Theoretical Substantiation of the Practical Need for the Sanitation of Spore Forms of Microorganisms in Raw Milk before Its Processing

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Abstract. In the production of sterilized dairy products, a relevant problem is the control of vegetative and spore forms of thermophilic bacteria, whose active growth by the end of the technological process in milk can reach a high number and form a biofilm on the equipment parts which have elevated temperatures. The results of the experiments showed the possibility of cleaning raw milk from spore forms of bacteria by provoking their germination by preliminary low-temperature heat treatment with their further destruction by pasteurization. Spores of Bacillus cereus, after primary heat treatment (65 or 72 °C), do not germinate both at low storage temperatures (at + 5 °C) and when stored in uncooled milk (51–65 °C), at least for six hours. To provoke the germination of spores of Bacillus cereus, it is necessary to lower the temperature to +37 °C and hold for 5-6 hours; at the end of the sixth hour, the proportion of spores germinated into the vegetative state is about 35%. Primary heat treatment of milk reduces the germination time of spores of Bacillus cereus by half: from 10-12 hours to six. With the help of further pasteurization at 75 °C, it is possible to reduce the concentration of spore forms of microorganisms in milk to 3.10^2 CFU/cm^3. The technology of double heat treatment of milk with its intermediate exposure for an hour and a half prevents the accumulation of metabolic products of a large group of bacteria: spore and vegetative, psychrophilic and thermophilic ones.

1. Introduction
In the production of sterilized dairy products, it is necessary to control vegetative and spore forms of thermophilic bacteria, the active growth of which by the end of the technological process in milk can reach up to 1.10^5 per 1 cm^3 of the quantity of mesophilic aerobic and facultative anaerobic microorganisms (QMAFAnM) [1, 2].

The main reason for this situation is the quality of raw milk. On the one hand, milk contains the facultatively thermophilic microorganisms Bacillus licheniformis, Bacillus coagulans and thermoactinomycetes, the low concentration of which in the final products has little effect on the overall picture of the microflora. On the other hand, the obligate thermophiles present in raw milk, surviving due to the formation of endospores and actively turning into a vegetative form with the formation of biofilms in the equipment parts that have elevated temperatures [3] create the main problem [4].
A decrease in the number of thermophilic spores is achieved by washing equipment, reducing production volumes, increasing the amount of rejected products and the cost of the hardware park.

To reduce the risk of residual microflora content in finished products, some researchers suggest a threshold for the content of thermophilic spores in milk powder up to $1.10^2$ per 1 cm$^3$ [4], although milk powder processors increase these requirements from $1.10^2$ to $5.10^2$ per 1 cm$^3$. In fact, production experience shows that the main cause of pasteurized products spoilage is secondary microbial contamination [5]. The increase in requirements for the quality of milk powder in terms of the content of thermophilic spores logically leads to an increase in the cost of final products: the markup above the standard price for milk powder containing $1.10^2$ spores per 1 cm$^3$ ranges from $70–100$ per ton.

Further studies of the reconstituted milk microflora showed that spores of mesophilic microorganisms have low thermal stability and therefore die during the production of sterilized products. The greatest danger to reconstituted milk is represented by thermophilic spore microorganisms.

According to preliminary studies, it was believed that the thermophilic microflora of dairy products contained, in most cases, only one type of microorganism - Bacillus stearothermophilus. Later experiments showed that in the thermophilic microbial community of milk powder, there are mainly two types of bacilli: Geobacillus spp. and Bacillus flavothermus, and only the first type can survive after sterilization [4]. Thus, the requirements for the content of thermophilic spores in milk powder are too rigorous and should include a selective method for the determination of Geobacillus spp.

