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Effect of holder pasteurization and storage of donor human milk on *Bacillus cereus* survival

Original Study

Kamila Strom[1], Sylwia Jarzynka[1]*, Anna Minkiewicz-Zochniak[1], Aleksandra Wesolowska[2], Gabriela Oledzka[1]

[1] Department of Medical Biology, Medical University of Warsaw, Warsaw, Poland
[2] Laboratory of Human Milk and Lactation Research at Regional Human Milk Bank in Holy Family Hospital; Department of Medical Biology, Warsaw, Poland

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Abstract

Introduction. Human milk, which constitutes the best composed nutrition for infants, is not a sterile biofluid. Besides nutritional and bioactive components, many microorganisms have been found in human milk, including *Bacillus cereus*. Those pathogenic bacteria can be responsible for causing pneumonia or septicemia in neonates. Although *Bacillus cereus* infections are not common, they represent one of the increasing factors of a high mortality rate in the preterm infants group. Donor human milk (DHM) may be a significant source of this pathogen.
Materials and Methods. Given the high ability of *Bacillus cereus* spores to survive under different thermal conditions, we tested the effect of the holder pasteurization and storage points (4 °C and –21 °C) on sporulation and vegetative forms' survival in inoculated human milk samples by culture method and light microscopy.
Results. Studies confirmed that holder pasteurization is effective against vegetative forms of *Bacillus cereus*. Additionally, during storage endospores were observed in all analyzed inoculated donor milk samples, both the pasteurized and unpasteurized samples, thus possibly promoting the occurrence of *Bacillus cereus* in DHM.
Conclusions. *Bacillus cereus* is considered a contamination in DHM and in the human milk banks' (HMB) environment. There is a need to further develop methods of identifying, transmitting, and preventing bacteria forming spores, which could be applied in HMB.

Keywords

donor human milk • human milk banks • Bacillus cereus • premature infants

1. Introduction

Human milk is the best composed nutrition for infants, recommended by the World Health Organization (WHO), for exclusive feeding for at least the first 6 months of life [1]. In situations when mothers cannot provide their own breast milk for their child, the best alternative for infant feeding is high quality donor human milk (DHM) from human milk banks (HMB) [2]. Human milk is not a sterile biofluid, besides nutritional and bioactive components, immunological factors and growth factors more than 820 taxa at a species level have been found in it, especially *Proteobacteria* and *Firmicutes* phyla [3,4]. Among the potentially pathogenic ones, the presence of *Bacillus cereus* has been confirmed in breast milk [5]. *Bacillus cereus* is a Gram-positive bacteria found in both vegetative and sporulating forms. It shows the ability to grow under aerobic or facultatively anaerobic conditions. Spores, commonly found in the outdoor environment, are a food contamination problem in the food industry [6]. *Bacillus cereus* is primarily responsible for causing severe inflammation of the gastrointestinal tract manifested by diarrheal and vomiting syndrome. Pathogenicity is due to tissue-destroying exotoxins produced by these bacteria such as hemolysins, phospholipases, and proteases [7]. In addition, they cause intraocular inflammation, pneumonia, and can cause central nervous system infections and septicemia in neonates and immunocompromised patients [6,8]. Preterm infants are highly susceptible to *Bacillus cereus* infections due to their underdeveloped immune systems and prolonged invasive procedures such as mechanical ventilation and catheterization. Although infant infections are not common, they have a high mortality rate [8] and DHM appears to be a significant source of infant infection with this pathogen [9,10].

The European Milk Bank Association (EMBA) recommends that milk should be pasteurized with a holder method before being given to a premature newborn [2]. This commonly used method of milk pasteurization effectively eliminates vegetative forms of microorganisms; however, it has been confirmed to be ineffective against heat-resistant spores [11]. Due to their unique structure and inactive metabolic state, spores are able to survive poor environmental conditions in a dormant state for many years while maintaining significant resistance to chemical and physical agents including elevated temperatures [12]. Heat treatment processes below 100 °C can also contribute to the activation of germination of spores already present in milk [13,14]. These properties are confirmed by the fact that many HMBs have shown

* *E-mail: sylwia.jarzynka@wum.edu.pl*

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contamination of DHM with *Bacillus* spp. both before and after pasteurization. It is one of the main reasons for rejecting batches of pasteurized DHM [9,15,16].

The aim of the study was to test the effect on HMBs of home temperature and storage time on the *Bacillus cereus* inoculated in human milk. The present study had the major objective of determining the effect of holder pasteurization and storage method and time on sporulation and survival of vegetative forms of *Bacillus cereus* in human milk samples inoculated with this strain. The project aimed to map the storage and processing conditions of DHM in HMBs and home conditions and the impact of these parameters on the presence of *Bacillus cereus* in human milk samples. We recreated the operating model of HMBs *versus* laboratory conditions (Figure 1).

