

## Occurrence of *Bacillus cereus* and *Bacillus licheniformis* strains in the course of UHT milk production

### Výskyt kmenů *Bacillus cereus* a *Bacillus licheniformis* v procesu výroby UHT mléka

M. VYLETĚLOVÁ<sup>1</sup>, P. ŠVEC<sup>2</sup>, Z. PÁČOVÁ<sup>2</sup>, I. SEDLÁČEK<sup>2</sup>, P. ROUBAL<sup>3</sup>

<sup>1</sup>Research Institute for Cattle Breeding, Rapotín, Czech Republic

<sup>2</sup>Czech Collection of Microorganisms (CCM), Faculty of Natural Science, Masaryk University, Brno, Czech Republic

<sup>3</sup>Dairy Research Institute, Prague, Czech Republic

**ABSTRACT:** Transfer of *Bacillus cereus* and *Bacillus licheniformis* strains from milk into final products (UHT milk) was studied. Phase milk samples were collected during the whole time of milk processing (one milk batch) in collaboration with the dairy. Representative *B. cereus* and *B. licheniformis* strains were characterised and compared by ribotyping with *EcoRI* enzyme and a probe complementary to *Escherichia coli* 16S and 23S rRNA as well as by phenotyping. According to 26 phenotypical traits the *B. licheniformis* strains formed almost a homogeneous group. In contrast, *B. cereus* strains exhibited variable results in several tests. The hybridisation profiles divided the analysed strains into two groups in full accordance with their species classification. Band patterns of *B. cereus* strains showed similarities ranging from 77 to 97%; this similarity values correlated with biochemical test results as well. *B. licheniformis* strains exhibited band pattern similarities from 82 to 100%. The hybridisation profiles of *B. licheniformis* strains B79 (pasteurized milk) were absolutely identical (100% similarity). Their phenotypical test results were identical as well. These results imply the identity of the strains isolated in the course of UHT milk production.

**Keywords:** *Bacillus cereus*; *Bacillus licheniformis*; raw milk; pasteurised milk; UHT milk; ribotyping

**ABSTRAKT:** Byl sledován možný průnik kmenů *Bacillus cereus* a *Bacillus licheniformis* ze syrového mléka do finálního výrobku, konkrétně UHT mléka. Ve spolupráci s mlékárnou byl proveden odběr fázových vzorků mléka během celého procesu zpracování jedné šarže mléka. Reprezentativní kmeny obou druhů byly charakterizovány a srovnány ribotypizací s enzymem *EcoRI* a sondou komplementární k *E. coli* 16S a 23S rRNA. Zatímco kmeny *B. licheniformis* tvořily na základě 26 fenotypových vlastností téměř homogenní skupinu, kmeny *B. cereus* se vzájemně lišily. Získané hybridizační profily rozdělily všechny analyzované kmeny do dvou skupin, které odpovídaly jejich druhovému zařazení. Hybridizační profily zástupců *B. cereus* vykazovaly vzájemně 77 až 97% podobnost, která rovněž korelovala s výsledky biochemického testování. Podobnost hybridizačních profilů kmenů *B. licheniformis* byla v rozmezí 82 až 100 %. Hybridizační profily kmenů *B. licheniformis* B79 (pasterizované mléko) a B82 (UHT mléko) byly naprosto totožné (100% shoda), stejně jako i výsledky biochemických a fyziologických testů. Podporují domněnku o identitě kmenů izolovaných v procesu výroby UHT mléka.

**Klíčová slova:** *Bacillus cereus*; *Bacillus licheniformis*; syrové; pasterované a UHT mléko; ribotypizace

The detection of spore-forming microorganisms in cow's raw milk is more difficult because of their occurrence in the phase of spores. Devitalisation of milk

samples in laboratory conditions (Anonymus, 1997) or practical pasteurisation in dairies enable germination of these spores resulting in relatively easier isolation

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of these bacteria on special media (Vyletřelová *et al.*, 2001). Their occurrence in final products is mostly caused by raw milk contamination and by subsequent transfer of spores in the course of milk processing. UHT temperature should extirpate vegetative cells as well as bacterial spores. Therefore sporadic occurrence of these food pathogens in UHT milk is usually attributed to milk contamination during technological processing.

Comparison of biochemical and physiological traits with the results of various molecular methods was used for *Bacillus* strains isolated from raw milk and from final products to specify the origin or identity of these food pathogens (Herman *et al.*, 1997, 1998; Nilsson *et al.*, 1998; Godič-Torkar and Smole-Možina, 2000; Helgason *et al.*, 2000; Svensson *et al.*, 2000).

Monitoring of the transfer of *B. cereus* and *B. licheniformis* strains from raw milk to final products (UHT milk), testing of ribotyping applicability to species classification and confirmation of *B. cereus* and *B. licheniformis* strains identity were the principal objectives of the present study.

