Prevalence Of *B.cereus* In Uncontrolled Fermented Cow-Milk And The Influence Of pH And Temperature On Its Survival During Storage

Bello, S. [1*], Whong, C.M.Z.[2] And Abdullahi, I.O.[2]

1. Department Of Food Technology, P.M.B. 2021, Kaduna Polytechnic, Kaduna, Nigeria.
2. Department Of Microbiology, Ahmadu Bello University, Zaria, Nigeria.

---**ABSTRACT**--

A study was conducted to determine the prevalence of B.cereus in uncontrolled fermented milk (nono) sold on some streets of Kaduna town, Nigeria, and to study the influence of pH and temperature on its survival during storage. Forty-three samples of nono were purchased from 10 different sellers and plated unto sterile plates of nutrient agar for total aerobic plate count (TAPC) and Mannitol Egg yolk Polymyxin (MYP) agar for B.cereus isolation. Mean TAPC ranged from 4.4 ± 0.7 to 5.4 ± 0.8 log_{10} cfu/ml for the nono samples analyzed while mean B.cereus counts ranged from 0.3± 0.7to1.3± 0.8 log_{10} cfu/ml. A B.cereus strain (about 8.5 log_{10} cfu/ml) isolated from retail nono was inoculated into sterile nono samples following pH adjustment to 3.7, 4.3 (challenge samples), 3.8 and 4.2 (control samples). Samples were stored at refrigeration temperature (4-10°C) for 72 h, and at ambient temperature (26-28°C) and 37°C for 24 h. Samples were analyzed for B.cereus counts on sterile nutrient agar at intervals of 24 h. Bactericidal effects were observed for samples at lower pH levels of 3.7 and 3.8 at 24 h of refrigeration and which were sustained for up to 72 h at pH 3.7. B.cereus appeared to be uninhibited during storage at ambient (26-28°C) temperature and at 37°C at all pH levels at the end of 24 h. The presence of B.cereus in nono, a widely consumed ready- to- eat product is of health significance owing to its ability to cause food poisoning. Findings of this study suggest B.cereus cells are suppressed in heat-treated nono during storage at refrigeration at pH level of 3.7 for up to 72 h.

I.   INTRODUCTION

Food poisoning is defined as an illness caused by the consumption of food or water contaminated with bacteria and/or their toxins, or with parasites, viruses, or chemicals[1]. The symptoms, varying in degree and combination, include abdominal pain, vomiting, diarrhoea, and headache; more serious cases can result in life-threatening neurologic, hepatic, and renal syndromes leading to permanent disability or death. Food-borne illnesses are caused by many different disease - causing pathogens that can contaminate foods, or by toxins produced by these pathogens that are present in food[2].*Bacillus cereus* or *B.cereus*, a gram positive, spore forming, rod shaped bacterium is known to cause a variety of non-gastrointestinal diseases[2].

The organism is widely distributed in the environment, mainly in the soil, from which it is easily spread to many types of foods especially those of vegetable origin, as well as meat, milks and dairy products [3,4,5,2,6]. *Bacillus cereus* can grow over a wide temperature range (8–55 °C), but it is not well suited to tolerate low pH values (minimum 5–6)[7].

Uncontrolled fermented milk or *nono* as it is called in northern Nigeria is a popular product produced by the womenfolk of Fulani herdsmen, and very popular especially around the northern part of the country. Its production generally involves storing of raw milk in calabashes overnight until it curdles, and then sold to consumers. In Nigeria, food vending is widely practiced in open places such as motor parks, offices and market places with little or no regard for regulations guiding the microbiological quality of such foods. Consumers and regulatory bodies most times portray a carefree attitude to the glaring hazard of improper handling and storage of ready-to-eat foods, hence effectively rendering such foods potentially hazardous for the large population of consumers that patronize them. There is therefore a perpetual need to assess microbial quality of ready –to- eat foods sold on Nigerian streets, in order to highlight the relevance of adhering to the use of standard techniques during preparation, storage and sale of such foods.

