

# Ultrasound pretreatment enhances the inhibitory effects of nisin/carvacrol against germination, outgrowth and vegetative growth of spores of *Bacillus subtilis* ATCC6633 in laboratory medium and milk: Population and single-cell analysis

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## ABSTRACT

This study evaluated the synergetic inhibitory effects of ultrasound and nisin/carvacrol on spore germination, outgrowth, and subsequent growth of vegetative cell of *Bacillus subtilis* in laboratory medium and milk. Ultrasound pretreatment (3.33 W/mL, 15 min) and nisin/carvacrol (0.01%, 0.02%) synergistically inhibited spore germination, outgrowth, and vegetative growth of spores in laboratory medium. Whereas no such inhibitory effect was observed in milk even with a 10-fold increase in the concentration (1%) of nisin. Flow cytometry analysis showed that the germination capacities of ultrasound pretreated spores combined with nisin/carvacrol (67.3% and 30.5%, respectively) was lower than that of the untreated spores (95.1%). These results quantitatively revealed the inhibitory effect of the combined treatments which were confirmed by phase-bright spore observations at single cell level. In general, the current work identified the combined ultrasound-carvacrol treatment as an effective strategy to control spores and vegetative cells of *B. subtilis* in the laboratory medium and milk during abusive storage.

## 1. Introduction

*Bacillus* spores can resist to physical and chemical treatments (heat, chemicals, UV, osmosis, desiccation) which are the main hurdles used in food industry to preserve food stability (Paredes-Sabja et al., 2010). The surviving spores under favorable conditions can rapidly revert to vegetative cells through germination and outgrowth leading to poisoning or spoilage issues (Nagler et al., 2016; Trunet et al., 2018). Thermal sterilization is an effective technique for the inactivation of bacterial spores, however, it can have detrimental effects on the nutritional quality and flavor of food, making it a less popular food-processing method (Fan et al., 2019; Georget et al., 2015). Thus, alternative strategies to the conventional thermal processing technology must be developed to fulfil customer demands whilst meeting microbiological inactivation standards.

Ultrasound as a non-thermal technology has attracted great attention in food preservation. A number of studies have reported the

antimicrobial activity of ultrasound against different spoilage and food-borne pathogenic bacteria (Duarte et al., 2018; Li et al., 2016a; Wang et al., 2018; Wu and Narsimhan, 2017). Microbial cells are sensitive to sonication treatment while sonication alone has little effect on highly resistant spores. However, its effectiveness can be enhanced by combining it with other treatments such as the use of natural antimicrobial agents. The enhanced decontamination capabilities of the ultrasound assisted treatments were attributed to the asymmetric cavitation near the cell surface (Sango et al., 2014). It generates microjets in the direction of the surface, thereby accelerating the diffusion of antimicrobial solutions in microbial cells producing an increased lethal efficacy (Wang et al., 2018). Nisin (a ribosomally synthesized peptide) and carvacrol (C<sub>10</sub>H<sub>14</sub>O) were reported to exhibit a broad-spectrum antimicrobial activity and are toxicologically safe (Onder et al., 2011; Sagong et al., 2013). Furthermore, a couple of studies have demonstrated the inhibitory activity of ultrasound with nisin or carvacrol against sporogenic bacteria such as *Clostridium perfringens*, *Bacillus*

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*subtilis*, and *Bacillus cereus* (Muñoz et al., 2012; Pol et al., 2001; Wang et al., 2018). Nevertheless, these studies could not reveal the effect of the combined treatments on specific phase of germination and outgrowth of bacterial spores, and more importantly, how homo-/heterogeneously the growing spores respond to these stressed conditions (Pandey et al., 2015). Even though a previous study by Van Melis et al. (2011) used flow cytometry (FCM) analysis to investigate the heterogeneity of germination and outgrowth of sorbic acid-stressed *Bacillus* spores, and verified its feasibility in exploiting individual stressed-spore behavior, they did not take the characteristics of processing medium into account (Alanazi et al., 2018).

Consequently, the current study is aimed at determining the effect of combining ultrasound pretreatment and nisin/carvacrol against germination, outgrowth and vegetative growth of spores of *B. subtilis* ATCC6633 in laboratory medium and in milk during storage at abusive conditions. Furthermore, a quantitative analysis was conducted to investigate the effect of these combined treatments on germination and outgrowth of *B. subtilis* spores at single cell level.

## 2. Materials and methods

### 2.1. Strain and spore preparation

Spores of *Bacillus subtilis* ATCC6633 (Hope Bio-Technology Co., Ltd., Qingdao, Shandong, China) was used in this study. Spores were generated in a defined solid medium containing (per litre) 33.0 g nutrient agar (Hope Bio-Technology Co., Ltd., Qingdao, Shandong, China), 2.0 g KCl, 0.5 g  $MgSO_4 \times 7H_2O$ , and 0.004 g  $MnCl_2 \times 4 H_2O$  (Moeller et al., 2006) for 10 days at 37 °C until it reached a sporulation frequency > 95% as confirmed by phase-contrast microscopy. Then the spores were harvested by washing with sterile distilled water at least eight times and centrifuged at 6000g and 4 °C for 15 min. The purified suspension was stored in a dark place at 4 °C until use within one month.

### 2.2. Ultrasound treatment

Ultrasound treatments were conducted with a probe-style Scientz-II D ultrasonic processor (Ningbo Scientz, Zhejiang, China) at 20 kHz frequency and 10-mm-diameter probe. An 85 mL cylindrical glass vial containing 30 mL of spore suspension was placed in a thermostatically controlled water bath and kept at 23 °C. A digital thermometer located in the center of the reaction vessel was used to measure the temperature. The emitter was always immersed in the centre of the spore suspensions and 1.5 cm below the surface. The power density (D, W/mL) and intensity (I, W/cm<sup>2</sup>) of ultrasound were calculated as shown in the equations:  $D = P/V$ , where P is the input power and V is the medium volume;  $I = P/(\pi r^2)$ , where r is the radius of probe tip (Li et al., 2016b). In this study, a 100 W input power dissipated in 30 mL-medium yielded a power density of 3.33 W/mL and a power intensity of 127.5 W/cm<sup>2</sup>. The spores that would be exposed to the combined treatment were first pretreated with ultrasound at a power density of 3.33 W/mL and 23 °C for 15 min. The spore suspensions without treatment were considered as the control samples (C) and all experiments were performed in at least triplicates.

