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Original Article

Influence of Heat Shock Temperatures and Fast Freezing on Viability of Probiotic Sporeformers and the Issue of Spore Plate Count Versus True Numbers

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ABSTRACT

Background and Objectives: The purpose of the present study was to investigate effects of various heat shock conditions and fast freezing and subsequent thawing on the viability and recovery of *Bacillus coagulans* and *Bacillus subtilis* as probiotic sporeformers, and also to compare spore plate and microscopic counts.

Materials and Methods: After preparing the final suspensions of *B. coagulans* and *Bacillus subtilis subsp. Natto* spores, they were spread-plated before and after fast freezing treatment (-70°C for about 1 min). Heat shock treatments of the spores were carried out at 68°C for 15, 20, and 30 min as well as at 80°C for 10 and 15 min. Concentrations of the examined probiotic sporeformers were determined simultaneously by plate enumerations and microscopically determined counts. Student's t-test and one-way analysis of variance (ANOVA) of SPSS were used for statistical analysis of the data. Analysis of DoE results was carried out using Minitab.

Results: The results presented here show that the highest recovery rates for *B. coagulans* (14.75 log CFU/mL) and *B. subtilis* spores (14.80 log CFU/mL) were under a heat shock condition of 68°C for 20 min in nutrient agar ($p<0.05$). In addition, the survival rates of *B. coagulans* and *B. subtilis* spores under the fast freezing and subsequent thawing condition were about 90% and 88%, respectively. Plate counts differed significantly from counts determined microscopically, with differences of almost 0.5 and 0.8 log for *B. coagulans* and *B. subtilis* spores, respectively ($p<0.05$). In addition, DoE results of the study revealed that both factors of spore count method and only freezing factor in fast freezing treatment have a significant effect on concentrations of the spores examined ($p<0.05$).

Conclusions: Heat shock conditions, freezing and subsequent thawing circumstances, and plate counts or enumerations determined microscopically have significant influences on the viability of probiotic sporeformers and should be considered in determining of their accurate concentrations.

Keywords: *B. coagulans*, *B. subtilis*, Freezing, Heat shock, Probiotic sporeformers

Introduction

Although the development and consumption of functional foods which provide health benefits in addition to nutrition as a growing, global consumer trend are on the rise, probiotic food products have captured a considerable percentage of functional food market (1, 2). The definition of the term *probiotic* has

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evolved throughout the years (3). The most recent definition says that probiotics are live microorganisms administered in amounts that positively affect the health of the host (human or animal) (3-5). Probiotics provide well-documented beneficial actions to human health including aid in lactose digestion, resistance to enteric pathogens, anti-colon cancer effect, small bowel bacterial overgrowth, allergy, and immune system modulation (1, 5-7). It is important to point out that organizations and factories interested in formulating products with probiotics are recognizing that probiotics must not only be supported by solid clinical data but for any probiotic to yield health benefits, the selected probiotic bacteria must survive the manufacturing process, the shelf life of the product and ultimately survive the passage through the digestive system to successfully colonize in the intestines (5, 8). Traditional probiotics (special species of *Lactobacillus* and *Bifidobacterium*) are very sensitive to normal physiological conditions such as the very low pH of the stomach and bile salts when consumed and their viability is affected by manufacturing methods as well as storage and shipping conditions (8-10). On the contrary, spore probiotics as novel probiotics have not the aforementioned disadvantages or limitations; i.e., they are able to resist the harsh manufacturing process and storage conditions, as well as acidity of the stomach and bile acids (11-15). Although probiotic application of spore-formers has not been established well in the world probiotic market, the use of spores of *Bacillus* species as probiotic dietary or food supplements is expanding rapidly with increasing number of studies demonstrating immune stimulation, antimicrobial activities and competitive exclusion and also few commercially available probiotic products containing spore-formers (16-18). Out of more than 100 *Bacillus* spp. known, only a few (including *B. coagulans* and *B. subtilis* subsp. *Natto*) have been most extensively examined and are being used as probiotics for human consumption (19-21). Since probiotics are live organisms, it is essential to enumerate accurately the population of viable microbes, especially fastidious ones or those having special nutritive requirements in food, and express this information to the consumer on the product label (19). Bacterial spores like probiotic sporeformers commonly require activation, which is usually accomplished by sublethal heating (heat shock) of water-suspended spores, to finish off dormancy and enable rapid germination to occur (22, 23). Indeed, conversion from the dormant spore to vegetative cell involves three steps: activation, germination, and outgrowth. Dormancy phase may be broken by heat treatment at a time and sublethal temperature appropriate to the organism concerned (24, 25). Further, there has been a revival of interest in validating and improving the procedure for enumeration of *Bacillus* spores by using different heat shock (or heat- activation) treatments (23, 26, 27). The heat shock step as an effective germinant has usually two functions: 1) activate the spores and so start or enhance of germination for all spores; 2) killing or destructing all kinds of vegetative cells in the sample, thereby to provide reliable counts of spores with no viability loss (24, 28, 29). Nevertheless, the amount of heat shock necessary to activate spores optimally varies greatly with the different organisms (30), the medium in which spores are suspended (23, 29, 31) and, if germinants are present, the particular germinants and their concentrations as well as the levels of at least phosphate, calcium and manganese ions present during sporulation (30, 32). Furthermore, it seems that the optimal temperature and time of heat shock vary from species to species and it is even very much strain dependent, though not all spores can be activated by heat. Instead, spores of some of strains germinate better if they are not heat activated (22, 25, 33). In other words, since heat shocking affects on variability and eliminates the background of non-spore formers on the non-selective plates, more consistent germination rates between different spore preparations would be achieved (29). To the best of our knowledge, this is the first study describing the viability and recovery of probiotic sporeformers during different sublethal heat-shock treatments. On the other hand, it is important to bear in mind that bacterial spores (especially Gram-positives like *Bacillus* species) are extremely resistant to a wide range of environmental stresses including freezing and storage at subzero temperatures (34). Freezing makes decline the numbers of viable spores by

