

**PJN**

ISSN 1680-5194

PAKISTAN JOURNAL OF  
**NUTRITION**

**ANSI***net*

308 Lasani Town, Sargodha Road, Faisalabad - Pakistan  
Mob: +92 300 3008585, Fax: +92 41 8815544  
E-mail: [editorpjn@gmail.com](mailto:editorpjn@gmail.com)

## Occurrence of *E. coli* O157:H7 and *Listeria monocytogenes* and Identification of Hazard Analysis Critical Control Points (HACCPs) in Production Operations of a Typical Tropic Cheese 'Wara' and Yoghurt

Victoria O. Adetunji and Mary I. Arigbede

Veterinary Public Health Unit, Department of Veterinary Public Health and Preventive Medicine, University of Ibadan, Ibadan, Oyo State, Nigeria

**Abstract:** Critical Control Points (CCPs) associated with the processing of cheese in five local cheese factories and yoghurt in two conventional processing factories, located in Ilorin, Kwara state, Nigeria were identified using flow diagrams and microbial assay for detection of *E. coli* and *Listeria monocytogenes*. Fifty-eight (58) samples were collected along the processing lines. The CCPs identified were reception of raw milk, addition of coagulant, curdling point and cheese in moulds for cheese processing, while for yoghurt processing, powdered milk, pasteurization, addition of inoculum, fermentation and the finished product were the CCPs. In all the factories evaluated, *E. coli* and *Listeria monocytogenes* were observed to be present throughout the processing line with the finished product containing a significant level ( $p < 0.05$ ) of these organisms which exceeded international standard. This depicts the inefficient method of processing of these ready to eat foods. Thus, presenting a risk of outbreak of infection caused by these microorganisms in the state evaluated.

**Key words:** Cheese, yoghurt, *Listeria monocytogenes*

### INTRODUCTION

Wara is an unripened cheese consumed in several parts of West Africa; the cheese is prepared by coagulating fresh cow milk with the leaf extract of the Sodom apple (*Calotropis procera*) or pawpaw (*Carica papaya*). Wara processing involves the use of rudimentary equipment, in many cases starter cultures are not used as processing conditions are not normally standardized or optimized. Although very recently an alternative coagulant "lemon juice" was introduced into the processing of 'wara' soft cheese by Adetunji *et al.* (2007) to reduce the microbial load.

Yoghurt is a fermented milk product produced by the acidification of milk, which results into changes in the physical and chemical properties of the yoghurt (Tamime and Deeth, 1980). Recently, "yoghurt markers" have been urged to produce yoghurt under controlled conditions (Tamime and Robinson, 1985).

Several microorganisms have been incriminated to be present in raw milk and milk products (cheese and yoghurt) that are of public health importance. They are capable of causing food borne illnesses after ingestion of contaminated milk of these products, these organisms ranges from; viruses, rickettsia, bacteria, protozoan and parasitic organisms to their toxins (Kaplan and Bertagna, 1955). Among the bacteria, *E. coli* and *L. monocytogenes* are important indicators of contamination of milk and milk products, thus resulting

in coliform infections; which presents as gastroenteritis and listeriosis respectively. Outbreak of listeriosis has been linked to the consumption of cheese in many parts of the world (Gellin and Broome, 1989; Goulet *et al.*, 2001; Makino *et al.*, 2005; Wehr, 1989).

Human listeriosis is largely attributable to food borne transmission of the microorganism (Meyer-Broseta *et al.*, 2003; Bemrah *et al.*, 1998; McLauchin *et al.*, 2004). In recent years, several outbreaks or cases of listeriosis associated with the consumption of contaminated food products by *Listeria monocytogenes* have been reported (McLauchin *et al.*, 1990; Lyytikainen *et al.*, 2000). In majority of cases, Mild symptoms including diarrhea, fever, headache and malagia are observed (FAO/WHO, 2004), but in case of invasive listeriosis, severe symptoms including septicemia, meningoencephalitis, abortion and stillbirths are seen in humans and animals, primarily in the risk groups are; pregnant, newborn and immunocompromised individuals (Pearson and Marth, 1990; Meyer-Broseta *et al.*, 2003). Other cases of listeriosis have been associated with the consumption of wide variety of foods including; dairy products especially cheese (Goulet *et al.*, 2001; Makino *et al.*, 2005), meat and fish products and ready to eat foods (Meyer-Broseta *et al.*, 2003; McLauchin *et al.*, 2004).

