HEAT RESISTANCE OF *Bacillus* spp. SPORES ISOLATED FROM COW'S MILK AND FARM ENVIRONMENT

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Abstract

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The aim of this study was to compare heat resistance of the spores of mesophile *Bacillus* spp. from raw milk (bulk tank milk and milk from individual cows) and farm environment (faeces, silage, litter, swabs from skin and udder) as related to their survival in heat- treated dairy products. Fifty-eight strains of 9 species of *Bacillus* were tested for termoresistance within the temperature range of 95 - 135 °C and various exposure times. The highest termoresistance was found in *B. licheniformis* spores, which survived the temperature of 135 °C. Except for the spores *B. coagulans* and *B. pumilus* all remaining spores were able to germinate after heat treatment of up to 120 °C. The greatest reduction of the spore numbers was found in *B. cereus*. The results indicate that some *Bacillus* spores may survive in milk even after heat treatment.

Bacillus spp., raw milk, faeces, silage, litter, swabs from skin and udder, spores, termoresistance

Aerobic and facultative anaerobic spore-forming bacteria of the genus *Bacillus* present a serious problem in milk industry. Because of the heat resistance of spores and ability of vegetative cells to produce extra-cellular enzymes they may cause milk and milk product deterioration. *Bacillus* spp. are quite common in the agricultural environment and may contaminate milk from various sources both during the production, storage and processing. Raw milk is most frequently contaminated under conditions of inadequate udder hygiene, from soil, feed, dust and faeces (Christiansson et al. 1999). Species such as *B. licheniformis, B. subtilis* and *B. cereus* (Crielly et al.1994; Páčová et al. 1996) are most commonly isolated from raw milk. *B. licheniformis* together with *B. subtilis* and *B. pumilus* belong to mesophiles, whereas *B. cereus* is rather a psychrophile able to grow in milk and milk products at cold-storage temperatures and even cause alimentary diseases (Christiansson 1992). It is known that the spores of bacilli survive pasteurisation and have suitable conditions for germination created in the pasteurised milk. *B. cereus* is a predominating micro-organism influencing the maintenance of pasteurised milk. It causes sweet curdling of milk and changes to milk odour and taste due to the production of the enzymes proteinase, lipase and phospholipase (Meer et al. 1991). Sterilisation and UHT milk treatment are the most effective means of spore destruction showing 99.99% success (Cox 1974).

Heat resistance is an important character of *Bacillus* spp. spores. Appropriate temperature selection and duration of heating are crucial for the required microbial quality of the milk product, as with a linear temperature increase the time needed to achieve nearly complete abiosis becomes shorter exponentially. When determining the heat resistance and thus the sufficient duration of thermic treatment, it is useful to define the D value (decimal reduction time), i.e., a temperature value resulting in a decrease of the number of live microorganisms by one order (Kyzlink 1980).

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This study deals with heat resistance of spores of bacilli occurring in raw milk and farm environment with regard to the pasteurisation and sterilisation temperatures.

Materials and Methods

Preparation of spore suspension

The examined strains were spread on the surface of Nutrient agar and incubated 24 h at 37 °C. The microorganisms were then transferred to 50 ml of peptone water and incubated at 37 °C for 14 d and then heated at 80 °C for 10 min in order to kill vegetative cells. Sporulation was checked microscopically. The spore content was between $10^4 – 10^7$ ml^{-1}. The spore suspensions were stored at 4 °C until processed.

Thermo-inactivation experiments

A. Determination of thermal resistance of spores

The following 58 strains of the *Bacillus* genus were selected for the experiments: 21 strains of *B. licheniformis,* 18 strains of *B. subtilis,* 6 strains of *B. cereus,* 5 strains of *B. sphaericus,* 3 strains of *B. megaterium,* 2 strains of *B. alvei* and 1 strain of *B. amylolyticus, B. coagulans, B. pumilus.* These strains were isolated from bulk milk tanks and individual samples of milk and agricultural environment. The suspension of spores of the given strain, characterised by the number of spores per 1 ml counted by plating on PCA, was diluted before the experiment 1:10 using UHT half-fat milk that was pasteurised at 85 °C for 10 min in the laboratory. The volume of 0.5 ml of this initial milk suspension was pre-treated by heating to 75 °C and then, contained in thin-walled capillaries, subjected to temperatures of 95, 100 °C and 105, 110, 115, 120, 135 °C in water and glycerol baths, respectively. Periods of exposure were selected according to the temperature used. Initial concentrations of spores in suspensions varied from 10^4 to $10^7{\cdot}ml^{-1}$. After cooling we determined the number of surviving spores using the Plate Count Agar (PCA). We determined D values for all temperatures used and periods of exposure as well as means specific for each *Bacillus* species. Thermo-inactivation curves were also plotted.

