Influence of the Preservative Material HOLDBAC^{TM} on the Growth and Proliferation of *Listeria* on the Surfaces of Cheeses

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Abstract

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Three varieties of cheese were selected for the experiment: Genuine Olomouc-brand soft ripened cheese, Loose-brand acid curd cheese, and Slovak-style string cheese. Their surfaces were inoculated with *Listeria* cells of a defined density and then treated with the preservative material HOLDBAC^{TM} at its optimal concentration. The cheeses were stored at two different temperatures for the periods of 2, 5, 9, and 14 days, after which the effectiveness of the treatment with the preservative material was determined. The results were compared with the effectiveness of the material on the growth and proliferation of *Listeria* when the application of the preservative material was made before the artificial contamination of the cheese surface with *Listeria*. The experiment demonstrated only a slightly greater effectiveness of HOLDBAC^{TM} when it was applied before the microbial contamination. Complete inhibition of *Listeria* was not observed when using this approach.

Keywords: *Listeria*; HOLDBAC^{TM}; soft ripened cheese; string cheese

Listeria comprise a group of Gram-positive, facultative anaerobic or aerobic non-spore forming bacilli of which the most dangerous is the pathogenic species *Listeria monocytogenes*. This is an invasive microbe that attacks the cells of the immune system and reproduces within them. The illness caused by these bacteria is very serious and strikes most seriously at individuals with low immunity, pregnant women, and children (McLaughlin 1987). While this illness does not occur often, it is unarguably a leading cause of the deaths attributable to food-borne pathogens. The most frequent source of infection is the consumption of contaminated foodstuffs, with the risk being the greatest in the case of soft ripened cheeses (Sharp 1987; Lunden *et al*. 2004), cheese produced from unpasteurised milk, seafoods, deli products, uncooked meat or insufficiently heat-processed meat products, and poorly washed vegetables. The survival and proliferation of *Listeria* is dependent upon the contaminating strain, type of cheese, and technological process involved

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(Marth & Ryser 1990). *Listeria* adheres well to various surfaces and consequently forms a biofilm. Studies have shown that *Listeria* can create biofilms on stainless steel, plastic materials, and hydrophobic surfaces, particularly in the presence of growth-supportive nutrients (Jeong & Frank 1994; Hood & Zottola 1997). This biofilm, therefore, may become a substantial source for contaminating food products with *Listeria*. Physical and chemical means (and frequently in combination with one another) are used to combat this dangerous microorganism. Among the effective means for inhibiting *Listeria* are high temperature, starter culture additives, the addition of lactic acid, bacteriocins, bacteriophages, and irradiation (Bradshaw *et al*. 1991; Farber & Peterkin 1991; Chen & Shelef 1992; Ennahar *et al*. 1998; Lossner *et al*. 2002; Osterholm & Norgan 2004). In recent years, the use of inhibitory additives that limit the growth of *Listeria* has become increasingly common. The Danish firm Danisco (Copenhagen) has developed a protective culture sold under the name HOLDBAC^{TM} Listeria dairy that provides immediate protection upon its application directly onto the surface of cheese. It exerts no influence upon the surface ripening flora. The product is a powdered, weakly acidic protective culture containing *Lactobacillus plantarum* (Loessner *et al.* 2002). This culture may be used for any foodstuff that is subject to contamination by *Listeria*. It does not change the sensory properties of the food. The culture may be applied to cheeses the day after they come out of the salt bath, during dry salting, or by spraying onto the cheese surface (Danisco A/S 2006). Such products include additional LISTEX^{TM} P100 and LMP 102, which are materials based on bacteriophages. LISTEX^{TM} P100 is a specially isolated culture of safe bacteriophages. It contains 2×10^{11} PFU/ml of active anti-*Listeria* bacteriophages. It works only against *Listeria monocytogenes* and does not influence the ripening process, growth of the cultures, or technical needs of the microorganisms. It is effective also in the presence of NaCl in a concentration of approximately 20%. It is used for the treatment of meat, cheeses, fish and other foodstuffs (Carlton *et al.* 2005). LMP 102 is produced by the company Intralytix (Baltimore, USA). This is a mix of six bacteriophages, each of which is focused upon a different strain of the *Listeria monocytogenes* pathogen (Leverentz *et al.* 2003). The aim of this study was to find how well various types of cheese are protected against *Listeria* contamination by the application of HOLDBAC^{TM}. Also examined was the potential for eliminating the contamination that had already occurred by means of a subsequent treatment with this preparation.

