Note

The Effect of Combined Treatment with Carbonation, Heating, and Monoglycerol Fatty Acid Esters on the Inactivation and Growth Inhibition of *Geobacillus stearothermophilus* Spores

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Received September 12, 2013 ; Accepted December 1, 2013

The effect of carbonation with heating (CH) on the number of viable *Geobacillus stearothermophilus* spores was investigated in the presence of monoglycerol-caprate (MC₁₀) or -laurate (MC₁₂). CH was performed at 90 and 100°C, 5 MPa, for 30 or 60 min. Spore counts were reduced by 1.4 log CFU/ml after CH at 90°C for 60 min in the presence of MC₁₀. On the other hand, after CH at 100°C for 60 min, spore counts decreased by 3 – 4 log CFU/mL or remained low after 7 days of storage. CH decreased the optical density of a spore suspension at 650 nm more than heat treatment alone, indicating that spore germination triggered by CH could be involved in the drastic decrease in spore count caused by CH in the presence of MC₁₀ or MC₁₂.

Keywords; carbonation, monoglycerol fatty acid ester, *G. stearothermophilus* spore

Introduction

Carbonation treatment is performed by solubilizing carbon dioxide (CO₂) into liquid foods under pressure. This is one of the most promising techniques for cold pasteurization and/or sterilization of liquid and solid materials, and is likely to replace or partially substitute currently and widely applied thermal processes (Spilimbergo and Bertucco, 2003). Over the past decades, many reports have demonstrated the substantial inactivation effect of carbonation under mild treatment conditions against various microorganisms in vegetative form, such as *Escherichia coli* (Dillow *et al.*, 1999), *Listeria monocytogenes* (Wei *et al.*, 1991), and *Lactobacillus plantarum* (Hong and Pyun., 1999). However, spores of various *Bacillus* species are metabolically dormant and are thus highly resistant to environmental stress factors (Ghosh *et al.*, 2008). Therefore, it is difficult to inactivate spores by

carbonation treatment alone without deteriorating the quality of the food.

Fatty acid esters (FAEs), which are used widely as food additives, are known to effectively inhibit the growth of various bacteria both in vegetative and spore forms (Shibasaki, 1979). Medium chain length monoglycerol FAEs, and especially monolaurin, show an antibacterial effect against bacterial spores (Kimsey *et al.*, 1981). We previously demonstrated that the presence of monoglycerol monocaprate (MC₁₀) and monoglycerol monolaurate (MC₁₂) substantially decreased the viable counts of *B. subtilis* spores after carbonation with heating (CH) (80°C, 5 MPa, 30 min) and prevented their growth during 30 days of storage (Klangpetch *et al.*, 2013). We also revealed the significant inactivation effect of combined treatment of CH with MC₁₀ or MC₁₂ on *B. cereus* and *B. coagulans*. In contrast, *Geobacillus*

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stearothermophilus spores were not affected by combined treatment with CH (80°C, 5 MPa, 30 min) and MC₁₀ or MC₁₂. The fact that *G. stearothermophilus* is known as one of the most heat resistant spores amongst aerobic microorganisms suggested that these conditions for heat treatment during CH were insufficient to substantially inactivate highly heat resistant spores. In this study, the combined effects of CH with MC₁₀ or MC₁₂ on *G. stearothermophilus* spores at higher temperatures (90 and 100°C) and for longer times (30 and 60 min) were studied.

Materials and Methods

Preparation of FAE solution The two types of FAE, MC₁₀ and MC₁₂, were kindly provided by Taiyo Kagaku Co. (Mie, Japan). Their average molecular weights are 246 and 274, and their hydrophile/lipophile balances are 6.5 and 5.3, respectively. They were dissolved in sterile water to a concentration of 0.5 % (w/v).

Bacteria and preparation of spore suspension *Geobacillus stearothermophilus* JCM 2501 was obtained from RIKEN BRC-JCM (Saitama, Japan). The spores were formed on nutrient agar (NA; Difco Inc., Detroit, USA) containing 0.1 mM MnSO₄ at 55°C for about 10 days. After more than 90% of the population appeared as reflective spores under phase contrast microscopy (BX 50, Olympus Co., Tokyo, Japan), the spores were harvested and washed three times by centrifugation at 7,000 × g at 4°C for 10 min in sterile water. The spore suspension was heated at 90°C for 30 min in order to inactivate the vegetative cells, then the suspension was lyophilized and stored at -20°C until use.

