

## Psychrotrophic *Bacillus* in Pasteurized Market Milk : A Threat

LOPAMUDRA B. HALDAR<sup>1\*</sup> AND R. K. KUILA

*Department of Dairy Bacteriology, West Bengal University of Animal & Fishery Sciences  
 Mohanpur 741252, India*

<sup>1</sup>*Faculty of Science & Technology, ICFAI University, Agartala, Tripura, India*

*E-mail : mohor7@gmail.com*

*\*Correspondence*

### Abstract

The incidence of psychrotrophic sporeforming bacteria in market milk supplies is a major determinant of the keeping and hygienic quality of milk and a threat for food poisoning in human beings. To investigate the incidence of psychrotrophic sporeforming organisms in pasteurized milk supplies, 51 milk samples from different organized dairies in and around Kolkata, West Bengal, India were collected and subjected to analysis. A total of 53 selected isolates from the samples were collected and examined. On the basis of different cultural, morphological and biochemical characteristics, all the isolates were found to belong under the genus *Bacillus* and tentatively classified into 9 types, of which *B. cereus* was the predominant psychrotrophic type (33.96%). *B. subtilis* showed the second highest in population (18.86%) followed by *B. megaterium* (11.32%) and *B. sphaericus* (9.43%), while *B. circulans* was 7.55%. *B. polymyxa*, *B. globisporus* and *B. macerans* represented 5.66% each in distribution. *B. licheniformis* was found to be the least in population (1.89%). The present study directed to make suitable technological strategies for eliminating the problem of psychrotrophic sporeforming bacteria in pasteurized milk.

**Key words :** Psychrotrophic sporeforming bacteria, *Bacillus* species, Pasteurized milk.

The incidence of sporeforming psychrotrophic bacteria in milk is one of the important bacteriological problems due to modern practices of higher pasteurization temperature and extended storage of raw and processed milk in dairy plants. The sporeforming psychrotrophic bacteria are troublesome in that they are difficult to eliminate from milk and milk products influencing markedly the keeping and hygienic quality of milk (1) and thus, subsequently posing a threat for food poisoning (2). The sporeforming psychrotrophic bacteria in milk most commonly include many species of the genus, *Bacillus* (3) and rarely to the genus, *Clostridium* (4). Since the incidence of sporeforming psychrotrophic bacteria in milk is seriously concerned with public health (5), the question whether psychrotrophic spore formers exist in the daily milk supplies really needs to be answered. Therefore, the present study was undertaken to investigate the occurrence of psychrotrophic spore forming bacteria in different milk supplies in the state of West Bengal, India.

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### Methods

#### *Samples*

Over the period of 9 months, a total of 51 samples of 500 ml retail pouches of pasteurized milk from 9 different Government and Private dairy plants in and around Kolkata, West Bengal were collected between 0600 to 0700 and put in the icebox and carried to the laboratory and then stored in the refrigerator at 4C until analysis. The samples from different dairy plants were designated by some confidential sample codes like MD, MTD, HD, CD, BD, KD, AND and TD.

#### *Culture Isolation*

The pasteurized milk samples (250 ml) were transferred aseptically to sterile conical flask and subjected

**Table 1.** Different morphological and biochemical characteristics of psychrotrophic sporeforming isolates for generic identification. + = Positive reaction, - = Negative reaction, SA = Strict aerobic, MA = Micro aerophilic.