### 2.3. Germination of *B. subtilis* spore in the presence of nisin/carvacrol after ultrasound pretreatment

Purified spore suspensions with or without ultrasound pretreatment were heat activated at 70 °C for 30 min, prior to germination as previously described (Nagler et al., 2016). Germination of heat-activated spores was triggered in germination media composed of LB medium plus 10 mM L-alanine-10 mM Tris-HCl buffer (pH 7.4) (Paidhungat and Setlow, 2000).

Forty µL of heat-activated spores were transferred to a 96-well microtiter plate containing 200 µL germination media alone, or

supplemented with various concentrations of nisin/carvacrol (0.01%, 0.02% (w/v)). These mixtures were incubated at 37 °C. The optical density drop of the cultures at 600 nm (OD<sub>600</sub>) was measured over time interval up to 1 h in a Multiskan GO OD reader (Thermo Scientific, United States). The phase-contrast microscopy (Leica, Germany) were used to confirm the drop which resulted from the rehydration of the spore core (Setlow, 2003). The germination rate was calculated by division of each reading by the initial OD value ( $t = 0$  min). Per condition, five technical replicates were conducted and two independent experiments were performed with one and the same spore crop.

### 2.4. Outgrowth of *B. subtilis* spore in the presence of nisin/carvacrol after ultrasound pretreatment

Purified spore suspensions with or without ultrasound pretreatment were heat activated as described in Section 2.3. After cooling down, the suspensions were inoculated into the germination medium alone or supplemented with nisin/carvacrol (0.01%, 0.02% (w/v)). The cultures were incubated at 37 °C and the change in OD<sub>600</sub> was measured every 10 min for 3 h post-inoculation. Per condition, two independent experiments were performed with at least five repeats with one and the same spore crop.

### 2.5. Vegetative growth of *B. subtilis* spore in the presence of nisin/carvacrol after ultrasound pretreatment

The ability of the combined ultrasound pretreatment-*nisin/carvacrol* to prevent the vegetative growth of *B. subtilis* spores were determined in the germination medium. A 0.5 mL of heat-activated spores as described in Section 2.3/2.4 was transferred into 10 mL germination medium, incubating at 37 °C for 3 h. Next, 0.5 mL of the 3-h growth culture was inoculated into 10 mL fresh germination medium alone or supplemented with nisin/carvacrol (0.01%, 0.02% (w/v)). The growth was monitored by measuring the OD<sub>600</sub> of germination medium-grown culture hourly for up to 24 h. All analyses were carried out at least five times with two independent spore preparations.

### 2.6. Flow cytometry analysis

The inhibitory effect of the combined ultrasound pretreatment-*nisin/carvacrol* on germination, outgrowth, and subsequent vegetative growth of *B. subtilis* spores at single cell level was assessed by flow cytometry (FCM) analysis. Flow cytometry (FCM) assessments were performed on a Gallios flow cytometer equipped with a 488 nm air-cool argon laser (Beckman Coulter Inc., Miami, FL, USA). Single staining method was employed using the green fluorescent nucleic acid dye SYTO 16 (Life Technologies, Carlsbad, CA, USA).

Samples were taken for FCM analysis after 40, 80, and 120 min. These samples were prepared by centrifugation at 12,000g (30 s), washed and then resuspended in sterile distilled water supplemented with 100 mM EDTA (Van Melis et al., 2011). After mixing with the freshly prepared fluorescent dye solution vigorously, samples were incubated in a dark place at ambient temperature for 15 min. The stained samples were kept in the dark on ice and used within 1 h for FCM analysis (Antolinos et al., 2014). A total of 20,000 events were set for data acquisition per sample, at a flow rate of 400–600 events per second. Kaluza software package (Beckman Coulter Inc., Miami, FL, USA) was used as the operation and acquisition software.

### 2.7. Phase contrast microscope analysis

The phase-contrast microscope was also employed to record the inhibitory ability of the combined ultrasound pretreatment-*nisin/carvacrol* against spore germination, outgrowth and vegetative growth of *B. subtilis* at single spore level. Samples were taken for microscope analysis after 40, 80 and 180 min. The specimens were observed with

100×/1.25 plan objective (Nikon, Japan) and images were acquired by a NIS-Elements BR 3.2 camera (Nikon, Japan). At least 3 different fields of view were observed in parallel per experiment. Three different microscopy experiments for each condition, with five technical replicates, were performed with one and the same spore crop.

### 2.8. Growth of *B. subtilis* spore in milk in the presence of nisin/carvacrol after ultrasound pretreatment

Sterile milk was purchased from a local supermarket in Hangzhou, Zhejiang, China. The spore cocktails of *B. subtilis* were prepared by mixing spores with sterile milk, and a final spore cocktails concentration of approximately  $10^6$  CFU/mL was obtained for further treatment and were stored at  $-20^\circ\text{C}$  until use.

To assess the impact of the combined ultrasound pretreatment-nisin/carvacrol treatment on growth of spores in milk, 30 mL of spore cocktails in sterile vessels were first treated with ultrasound at a density of 3.33, 6.67, and 13.3 W/mL for 15 min. After which the treated spore cocktails were transferred into sterile bags. Appropriate amounts of nisin (0.01–1% (w/v)) or carvacrol (0.05–0.8% (w/v)) were added into the bags and sealed. This was followed by a manual mixing for 1 min. Negative controls (C-) were also included using the milk without the introduction of spores. Samples without any treatment were regarded as the positive control (C+). For each condition, two bags were needed to either determine the initial population of spore cocktails or incubate at  $37^\circ\text{C}$  for 6 h. The populations of *B. subtilis* in the milk was determined using the plate count method (Evelyn and Silva, 2015), and results were calculated as Log CFU/mL. All experiments were done at least in triplicate.