The old method for determining the quality of milk powder includes the preliminary destruction of vegetative forms of microorganisms by pasteurization at a temperature of 80–100 °C for 10–30 minutes. Then the raw material under study is sown on agar with exposure in a thermostat at a temperature of 55°C for 48–72 hours, followed by counting the colonies. The new method for the determination of spores in milk powder includes sterilization at 108 °C for 30 minutes, which ensures the destruction of Bacillus flavothermus spores and the preservation of Geobacillus spp. Thus, the new limit for the content of thermophilic spores in milk powder can be reduced to $1.10^2$ spores per cm$^3$ [4].

To remove spores of Bacillus licheniformis and Geobacillus spp. from skim milk, some researchers suggest using cold microfiltration using membranes with pore sizes of 1.4 and 1.2 μm [6].

One of the most serious quality defects of hard and semi-hard cheeses, late blowing defect, is caused by endospore-forming bacteria of the genus Clostridium. To minimize financial losses and waste of resources due to cheese spoilage, raw milk with an increased amount of Clostridial spores should not be used for the production of certain types of cheese. In this context, the threshold values of the concentration of Clostridial spores, which cause defects in cheese quality, are discussed [7].

Traditional microbiological detection methods used in the dairy industry to detect spores show time constraints (they are time-consuming), effectiveness and sensitivity. Therefore, the use of food probing for the detection of Bacillus cereus spores in dairy products by molecular biology methods based on biosensors is of great importance [8].

The study of the milk microbiome in reservoirs in the relationship between bacterial groups and milk quality parameters showed the presence of spore-forming and pathogenic bacteria including Corynebacterium, Streptococcus, Lactobacillus, Coxiella, Arthrobacter and Lactococcus [9].

The ability of spores to cycle between the state of the spores and the germinating cell makes them attractive as biosensor systems for detecting pollutants in milk, in particular: antibiotics, aflatoxins, and bacteria [10].

A research on the toxic effect of aerobic spore-forming isolates obtained from raw milk on the quality and safety of dairy products showed that strains of Bacillus subtilis, Bacillus cereus group, Paenibacillus polymyxa and Bacillus amyloliquefaciens have a strong proteolytic effect, in contrast to the less active Bacillus licheniformis, Bacillus pumilus and Lysinibacillus fusiformis [11].

The late blowing defect affects the production of hard cheeses and Clostridium tyrobutyricum is the main cause of spoilage. The use of quantitative real-time PCR method for the detection of this agent is a relevant detection method [12].

The characterization of the spore-forming bacteria of the Bacillus cereus sensulato group and Clostridium perfringens isolated from the environment of an Australian dairy farm shows that they are
significantly different for each farm. Enterotoxin production by representative isolates of each identified species of Bacillus cereus sensulato was usually reduced in milk compared to broth [13].

Homogenization at ultrahigh pressure opens up new possibilities for the inactivation of Bacillus amyloliquefaciens spores in raw milk [14].

It has been empirically found that the spores of Geobacillus spp. do not germinate into a vegetative form if the ambient temperature is below 37 °C. Therefore, tests for the presence of thermophilic spores for sterilized dairy products are justified only if during their technological processing the milk is heated above 37 °C [4].

The greatest threat is posed by spore-bearing microorganisms that release toxins. When spores of Bacillus cereus germinate into vegetative forms, these bacteria release enterotoxin, which causes food poisoning in people. Often, infection with Bacillus cereus can cause bacteremia, endocarditis and meningitis against the background of symptoms of secondary immunodeficiency and depression, especially in people with prosthetic organs and hemodynamic disorders.

The results of the experiments showed that the most favorable conditions for the development of vegetative forms of Bacillus cereus are 30–32 °C; extreme temperature limits of their development range from 10 °C to 48 °C. The division process of Bacillus cereus takes place at a temperature of 17–18 °C, but the fastest is at 32 °C. A dangerous concentration of Bacillus cereus in food is considered to be exceeding $10^5$ bacteria per cm$^3$, and for wastewater, feces and vomit – $10^2$–$10^3$ bacteria per cm$^3$ [15, 16].

