

ISOLATION AND IDENTIFICATION OF *BACILLUS SPP* AT THE LEVEL OF MILK PRODUCTION AND IN SUPPLY CHAIN

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ABSTRACT

The study was conducted to track *Bacillus* species in the production - supply chain of fluid milk. A total of 20 milk samples were collected viz., raw milk samples, pasteurized milk immediately after pasteurization and pasteurized samples stored under refrigeration for two days from a private dairy farm and its milk processing unit, in Thrissur district. The samples were evaluated for microbiological quality parameters like Total Viable Count (TVC) and *Bacillus* count. The TVC observed in both raw and pasteurized milk samples were higher when compared to the prescribed standards by FSSA (2006). Raw milk samples and stored pasteurized milk samples showed presence of *Bacillus* organisms. But they were totally absent in pasteurized milk samples. Isolates showing peacock blue colored colonies surrounded by a yellow zone of lysis in *Bacillus cereus* agar (BCA) were identified as *Bacillus cereus* organisms. The enteropathogenic

effect of the isolates was studied by polymerase chain reaction (PCR) but none of the isolates from the milk samples were found to be pathogenic strains of *B. cereus*. The occurrence of *Bacillus spp.* and higher TVC in the milk samples, suggests that the protective measures are inadequate and good hygienic practices (GHP) to be followed especially at the farm level, and during post pasteurization .

Keywords: Milk, TVC, *Bacillus spp.*

INTRODUCTION

Milk has high nutritious value and is also a good growth medium for a variety of spoilage and potentially pathogenic microorganisms like *Pseudomonas*, *Bacillus*, *Enterococcus*, *Microbacterium*, *Micrococcus*, *Proteus*, coliforms etc. The genus *Bacillus* is ubiquitous and is generally considered as harmless microbial contaminant. Among this *Bacillus cereus* has more importance in dairy industry. It is a thermophilic microorganism and can

produce spores and toxins which are capable of surviving pasteurization (Frank, 1997). They affect the shelf life of thermized milk dairy products. The organism can cause defects such as off flavours, sweet curdling and bitty cream due to the effect of enzymes like proteinase, lipase and phospholipase (Heyndrickx and Scheldeman, 2002). Other than these defects in dairy products, *B. cereus* has also been associated with outbreaks of food poisoning due to the effect of its enteropathogenic strains (Logan, 2011). In this study, investigation has been conducted to trace the presence of *Bacillus cereus* in milk from farm to table, which provided a general idea about the source of contamination and this would offer a comprehensive approach with reference to food safety and public health perspective.

MATERIALS AND METHODS

Collection of milk samples

A total of 20 milk samples viz, 10 raw milk samples and five each of pasteurized and pasteurized stored (under refrigeration for two days) milk samples were collected from a private dairy farm and its dairy processing unit, situated in Thrissur district.

Estimation of TVC and Bacillus count

The microbiological quality parameters like Total Viable Count (TVC) and *Bacillus* count of the samples were

estimated by the pour plate technique described by Wehr and Frank (2004). Plate count agar was used for TVC and Hicrome *Bacillus* agar with polymixin B as selective supplement was used for *Bacillus* count. The number of colony forming units (cfu) in the agar plates was expressed as cfu/ml and was compared with the microbiological standards for raw and pasteurized milk prescribed by FSSA (2006).

Isolation and identification of Bacillus cereus from the milk samples

B. cereus organisms from the milk samples were isolated by culturing on *Bacillus cereus* agar (BCA) with selective supplements like egg yolk emulsion and Polymixin B. The isolated colonies of *B. cereus* were tentatively identified by the morphological, cultural and biochemical characteristics given in Bergey's manual of determinative bacteriology (1974).