CH and heat treatment (HT) Lyophilized spores of *G. stearothermophilus* were suspended in nutrient broth (NB; Difco Inc., Detroit, USA) at an optical density of 1.5 at 650 nm. After heating the spore suspension at 90°C for 30 min, it was cooled immediately and diluted with NB to yield a final spore concentration of about 10⁵ CFU/mL. The spore suspension (2.7 mL) was mixed with 0.3 mL of the FAE solution described above, giving a final FAE concentration of 0.05%. The pH of the spore suspensions was 7.0 without FAE and 6.6–6.8 with FAE. The spore suspensions with and without each FAE were subjected to CH and HT.

CH and HT were conducted using a method similar to that reported previously (Klangpetch *et al.*, 2013). In this study, an oil

bath (OBS-200AM, AS ONE Co., Osaka, Japan) was used to control the processing temperature. CH was performed at 90 or 100°C and at a pressure of 5 MPa for 30 or 60 min. HT was performed under atmospheric conditions at the same temperature and for the same duration as CH. The spore suspensions subjected to CH and HT were immediately cooled by immersion in ice-cooled water.

Determination of viable counts After CH or HT, viable counts were determined as described previously (Klangpetch *et al.*, 2013). To study the changes in spore count during storage, each spore suspension was stored at 55°C in sterile plastic tubes. After 1, 3, and 7 days, viable counts were enumerated.

Measurement of optical density The optical densities of the spore suspensions (about 10⁷ CFU/mL) were measured at 650 nm (OD₆₅₀) using a spectrophotometer (UV-1700, Shimadzu, Kyoto, Japan) before and just after CH or HT to evaluate spore germination.

Statistical analysis Significant differences were determined by the Student's or Welch's *t*-test (Microsoft Excel, Microsoft Corporation, Redmond, USA) after the *f*-test to ensure the equality of variance (*p* < 0.01). Fisher's least significant difference was employed to determine statistically significant differences in OD₆₅₀ among the treated samples (*p* < 0.05).

Results

Combined effect of CH with MC₁₀ or MC₁₂ on the viability of *G. stearothermophilus* spores Fig. 1 shows the viable counts of *G. stearothermophilus* spores after CH (90°C, 5 MPa, 30 or 60 min) with and without MC₁₀ or MC₁₂ at a concentration of 0.05% (w/v). Without FAEs, spore counts were not affected by HT or CH (0-day storage) and increased by about 2 log CFU/mL after 1 day of storage. After that, spore counts in all samples decreased to almost the same level as that on day 0. The reason for this is not clear. One possible explanation for this decrease is that spore vegetative growth after outgrowth reached stationary phase, and a part of the culture was died. In the presence of MC₁₀ or MC₁₂, growth of both untreated and HT- or CH- treated spores was inhibited during 7 days of storage. Although CH for 60 min decreased spore counts by 1.4 log CFU/mL in the presence of MC₁₀, under the other treatment conditions, neither HT nor CH had any effect on spore

Fig. 1. Changes in viable counts of *G. stearothermophilus* during 7 days of storage. Spore counts at day 0 were obtained immediately following treatment.

Fig. 2. Changes in viable counts of *G. stearothermophilus* during 7 days of storage. Spore counts at day 0 were obtained immediately following treatment.

counts. As a result, CH at 90°C did not effectively induce inactivation.

At 100°C, spore counts were also not affected by HT or CH without MC₁₀ or MC₁₂ (Fig. 2). However, spore counts decreased significantly after CH for 60 min in the presence of MC₁₀ or MC₁₂ ($p < 0.01$), while HT caused no reduction in viable counts. In the presence of MC₁₀, CH for 30 or 60 min induced a 1.6 and 3.9-log reduction, respectively. In the presence of MC₁₂, CH for 60 min induced a 3.6-log reduction, while CH for 30 min did not induce any reduction. During storage after CH for 60 min, MC₁₀ or MC₁₂ inhibited spore outgrowth and/or vegetative growth, and kept spore counts below 2 log CFU/mL during 7 days of storage.

