, determination of the antibiotic susceptibility profiles and evaluation of the proteolytic activity of the isolates.

MATERIALS AND METHODS

The study was carried out in Science Laboratory Technology Department, Ladoke Akintola University of Technology, Ogbomoso, Oyo State, Nigeria between January, 2009 and November, 2009.

Collection of samples: A total of ten canned evaporated milk samples were purchased from different selling point in Ogbomoso, South West Nigeria in January, 2009. The samples were taken to the laboratory for analysis. The samples were incubated for 14 days at 35°C after which the cans were examined as to the type of swells produced as a result of microbial activity.

Test for carbondioxide and hydrogen: The can end to be opened was sanitized with 4% iodine in 70% ethanol for 30 min and it was wiped off with sterile cotton wool. A plastic tubing was attached to a hollow punch fitted with a large rubber stopper. The free end of this apparatus was

inserted into a test tube filled with dilute KOH; it was then inverted in a beaker filled with dilute KOH. An opening was made in one end of the can with the hollow punch; the gases displaced the dilute KOH inside the tube. The tube was closed by placing the thumb over the end before removing the open end from the beaker. The tube was then shakened, a vacuum as evidenced by suction against the finger was indicative of the presence of CO_2 gas. A match was applied near the top of the tube and then the thumb was quickly removed. A pop indicated the presence of hydrogen.

Test for hydrogen sulphide (H_2S): When the can was opened, the sense of smell was used to detect the presence of the characteristic rotten egg smell of H_2S gas (USFDA, 2001).

Isolation of microorganisms: One milliliter of each sample of evaporated milk was serially diluted and 1 m of an appropriate dilution was inoculated on sterile MacConkey agar and Nutrient agar, the plates were incubated for 24 h at 37°C. After 24 h, sterile wire loop was used to pick the isolate from the plate and was streaked on a freshly prepared nutrient agar then incubated for 24 h at 37°C in order to get pure culture. The routine laboratory method of Cruickshank *et al.* (1975) was used to characterize different isolates. The isolates were identified using their macroscopic, cellular, physiological and biochemical characteristics.

Antibiotics susceptibility test: Sterile nutrient agar medium was poured into sterile petri dishes and allowed to solidify. A suspension of the isolated organisms was transferred into petri-dishes accordingly and swab over the entire plate, it was then incubated for 1 h at 37°C and a forcep was used to transfer each sensitivity disc on the plate and incubated for 24 h at 37°C. The antibiotics used included Amoxyllin, Ampicillin, Tetracycline, Gentamycin, Ofloxacin, Augmentin, Ciprofloxacin, Cotrimoxazole, Nitrofurantion, Ampiclox, Cefroxine and Erythromycin.

Proteolysis: Sterile lactose egg yolk milk medium was poured aseptically into Petri dish, after solidifying a sterile cock-borer was used to bore holes in the plate and a suspension of the test organisms was introduced into the holes with the aid of sterile pipette and incubated for 24 h at 37°C. The plates were examined for zones inhibition after 24 h.

Casein hydrolysis: Nutrient agar containing 1% skimmed milk was poured aseptically into petri dishes, after solidifying a sterile Cork-borer was used to bore holes and a suspension of the isolates were introduced into the holes with the aid of a sterile pipette and incubated for 24 h at 37°C. The plates were examined for zones of inhibition after 24 h.

RESULTS

Out of the 10 samples of evaporated milk used for this study only 2 (mil and crn) tested positive for the presence of presence of hydrogen and carbondioxide gas while the rest were negative. All the samples were negative when tested for the presence of hydrogen sulphide. The cans of the samples were also examined and it was discovered that MIL had springer swell, CRN and NUN had flipper swell and the rest of the cans were flat. All the samples were also found to have normal odour when perceived with sense of smell (Table 1).