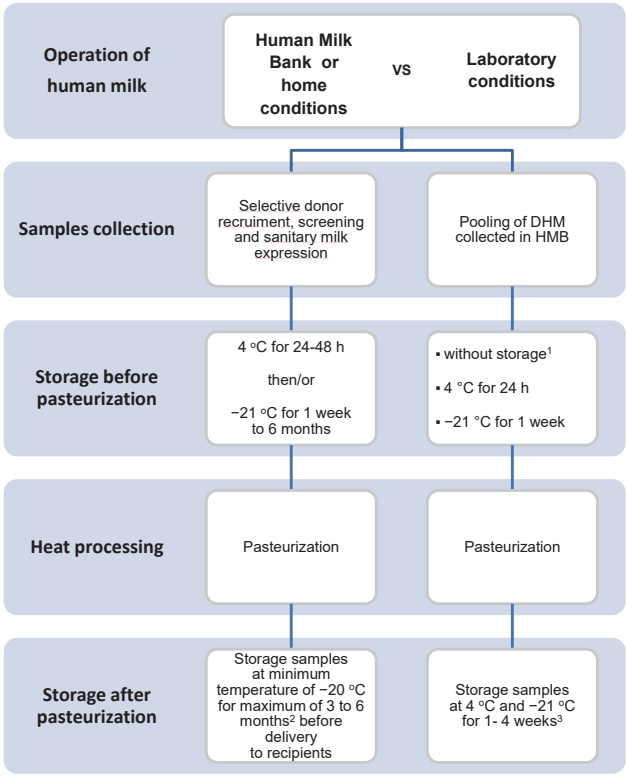


Figure 1. Mapping of the HMBs'/home procedures under laboratory conditions
[1]In situations when sample was pasteurized immediately after collection of DHM; [2,3]the most samples were used during first month of storing

2. Materials and methods

2.1. Milk samples

DHM (2nd–6th week of lactation) was obtained from the Regional Human Milk Bank in Warsaw. Before milk samples were delivered to the institution, donors were instructed in hygienic pumping. Until the analyses were performed, milk was stored at –21 °C for no longer than 6 months.

After thawing, the collected milk samples were pooled for the experiment. A pool of 1200 mL was divided for three independent analyses.

2.2. Microbiological purity analyses after inoculation and next pasteurization

The pooled milk was divided into equal parts and then bottled into 100 ml bottles, and then used in three independent experiments (Figure 2). The samples prepared this way were pasteurized to eliminate native microbiota and possible contamination. After microbiological purity was confirmed by serial dilution method, samples were inoculated with a *Bacillus cereus* ATCC 14579 standard strain to a final concentration of 10^5 CFU/ml (1 – 3). Milk samples not inoculated with the reference strain served as controls to confirm the purity of the milk used in the experiment (1* – 3*). After 12 h of incubation at 37 °C, the samples were subjected to the storage processes:

- without storage (1, 1*);
- in a refrigerator (4 °C) for 24 h (2, 2*);
- and in a freezer (–21 °C) for 7 days (3, 3*).

Next, they underwent the final pasteurization.

The above parameters are related to the time and temperature of milk storage at home before being transferred to the HMB or with storage time at the HMB before pasteurization [17].

All experiments were performed in four replicates. Before and after final pasteurization, quantitative evaluation of viable bacteria cells was performed by a serial dilution method on the selective-differentiation medium *Bacillus* ChromoSelect Agar (BACARA®, bioMérieux SA, Marcy l'Étoile, France). After final pasteurization, microbiological analyses were performed at weekly intervals for a period of 1 month simultaneously on samples stored under refrigeration (4 °C) and in the freezer (–21 °C). Before seeding, we performed a pre-incubation step (overnight at 37 °C). One week to six months is the period of the average shelf life of pasteurized milk in HMBs [18].

Control samples 1C, 2C, and 3C, were not subjected to the final pasteurization process, but only to inoculation. Additionally, analysis of *Bacillus cereus* concentrations in samples with pure milk—non-inoculated and non-pasteurized—(1C*, 2C*, 3C*) were done. Samples 1C – 3C and 1C* – 3C* underwent the same processes of storage as samples (1 – 3; 1* – 3*).

At each stage of the experiment, Gram staining and Schaeffer-Fulton staining were performed to assess the presence/absence of endo- and exospores in the tested samples. The microscopic observation of vegetative cells and endospores was monitored using the microscope OPTA-TECH MB200 (OPTA-TECH, Poland) with a digital camera.