Isolate no.	Shape of cell	Motility	Gram reaction	O <sub>2</sub> requirement	Catalase test	Marked acidity from glucose	Tentative genus identification
IS-1	Rod	+	+	MA	+	+	<i>Bacillus</i>
IS-2	Rod	+	+	MA	+	+	<i>Bacillus</i>
IS-3	Rod	+	+	SA	+	+	<i>Bacillus</i>
IS-4	Rod	+	+	MA	+	+	<i>Bacillus</i>
IS-5	Rod	+	+	SA	+	+	<i>Bacillus</i>
IS-6	Rod	+	+	MA	+	+	<i>Bacillus</i>
IS-7	Rod	+	+	SA	+	+	<i>Bacillus</i>
IS-8	Rod	+	+	SA	+	-	<i>Bacillus</i>
IS-9	Rod	+	+	SA	+	+	<i>Bacillus</i>
IS-10	Rod	+	+	MA	+	+	<i>Bacillus</i>
IS-11	Rod	+	+	SA	+	-	<i>Bacillus</i>
IS-12	Rod	+	+	MA	+	+	<i>Bacillus</i>
IS-13	Rod	+	+	MA	+	+	<i>Bacillus</i>
IS-14	Rod	+	+	MA	+	+	<i>Bacillus</i>
IS-15	Rod	+	+	MA	+	+	<i>Bacillus</i>
IS-16	Rod	+	+	MA	+	+	<i>Bacillus</i>
IS-17	Rod	+	+	SA	+	+	<i>Bacillus</i>
IS-18	Rod	+	+	SA	+	-	<i>Bacillus</i>
IS-19	Rod	+	+	MA	+	+	<i>Bacillus</i>
IS-20	Rod	+	+	MA	+	+	<i>Bacillus</i>
IS-21	Rod	+	+	MA	+	+	<i>Bacillus</i>
IS-22	Rod	+	+	MA	+	+	<i>Bacillus</i>
IS-23	Rod	+	+	MA	+	+	<i>Bacillus</i>
IS-24	Rod	+	+	SA	+	+	<i>Bacillus</i>
IS-25	Rod	+	+	SA	+	+	<i>Bacillus</i>
IS-26	Rod	+	+	MA	+	+	<i>Bacillus</i>
IS-27	Rod	+	+	MA	+	+	<i>Bacillus</i>
IS-28	Rod	+	+	SA	+	+	<i>Bacillus</i>
IS-29	Rod	+	+	SA	+	+	<i>Bacillus</i>
IS-30	Rod	+	+	MA	+	+	<i>Bacillus</i>
IS-31	Rod	+	+	MA	+	+	<i>Bacillus</i>
IS-32	Rod	+	+	SA	+	+	<i>Bacillus</i>
IS-33	Rod	+	+	MA	+	+	<i>Bacillus</i>
IS-34	Rod	+	+	MA	+	+	<i>Bacillus</i>
IS-35	Rod	+	+	SA	+	+	<i>Bacillus</i>
IS-36	Rod	+	+	SA	+	-	<i>Bacillus</i>
IS-37	Rod	+	+	MA	+	+	<i>Bacillus</i>
IS-38	Rod	+	+	MA	+	+	<i>Bacillus</i>
IS-39	Rod	+	+	MA	+	+	<i>Bacillus</i>
IS-40	Rod	+	+	MA	+	+	<i>Bacillus</i>
IS-41	Rod	+	+	SA	+	+	<i>Bacillus</i>
IS-42	Rod	+	+	SA	+	+	<i>Bacillus</i>
IS-43	Rod	+	+	MA	+	+	<i>Bacillus</i>
IS-44	Rod	+	+	MA	+	+	<i>Bacillus</i>
IS-45	Rod	+	+	MA	+	+	<i>Bacillus</i>
IS-46	Rod	+	+	SA	+	+	<i>Bacillus</i>
IS-47	Rod	+	+	SA	+	-	<i>Bacillus</i>
IS-48	Rod	+	+	SA	+	+	<i>Bacillus</i>
IS-49	Rod	+	+	MA	+	+	<i>Bacillus</i>
IS-50	Rod	+	+	MA	+	+	<i>Bacillus</i>
IS-51	Rod	+	+	SA	+	+	<i>Bacillus</i>
IS-52	Rod	+	+	MA	+	+	<i>Bacillus</i>
IS-53	Rod	+	+	MA	+	+	<i>Bacillus</i>

**Table 2.** Tentative classification of isolates for species identification on the basis of biochemical characteristics. V-P test = Voges-Proskauer test, Ar = Arabinose ; Ma = Manitol ; Malt = Maltose ; Sa = Salicine ; Su = Sucrose ; Glu = Glucose ; RCP = Reduction of litmus, coagulation, proteolysis ; cP = Complete proteolysis ; pR = Partial Reduction ; nC = No coagulation ; cR = Complete reduction ; pP = Partial proteolysis ; + = Positive reaction ; - = Negative reaction.

