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The Effect of Lipolytic Enzymes of *Bacillus* spp. on Quality of Ultra-High-Temperature-Treated Milk

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Abstract

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Lipolysis was monitored based on determining the concentration of free fatty acids in milk, on the model case of UHT milk contamination with spores of 15 *B. licheniformis, B. subtilis* and *B. cereus* strains isolated from farm environment and raw milk. Lipolysis was not recorded at storage temperature of 4 °C, whereas significant changes in levels of free fatty acids were shown at storage temperature of 24 °C. After 3 weeks of storage the initial content of 41.97 mmol·kg^{-1} of fat rose to as much as 1,617.22 mmol·kg^{-1} of fat. The extent of the change depended mainly on the *Bacillus* spp. species and the storage period and, to a certain degree, also on the initial number of microorganisms. Significant lipolytic activity was detected in association with *B. licheniformis* and *B. cereus* species. It was found that spores of resistant *B. licheniformis* strains may survive 100 °C/10 min and 135 °C/5 s heating and show lipolytic activity.

Lipolysis, spores, Bacillus spp., free fatty acids, milk

Bacillus spp. microorganisms represent important contaminants of raw milk from the point of view of both hygiene and technology, and in some cases, human health.

Bacillus spp. spores are commonly present in stables, causing mainly secondary milk contamination during milking. A number of representatives of the *Bacillus* family are present in milk as psychrotrophic microflora. The species of the *Bacillus* family most frequently isolated from raw milk are *B. licheniformis* and *B. cereus* (Crielly et al. 1994). Páčová et al. (1996) report that the species with the widest distribution is *B. licheniformis*. The distribution of other species is lower (Lukášová et al. 2001; Vyletělová et al. 2001). Spores of thermostable bacteria may pollute a product even during the technological process, and they may be present in UHT milk, too, as reported e.g. by Bahout (2000), who detected the spores at concentrations of up to 2.6×10^2·ml^{-1} in 18.3% of tested samples.

Bacillus spp. microorganisms are an extremely burdensome part of raw milk microflora, as removing their spores is very difficult due to their thermoresistance. The spores may be partly damaged by pasteurization, but mostly cannot survive sterilization and the UHT process. One important characteristic of *Bacillus* spp. is the ability of the vegetative cells to produce thermostable extracellular enzymes after proliferation (Meer et al. 1991; Ipsen et al. 2000). The proteolytic and lipolytic activity of these enzymes bears on nutritional and sensory properties of milk products even when no live bacteria are present (Boor et al. 1998). Brown (2000) refers to *Bacillus* spp. as microorganisms causing significant economic loss.

Some *Bacillus* spp. species, especially *B. cereus*, show a high degree of lipolytic and proteolytic activity (Janštová et al. 2004). The enzyme activity is demonstrated in a number of milk and milk-product defects, such as those described by Silveira et al. (1999). Lipolytic enzymes produced by *Bacillus* spp. microorganisms cause fat hydrolysis

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and production of free fatty acids after germination of spores in milk and milk products. Microbial enzymes are strictly specific and much more active than native lipases (Murray et al. 2001). Johnston and Bruce (1982), who studied lipolytic properties of *Bacillus* spp., detected fat lipolysis in 52% of strains and a 77% prevalence of lecithinase-positive strains.

Since lipases are largely produced by microorganisms in the late lag stage and the early stationary growth stage, there is no direct proportional correlation between the number of microorganisms and the enzyme concentration. Defects are detected when the concentration of microorganisms reaches 5×10^5 - 10^7 CFU·ml^{-1} (Vyletělová et al. 2000; Marth and Steele 1998).

Unlike milk lipases, microbial lipases are thermoresistant and remain active despite heating including UHT milk treatment, being able to cause the development of rancid taste and flavour, contributing thus to product degradation. Lipase activity thus presents an obstacle to successful implementation of UHT products where lipases are contained in the raw material, and their activity in the UHT product is preserved.

It is low-carbon fatty acids (C_4 - C_{12}), especially the butyric acid, that contribute to the development of sensory defects the most. Fatty acids C_4 - C_8 are to blame for the rancid flavour while the foul, bitter and soapy flavour is due to C_{10} - C_{12} fatty acids (Champagne et al. 1994).

Lipase activity is enhanced by physical phenomena applied in milk processing such as homogenisation, sudden temperature change, intensive stirring or milk turbulence in the pipes, as they may damage the lipoprotein membrane of fat globules, making the fat vulnerable to lipase activity.