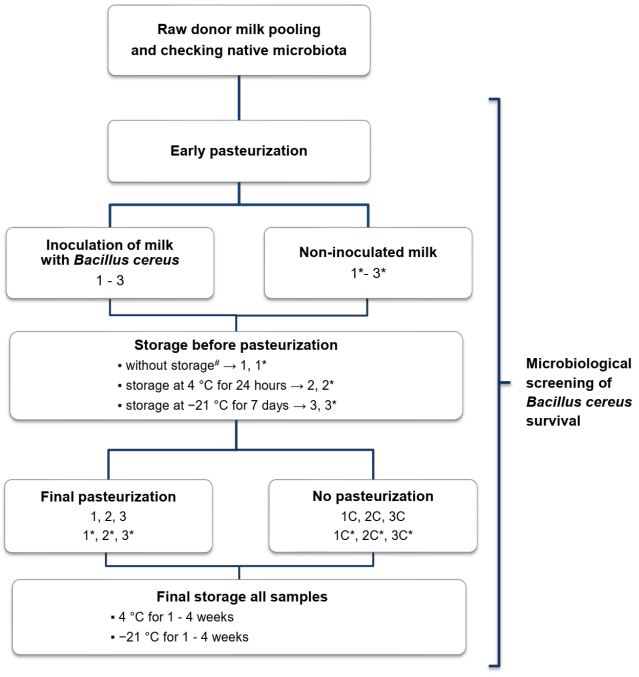


Figure 2. Study design of Bacillus cereus survival
1, 2, 3—testing samples (inoculated, pasteurized); 1C, 2C, 3C—control of bacterial viability (inoculated, unpasteurized); 1, 2*, 3*—pasteurization process control (non-inoculated, pasteurized); 1C*, 2C*, 3C*—(non-inoculated, unpasteurized). [1]In situations when sample was pasteurized immediately after collection of DHM*

2.3. *Bacillus cereus* bacterial culture

Bacillus cereus ATCC 14579 strain was cultured in brain heart infusion medium (BHI medium, Oxoid™, CM1135, United Kingdom) at 37 °C for 24 h. To evaluate the average concentration of the culture, the optical density was measured using a McFarland densitometer (DEN-1B, Biosan, Latvia). To obtain a culture having 0.5 McF (McFarland Units), corresponding to a bacterial concentration of 10^8 CFU/ml, the culture was diluted with sterile BHI. The bacterial concentration was confirmed by the serial dilution method. The culture thus obtained was used for further analysis.

2.4. Holder pasteurization

Human milk samples were pasteurized in a closed water bath in sterile 100 ml bottles. To monitor the temperature of the process, a control bottle was placed in the water bath with a thermometer submerged in the milk. As the temperature reached 62.5 °C in the control bottle, the process was carried out for 30 minutes. After heat treatment, all samples including the control bottle were rapidly cooled to 4 °C in an ice-cold water bath. The cooling process took no more than 15 minutes. At each stage, the milk bottles were manually stirred every 2 – 3 minutes.

2.5. pH assay

Additionally, pH measurements of DHM samples fortified with *Bacillus cereus* were performed (samples: 1 – 3, 1C – 3C, 1* – 3*).

Furthermore, an analysis examining the effect of pH on the growth of *Bacillus cereus* over time was performed. DHM in standard pH 7.0 was swirled and filtered through a filter to remove excess fat. The sample prepared in this way was used for further analysis. Liquid BHI medium was prepared in three pH variants: 6.0, 6.5, and 7.0. pH values, which was determined by the Accument Basic AB 15 pH meter (Fisher Scientific, Pittsburgh, PA, USA) before the sterilization process using hydrochloric acid. BHI's pH value corresponds to the range of pH values of milk from the experiment in section 2.2. Each of the media and breast milk were inoculated with a *Bacillus cereus* ATCC 14579 strain to a final concentration of 10^5 CFU/ml. The samples thus prepared were applied to 12 well plates (TPP, Switzerland) in triplicate; 1.5 ml to each well. Bacterial growth kinetics were measured, at 4 °C, RT, and 37 °C using a microtiter plate reader (Synergy HTX multimode reader, Biotek®, Winooski, VT, USA). Absorbance measurements (OD 600) were taken at 30 min intervals. Clean medium and uninoculated milk served as controls for each variant. Results were given as the difference of OD 600 of the test sample and OD 600 of the control. The Gen5 Data Analysis Software (Biotek®, Winooski, VT, USA) was used for data analysis.

2.6. Statistical analysis

All statistical analyses were performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, California USA, www.graphpad.com). The differences between the results of all evaluations were analyzed by t-test. Statistical significance was assumed at a p-value of < 0.05. All graphs and table were completed using Microsoft Excel 365 (Washington, DC, USA) licensed to the Medical University of Warsaw.

3. Results

3.1. Inoculation with *Bacillus cereus*

No growth of *Bacillus cereus* was observed in the uninoculated milk pool (1* – 3*). After inoculation of the early pasteurized milk, a *Bacillus cereus* growth of 10^5 CFU/ml (range 3.40×10^5 – 8.98×10^5 CFU/mL) was obtained in all milk samples inoculated with a *Bacillus cereus* strain ATCC 14579.