## 3. Results and discussion

### 3.1. Inhibition of *B. subtilis* spores' germination via the combination of ultrasound pretreatment and nisin/carvacrol

The impact of combined ultrasound pretreatment (3.33 W/mL,  $23^\circ\text{C}$ ) and nisin/carvacrol (0.01%, 0.02%) on germination were first analyzed by monitoring the relative drop in  $\text{OD}_{600}$ . Within 60 min, the  $\text{OD}_{600}$ -values of the control spores decreased by approximately 55% of the initial OD value (Fig. 1). This drop in OD represents close to 100% germination (Paredes-Sabja et al., 2010). *B. subtilis* spores incubated in germination medium plus 0.01–0.02% of nisin alone germinated normally after 60 min of incubation at  $37^\circ\text{C}$  (Fig. 1A). This was in accordance with a study by Pathima et al. (2012) which reported that 100  $\mu\text{M}$  (equivalent to 0.035%) nisin alone had no effect on germination of both *Clostridium perfringens* type A isolates after 60 min of incubation. Spores treated with ultrasound alone germinated completely with the same OD-drop ( $\sim 55\%$ ) as control spores after 60-min incubation in germination medium (Fig. 1). These results indicated that neither nisin nor ultrasound pretreatment has an effect against germination of *B. subtilis* spores. In contrast, the combination of ultrasound and nisin incubation resulted in a strong germination inhibition (Fig. 1A). This indicated that ultrasound pretreatment drastically improved the effectiveness of nisin in inhibiting the germination of spores. This phenomenon may be attributed to the acceleration of the bioactive compounds' action induced by the ultrasound application (Cárcel et al., 2007; Knorr et al., 2004). Fig. 1B showed greater germination inhibition was achieved when ultrasound pretreatment in combination with carvacrol at the same concentrations as nisin (0.01%, 0.02%). Moreover, an increase in the concentration of carvacrol in the germination medium after ultrasound pretreatment increased inhibition of the spore germination (Fig. 1B). Incubating *B. subtilis* spores in the presence of carvacrol alone could not observe any apparent differences in the  $\text{OD}_{600}$  drop when compared to the control condition (Fig. 1B). This indicated that carvacrol alone exhibited no clear effects on spore germination. Spore germination was triggered by the interaction of germinants with

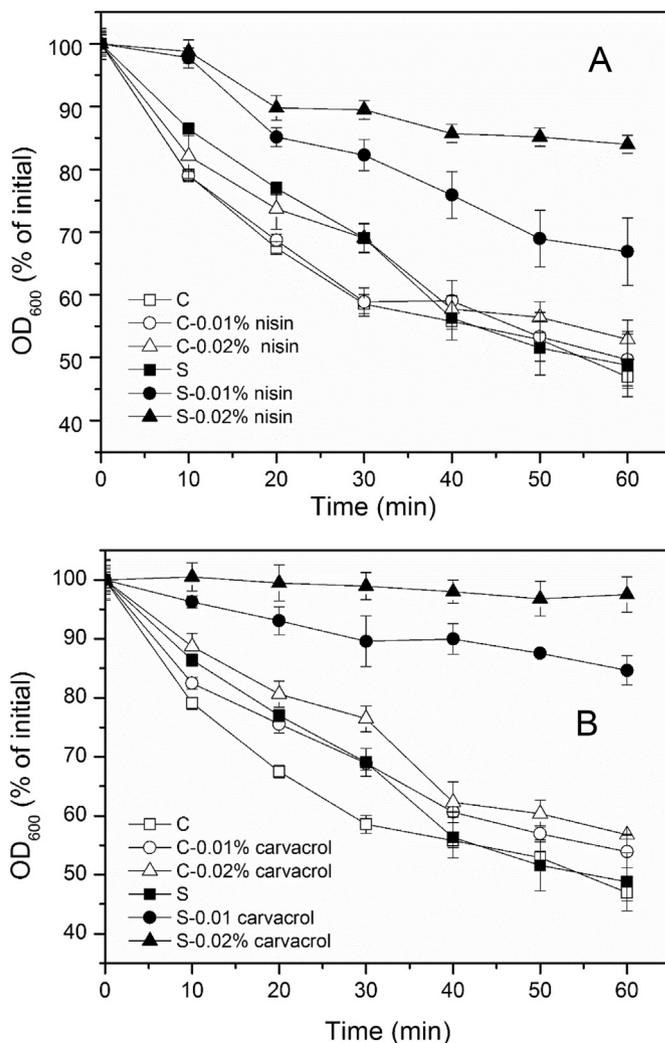
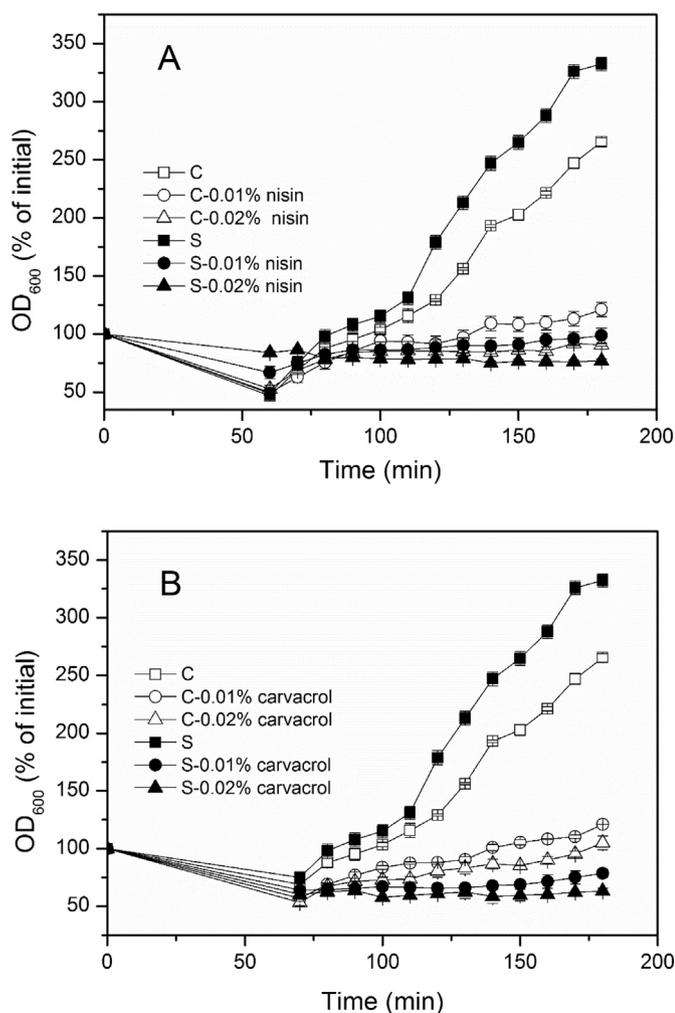