II.   METHODOLOGY

Isolation and enumeration of *Bacillus cereus*: A total of 43 samples of *nono* were purchased from 10 fulani milkmaids from Unguwar rimi, Unguwar Sarki, and Tudun Wada districts of Kaduna town. Samples were collected in sterile containers and transported in ice pack containers to the laboratory for analysis. Food samples were prepared by homogenizing 25ml of each sample in 225 ml of sterile 0.1% peptone water. The homogenates were then subjected to 10 fold serial dilutions in sterile 0.1% peptone water. From these 10 fold dilutions, 1 ml of dilutions 10^{-1}, 10^{-3}, and 10^{-5} were each plated on sterile plates of Mannitol Egg yolk Polymyxin Agar (MYP) (OXOID), a highly selective *Bacillus cereus* medium[8]. MYP plates of the food samples were incubated at 37°C for 24-48 h. Gram positive colonies that appeared surrounded by zones of clearance on red/pink backgrounds on the MYP plates were counted and recorded as presumptive *Bacillus cereus* counts[9]. Colonies were streaked on nutrient agar slants, incubated at 37°C for 24 h, and stored at 4°C until required for characterization. Stored, presumptive *Bacillus cereus* isolates obtained from food samples above were characterized using a Microgen[TM] *Bacillus* ID identification kit. This kit is a miniaturized biochemical identification system designed to identify mesophilic *Bacillus* species and related genera associated with food and beverage spoilage and food poisoning. Results recorded on the report forms generated an octal code for each isolate. These codes were then analyzed using the Microgen Identification System Software.

Enumeration of Aerobic Mesophilic bacteria: *Nono* samples were analyzed for Total Aerobic Plate Count (TAPC) by plating 1 ml of dilutions 10^{-1}, 10^{-3}, and 10^{-5} on to sterile nutrient agar plates (OXOID). The plates were incubated at 37°C for 24 h. Colonies were then counted and recorded as Total Aerobic Plate Count [10] as also shown in tables 1 and 2.

Growth and Enumeration of *B.cereus* in nono at Varying pH levels and temperature: *Nono* was purchased from Samaru market, Zaria, and tyndallized by heat treating at 63°C for 30 mins for a period of three days to sterilize the sample. A lower temperature was adopted to avoid coagulation of the fermented milk. The sample was then checked for sterility after which 200ml of sterile nono was then dispensed in each of four 250ml conical flasks. The pH levels were adjusted to 3.7, 3.8, 4.2 and 4.3 using 0.1M each of lactic acid and sodium hydroxide with a JENWAY 3150 series pH meter. Lactic acid was used to lower and sodium hydroxide to increase pH levels as required. Twenty millilitre aliquots of *nono* sample from each conical flask were then dispensed in each of 9 sterile glass bottles. The samples were then inoculated with an approximate cell count of 3×10^8 cfu/ml of *B. cereus* isolate F5M1 (isolated from retail *nono* as described above). Following inoculation, samples were divided into three sets, each set consisting of triplicate samples at pH levels of 3.7, 3.8, 4.2 and 4.3. One set was kept at optimum temperature (37°C) for 24 h, one at ambient temperature for 24 h and the last set at refrigeration temperature for 72 h.

III.   RESULTS AND DISCUSSION

B.cereus counts and TAPC of retail *nono* : Mean counts of *B.cereus* isolated from *nono* are shown in Table 1. *B.cereus* counts ranged from 0.3 ± 0.7 to 1.3 ± 0.8 $\log_{10}$ cfu/ml for samples. Mean TAPC also shown in Table 1, ranged from 4.4 ± 0.7 to 5.4 ± 0.8 $\log_{10}$ cfu/ml for the *nono* samples analyzed.Following biochemical characterization of presumptive *B.cereus* isolates, 30% (13 samples) of *nono* had *B.cereus* isolated from them. Statistical analysis using the one-way ANOVA (SPSS version 16) showed no significant differences in *B.cereus* counts (P=0.51) and TAPC counts (P=0.71) of *nono* samples. Level of Significance was set at P<0.05.