Although the first confirmed case of human *L. monocytogenes* infection in Nigeria was reported in

1982 (Onyemelukwe *et al.*, 1983), food borne listeriosis has not been much documented. However, recent reports have confirmed the presence of *Listeria monocytogenes* in milk and milk products processed in Nigeria including ice cream, fermented milk and local butter (Adetunji *et al.*, 2003).

*Listeria monocytogenes* is ubiquitous in farm and food industrial environment where it frequently contaminate foods including; a wide variety of dairy products, vegetables, fish and meat products (Bemrah *et al.*, 1998; Pak *et al.*, 2002; Meyer-Broseta *et al.*, 2003). *Escherichia coli* O157:H7 was first recognized as a human pathogen in 1982 during the investigation of two outbreaks of bloody diarrhea in Oregon and Michigan (USA) (Riley *et al.*, 1983). Most food borne outbreaks involving this organism have been associated with consumption of foods from bovine origin; including ground beef and raw milk (Griffin and Tauxe, 1991). Also, low levels of *E. coli* O157:H7 surviving in moderately acid products, such as apple juice and cider, yoghurt and mayonnaise, have been the cause of several outbreaks of hemorrhagic colitis (Besser *et al.*, 1990; Morgan *et al.*, 1993; Zhao *et al.*, 1993; Centers for Disease Control and Prevention, 1995).

The contamination of raw and pasteurized milk by these microorganisms suggests the possibility of their transmission to the human population via consumption of milk products. Even yoghurt, which has always been considered safe because of its acidic nature, was involved in a fatal infection (Morgan *et al.*, 1993). Oral transmission of *E. coli* (strain O157:H7) present in fermented foods e.g. cheese and yoghurt suggests that, pH reduction and or competitive inhibition by starter culture may not ensure the elimination of this pathogen from fermented food products (Dineen *et al.*, 1998). Several authors have demonstrated the survival of these microorganisms in dairy products over several weeks: yoghurt and colby, romano and feta cheeses (Hudson *et al.*, 1997), cheddar cheese (Mossel *et al.*, 1995), sour cream, buttermilk and cheese (Arocha *et al.*, 1992; Dineen *et al.*, 1998). It is known that *L. monocytogenes* is able to survive the manufacture and storage conditions of several cheeses (Yousef and Marth, 1990; Buazzi *et al.*, 1992; Erkmén, 2001; Carminati *et al.*, 2004; Manfreda *et al.*, 2005; Anonymous, 2006). These further confirms the potential health risks associated with post processing contamination of even low levels of *E. coli* O157:H7 (Dineen *et al.*, 1998) and *L. monocytogenes* in several dairy products, thus necessitating the need to critically identify the points where occurrence of contamination are likely to occur.

The Hazard Analysis Critical Control Point system (HACCP) aims to prevent food safety problems in several different points rather than finding them after they have occurred. In this system, processors develop plans, detailing critical points and document their

monitoring of these critical areas. Critical Control Points (CCP) are steps or procedures in production where food safety hazard are likely to occur if precautions are not taken, it is a rational approach to food systems. The objective of HACCP is to ensure that safe wholesome and unadulterated food reaches the consumer. It depends on process control throughout product life, identification of potential hazards and establishment of CCPs in the food systems to minimize the presence of unacceptable health risks.

The concept of HACCP is a preventive, structured, systematic and documented approach to ensure food safety (Buchana, 1990; Montarjemi *et al.*, 1996). In recent years, the hazards analysis critical control point concept has been proposed as the best approach to assure food safety. Inclusion of *L. monocytogenes* in the list of microorganisms subjected to HACCP has recently driven the search for detection methods suitable for its monitoring (Almeida and Almeida, 2000).

Generally, the production of cheese and yoghurt with desirable organoleptic characteristics safe for consumption, can only be assured when certain factors are continuously controlled and tested such as; the microbial quality of the raw milk, pasteurization of the raw milk prior to processing, prevention of recontamination after pasteurization of the milk and predominance of desirable microbial flora during storage (Zottola and Smith, 1993).