Fig. 1. Inhibitory effects of the combined ultrasound pretreatment-nisin (A)/-carvacrol (B) on germination of spores of *B. subtilis*. After being untreated (C)/treated with ultrasound (S) at 3.33 W/mL and  $23^\circ\text{C}$ , spores of *B. subtilis* were heat activated and then incubated with germination medium alone or supplemented with nisin/carvacrol (0.01%, 0.02%).  $\text{OD}_{600}$  was measured every 10 min for 1 h as described in Materials and methods. Error bars represent standard deviations from the mean of five technical replicates.

germinant receptors (GRs), which are located in the spore's inner membrane (IM) (Setlow, 2003). It is possible that ultrasound pretreatment promotes the accumulation of nisin or carvacrol in spore's inner membrane where these agents prevent the spore germination by specifically damaging the L-alanine germinant receptors (GerA GR) (Cortezzo et al., 2010; Van Melis et al., 2011). Collectively, the results suggested that ultrasound pretreatment has a pronounced effect on enhancing the inhibitory effect of nisin/carvacrol against *B. subtilis* spore germination.

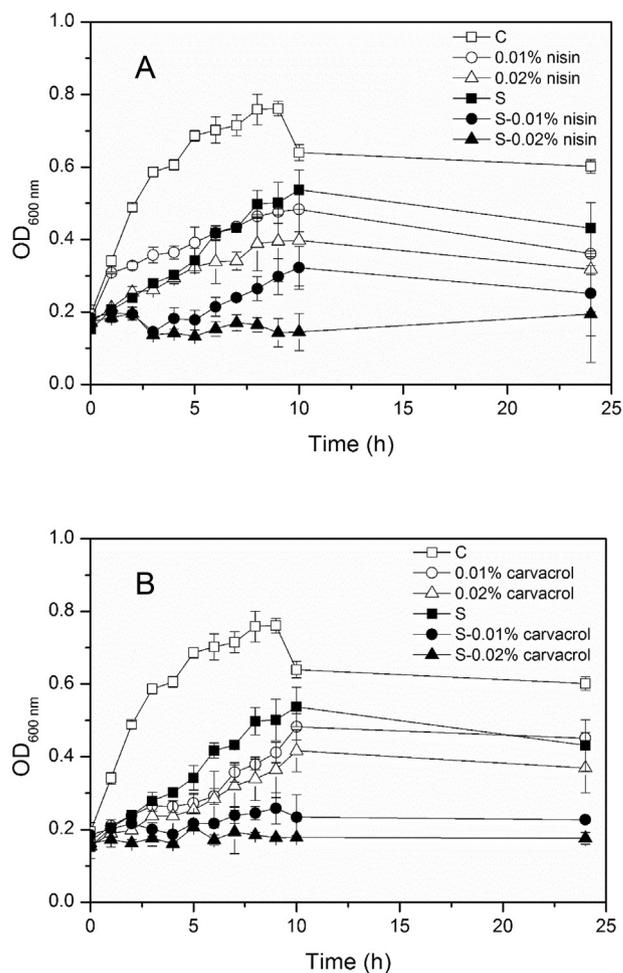
### 3.2. Combination of ultrasound pretreatment and nisin/carvacrol blocks *B. subtilis* spore outgrowth

The outgrowth of *B. subtilis* spores was investigated at the same experimental conditions as presented in Fig. 1A-B. The spore outgrowth in the germination medium was initiated after incubation at  $37^\circ\text{C}$  for  $\sim 100$  min and this continued for up to 3 h. As shown in Fig. 2, the inhibitory effect of nisin/carvacrol (0.01%, 0.02%) alone on outgrowth of the spores was stronger than that on the germination. This indicated that the outgrowth of spores is a more susceptible stage for the



**Fig. 2.** Inhibitory effects of the combined ultrasound pretreatment-nisin (A)/-carvacrol (B) on outgrowth of spores of *B. subtilis*. After being untreated (C)/treated with ultrasound (S) at 3.33 W/mL and 23 °C, spores of *B. subtilis* were heat activated and then incubated with germination medium alone or supplemented with nisin/carvacrol (0.01%, 0.02%). Error bars represent standard deviations from the mean of five technical replicates.