MATERIAL AND METHODS

Samples of cheeses. Two specimen cheeses were purchased in a retail store, that is Genuine Olomouc-brand soft ripened cheese (Pravé olomoucké tvarůžky), Loose-brand acid curd cheese (Sauermilchkäse), and Slovak-style string cheese (Korbáčiky).

Testing microorganism and nutrient media. The experimental strain was *Listeria innocua* CCM 4030 from the Czech Collection of Microorganisms belonging to the Masaryk University in Brno. The culture was stored on slanted blood agar (HIMEDIA, Mumbai**,** India) and held at a refrigerated temperature. Re-inoculation was made once monthly. The media used were: Listeria Enrichment Broth (LEB), TSYEA agar, PALCAM agar, Nutrient Agar No. 2, and Blood Agar (all from HIMEDIA, Mumbai**,** India) as well as ALOA agar (BioRaD, Mames-la-Coquette Francie). The preservative material HOLDBAC^{TM} was obtained from Danisco Czech Republic a.s., Smiřice, Czech Republic.

Preparation of the preservative material HOLDBAC^{TM}. The material contains *Lactobacillus plantarum* and glucose, with 97.9 g corresponding to 10 IP. The amount of HOLDBAC^{TM} Listeria dairy calculated to achieve a concentration of 3 IP/l was dissolved in 100 ml of sterile distilled water. A sterile plastic sprayer was used for spraying the prepared preservative material. Before each use, the sprayer was immersed for 45 min into the disinfecting agent Septoderm and was subsequently rinsed three times with sterile distilled water. The cleanness of the final rinse water was confirmed by sprinkling it onto Nutrient Agar No. 2, spreading with an L-shaped stirring rod, and subsequent incubation for 24–48 h at 37°C.

Procedure. A suspension from 24 h old *Listeria innocua* culture was prepared in a physiological solution using the McFarland turbidity scale at a density of 1.5×10^8 CFU/ml. It was diluted by decimal dilution to the value of 10^3 CFU/ml. The actual number of *Listeria* was confirmed by inoculation on Blood Agar (incubation at 37°C for 24 h).

The specimens were aseptically placed into large Petri dishes in sets of five pieces. From various possible concentrations of the HOLDBAC™ preservative material the optimal concentration of 3 IP/l (Danisco A/S 2006) was selected. The HOLDBAC™ *Listeria dairy* was applied using two methods: spraying the cheese surface before inoculation with *Listeria*, and spraying the cheese after inoculation.

In all cases, five pieces of the respective cheese specimens were placed into sterile Petri dishes. Each specimen piece was inoculated with 100 µl of the *Listeria* cell suspension at a density of 1.5×10^5 CFU/ml. Afterwards, the same amount of HOLDBAC™ was applied by spraying in every case. Immediately after inoculation, the actual number of *Listeria* on the cheeses in CFU/cm^2 (in the case of the string cheese CFU/g) was confirmed by the culture method. The Petri dishes were wrapped in aluminum foil and held at temperatures of 20°C and 5–6°C for 14 days. After 2, 5, 9, and 14 days of incubation, one piece of specimen was removed from each dish and analysed for the number of *Listeria* in accordance with the norm CSN ISO 10560 (PALCAM, ALOA). The same procedure was applied again, but in the reverse order: the cheeses were first sprayed with the HOLDBAC™ which was followed by the inoculation with *Listeria*. The entire experiment was repeated five times for each specimen type and the average number of *Listeria* was determined after the treatment with the preservative material both before the inoculation with *Listeria* and after the inoculation with *Listeria*. The control samples of cheeses inoculated with *Listeria* but untreated with HOLDBAC were stored at the same temperatures and for the same periods of time.