Materials and Methods

The experiments were conducted on collection strains of the Department of Milk Hygiene and Processing of the University of Veterinary and Pharmaceutical Sciences Brno. A total of 15 *Bacillus* spp. strains isolated from farm environment and raw milk were used. Each experiment involved 5 *B. licheniformis*, 5 *B. subtilis* and 5 *B. cereus* strains. All the isolated strains were identified by Lukášová et al. (2001). Durable UHT milk distributed on the market was used as the medium for *Bacillus* spp. spore contamination. The UHT milk originated from the same batch.

Milk was inoculated with *Bacillus* spp. spore suspensions, without using thermic inactivation, in such an amount as to reach spore concentrations of 10^2, 10^1, $< 10^1$ in 1 ml of milk. Besides that, *Bacillus* spp. spore suspensions heated to 100 °C in water bath (exposure time 10 min) and to 135 °C in glycerol bath (exposure time 5 s) were used.

Thermoinactivation of spore suspension (5 ml) was performed after heating to 70 °C. The complete inactivation of *B. subtilis* and *B. cereus* spores was performed after heating, at 100 °C for 10 min and 135 °C for 5 s. The initial concentration of *B. licheniformis* thermoresistant strain was reduced by 1 logarithmic order.

Milk samples were stored in closed sterile glass sample bottles at 24 °C in a thermostat and at 4 °C in a refrigerator.

The proof and quantification of lipolysis due to *Bacillus* spp. microorganisms was based on the extraction/titration method for determining substance content of free fatty acids (FFA) in accordance with ČSN 57 0533 "Determination of Substance Content of Free Fatty Acids" (1997). The amount of substance for FFA was expressed in mmol·kg^{-1} of milk fat. The analysis was performed at 1-week intervals for 3 weeks. All samples were analyzed in triplicate.

To be able to compare the dynamism of growth of the microorganisms and change the values of observed indicators, we performed a microbiological analysis of milk samples, namely determination of the total number of spore-forming microorganisms (ČSN ISO 6887, 1994). Cultivation was performed on Plate Count Agar (HiMedia, India). Sensory evaluation (colour, coagulum, and odour) was performed, too, when the samples were taken for analysis.

The STAT Plus Statistical and Graphic System (Matoušková et al. 1992) was used to assess the significance of variances of individual indicators at levels $p < 0.01$ and $p < 0.05$. The data processing was based on dispersion analysis and Scheffe's contrasts the Turkey's test of variance significance. To obtain correct results, the data were subjected to the Box-Cox transformation prior to variance analysis so as to ensure that they meet the condition of basic distribution normality. Before the analysis proper, homogeneity of variances of the tested samples had to be verified. The Bartlett's test was used for this purpose.

Results and Discussion

Microorganisms of the *Bacillus* family are important producers of lipolytic enzymes. Thermoresistant bacterial lipases may represent a serious obstacle to UHT processing of milk products containing fat.

No increase in FFA content due to any of the considered *Bacillus* spp. species compared with the initial FFA levels occurred during sample storage at 4 °C; the detected levels were consistent with control samples (the results are not mentioned). None of the considered strains proliferated at 4 °C. Our results achieved by FFA content determination in samples stored at this temperature are in line with the conclusions of Burdová (2002), who stressed the importance of storage temperature based on her finding that milk storage temperature of 4 °C was not high enough to ensure production of an amount of lipolytic and proteolytic enzymes comparable to that produced in samples stored at 10 °C for 2 - 3 days despite the storage period being three times longer.

An increase in FFA caused by lipolytic enzyme activity was recorded only in association with sample storage at 24 °C and correlated with the increase in the number of microorganisms. Some change was detected already when levels 10^4 - 10^5 CFU·ml^{-1} were achieved; CFU peaked in the 3rd week of storage and was identical for all *Bacillus* spp. thermally non-inactivated strains, namely 10^8·ml^{-1} of milk. A lower number of CFU was found when we used non-inactivated *B. licheniformis* spores. *B. subtilis* and *B. cereus* were completely inactivated. No germination or growth was observed. The dynamics of changes of *B. licheniformis* number depending on time storage is demonstrated in Fig. 1. No increase of FFT content was found in control samples during the experiment.