inhibitory actions of nisin/carvacrol. Based on the comparative analysis, the extent of inhibition in the outgrowth of spores exposed to the combined ultrasound pretreatment-carvacrol was greater as exposed to the combined ultrasound pretreatment-nisin at some concentration (0.01%–0.02%) (Fig. 2A). The discrepancy may be attributed to their different mechanisms of action. The main target of carvacrol is the cytoplasmic membrane of microorganisms, where carvacrol could interact with membrane proteins and enzymes, as well as intracellular targets (Burt, 2004; Hyldgaard et al., 2012). While nisin exerts its antimicrobial properties against Gram-positive microorganisms by forming pores in the lipid membranes and inhibiting cell wall synthesis (Hasper et al., 2006; Ruhr and Sahl, 1985). More interestingly, ultrasound treatment alone effectively promoted the outgrowth of spores (Fig. 2), even though it didn't affect the stage of spore germination (Fig. 1). It is assumed that the chemical bonds between molecular components in the cell wall were ruptured by ultrasound, facilitating the escape of the hydrated core from the spore coat in outgrowth stage. Unfortunately, the specific mechanism remains unclear and further work will be required to validate it. Furthermore, ultrasound pretreatment increased the inhibitory effects of nisin or carvacrol against the outgrowth of spores (Fig. 2A–B). Collectively, these results indicated that nisin/carvacrol at their present concentrations combined with

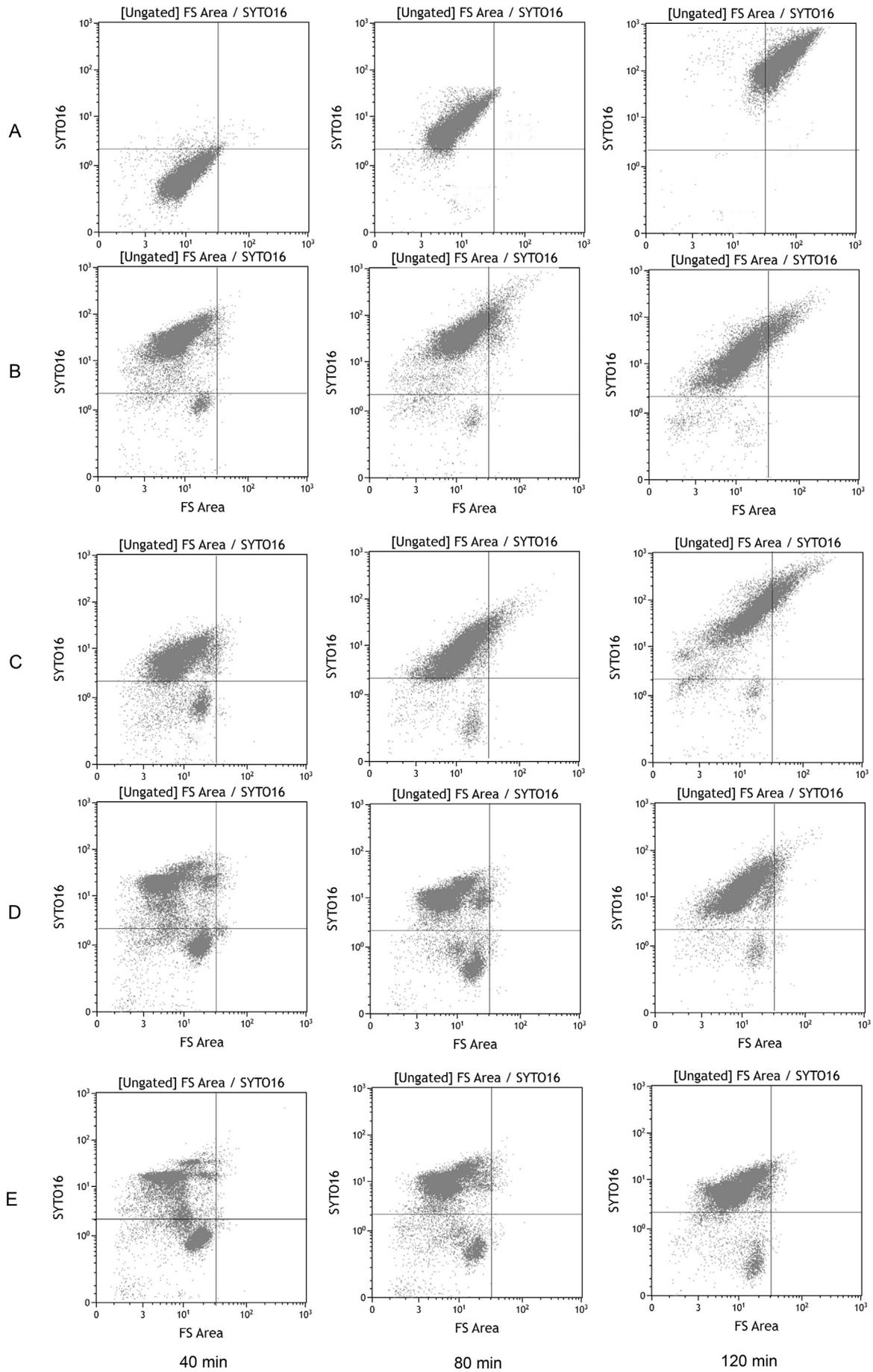


**Fig. 3.** Inhibitory effects of the combined ultrasound pretreatment-nisin (A)/-carvacrol (B) vegetative growth of spores of *B. subtilis*. 3-h germination medium grown culture (vegetative cells) of *B. subtilis* spores were untreated (C)/treated ultrasound (S) at 3.33 W/mL and 23 °C, and then incubated into germination medium containing nisin/carvacrol (0.01%, 0.02%). OD<sub>600</sub> readings of vegetative cells were monitored at different time intervals for 24 h. Error bars represent standard deviations from the mean of five technical replicates.

ultrasound pretreatment could effectively block the outgrowth of germinated spores in the laboratory growth medium.