Type	No. of isolates	V-P test	Nitrate reduction test	Carbohydrate fermentation						Gas from glucose
				Ar	Ma	Malt	Sa	Su	Glu	
I	15	+	+	-	-	+	+	+	+	-
	1	+	+	-	-	+	+	-	+	-
	2	-	+	-	-	+	+	+	+	-
II	9	+	+	+	+	-	-	+	+	-
	1	+	+	+	+	-	-	-	+	-
III	5	-	-	-	+	-	-	-	+	-
	1	-	-	-	+	-	-	-	+	-
IV	4	-	-	-	-	-	-	-	-	-
	1	-	-	-	-	-	-	-	-	-
V	4	-	-	+	+	-	-	+	+	-
VI	2	+	+	+	+	+	+	+	+	+
	1	+	+	+	+	+	+	+	+	+
VII	3	-	-	-	-	-	-	+	+	-
VIII	3	-	+	+	+	+	+	+	+	+
IX	1	+	+	+	+	-	-	-	+	-

**Table 2.** Continued.

Type	Reaction in litmus milk	Gelatin hydrolysis	Starch hydrolysis	Salt tolerance (%)			
				2	5	7	10
I	RCP (20% from top)	+	+	+	+	-	-
	RCP (20% from top)	+	+	+	+	-	-
	RCP (20% from top)	+	+	+	+	-	-
II	cP (90% from top) Alkaline	+	+	+	+	+	-
	cP (90% from top) Alkaline	+	+	+	+	+	-
III	pR nC	+	+	+	+	-	-
	pR nC	-	+	+	+	-	-
IV	No change	-	-	+	+	-	-
	No change	-	+	+	+	-	-
V	cR nC	-	+	+	-	-	-
VI	pR pP	+	+	+	-	-	-
	pR pP	+	+	+	+	-	-
VII	pR	+	-	+	+	-	-
VIII	cR	+	+	+	-	-	-
IX	pR cP	+	+	+	+	+	-

to heat treatment in water bath at 80 C for 10 min to destroy all non-spore forming organisms, as well as, the vegetative forms of spore forming bacteria (6). Immediately after the heat treatment, the milk sample was cooled rapidly in ice water (7). The sample was then incubated at  $7 \pm 1$  C for 7 d in a refrigerator (8). One ml of sample was then transferred aseptically and sequentially to 9 ml sterile dilution blanks (physiological saline) for making different dilutions as per requirements. One ml of each diluted sample was used for planting on Tryptone-Glucose-Yeast Extract Agar

(TGYA). The plate was then incubated at  $7 \pm 1$  C for 10 d (9). Representative distinct colonies were picked and transferred to sterile reconstituted skim milk (10%), nutrient broth and TGYA slants and incubated at  $7 \pm 1$  C for 10 d for further study.

#### *Identification of Isolates*

The isolates were identified on the basis of cultural and morphological studies and biochemical tests viz., catalase test, oxygen requirement test, carbohy-

drate fermentation test (10, 11). The species were identified using biochemical tests (voges-proskauer test, nitrate reduction test, salt tolerance test, carbohydrate fermentation test, gelatine liquefaction test, starch hydrolysis test and reaction on litmus milk) as per the standard procedures (11). All the results were compared with Bergey's Manual of Systematic Bacteriology (12) for tentative identification of genus and species.

### Results and Discussion

A total of 51 pasteurized market milk samples were investigated and 53 isolates were collected in the present study.