Germination of *G. stearothermophilus* spores by CH Germination of *G. stearothermophilus* spores by CH or HT was investigated by measuring the OD₆₅₀ of the spore suspensions (Fig. 3). The decrease in OD₆₅₀ after CH (90°C, 60 min and 100°C, 60 min) was significantly greater than that after HT at the corresponding treatment temperature and time for each CH ($p < 0.05$). This result indicates that CH induces spore germination. Additionally, increasing the CH temperature from 90 to 100°C and the treatment time from 30 to 60 min tended to enhance the decrease in OD₆₅₀, indicating spore germination.

Discussion

In our previous study, CH (80°C, 5 MPa, 30 min) in the

presence of MC₁₀ or MC₁₂ decreased the number of viable spores of *B. subtilis* by 3.9 and 2.9 log CFU/mL, respectively, and inhibited spore outgrowth and/or vegetative growth during storage for 30 days, while CH or FAEs alone showed no effect on spore counts (Klangpetch *et al.*, 2013). Monoglycerol FAEs had a more pronounced combined effect with CH than did di- and polyglycerol FAEs, consistent with the primary bacteriostatic effect of FAEs reported earlier (Kato and Shibasaki, 1975; Nakayama *et al.*, 2003). A previous study also revealed greater susceptibility of *B. cereus* to CH and FAEs compared to *B. subtilis* (Klangpetch *et al.*, 2013). The heat-resistant spores of *B. coagulans* were substantially inactivated by combined treatment with CH and FAEs, although the spore counts increased after 7 days of storage. In contrast with these *Bacillus* spores, *G. stearothermophilus* spores were not affected by combined treatment with CH (80°C, 5 MPa, 30 min) and FAEs. The spore of *G. stearothermophilus* is the most heat-tolerant of all aerobic spore-forming bacteria, and is often used as a biological indicator to evaluate sterilization processes because of its high heat resistance (Furukawa *et al.*, 2000). It has been reported that high temperature and long treatment times with CH were needed to cause a 5-log reduction of *G. stearothermophilus* viable spore count (95°C, 30 MPa, 120 min) compared to the same reduction of *B. cereus* spores (50°C, 30 MPa, 60 min) (Ishikawa *et al.*, 1997). These results suggest that more severe treatment conditions are needed for obtaining the combined effect of CH and

Fig. 3. Effects of HT and CH on the optical density of spore suspensions at 650 nm (OD₆₅₀). Data represent the mean of four determinations, and error bars indicate the standard deviation of the mean. The same characters represent no significant difference ($p > 0.05$), and different characters represent a significant difference ($p < 0.05$) in Fisher's LSD among treated samples.

FAEs with *G. stearothermophilus* because of their extremely high heat resistance.

The spore counts of *G. stearothermophilus* were substantially decreased by CH at 100°C for 60 min in the presence of MC₁₀ or MC₁₂ (Fig. 2). Significant inactivation caused by high temperatures and long treatment times indicates the important contribution of heat to the combined effect of CH with FAEs. Kato and Shibasaki (1975) revealed that MC₁₀ and MC₁₂ improve the inactivation effect of HT at 50°C for 5 min by about 3 log CFU/mL on *E. coli* and *Pseudomonas aeruginosa*. Fujimoto *et al.* (2006) found that the combined use of FAEs and ultrahigh temperature (UHT; 125°C, 10 s) served as a substitute for conventional UHT treatment at a higher temperature (135°C, 30 s) against *G. stearothermophilus* spores in chicken extract. On the other hand, while the combined effect of CH with FAEs was much more prominent after CH at 100°C for 60 min, the effect was much decreased at milder heat conditions (Fig. 1, 2). Consistent with these results, we previously revealed that the combined effect of CH with FAEs was prominent only above 80°C on *B. subtilis* spores (Klangpetch *et al.*, 2013). It has also been reported that carbonation treatment at 30 MPa decreased spore counts of *G. stearothermophilus* drastically at 95°C, although inactivation was not induced by temperatures between 35 to 85°C (Watanabe *et al.*, 2003). These results indicate that there is a threshold of heat impact for each kind of spore, suggesting that heating above a certain threshold temperature with CH and FAEs could enhance the inactivation effect. Stecchini *et al.* (2006) suggested that the glassy state of the cortex could be associated with the heat resistance of spores. In that study, a reversible heat capacity shift ascribable to the transformation of cortex from a rigid, glass-like amorphous solid to a visco-elastic rubber glassy state was observed at 90–115°C with *B. subtilis* spores by differential scanning calorimetry. This result indicates that structural changes in the cortex may occur at 90–100°C in *G. stearothermophilus* spores, resulting in increased sensitivity to FAEs. Additionally, a prominent combined effect of CH upon longer treatment (60 min) indicates the importance of treatment time. Increased duration of CH treatment could also help improve the combined effect with FAEs on spores. However, to date there have been few studies on the effect of treatment time on spore inactivation.