Effects of pH and temperature on B.cereus survival during storage in nono: Mean *B. cereus* counts decreased for all *nono* samples at 24 h of storage from initial levels of $8.5 \pm$ $\log_{10}$ cfu/ml to a lowest of 6.9 ± 0.3 $\log_{10}$ cfu/ml at pH 4.2 during refrigeration temperature (4-10°C) (Fig 1). Counts further decreased at 48 h for all samples to a lowest of 6.3 ± 0.1 $\log_{10}$ cfu/ml at pH 3.8. At 72 h, counts increased from their levels at 48 h in all samples except at pH 3.7, where counts decreased from 7.0 ± 0.2 $\log_{10}$ cfu/ml to 6.2 ± 0.6 $\log_{10}$ cfu/ml. Fig 1 also shows mean *B.cereus* counts in *nono* samples stored at ambient (26°C-28°C) and elevated (37°C) temperatures at different pH levels for 24 h. Mean counts generally increased from initial levels of 8.5 $\log_{10}$ cfu/ml for samples, except for samples at pH 4.2 (control), where mean counts decreased to 1.5 ± 0.4 $\log_{10}$ cfu/ml at the end of 24 h. Statistical analysis using one-way ANOVA revealed significant differences at 5% level (P<0.05), in *B.cereus* counts in *nono* samples during storage across the different temperatures used. Lowest mean counts were recorded for samples stored at refrigeration temperature (4-10°C) for 48 and 72 h, while highest value was recorded for samples stored at ambient temperature (26°C-28°C) and at refrigeration temperature (4-10°C) for 24 h. Similarly, significant differences were recorded for the above samples across the different pH levels used (P<0.05), with lowest counts for samples at pH 4.2, and highest counts at pH 3.8.

Table 1: Mean* Aerobic and presumptive *B. cereus* Counts (cfu/ml) of Retail *Nono*

Product name	Mean *B. cereus* count (log $_{10}$ cfu/ml)	Mean TAPC (log $_{10}$cfu/ml)
FM1	0.6± 0.9	5.4± 0.8
FM2	0.5± 0.6	5.0± 0.5
FM3	1.3± 0.8	4.8± 0.7
FM4	0.3± 0.7	4.6± 1.0
FM5	0.5± 0.7	4.4± 0.7
FM6	0.8± 0.9	4.9± 0.8
FM7	1.1± 0.8	4.9± 0.8
FM8	0.9± 1.0	4.8± 1.0
FM9	0.9± 1.0	5.2± 0.7
FM10	1.0± 1.1	5.3± 0.8

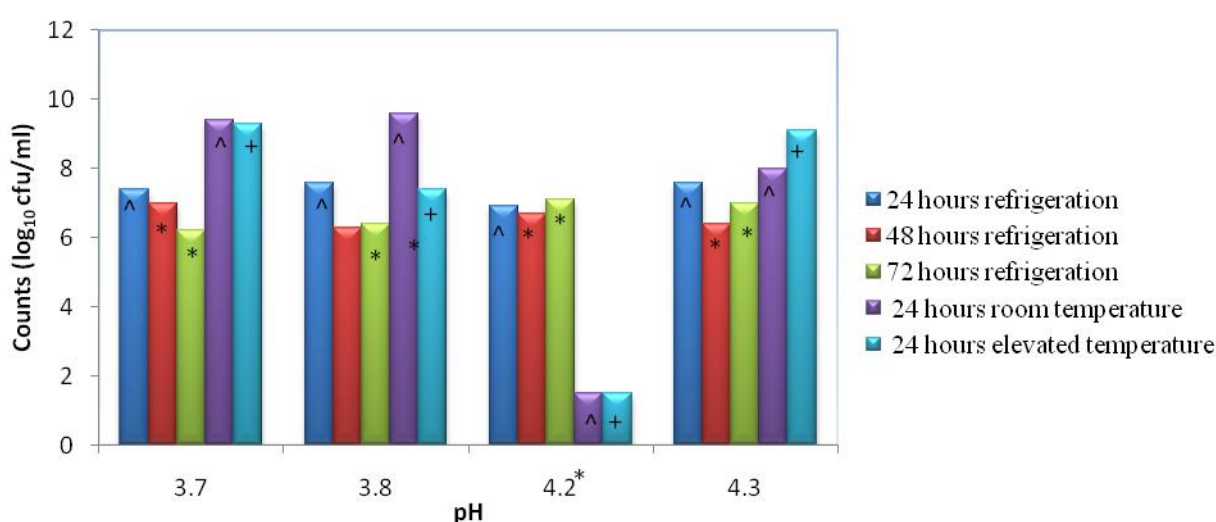cfu/ml: Colony forming unit per millilitre, FM: fermented milk, *: mean counts of at least 4 samples per product.