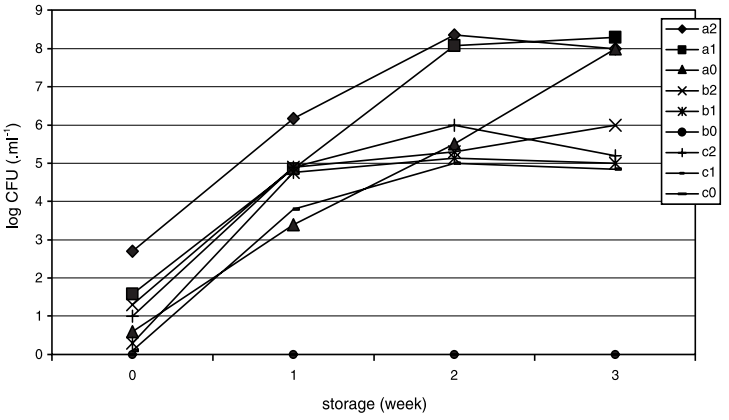

Fig. 1. Dynamics of changes of CFU number in milk containing *B. licheniformis* spores stored at 24 °C depending on inactivation method (a - no inactivation, b - inactivation by heating to 100 °C/10 min, c - inactivation by heating to 135 °C/5 s) and initial spore concentration (2 = 10^2 in l ml, 1 = 10^1 in 1 ml, 0 = < 10^1 in 1 ml).

Significant lipolysis was detected in samples with higher initial content of inoculated, thermally non-inactivated spores in milk; small-scale lipolysis was detected in samples containing few individual spores. The average initial FFA content in the milk used for the experiments was 41.97 mmol·kg^{-1} of milk fat.

As shown in Table 1, the use of the highest concentration of thermally non-inactivated and a 3-week storage period led to an increase of FFA content in milk in relevant *B. licheniformis* strains depending on the initial concentration. The initial number of spores being 10^2·ml^{-1}, the average content was 330.53 ± 80.98; 940.74 ± 482.77; and 1,129.61 ± 387.42 mmol·kg^{-1} of

Table 1. Average FFA content levels (mmol·kg^{-1} of milk fat) in milk containing non-inactivated *B.licheniformis, B. subtilis, B. cereus* spores. Dependence on storage period (week) at 24 °C

Species	Week	Initial No. of spores/1 ml of milk		
		$10^2 \pm$ SD min max	$10^1 \pm$ SD min max	$< 10^1 \pm$ SD min max
Initial FFA content 41.97				
B. licheniformis	1	330.53 ± 80.98	255.89 ± 138.79	212.12 ± 131.73
		239.68	106.29	58.81
		443.60	446.4	353.21
	2	940.74 ± 482.77	758.80 ± 389.69	459.22 ± 294.36
		417.10	329.29	147.61
		1513.40	1265.90	942.75
	3	1129.61 ± 387.42	1104.82 ± 366.18	787.68± 144.75
		598.40	679.29	565.20
		1665.40	1504.40	1109.80
B. subtilis	1	145.16 ± 70.26	124.02 ± 97.89	79.59 ± 21.77
		54.67	42.94	42.98
		219.44	294.55	95.50
	2	467.78 ± 161.78	227.74 ± 89.04	203.24 ± 38.89
		118.32	86.99	43.25
		515.92	332.96	138.25
	3	488.61 ± 67.67	408.59 ± 53.57	354.22 ± 18.54
		280.60	191.52	43.11
		536.46	617.75	582.16
B. cereus	1	241.79 ± 160.48	202.63 ± 129.53	157.71 ± 147.84
		62.24	67.13	64.69
		412.35	382.80	364.72
	2	771.44 ± 485.22	838.98 ± 559.94	701.96 ± 438.31
		130.59	118.60	110.41
		1240.70	1381.00	1109.80
	3	1617.2 ± 68.17	1261.1 ± 31.11	895.0 ± 13.01
		1502.55	1068.25	885.60
		1665.40	1425.50	904.40

fat, after 1, 2 and 3 weeks, respectively. The maximum level recorded for the individual strains was 1,665.22 mmol·kg^{-1} of fat. The initial content of 10^1·ml^{-1} led to similarly high levels. The initial spore concentration of $< 10^1$·ml^{-1} produced a high determined amount of FFA, too (787.68 ± 144.75 mmol·kg^{-1} of fat).

