

Full Length Research Paper

Prevalence of enteropathogenic and lactic acid bacteria species in *wara*: A local cheese from Nigeria

T. E. Sangoyomi^{1*}, A. A. Owoseni² and O. Okerokun²

¹Department of Crop Production, Soil and Environmental Management, Faculty of Agriculture, Bowen University, Iwo, Osun State, Nigeria.

²Department of Biological Sciences, Faculty of Science and Science Education, Bowen University, Iwo, Osun state, Nigeria.

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Eight bacteria genera and yeasts were isolated from *wara* - a local soft cheese from Nigeria, the bacteria genera were made up of 76% lactic acid bacteria (LAB), 17% Enterobacteria and 7% *Staphylococci*. The LAB group was made up of the genera *Lactobacillus*, *Leuconostoc*, *Streptococcus* and *Pediococcus* with *Lactobacillus* being the most frequently occurring genus. *Escherichia coli*, *Klebsiella* and *Enterobacter* made up the Enterobacteria group. A protease enzyme produced by the *E. coli* was characterized. Its activity was found to be highest at 60°C and pH 5.4. The protease activity was highest at 5 mmol/l and was inhibited at 10 mmol/l concentration of EDTA.

Key words: *Wara*, lactic acid bacteria, *Escherichia coli*, protease.

INTRODUCTION

Milk is an extremely nutritious food. It is an aqueous colloidal suspension of proteins, fats and carbohydrates that contains numerous vitamins and minerals. Many of the pathogenic bacteria encountered do not grow well in milk but remain viable for undesirable lengths of time (Frazier and Westhoff, 1998). Processing and utilization of dairy products in African countries is not well developed. Cheese is an excellent source of protein, fats and minerals such as calcium, iron and phosphorus, vitamins and essential amino acids, thus making it an important food in the diet of both old and young. Cheese making is largely dictated by tradition. Due to shortage of milk, cheese production is expensive and powdered milk and cheese may be imported.

Wara has been classified as a natural cheese made from milk. The soft *wara* cheese produced in Nigerian farms especially in the northern part makes use of local ingredients. The vegetable rennet used for the production is made from a native plant *Calotropis procera* (sodom apple), which can be cultivated, all year round

(FAO, 1994). *Wara* is a fresh cheese, that is, soft, moist curd that has been cut and drained of the whey but never ripened. The number of microorganisms present at the time of milking has been reported to range between several hundreds and several thousand per milliliter (IDF, 1981).

Microorganisms found in milk can be classified based on their biochemical types, temperature response and ability to cause infection and disease (Marth, 1978). If maintained under normal conditions that permit bacterial growth, raw milk of good sanitary quality will develop a sour flavour and this is brought about mainly by *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris* and certain lactobacilli (Morse et al., 1968). Organisms associated with milk and milk products include *streptococci*, *lactobacilli*, coliform bacteria especially *E. coli*, *micrococci*, *Clostridium butyricum*, *Bacillus* sp., *Brucella abortus*, *Salmonella* sp., *Pseudomonas* sp., and some fungi. All these organisms are from various sources and they act on different substrates in milk, thereby producing various end-products (Milk pasteurization Controls and Tests, 1974). *Wara* is eaten in various forms either as normal cheese, a flavoured snack and meat substitute in sauces or as fried cake or sandwich filling (FAO, 1998).

*Corresponding author. E-mail: t.sangoyomi@yahoo.com. Tel: 234-803 451 0695.

LAB widely distributed in nature and occurring naturally as indigenous microflora in raw milk are gram positive bacteria. The lactic acid fermentation that these bacteria carry out has long been known and applied by humans for making different food stuffs (Guessas and Kihal, 2004). LAB have the ability to reduce pH by production of lactic acid from carbohydrates (Hammes and Knauf, 1994; Parente et al., 2001). LAB have been isolated from *wara* and the cell free supernatant used to treat urinary tract infections (Adeniyi et al., 2006).