### 3.3. Inhibition of growth of *B. subtilis* vegetative cells by the combination of ultrasound pretreatment and nisin/carvacrol

The inhibitory effect of the combined ultrasound pretreatment and nisin/carvacrol against germination and outgrowth of spores prompted us to speculate that these treatments might also inhibit spore vegetative growth. Results demonstrated that nisin at either 0.01% or 0.02% concentration arrested the growth of vegetative cells of *B. subtilis* after 5 h-incubation and continued up to 24 h (Fig. 3A). The inhibitory effects of nisin at test concentrations against vegetative cells of *B. subtilis* were consistent with the findings of previous studies (Natrajan and Sheldon, 2000; Pathima et al., 2012). Furthermore, an enhanced growth inhibition of *B. subtilis* vegetative cells was obtained when nisin was combined with ultrasound pretreatment (Fig. 3A). This also happened when ultrasound pretreatment combined with carvacrol at either 0.01% or 0.02%. It is possible that the asymmetric cavitation near the cell surface resulted in the weakening of the cell membrane (Li et al., 2016b). Such behavior could further enhance the effectiveness of nisin/carvacrol treatment, which produced rapid membrane damage resulting in the inhibition of vegetative growth of *B. subtilis*. Additionally,



(caption on next page)

**Fig. 4.** Fluorescence scatterplots versus forward-scatter intensities (FS Area) of SYTO16 stained *B. subtilis* spores during germination, outgrowth. A: the population from left to right is pure dormant spores, germinating spores and vegetative cells, respectively. B: control, C: ultrasound at 3.33 W/mL and 23 °C, D: ultrasound (3.33 W/mL, 23 °C)-0.02% nisin, E: ultrasound (3.33 W/mL, 23 °C) -0.02% carvacrol.

the vegetative growth of *B. subtilis* spores was increasingly affected (decreased) with higher concentrations of the combined carvacrol (Fig. 3B).

### 3.4. Inhibitory effect of the combination of ultrasound pretreatment and nisin/carvacrol on germination, outgrowth, and subsequent vegetative growth of spores at single cell resolution

Since OD<sub>600</sub> readings from cultures reflected the result of the whole population, it cannot present the variation within each phase of germination and outgrowth, and how heterogeneously the individual spore respond to a given stress (Pandey et al., 2015). To get a better understanding of the heterogeneity in the germination and outgrowth process in more details, the increase in green fluorescence level and particle size were measured at the single spore level by flow cytometry (Fig. 4). As illustrated in the dot plots (Fig. 4A), the dormant spores are located in the lower left quadrant. Upon germination, followed by hydrolysis of the spore cortex, the membrane permeant SYTO16 was able to cross the inner spore membrane and bound to nucleic acids in the spore core. This was highlighted by the increase in green fluorescence level (Fig. 4A, upper left quadrant). The population in the upper right quadrant represented the outgrowth of germinated spores with the increase in particle size (Fig. 4A).

As shown in Fig. 4B-E, after 40 min, part of the spore populations for all conditions become permeable to SYTO16. After 40-min incubation, 93.2% of the ultrasound treated spores emerged in the upper left quadrant as fluorescent particles (Fig. 4B). For the combined treatment, the germination capacities of the ultrasound stressed spores with nisin/carvacrol (67.3% and 30.5%, respectively) was lower than that of unstressed spores (95.1%). The obtained results indicated that the combined treatments significantly extended the mean time needed for spore germination ( $P < 0.05$ ). Incubating spores under the unstressed condition for 80 min, a substantial part of the spore population was transferred to the upper right quadrant (Fig. 4A). Whereas the majority of the combined treatment-stressed spores was located in the upper left (Fig. 4C-D). These results suggested that the ability of spores to grow out was affected when spores were stressed with the combined treatments. After 120 min, compared to unstressed spores (outgrowth efficiency, 38.8%), ultrasound alone had the least effect (40.2%) whereas the combined ultrasound-carvacrol at concentration of 0.02% had the largest effect (8.6%) on the ability to grow out. Overall, the results from all individual data specially revealed a strong effect of the combined treatments on the germination and outgrowth of spores. This further confirmed the microtiter plate results discussed above.

The phase contrast microscopy images reflect intuitively the heterogeneity in the behavior of individual cells, and further confirmed the results of the FCM analysis. The results (Fig. 5A-D) showed that not all spores have germinated or outgrown within the experimental period. Also, the germination and outgrowth occurred heterogeneously in all samples particularly the spores stressed with the combined treatments. This coincided with the appearance of various subpopulations within the combined ultrasound pretreatment-nisin/carvacrol treated-population (Fig. 5C-D). Moreover, the unstressed spores and ultrasound (without nisin/carvacrol) treated-spores have an aggregation of long filamentous cells clearly visualized by microscopic observation after ~6 h (Fig. 5A-B). This unique cell deformation could be explained by the increase in OD<sub>600</sub> recorded during the growth in Fig. 2. However, a small number of elongated cells were observed in the condition where spores were stressed with the combined ultrasound-carvacrol treatment (Fig. 5D). The poorly elongated characteristic observed could be explained by i) the stress conditions did inhibit the vegetative growth of