The number of microorganisms in milk being roughly the same, lipolytic activity of *B. subtilis* enzymes was the least significant compared with other considered bacterial strains. At spore concentration of 10^2 per 1 ml of milk, the average FFA levels were 467.78 ± 161.78 mmol·kg^{-1} of fat and 488.61 ± 67.67 mmol·kg^{-1} of fat after 2 and 3 weeks of storage respectively. The initial spore concentration had a significant differentiating effect on FFA milk content: FFA content was significantly lower at spore content $< 10^1$ per 1 ml of milk and no FFA content increase at all occurred in 2 of the 5 tested strains.

The *B. cereus* strain was generally characterized by strong lipolytic activity, to which the high FFA levels found even in samples with the lowest initial spore content testify. The values for the individual strains however varied considerably. With the initial number of spores 10^2 per ml^{-1}, the determined levels of FFA were 241.79 ± 160.48, 771.44 ± 485.22

and 1617.22 ± 68.17 mmol·kg^{-1} of fat. The final FFA content levels at lower spore concentrations were 1,261.1 ± 31.11 and 895.00 ± 13.01 mmol·kg^{-1} of fat.

Significant variances ($p < 0.05$) and highly significant variances ($p < 0.01$) were found between the activities of individual *Bacillus* spp. species tested. FFA content for *B. subtilis* was lower ($p < 0.05$) compared with *B. licheniformis* and lower ($p < 0.01$) compared with *B. cereus*.

When spores inactivated by heating to 100 °C for 10 min were used in samples stored at 24 °C, lipolysis was observed only in the *B. licheniformis* thermoresistant strain. Thermoresistance of *Bacillus* spp. spores was described by Janštová and Lukášová (2001). At higher initial concentrations of inoculated spores, the survivor spores of the strain germinated and FFA content increased to 206.36, 417.80 and 605.21 mmol·kg^{-1} of fat (after 1st, 2nd and 3rd week of storage). The average FFA content levels after 3 weeks of storage were 230.33 ± 324.47 mmol·kg^{-1} (initial concentration of spores was 10^2·ml^{-1}) and 144.25 ± 6.26 mmol·kg^{-1} of fat (initial concentration of spores was 10^1·ml^{-1}) (see Table 2a). When spores of other *B. licheniformis* strains and also *B. subtilis* and *B. cereus* spores were heated, they were eradicated and no lipolysis was recorded (see Table 2b).

Table 2a. Average FFA content levels (mmol·kg^{-1} of milk fat) in milk containing *B. licheniformis* spores inactivated by heating to 100 °C/10 min. Dependence on storage period (week) at 24 °C

Species	Week	Initial No. of spores/1 ml of milk		
		10^2 ± SD min max	10^1 ± SD min max	< 10^1 ± SD min max
Initial FFA content 41.97				
B. licheniformis	1	97.19 ± 95.55	84.12 ± 0.28	42.11 ± 0.16
		42.05	41.59	42.00
		206.36	70.18	42.68
	2	168.06 ± 216.29	112.11 ± 0.31	43.02 ± 0.22
		42.09	42.06	42.42
		417.80	72.56	45.02
	3	230.33 ± 324.47	144.25 ± 6.26	43.38 ± 0.08
		44.28	42.06	42.11
		603.25	344.72	45.02

Table 2b. Average FFA content levels (mmol·kg^{-1} of milk fat) in milk containing *B. subtilis* and *B. cereus* spores inactivated by heating to 100 °C/10 min. Dependence on storage period (week) at 24 °C

Species	Week	Initial No. of spores/1 ml of milk		
		10^2 ± SD	10^1 ± SD	< 10^1 ± SD
Initial FFA content 41.97				
B. subtilis	1	42.35 ± 0.49	42.85 ± 1.37	42.52 ± 0.57
	2	43.11 ± 1.51	42.63 ± 1.16	42.88 ± 1.12
	3	43.05 ± 2.04	42.54 ± 1.12	42.96 ± 2.08
B. cereus	1	42.58 ± 0.38	43.25 ± 0.71	43.08 ± 0.35
	2	42.62 ± 0.71	42.15 ± 1.07	42.42 ± 1.68
	3	42.55 ± 2.11	42.32 ± 0.98	42.60 ± 1.25

When inactivated by heating to 135 °C for 5 s (see Table 3a), a limited amount of the same *B. licheniformis* thermoresistant strain germinated. With the initial spore concentrations 10^1 and 10^2·ml^{-1}, the spores germinated to reach 10^5 - 10^6 CFU·ml^{-1} of milk. Lipolytic activity

