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AN INVESTIGATION OF THE CYTOTOXIC EFFECT OF *BACILLUS SPP.* ISOLATED FROM RAW MILK ON VERO CELL CULTURES

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Members of the genus Bacillus are often present as contaminants in raw milk. The sources of milk contamination are in the environment. The average level of raw milk contamination with Bacillus spp. is 3.42 x 10^2/ml during the winter and 1.03x10^2/ml in the summer. Reduction of the number of Bacillus in thermally processed milk directly depends on the applied treatment. The thermic treatments which are used for pasteurization do not lead to a reduction of Bacillus number. Thermic treatments aimed at sterilization, decrease Bacillus numbers in direct cerrelation with their thermoresistance (D-values). Bacillus cureus is known as a cause of food poisoning. Intensive examination in the last ten years has shown that other Bacillus spp. can also produce toxins. Besides biological assays and immunological methods toxin production has been measured by determine cytotoxicity to suitable cell cultures. Thus, 15 strains of Bacillus spp. have been screened for cytotoxicity to Vero cell cultures. Out of this 13 were from the mesophyl group and 2 from the psychrotrophic group. All the examined strains were lecithinase positive, haemolytic and hydrolysed casein. The strains were cultivated in brain-heart infusion broth, supplemented with 0.1% glucose (BHIG broth) at 20$\pm$1^{0}C and 30$\pm$1^{0}C for 24-96 hours. Cytotoxicity was estimated after incubation for 1-48 h of the broth culture supernatant inoculum on Vero cells. It was found that the Bacillus spp. population after 24 h of incubation at 20$\pm$1^{0}C achieved a level of log 9.888$\pm$0.909 and at 30$\pm$1^{0}C a level of log 9.739$\pm$1.001.

There was no statistically significant difference in the Bacillus spp. numbers during incubation for 96 hours at 30$\pm$1^{0}C. A statistically significant difference in Bacillus spp. numbers ($p<0.05$) occurred between the first and the fourth day during the incubation

for 96 hours at 20+1^{0}C. The appearance of a cytotoxic effect on Vero cells was found in 8 of the 15 investigated broth culture supernatants obtained after the first day of incubation of Bacillus strains at 30±1^{0}C in 9 after the second day and in 11 strains affter the third and tourth day. After incubation of Bacillus strains in BHIG broth for 24-96 h at 20±1^{0}C, cytotoxicity of supernatants on Vero cells was noted in 7 out of 15 strains after the first day and in 10 strains after the second, third and the fourth days.

Key words: raw milk, Bacillus spp., cytotoxic effect

INTRODUCTION

It has been recognized for some time that strains of *Bacillus spp.* exist as contaminants in raw milk. Sources of milk contaminantion with Bacillus spp. are in the environment, and milk contamination level varies depending on the place where the sample is taken (Mijačević et al., 1998). According to Waes (1976), the average level of raw milk contamination with *Bacillus spp.* was 3,42x10^2/ml during the winter and 1.03x10^2/ml in the summer. The most frequently isolated microorganisms were *B. licheniformis* and *B.brevis.* Moreover, contamination of buffalo cow milk with aerobic mesophylic sporogenic bacteria was 3.2x10^2 - 1.2x10^4/μ ml in the summer and 9.2x10^2 - 6.4x10^2/ml in the winter (Khalafalla et al., 1976). According to the same authors, isolated aerobic sporogenic bacteria were mostly *B. brevis, B. megaterium, B. subtilis* and *B. firmus.* Becker and co-workers (1994) reported that even 54% infant milk formula is contaminated with diarrheogenic *B. cereus* at the level of 0.3 - 600 cfu/g.

Bacillus spp. reduction in thermally processed milk is directly dependent on the applied treatment. *B. cereus* strains have a D_{100} value ranging from 2.16-3.06 min, *B. polymyxa* 4.36 min, and *B. circulans* 3.54 min (Ivanović et al., 1990). Thermoresistance of *B. cereus* spores in phosphate buffer was noted by Johnson and co-workers (1982). The estimated D_{95} values ranged from 1.2 up to 20.2 min for eight B.cereus strains with an average 2. value of 92^{0}C. Bradshaw and co-warkers (1975) reported about 2 highly thermoresistant *B. cereus* isolates from canned soups with D_{95} values ranging from 256.7 up to 5122.3 min.