Fig 1: *B.cereus* counts in *Nono* at different pH levels during storage at refrigeration (4-10 ^{o}C), room (26-28°C) and elevated (37°C) temperatures

Mean counts of triplicate samples. Counts across storage conditions and pH levels with the same symbols are not significantly different (P>0.05)

Results obtained from the prevalence study of *B.cereus* in *nono* showed a rather high prevalence rate (30%). This may be attributed to the processing and storage conditions and also method of sale of the product. These include the use of raw milk for processing, contamination from utensils like calabashes, wooden spoons and straw mats, unclean udder and milking environments littered with cow dung (Plates I and II)), as well as the storage environment during fermentation, and methods employed at sale . Similar results were observed in a previous study[11], where HACCP of traditional processing of uncontrolled fermented milk revealed hazards associated with most steps of the production. An important observation made at that time was the addition of water to the fermented milk, which was then agitated by shaking vigorously to separate fat globules from the fermented milk. In addition during sale of the product, small wooden calabashes or plastics exposed to open air were repeatedly dipped into the product to serve consumers (Plate III). These practices could contaminate the products with more *B. cereus*, as such would explain the rather high prevalence rate of the organism in the product. High aerobic plate counts recorded for samples could also be attributed to the practice of uncontrolled fermentation of raw milk by the microflora present, allowing for increase in microbial population in fermented milk samples.

Plate I: Cattle herd in an outdoor open air housing system, observe the cow dung littering the area (arrow)

Plate II: Hand-milking in process from unclean udder (arrow)

Plate III: *Nono* **during sale**

Results of this study agree with those of other researchers[12, 13] some of who recorded a higher prevalence rate of 93.3% of *B.cereus* in locally fermented milk[12.]. *B.cereus* counts in **nono** samples in this study were however within expected microbiological levels for ready- to- eat meals[14]. TAPC for samples in this study were however at levels assumed to be unsatisfactory ($>10^5$) ready- to- eat meals[14]. Insignificant differences in both *B.cereus* counts and TAPC (P>0.05) may be attributed to similarities in methods employed during milking, proximity of milking environments to each other which could result in having similar microbial associations. It has been hypothesized that differences in feeding and housing strategies of cows may influence the microbial quality of milk[15]. The findings of this study reaffirm those of previous researchers documenting the presence of *B.cereus* in uncontrolled fermented milk products in some Nigerian towns. In addition , the rather unsatisfactory levels of aerobic mesophilic bacteria in the products analyzed in this study highlights the potential hazard associated with the consumption of *nono*, as it could harbour a variety of pathogens associated with raw unpasteurized milk, used in *nono* production. A number of studies have reported the isolation of microorganisms, including pathogens from raw milk[16, 17, 18] .

B.cereus counts dropped consistently in all *nono* samples over 48 h of refrigerated storage (4-10 ^{o}C), and further at 72 h at pH 3.7. *B.cereus* has been shown to be affected by low levels of acidity by rapidly dying in yoghurt as acidity of fermented milk increased[19]. In addition, bacteriocins which could have been produced during uncontrolled fermentation of milk would exert additional inhibitory effects on *B.cereus* cells. The broad spectrum bacteriocin, Enterocin AS-48 has been documented to decrease cell counts of toxigenic psychrotrophic strains of *B.cereus* in food systems consisting of boiled rice, commercial infant rice-based gruel in whole milk stored at 37°C, 15°C and 6°C[20]. Resistance of bacteriocins from lactobacilli isolated from raw cow milk to boiling for 10 minutes have been documented [21]. Stability of inhibitory activity of bacteriocins following heat treatment at 95°C and even at 120°C for 20 minutes have also been reported; as well as activity at pH range 3-10 for bacteriocins (obtained from uncontrolled fermented milk) that inhibited *B.cereus* [22]. The bacteriocins were more active at lower pH, and activity decreased with alkalinity. *Nono* samples in this study were heat treated at 63°C for 30 minutes over a three day period, and bacteriocins associated with the product would still have retained their inhibitory effects on *B.cereus* cells. Another striking observation was made with samples in this study ; there appeared to be a positive correlation between increasing pH levels and *B.cereus* cells ability to overcome previously inhibitory effects during refrigerated storage (4-10 ^{o}C) at the end of 72 h. Results of this study also suggested higher temperatures (26-28°C and 37°C) allowed for increases in counts, as bactericidal effects were observed for samples at refrigeration temperature (4-10°C) at the same pH levels following 24 h of