RESULTS AND DISCUSSION

In the control samples, the final number of *Listeria* was approximately 3–4 logarithm higher than the initial number after 14 days at 20°C. If cheeses were stored at 15°C, the number of *Listeria* in the control samples increased in 1–2 logarithm (according to the kind of cheese).

Figure 1a shows the relationship between the count of *L. innocua* (CFU/cm^2) in the Olomouc-brand cheese and the days of storage at the refrigerated temperature, and Figure 1b shows this relationship at 20°C. This cheese was treated with the preservative material only after the contamination with *Listeria*. It is clear from the results that at the refrigerated temperature the action of the preservative material resulted in a reduction in the number of *Listeria* cells. In the course of 14 days, there occurred a reduction in the number of viable *Listeria* cells by an order of 4. In a similar study by Bizani *et al.* (2007), in which they treated the surface of *Listeria*-contaminated cheese with Cerein 8A, a reduction was observed in the number of viable cells by an order of 2 in the course of 30 days at 4°C. During the storage of the Olomouc-brand cheese at 20°C, a reduction in the cell count by three logarithmic units was observed, however, over the following days a perceptible and gradual growth in the number of cells took place so that after 14 days of storage the number of *Listeria* was only two logarithmic unites lower than the original level (Figure 2).

Figure 2a shows the results concerning the number of *Listeria* in Loose-brand acid curd cheese that had been inoculated with the suspension of *L. innocua* before the treatment with the preserva-

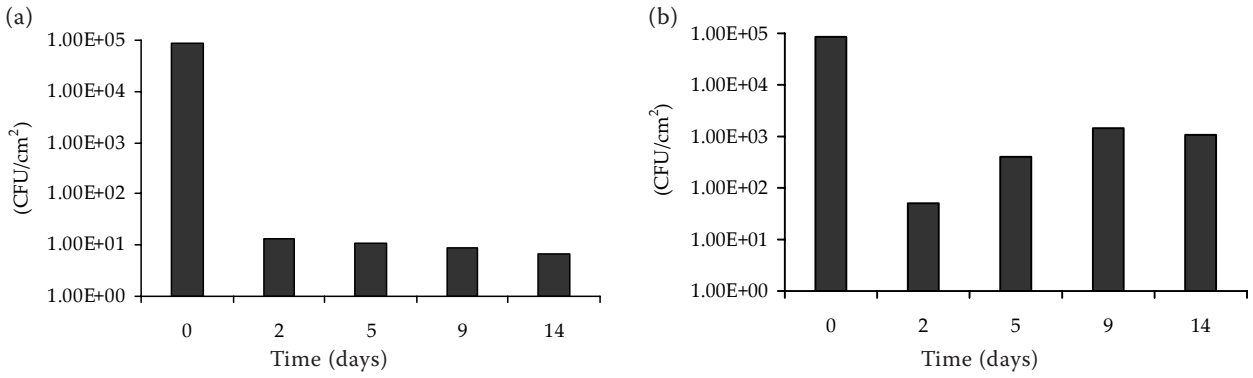

Figure 1. Relationship between the count of *L. innocua* in the Olomouc-brand cheese by the days in storage at the refrigerated temperature (a) and at 20°C (b) – cheese was treated with the preservative material only after the contamination with *Listeria*