Enterobacteriaceae is a family of gram negative enteric bacteria. They are a consistent inhabitant of the human intestinal tract and the regular presence of *E. coli* in the human intestine and faeces has led to tracking the bacterium in nature as an indicator of faecal pollution. Retail surveys of soft and semi cheese have found *E. coli* in 40 - 50% of these cheeses (Aureli et al., 1992) demonstrating that a mode of contamination of *E. coli* exists during production or processing. *Salmonella typhimurium* and *Listeria monocytogenes* have been isolated from pasteurized milk and the contamination was found to have occurred post pasteurization. Shigella infections have been reported to be spread by contaminated water, milk and food.

The objectives of this study were to characterize the microflora of *wara*, to detect the presence of indicators of faecal contamination (*E. coli*) and to partially characterize a protease enzyme of the isolated *E. coli*.

MATERIALS AND METHODS

Sample collection

Samples of *wara* were bought from hawkers at 3 different locations within Iwo, Osun state, south west of Nigeria. The sampling was done at 12.00 h (immediately after production), at 14.00 h (after about half of the contents have been sold) and finally at 18.00 h (when the *wara* is almost exhausted). The samples were collected in new polyethylene bags, kept in food flasks containing ice cubes and taken to the laboratory for immediate analyses.

Isolation of microorganisms

10 g of *wara* were homogenized in 90 ml sterile diluents [5 g peptone (Oxoid), 8.5 g NaCl and 1000 ml of distilled water, pH 7.2] using a stomacher (Lab Blender 400; Seaward medical, London, UK) for 1 min, normal speed. 1 ml from 10 fold dilutions of the homogenised samples were inoculated in nutrient agar (NA; LabM) for total aerobic count, de Man, Rogosa and Sharpe (MRS; LabM) agar for lactic acid bacteria (LAB), MacConkey agar (MCA; LabM) for Enterobacteria, Eosin methylene blue agar (EMB; LabM) for *E. coli* and *Salmonella-Shigella* agar (SSA; LabM) for *Salmonella* or *Shigella*. Yeast extract (5 g/l), peptone (10 g/l), dextrose (20 g/l) and agar-agar (20 g/l) (YEPD) was compounded for yeast isolation. The NA, MCA, EMB and SSA plates were incubated for 48 h at 37°C; MRS plates were incubated for 3 days at 30°C and YEPD plates at 25°C for 3 days. Counts were carried out on plates having between 30 and 300 colonies. For each sample, 5 different colonies were picked at random and purified by successive streaking on the same agar substrate (Vieira-Dalode et al., 2007).

Identification of isolated microorganisms

Bacterial colonies on NA, MRS, MCA were examined by microscopy, tested for gram staining and other biochemical tests. Colonies with a green metallic sheen on EMB plates were picked and purified and identified as *E. coli*. Yeast colonies on YEPD agar were examined by microscopy. Pure cultures were kept on appropriate agar slants.

Characterization of isolates

The bacterial colonies were differentiated first on the basis of colonial morphology followed by microscopic examination after gram staining. Gram and catalase tests were carried out for presumptive lactic acid bacteria identification. Gram staining followed the procedure of Pollack et al. (2002).

Biochemical tests including growth at various temperatures (10, 15 and 45°C), tolerance to different salt levels (2, 4 and 6.5% NaCl), hydrolysis of arginine and production of acid and gas from glucose were carried out to characterize the isolates as described by Olutiola et al. (1991) and Pollack et al. (2002).

Test for milk digestion (Protease production)

Isolated *E. coli* colonies were tested for protease production by streaking into solid skimmed milk agar medium (Skimmed milk powder 50 g/l; nutrient agar 28 g/l). Incubation was done at 35°C for 3 days. Proteolytic activity was noted as a zone of clearing around the stab (Rajmohan et al., 2002) or after flooding the plates with mercuric chloride solution (mercuric chloride, 15 g; Conc. HCl, 20ml; distilled water, 100 ml). Unhydrolysed casein form white opaque precipitate, clear zones indicate casein hydrolysis (Olutiola et al., 1991). Colonies with clear zones were picked for protease characterization.