Table 3a. Average FFA content levels (mmol·kg^{-1} of milk fat) in milk containing *B. licheniformis* spores inactivated by heating to 135 °C/5 s. Dependence on storage period (week) at 24 °C

Species	Week	Initial No. of spores/1 ml of milk		
		$10^2 \pm$ SD min max	$10^1 \pm$ SD min max	$< 10^1 \pm$ SD min max
Initial FFA content 41.97				
B. licheniformis	1	100.42 ± 82.26	100.08 ± 81.89	42.37 ± 0.16
		42.25	42.17	42.25
		158.58	157.98	42.48
	2	302.74 ± 367.82	199.40 ± 221.49	42.78 ± 0.31
		42.65	42.78	42.41
		562.83	356.02	43.15
	3	537.32 ± 699.01	473.76 ± 556.01	42.80 ± 0.30
		43.04	43.12	42.24
		1031.60	904.40	43.41

was subsequently demonstrated as an increase in average FFA levels was to 537.32 ± 699.01 (initial concentration of spores was 10^2 ml^{-1}) 473.76 ± 556.64 mmol·kg^{-1} (initial concentration of spores was 10^1·ml^{-1}) of fat in the 3 rd week of storage. No increase in FFA content was recorded in samples with the lowest initial inactivated spore concentrations. Spores of other *B. licheniformis* strains and *B. subtilis* and *B. cereus* spores were completely inactivated; no lipolysis was recorded (see Table 3a, Table 3b).

Table 3b. Average FFA content levels (mmol·kg^{-1} of milk fat) in milk containing *B. subtilis, B. cereus* spores inactivated by heating to 135 °C/5 s. Dependence on storage period (week) at 24 °C

Species	Week	Initial No. of spores/1 ml of milk		
		$10^2 \pm$ SD	$10^1 \pm$ SD	$< 10^1 \pm$ SD
Initial FFA content 41.97				
B. subtilis	1	42.85 ± 0.50	42.25 ± 0.08	42.68 ± 0.59
	2	43.14 ± 0.86	43.91 ± 2.11	42.68 ± 0.69
	3	43.32 ± 0.40	43.24 ± 0.25	42.78 ± 0.83
B. cereus	1	43.08 ± 0.85	42.38 ± 0.99	42.73 ± 0.24
	2	43.32 ± 0.08	43.03 ± 0.33	43.07 ± 0.38
	3	43.29 ± 0.20	43.04 ± 0.05	43.09 ± 0.03

The dynamics of FFA content growth depending on thermo-inactivation mode and initial *B. licheniformis* spore concentration is shown in Fig. 2.

The significance of the variances between FFA levels detected in samples inoculated with thermoresistant *B. licheniformis* strain spores inactivated by heating to 100 °C for 10 min and to 135 °C for 5 s on the one hand and the other strains of the same species on the other is highly significant ($p < 0.01$). Just as highly significant variances ($p < 0.01$) were found when average FFA content levels in samples with *B. licheniformis* inoculation were compared with those of other tested *Bacillus* spp. species.

Our experiments have proved that thermo-inactivation of spores, depending on the temperature, causes reduction or cessation of lipase production as a consequence of sublethal or lethal spore damage. However, if the strain is a thermoresistant one or if

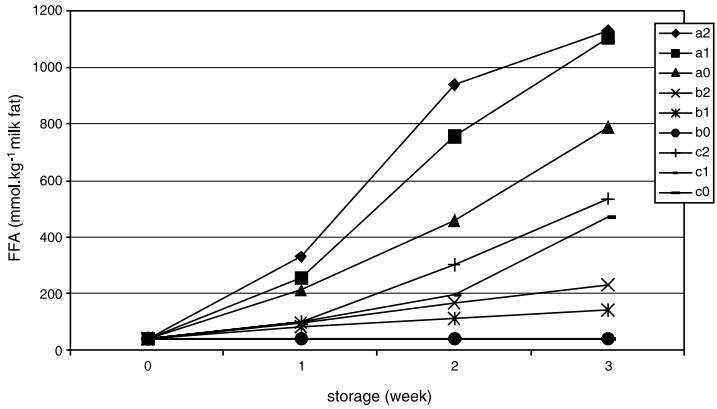

Fig. 2. FFA content in milk containing *B. licheniformis* spores stored at 24 °C depending on inactivation method (a - no inactivation, b - inactivation by heating to 100 °C/10 min, c - inactivation by heating to 135 °C/5 s) and initial spore concentration (2 = 10^2 in l ml, 1 = 10^1 in 1 ml, 0 = < 10^1 in 1 ml)

enzymes are produced before thermic treatment of milk, the spores remain active or partly active even in the finished product. Grieger et al. (1990) report that heating milk above 130 °C for 5 - 10 min leads to reduction of lipases to 10% and that inactivation of some lipases may require temperatures as high as 150 °C.

