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Short communication

Influence of carvacrol on growth and toxin production by *Bacillus cereus*

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Abstract

The natural antimicrobial compound carvacrol was investigated for its effect on diarrheal toxin production by *Bacillus cereus*. Carvacrol (0–0.06 mg/ml) reduced the viable count and the maximal specific growth rate (μ_{max}) of *B. cereus* in BHI broth. The total amount of protein was not affected by carvacrol. However, a sharp decrease (80%) in diarrheal toxin production was observed in the presence of 0.06 mg/ml carvacrol. Carvacrol also inhibited toxin production in soup, but approximately 50-fold higher concentrations were needed to achieve the same effect as in broth. From this study it can be concluded that carvacrol can be added to food products at doses below the MIC value, thereby reducing the risk of toxin production by *B. cereus* and increasing the safety of the products.

Keywords: Diarrheal enterotoxin; *B. cereus*; Carvacrol; Soup

1. Introduction

Bacillus cereus is a motile, spore forming, facultatively anaerobic, gram-positive rod. Some strains have the ability to grow at low temperatures and can be regarded as psychrotrophs with a minimal growth temperature of 4°C (Granum, 1997). In 1997, *B. cereus* was rated as the number one cause of food poisoning in The Netherlands. However, the number of cases reported is probably an underestimation as a consequence of the short duration and relative mildness of the illness (Duynhoven and Wit, 1997; Granum, 1997). *B. cereus* is associated with two kinds of foodborne illnesses: a diarrheal and an emetic type, caused by two distinct toxins (Johnson, 1984). Strains causing the diarrheal type outbreaks produce a labile enterotoxin, which is easily inactivated by heat, low pH and proteases. The diarrheal enterotoxin is produced in the exponential phase, but maximum toxin is found in the early stationary phase (Griffiths, 1990). The main symptoms of this illness are abdominal pain and diarrhea. The foods most implicated with *B. cereus* contamination are meat products, soups, vegetables, puddings, sauces, milk and other dairy products (Granum, 1997). A second

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illness, the emetic type, mainly causes vomiting due to the production of the heat stable emetic toxin. The products involved are mainly rice, pasta and noodles.

Several food preservation systems such as heating, refrigeration and addition of antimicrobial compounds can be used to reduce the risk of outbreaks of *B. cereus* food poisoning. A novel way to preserve foods is the use of plant essential oils. The antifungal and antibacterial effects of these volatile oils towards different microorganisms have been described in several studies (Knobloch et al., 1986; Thompson, 1990, 1996; Conner, 1993; Juven et al., 1994; Kim et al., 1995a,b; Sivropoulou et al., 1996; Ultee et al., 1998). An example of an antimicrobial compound present in the essential oil fraction of oreganum and thyme is carvacrol. Carvacrol has a characteristic pungent, warm odor and has a 'pizza-like' taste. In earlier studies, we demonstrated the bactericidal action of carvacrol towards *B. cereus*. In addition, we showed the interaction of this compound with the cytoplasmic membrane by changing its permeability for protons and potassium ions (Ultee et al., 1998, 1999). Since toxin is an essential factor in outbreaks of food poisoning, it is important to know the effect of antimicrobial compounds on toxin production. This is the first paper describing the effect of carvacrol on toxin production by *B. cereus* in both broth medium and mushroom soup.

2. Materials and methods

2.1. Bacterial strain and growth conditions

2.1.1. Pre-incubation

Bacillus cereus IFR-NL94-25 (obtained from the Institute of Food Research, Norwich, UK) was used in all experiments. Cells were grown in Brain Heart Infusion (BHI) medium (Oxoid) supplemented with 0.5% (w/v) glucose (initial pH 6.7). Cell cultures were maintained at −80°C in 15% glycerol as a cryoprotectant.

2.1.2. Growth in BHI

To determine growth of *B. cereus* in BHI, an overnight culture of *B. cereus* was incubated (17°C) in BHI, supplemented with 0 to 0.06 mg/ml carvacrol in a microtiterplate. The starting optical density (OD) at 660 nm (OD_{660}) (light path 1 cm) of the cell suspension was set at 0.02. The OD_{660} was measured at different time intervals until a constant reading was obtained. For toxin measurements, an overnight culture of *B. cereus* was diluted 200 times in fresh BHI + 0.5% glucose to which 0–0.06 mg/ml carvacrol was added. Toxin production was determined after incubation for 24 h at 17°C.