#### Generic Identification

All the selected isolates (53) were found to belong to the genus, *Bacillus* on the basis of the morphological, cultural characteristics and biochemical tests (Table 1). All the isolates were gram positive, rod-shaped organisms, capable of forming spore. They were all either strict aerobic or microaerophilic, gram positive, catalase positive, motile in nature, capable of producing acid from glucose (with exception of only five isolates). The incidence of psychrotrophic spore formers in pasteurized milk samples appeared close to the earlier findings (13, 14). In spite of the low population of psychrotrophic spore forming bacteria, this group was considered as the most important one because of its faster growth, metabolism and pathogenicity as compared to any other bacteria present in the milk (15). Psychrotrophic spore formers were reported to cause perceptible spoilage de-

fects (16), and pose a health risk in the form of food poisoning (17) by producing different types of enterotoxins (18).

#### Species Identification

This selected isolates were subjected to undergo a series of biochemical tests for the tentative species identification (Table 2). The results of the biochemical tests were compared with Bergey's Manual of Systematic Bacteriology (12) and the selected isolates were found to belong into different 9 types (I to IX), which were assigned to different species under the genus, *Bacillus* (Table 3). The relative distributions of the species in percentage (%) indicated that *B. cereus* was the predominant psychrotrophic type (33.96%) in the pasteurized market milk samples. The psychrotrophic type *B. subtilis* was second highest in population (18.86%) followed by *B. megaterium* (11.32%), *B. sphaericus* (9.43%), *B. circulans* (7.55%), *B. polymyxa*, *B. globisporus* and *B. macerans* represented 5.66% each in population distribution in pasteurized milk samples, while *B. licheniformis* was found to be the least psychrotrophic type (1.89%) in the present study. As observed in previous studies (19), we also noted the presence of *Bacillus* species in the milk samples in the current study. We recorded the highest occurrence of *B. cereus* in the milk samples as also found in the previous findings (20, 21). However, few other reports indicated that *B. subtilis* was the predominant in pasteurized milk (22, 23). Similarly, it was also reported (4) that the US market milk was contaminated with *Bacillus* species, of which 43% belonged to the species *B. licheniformis* followed by *B. cereus* (37%) and the remaining 20% comprised of

**Table 3.** Tentative identification of psychrotrophic sporeforming isolates.

Type	No. of isolates	Tentative species	Percent of total isolates	Variation from Bergey's manual
I	18	<i>Bacillus cereus</i>	33.96	2 isolates produced negative V-P test and 1 isolate failed to ferment sucrose
II	10	<i>Bacillus subtilis</i>	18.86	1 isolate failed to ferment sucrose
III	6	<i>Bacillus megaterium</i>	11.32	1 isolate failed to hydrolyze gelatin
IV	5	<i>Bacillus sphaericus</i>	9.43	1 isolate hydrolyzed starch
V	4	<i>Bacillus circulans</i>	7.55	-
VI	3	<i>Bacillus polymyxa</i>	5.66	1 isolate grew at 5% salt level
VII	3	<i>Bacillus globisporus</i>	5.66	-
VIII	3	<i>Bacillus macerans</i>	5.66	-
IX	1	<i>Bacillus licheniformis</i>	1.89	-

other *Bacillus* species. *B. licheniformis* was reported to be the common contaminant in pasteurized milk (7), whereas, the present finding showed the least occurrence of the same species. Some *Bacillus* isolates in the present study failed to show identical biochemical reactions for species identification and designation (Tables 2 and 3). This might have happened due to mutagenic changes during serial transfer of the isolates (24) or, due to alteration in the pathway of various biochemical reactions under the environmental influences (25).

### Conclusion

The existence of psychrotrophic *Bacillus* spore formers in pasteurized milk posed a great threat to the dairy industry, as the psychrotrophic spore forming bacteria were the major determinant of the shelf life of pasteurized milk. It also indicated a potential public health hazard. In the present study, *B. cereus* appeared to be the most common in pasteurized market milk supplies. *B. subtilis* and *B. megaterium* were also present in significant numbers. It warranted further research to make the technological strategies for eliminating the problem of sporeforming psychrotrophic bacteria in pasteurized milk.

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