3.2. Microbiological analyses after final pasteurization

The concentration of bacilli after additional storage before the final holder was close to the baseline concentration after incubation for samples 1, 2, 1C, and 2C. The storage of 3, 3C samples before the final holder reduced the concentration by three orders. Immediately after final pasteurization, a 100% reduction of vegetative *Bacillus cereus* was observed in each of the three experiments (1, 2, 3). In samples stored under refrigeration

Table 1. Viability of Bacillus cereus after processes in the study

Phase / Sample	Raw milk	Early pasterurization	Inoculated milk[1]	Storage before final pasteurization		Final pasteurization	Final storage conditions							
							-22 °C				4 °C			
							1 wk	2 wks	3 wks	4 wks	1 wk	2 wks	3 wks	4 wks
1	0.00	0.00	5.81 ± 0.19	Processed immediately after inoculation[2]	5.81 ± 0.19	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1*			0.00		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1C			5.53 ± 0.01		5.53 ± 0.01	x	3.05 ±0.21	0.00	0.00	0.00	5.42 ± 0.02	0.00	0.00	0.00
1C*			0.00		0.00	x	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2			5.81 ± 0.08	24 h at 4 °C	5.72 ± 0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2*			0.00		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2C			5.58 ± 0.02		5.54 ± 0.11	x	3.14 ± 0.21	0.00	0.00	0.00	5.25 ± 0.05	0.00	0.00	0.00
2C*			0.00		0.00	x	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
3			5.74 ± 0.47	7 days at -21 °C	2.82 ± 0.13	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
3*			0.00		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
3C			5.54 ± 0.12		2.39 ± 0.39	x	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
3C*			0.00		0.00	x	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

At each stage, microbiological analyses were performed. 1, 2, 3—testing samples (inoculated, pasteurized); 1C, 2C, 3C—control of bacterial viability (inoculated, unpasteurized); 1, 2*, 3*—pasteurization process control (non-inoculated, pasteurized); 1C*, 2C*, 3C*—evaluation of the viability of the native microbiota (non-inoculated, unpasteurized). The data (log CFU ml^{-1}) are presented as mean values with the standard error of the mean. Statistically significant differences are marked in color, depending on the degree of significance: green: $p < 0.001$, violet: $p < 0.05$. Non-significant differences ($p > 0.05$) are not marked. [x]Samples not-processed. [1]Concentration after 12 h incubation with bacteria; [2]In situations when sample was pasteurized immediately after collection of DHM*

and in the freezer, no growth of bacilli was observed at any of the analyzed four time points (1 – 4 weeks). Detailed results are presented in Table 1.

After the final holder stage, in inoculated control samples after 1 week of storage at –21 °C, a decrease in bacterial concentration by two orders of magnitude was observed for 1C, 2C. Storage in the fridge did not change the concentration in 1C and 2C after 7 days. At subsequent time points, i.e., after 2, 3, and 4 weeks of storage, no growth of *Bacillus cereus* was observed in the pasteurization control samples. For the third type of experiment, 3C in four time analyzed points, both at –21 °C and at 4 °C, no growth of vegetative *Bacillus cereus* in all testing samples was observed. Samples 1* – 3* and 1C* – 3C* did not grow any *Bacillus cereus*.

Gram staining visualized vegetative forms of the analyzed bacterium at each of the analyzed stages after inoculation. In addition, in samples inoculated with *Bacillus cereus*, after 12 h of culture and at each subsequent analyzed point after final pasteurization, staining highlighted endospores were marked by the green arrow on Figure 3. Schaeffer-Fulton staining identified the vegetative forms of bacteria and endospores as well, but endospores did not stain green.

3.3. pH measurement

Examination of the pH of the milk pool before pasteurization showed a value of 7.0. After the first pasteurization process, a slight decrease in pH value to 6.84 was observed. Inoculation with *Bacillus cereus* strain and carrying out another final pasteurization process caused a decrease in pH value in the analyzed points to an average of 6.37 in the range from 6.27 to 6.46 for samples' storage in –21 °C and from 6.26 to 6.47 for 4 °C. Detailed results are shown in Figure 4. Compared to the samples 1* – 3*, in which the pH values were 6.76 ± 0.04 after final pasteurization, it can be concluded that there was an increase in bacteria-caused acidification and a decrease in pH by 0.38 ± 0.09 for 4 °C and 0.38 ± 0.06 for –21 °C.

