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Prevalence of *B.Cereus* in Retail Raw Milk and Influence of Ph on its Survival in *Pasteurized Milk During Low Temperature Storage*

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Abstract:
A study was conducted to determine the prevalence of B.cereus in retail raw milk samples and the influence of pH on survival of the organism during low temperature storage. A total of 43 samples of raw milk were purchased from 9 Fulani milkmaids in Unguwar Rimi district of Kaduna, Nigeria. Samples were collected at the point of milking, in sterile bottles, and plated unto sterile Mannitol Egg yolk Polymyxin (MYP) agar (Oxoid) plates for B.cereus isolation . Counts ranged from <1.4 ± 0.0 to 0.4 ± 0.6 log_{10} CFU/ml for B. cereus in the raw milk samples. Results also revealed a low prevalence rate (7%) for the raw milk samples. B.cereus counts fell within expected microbiological levels for ready-to-eat meals (<10^2). Analysis of variance revealed non significant differences in counts amongst samples at 5% level (P>0.05). To study the influence of pH on survival of B.cereus in milk during low temperature storage, Mcfarlands standard 1 of a B.cereus strain (about 8.5log 10 CFU/ml) isolated from raw milk during the prevalence study, and identified using a microgen™ Bacillus ID, was inoculated into pasteurized milk samples (72°C for 15min) following pH adjustment to 6.7, 6.4, (control samples) 6.3 and 6.8 with 0.1M lactic acid/Sodium hydroxide. Samples were stored at low temperature (4-7°C) for a period of 72 hrs, and analyzed for B.cereus counts on sterile nutrient agar at intervals of 24 hours. At the end of 24 hours, mean counts were observed to decrease from initial levels in samples especially at pH 6.8, except at pH 6.3, where counts remained almost unchanged. Further decreases were also observed at 48 hours, in samples at pH extremes of 6.3(about 2 log units) and 6.8, whereas slight increases were observed (less than 1 log unit) in control samples. At 72 hours, mean counts decreased in control samples while samples at 6.3 and 6.8 had increases of about 1 log unit in counts. Mean counts were not significantly different across samples during the storage period (P>0.05), but significant differences existed when counts were analyzed across pH levels (P<0.05) at 5% level. Findings of this study suggest B.cereus cells are inhibited in pasteurized whole cow milk during storage at less than 10°C at pH levels 6.3. 6.4 and 6.7 for 24 hours, while sustained bactericidal effects are exerted for up to 48 hours at a higher pH level of 6.8. Longer storage periods however allowed for growth as a possible consequence of change in milk composition due to lysis of dead cells, and proteolytic activity of B.cereus cells. Findings also infer that B.cereus cells from mesophilic environments in raw milk are capable of psychrotrophic growth.

Keywords: *B.cereus , raw milk, survival, pH, refrigeration, pasteurized milk*

1. Introduction

Food-borne diseases (FBD) or illnesses are defined as diseases of infectious or toxic nature caused by agents that enter the body through the consumption of food. (22). FBDs are caused by many different disease - causing pathogens that can contaminate foods, or by toxins produced by these pathogens that are present in food (8). *Bacillus cereus* , the model species of the "*Bacillus cereus* group", also known as *Bacillus cereus* sensu lato (18), is a gram positive, rod shaped bacterium capable of facultative aerobic metabolism. 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 (15). *B. cereus* may cause flavors to go off and become putrid, rancid, bitter, unclean, fruity and yeasty in fluid milk products (9). *B. cereus* can grow over a wide temperature range (8–55 °C), and its been reported to grow at temperatures as low as 4°C (10).. *B. cereus* sensu lato has recently been divided into seven major phylogenetic groups (I to VII) with clear-cut differences in growth temperature ranges, suggesting that the genetic structure corresponds to "thermotypes" and showing the emergence of multiple psychrotrophic groups within *B. cereus* sensu lato (6). It's been shown to be not well suited to tolerate low pH values (minimum 5–6), (*18).*