Despite the risk presented by *L. monocytogenes* and *E. coli* in dairy products (cheese and yoghurt), there are few studies on the incidence of this microorganisms at critical control points along the processing lines of these dairy products in Nigeria. Thus, having all these in mind, it is important to critically examine the processing stages of cheese and yoghurt to identify the critical control points that should be put into consideration using *L. monocytogenes* and *E. coli* as indicators.

The objective of this study was to identify the CCP of *L. monocytogenes* and *E. coli* occurrence along the processing lines of cheese and yoghurt at Ilorin Kwara state, Nigeria.

## MATERIALS AND METHODS

**Sample collection and laboratory procedure:** Cheese samples were collected from a Fulani settlement; Gaa Bolorunduro village at the University of Ilorin Kwara state, Nigeria. The yoghurt samples were collected from two factories namely; Factory F at Offa garage and Factory G at Oko-Erin both in Ilorin Kwara state, Nigeria. The raw milk used for processing was obtained from the white Fulani and Gudali local breeds of cattle.

Samples were collected at each step of yoghurt and cheese processing line. At each sampling point 10mls of milk or 10 g of cheese/powdered milk were drawn aseptically into sterile bijoux bottles. Samples were stored on ice and transported to the laboratory for

immediate analysis. The pH of samples was taken using a pH meter (vwr model 8000). Samples were homogenized in 10 mls of sterile 1% peptone water and serially diluted to  $10^4$ - $10^6$  appropriate dilutions were then surface plated on sorbitol macConkey agar for *E. coli* O157:H7 count and on Modified Oxford Agar (MOA) with antibiotic supplements (acriflavine, nalidixic acid and cycloheximide) (Becton, Dickinson and company) for isolation of *Listeria monocytogenes*. All plates were incubated at 37°C for 24 h, colonies were counted using a digital colony counter to evaluate the level of contamination and expressed in Log<sub>10</sub> cfu/ml or gm. With respect to *E. coli* organism, the *E. coli* O157:H7 strain was detected from the isolates by sub-culturing on Sorbitol Macconkay Agar (SMAC). Its presence was observed by the production of colourless colonies on the SMAC agar plates while the *Listeria monocytogenes* strains isolated were purified using the MOA incubating at 37°C for 24 h. Colonies were observed on agar plates with the production of black halo from esculin hydrolysis. Biochemical tests were carried out for further confirmation of the isolate according to Monica (1984).

**Serology:** Serological slide agglutination tests was carried out according to Seeliger and Holhne (1979) on all the isolates observed to be *Listeria spp* using commercially prepared antisera (Difco), *E. coli* O157:H7 antiserum to confirm the *E. coli* O157:H7 among the *E. coli* and polyserotypic antiserum in subcultures of the test *Listeria monocytogenes* inoculum.

**Determination of CCPs:** The processing stages were represented using flow diagrams, samples were taken along the processing line for microbial assay to determine the level of contamination at each stage of processing. This was to determine the stages of processing where maximal contamination occurred and hence where CCPs should be effected.

**Statistical analysis:** The results analyzed using the Statistical Package for Social Sciences (SPSS) database (2003). From these, data descriptive summaries were carried out (Marsh and Martin, 1999), the relationship between each stages and correlation between factories were tested for.

## RESULTS

**Serology:** Five of the *E. coli* isolated was positive to *E. coli* O157:H7 antiserum the isolates were majorly from stage IV of processing (Finished product) of cheese factory B, C, D and stages III and IV of yoghurt factory G. For the *L. monocytogenes* isolates, twelve isolates were positive to the *L. monocytogenes* polyserotypes antiserum: stage I of factories; A, B and D, stage II of factories C, D and E, stage III of factory; D, stage IV of factories; A, C, D and E.

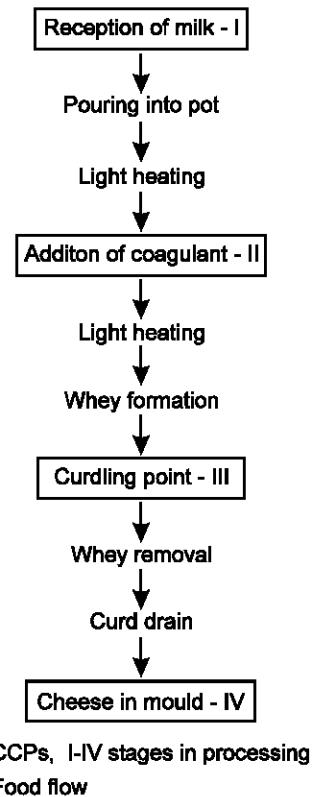


Fig. 1: Flow chart showing 'wara' (cheese) processing and CCPs in factories A-E

**Flow diagrams for cheese and yoghurt processing:** Flow charts and CCPs identified along the processing line of cheese and yoghurt in the factories evaluated are presented in Fig. 1 and 2. Microbial assay of samples collected at various stages of processing, to determine the extents of contamination along the processing lines are shown in the Table 1 and 2.