3.4. Dynamics of bacterial growth on different pH media and in milk

The lag phase and exponential growth phase (log phase) lasted longest for the BHI medium with pH 6.0 and shortest for pH 7.0 at room temperature (RT) and 37 °C. Bacterial cultures in the BHI medium with a higher pH reached a higher OD600 value regardless of the temperature (Figure 5). At 4 °C, there was no increase in OD600 in any of the samples analyzed (data not

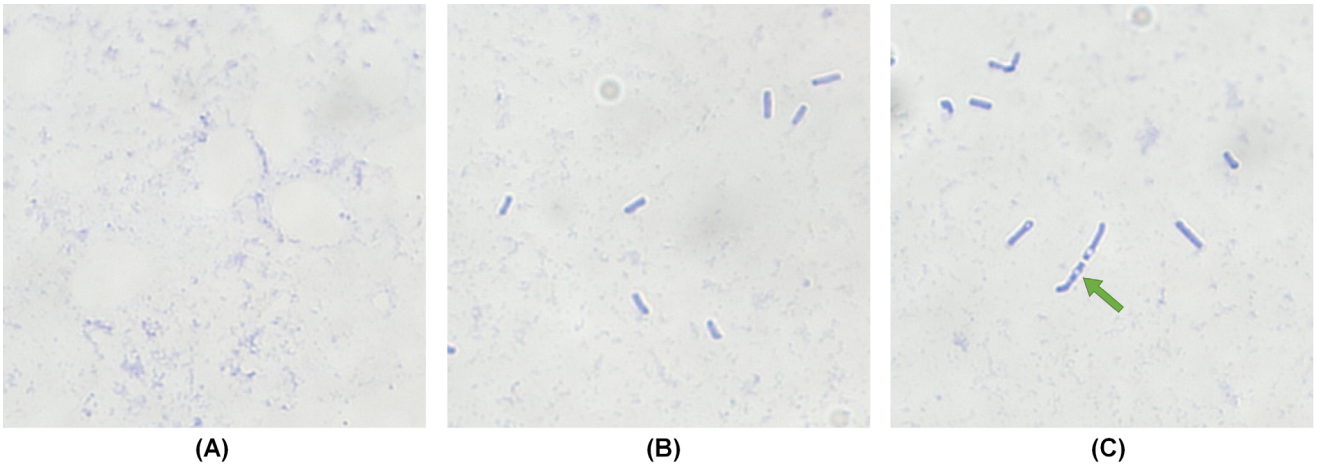


Figure 3. Gram stain of Bacillus cereus viewed by light microscopy
Microscopic images of human milk before inoculation of Bacillus cereus (A), vegetative cells in Bacillus cereus culture (B), and pasteurized milk inoculated with Bacillus cereus (C); ×1.000 magnification, oil immersion

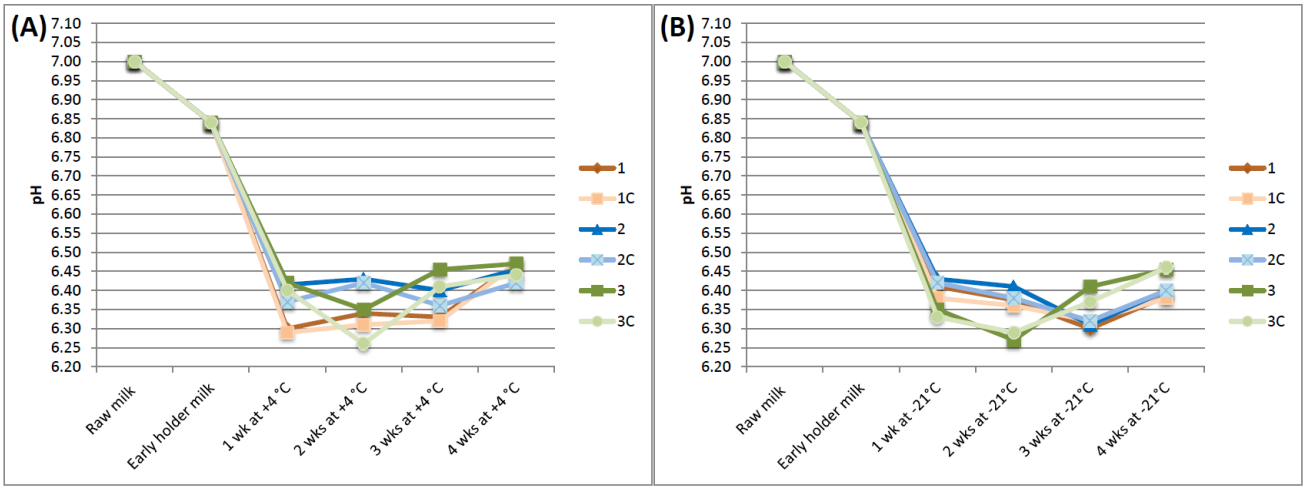


Figure 4. The pH level analysis of the inoculated human milk
Milk storage at 4 °C after final holder (A), Milk storage at –21 °C after final holder (B). Mean values of 3 readings. 1, 2, 3—testing samples (inoculated, pasteurized samples); 1C, 2C, 3C—control of bacterial viability (inoculated, unpasteurized samples)

shown in graph). The inoculated breast milk sample showed a longer lasting lag phase than any of the analyzed cultures in the BHI medium, both at RT and at 37 °C. At RT, log phase started for pH 6.0 was the highest at 24 h, for pH 6.5 at 22 h 30 min, and for pH 7.0 the lowest was at 19 h. At 37 °C, log phase started after: 9 h, 8 h, and 7 h for pH 6.0, 6.5, and 7.0, respectively. For inoculated milk, the log phase started after 30 h at RT and after 14 h at 37 °C. Only breast milk samples went into the death phase, after 15 h (37 °C) and 34 h (RT).