A number of food poisoning incidents can be attributed to *B. cereus,* and this bacterium is known to cause a variety of non-gastrointestinal diseases, as well as two different types of food poisoning which are characterized by either diarrhoea or emesis. The most common is a diarrhoea illness caused by a heat - labile toxin, accompanied with abdominal pain. An incubation period of 4 - 16 hours is followed by symptoms lasting 12 - 24 hours. The second type of disease state is an emetic illness caused by a

heat stable toxin, often associated with ingestion of rice that is not properly refrigerated after cooking. This illness is characterized by nausea and vomiting that usually occurs within 1 - 5 hours upon ingestion of the contaminated food. The relevance of the *Bacillus* species as food poisoning organisms and as etiological agents in non gastrointestinal infections including local deep tissue systemic infections is however being increasingly recognized. Infections in individuals who are intravenous drug abusers or immune compromised as a consequence of infection with human immunodeficiency virus, chemotherapy or malignancy and neonates are sometimes severe and life threatening (17, 4, 18). Researching and Understanding the ability of *B. cereus* to grow under various storage conditions is therefore paramount to help to control multiplication during food storage and prevent outbreaks of food-borne poisoning. *Against this backdrop, t*he survival pattern of *B.cereus* in pasteurized whole cow milk at varying pH levels was carried out to study the effect of pH on survival of *B.cereus* in milk, during low temperature storage.

2. Materials and Methods

2.1. Enumeration of B.cereus in raw milk samples

A total of 43 samples of raw unpasteurized milk were purchased from 9 Fulani milkmaids at their settlements during early morning milking. 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 B Agar (MYP) (OXOID), a highly selective *Bacillus cereus* medium (7). MYP plates of the food samples were incubated at 37°C for 24-48 hours. Gram positive colonies that appeared surrounded by zones of clearance on the MYP plates were counted and recorded as presumptive *Bacillus* cereus counts(11) as shown in table 1. Presumptive isolates were characterized using Microgen^{TM} *Bacillus* ID identification kits. 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.

2.2. Survival of B.cereus isolates in pasteurized milk during low temperature storage

Fresh cow milk was obtained from the Dairy processing unit of National Animal Production and Research Institute (NAPRI) in Shika, Kaduna state. This was then pasteurized at 72^0C for 15 minutes (HTST) in a Grant JB series water bath, by placing a sterile mercury thermometer into the sample until it attained the temperature of 72^0C which was then maintained for 15 minutes. 0.1 ml of the sample was then spread on sterile nutrient agar plate and incubated for 24 hours at 37^0C to test for sterility. 100ml of the pasteurized milk was then dispensed into each of four sterile 250ml conical flasks. The milk in each of these conical flasks had its pH adjusted to 6.3, 6.4, 6.7 and 6.8 respectively, 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. 20ml of milk sample from each conical flask was then dispensed in each of four sterile glass bottles. These bottles were each inoculated with 1 ml of Mcfarland standard 1 of *B. cereus* isolate R3M1 (isolated from retail raw milk above) using sterile 2ml syringes. Samples were stored at low temperature (4-7^{o}C) for a period of 72 hrs, and analyzed for *B.cereus* counts on sterile nutrient agar at intervals of 24 hours. Analysis was carried out in triplicates, and results were subjected to statistical analysis using the two- way analysis of variance (ANOVA).

3. Results and Discussion

3.1. Enumeration of B.cereus in raw milk samples

Mean counts of presumptive *B.cereus* isolates from raw milk are shown in table 1. Counts ranged from $<1.4 \pm 0.0$ to 0.4 ± 0.6 $\log_{10}$ CFU/ml. Statistical analysis using the one-way ANOVA (SPSS version 16) showed no significant differences in *B.cereus* counts of the raw milk samples ($P = 0.99$). Following biochemical characterization of presumptive isolates however, only 3 % (7 samples) of samples had confirmed *B.cereus* isolates.

B. cereus counts in raw milk samples in this study were within expected microbiological levels ($<10^2$ CFU/g) that presented no food safety concern for ready- to- eat meals (13). Prompt cooling and analysis of the raw milk samples immediately after milking could account for the low level of counts. Insignificant differences in these counts ($P>0.05$) for samples may be attributed to similarities in methods employed during milking (hand milking and no prior cleaning of the udder), proximity of milking environments from where samples were collected to each other, which could all have resulted in similar microbial associations in samples.

Product name	Mean *B. cereus* count ($\log_{10}$ CFU/ml)
RM1	< 1.4± 0.0
RM2	< 1.4± 0.0
RM3	0.3± 0.5
RM4	<1.4± 0.0
RM5	0.3± 0.6
RM6	0.4± 0.6
RM7	0.3± 0.6
RM8	< 1.4± 0.0
RM9	<1.4± 0.0

Table 1: Mean presumptive B. cereus counts ((log $_{10}$ CFU/ml) of retail Raw milk from CFU/ml; Colony forming unit per milliliter, RM; raw milk, *; mean counts of at least 4 samples per product*

It has been hypothesized that differences in feeding and housing strategies of cows may influence the microbial quality of milk (21). A number of studies have also reported the isolation of microorganisms, including pathogens from raw milk (23,12, 21).