PROTEASE ENZYME PREPARATION

Production of protease

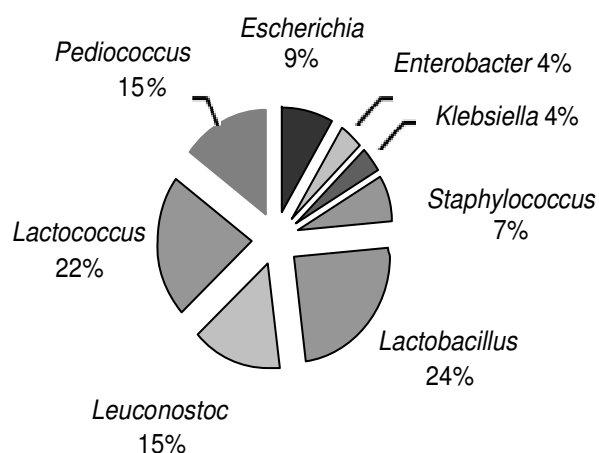
Each isolate was grown in semi synthetic medium containing 10 g/l casein - peptone, 5 g/l NaCl, 3.15 g/l Na₂HPO₄ and 1.5 g/l NaH₂PO₄ for 24 h at 37°C (Tondo et al., 2004). The culture supernatant obtained by centrifugation at 10 000 x g for 5 min, were used as crude enzyme preparation (Tondo et al., 2004). Protease activity was measured by the modified azocasein assay of Sacherer et al., (1994).

Protease assay

Reaction mixture contained 0.5 ml azocasein (0.5% w/v) dissolved in 0.1 M citrate buffer, pH 6, by boiling for 15 min in a water bath and then filtered, 0.5 ml 0.1 M citrate buffer, pH 6 and 0.1 ml culture supernatant fluid (Rajmohan et al., 2002). The reaction mixture was incubated at 37°C for 30 min, then stopped with 1.1 ml 10% (w/v) trichloroacetic acid (TCA) and left on ice for 15 min. The samples were clarified by centrifuging at 5000 x g for 10 min and optical density readings of the supernatant fluid measured at 600 nm in a colorimeter (Jensway, Ersex, UK). 1 unit of enzyme activity is defined as the amount of protease required to produce an absorbance increase of 0.01 under the described assay conditions. The blank contained azocasein with citrate buffer (De Azeredo et al., 2006).

Table 1. Number and distribution of groups of Isolated microorganisms.

Groups	Number of organisms (x10 ⁴ cfu/ml)		
	12 h	14 h	18 h
Aerobic counts	250	185	68
Lactic acid bacteria	149	230	268
Enterobacteria	30	35	30
<i>E. coli</i>	30	75	20
<i>Salmonella/Shigella</i>	-	-	-
Yeasts	10	28	68

**Figure 1.** Percentage of bacteria genera isolated from wara.

CHARACTERIZATION OF ENZYME

Effect of pH on protease activity

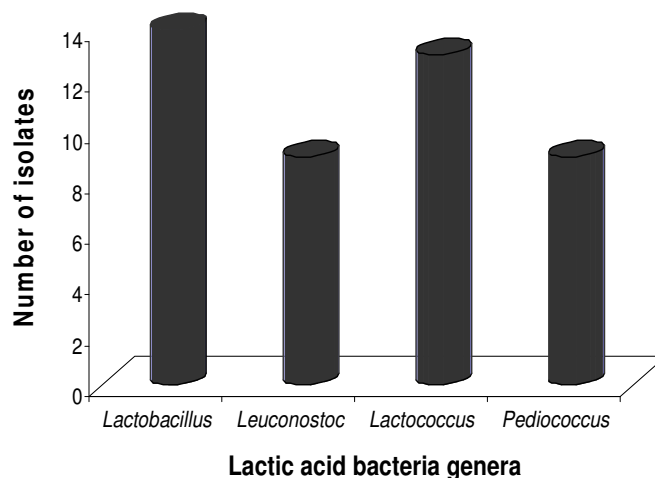
This was determined with 0.1 M citrate phosphate buffer at varying pH values ranging from 5.0 - 7.8. 0.5 ml casein mixture was boiled for 15 min and then filtered. 0.5 ml of 0.1M citrate phosphate buffer (at the different test pH values) was added to 0.1 ml culture supernatant fluid and the mixture incubated at 37°C for 30 min. The assay proceeded as described above.