B. Resuscitation of spores

The possibility of resuscitation of microorganisms was checked in 3 strains of *B. cereus, B. subtilis,* and *B. licheniformis.* Samples were processed in the same way as in the experiment to determine the thermo-resistance (cf. A). The initial concentration of spores in each strain examined amounted to 10^1-10^5 $\cdot ml^{-1}$ and temperatures of 100, 110, 120 and 135 °C were used for inactivation. The experiment, including a transfer of 0.1 ml of a sample into 0.9 ml of peptone water for the purpose of resuscitation, was performed in duplicate. The first set of samples was plated immediately after the thermal treatment and cooling, while the other one was kept in a refrigerator and the corresponding samples used only in those cases when there was no growth on the PCA detected during the first phase of the experiment. After 24 h of incubation at 37 °C we determined the number of spores germinating on the PCA, recounted the value per 1 ml and made a plot.

C. Spore survival dynamics

Samples were processed as in the above-mentioned experiments. The initial concentration used in strains of all 9 species amounted to 10^4-10^5. ml^{-1}. Periods of heating at the temperatures of 95 and 100 °C; 105, 110, 115 and 120 °C; and 135 °C lasted for 1, 2, 3, 4, 5 min; 5, 10, 20, 30, 60 s; and 5 s, respectively.

Results were evaluated as the number of surviving microorganisms per 1 ml expressed in %.

Results and Discussion

A total of 58 strains of 9 species of the genus *Bacillus* were examined, i.e., 21 strains of *B. licheniformis,* 18 strains of *B. subtilis,* 6 strains of *B. cereus,* 5 strains of *B. sphaericus,* 3 strains of *B. megaterium,* 2 strains of *B. alvei* and 1 strain of *B. amylolyticus, B. coagulans,*

B. pumilus. The number of strains of individual species examined was influenced by their incidence in raw milk and agricultural environment. Heat resistance of spores was evaluated using the D-value. Mean, minimal and

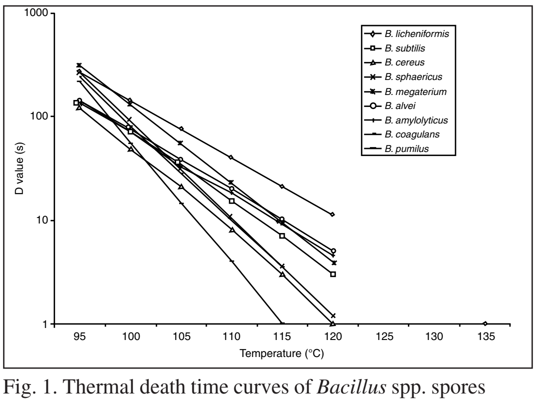

Fig. 1. Thermal death time curves of *Bacillus* spp. spores

Table 1
Heat resistance of *Bacillus* spp. spores

Strain		Heating temperature (°C)						
		95	100	105	110	115	120	135
Spp/number of strains		**D value** (min)						
B. licheniformis 21	x	**4.51**	**2.37**	**1.26**	**0.68**	**0.38**	**0.18**	**0.02**
	min.	2.01	1.12	0.51	0.27	0.11	0.03	0
	max.	11.8	5.06	3.37	1.84	1.61	0.47	0.18
B. subtilis 18	x	**2.24**	1.18	0.56	0.25	0.14	0.05	0
	min.	1.78	0.96	0.29	0.10	0.05	0	0
	max.	3.26	2.13	1.16	0.74	0.39	0.23	0
B. cereus 6	x	**2.02**	0.80	0.38	0.13	0.07	0.02	0
	min.	1.78	0.71	0.26	0.07	0.03	0	0
	max.	2.76	1.48	0.75	0.41	0.18	0.08	0
B .sphaericus 5	x	**4.40**	1.58	0.54	0.18	0.07	0.01	0
	min.	3.33	1.32	0.32	0.14	0.06	0	0
	max.	6.84	1.98	0.71	0.18	0.08	0.60	0
B. megaterium 3	x	**5.13**	2.16	0.98	0.37	0.18	0.03	0
	min.	4.77	1.95	0.74	0.33	0.09	0	0
	max.	5.47	2.28	1.11	0.43	0.28	0.05	0
B. alvei 2	x	**2.34**	1.33	0.62	0.32	0.15	0.08	0
	min.	2.13	1.16	0.51	0.29	0.13	0.07	0
	max.	2.55	1.50	0.72	0.34	0.16	0.08	0
B. amylolyticus 1	x	**2.23**	1.19	0.58	0.34	0.15	0.07	0
B. coagulans 1	x	**3,.96**	1.26	0.42	0.17	0.06	0	0
B. pumilus 1	x	**3.59**	0.83	0.27	0.10	0.02	0	0

