Letter to the Editor


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In the July issue of the Journal of Food Protection, Muñoz et al. described the application of a bacteriocin-producing strain of Enterococcus faecalis to prevent the growth of Bacillus cereus in cheese. The bacteriocin, AS-48, also known as enterocin 4 (2), is active against a wide range of gram-positive microorganisms and had been applied previously to control undesirable microorganisms in milk (1). At first sight, it is not surprising that it would also inhibit B. cereus in cheese. However, a detailed evaluation of the presented data casts serious doubts on the reliability and significance of the results.

GROWTH OF B. CEREUS LWL1 IN MILK

The authors stated that the efficacy of the bacteriocin-producing strain against B. cereus was first examined in skim milk. B. cereus LWL1 was reported to have reached concentrations between $10^{10}$ and $10^{11}$ CFU/ml in the control experiment (pure culture without added enterococci). Because these concentrations were much higher than those ever reported for B. cereus in milk, we decided to repeat this experiment in our laboratory with the same strain of B. cereus, LWL1, which was kindly supplied by Dr. F. M. van Leusden (Microbiological Laboratory for Health Protection, National Institute of Public Health and the Environment, Bilthoven, The Netherlands). Using Trypticase soy agar and mannitol egg yolk polymyxine agar, we found that B. cereus LWL1 counts did not exceed $2 \times 10^8$ CFU/ml, the same order of magnitude as reported for other strains of B. cereus (4).

GROWTH OF B. CEREUS IN CHEESE

According to the authors, B. cereus LWL1 was able to grow well in the control cheese. They reported a substantial increase (ca. 2-log increase) between day 10 and day 15, even though the pH of the cheese was about normal for a nonfat hard cheese (around 5.0). However, B. cereus normally does not survive during storage of hard or semihard cheese (3, 5), and to our knowledge cheese has never been incriminated as a vehicle for B. cereus outbreaks, which makes this finding remarkable. We investigated whether this result could be due to unexpected properties of this particular strain by inoculating B. cereus LWL1 into skim milk acidified with lactic acid to a pH of 5.0 and incubating the mixture at 30°C. After 24 h, B. cereus was cultured using two different media (Trypticase soy agar and mannitol egg yolk polymyxine agar), but neither of these media produced colonies of B. cereus despite the relatively high inoculum concentration of ca. 1,000 CFU/ml. Repeated culture attempts after incubation for 48, 72, and 96 h produced the same result (<10 CFU/ml).

Assuming that the results obtained with this simple model system are representative for cheese, it appears that there is a discrepancy between our data and the results reported by Muñoz et al. We suspect that the difference is due to the method used to enumerate B. cereus in the cheese. According to the information provided in the “Materials and Methods” section, Muñoz et al. used Trypticase soy agar. However, this agar is not selective, and although it has a low fermentable carbohydrate content, it will probably also support some growth of the starter bacteria, the bacteriocin-producing enterococci, and in particular the nonstarter lactic acid bacteria.

Considering the lack of reliability of the analytical methods used by Muñoz et al., we do not agree with the conclusion that this study has demonstrated that the bacteriocin-producing E. faecalis strain A-48-32 could be useful as an adjunct culture for the control of B. cereus in cheese.

REFERENCES

Response

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The authors of the Letter to the Editor severely criticize some of the results presented by Muñoz et al. in the Journal of Food Protection (67:1517–1521) and presumably repeated some of our experiments. We are thankful for the authors’ renewed interest in our work, which they have repeated several times over the years. We gladly respond to their queries and explain several points.

Bacteriocin AS-48 (2, 3) is not known as enterocin 4 (6). There is an enterococcal strain (INIA 4) that, according to Joosten et al. (6), produces AS-48, a feature that we have never questioned although we have not corroborated. AS-48 was described for the first time in 1986 (3), and it does not seem proper to make a capricious change of name 10 years later. Giving different names to the same thing is puzzling, and trying to reinvent what has already been described may induce the wrong conclusions, as we have suggested (7).

GROWTH OF B. CEREUS LWL1 IN MILK

Joosten et al. state in their letter that counts of B. cereus LWL1 reported by Muñoz et al. in the July issue of the Journal of Food Protection seem too high. Therefore, Joosten et al. decided to repeat the experiment with the same strain, LWL1. Because this strain is available only from a particular research group, Joosten et al. did not have much time between July and the present time to acquire strain LWL1 and repeat conscientiously the experiments reported by Muñoz et al. Therefore, their arguments must be based on very preliminary results and should be reviewed with all necessary precautions.