The presence of *Bacillus spp.* spores in milk leads to the risk of their appearance in final products. Thus Bacillus spp. isolated from evaporated milk (*Bacillus subtilis,B.licheniformis, B. circulans, B.brevis, B.alvei*) had D_{120} values ranging from 0.16- 0.86 min (Skrebkova, 1982). Milk processing temperatures of 85 and 95^{0}C can only reduce the number of *Bacillus spp.* because it has been experimmentally confirmed that the $D_{85\,°C}$ values for the same bacilli are 2.04, 1.71 and 1.40 min, respectively (Oljačić, 1996).

Investigations showe that spores germination ability after thermical tretment at 80^0C for 10 min was greater than after 10 min at 90^0C or 1 min at 100^0C (Moran et al., 1990). The decimal reduction period of mesophylic spores isolated from UHT milk was D_{100} 5.09 min, D_{121} 8.2 - 34 sec. Hammer and co-workers (1995) found that for certain isolated *Bacillus spp.* D_{120} was 10 min, D_{126}- 1 min and D_{147} - 5 sec. The significance of contamination of raw milk with psychrotrophic *Bacillus spp.* has not been fully investigated. Mesophylic sporogenic Bacillus spp. can grow even in psychrotrophic conditions. Chung and co-workers (1971) studied the frequency, cultural characteristics, spore germination rate and outgrowth of psychrotrophic sporogenic bacteria present in raw milk. The bacterial number ranged from 0.8 to $120x10^3$/ml and the spore number was 2 - 900/ml. It was determined that in 46.3% of the samples there were B.firmus, in 23% *B.megaterium*, 15.7% *B. brevis* and the rest contained *B.coagulans, B. polymyxa, B.mascerans, B.circulans* and *B.cereus*. More than 85% of *B. firmus and B.brevis* spores inoculated in milk, germinated within 2 days at the temperature of 7^0C. At temperatures of 7 ± 1^0C the *Bacillus spp.* population grew from log 0.81 $\pm$ 0.78 up to log 2.07 $\pm$ 0.47 during 10 days (Mijačević et al., 1998). Bacer and co-workers (1992) stated that *B. cereus* was isolated as a cause in many cases of food poisoning. Many *B. cereus* strains are capable to grow and produce enterotoxins at low temperatures (6^0C). Intensive examinations in the last ten years have shown that certain *Bacillus spp.* strains can produce toxins. The toxin production was determined using immunological methods and by determining cytotoxicity on Vero and Hep - 2 cells. Christiansson and co-workers (1989) screened 136 psychrotrophic strains of *Bacillus cereus* strains isolated from milk and cream for cytotoxicity in vitro on HeLa S3, Vero and HEL cells. Of these, 37,8% showed citotoxicity, to Hel a S3, 37% to Vero, and 43% to HEL cells after growth in brain-heart infusion broth (BHI) at 30^0C under aeration. Griffiths (1990) used a reversed passive latex agglutination assay to show that about 85% of the psychrotrophic *Bacillus spp.* tested produced diarrheogenic toxin during growth in BHI broth at 25^0C. The majority (92%)of these strains were *Bacillus cereus* and *B. cereus*-related strains, including *B. mycoides* and *B. thuringiensis.* The other strains capable of producing the toxin were *B. circulans, B. lentus, B. pumilus, B. polymyxa* and *B. carotarum.* Turnubull (1981) concluded that *B.thuringensis* and *B.mycoides*, which were closely related to *B. cereus*, could produce diarrheogenic toxin, whereas the other *Bacillus spp.* either did not do so or synthesized it to only a limited extent. The diarrheogenic toxin was found to be synthesized during the exponential phase of growth, but maximal concentrations of the toxin were detected early in the stationary phase (Glatz and co-workers). According to Griffiths (1990) bacterial couns exceeding 1 x 107 cfu/ml were required before appreciable levels of toxins were produced in milk. Christiansson and co-workers (1990) observed that the value of 2x107 cfu/ml was necessary for appreciable synthesis of toxin by *B. cereus* grown in milk.