maximal D-values are presented in Table 1. D-values determined and thermo-inactivation curves plotted (Fig. 1) show differences in the speed of inactivation, great variability of spore resistance of individual species as well as individual strains of the *Bacillus* genus at temperatures examined. The rise of temperatures from 95 to 135 °C resulted in a continual decrease of the D-value of spores of all species of the *Bacillus* genus. Fig. 1 clearly shows that the temperature of 135 °C is adequate for the inactivation of spores even in high initial concentrations (up to 10^7. ml^{-1}) of all strains studied except for *B. licheniformis*, spores of which were on exception able to germinate even after heating to this temperature. The temperature which spores of all strains studied remained active at was 115 °C. The temperature of 120 °C was survived by spores of *B. licheniformis, B. subtilis, B. cereus, B. sphaericus, B. megaterium*, *B. alvei* and *B. amylolyticus.* Spores of *B. coagulans* and *B. pumilus* were not able to germinate at this temperature.

B. licheniformis showed the greatest heat resistance as compared to other species examined. The respective D values at temperatures of 95, 100, 105, 110, 115, 120 and 135 °C amounted to 4.51, 2.37, 1.26, 0.68, 0.38, 0.18 and 0.02 min. There was also the greatest heat-resistance variability of strains in this species. Our results are in good agreement with data of Pendurkar et al. (1989). The revival of germination of spores that were only sub-lethally impaired (i.e., resuscitation) was found in 18 cases out of 42 (i.e., 42.9%) experiments at temperatures up to 120 °C. There was no resuscitation found at the temperature of 135 °C. The success rate of spore resuscitation of individual species at temperatures of 100, 110, 120 and 135 °C can be seen in Fig. 2. Table 2, presenting the spore destruction dynamics, shows again greater resistance of *B. licheniformis* spores in comparison with other species.

The second most commonly isolated species from milk and agricultural environment, i.e. *B. subtilis* (Páčová et al. 1996), was more susceptible to the temperatures used than *B. licheniformis*. The D value at 95 °C was 2.24 min, similar to the study by Stojanovic et al. (1997). After heating the suspension of spores to 135 °C we noticed no growth; active spores, however, were found at the temperature of 120 °C. Pendurkar et al. (1989) also mentioned its lower heat resistance.

B. cereus species received great attention, the fact being documented by many authors, because it is an important pasteurised milk contaminant. Vegetative cells are devitalised by

Table 2

Effect of different temperature/ time combinations on the surviving of spores *B. licheniformis, B. subtilis, B. cereus*

Temperature	Heating time	*B. licheniformis*	*B. subtilis*	*B. cereus*
°C	min	%	%	%
95	1	47.08	35.66	31.82
	2	25.03	12.96	10.16
	3	13.09	4.66	3.48
	4	8.13	1.81	1.27
	5	4.97	0.69	0.38
100	1	51.56	13.98	8.69
	2	27.88	3.00	1.22
	3	8.23	0.59	0.34
	4	4.10	0.12	0.05
	5	1.81	0.03	0.01
	Sec.			
105	5	80.05	67.67	55.58
	10	64.96	46.78	34.27
	20	44.49	23.69	13.62
	30	28.58	12.02	8.69
	60	11.96	2.52	1.66
110	5	64.94	40.78	17.69
	10	45.57	20.25	6.68
	20	24.12	6.42	2.43
	30	14.04	2.14	0.81
	60	3.72	0.27	0.07
115	5	41.96	19.83	6.71
	10	22.71	6.10	1.69
	20	11.41	1.23	0.13
	30	4.95	0.39	0.03
	60	0.98	0.02	0
120	5	21.58	5.12	0.81
	10	9.27	1.44	0.13
	20	3.58	0.14	0
	30	1.75	0	0
	60	0.29	0	0
135	5	2.33	0	0