The most favorable conditions for the development of vegetative forms of Bacillus anthracis are 37 °C; at 45.5 °C, their growth stopped, and spores, under optimal conditions, opened up and actively split after 10-14 hours of incubation, and all cultural signs appeared after 18–24 hours [1].

Summing up the research literature review on the metabolism of spore forms of microorganisms in raw milk, we can conclude that their germination temperature is 37–55 °C, and the time is 10–48 hours [1, 4, 17].

In this regard, the goal of our further research was the development of production modes for milk processing, including temperature, exposure time and frequency of processing, which allow reducing the number of spore forms of bacteria, preventing the accumulation of metabolic products, while maintaining biochemical and improving the technological properties of raw material.

2. Materials and methods

The study of methods for the destruction of spore forms of bacteria using various modes of heat treatment was carried out in cooperation with Stavropol Antiplague Scientific Research Institute (SAPRI), which permits experiments with conditionally infectious and pathogenic groups of microorganisms.

The Bacillus cereus 16 strain was used as a model microorganism; the immunofluorescence test by the direct staining method was used. The equipment used included thermostats, refrigerating chamber, K-24 centrifuge, luminescent microscope “Lumam P8”, light microscope “Biolam”, and glassware. For germination and microbiological analysis of Bacillus cereus spore forms, we used Gladstone-Fields nutrient medium. Microbiological preparations of Bacillus cereus spore forms were stained by a direct method according to A. H. Coons, M. H. Kaplan (1950) with antispore fluorescent immunoglobulins; and vegetative forms were stained with antisomatic fluorescent immunoglobulins [18]. Experimental modes of heat treatment such as thermization at 65 °C and pasteurization at 72 °C, as well as the storage temperature (with a temperature range of +5, +37, +51, +58 and +65 °C) were chosen taking into account the modeling of actions, which were the closest to the technological processes of dairy production.

3. Results and discussion

The results of the experiments show that:

1. Spores of Bacillus cereus, after primary heat treatment (65 or 72 °C), do not germinate both at low storage temperatures (at + 5 °C) and when stored in uncooled milk (51–65 °C), at least within six hours;
2. To provoke the germination of Bacillus cereus spores, after the primary heat treatment of milk, it is necessary to lower the temperature to +37 °C and expose for 5–6 hours; at the end of the sixth hour the proportion of spores germinated into a vegetative state is about 35% (figure 1);

3. Primary heat treatment of milk (both thermization at 65 °C and pasteurization at 72 °C) reduces the germination time of Bacillus cereus spores by half: from 10–12 hours mentioned in the research literature [19] to six (figure 2);

Figure 1. Dynamics of germination of Bacillus cereus spores after heat treatment of milk.

| Temperature, °C | 1  | 2  | 3  | 4  | 5  | 6  | 7  |
|-----------------|----|----|----|----|----|----|----|
| Spore forms of Bacillus cereus, % | 100 | 100 | 100 | 100 | 100 | 100 | 65 |
| Vegetative forms of Bacillus cereus, % | 0 | 0 | 0 | 0 | 0 | 0 | 35 |
| Time, hours | 0 | 1 | 2 | 3 | 4 | 5 | 6 |

Figure 2. Spore and vegetative forms of Bacillus cereus.

Bacillus cereus spores stained with antispore fluorescent immunoglobulins (magnification 450 times)
4. After provoking the spores of *Bacillus cereus*, with the help of further pasteurization at 75 °C, it is possible to reduce the concentration of spore forms of microorganisms in milk to 3.10^2 CFU/cm³.

5. The technology of double heat treatment of milk (both thermization at 65 °C and pasteurization at 72 °C), with an intermediate exposure for one and a half hours, prevents the accumulation of metabolic products of a large group of bacteria: spore and vegetative, psychrophilic and thermophilic ones.

**4. Conclusion**

The results of the experiments showed the possibility of cleaning raw milk from spore forms of bacteria by provoking their germination by preliminary low-temperature heat treatment with their further destruction by pasteurization.

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