2.1.3. Growth in soup

Different (sterile) soups were bought at a local supermarket. Mushroom soup, goulash soup, vegetable soup and chicken soup (Uno® soup, Unox®, Rotterdam, The Netherlands) were ready to eat. Bouillon (Libra, IJsselstein, The Netherlands) and chicken bouillon (Knorr Best Foods Benelux®, Hilversum, The Netherlands) were prepared by dissolving one tablet in 500 ml water and heating until the tablet was completely suspended. The soups were cooled to room temperature (when necessary) and 100 μl vegetative cells of an overnight culture of *B. cereus* was added. The soups were incubated for 24 h at 17°C. Because best growth of the organism was observed in mushroom soup, carvacrol (0–3 mg/ml) was added to 50 ml of this soup. Vegetative cells of *B. cereus* were washed in water, and diluted to an OD_{660} (light path 1 cm) of 0.025. The soup was inoculated with a 250 μl cell suspension (130×10^2 cfu/ml) and incubated for 5 days at 17°C.

2.2. Determination of viable counts

Viable counts were determined by plate counting. Samples of 1 ml were taken and directly diluted in peptone physiological salt (1 g/l peptone and 8.5 g/l NaCl). Serial dilutions were plated on BHI agar plates (BHI samples) (Oxoid) or *Bacillus cereus* selective agar (soup samples) (Oxoid) and incubated for 24 h at 30 and 37°C, respectively, following the instructions provided by the manufacturer. The *Bacillus cereus* selective agar plates were incubated a further 24 h at room temperature before they were enumerated.

2.3. Determination of toxin production

Analysis of the excreted toxin production was carried out using an enzyme linked immunosorbent assay (ELISA) developed by Tecra (Roseville, NSW, Australia) for the detection of *B. cereus* diarrheal

enterotoxin. Measurement of toxin production was performed as specified by the instructions provided by the manufacturer. Toxin quantities were defined as extinction at 405 nm. An uninoculated sample was used as a negative control; an enterotoxin positive control was provided with the kit.

2.4. Protein determination

To determine the amount of cell protein, the sample was centrifuged, washed and protein analysis was carried out according to Lowry et al. (1951) using bovine serum albumin as a standard.

2.5. Chemicals

Purified carvacrol was obtained from Fluka Chemie AG (Buchs, Switzerland). A stock solution (1 M) was prepared in 95% ethanol. The final ethanol concentration in the experiments was always kept below 2% ethanol (v/v).

3. Results

3.1. Toxin production in BHI

To investigate the effects of carvacrol on diarrheal enterotoxin production, cells were incubated in BHI in the presence of 0 to 0.06 mg/ml carvacrol. Toxin production was measured after 24 h incubation (17°C). Toxin quantities were determined as a function of the carvacrol concentration and related to the amount of cell protein. An increase of the carvacrol concentration from 0 to 0.015 mg/ml reduced the specific toxin production from 100 to 79 a.u./mg cell protein (Fig. 1A). A further increase of carvacrol to 0.06 mg/ml resulted in a reduction of the specific toxin production to 24 a.u./mg. Interest-