3.2. Survival of B.cereus isolates in pasteurized milk during low temperature storage

At the end of 24 hours, mean counts were observed to decrease from initial levels in samples especially at pH 6.8, except at pH 6.3, where counts remained almost unchanged (fig1). Further decreases were also observed at 48 hours, in samples at pH extremes of 6.3(about 2 log units) and 6.8, whereas slight increases were observed (less than 1 log unit) in control samples. At 72 hours, mean counts decreased in control samples while samples at 6.3 and 6.8 had increases of about 1 log unit in counts. Mean counts were not significantly different across samples during the storage period ($P>0.05$), but significant differences existed when counts were analyzed across pH levels ($P<0.05$) at 5% level.

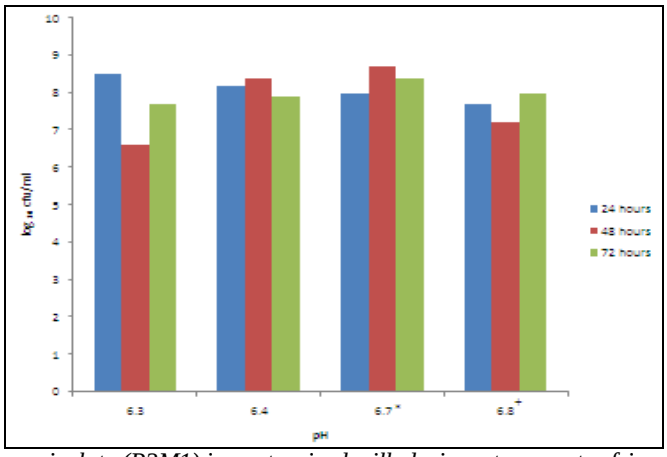

Figure 1: Counts of B.cereus isolate (R3M1) in pasteurized milk during storage at refrigeration (4-7^{o}c) for 72 hours.

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

Results of this study suggest bacteriostatic and bactericidal effects on *B.cereus* at 24 hours at temperature range of 4-6^{o}C, and inhibitory effects seemed to increase as pH increased, this could be attributed in part to natural inhibitory systems including the lactoperoxidase/thiocyanate/hydrogen peroxide (LP) system, lactoferrins, Lysozymes, immunoglobulus and free fatty acids (5,2) in milk. Increases in inhibitory effects at higher pH levels may be due to a combined effect of temperature and pH. Increases observed at 48 hours in control samples could be as a result of change in composition and pH due to lysis of dead cells and proteolytic activity of surviving cells (10, 3) that could reduce inherent antimicrobial activity of milk. Likely loss of activity of some of the antimicrobial properties of the milk medium, as increasing numbers of *B. cereus* cells breakdown milk components. *Bacilli* are known for their abilities to secrete large amounts of alkaline proteases having significant proteolytic activity and stability at considerably high pH and temperatures (14, 1). Breakdown of milk would result in production of metabolites such as amines, amino acids; release of these metabolites would affect the ionic strength and concentration of metal ions of the milk medium to levels which could reduce lysozyme activity. High concentrations (>50mm) of cations such as Na^+, K^+, NH_4^+ has been shown to reduce lysozyme activity (19). The resulting increase in pH (10, 19) would permit growth of previously inhibited *B.cereus* cells in the medium. The likely exhaustion of nutrients in both milk media after 72 hours, as well as increased pH levels would explain the decrease in counts observed. Unlike in control milk samples, increase in counts in samples at pH extremes of 6.3 and 6.8 after 72 hours could be attributed to a slower increase in pH which permitted growth, lower levels of inhibitory metabolites , as well as higher levels of available nutrients owing to suppressed growth during the initial storage period. These variations in counts would explain the significant difference in *B.cereus* counts across the samples when analyzed across pH levels.

4. Conclusion

Results of this study reveal the presence of *B.cereus in* some retail raw cow milk products sold around Kaduna metropolis by Fulani milk maids, Nigeria. Although counts were found to be within acceptable limits, it is nevertheless of significance as subsequent sub standard handling methods could allow for increase to levels that could affect quality of milk products produced from such raw milk, such as locally fermented milk (*Nono*), sold by fulani milk maids.

Findings of this study also suggest *B.cereus* cells from raw cow milk, are inhibited in pasteurized whole cow milk during storage at less than 10^{o}C at pH levels between 6.3-6.8 for 24 hours, and for as long as 48 hours at pH 6.8.. Longer storage periods however allow for growth as a possible consequence of change in milk composition due to lysis of dead cells, and proteolytic activity of surviving *B.cereus* cells. Findings of this study also infer that *B.cereus* from mesophilic environments in raw milk, are capable of psychotrophic growth.

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