Effect of inhibitors

An overnight culture was grown and centrifuged at 5000 x g for 20 min. The supernatant fluid was pre-incubated at 27°C for 1 h at pH 7 with sodium-EDTA salt dissolved at different concentrations (0, 5, 10, 15, 20 and 25 10 mmol/l). A protease assay was performed as described above with a control containing citrate buffer without inhibitor.

Effect of temperature

The effect of temperature on the activity of the enzyme was determined. The reaction mixtures (0.5 ml casein, 0.5 ml 0.1M

**Figure 2.** Occurrence of lactic acid bacteria genera identified from wara.

citrate phosphate buffer and 0.1 ml culture supernatant fluid) were incubated for 30 min at 40 to 80°C, respectively. Protease activity was determined as described above (Lee et al., 1997).

Statistical analysis

All experiments were performed in triplicates and the mean values of each experiment were used as data. A statistical analysis (ANOVA) was carried out. Significance was set at the 5% level of probability.

RESULTS

Table 1 shows the number and distribution of groups of organisms isolated from the wara samples. At 12 h (immediately after production), Total aerobic count (TAC) was 250 x 10⁴ cfu/ml, LAB counts was 149 x 10⁴ cfu/ml, *E. coli* count was 30 x 10⁴ cfu/ml Yeast cells were 10 x 10⁴ cfu/ml. TAC decreased to 185 x 10⁴ cfu/ml at 14 h and to 68 x 10⁴ cfu/ml at 1800 h, but LAB count increased to 230 x 10⁴ cfu/ml at 14 h and 268 x 10⁴ cfu/ml at 18 h. *E. coli* was most abundant (75 x 10⁴ cfu/ml) at 14 h but reduced to 20 x 10⁴ cfu/ml at 18 h. The number of yeast cells kept increasing, it was 28 x 10⁴ cfu/ml at 14 h and increased to 68 x 10⁴ cfu/ml at 18 h. No *Salmonella* or *Shigella* was isolated throughout the sampling times though they were screened for.

Eight genera of bacteria were isolated from wara samples. *Lactobacillus* was the highest occurring genus (24%), *Leuconostoc* and *Pediococcus* both occurred at 15%. *E. coli* occurred at 9%, *Staphylococcus* 7%, *Klebsiella* and *Enterobacter* occurred at 4% each (Figure 1). The occurrence of lactic acid bacteria genera isolated from wara (Figure 2). 13 isolates of *Lactobacillus* and 8 each of *Leuconostoc* and *Pediococcus* were identified. 12

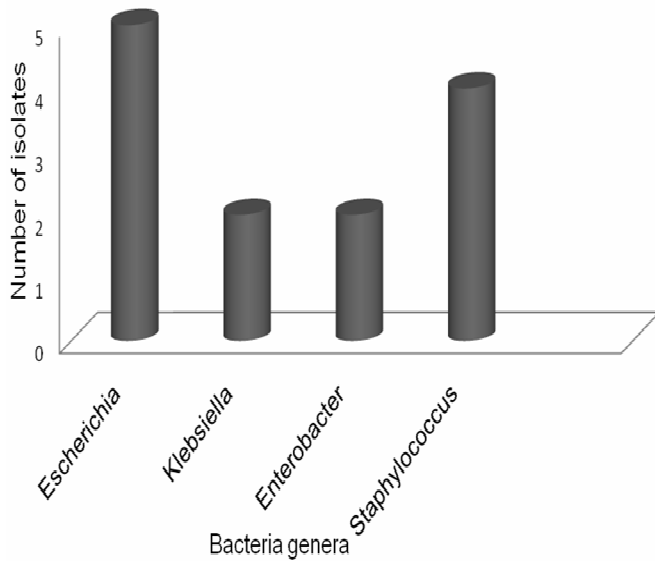


Figure 3. Occurrence of genera of pathogenic bacteria identified from wara.