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reducing only about 10% (34, 35). The rate of freezing, the medium composition in which freezing occurs, physiological adaptation and intrinsic differences among microorganisms in their susceptibility to damage from freezing are among the important factors to be considered (34, 36). The overall objectives of the present study were to determine the effectiveness of different heat shock treatments in recognition of accurate viable count of *Bacillus* probiotics and the influence of fast freezing and subsequent thawing on the recovery of the spores examined too. To our knowledge, no extensive study on evaluating heat shock temperature/time combinations for probiotic sporeformers and finding an optimum heat shock treatment yielding the greatest spore recovery has been published so far. So, we examined the degree of tolerance and recovery of *B. coagulans* and *B. subtilis* spores to various sublethal heat shock temperature/time combinations recommended among the most frequently cited in the literature (68^{o}C and 80^{o}C for different times). Effect of fast freezing and subsequent thawing on the recovery of studied spores and comparison between two common and routine enumeration methods (i.e. plate and direct microscopic counts of the examined spores) were also investigated. In addition, the single and interaction effects of freezing and count method treatments on the number (CFU/mL) of studied *Bacillus* probiotics were determined.

Materials and Methods

Materials and chemical reagents: All the chemical and reagents used for the study were of analytical grade and purchased from Merck (Darmstadt, Germany).

Bacterial spores and spore preparation: Commercial spore probiotic strains of *B. coagulans* and *Bacillus subtilis* subsp. *natto* were obtained from Natures Only, INC., USA and World Intellectual Resource Co., Taiwan, respectively. In order to assure enumeration and purity of the spores, they were separately cultured on Müller-Hinton agar (Merck, Germany). Gram staining and direct microscopic examination were then conducted after 7-10 days to approve whether or not sporulation was completed in the plates. Preparation and purification of the studied spores were done according to Alebouyeh et al. (37). The final suspensions of the studied spores were titrated (spores/mL) and then frozen in sterile deionized water at -70°C till use (for maximum 72 h). In addition, in order to determine the effect of fast freezing on the recovery of the *Bacillus* probiotics examined, they were spread-plated on Müller-Hinton agar (Merck, Germany) before and after the freezing treatment, followed by aerobically incubation for 2 days at 37^{o}C. The colonies were manually counted after 48 h of incubation and the numbers of the spores were calculated from duplicate plating.

Fast freezing and subsequent thawing: In order to determine effect of fast freezing and subsequent thawing on the viability of examined *Bacillus* probiotics, the spores were spread-plated on Müller-Hinton agar (Merck, Germany) before and after the fast freezing treatment and subsequent thawing, followed by aerobically incubation for 2 days at 37^{o}C. The colonies were manually counted after 48 h of incubation and the numbers of the spores were calculated from duplicate plating.