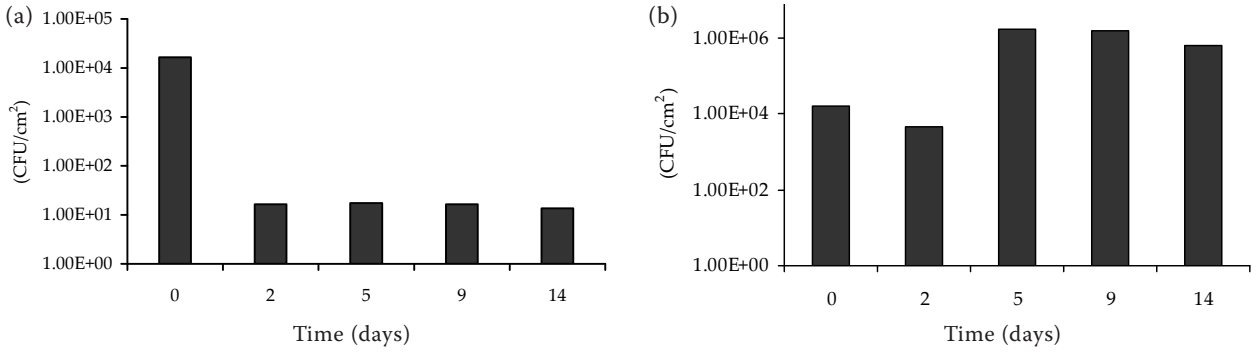

Figure 2. Relationship between the count of *L. innocua* in the Loose-brand acid curd cheese by the days in storage at the refrigerated temperature (a) and at 20°C (b)– cheese was treated with the preservative material only after the contamination with *Listeria*

tive material and stored at the refrigerated temperature, while Figure 2b shows the result achieved at 20°C. After 14 days of storage at the refrigerated temperature, a decline was observed by an order of 4, in viable *Listeria* cells count although complete inhibition did not occur. In a similar study, Carlton *et al.* (2005) used the material P 100 on artificially contaminated cheese stored at 6°C. With the repeated use of the preservative material, the *Listeria* count was reduced by an order of 2 to 3, but again there was no complete inhibition. The number of *Listeria* in the acid curd cheese stored at 20°C (Figure 2b) was reduced by an order of 1 after two days of storage, but already during days 5 and 10 of storage, the number of *Listeria* increased by an order of 2 relative to the original count. In this case, the preservative material HOLDBAC™ was ineffective. Similar results were obtained by Stecchini *et al.* (1995) after the application of the preservative material, demostrating reduction in the number of *Listeria* followed by a repeated increase in the *Listeria* count. These followed the behaviour of *L. monocytogenes* on Italian Mozzarella in the presence of a bacteriocin produced by *Lactococcus lactis* spp. *lactis.* The addition of the bacteriocin to the *L. monocytogenes* culture reduced the number of cells at 5°C but not at 30°C.

In the case of the Slovak-style string cheese, the *Listeria* count was reduced only by an order of 1 in the course of 14 days while stored at the refrigerated temperature (Figure 2a). When stored at 20°C (figure 3b), a slow and gradual decrease occurred in the number of viable cells. After 2 days, the count fell by an order of 1, and during days 5, 9, and 14 by an order of 2. Neither in this case was total inhibition of the cells observed. Also contributing to the reduction in the *Listeria* count was the low moisture activity in this type of cheese and its high salt content.

Buyong *et al.* (1998) reported interesting findings when they used the strain *Lactococcus lactis* subsp. *lactis* MM217 that produces pediocin PA-1 during the manufacture of Cheddar cheese. They monitored the viability of *Listeria* cells during the production process and aging at 8°C over a period of 6 months. During the first week of aging, the