On the basis of literature data, concerning *Bacillus spp.* as potential causes of food poisoning, in this work we aimed to investigate the cytotoxicity of lecithinase positive strains of *Bacillus spp.* isolated from raw milk.

MATERIALS AND METHODS

The material consisted of 15 *Bacillus spp.* strains, isolated from raw milk, out of which 13 were from the mesophyl group and 2 from the psychrotrophic group. All the examined strains were lecithinase positive, haemolytic and hydrolysed casein. Not one of the strains broke down gelatin, but 4 of them hydrolysed starch.

The strains were cultivated in 10 ml of BHI broth supplemented with 0,1% glucose (BHIG) for 16 hours at 30^0C. After the incubation, the concentration of 10^8 cfu/ml was reached - 1% broth culture was added in 20 ml of BHIG to an Erlenmeyer flask - (250 ml). The inoculated broths were shaken at 250 rpm in shaker (type THYS2 WLW) at room temperature (20 ± 1^0C) and at a temperature of $30+1^0$C. During 4 days 5 ml of BHGI broth was taken by a sterile pipette every 24 hours. The number of sporogenic bacteria was determined after 24 hours incubation at 30^0C of decimal dilutions sown on nutritive agar.

Cytotoxicity was estimated in the supernatant after centrifugation of broth at 12000 revolutions/min. The obtained supernatant was filtered through a Millipore filter. The same procedure of cultivation, shaking and centrifugation was carried out also with a laboratory reference strain for producing emetic toxin (*B.cereus* ATCC-14579), which served as the control in the cytotoxicity investigation. Cytotoxicity was estimated after incubation for 1-48 h of the broth culture supernatant inoculum on Vero cells. A cytotoxic effect (CTE) was manifested by the appearance of vacuoles, cells pigmentation, more intensive refraction of light, cells rounding off and creation of syncytia and plaques. Supernatants of certain *Bacillus spp.* broth cultures after 24 h provoked complete cell destruction in the layer. We marked the intensity of changes on Vero cells which were the consequence of toxin activity, -; +; ++; +++.

RESULTS AND DISCUSSION

Bacillus spp. are ubiquitous microorganisms. They grow and proliferate at environmental temperatures and often contaminate food. Spores of *Bacillus spp.* are thermoresistant and survive thermic treatments in milk processing, so their number in final products directly depends on the level of raw material contamination.

In final products they can proliferate during storage both at refrigerator temperatures (7 ± 1^0C) and at room temperatures (20 ± 1^0C). Bacilli proliferation in BHIG broth at the temperatures of 20 ± 1^0C and 30 ± 1^0C during a period of 24 hours, is presented in Table 1.

Table 1. The changes of log number of bacilli during 96 hours of incubation in BHIG broth

conditions of incubation	statistical parameters	Log number of bacilli after incubation for			
		24 h	48 h	72 h	96 h
20 ± 1°C	n	15	15	15	15
	x ± s	9.888±0.909	9.577±0.946	9.438±0.794	8.995±1.137
	Cv (%)	9	9	8	12
30 ± 1°C	n	15	15	15	15
	x ± s	9.739±1.001	9.693±0.730	9.676±0.513	9.234±0.649
	Cv (%)	10	7	5	7

The mean value for *Bacillus spp.* number in BHIG broth incubated at 20°C ranged from log 8.995±1.137 to log 9.888±909. The number was greatest after the first day of incubation. The coefficients of variation ranged from 8-12% in the samples incubated at 20±1°C, and from 5-10% in the samples at 30±1°C.

Analysis of variance, confirmed by individual LSD tests, concerning the changes of bacilli population after 24-96 hours at 20±1°C and 30±1°C, showed that there was no statistically significant difference in *Bacillus spp.* populations during the incubation of 96 hours at 30±1°C. A statistically significant difference in *Bacillus spp.* number ($p < 0.05$) in BHIG broth samples was found between the first and the fourth day of incubation in the environmental conditions (20±1°C).