## MATERIAL AND METHODS

### Sampling and processing of samples

Milk samples (one batch) were collected (250 ml in sterile samplers) in the course of the whole production process (transport – final product) from the following sampling sites: tank RMT<sub>x</sub> 100 000 1 – raw milk; surge tank before the pasteur – raw milk; cream behind the centrifuge; milk behind the pasteur; 1.5% milk fat; pasteurised milk in the tank before UHT; surge tank before UHT; final products (8 boxes) after UHT (138°C, 4 s); 8 final products (UHT milk) after thermostat testing (37°C, 14 days). Milk samples were frozen, transported to the laboratory and analysed.

### Thermostat testing of final products

Samples of UHT milk consumer's package were cultivated in an incubator (37°C). Milk samples (200 ml) were taken after 14 day-incubation; samples were frozen immediately and transported to the laboratory for microbiological analyses.

### Isolation of *Bacillus* strains

Modified MYP Agar complemented with Egg Yolk emulsion and Polymyxin B sulphate (HiMedia) and

Milk Agar (Merck) were used for the isolation of *Bacillus* strains. Milk samples were devitalised (85°C, 10 min) and consequently cultivated 3 days at 30°C as described by Havlová *et al.* (1993).

### Morphological characteristics

Colony morphology on blood agar and production of spores on the nutrient agar complemented with 10 mg MnSO<sub>4</sub> · H<sub>2</sub>O/l were studied for 1–7 days.

### Biochemical and physiological characteristics

Tested strains were cultivated on blood agar (24 h, 37°C). Conventional tests described by Gordon *et al.* (1973) were used for production of catalase, haemolysis, urease, production of acetoin, indole, reduction of nitrates, hydrolysis of esculin, starch and tyrosine, growth at 5, 40, 50 and 55°C, growth in 7 and 10% NaCl, and for acidification of carbohydrates. Arginine dihydrolase was tested according to Brooks and Sodeman (1974), ONPG according to Lowe (1962), phosphatase, hydrolysis of gelatine and Tween 80 according to Páčová and Kocur (1978, 1984). Growth on commercial media Simon's Citrate Agar (Oxoid), *Bacillus cereus* Agar Base (HiMedia) and BBL Anaerobic Agar was characterised.

Identification was made according to previously published differentiation tables (Páčová *et al.*, 1996) on the basis to 26 phenotypical traits and morphological characteristics.

### Ribotyping

Ribotyping with *EcoRI* restriction enzyme and the probe complementary to *Escherichia coli* 16S and 23S rRNA was made according to Švec *et al.* (2001). Lambda DNA cleaved by *EcoRI* and *HindIII* (Promega) was used as a molecular weight marker. Band-pattern analysis and cluster analysis were carried out using GelCompar II software (Applied Maths, Belgium). *B. cereus* CCM 2010<sup>T</sup> and *B. licheniformis* CCM 2145<sup>T</sup> obtained from the Czech Collection of Microorganisms were used as reference strains.

## RESULTS AND DISCUSSION

Proteolytic and lipolytic enzymes affect the quality and sensory properties of foods. These groups of

enzymes are also produced by various *Bacillus* species; therefore the microbiological control of milk product quality includes monitoring of this genus. Bacilli are classified as health-risky microorganisms that are able to produce thermoresistant endospores facilitating the transfer of bacilli to final milk products.

Aerobic growth together with positive catalase reaction enable to distinguish this genus easily from other gram-positive bacteria producing endospores.

As for milk and milk products, *B. licheniformis*, *B. subtilis* and *B. cereus* species are the most frequently isolated bacilli (Griffith and Phillips, 1990; Crielly *et al.*, 1994; Páčová *et al.*, 1996). In total, 158 strains were isolated from 396 milk samples in the period 1999–2000. Hundred and two strains (64.6%) were identified as *B. licheniformis*, 43 (27.2%) as *B. cereus*, the remaining 8.2 % were classified as different species of *Bacillus* and *Paenibacillus* (Vyletělová *et al.*, 2001). Therefore, the two most frequent species – *B. licheniformis* and *B. cereus* – were chosen to monitor the transfer of spore-forming bacteria from raw milk to the final product (UHT milk).

*B. licheniformis* strains were found in milk samples collected from all sampling sites. In contrast *B. cereus* strains were seldom isolated from raw milk, pasteurised milk and UHT milk (Table 1).