Heat shock procedure: For this part of the study, heat shock (or heat-activation) treatments of the spore probiotics (at 68^{o}C for 15, 20, and 30 min, and at 80^{o}C for 10, and 15 min) were selected on the basis of reports in literature (22, 23, 38). It is worth noting that that temperatures above 80^{o}C (especially 90^{o}C) have sporicidal effect (23, 39). In practice, the spore suspension sample was placed in a 68^{o}C and/or 80^{o}C water bath (Memmert, Germany) for the aforementioned times to stimulate germination of the spores and to kill vegetative cells. After heat treatment, the suspension was immediately cooled in a crushed ice water bath and then serially diluted in sterile 0.1% peptone water. 0.1 mL of each serially diluted sample was spread-plated using nutrient agar (Merck, Germany) as a general culture medium. Inoculated plates were incubated for 2 days at 37^{o}C, and then the colonies were manually counted. The number of spores was calculated from duplicate plating. In order to improve the situation to determine the counts of spores (i.e. to prevent an underestimation), the time between preparation of the primary dilutions and the heat shock step was

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considered less than 10 min according to the studies conducted by Te Giffel et al.(40).

Comparison of plate and microscopically determined counts: Viable counts of B. *coagulans* and *B. subtilis* spores were done by the spread plate method before freezing on Müller-Hinton agar plates (Merck, Germany), followed by incubation at 37°C for 48h. The plates were spread with 100 µl volumes of the relevant dilutions and spreading was done with 10 µl loops to avoid the losses inherent with conventional L-shaped spreaders (23). Generally, the 10^{-3} dilution was appropriate for the microscopic counts. For this purpose, the spores were suspended by pipetting up and down, and 10 µl was inserted under the coverslip of a hemocytometer. This was allowed to stand for a few minutes to allow the spores to settle and then placed under the 20× objective of a Nikon Eclipse 90i microscope. The numbers of spores in each of ten 250 μm^2 squares as seen needed through the eyepiece were recorded and the total numbers calculated accordingly (23).

Statistical analysis: All data are presented throughout as mean value with their standard deviation (mean ± SD) for each treatment. Colony-forming units (CFUs) in all experiments were converted to $\log_{10}$ values. All Experiments were replicated. Statistical analysis of the data was carried out using Student's t-test and one-way analysis of variance (ANOVA) of SPSS (Version 20, SPSS Inc, Chicago, IL, USA). The differences among the mean values were detected using Duncan's Multiple Range test at a significance level of $p \leq 0.05$. In order to determine the single and interaction effects of freezing parameters (type of spore probiotics: X_1 and before and after freezing: X_2) and count method factors (type of spore probiotics: X_1 and plate and microscopic count: X_3) on the response (CFU of spore probiotics examined), a full factorial design of experiments was used. All the analyses of DoE (Design of Experiment) were carried out using the statistical software, MINITAB 17 (Minitab Inc., State College, PA, USA).

Results

The results of heat shock/activation are presented in Fig.1. In this experiment, the viable counts of B. *coagulans* and *B. subtilis* spores were 14.34 and 14.67 log CFU/mL in a control suspension (without heat shock), respectively. In comparison to the controls, the spore counts of *B. coagulans* (14.75 log CFU/mL) and *B. subtilis* (14.80 log CFU/mL) were the greatest only after a heat shock at 68°C for 20 min ($p<0.05$). Moreover, it seems that viable spore counts of B. *coagulans* enumerated after other studied heat shock temperature/ time combinations, except the treatment of 80°C for 15 min, were found to be significantly different from the control ($p<0.05$). However, for *B. subtilis* spore, no viability benefit was gained by other examined heat shock temperature/ time combinations compared to the control ($p<0.05$). In other words, the exposure to a temperature of 80°C and above brought about significantly lowering viability of *B. subtilis* spores compared to the control, whereas CFU of *B. coagulans* spores was higher even after a heat shock of 80°C for 10 min (14.43 log CFU/mL) compared to the spore suspension without heat shocking (14.34 log CFU/mL) ($p< 0.05$). It is worth noting that exposure to 80°C for 15 min caused damaging of the *B. coagulans* spores.

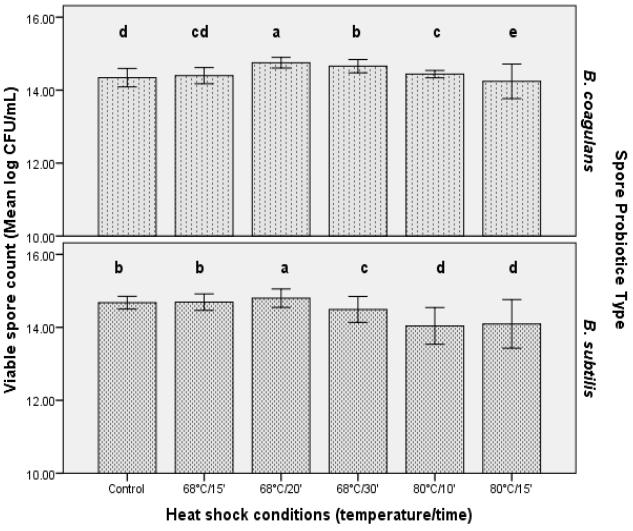

Fig 1. Comparison of viable counts of *B. coagulans* and *B. subtilis* spores activated under different heat -shock conditions. values were determined from serially decimally diluted samples to approximately 10^3 CFU/mL in sterile 0.1% peptone water and then nutrient agar to give a statistically valid range of colonies, and independent experiments were performed in duplicate; Mean ± SD with different letters (a-e) are significantly different; $p<0.05$. Error bars indicate the standard errors of experiment.