Confirmation of B. cereus isolates by PCR

Pure culture of *B. cereus* (1306) was purchased from Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh which was used as the standard organism for PCR technique. For molecular identification of genus *Bacillus* by PCR technique, *BCFomp1* and *BCRompl* primer pairs were used. Primers *BalF* and *BalR* were employed for species

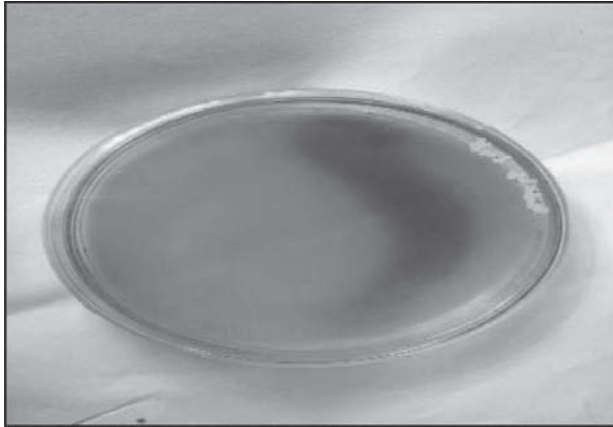


Fig. 1. Appearance of *B. cereus* colony in BCA

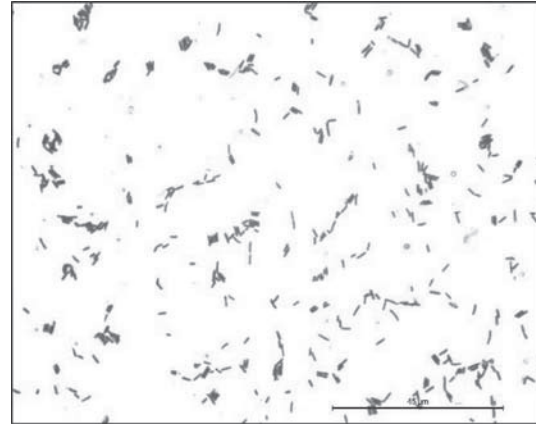


Fig. 2. Grams reaction of *B. cereus*

identification of *B. cereus*. For detecting the enteropathogenic effect of the isolates *Hbla1/Hbla2* primers were used. The primer sets used in this study was procured from Sigma Aldrich, Bangalore.

Statistical analysis

Data obtained from this study was statistically analysed using SPSS version 21 (Snedecor and Cochran, 1994).

RESULTS AND DISCUSSION

TVC and Bacillus count of the milk samples

Total viable count observed in the raw, pasteurized and stored milk samples, given in table 1, was compared with their respective standard values prescribed by FSSAI (2017). The TVC of both raw and pasteurized milk samples were found to be higher than their respective FSSAI standards. As per the observation of O'Connor (1994) milk produced under hygienic conditions

from healthy animals should not contain more than 5×10^5 cfu/ml (i.e. 5.70 log cfu/ml) organisms. The increase in the TVC count of raw milk samples in the present study might be due to the contamination of milk during handling, from the contaminated utensils and from the farm atmospheric conditions. The quality of raw milk is important for the production of pasteurized milk with higher quality and longer shelf life. In the present study, higher microbial load of raw milk might have influenced the quality of pasteurized milk and its shelf life.

The mean count of *Bacillus* obtained from the milk samples were given in table 2. In pasteurized milk samples *Bacillus* organisms were totally absent which is suggestive of an efficient pasteurization procedure in the dairy plant. However, both raw milk and stored milk samples showed the presence of *Bacillus*.

Table.1 Total Viable Count of milk samples

Sample	Count (Mean±SE) Log cfu/ml	Standard value by FSSA
Raw milk	7.86 ±0.06	≤ 5.30 log cfu/ml
Pasteurized milk	6.88±0.16	m=4.48 log cfu/ml M= 4.69 log cfu/ml
Pasteurized stored milk	7.69±0.04	m=4.48 log cfu/ml M= 4.69 log cfu/ml

'm' represents an acceptable level and values above it are marginally acceptable in terms of sampling plan
Values above 'M' are unacceptable in terms of the sampling plan

According to Christiansson *et al.* (1989) *B. cereus* spores have the ability to resist the high pasteurization temperature, and contaminate the dairy products. The count of *Bacillus* in raw milk was found to be 5.16 ± 0.10 log cfu/ml and it was significantly higher than the count obtained for pasteurized stored milk samples. TeGiffel *et al.* (1995) also reported that *B. cereus* spores are frequently found in raw milk.