These two species are represented by facultatively anaerobic rods belonging to the 1st morphological group with oval spores not swelling the cell. Vegetative cells of *B. licheniformis* differ from *B. cereus* cells (large rods, in strings); they are smaller and form irregular clusters. Significant differences are also found in colony morphology. *B. licheniformis* strains form almost rough colonies adhering to the agar, with mucose droplets in young cultures. In contrast, typical colonies of *B. cereus* are round, matt and granular with greenish

tint on the blood agar. In addition to the above described morphology, the following tests can be used for their species differentiation: production of lecithinase, growth at 50°C and in 10% NaCl, ONPG test, and acidification of mannitol and xylose (Table 2).

In regard to biochemical and physiological traits, all tested strains of *B. licheniformis* formed a relatively homogeneous group; inability to utilise citrate (strains B77 and B78) and negative arginine dihydrolase activity (strain B75) were the only variable results obtained. *B. cereus* strains were characterised by variability in the following characteristics: Simmon's citrate, arginine dihydrolase, growth in 7% NaCl, acidification of cellobiose. Strain B71 was completely atypical: it did not produce acetoin and lecithinase. The negative egg-yolk reaction exhibited by *B. cereus* strain is a remarkable trait because the production of lecithinase forms a basis for selectivity of media recommended for the isolation of this species (Anonymous, 1997).

Ribotyping was made to confirm the species identification and identity of *B. cereus* and *B. licheniformis* strains isolated in the course of UHT milk production.

Ribotyping with restriction enzyme *EcoRI* separated unequivocally representative strains of *B. cereus* and *B. licheniformis* into two groups (Figure 1) in full agreement with species identification based on biochemical and physiological tests. Band patterns of the reference type strains (*B. cereus* CCM 2010<sup>T</sup> and *B. licheniformis* CCM 2145<sup>T</sup>) showed high coincidences with both species-specific clusters.

Similarities of analysed *B. cereus* strains ranged from 77 to 97% (Figure 1). The highest difference was found for strain B71 isolated from raw milk in tank RMT<sub>x</sub> (77% similarity with the type culture of *B. cereus* CCM 2010<sup>T</sup>). This result is in full agreement with pheno-

Table 1. Total counts (CFU/ml) of *B. cereus* and *B. licheniformis* in phase samples

Sampling site	<i>B. cereus</i>	<i>B. licheniformis</i>
Tank RMT <sub>x</sub> , 100 000 l – raw milk	10	30
Surge tank before pasteur – raw milk		30
Cream behind centrifuge		20
Milk behind pasteur, 1.5% milk fat		20
Pasteurised milk in tank before UHT		50
Surge tank before UHT	10	80
Final products (boxes) after UHT (138°C, 4 s)	10	10
Final products – UHT milk after thermostat testing (37°C, 14 days)		10

Table 2. Characteristics of the tested strains of *B. cereus* and *B. licheniformis*

Strain No.	LEC	VPT	SCI	ADH	ONP	HEM	TWE	TYR	C50	C55	NAC7	NAC10	MAN	XYL	CEL	Sampling site*	Identification
B 70	+	+	-	+	-	+	sl.	+	-	-	sl.	-	-	-	+	6	<i>B. cereus</i>
B 71	-	-	+	+	-	+	+	-	-	-	-	-	-	-	-	1	
B 72	+	+	-	-	-	+	+	-	-	-	-	-	-	-	+	7	
B 73	+	+	+	+	-	+	+	sl.	-	-	sl.	-	-	-	sl.	1	
B 75	-	+	+	-	+	-	-	+	+	+	+	+	+	+	+	1	<i>B. licheniformis</i>
B 76	-	+	+	+	+	-	-	+	+	+	+	+	+	+	+	2	
B 77	-	+	-	+	+	-	-	+	+	+	+	sl.	+	+	+	3	
B 78	-	+	-	+	+	-	-	+	+	+	+	+	+	+	+	4	
B 79	-	+	+	+	+	-	-	+	+	+	+	+	+	+	+	5	
B 80	-	+	+	+	+	-	-	+	+	+	+	+	+	+	+	6	
B 81	-	+	+	+	+	-	-	+	+	+	+	+	+	+	+	7	
B 82	-	+	+	+	+	-	-	+	+	+	+	+	+	+	+	8	

\* = according to Table 1

+ = positive; - = negative; sl. = slightly positive

LEC = lecithinase; VPT = acetoin production; SCI = Simmons' citrate; ADH = arginine dihydrolase; ONP =  $\beta$ -galactosidase; HEM = haemolysis;

TWE = hydrolysis of Tween 80; TYR = hydrolysis of tyrosine; C50 and C55 = growth at 50 and 55°C; NAC7 and NAC10 = growth in 7 and 10% NaCl; MAN = mannitol;

XYL = xylose; CEL = cellobiose

Positive reaction: catalase, phosphatase, reduction of nitrates, hydrolysis of gelatine, starch, casein and esculin, growth at 40°C, growth under anaerobic conditions, acidification of glucose and fructose