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The result of rapid freezing and subsequent thawing effect on the examined spore probiotics is shown in Fig. 2. As can be seen, there was a decline in the viable spore count of *B. coagulans* from 15.39 to 13.88 log CFU/mL, whereas the viable enumeration of *B. subtilis* spore decreased from 15.59 to 13.96 log CFU/mL. The experiment also indicated that loss of spore enumeration of *B. subtilis* was slightly more than that of *B. coagulans*.

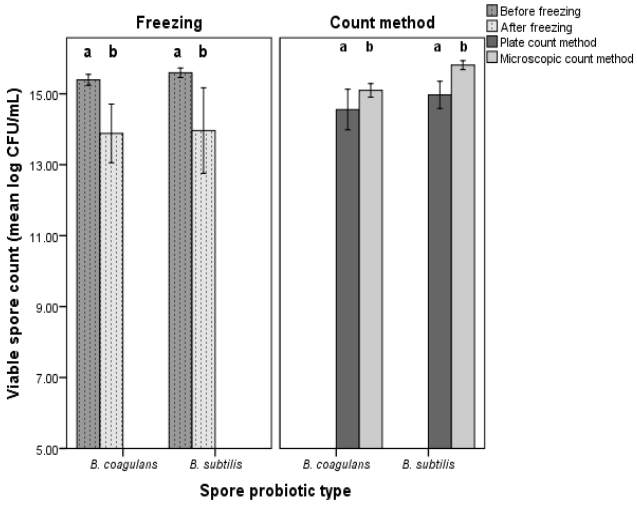

Fig 2. Comparison of viable counts (log CFU/mL) of *B. coagulans* and *B. subtilis* spores before and after fast freezing (-70 °C for about 1 min) via plate count assay and also simultaneous microscopic and plate count of the spores before freezing. values were determined from serially decimally diluted samples to approximately 10^3 CFU/mL in sterile 0.1% peptone water and then Müller-Hinton agar to give a statistically valid range of colonies. Independent experiments were performed in duplicate; Mean ± SD with different letters (a&b) are significantly different; $p<0.05$.

As shown in Fig. 2, in comparative determinations of the concentrations of spore probiotics by plate counts or microscopically, plate counts were significantly lower, with differences of almost 0.5 and 0.8 log for *B. coagulans* and *B. subtilis* spores, respectively. In other words, the viable spore counts of *B. coagulans* were 15.09 and 14.55 log CFU/mL and of *B. subtilis* were 15.81 and 14.97 log CFU/mL for microscopically and plate count assays, respectively. In addition, the DoE results showed that the freezing factor alone, and not the spore type(s), had a significant effect on CFU of the spores examined in freezing treatment ($p<0.05$). However, in count method treatment, both main factors (i.e. type of spore and count method) influenced on the response of the experiments ($p<0.05$) (Table 1).

Table 1. Statistical analysis for freezing treatment and count methods using Full Factorial Design.

Experiment parameters [1, 2]		Effect	F-value	P-value
Freezing	X_1	0.141	0.37	0.554
	X_2	1.569	45.79	< 0.001
	X_1X_2	0.059	0.06	0.804
Count method	X_1	0.565	392.89	< 0.001
	X_3	0.690	585.91	< 0.001
	X_1X_3	0.150	27.069	0.006

[1] X_1, X_2 and X_3 represent the main effects of spore probiotic type, freezing treatment (before and after), and count method (plate and microscopic), respectively. X_1X_2 and X_1X_3 represent their interaction effects. Significant effect ($P \leq 0.05$).

[2] Regression equations in uncoded units for freezing and count method experiments are

$Y = 14.708 + 0.071\ X_1 + 0.784\ X_2 + 0.029\ X_1X_2$ ($R^2 = 79.39\%$; R^2 (adj) = 74.24%) and

$Y = 15.1075 + 0.2825\ X_1 + 0.3450\ X_3 + 0.0750\ X_1X_3$ ($R^2 = 99.60\%$; R^2 (adj) = 99.31%), respectively.