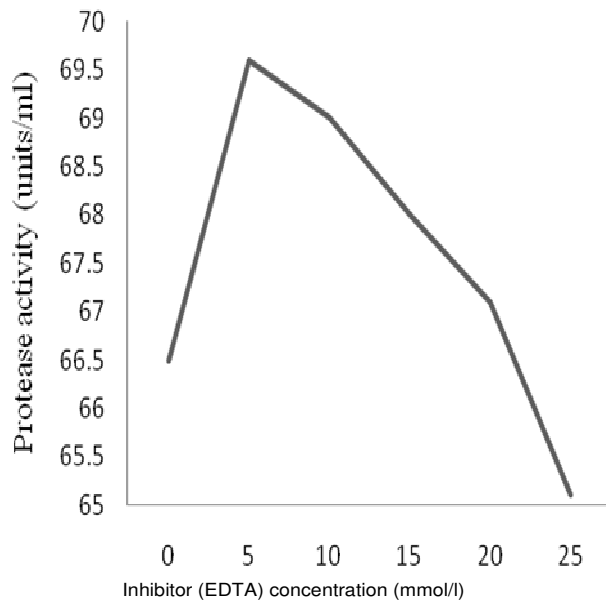


Figure 4. Effect of inhibitor concentration on protease activity of *E. coli* isolated from wara.

isolates of *Lactococcus* were identified. The presence of faecal coliforms especially *E. coli* shows the poor production conditions of food items. 5 strains of *E. coli* were identified. *Klebsiella* and *Enterobacter* had 2 isolates each identified (Figure 3). 4 *Staphylococcus* isolates were identified from wara. A protease of the *E. coli* isolated was partially characterized by studying the effects of temperature, pH and concentration of EDTA as an inhibitor. Figure 4 shows that the protease activity was

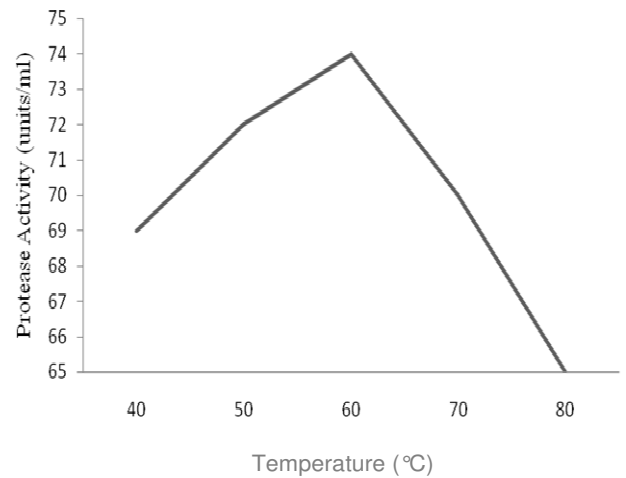


Figure 5. Effect of temperature on protease activity of *E. coli* isolated from wara.

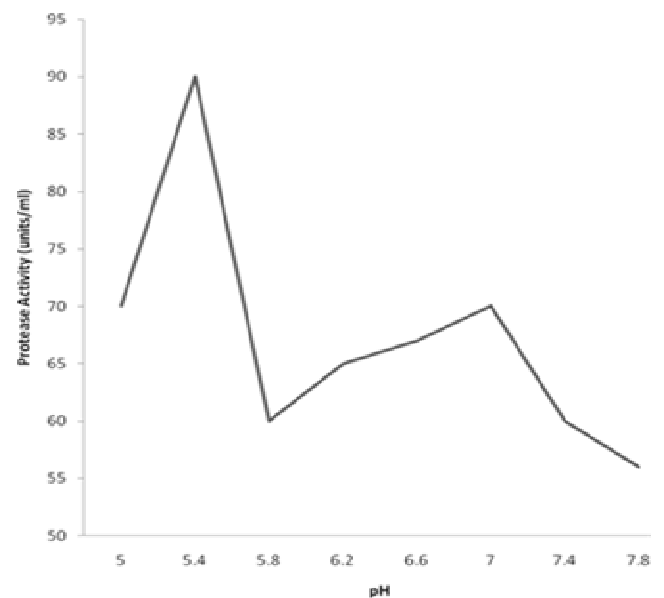


Figure 6. Effect of pH on protease activity of *E. coli* isolated from wara.