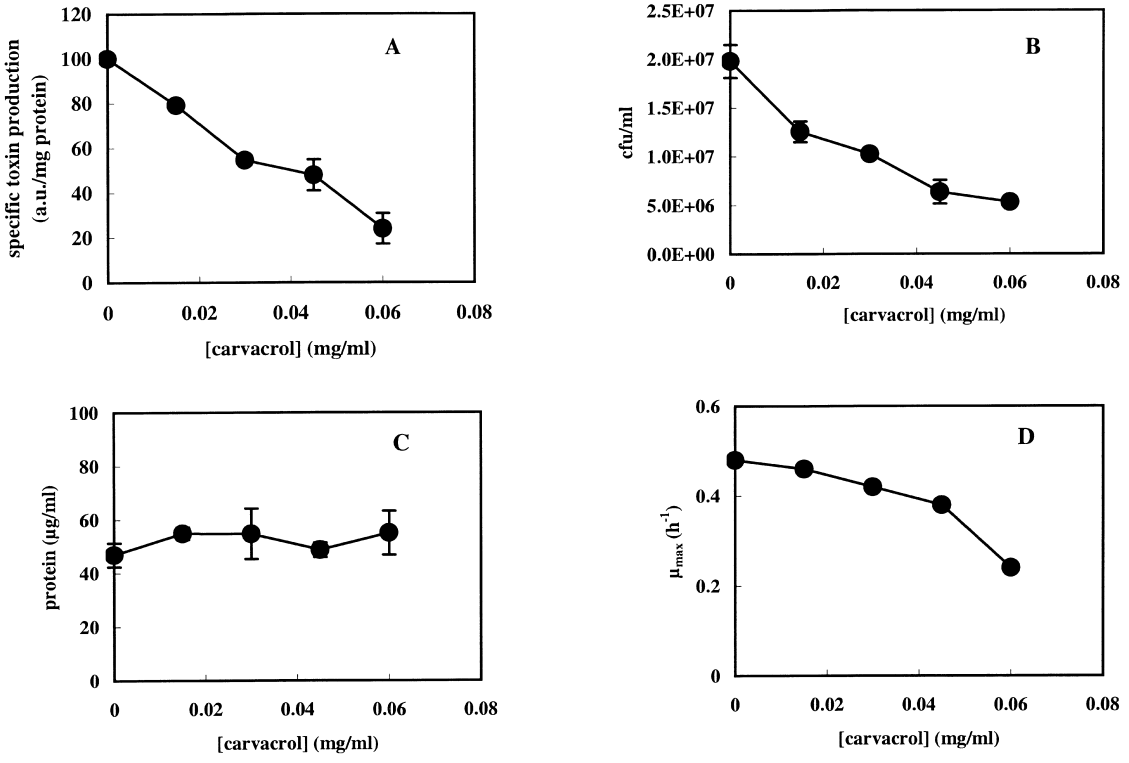

Fig. 1. The effect of carvacrol on different parameters of *B. cereus* after 24 h growth in Brain Heart Infusion (BHI) (17°C). (A) Specific toxin production (a.u./mg cell protein) (a.u., arbitrary units). (B) Viable counts of *B. cereus* (cfu/ml). (C) Total amount of protein in the culture (µg/ml). (D) The maximal specific growth rate (h^{-1}). Data represent mean values of duplicate measurements and error bars are indicated.

ingly, viable counts of the cultures were reduced in the presence of 0.06 mg/ml carvacrol from 2×10^7 to 5.3×10^6 cfu/ml (Fig. 1B), whereas no significant differences in total amount of cell protein were found in the cultures (Fig. 1C). The addition of 0.06 mg/ml carvacrol reduced μ_{max} from 0.48 h^{-1} (in the absence of carvacrol) to 0.24 h^{-1}.

3.2. Growth in soup

To find a suitable product to measure toxin production by *B. cereus*, different soups were inoculated with vegetative cells (17°C). Samples were taken after 24 h incubation, diluted and plated on *B. cereus* selective agar plates (Fig. 2A). There were no significant differences in the viable counts at the start of the experiment (2.5×10^5 cfu/ml). Growth occurred in all soups, except vegetable and goulash soup. Viable counts in these two soups were reduced to levels below the detection limit (10^3 cfu/ml) after 24 h. The highest viable counts were observed in mushroom soup. To determine if the pH of the soups played a role in the capacity of *B. cereus* to grow, pH values were measured (Fig. 2B). No growth and even a decline of the viable counts occurred in soups which had an initial pH below 5. In chicken bouillon, no pH decrease was observed. The pH values after 24 h in bouillon, chicken soup and mushroom soup were all around 5. These results indicate that no growth occurred below pH 5.