A total number of 12 organisms were isolated from the evaporated milk samples. The organisms were subjected to physiological and biochemical test and the organisms were identified to be

Table 1: Gas and swell detection in cans of evaporated milk

Sample code	Odour	CO_2 Gas	H_2 Gas	H_2S Gas	Type of swell
GRF	Normal	-	-	-	Flat
OLY	Normal	-	-	-	Flat
LUN	Normal	-	-	-	Flat
MIL	Normal	+	+	-	Springer
PKE	Normal	-	-	-	Flat
JGO	Normal	-	-	-	Flat
CST	Normal	-	-	-	Flat
CRN	Normal	+	+	-	Flipper
HDA	Normal	-	-	-	Flat
NUN	Normal	-	-	-	Flipper

-: Not detected, +: Detected

Table 2: List of sources of isolates

Sample code	Isolates
PKE	*Bacillus circulans, Klebsiella pneumoniae*
OLY	*Bacillus coagulans, Bacillus circulans*
CST	*Bacillus licheniformis, Proteus mirabilis*
CRN	*Micrococcus varians, Micrococcus luteus*
NUN	*Paracoccus denitrificans, Micrococcus luteus*
GRF	*Staphylococcus saprophyticus*
HDA	*Pseudomonas fluorescens, Enterobacter aerogenes*
JGO	*Micrococcus varians*
LUN	*Micrococcus varians*
MIL	*Pseudomonas fluorescens*

Bacillus coagulans, *Bacillus licheniformis*, *Bacillus cereus*, *Bacillus circulans*, *Paracoccos denitrificans*, *Micrococcus luteus*, *Micrococcus varians*, *Proteus mirabilis*, *Enterobacter aerogenes*, *Staphylococcus saprophyticus*, *Klebsiella pneumoniae* and *Pseudomonas fluorescens* (Table 2).

Agar diffusion method was used to carry out antibiotics susceptibility test and it was observed that *Bacillus cereus*, *Micrococcus varians*, *Bacillus circulans*, were sensitive to gentamycin (GN) with inhibitory zones of 18.0, 17.5, 20.0 mm, respectively while the other isolates were resistant to it. *Bacillus circulans* was sensitive to cotrimoxazole (CO), amoxylin (AMX) and augmentin (AU) with inhibitory zones of 17.5, 22.0 and 20.0 mm, respectively. It was also noticed that *Bacillus licheniformis*, *Bacillus cereus*, *Micrococcus varians* and *Bacillus circulans* were sensitive to ofloxacin (OF), erythromycin (E) and ciprofloxacin (CIP) but the other isolates were resistant to these antibiotics. All organisms were resistant to Ampicillin (AM), Nitrofurantion (N) and Ampiclox (AX). *Pseudomonas fluorescens*, *Enterobacter aerogenes*, *Proteus mirabilis* and *Klebsiella pneumoniae* where sensitive to tetracycline (TE) with inhibitory zones of 13.0, 16.5, 16.5 and 18.5 mm, respectively while others where resistant to it. *Enterobacter aerogenes* and *Klebsiella pneumoniae* were sensitive to cefroxine (CF) with zones of inhibition of 16.0 and 12.5, respectively while the other isolates were resistant to it (Table 3).

The isolates were also grown on skim milk agar to evaluate their protease activity and ability to hydrolyse casein, it was found out that *Bacillus licheniformis*, *Bacilllus circulans*, *Bacillus coagulans*, *Staphylococcus saprophyticus*, *Pseudomonas fluorescens* and *Klebsiella pneumoniae* were highly proteolytic as a result of their ability to produce clear zones around their line of growth

Table 3: Antibiotics susceptibility profile of the isolates

Isolates	GN	CO	AMX	AU	OF	E	CIP	AM	N	TE	AX	CF
Bacillus licheniformis	-	-	-	-	17.5	15.0	20.0	-	-	-	-	-
Bacillus cereus	18.0	-	-	-	20.0	17.5	19.0	-	-	-	-	-
Micrococcus varians	17.5	-	-	-	16.0	12.5	20.0	-	-	-	-	-
Bacillus circulans	20.0	17.5	22.0	20.0	17.5	17.5	18.5	-	-	-	-	-
Bacillus coagulans	-	-	-	-	-	-	-	-	-	-	-	-
Micrococcus leuteus	-	-	-	-	-	-	-	-	-	-	-	-
Paracoccus denitrificans	-	-	-	-	-	-	-	-	-	-	-	-
Staphylococcus saprophyticus	-	-	-	-	-	-	-	-	-	-	-	
Pseudomonas fluorescens	-	-	-	-	-	-	-	-	-	13.0	-	-
Enterobacter aerogenes	-	-	-	-	-	-	-	-	-	16.5	-	16.0
Proteus mirabilis	-	-	-	-	-	-	-	-	-	16.5	-	-
Klebsiella pneumoniae	-	-	-	-	-	-	-	-	-	18.5	-	12.5