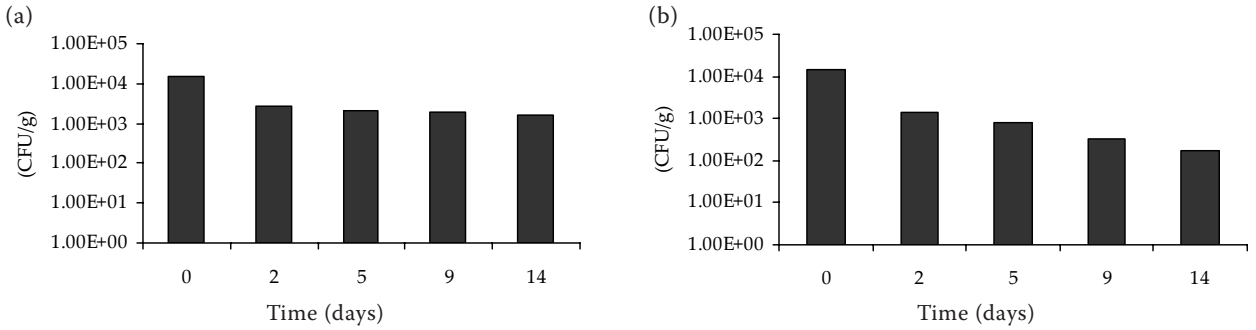

Figure 3. Relationship between the count of *L. innocua* in the Slovak-style string cheese by the days in storage at the refrigerated temperature (a) and at 20°C (b) – cheese was treated with the preservative material only after the contamination with *Listeria*

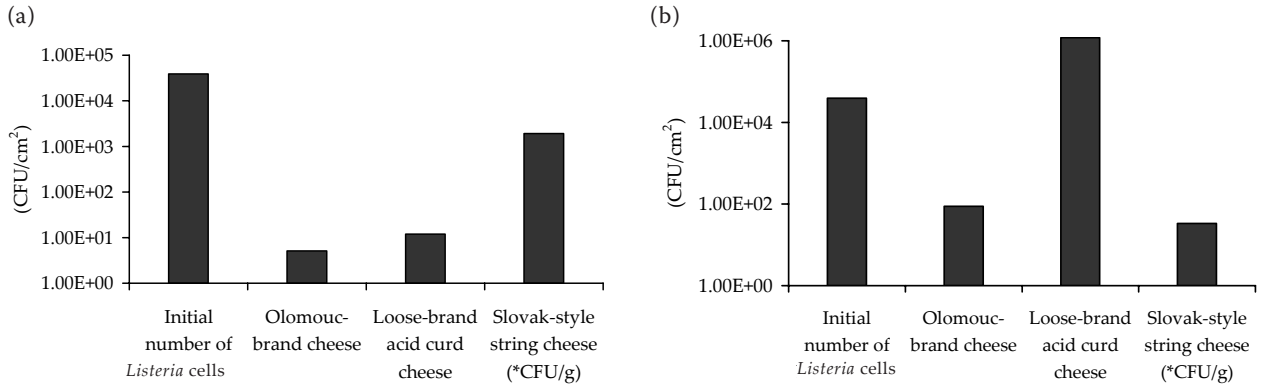

Figure 4. The count of the *L. innocua* in the all of the using cheese after 14 days in storage at the refrigerated temperature (a) and at 20°C (b) – cheese was treated with the preservative material only after the contamination with *Listeria*

Listeria count decreased to 10^2 CFU/g, and over 3 months it was reduced to 10 CFU/g.

Comparing the effectiveness of the preservative material on individual types of cheese

From Figure 4, the results can be compared as obtained with the individual types of cheeses that had been inoculated with *Listeria* before spraying with the preservative material. From Figure 5, the results can be compared concerning the number of *Listeria* in the individual cheeses that were inoculated with *Listeria* after spraying with the preservative material.

At the refrigerated temperature, HOLDBAC™ worked after 14 days of storage with all specimens. The *Listeria* count was reduced in Olomouc-brand and Loose-brand cheeses by an order of 4. In the case of the string cheese, the reduction in *Listeria* was by an order of 1 (Figure 4a).

HOLDBAC™ worked most effectively on the Slovak-style string cheese, where the number of viable *Listeria* cells was reduced by an order of 2. In the case of the Olomouc-brand soft ripened cheese, the *Listeria* count was reduced by an order of 1. For artificially contaminated Loose-brand acid curd cheese, there was no inhibition of viable *Listeria* cells. On the contrary, the *Listeria* count increased (Figure 4b).