storage (except at pH 4.2). This bactericidal effect was sustained in samples stored at refrigeration temperature (4-10°C) for up to 48 h. A number of factors, including bacterial strains and environmental factors such as temperature have been shown to affect the antimicrobial activity of organic acids in microbial cultures[23,24,25]. In addition, The use of different stresses at the same time (combination treatment) may prevent the synthesis of protective proteins because simultaneous exposure to different stresses will require energy-consuming synthesis of several protective stress shock proteins which in turn may cause the microorganisms to become metabolically exhausted[26]. Similar findings have been reported[24] where effects of organic acids were observed to be less inhibitory on *E. coli* 0157:H7 at temperatures of 25°C and 37°C, than at 10°C. Increase in *B.cereus* counts at 72 h in refrigerated samples (4-10°C) could have been as a result of rise in pH of the fermented milk samples due to lysis of dead cells, and activity of "fitter" cells[27]. These would have led to a subsequent decrease[22] or loss of antimicrobial properties of the fermented milk samples. It was also observed in this study that samples at pH 4.2 had a bactericidal effect that resulted in reduction of *B.cereus* cells by about 4.0 log units, during storage at room (26-28°) and elevated temperatures (37°C) for 24 h. Colonies were also observed to be much smaller, appearing as pinpoint colonies. Storage at refrigeration temperature at the same pH had less bactericidal effect, resulting in reduction of cells by only 2 log units. These observations therefore suggest that *B.cereus* cells are killed at pH 4.2 in uncontrolled fermented milk, at both low and elevated temperatures. This would explain why *B.cereus* counts were significantly different across the pH levels, with samples at pH 4.2 having the lowest mean counts ($P<0.05$). Similarly, the significant difference in counts when analyzed across different temperatures could be explained by the increased bactericidal effect observed in refrigerated samples.

IV. CONCLUSION AND RECOMMENDATIONS

Results of this study have established the presence of *B.cereus* in uncontrolled fermented milk (*Nono*) sold in some streets and markets of Kaduna metropolis, although within acceptable limits, but nevertheless of significance. Sustained inhibitory effects are exerted on *B.cereus* cells in heat treated *nono* at pH level of 3.7 during refrigerated storage for up to 72 h. There is the need to enlighten local food service operators on the hazards associated with sale of unsafe foods, which for the most part could be attributed to use of substandard methods of processing. There is also the need to enact and enforce regulations for food service operators and vendors by the appropriate agencies with a view to providing safer RTE foods in Nigeria. In addition there is the need to partake in studies to understand the mechanisms of survival of food –borne pathogens in locally consumed foods. Results of such studies could be exploited with a view to controlling food –borne diseases associated with consumption of such foods.

V. ACKNOWLEDGEMENT

REFERENCES

[1]   Gamarra RM, Manuel DM, Piper MH, Nachimuthu and Balasundaram P, Food Poisoning. http://Medicine.medscape.com (accessed 6/2/ 2014)
[2]   Keith RS, Micky EP, Renee MG, and Taylor, C. (2004). Preventing food-borne illness: *Bacillus cereus* and *Bacillus anthracis*. A publication of the Institute of Food and Agricultural Science,University of Florida.
[3]   Notermans SJ, Dufrenne P, Teunis R, Beumer M, te Giffel and Weem, P. (1997). A risk assessment study of *Bacillus cereus* present in pasteurized   milk, *Food Microbiology,* 14:143 - 151.
[4]   Rowan NJ, Deans JO, Anderson CO, Gemmell CO, Hunter IS and Chaithong T. (2001) Putative virulence factor expression by clinical and food isolates of *Bacillus* spp. After growth in reconstituted infant milk formulae, *Appl. Environ Microbiol*, 67: 3873 - 3881.
[5]   Werner BO and Hotchkiss JH. (2007). Effects of carbon dioxide on the growth of *Bacillus cereus* spores in milk storage, *Dairy Sci*. 85:15 - 18.
[6]   Prestamo O, Pediazuela A, Gugno B and Sanz PD. (2007). Synergy between high-pressure, temperature and ascorbic acid on the inactivation of *Bacillus cereus*, *European Food Research and technology*, 225:693 - 698.
[7]   Senesi S and Ghelardi E. (2010) Production, Secretion and Biological Activity of *Bacillus cereus* Enterotoxins, *Toxins*, 2:1690-1703, doi: 10.3390/toxins2071690
[8]   Holbrook R and Anderson JM.(1980). An improved selective and diagnostic medium for the isolation and enumeration of *Bacillus cereus* in foods, *Canadian Journal of Microbiology*, 26:753-759
[9]   MFLP-42 (Laboratory Procedures For The Microbiological Analysis Of Foods) Isolation and Enumeration of *Bacillus cereus* in Foods, Http:// www.hc- sc.gc.ca (Accessed 27/7/2007)
[10]   Maturin L and Peeler JT. (2001). Aerobic Plate Count: Bacteriological Analytical Manual: US food and Drug Administration and Control,.
[11]   Bello S. (2002). Prevalence of spoilage and   pathogenic organisms and the survival   pattern of yeasts in locally fermented milk, M.Sc. thesis, Department of Microbiology, Ahmadu Bello University, Zaria, Kaduna, Nigeria.
[12]   Whong CMZ, Kwaga JKP, Umoh VJ and Ameh JB. (2006). Densities of *Bacillus cereus* in some Nigerian foods and food ingredients, *Nigerian Journal of Microbiology*, 20(2): 960-970
[13]   Uzeh RE, Ohenhen RE, and Rojugbokan AK. (2006). Microbiological and Nutritional Qualities of Dairy Products: Nono and Wara. *Nature and Science*, 4(3), 37-40