Regarding the high density of B. cereus we found in cultures of skim milk, those results were obtained in two independent experiments. One explanation for the difference in the results mentioned by Joosten et al. in their letter may be the type of commercial milk they used. As an alternative, some nutrients may have sedimented out, if they used a large container for the work to which they refer; this may occur frequently in a dry powder formula such as powdered milk. However, this issue does not seem to be a question of great relevance to our report. Regardless of whether B. cereus reaches 10⁶ or 10¹⁰ CFU/ml in milk, viable counts were reduced to below detection limits when the bacteriocinogenic E. faecalis strain A-48-32 was present and bacteriocin AS-48 was being produced.

COUNTS OF STARTERS AND B. CEREUS IN CHEESE

The lower counts for starter bacteria and B. cereus detected in curd compared with whey probably are due to loss of inoculum during whey drainage. This was our initial assumption, and we were not concerned because this question is far from the main issue of the article, which is whether B. cereus can be inhibited by the bacteriocinogenic strain. In our experiments, milk was inoculated strictly as recommended by the manufacturer.

With respect to the assertion that Tryptase soy agar is not a selective culture medium, the morphology of B. cereus colonies in this medium is totally different from that of colonies of lactic acid bacteria, making the use of selective medium unnecessary (Fig. 1). Furthermore, microscopic examination of stained colonies always gave confirmatory results.

The way that Joosten et al. repeated the cheese experiments seems wrong, because skim milk acidified to pH 5.0 is a very simple model system that is very different from real cheese conditions. In our study, a pH value of approximately 5.0 was reached after 5 days of ripening at 12°C. In the experiment of Joosten et al., B. cereus was inoculated (at a much lower concentration of 10³ CFU/ml compared with the 10⁴ to 10⁵ CFU/ml we used) into precodified milk and incubated at 30°C for 24 h. Because of these differences in culture matrix (skim milk versus cheese), initial pH, lactic acid content, and time and temperature of incubation, only very optimistic people would compare both results and venture to use these data to question results obtained from a real cheese-making process. To our knowledge, this is the first time that precodified skim milk has been suggested as a cheese model. The cheese environment is much more complex than that of simple acidified milk, in which no ripening takes place. Because cheese-making experiments are expensive and time-consuming, researchers sometimes use cheese curd slurries as cheese models when controlled bacteriological conditions are needed. However, cheese slurries cannot entirely replace cheese in these trials (10).

Inactivation of B. cereus vegetative cells at pH below 6.15 is frequently attributed to pH reduction itself or lactic acid production and to the synergistic effects of these and other factors (such as competition for nutrients or changes in reduction potential) that were not clearly described by Joosten et al. Some authors have attributed inhibition of Bacillus mainly to undissociated lactic acid (1). Therefore, direct addition of lactic acid to milk (as Joosten et al. report) may simply have a killing effect on B. cereus.

B. CEREUS NORMALLY DOES NOT SURVIVE DURING STORAGE OF HARD OR SEMIHARD CHEESE

We were unable to obtain a copy of the article by Northolt (8) and therefore do not know what type of hard cheese they evaluated. However, examination of the article by Rukure and Bester (9) on Gouda cheese revealed that the manufacturing process is quite different because milk is inoculated with spores and subjected to slow pasteurization (63°C for 30 min). The extent of acid production in the curds is controlled by replacing part of the whey with the same amount of water. Brine salting is also different: 24 h for Gouda versus 5 h in our study.

The two references provided by Joosten et al. do not
seem to substantiate their assertion that *B. cereus* does not represent a risk in hard or semihard cheese based on the following.

(i) Milk and dairy products are among the foods reported most frequently as vehicles for this bacterium.

(ii) Pasteurization processes used in making this type of cheese kill only vegetative cells and not spores.

(iii) The number of *B. cereus* foodborne illness outbreaks is greatly underestimated, mainly because of the relatively short duration of illness and the likelihood that foodborne illness involving *B. cereus*-contaminated milk is limited to one or two cases within a family and thus is not identified as an outbreak (4, 5). In Spain, 2,007 of the 5,517 foodborne outbreaks reported in the period 1993 through 1998 were attributed to unknown or unidentified agents.

(iv) Cereulide produced by *B. cereus* may produce severe adverse effects, such as liver damage (11), that are very difficult to quantify from current outbreak statistics.

We conclude that the arguments presented by Joosten et al. in their Letter to the Editor lack the necessary sound scientific basis to compromise the main conclusions drawn by Muñoz et al. in the article on the control of *B. cereus* LWL1 by the bacteriocinogenic strain *E. faecalis* A-48-32.

**REFERENCES**