highest (69.5 units/ml) at EDTA concentration of 5 mmol/l. In the absence of EDTA, the activity was 66.5 units/ml. The activity fell sharply at 10 mmol/l and continued declining till 65 mmol/l. Effect of temperature on the protease activity was studied. At 40°C, the protease activity was 69 units/ml, the activity peaked at 60°C (74 units/ml) and fell sharply to 65 units/ml at 80°C as shown in Figure 5. At pH 5.4, the activities peaked at 90 units/ml and fell to 55 units/ml at pH 5.8. The protease activity increased to 70 units/ml at neutral pH of 7 but began to decline as from pH 7.4 (Figure 6).

DISCUSSION

Wara samples sold in 3 different locations within Iwo town were tested for the bacterial load, presence of faecal contaminations and a protease of the faecal coliform *E. coli* isolated was partially characterized. The presence of Lactic acid bacteria (LAB), Enterobacteria and yeast indicate that the *wara* was not sold sterile. LAB had the highest percentage occurrence (76%) followed by Enterobacteria (17%) and Staphylococci (7%). 4 genera of LAB were isolated. They were *Lactobacillus*, *Lactococcus*, *Leuconostoc* and *Pediococcus*. This is in line with the work of Guessas and Kihal (2004) that isolated *Lactobacillus* and *Leuconostoc* from raw goats' milk. LAB improve food quality and also play an important role in preventing the growth of undesirable bacteria. Steinkraus (1996) proposed that LAB create an acidic environment conducive to proliferation of yeasts, while yeasts provide growth factors such as vitamins and amino acids for LAB. Many researchers have reported the simultaneous increase in the numbers of both LAB and yeasts in indigenous fermented foods (Lu et al., 2008; Odunfa and Adeyeye, 1985). In this study, the yeast counts were present at the 3 sampling periods. It is evident that yeast strains which have activities of amylase, protease and lipase will have an impact on the textural and taste profile of *Wara*.

Coliforms are known to be acid intolerant (Steinkraus, 1996). The coliforms were present in increasing amounts probably because this food is not a fermented food though LAB were present. Viganò et al. (2007) studied 300 samples of ready-to-eat food in Tanzania. 58% Coliforms were isolated and 98% in milk samples. *E. coli* was the most frequently isolated species. The results showed that the samples contained *E. coli*. It is a consistent inhabitant of the human intestinal tracts and the regular presence of the bacterium in the human intestine and faeces has led to tracking the bacterium in nature as an indicator of faecal pollution. Through this, it means wherever *E. coli* is found, there may be faecal contamination (Todar, 2008). The methods of transportation, handling and sale of milk and milk products are not hygienic/sterile hence unclean hands of workers, poor quality of materials used and water supplied for washing utensils could be the source of accelerating the bacterial contamination of milk products (Bhat et al., 1984). It has been reported that contamination of raw milk and cheese poses a significant risk to humans (Wells et al., 1991). The isolation of non-pathogenic *E. coli* from cheeses (Aureli et al., 1992) also demonstrates that contamination occurs at some point during cheese production and processing. Retail surveys by Aureli et al. (1992) in Italy of soft and semi cheeses found *E. coli* in 40 - 50% of these cheeses demonstrating that a mode of contamination of *E. coli* exists during production or processing.

During the course of this work, it was observed that

wara was not dispensed under sterile/standard hygienic conditions because the producers did not practice sanitary procedures such as disinfection of hands and sterilization of equipment/materials. Materials used for the production of *wara* were under unhygienic conditions and in this manner, domestic animals and houseflies had access to bowls, Sodom apple leaves and the uncovered raw milk. Even after production, the *wara* is covered and opened intermittently to air with the same spoon used to scoop and sell throughout the day without any form of washing or sterilization before the next sale. Hence there could be faecal contamination of *wara* due to this careless condition/environment. It has been reported that pasteurization was effective for the eradication of *E. coli* without requiring the more vigorous sterilization (Kalab, 1992).