The microorganisms survived throughout the processing stages, at stage III (curdling point) the microbial counts decreased and later increased in the final stage of processing (stage IV) in all the factories evaluated (Fig. 3 and 4).

For yoghurt processing, the factories had effective pasteurization process, which led to the reduction in the counts of *L. monocytogenes* and *E. coli* at the stage II of processing however, pasteurization process in factory G was not effective against *E. coli*. Post pasteurization contamination from inoculum may account for the increase in counts observed in stage III, there was increase in counts till the final stage in factory G, although, there was a decrease in counts in factory F after fermentation (Fig. 5 and 6).

## DISCUSSION

From the study, the raw milk used in processing of cheese and yoghurt was contaminated with the indicator

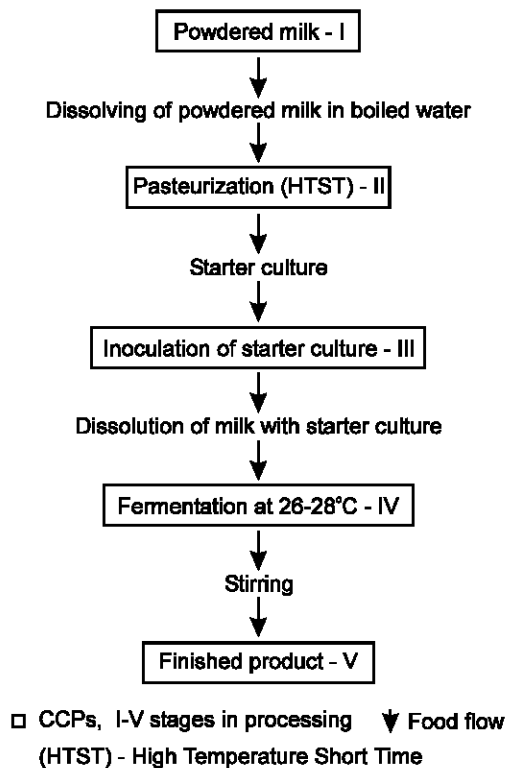


Fig. 2: Flow chart showing yoghurt processing and CCPs in factories F and G

organisms. Contamination of raw milk by *L. monocytogenes* and *E. coli* can occur from mastitic animals and from environmental sources such as silage and soil during milking. The presence of *L. monocytogenes* in improperly fermented silage has been documented (Fenlon, 1986) thus, animal feed must be controlled. Additional sources of contamination can occur from handling milk enroute and at the dairy factory.

Raw milk should be obtained from healthy animals under hygienic conditions, cleaning and antiseptics of udder and hands of the milkmen before and after milking with appropriate antiseptics constitute preventive measures. The reception of raw milk is a stage to be critically considered because; the presence of any organism at this stage will help determine the extent of survival of the organism during the processing and at the end of processing. Thus, time and temperature at this stage of processing should be established and systematically monitored to prevent possible hazard.

For the cheese processors (Factory A-E), the increase in microbial load (Fig. 3 and 4) explain the fact that the temperature used was too low for microbial destruction or death which lead to their continued growth and survival. The coagulation stage was considered as a CCP, the use of extract from Sodom apple leaves to achieve coagulation could serve as a means of introducing contaminant into the cheese this accounted for the increase in microbial growth in stage II of cheese processing. Therefore some preventive measures should be taken at this stage such as; washing the leaves thoroughly before extraction of the juice, monitoring the temperature of the milk and controlling the development of acidity.

The reduction in microbial count at the curdling point of cheese in all the factories is similar to results obtained by Adegoke *et al.* (1992), who observed reduction in population of total aerobes at the curdling point using the manufacture of wara. Cheese in mould was considered CCP because of the possibility of recontamination of the finished product from utensils used during the processing or workers handling the products.