4. Discussion

HMBs in Europe and around the world, in addition to the stringent selection criteria for donors, are placing increasing emphasis on the microbiological safety of milk administered to preterm infants [19,20]. Although HMBs have inconsistent guidelines for screening or microbiological analysis of breast milk before it is given to the newborn, a large role is attributed to various types of donors and breast milk testing [2,9,19]. Microbiological analyses of breast milk are based primarily on the assessment of the presence of vegetative forms of bacteria in milk samples before and after pasteurization. Recently, increasing attention has been paid to bacterial strains that are able to survive the pasteurization process due to their ability to spore. One such bacteria is *Bacillus cereus*, which is an increasingly common cause of milk rejection after heat treatment in HMBs [15,21,22]. Therefore, testing for *Bacillus cereus* in milk after pasteurization is necessary [23].

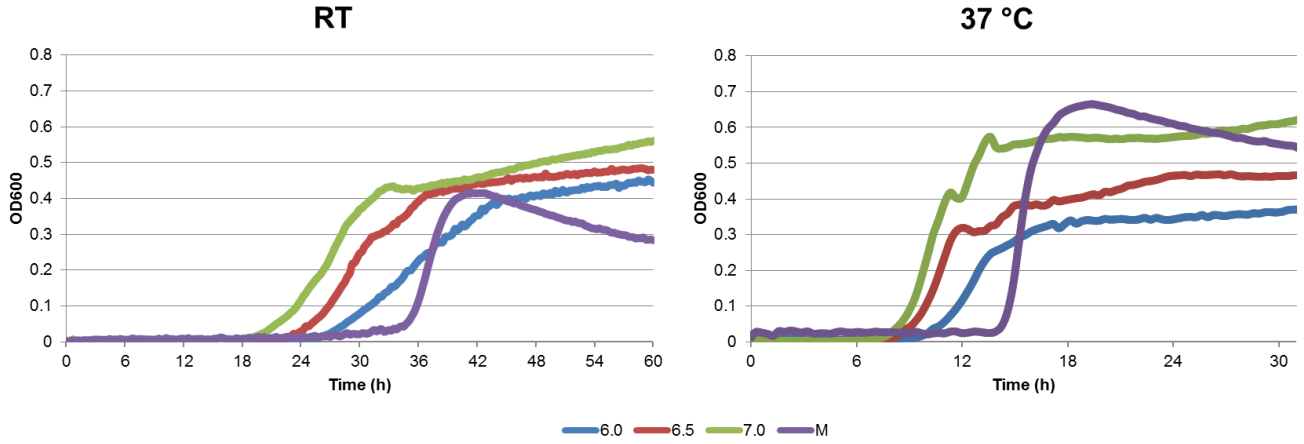


Figure 5. Bacillus cereus growth curves on BHI broth cultures and in donor milk samples
Mean values of 3 analyses. BHI—brain heart infusion medium; RT—room temperature; 6.0—BHI 6.0 pH, inoculated; 6.5—BHI pH 6.5, inoculated; 7.0—BHI pH 7.0, inoculated; M—inoculated human milk

It has been proven that different strains of *Bacillus cereus* are identified in DHM, which confirms that this bacterium enters milk from different sources [21]. The mortality rate due to *Bacillus cereus* infections among preterm infants with poorly developed immune systems is high. It is believed that hospital outbreaks may be caused by *Bacillus* sp. strains found in the hospital environment, e.g., alcohol-based hand washing solutions, gloves, in ventilator humidifier water, freshly washed bedding, or from the staff themselves. However, DHM is also indicated as one of the potential routes of infection [6,10]. Although no similarities in genetic profiles were found between strains isolated in the hospital environment or from maternal skin and strains isolated from donor milk, it is suspected that *Bacillus cereus* may enter DHM from the banking environment [21]. On the other hand, the lack of association may be due to the fact that spores are more likely to enter the milk much earlier than during the processing of milk in HMBs, e.g., from contaminated breast pumps used at home [16,24]. To avoid the potential transmission of *Bacillus cereus* through DHM, basic hygienic principles should be followed when preparing portions/batches of milk in the HMB by conducting careful monitoring of conditions during milk collection, storage, and administration [25]. Milk, due to its composition, provides a good medium for bacterial growth. If milk is stored under inappropriate conditions, bacterial counts may increase [26]. Slow cooling, too much storage at RT, and even keeping milk at elevated temperatures for too long after pasteurization should be avoided [10,19,22]. Focusing on good practices to better control the cleanliness of the bank environment and the better education of donors are other aspects that influence the increase of the cleanliness of milk in banks [27]. Modification of currently existing contamination detection methods and the use of more advanced diagnostic methods focusing on the analysis of the presence of spores in DHM can also help to increase the safety of milk transferred to newborns. The experience of a French bank, where after an outbreak of *Bacillus cereus* among newborns, existing analytical methods were modified, resulted in increased sensitivity in the detection of *Bacillus cereus* contamination [8,16].