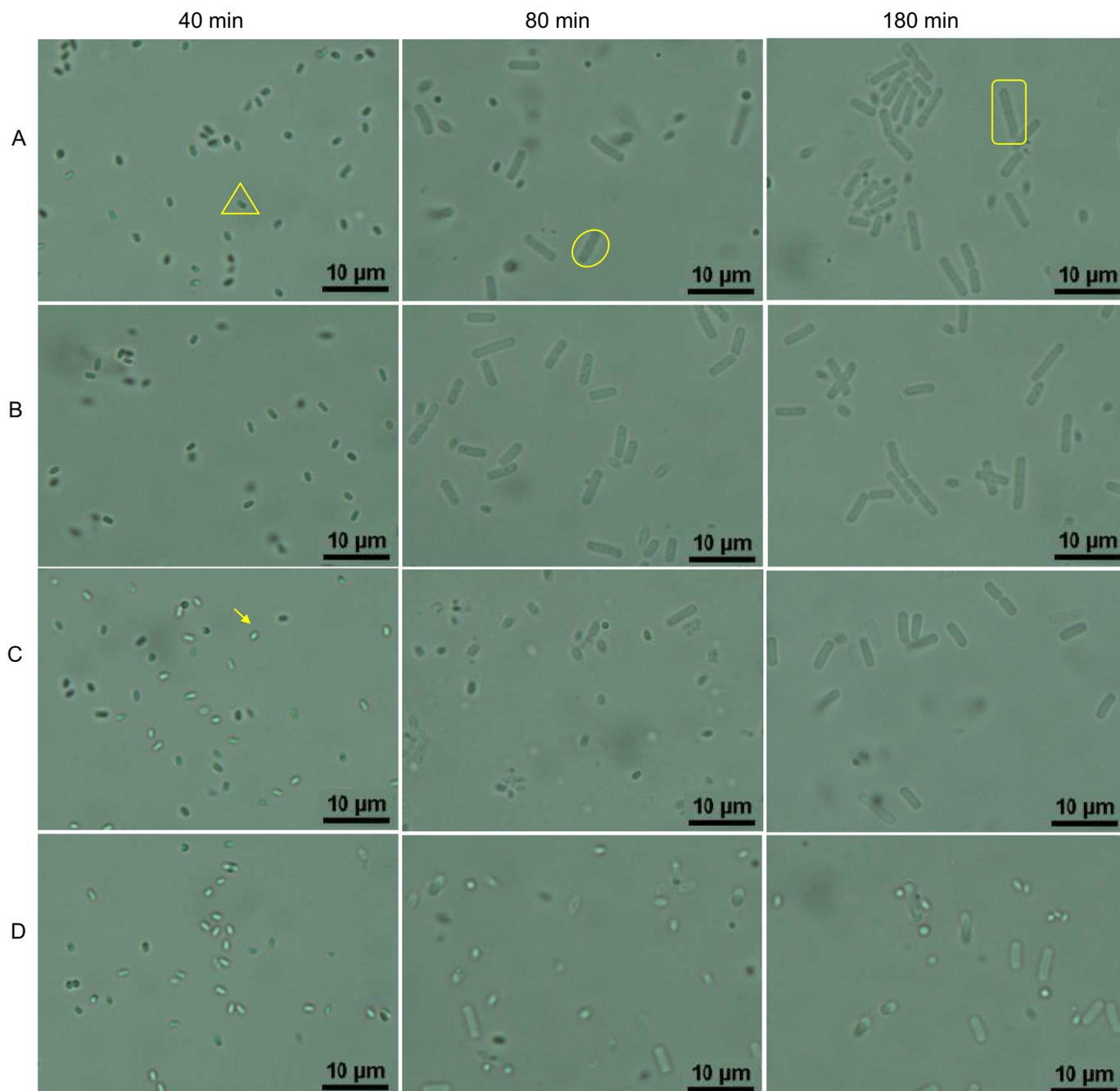
cells, and ii) these aberrant-growing cells were lysed prior to be normal cells (Seward et al., 1982).

### 3.5. Control of germination and outgrowth of *B. subtilis* spores in milk

In order to evaluate the effectiveness of the combined ultrasound pretreatment-nisin/carvacrol on the growth of *B. subtilis* spores in milk under abusive storage conditions, a model system for the contaminated milk was used as reported in previous studies (Evelyn and Silva, 2015; Gera and Doores, 2015). It should be noted that the main reason for choosing the abusive storage conditions tested in the present study was to simulate the least desirable situation that could happen if the milk was to be kept in a favorable temperature that supports the optimum growth of *B. subtilis* before consumption. The results showed that the combined ultrasound pretreatment-nisin/carvacrol (3.33 W/mL, 0.01%, 0.02%) significantly inhibited the growth of *B. subtilis* in laboratory media. However, the combined treatments failed to block the growth of *B. subtilis* in milk after 6-h incubation at 37 °C (Fig. 6A-B). This discrepancy in the two media could be attributed to the protective effect of milk components on bacteria. For example, the high sugar content of milk was reported to protect bacteria by stabilizing the protein and membranes (Leslie et al., 1995). Furthermore, Gera and Doores (2015) have previously elucidated that the presence of lactose in milk could increase the resistance of spores to ultrasound. After being pretreated with ultrasound at 3.33 W/mL, the *B. subtilis* spores were mixed with milk containing nisin of different concentrations. The spore cocktails exhibited an increase of ~2 Log CFU/mL in *B. subtilis* cell counts after 6-h incubation at 37 °C, which is similar to those observed in positive control samples (Fig. 6A). Moreover, increasing the power density of ultrasound to 6.67–13.3 W/mL has no effect in reducing *B. subtilis* cell counts in the milk with nisin/carvacrol at lower concentration ( $\leq 0.02\%$ ,  $\leq 0.1\%$ , respectively). However, after ultrasound pretreatment at 13.3 W/mL, > 2 Log CFU/mL reduction in *B. subtilis* cell counts was obtained in the milk containing carvacrol concentrations of 0.2%–0.8% after 6 h of storage at 37 °C (Fig. 6B). The presence of 0.8% carvacrol alone only resulted in 0.32 Log CFU/mL decrease in viable cells generated from spore cocktails of *B. subtilis* compared to the positive control sample (C+) (Fig. 6B). This is consistent with previous study where a higher level (7.5%) of carvacrol alone failed to control the growth of *Bacillus* viable cells in skimmed milk (Pol et al., 2001). These data suggested that ultrasound and carvacrol provided synergistic effects and this could pave the way for the development of a safe and effective strategy to control *B. subtilis* growth in milk during abusive storage. More interesting, the same ultrasound treatment conditions even combined with nisin at 0.5%–1% levels, still produced no clear effect on the number of *Bacillus* viable cells (Fig. 6). These findings indicated that the antimicrobial activity of the combined treatments in milk samples was dependent on inhibitor concentration and inhibitor type. Collectively, these results suggested that ultrasound pretreatment combined with high level (0.8%) of carvacrol exerted significant inhibitory effect against *B. subtilis* spores in milk by affecting the germinated spores and further inhibiting the outgrowth under abusive temperature condition.