-: Resistant to antibiotic

Table 4: Proteolysis of the isolates

Isolates	Proteolytic activities (mm)	Casein hydrolysis (mm)
Bacillus licheniformis	50.0	11.0
Bacillus cereus	-	12.0
Micococcus varians	50.5	-
Bacillus circulans	33.5	18.0
Bacillus coagulans	63.5	12.0
Micrococus luteus	67.5	-
Paracoccus denitrificans	50.0	-
Staphylococcus saprophyticus	45.0	15.0
Pseudomonas fluorescens	50.0	18.5
Enterobacter aerogenes	-	-
Proteus mirabilis	-	17.0
Klebsiella pneumoniae	42.5	11.0

-: Not dedicated

on the two skim milk medium used. On the other hand, *Bacillus cereus*, *Micrococcus varians*, *Micrococcus luteus*, *Paracoccus denitrificans* and *Proteus mirabilis* were found to be weakly proteolytic as indicated by their ability to produce clear zones around their lines of growth on one of the two medium used. *Enterobacter aerogenes* was to be non- proteolytic (Table 4).

DISCUSSION

All the evaporated milk samples collected were contaminated with bacteria; the identified bacteria, *Bacillus* sp., *Pseudomonas* sp., *Enterobacter aerogenes* and so on occur in different proportion and has being implicated in milk related diseases and in the spread of some food borne diseases.

The presence of *Bacillus* sp., which is a versatile organism in milk, could be as a result of poor hygiene or contamination from poor handling of the milk samples by workers. Also, the *Bacillus* sp. do produce enterotoxin, which could be deadly when ingested into the body. In addition, the presence of pseudomonas known for it versatility and high incidence of antibiotic resistance is a cause for concern.

When microorganisms grow and produce gases, the can goes through series of changes visible from outside; in this study only three changes was noted in the cans of evaporated milk, i.e., the springer, the flat and the flipper swell. It has being established that the two most common gases in cans of spoiled foods are carbondioxide and hydrogen sulphide that is as a result of metabolic activities of microorganisms. Hydrogen is released by the action of food acids on the iron of the cans.

The result of proteolytic activity test shows that six of the isolates were highly proteolytic, five were weakly proteolytic while only one was non proteolytic. The six organisms were able to effectively degrade proteins present in skim milk agar using their cellular enzymes called proteases. These proteases are heavily involved in many normal biological processes. Three out of four species of *Bacillus* isolated were highly proteolytic while one was weakly proteolytic, this supports the work of Almeida *et al.* (2000) who reported that 92% of *Bacillus* isolates were either proteolytic or lipolytic in nature.

However, from the conducted antibiotic susceptibility test, it was observed that 83.33% of the isolated microorganisms were resistant to antibiotics used while 16.67% of the organisms were sensitive. Most of the antimicrobial resistance microorganism's emergence was due to the extensive use and misuse of antibiotics. Bacteria become resistant to antimicrobial agents by a number of mechanisms which are; production of enzymes which inactivate or modify antibiotics, changes in the bacterial cell membrane, preventing the uptake of an antimicrobial and development of metabolic pathways by bacteria which enable the site of antimicrobial action to be passed; this is as reported by Monica (2000).

Conclusively, Levy (1992) reported that antimicrobial agents resistance bacterial pathogen is a major impediment to successful therapy and in several instances bacteria strains have arisen that are resistant to most available antimicrobial agents. The public health consequences of antimicrobial resistance to many antibiotics have been debated; however until recently clear evidence of health risk was not available. To prevent transmission of milk related diseases, Farzana *et al.* (2009) suggested that strict control measures must be applied to minimize and eliminate the contamination possibilities through milk and its products leading to minimized use of various antibiotics, which are excessively used and becoming ineffective against antibiotic resistant bacteria strain.

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