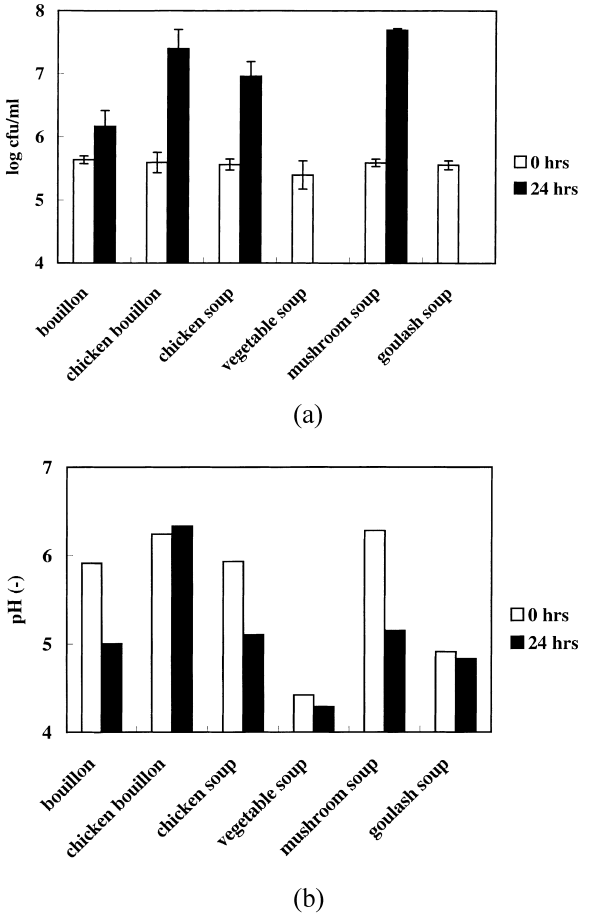

Fig. 2. Viable counts (A) of *B. cereus* and pH values (B) in different soups after 0 or 24 h incubation at 17°C. Data represent mean values of triplicate measurements. Error bars are indicated.

3.3. Toxin production in soup

B. cereus was inoculated into mushroom soup in the presence of 0 to 3.0 mg/ml carvacrol. The addition of 0 to 2.0 mg/ml did not significantly affect the viable count of *B. cereus* after 5 days of incubation (Fig. 3). The addition of 3.0 mg/ml carvacrol reduced the viable count by one order of

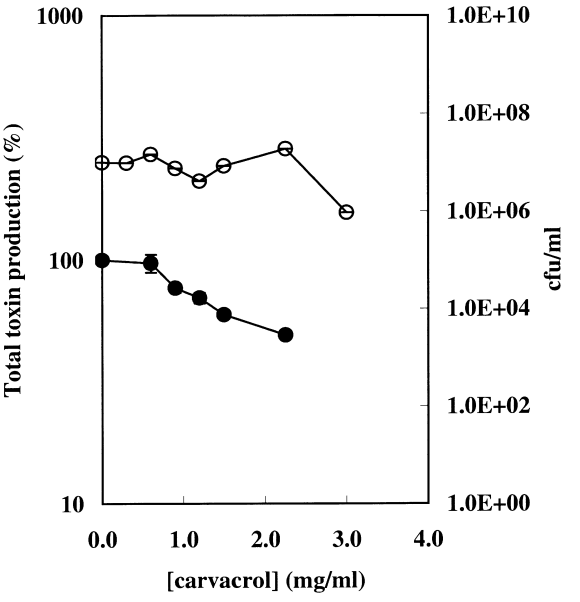

Fig. 3. Influence of carvacrol on total toxin production (●) by *B. cereus* in mushroom soup after 5 days of incubation (17°C) as a percentage of the control (no carvacrol added (= 100%)) and on the viable count (○). Data represent mean values of duplicate measurements and error bars are indicated.

magnitude. No toxin was found in all samples after 2 days of incubation, however after 5 days of incubation, toxin could be detected (17°C). The presence of 0.5 mg/ml carvacrol did not result in a significant decrease in total toxin production (Fig. 3). However, at carvacrol levels of 0.9 mg/ml and above, toxin production decreased progressively. At 3.0 mg/ml, the amount of toxin in the sample was below the detection threshold. Thus, carvacrol inhibited toxin production in mushroom soup at concentrations which did not have a significant effect on the viable counts of *B. cereus*.