[14] NSW Food Authority (2009). Microbiological quality guide for ready-to-eat food. A guide to interpreting microbiological result.http:/www.foodauthority.nsw.gov.au

[15] Torkar KG and Teger SG. (2008).The microbiological quality of raw milk after introducing the two day's milk collecting system, *Acta Agri. Slovenica,* 92(1), 61– 74.

[16] Mubarack, H. M., Doss, A., Dhanabalan, R. and Balachande, S. (2010). Microbial quality of raw milk samples collected from different villages of Coimbatore District, Tamilnadu,South India. *Indian Journal of Science and Technology*, 3 (1) 61-63

[17] Tassew A and Seifu E. (2011). Microbial quality of raw cow's milk collected from farmers and dairy cooperatives in Bahir Dar Zuria and Mecha district, Ethiopia, *Agriculture and Biology Journal of North America*, (1): 29-33

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

[19] Hassan GM, Al-ashmawy MAM, Meshref AMS, Afify SI, (2010). Studies on enterotoxigenic *Bacillus cereus* in raw milk and some dairy products, *Journal of Food Safety*, 30(3): 569-583.

[20] Grande MJ, Lucas R, Abriouel H, Valdivia E, Omar, NB, Maqueda M, Martinez-Bueno M, Martinez-Canamero M and Galvez, (2006). Inhibition of toxicogenic Bacillus cereus in rice- based foods by enterocin AS-48 *International Journal of Food Microbiology*, 106:185-194.

[21] Mohankumar A and Murugalath N. (2011). Characterization and Antibacterial Activity of Bacteriocin Producing Lactobacillus Isolated from Raw Cattle Milk Sample, *International Journal of Biology,* 3(3): 128-143

[22] Abdelbasset M and Djamila K. (2008). Antimicrobial activity of autochthonous lactic acid bacteria isolated from Algerian traditional fermented milk "Raïb", *African Journal of Biotechnology,* 7(16): 2908-2914.

[23] Buchanan RL and Edelson SG. (1996).Culturing enterohemorrhagic *Escherichia coli* in the presence and absence of glucose as a simple means of evaluating the acid tolerance of stationary-phase cells, *Applied and Environmental Microbiology,* 62: 4009-4013.

[24] Conner DE and Kotrola JS. (1995). Growth and survival of *Escherichia coli* O157:H7 under acidic conditions, *Applied and Environmental Microbiology*, 61: 382-385.

[25] Cheng HY, Ye RC and Chou CC. (2003). Increased acid tolerance of *Escherichia coli* O157:H7 by acid adaptation time and conditions of acid challenge. *Food Research International,* 36: 49-56.

[26] Leistner L. (2000). Basic aspects of food preservation by hurdle technology. *International Journal of Food Microbiology*, 55:181– 186.

[27] Mossel DAA, Corry JEL, Struijk CB, Baird RM. (1995). Essentials of the microbiology of foods: a textbook for advanced studies, John Wiley and Sons, England, pp 83-110.