From the present work, it was observed that *E. coli* fermented both D-glucose and lactose producing acid and gas. This may cause rapid spoilage of *wara* and reduce the shelf life. The strains of *E. coli* obtained from this work secrete proteases to hydrolyze the peptide bonds in proteins and therefore break the proteins down (Hooper, 2002). Proteases are also a type of exotoxin which is a virulence factor in bacteria pathogenesis. Exotoxins are susceptible to antibodies produced by the immune system, but many exotoxins are so toxic that they may be fatal to the host before the immune system has a chance to mount defenses against it. *E. coli* exotoxic proteases destroy extracellular structures (Hooper, 2002). Room temperature, high water activity and a pH of > 4.6 provides an environment conducive to pathogen growth. The optimal pH for the enzyme studied was 5.4 and optimal temperature was 60°C. Many reports explained the problems caused by thermal-resistant proteases and lipases in milk processing (Boor et al., 1998; Craven and Macauley, 1993), but only few characterized specific microorganisms and their respective enzymes.

The highest protease activity was observed at 60°C meaning that this protease is heat stable - this agrees with the results of Banerjee et al. (1999) who found out that the optimal temperature of a protease of *Bacillus* was 60°C and this temperature was described for other *Bacillus* proteases. Thermostable proteases are advantageous in some applications because higher processing temperatures can be employed, resulting in faster reaction rates, increase in the solubility of nongaseous reactants and products and reduced incidence of microbial contamination by mesophilic organisms (Zeikus et al., 1998). The enterotoxin produced by *E. coli* has been reported to be heat labile which is similar to the cholera toxin (Todar, 2008). Protease activity peaked at 5 mmol/l of EDTA. The activity declined sharply (that is, inhibition) as concentration increased. Thys et al. (2004) observed that the protease produced by *Micobacterium* sp was inhibited by EDTA at a concentration of 5 mM. This demonstrates

that the enzyme is a metallo-protease and not a serine protease (Rajmohan et al., 2002). Sodium-EDTA is a metalloprotease inhibitor and has been reported to inhibit proteases in *E. coli* and *S. aureus* (Ulvatne et al., 2002). Proteases are of vital importance to all bacteria. Juretic et al. (1989) have suggested that one of the bacterial resistance mechanisms against magainins is mediated by proteases present on the microbial surface. Protease of the serine-, cysteine and metallo types are widely spread in many pathogenic bacteria, where they play critical functions related to colonization and evasion of host immune defenses, acquisition of nutrients for growth and proliferation, facilitation of dissemination, or tissue damage during infection. Bacterial protease inhibitors constitute an interesting possibility due to the fact that many specific and ubiquitous proteases have recently been characterized in some detail in Gram positive and Gram negative pathogens. Unfortunately, few potent specific inhibitors for such bacterial proteases have been reported (Supuran et al., 2001).

The wara was bought from hawkers who prepared it themselves using traditional methods and basic equipment. There is a need for further studies on standardizing the production methods and the seasonal prevalence of these pathogenic bacteria in this food commodity. Data from the current study on wara cheese will be helpful to public health professionals to identify this as a high risk food item. These pathogenic bacteria species are foodborne and their presence in ready-to eat food illustrates the need to keep a careful watch over which pathogens are causing human disease, determine where they are coming from and then develop methods to control them.

Conclusively, regular sterilization of dairy equipments, washing of utensils and proper hygiene during production of wara are some of the preventive measures that could be applied to check contamination of wara by *E. coli* and other coliforms. This can be achieved by educating the major distributors of raw milk and the wara producers on the benefits of disinfection and sterilization of the equipments. It is then recommended that before distribution for sales, wara should be screened for the presence of the different strains of pathogenic *E. coli* before being allowed to be sold.

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