Emphasis have been made that, the contamination of cheese or end-products with *L. monocytogenes* is most likely due to; contamination during the ripening stage (Pak *et al.*, 2002), post process contamination from environmental sources, cross-contamination in the dairy

Table 1: Microbial assay of *E. coli* and *L. monocytogenes*, along the processing line of cheese in factories A-E

Stages	Factory				
	A	B	C	D	E
<b><i>E. coli</i></b>					
I	7.037±0.064 <sup>Aabc</sup>	7.329±0.109 <sup>Ab</sup>	6.976±0.006 <sup>Aabc</sup>	6.490±0.302 <sup>Aac</sup>	7.342±0.215 <sup>Aab</sup>
II	9.114±0.132 <sup>Ba</sup>	9.089±0.320 <sup>Ba</sup>	8.804±0.088 <sup>Ba</sup>	8.929±0.096 <sup>Ba</sup>	9.626±0.042 <sup>Cb</sup>
III	7.121±0.049 <sup>Ab</sup>	7.371±0.225 <sup>Ab</sup>	7.039±0.125 <sup>Ab</sup>	6.284±0.005 <sup>Ac</sup>	6.770±0.023 <sup>Ba</sup>
IV	9.211±0.077 <sup>Ba</sup>	9.347±0.207 <sup>Ba</sup>	9.148±0.099 <sup>Ba</sup>	9.260±0.077 <sup>Ba</sup>	9.329±0.059 <sup>Ca</sup>
<b><i>L. monocytogenes</i></b>					
I	6.211±0.132 <sup>Aa</sup>	6.239±0.151 <sup>Aa</sup>	6.632±0.100 <sup>Ab</sup>	6.716±0.017 <sup>ab</sup>	6.000±0.000 <sup>Aa</sup>
II	7.841±0.239 <sup>Ba</sup>	8.190±0.190 <sup>Bab</sup>	8.460±0.120 <sup>BbC</sup>	8.881±0.012 <sup>Bc</sup>	7.778±0.000 <sup>Bab</sup>
III	6.491±0.066 <sup>Aa</sup>	5.778±0.477 <sup>Aa</sup>	6.681±0.234 <sup>Aa</sup>	6.640±0.193 <sup>Aa</sup>	5.952±0.349 <sup>Aa</sup>
IV	8.709±0.0205 <sup>CaB</sup>	8.322±0.021 <sup>BabC</sup>	8.025±0.122 <sup>BbD</sup>	8.831±0.830 <sup>BaE</sup>	8.000±0.000 <sup>Bb</sup>

Values represents: mean±standard error of mean in log<sub>10</sub> CFU/ml. Processing stages: I = Raw milk, II = Addition of coagulant, III = Curdling point, IV = Cheese in mould. Values not containing a common uppercase letter in the same column are significantly different with respect to the factory along the processing stages. Values not containing a common lowercase letter in the same row are significantly different with respect to the stage across the factory

Table 2: Microbial assay of *E. coli* and *L. monocytogenes*, along the processing line of yoghurt in factories F and G

Stages	Indicator organism			
	<i>E. coli</i>		<i>L. monocytogenes</i>	
	F	G	F	G
I	6.577±0.046 <sup>a</sup>	6.489±0.042 <sup>a</sup>	5.778±0.000 <sup>a</sup>	0.999±0.000 <sup>a</sup>
Sig.	0.239	0.294	NC	NC
II	0.999±0.000 <sup>b</sup>	7.097±0.004 <sup>a</sup>	0.999±0.000 <sup>b</sup>	0.999±0.000 <sup>a</sup>
Sig.	0.000	0.000	NC	NC
III	8.954±0.000 <sup>d</sup>	7.228±0.046 <sup>a</sup>	8.146±0.000 <sup>d</sup>	5.452±0.151 <sup>b</sup>
Sig.	0.662	0.699	0.046	0.140
IV	7.205±0.006 <sup>c</sup>	7.140±0.693 <sup>a</sup>	4.778±0.000 <sup>c</sup>	7.602±0.000 <sup>c</sup>
Sig.	0.120	0.232	NC	NC
V	9.244±0.151 <sup>e</sup>	9.072±0.055 <sup>b</sup>	7.991±0.088 <sup>a</sup>	7.690±0.389 <sup>c</sup>
Sig.	0.397	0.448	0.529	0.579