## 4. Conclusion

In this study, the combined ultrasound pretreatment and nisin/carvacrol exerted their inhibitory effects against spore germination and outgrowth, and vegetative growth of *B. subtilis* in laboratory medium. It confirmed the assertion that ultrasound enhanced the antimicrobial activity of nisin/carvacrol during processing. Our results also extended



**Fig. 5.** Microscopy images showing heterogeneous germination, outgrowth and vegetative growth of *B. subtilis* spores in response to the combined ultrasound pretreatment-nisin/carvacrol. A: control, B: ultrasound at 3.33 W/mL and 23 °C, C: ultrasound (3.33 W/mL, 23 °C)-0.02% nisin, D: ultrasound (3.33 W/mL, 23 °C)-0.02% carvacrol. Dormant (arrow, C), Germination (triangle, A), outgrowth (oval, A) and vegetative growth (rectangle, A) were observed using phase-contrast microscopy for 6–8 h.

the analysis to a single spore level, thus facilitating the assessment of the heterogeneity in each life cycle phase in response to the combined ultrasound pretreatment-nisin/carvacrol treatment. Although their inhibitory effectiveness in laboratory medium was superior. At same treatment conditions, the combined treatments failed to prevent the germination and outgrowth of *B. subtilis* spores inoculated into the milk, except when the combined concentration of carvacrol was increased. Thus, our results showcased the possible application of the combined treatments in food preservation. Further work would be required to examine spores of various species and to identify the suitable stress response related genes, elucidating the key factors contributing to spore germination and outgrowth under the combined ultrasound

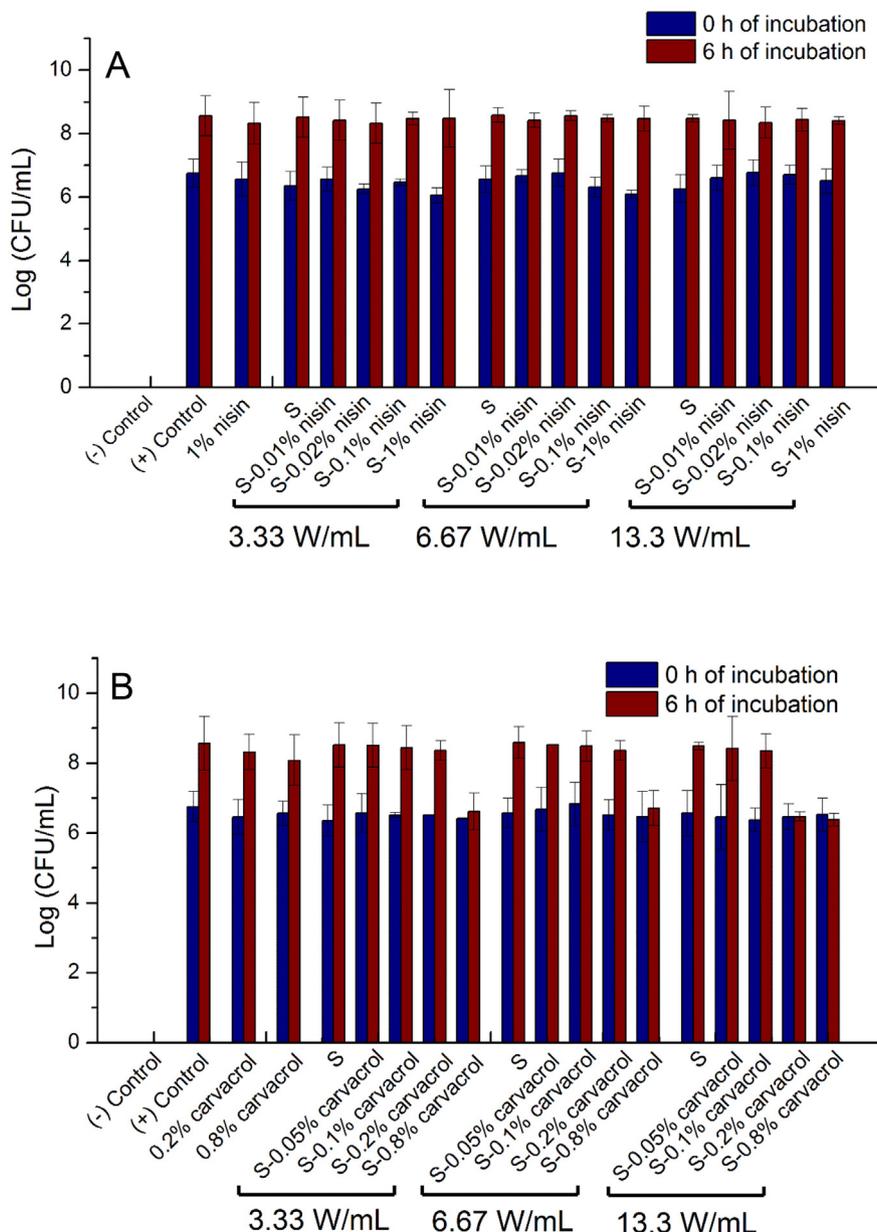
pretreatment-nisin/carvacrol stress conditions.

#### Declaration of competing interest

None.

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**Fig. 6.** Effects of the combined ultrasound pretreatment-nisin/carcacrol on the growth of *B. subtilis* spores in milk. Spores cocktail pretreated with ultrasound (S) were added different concentrations (% v/v) of nisin (A) or carvacrol (B) as indicated. Numbers of colony forming unit per mL (Log CFU/mL) of *B. subtilis* were determined before and after 6 h of incubation at 37 °C. Error bars represent standard deviation from the mean of three technical replicates.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijfoodmicro.2019.108329>.

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