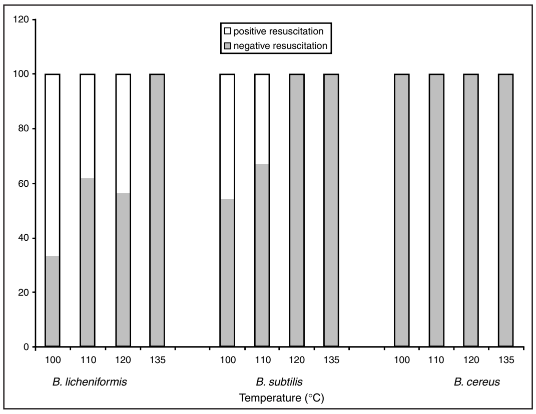

Fig. 2. Recovery of spores *B. licheniformis, B. subtilis, B. cereus* from heat injury

pasteurisation temperatures, the ability of spores to germinate, however, remains unaffected. Spores, nevertheless, mostly do not survive sterilisation temperatures. Out of the six strains examined, only one survived the temperature of 120 °C. The temperature of 135 °C was sufficient for the destruction of spores. Nagarajan et al. (1990) found that *B. cereus* spores survived 100 °C for 10 min in sterile milk. We, however, did not prove this. Our results, nevertheless, are comparable to those by other authors such as Griffiths et al. (1986). The destruction of spores in dependence on the temperature and time was much quicker as compared to *B. subtilis* and *B. licheniformis* (Table 2), while the germination of spores was not revived by resuscitation (Fig. 2).

As far as other species are concerned, we examined a smaller number of strains, because their occurrence in milk samples was low. These findings, therefore, are only complementary. It is clear that, should we examine more numerous samples, there would have been greater variability in heat resistance. The heat destruction of spores in strains of *B. sphaericus* and *B. megaterium* at temperatures up to 100 °C was slower than in other species, similarly as in the spores of *B. licheniformis.* Great resistance of *B. sphaericus* was found by Kamei et al. (1990). We did not confirm the results reporting survival of spores at the temperature of 140 °C. Spores of *B. coagulans* and *B. pumilus* strains did not survive the temperature of 120 °C.

Mikolajcik (1969) reports the respective D values at 95 and 100 °C in *B. pumilus, B. coagulans, B. megaterium* and *B. sphaericus* being 4.03 and 0.875, 6.90 and 1.97, 6.50 and 2.35, and 7.60 and 2.25 minutes. In *B. pumilus, B. coagulans, B. megaterium,* and *B. sphaericus* we found at the above-mentioned temperatures the following the respective D values: 3.59 and 0.83, 3.96 and 1.26, 5.13 and 2.16, 4.40 and 1.58 min. The finding of *B. amylolyticus* in raw milk is interesting. Even when we examined only one strain, it can be stated that its spores may survive the temperature of 120 °C.

Evaluating our results on the heat resistance of species of the *Bacillus* genus, we may conclude that especially the spores of *B. licheniformis* are able to survive the UHT process. Considering the concentration of spores in raw milk and the dynamics of their destruction during heating, *B. licheniformis* is not much dangerous for the UHT milk quality.

Spores of other bacilli surviving the temperatures from 95 to 120 °C, may play their important role in the contamination of cream and high pasteurised milk for the production of some milk foods.

When evaluating the heat resistance of spores, it is necessary to consider the fact that in the laboratory we cannot create the same environment and conditions for the sporulation as in the natural substrate. We, therefore, have to take into consideration some differences in the heat resistance as compared to spores in the natural environment of foods.

Termorezistence spór mikroorganismů rodu *Bacillus* izolovaných z mléka a zemědělského prostředí

Cílem práce bylo porovnání termorezistence spór mezofilních druhů rodu *Bacillus* vyskytujících se v syrovém mléce ve vztahu k přežívání v tepelně ošetřených mléčných výrobcích. U 58 kmenů 9 druhů rodu *Bacillus* byla stanovena termorezistence spór v UHT mléce v rozsahu teplot 95 - 135 °C při různé expozici. Nejvyšší termorezistence byla zjištěna u *B .licheniformis*, jehož spóry přežily teplotu 135 °C. Spóry schopné klíčit byly u ostatních druhů zaznamenány při teplotě 120 °C, s výjimkou *B. coagulans* a *B. pumilus.* Redukce spór byla nejrychlejší u *B. cereus* ve srovnání s *B licheniformis* a *B. subtilis.* Výsledky poukazují na možnost přežívání některých spór rodu *Bacillus* v mléce ošetřeném vysokými teplotami.

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