Values represents: means ± standard error of mean in log<sub>10</sub> CFU/ml, Sig. = Level of significance, NC = Not Computable. Processing stages: I = Powered milk, II = Pasteurization, III = Inoculation of starter culture, IV = Fermentation, V = Finished product. Values not containing a common lowercase letter in the same row are significantly different with respect to the factory along the processing line. Only stage II is significantly different (0.000) in all the stages of processing for *E. coli* in the yoghurt factories

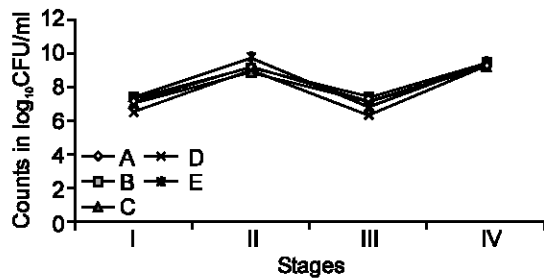


Fig. 3: Charts showing growth and survival of *E. coli* along the processing line of cheese in the five factories

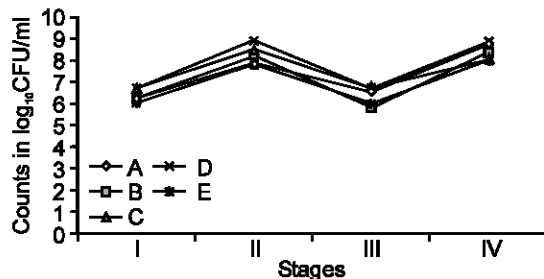


Fig 4: Charts showing growth and survival of *L. monocytogenes* along the processing line of cheese in the five factories

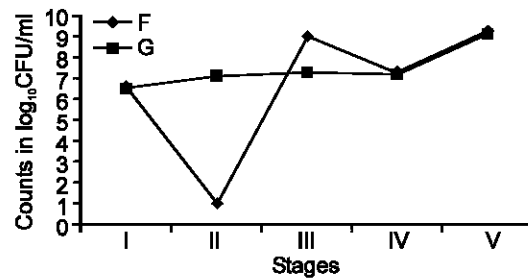


Fig. 5: Charts showing growth and survival of *E. coli* along the processing line of yoghurt in the two factories

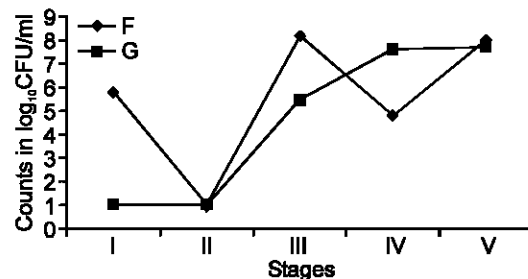


Fig. 6: Charts showing growth and survival of *L. monocytogenes* along the processing line of yoghurt in the two factories

plant and/or retail stores, inadequate processing (FAO/WHO, 2004; Pak *et al.*, 2002; Rudolf and Seigfried, 2001; Sagun *et al.*, 2001) and colonization of in retail stores (Sergelus *et al.*, 1997).

*L. monocytogenes* can survive a number of cheeses-making processes and can remain viable in the final product for a considerable length of time (Griffiths, 1989). Therefore, the high incidence of these indicator organisms observed in cheese in this study, with

respect to the various stages of processing may be accounted for by, insufficient hygiene during milking and manufacturing process. In effective pasteurization is also a crucial cause of the survival of these organisms throughout the manufacturing process. Report has shown that, the production of microbiologically safe cheese depends on primarily pasteurization of milk, as well as strict periodic controls (Klinger and Rosenthal, 1997).

For the yoghurt processors (Fig. 5 and 6), factory F had an effective pasteurization process which led to the drastic reduction in microbial counts (<1.00 log) for the indicator organisms evaluated. It is expected that the microbial load should increase at the inoculation (stage III) stage due to the introduction of starter culture into the yoghurt, however the purity of the starter culture used cannot be guaranteed. Thus, serves as another means of recontamination of the yoghurt by these indicator organisms.