The cytotoxic effect (CTE) of *Bacillus spp.* broth culture supernatants obtained after the incubation for 24-96 hours at 20±1°C is presented in Table 2.

Table 2. Cytotoxic effect (CTE) of broth culture supernatants of 15 *Bacillus strains* after incubation for 24-96h at 20±1°C.

Cytotoxicity	Cytotoxic effect (on Vero cell culture) of broth culture supernatants of Bacillus strains obtained after the incubation for							
	24 h		48 h		72 h		96 h	
	number of strains	%	number of strains	%	number of strains	%	number of strains	%
−	8	53.33	5	33.33	5	33.33	5	33.33
+	3	20.00	3	20.00	2	13.33	2	13.33
+	0	0	0	0	1	6.66	1	6.66
+++	4	26.66	7	46.66	7	46.66	7	46.66
total	15	99.99	15	99.99	15	99.98	15	99.98

Cytotoxicity was not detectted in 8 out of the 15 examined supernatants of *Bacillus spp.* broth cultures during the first day and in 5 strains after the second, third and fourth days. Extreme cytotoxicity (+++) was shown for 4 strains of *Bacillus spp.* the first day, and in 7 strains after the second, third and fourth days of incubation at 20±1°C.

Cytotoxicity of the supernatant obtained after incubation of *Bacillus spp.* for the same period at 30 ± 1°C is presented in Table 3.

Table 3. Cytotoxic effect of broth culture supernatants of 15 *Bacillus* strains after incubation for 24-96h at 30 + 1°C.

Cytotoxicity	Cytotoxic effect (on Vero cell culture) of broth culture supernatants obtained after the incubation for							
	24 h		48 h		72 h		96 h	
	number of strains	%	number of strains	%	number of strains	%	number of strains	%
–	7	46.66	6	40.00	4	26.66	4	26.66
+	5	33.33	5	33.33	5	33.33	4	26.66
+	1	6.66	1	6.66	0	0	0	0
+++	2	13.33	3	20.00	6	40.00	7	46.66
total	15	99.98	15	99.99	15	99.99	15	99.98S

Cytotoxicity was not found for 7 out of the 15 *Bacillus spp.* broth culture supernatants after the first day, for 6 of them after the second day, and for 4 strains after the third and the fourth days of ilncubation in BHIG broth at 30±1°C. On the first day extreme cytotoxicity (+++) was shown in 2 out of 15 *Bacillus spp.* broth culture supernatants, on the second day for 3 of them, the third day for 6, and the fourth day for 7.

According to other authors, *Bacillus spp.* enterotoxins are produced in measurable quantities at the number of 10^7/ml (Christiansson and co. workers, 1990; Griffiths 1990). The possibility that bacilli grow and produce enterotoxins at low temperatures enlarged the interest for investigations concerning the presence of *Bacillus spp.* in food. Christiansson and coworkers (1990) found that out of 136 psychrotrophic strains of *B.cereus isolated* from milk and cream, 37.8% showed cytotoxicity to HeLa S3, 37% to Vero and 43% to HEL cells. According to Griffiths (1990), the majority (92%) of strains producing toxins are *B.cereus* and similar strains. The author also observed that *B. mycoideus* Pm 57 produced toxin during growth in milk at all the tested temperatures: 6,10, 15 and 21°. Christiansson and co-workers (1990) confirmed that toxin was produced during cultivation in milk, but only under aeration conditions, while Wong and co-workers (1988) recorded the presence of toxin in milk after the strains had been grown but without aeration. In this work it was observed that cytotoxicity of 15 lecithinase positive *Bacillus spp.* strains isolated from milk to Vero cells depended on the temperature. After the first day of BHIG broth incubation at 30±1°C, 53.34% of *Bacillus spp.* strains showed a cytotoxic effect on Vero cells, after the second day 60.0%, the third 73.33% and the fourth 73.32%. The percent of *Bacillus spp.* which showed cytotoxicity at 20±1°C was 7% lower in the investigated period.

The number of *Bacillus spp.* strains showing cytotoxicity increased when supernatant was obtained from a broth culture incubated for a longer period

(3 or 4 days) at 30±1°C. However, the maximal number of cytotoxical positive strains was obtained after 48 hours of incubation at 20+1°C.