Discussion

The activation of dormant spores by heat shock is a process that progresses with time and involves an abrupt change in individual spores rather than a gradual change in all spores, as indicated by increased colony counts with time on conventional plating media (41). On the other hand, if heat-shock treatment is too severe, the damage will be so strong that the spores cannot repair themselves and the viable spore counts will be smaller, compared with the conventional and mild heat-shock conditions (27). Our results revealed that heat shock of 68°C for 20 min was above the critical minimum heat activation temperature/time combinations for the spores examined (Fig. 1). Due to this, a higher percentage of germinating spores and, consequently, a higher count were obtained. However, as shown in Fig. 1, application of heat shock of 80°C especially for long time (> 10 min) caused lowering the viable counts of both studied spores because heating to an insufficiently high temperature deactivates rather than activates the spores (23, 31). This was termed "heat induced dormancy" (23). Another obvious conclusion to be drawn from the findings is that for heat shock of

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the examined *B. subtilis* spore, temperatures should be kept to ≤ 70°C (e.g. 68°C) with holding times not needing to exceed 15-20 min. This outcome affirms that the optimal temperature and time of heat shock vary from species to species, though not all spores can be activated by heat. In addition, it is important to bear in mind that bacterial spores (especially Gram-positives) are usually very resistant to freezing and storage at subzero temperatures; therefore, high proportions (about 90%) survive in most conditions (36, 42). In this study, following the rapid freezing of the studied spores (-70°C for about 1 min) in deionized water and subsequent thawing; about 90% and 88% of the *B. coagulans* and *B. subtilis* spores remained viable, respectively. This result is in agreement with previously reported data (36, 42, 43). Fairhead et al. (44) revealed that small acid-soluble proteins bound to DNA protected spores like *B. subtilis* from being killed by freeze drying. Undoubtedly, in comparison to the type of spore probiotics, the fast freezing had a significant influence on their counts (Table 1). Indeed, behavior of the examined spores in fast freezing experiment had a similar pattern and type of spores as one of the main factors could not influence on their CFUs. Another experiment was set up then to determine variation and error in the technique of enumeration viz. comparison between plate and direct microscopic counts for the spore probiotics examined. Microscope counts of bacteria in chambers of known depth have generally been accepted and used as the most accurate total count technique available (45). On the contrary, plate count is also known as a common and rather simple method. By referring to Fig. 2, plate counts were significantly lower than microscopically determined counts for both spores examined at the 5% significance level. In general, there are a number of reasons why enumeration of spores by the two methods may give varying results, and also why plate counts may generally be lower than counts determined microscopically. It is evident that a proportion of spores in a population may fail to germinate or may be genuinely non-viable or may have become viable but not cultivable (23, 45). Plate counts fail to take into account the difference between single cells and groups of cells. Of course, spores have the reputation of tending to clump during the growth, which makes a high probability of being missed (45). Additionally, methods using spreaders suffer some loss of the spores on the spreaders, even though, in the experiments done here this was minimized by using 10 µl loops in place of spreaders. Time-to-time and operator-to-operator differences in counts on any one sample are also generally to be expected in biological tests. In other words, culture-based enumeration of specific organisms requires specialized and standardized methodologies, which will only detect bacteria that are able to replicate on synthetic media and under specific conditions. This finding is in agreement with the studies conducted by Turnbull et al. (23) and Davis (45). Nonetheless, DoE results of the present study revealed that not only kind of spore count (viable counts or microscopically) but type of spores (*B. coagulans* or *B. subtilis*) as a main factor have significant influences on their counts (Table 1). It seems that movement of spores or distribution of spores in the counting chamber, tendency level of spores to form clumps, and the amount of spore adherence to solid surfaces are not the same among different species of spore forming bacteria (43).

Collectively, it can be concluded that optimal heat activation/shock conditions vary with species and/or even strains, media and germinants. Under the conditions described in the current study, the findings indicated that in comparison to other evaluated heat shock temperature/time combinations, a heat-shock of 68°C for 20 min would be the most appropriate to quickly activate spore suspensions of B. *coagulans* and *B. subtilis* and to eliminate the possibilities of the viability loss of spores and survival of vegetative cells, which is likely to be of advantage in enumerating the viable counts of the spores examined. In addition, the results summarized in this study revealed that following the rapid freezing of *B. coagulans* and *B. subtilis* spores in deionized water and subsequent thawing, their viability was consistent with the survivability rate found in the literatures. In comparative determinations of concentrations of the spore suspensions by viable counts or microscopically, plate counts were significantly lower than microscopically determined counts.

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