The results of our study confirmed that holder pasteurization is effective against vegetative forms of *Bacillus cereus*. The method of the storage of milk inoculated with bacteria before pasteurization did not affect the efficiency of sterilization, as no bacterial growth on BACARA medium was shown after processing in any of the samples tested (samples: 1, 2, 3). However, microscopic examination showed the presence of endospores in the bacilli present in the samples of human milk tested, but their numbers in samples 1, 2 and 3 were comparable. This shows that pasteurization may not be effective against *Bacillus cereus* spores. Although in our study we performed a pre-incubation step before seeding the samples, we did not confirm the growth of germinated spores on a solid medium. The reason was probably that the concentration of spores was too low. Scientific studies show that classical culture methods have major limitations: they are time consuming, and have low sensitivity and efficiency [14]. This fact may have additionally affected the inability to confirm the presence of bacterial growth in the samples. Raso et al. obtained 90% sporulation efficiency of *Bacillus cereus* under culture conditions after 2 days at 37 °C and higher. At lower temperatures, 15 °C (the lowest temperature analyzed), sporulation was unsuccessful [28]. The microscopic images of the milk samples we examined confirm the appearance of endospores, but they did not form from all vegetative cells. The number of endospores did not increase with the time of storage after processing, which would suggest that the critical moment for their production was the stage of multiplication of bacteria

before holder pasteurization, because in the bacterial culture that was inoculated into the samples the presence of endospores was not found.

Studies indicate that refrigerated storage of milk can affect the number of vegetative forms of bacteria [20,29]. It is recommended that the storage time of milk before the pasteurization process should not exceed 24 – 48 h at 4°C, then milk should be frozen [2,29]. According to polish Regional Human Milk Banks, unpasteurized DHM should be stored frozen in home conditions between 1 week and 3 months and in HMB conditions for up to 6 months [18]. Therefore, data collected by E. Kontopodi et.al showed that donors stored milk in the freezer before holder pasteurization from 1 week up to 6 months [17]. Meanwhile, the shelf life of milk after pasteurization is 3 to 6 months [18]. However, data from worldwide HMBs indicate that in some institutions the time of storage for the frozen milk after holder pasteurization is extended up to 2 years [17]. Pandya et al. showed that storing milk at negative temperatures reduces the number of vegetative forms of bacteria by nearly 100%. Analyses indicated that the greatest decreases were observed for up to 12 weeks of storage [20,29]. In our control samples, 1C, 2C, and 3C, we observed a similar relationship. As early as 2 weeks after processing, no bacterial growth was shown in samples stored in the freezer. Also, in the milk stored in the refrigerator, a 100% reduction in the number of bacteria was observed 2 weeks after processing. The possible thermal shock during freezing is responsible for the decrease in the number of bacteria [8]. The slow freezing probably causes the formation of ice crystals inside the bacterial cell by which the cell wall is destroyed [29]. The control samples showed a similar amount of spores as the samples that underwent pasteurization. Storing the samples under refrigeration conditions also did not promote the formation of more endospores.

Bacillus cereus spores, which lack metabolic activity, are resistant to extreme environmental conditions such as higher temperatures, freezing, drying, and radiation [6]. A 30-minute incubation at 65 °C and 70 °C has been confirmed to be sufficient for heat activation of dormant spores, but incubation parameters to awaken spores may vary within spores of the same strain [30]. Such temperature parameters are similar to the conditions achieved during holder pasteurization. Therefore, if *Bacillus* spores are present in milk samples before heat treatment, they may be germinated during the sterilization process. Storing such milk under refrigerated conditions may cause *Bacillus cereus*, which is a psychrotrophic bacterium, to lead to reduced microbial stability of DHM [26]. On the other hand, the refrigeration process may lead the cell to revert to the spore form since sporulation is a reversible process [30]. Therefore, without a sufficiently sensitive spore detection method preceded by spore germination, sporulation may not be detected despite its presence. For now, our research focuses more on growth conditions, but in the future it is important to expand it to sporulation and germination.