Negative reaction: urease, production of indole, growth at 5°C, acidification of lactose and inositol

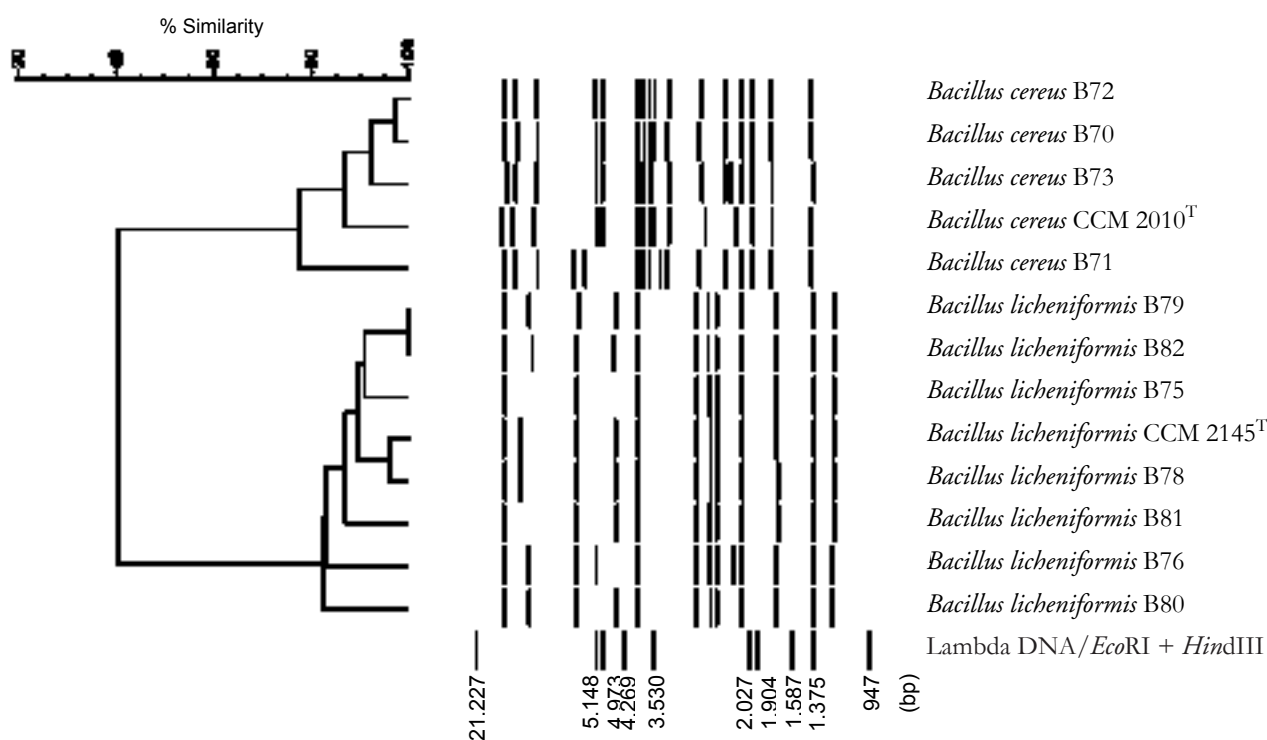


Figure 1. Band-pattern profiles of *B. cereus* and *B. licheniformis* strains. The dendrogram was constructed with Jaccard coefficients using UPGMA clustering method

typic characteristics (atypical reaction in VP test and lecithinase as discussed above). In contrast, another strain isolated from the same sample (B70) showed high similarity with the other strains isolated from pasteurised milk and UHT milk (97 and 92%). The results of ribotyping and biotyping of these two strains (B70 a B71) confirmed the occurrence of two different *B. cereus* strains in the same milk sample.

Similarity of analysed *B. licheniformis* strains ranged from 82 to 100%. Band-pattern profiles of strains B79 (pasteurised milk) and B82 (UHT milk) were identical. However, our results imply that these strains are identical strains passing through the studied technological process. This hypothesis (strain identity) should be confirmed by ribotyping with more restriction enzymes or by other methods based on the genomic DNA analysis.

Although only a small number of strains was analysed in this study, it is evident that these two species give well detectable and easily distinguishable band-pattern profiles (Figure 1). It seems that ribotyping with *EcoRI* is a useful method for the of species identification of *B. cereus* and *B. licheniformis* mainly in case atypical results of biochemical test were obtained.

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*Corresponding Author*

RNDr. Marcela Vylet lov, Vzkumn stav pro chov skotu Rapotn, Vzkumnk 207, 788 13 Vikřovice,  esk republika  
Tel. +420 649 39 24 30, e-mail: marcela.vyletelova@vuchs.cz

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