It is apparent from Figure 5a that at the refrigerated temperature, the preservative material worked with all the specimens inoculated with *Listeria* after the treatment. After 14 days of storage, the *Listeria* count in all specimens except Slovak-style string cheese was reduced by an order of 3. HOLDBAC™ performed best on the Olomouc soft ripened cheese.

Figure 5b shows the results obtained after 14 days of storage at 20°C and in the case that the specimens were inoculated with *Listeria* cells only after spraying with the preservative material. The preservative material worked at the best with the string cheese,

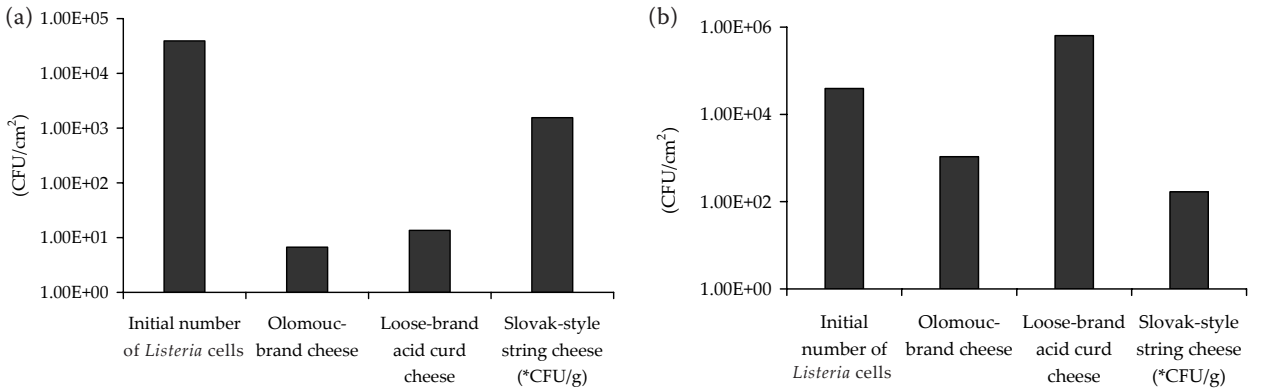

Figure 5. The count of the *L. innocua* in the all of the using cheese after 14 days in storage at the refrigerated temperature (a) and 20°C (b) – cheese was treated with the preservative material only before the contamination with *Listeria*

where a reduction occurred in the *Listeria* count by an order of 3 but complete inhibition of *Listeria* was not observed. In the case of Olomouc-brand cheese, the number of viable *Listeria* cells decreased by an order of 2. With Loose-brand cheese, the preservative material was not effective and there occurred an increase by an order of 2 as compared to the original concentration.

In examining the number of *Listeria* in the reverse approach wherein the *Listeria* contamination was performed only after the HOLDBAC™ had been applied, no meaningful differences were observed. Viewed graphically, the progressive relationship between the *Listeria* count and the days of storage at both temperatures examined was nearly identical, but somewhat better results were obtained if the specimens were first treated with the preservative material HOLDBAC™, then inoculated with the *Listeria* suspension and stored at the refrigerated temperature. It was with this method that the lowest count of viable *Listeria* cells was achieved.

CONCLUSION

The method of applying the preservative material did not demonstrate any substantial influence on its effectiveness. The preservative material worked effectively in reducing the number of *Listeria* cells with all cheese types stored at the refrigerated temperature. The lowest *Listeria* count among the cheeses stored at the refrigerated temperature (reduction by an order of 4) was found in Olomouc-brand cheese, while at 20°C it was in Slovak-style string cheese (with inoculation after the treatment). In view of the fact that complete inhibition of *Listeria* was not observed, one can recommend to apply the material repeatedly or to apply the anti-*Listeria* culture together with the smear culture during the technological process. The findings obtained could be helpful in advising on measures for preventing the contamination with *Listeria* in a Hazard Analysis and Critical Control Points (HACCP) system for the production of soft cheeses.

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