CONCLUSION

1. *Bacillus spp.* populations after 24 h of incubation at 20±1°C achieved a level of log 9.888+0.909 and at 30±1°C a level of log 9.739±1.001.

2. There was no statistically significant difference in the Bacillus spp. population during incubation for 96 hours at 30±1°C. A statistically significant difference in *Bacillus spp.* numbers ($p<0.05$) was found between the first and the fourth day during incubation for 96 hours at 20±1°C.

3. A cytotoxic effect on Vero cells appeared in 8 out of 15 investigated broth culture supernatants obtained after the first day of incubation of *Bacillus spp.* strains at 30±1°C, in 9 after the second day, in 11 strains after the third and the fourth days.

4. After incubation of *Bacillus spp.* in BHIG broth for 24-96 h at 20±1°C, cytotoxicity of supernatants on Vero cells was demonstrated in 7 out of 15 strains after the first day and in 10 strains after the second, third and the fourth days.

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ISPITIVANJE CITOTOKSIČNOG EFEKTA *BACILLUS SPP.* IZOLOVANIH IZ SIROVOG MLEKA NA KULTURI VERO ĆELIJA

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SADRŽAJ

Predstavnici roda *Bacillus* su često prisutni kao kontaminenti u sirovom mleku. Izvori kontaminacije mleka nalaze se u okolini. Nivo kontaminacije sirovog mleka u zimskom periodu je prosečno $3.42x10^2$/ml, a u letnjem periodu $1.03x10^2$/ml.

Redukcija broja *Bacillus spp.* u termički obrađenom mleku je u direktnoj vezi sa primenjenim tretmanom. Termički tretmani koji se koriste prilikom pasterizacije ne dovode do redukcije broja *Bacillus spp.* Primenom termičkih treetmana pri sterilizaciji, redukcija broja *Bacillus spp.* direktno zavisi od njihove termorezistencije (D-vrednosti).

Bacillus cereus je poznat kao uzročnik trovanja hranom. Intenzivnim ispitivanjima poslednje decenije utvrđeno je da i druge vrste roda *Bacillus* mogu stvarati toksine. Produkcija toksina se može odrediti, pored bioloških ogleda i imunoloških metoda, i utvrđivanjem citotoksičnosti na odgovarajućim ćelijskim kulturama.

Citotoksični efekat je određivan kod 15 sojeva *Bacillus spp.* izolovanih iz sirovog mleka. Od ispitivanih sojeva, 13 sojeva je iz grupe mezofilnih, a 2 iz grupe psihrotrofnih, svi su lecitinaza pozitivni, hemolitični i razlažu kazein. Sojevi su inkubirani u BHI bujonu sa 0.1% glukoze (BHIG) na 20 ± 1^0C i 30 ± 1^0C u vremenu od 24-96h. Citotoksični efekat je određivan inokulacijom supernatanta bujonskih kultura na Vero ćelije.

Utvrđeno je da je populacija *Bacillus spp.* posle 24 h inkubacije na 20 ± 1^0C dostigla nivo od log 9.888 ± 0.909 a na 30 ± 1^0C nivo od log $0,739 \pm 1.001$. Ne postoji statistički značajna razlika u promeni populacije *Bacillus spp.* tokom

inkubacije od 96 časova na 30±1^{0}C. Dokazana je statistički značajna razlika u broju *Bacillus spp.* ($p<0.05$) tokom inkubacije na 20 ± 1^{0}C između prvog i četvrtog dana.

Pojava citoksičnog efekata na Vero ćelijama dokazana je kod 8 od 15 ispitivanih supernatanata bujonskih kultura koji je dobijen posle prvog dana inkubacije sojeva *Bacillus spp.* na 30 ± 1^{0}C, drugog dana kod 9, a trećeg i četvrtog dana kod 11 sojeva. Inkubacijom *Bacillus spp.* u BHIG bujonu 24–96 h na 20 ± 1^{0}C, citotoksičnost supernatanta na Vero ćelijama je dokazana kod 7 od 15 sojeva prvog dana a drugog, trećeg i četvrtog dana kod 10 sojeva.