Characteristics of microbial lipases and their role in food spoilage was studied e.g. by Stead (1986). In consistence with our findings, Vlachos and Litopoulou-Tzanetaki (1985) report differences as to production of lipolytic enzymes between individual *Bacillus* spp. species including between strains of the same species. They found no proofs of lipolytic activity of some *B. licheniformis* strains.

Despite the fact that due to differences of focus and use of different determination methods our findings could not be compared with data published by other authors, it is clear that the process of lipolysis can be recorded by the method we used. In contrast to the requirement of ČSN 570529 (1993) - requiring that the FFA content in raw milk for dairy technologies and processing determined by the titration/extraction method does not exceed 32 mmol·kg^{-1} - a mild increase in FFA content in UHT milk and a subsequent growth due to lipolysis caused by *Bacillus* spp. enzymes was observed.

Action of bacterial enzymes produced by microorganisms including some microorganisms from the *Bacillus* family is the cause of sensory defects of milk and milk products. Enzymes isolated from *B. licheniformis, B. coagulans* and *B. subtilis* were studied by Kalogridou-Vassiliadou (1992), reporting an association with milk spoilage. Matta and Punj (1999) speak of a connection between flavour defects and the 48% prevalence of lipolytic bacillary strains in raw milk samples they detected. Defects originate upon reaching a certain concentration of microorganisms; sensory alterations of milk can be detected at concentrations of 10^5 - 10^7 CFU·ml^{-1} (Vyletělová et al. 2000; Marth and Steele 1998; Šilhánková 1999). In contrast to that, the content of psychrotrophic CFU sufficient to initiate lipolysis reported by Silveira et al. (1999) is 2.7×10^4 CFU·ml^{-1}.

No sensory alterations were recorded in samples stored at 4 °C, despite 3 months of storage, in association with any of the *Bacillus* species. Deterioration of sensory qualities of milk (bad odour, running) was recorded in samples containing non-inactivated spores stored at 24 °C in correspondence to the process of lipolysis and proteolysis. No sensory change was detected in thermically inactivated samples, with the exception of milk samples inoculated with *B. licheniformis* thermoresistant strain spores. In consistence with our

results, milk defects such as running, gelation, ropiness, colour and flavour defects, fat clotting, and cream bitterness are described by Te Giffel et al. (1996), Bassette et al. (1986), Walstra et al. (1999). Cream bitterness is the most widely spread defect caused by *B. cereus*, the cause being fat destabilization due to phospholipase C, degrading fat globule membranes by enzymatic processes (Al-Kanhal 1985; Meer et al. 1991). Lecithinase, too, causes cream flocculation; the process involves aggregation of fat globules after their envelopes are destructed (Harrigan 1998).

Vliv lipolytických enzymů Bacillus spp. na kvalitu UHT mléka

Sledování lipolýzy bylo provedeno na základě stanovení látkového obsahu volných mastných kyselin v mléce a to na modelovém případě kontaminace trvanlivého mléka sporami 15 kmenů *B. licheniformis, B. subtilis* a *B. cereus* izolovaných z prostředí farmy a ze syrového mléka. Při skladovací teplotě 4 °C nebyla lipolýza zaznamenána, zatímco při skladovací teplotě 24 °C byly zjišťovány výrazné změny obsahu volných mastných kyselin. Po 3 týdnech skladování došlo ke zvýšení obsahu volných mastných kyselin z počáteční hodnoty 41.97 mmol.kg^{-1} tuku na hodnotu až 1617.22 mmol·kg^{-1} tuku. Rozsah změn závisel především na druhu *Bacillus* spp. a době skladování a v určité míře také na výchozím počtu mikroorganismů. Výrazná lipolytická aktivita byla zjištěna u druhů *B. licheniformis* a *B. cereus*. Bylo zjištěno, že teplotní a časový parametr záhřevu na 100 °C 10 min a 135 °C 5 s mohou spory rezistentních kmenů *B. licheniformis* přežít a vykazovat lipolytickou aktivitu.

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