4. Discussion

The main problem of contamination of food products with *B. cereus* is toxin production by this pathogen. It was of great interest to determine if the toxin production could be inhibited at concentrations at which growth of *B. cereus* could still occur. This study evaluated the influence of carvacrol on toxin production by *B. cereus* at 17°C, and found that carvacrol reduced diarrheal toxin production by *B. cereus* in a broth medium. The total amount of biomass, expressed as cellular protein, remained unaffected by carvacrol, while the viable count was reduced. Most likely, this observation can be explained by the lysis of carvacrol treated cells in the stationary phase. Since production of diarrheal enterotoxin by *B. cereus* starts in the exponential phase and maximum toxin levels are reached in the early stationary phase, maximum amounts of toxin are expected to be produced after 24 h of incubation in all cultures. However, events occurring in the late stationary phase, such as cell lysis, could affect the final toxin levels by release of proteases, degrading the protease-sensitive enterotoxin.

Paster et al. (1988) showed a reduction of aflatoxin B1 production by *o*-coumaric acid and caffeic acid at concentrations which did not influence the final yield in mycelial dry weight of *Aspergillus niger*. A comparable effect was described by Bullerman (1974). Cinnamon reduced aflatoxin production by *Aspergillus parasiticus* more than final mycelial weight. Tassou and Nychas (1994) observed a similar effect when a phenolic extract of olives was added to a culture of *Staphylococcus aureus*. In all these studies, toxin production was more affected than growth yield. However, Buchanan and Shepherd (1981) showed that this is not valid for all (antimicrobial) compounds. They observed that thymol inhibited aflatoxin production by *Aspergillus parasiticus* to a smaller extent than growth of the fungus (mycelium wet weight). It seems that this phenomenon is dependent on the organism and antimicrobial compound.

Growth of *B. cereus* was observed in different soups, depending on the pH of the medium. No growth occurred in soups when the pH was below approximately 5. This phenomenon has been described earlier (Sutherland and Limond, 1993; Setlow and Johnson, 1997). However, the lack of essential nutrients could also play a role. While good growth was observed in mushroom soup, toxin production was detected in this medium at carvacrol concentrations up to 2.0 mg/ml. In contrast to growth in BHI, carvacrol inhibited toxin production in mushroom soup at concentrations which did not significantly influence the viable count. Our observation that 50 times more carvacrol is needed to have the same effect in soup as in BHI agrees with former observations in rice (Ultee et al., 2000) and shows that carvacrol is less effective in a food matrix, most likely as a result of interaction with components in the product.

The mechanism of inhibition of toxin production by carvacrol in the cell is still unclear. It could be at the level of gene regulation, transcription or translation, or by carvacrol affecting transport and excretion of the toxin. The diarrheal toxin is produced in the cell and excreted. Excretion may be an active process and therefore energy dependent. Carvacrol makes the cell membrane permeable for K^+ and H^+ and, consequently, inhibits ATP synthesis by dissipating the proton motive force (Ultee et al., 1999). Based on this, we hypothesize that, during exposure to carvacrol, the driving force for optimal secretion of the toxin (ATP or the proton motive force) is not sufficient, resulting in accumulation of the toxin inside the cell. Consequently, intracellular toxin might inhibit its own synthesis (feedback inhibition). However, it is also possible that, as a result of a lower specific growth rate, toxin synthesis is directly inhibited. Production of the toxin requires metabolic energy and, in the presence of carvacrol, the cell uses the limited amount of metabolic energy for maintaining its viability and not for toxin production. The

results of *B. cereus* growth in soup support this hypothesis. Further studies, where the intracellular toxin concentration is examined during exposure to carvacrol, could give more insight into the mechanism(s).

In conclusion, carvacrol inhibited toxin production by *B. cereus* in both BHI and mushroom soup. This interesting observation can be exploited for food preservation purposes. Carvacrol can be added to food products at doses below the MIC value, thereby reducing the risk of toxin production by *B. cereus* and increasing the safety of the products. At the same time, low doses of carvacrol do not affect the flavor and taste of the products.

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