Isolation and identification of *B. cereus*

A total of 20 milk samples from three sources were tested for the presence of *Bacillus* using Hicrome *Bacillus* agar. The colony characteristics of various *Bacillus* species is given in table 3. From the colony characteristics, seven of the raw milk samples were suspected to have contamination with *Bacillus cereus*. Upon culturing suspected colonies in *Bacillus cereus* agar (BCA), they appeared as peacock blue coloured colonies surrounded by a yellow zone of lysis (Fig. 1). Similar result was observed in the study conducted by Peng *et al.* (2001).Such isolates

were taken for further investigative and confirmatory studies.

Gram staining revealed that the organisms were Gram positive rods (Fig. 2). The results of further identification studies done by the biochemical reaction tests were given in table 4. By comparing those results with the Bergey's manual of determinative bacteriology (1974), it was tentatively identified that the isolates were of *B. cereus* organisms.

Confirmation of *B. cereus* isolates by PCR

Confirmative identification of the *B. cereus* isolates was done by molecular technique. *BCFomp1* and *BCRompl* primer

Table. 2 *Bacillus* count in milk samples

Sample	<i>Bacillus</i> Count (Mean±SE) log cfu/ml
Raw milk	5.16±0.10 ^a
Pasteurized milk	Nil
Pasteurized stored milk	4.79±0.18 ^b

Means having different small letters as superscript differs significantly within a column (p < 0.01)

Table 3. Colony characteristics of *Bacillus*

Sl. No	Name of the organism	Colony characteristics
1	<i>B. cereus</i>	Light blue, large, flat colonies with blue centre
2	<i>B. subtilis</i>	Yellowish green to green colonies
3	<i>B. thuringiensis</i>	Light blue, large, flat colonies with irregular margins
4	<i>B. megaterium</i>	Yellow, mucoid colonies

Table 4. Results of Biochemical reactions

Sl. No	Biochemical reactions	Results
1	Gram staining	Gram positive rods
2	Catalase test	Positive
3	Oxidase test	Negative
4	Motility test	Motile
5	Spore staining	Pink rods with Green coloured central spherical spores
6	Indole test	Negative
7	Methyl red test	Negative
8	Voges-proskauer test	Positive
9	Citrate utilization test	Positive
10	Urease test	positive

pairs with an amplified product of 575 bp gave the confirmatory result for *Bacillus* genus. The species confirmation of *B. cereus* was performed with primer pair *BalF* and *BalR*. Among the seven suspected *B. cereus* isolates from raw milk samples, 5 isolates gave a product size of 533 bp confirming that 5 among the 7 isolates are *B. cereus* organisms. For detecting the enteropathogenic effect of these isolated *B. cereus* strains, the primers *Hbla1/Hbla2* were used to identify the pathogenic *hbla* gene. None of the isolated strains gave positive result for this reaction, which

indicates that the *B. cereus* present in the raw milk samples were not pathogenic.

SUMMARY

This study was carried out to trace the *Bacillus* species in fluid milk production-supply chain. A total of 20 samples under different stages of production, processing and storage were screened for the study. The parameters studied were TVC, *Bacillus* sp. count and presence of *Bacillus cereus* in the samples. Since *B. cereus* strains are important as it affects the shelf life of pasteurized milk and heat treated dairy

products, due importance was given for their isolation from different regions of milk supply chain.

The high microbial count obtained for raw, pasteurized and stored milk samples indicated the necessity for improving the hygienic practices at production and processing point. Since none of the *Bacillus* isolates were recovered from pasteurized milk samples, it can be assumed that vegetative cells might have been destroyed by pasteurization process and the process adopted at processing plant is effective. The presence of *B. cereus* in farm milk samples indicated that the farm environment might have acted as an entry path for potentially highly heat-resistant spores into raw milk. Effective cleaning and disinfection procedures at farm level can avoid build-up of high levels of *B. cereus*.

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