The reduction in counts at the end of the fermentation observed in factory F is in accordance with reports by (Pearson and Marth, 1990; Benkerroun *et al.*, 2003; Arques *et al.*, 2005), where it was observed that, the growth of lactic acid bacteria (especially, during fermentation) in turn produces bacteriocins which led to the reduction in counts of *L. monocytogenes* in cheese and yoghurt. Fermentation failure was observed in factory G; the temperature used (26-28°C) was quite low as compared to standard temperature of 43°C (short incubation method) or 30°C (longer incubation method). Hence, factory G should be monitored closely to ensure that sufficient, pure starter culture was inoculated and the milk was actually fermented. An increase in fermentation temperature and holding time would be suggested to factory F yoghurt processor to further achieve a decreased microbial growth.

Although, survival of *L. monocytogenes* in yoghurt depends on the acidity, the microorganism disappears when the pH falls to 3.5 (Cottin *et al.*, 1990). Among the dairy products, yoghurt has received the least attention, due to the fact that, high acidity and pasteurization involved in its processing are regarded to be effective barriers to the growth of pathogens including *L. monocytogenes* (Benkerroum *et al.*, 2003). For the final stage (V) increased microbial growth was observed this could have resulted from recontamination of the product during packaging handling and failure to maintain refrigerator temperature during storage before sales or after sales to retail stores.

The temperature of many home refrigerators ranges from 7-10°C (Rhodehamel, 1992), the increase in refrigeration temperature could lead to a high risk, if the yoghurt was previously contaminated by the microorganism (Bachrouri *et al.*, 2005). Thus, the presence of *L. monocytogenes* and *E. coli* in the finished yoghurt product reveals, the failure in the processing stages and the survival of the organism throughout the processing in factories F and G as seen in Fig. 6.

Our results, coincides with report by Arocha *et al.* (1992) and Reitsma and Henning (1996) where it was observed that *E. coli* O157:H7 can survive and even grow during the manufacturing of cottage cheese, cheddar cheese and yoghurt (McDonough *et al.*, 1991; Morgan *et al.*, 1993) Unlike reports from Massa *et al.* (1997) and Bachrouri *et al.* (2005), who observed that *E. coli*

O157:H7 grew during the fermentation of milk into home-made yoghurt, adding that, the temperature of fermentation has some influence on the behaviour and survival of *E. coli* O157:H7 during both coagulation and refrigeration periods. *E. coli* O157:H7 survived the processing stages in yoghurt (factory G) with a decreased growth during fermentation, this affirms the ability of the organisms to survive (Weagant *et al.*, 1994) in acidic environment or adapt in strongly acidic environment (Hill *et al.*, 1995). The prevalence of *L. monocytogenes* in the samples collected is in agreement with reports by Fenlon and Surendran (1989) and Gabis *et al.* (1989) where the occurrence of *L. monocytogenes* in raw milk and manufacturing plant was observed.

Based on data from the United States of America, the current allowable limit for *L. monocytogenes* in ready-to-eat foods is effectively from 0.04 CFU/g, a level that if consistently achieved will result in less than one case of listeriosis. The counts observed in our finished products was far more than this, However, the current risk assessment of food borne listeriosis indicates that as the limit increases from 0.04-1000 CFU/g, there will be a proportional increase in outbreak of food borne listeriosis (WHO/FAO, 2004).

From the study, the occurrence of *L. monocytogenes* and *E. coli* at the various stages of processing affirms, the need for CCP at these stages in order to control the level of contamination of these commonly consumed dairy products. A CCP in the dried milk (powdered milk), after pasteurization, will help to determine the effectiveness of pasteurization. The purity of the starter culture is highly considered has a major CCP. The fermentation stage should also be put into consideration since contaminating flora will *multiply*. Lastly, a CCP in the finished product will help assess the microbial quality of the finished product and potential hazards that can ensue after consumption of this product.

**Conclusion:** The results of this study demonstrates that consumers of these dairy products in Ilorin, Kwara state of Nigeria are at serious risk, since these indicator organisms; *L. monocytogenes* and *E. coli* were isolated from the finished product. In addition, all the factories evaluated showed inefficient method of processing in the reduction of growth of these organisms at the CCPs, this further explains the poor standard of processing in the dairy industries.

Therefore, efforts should be concentrated to control these organisms in dairy industries by; safe handling of raw milk, effective destruction of microorganism through heat processing and proper cleaning and sanitation. It is evident that, development and use of the HACCP program is needed urgently and should be enforced for all processing plants, going by the degree and level of contamination observed in this study.

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