The combined effect of CH with FAE against *G. stearothermophilus* spores was larger with MC₁₀ than with MC₁₂ (Fig. 2), although both MC₁₀ and MC₁₂ completely inhibited proliferation of *G. stearothermophilus*. These results are consistent with those reported earlier for *B. subtilis* spores (Klangpetch *et al.*, 2013). The primary bacteriostatic effects of FAEs likely reflect the intensity of the combined effects with CH, although conclusive proof will require detailed comparison of the bacteriostatic effect between MC₁₀ and MC₁₂ in NB.

It is possible that pressurized CO₂ enhanced the bacteriostatic effect of FAEs against spores. The decrease in OD₆₅₀ of *G.*

stearothermophilus spore suspensions after CH was significantly greater than after HT at the same temperature and for the same duration (Fig. 3). Therefore, CH could induce more spore germination than HT, thereby enhancing the bacteriostatic effect of FAEs against germinated spores and causing significant inactivation. It has been reported that spore germination is initiated by carbonation (35°C, 6.5 MPa, 30 min) despite almost no initiation of germination by hydrostatic pressure under the same conditions with *B. coagulans* (Furukawa *et al.*, 2004). The authors described that carbonation might initiate spore germination due to lytic germination enzymes, which are known to be initiated by hydrostatic pressure; however, lytic enzymes could not contribute to carbonation-germination due to the low pH used during treatment. One explanation of the effect of CH on bacterial spores is that the protons generated during CH neutralize the negative charge on the cortex layer, inducing germination (Noma *et al.*, 2011). Germinated spores might be more permeable to various substances, indicating the possibility that CH-induced germination could contribute to the combined effect of CH with FAEs. Spore germination induced by CH could increase permeability towards FAEs. Additionally, CH was shown to increase the hydrophobicity of spores (Furukawa *et al.*, 2006). MC₁₀ and MC₁₂ have a low hydrophile-lipophile balance of 6.5 and 5.3, respectively. Therefore, CH may enhance the adhesion of MC₁₀ and MC₁₂ to the spore surface. Another possibility is that the CO₂ molecules saturating the spore suspension under pressure act as a carrier of FAEs to the spore surface. These possibilities lead to the consideration that the hydrophobicity of FAEs and of spore surface play a role in enhancing the combined effects of CH with FAEs.

Conclusion

Severe CH conditions are required (95°C, 30 MPa, 120 min) to inactivate one of the most heat resistant spores, *G. stearothermophilus* (Watanabe *et al.*, 2003). In this study, the use of MC₁₀ or MC₁₂ reduced the required CH pressure and treatment time to 5 MPa and 60 min at 100°C. This result could help solve problems caused by the harsh conditions used to inactivate microorganisms in food, such as loss of food quality. For this approach to be put to practical use, further studies are needed to elucidate the mechanisms of spore inactivation by combined treatment with CH and FAEs.

Acknowledgements The authors would like to thank Taiyo Kagaku Co., Ltd., Mie, Japan, for kindly providing MC₁₀ and MC₁₂.

Part of this research was supported by the Towa Foundation for Food Research.

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