A study by Nakata et al. showed that, reaching a minimum pH during *Bacillus* growth was associated with the end of exponential growth [31]. The pH value reached by the milk samples after bacterial growth was maintained at a similar level throughout the storage period in both refrigerated and freezer conditions. Reaching such a value could, under the conditions we created, be a value indicative of the end of growth. Moreover, our study showed that the *Bacillus cereus* strain analyzed achieves slower growth at a lower pH. Maximum absorbance during the stationary phase is statistically significantly lower at pH 6.0 and 6.5 than at pH 7.0, regardless of the growth temperature. This confirms that the observed decrease in pH in the milk samples during the bacterial growth phase could be one of the reasons for the slower bacillus growth in the control samples. Inoculated milk had the longest lag phase relative to bacterial cultures on BHI medium. However, the exponential phase was more dynamic. The inoculated milk reached higher OD600 at 37 °C than the culture at pH 7.0, however, the stationary phase was very short and lasted about 1 h. After this time, the death phase started for the inoculated milk. This result indicates that the growth of vegetative forms in milk is not stable and stops very quickly. This is confirmed by the results of the control trials. The combination of lower temperature, lower pH values, and additional anaerobic conditions during *Bacillus* growth reduces its growth [32]. Possible vacuum packaging of milk portions could further increase the safety of DHM in banks. Furthermore, it has been noted that *Bacillus* spores are more thermally unstable at a lower pH. In the researchers' case, in samples at a pH similar to ours achieved after pasteurization, the lag phase of spore growth was longer than in samples at a higher pH [30].

Isolation of *Bacillus* and even confirmation of the presence of spores does not necessarily mean that it is a highly virulent strain. Some strains are both harmless and highly lethal [19,33]. Studies show that despite confirmation of the presence of this Gram-positive bacilli in milk samples after pasteurization, additional quantitative analyses do not necessarily confirm the presence of toxins [19]. Culture methods do not show the presence of toxins, nor can they distinguish *Bacillus* strains from each other. Unfortunately, there are not yet well standardized alternative molecular methods for the detection of food spores, as there are difficulties, for example, in isolating genetic material from spores. From a clinical point of view, it would be worthwhile to introduce complementary tests to the screening of milk samples; however, they cannot completely replace the currently used methods because of the group of premature infants to whom milk is administered [14]. All these difficulties translate into underestimation and under-documentation of all *Bacillus* infections. The number of toxigenic *Bacillus cereus* is increasing, and the effective methods to identify them are limited, so they should be considered as a serious threat for populations [33].

In the future, when new methods become more common in diagnostics, so-called growth predictors, which allow computer simulations of how bacteria grow and behave in a given environment, may help [34]. This would make it easier to determine the growth trend and behavior of bacteria in, for example, milk samples, after first assessing the concentration of bacteria in a given volume and in HMB environment.

5. Limitation of the study

The main limitation of our experiment was the problem with spore germination. More detailed analysis or testing of germinant receptors are needed. Additionally, examination of growth on the BACARA medium gives the answer about the presence of only vegetative forms of bacteria. The inability of potentially present spores in the sample to germinate could result in false-negative culture results.

Because of the ability of spores to survive in adverse conditions, it is worth repeating such analyses in the future on strains growing at low temperatures.

In the future, the research should be expanded to include experiments using wild *Bacillus cereus* strains, the epidemiological analyses of the *Bacillus cereus* occurrence in the HMB environment, and the assessment of the biological properties of these strains, including antibiotic resistance.

6. Conclusion

Bacillus cereus vegetative cells and spores are considered a contaminant when isolated from clinically relevant samples such as DHM before and after pasteurization. Due to the problem of isolating this strain from samples and its transmission in medical care, further modifications of existing identification methods should be undertaken and new analytical and sanitizing techniques should be introduced into the operations of HMBs. Additionally, greater emphasis should be placed on HMBs to test samples for the presence of *Bacillus cereus* in milk not only before pasteurization but also after.

Abbreviations

BACARA® – *Bacillus* ChromoSelect Agar; **BHI medium** – brain heart infusion medium; **DHM** – donor human milk; **EMBA** – European Milk Bank Association; **HMB** – human milk banks; **McF** – McFarland units; **RT** – room temperature; **WHO** – World Health Organization.

Authors' contribution

K.S., S.J.: research concept and design; **S.J., G.O.**: supervising the project; **K.S.**: carrying out the experiments; **K.S.**: acquisition of data; **K.S., S.J.**: data analysis and interpretation; **K.S.**: writing - original draft preparation; **S.J., A.M.-Z.**: writing—review and editing; **K.S., S.J.:** visualization and literature review; **G.O., A.M.-Z., A.W.**: final proofreading and approval of the version for publication.

ORCID

Kamila Strom https://orcid.org/0000-0003-4109-0391
Sylwia Jarzynka https://orcid.org/0000-0002-9269-3494
Anna Minkiewicz - Zochniak https://orcid.org/0000-0002-1249-7670
Aleksandra Wesołowska https://orcid.org/0000-0002-7270-5910
Gabriela Oledzka https://orcid.org/0000-0001-7090-6156

Conflicts of Interest

The authors have no potential conflicts of interest to declare.

Ethics approval

Approval of the Bioethics Committee at the Medical University of Warsaw was obtained, No. KB/65/2019.

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