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Ultraviolet-C inactivation and hydrophobicity of Bacillus subtilis and Bacillus velezensis spores isolated from extended shelf-life milk

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Abstract:	Bacterial spores are important in food processing due to their ubiquity, resistance to high temperature and chemical inactivation. This work aims to study the effect of ultraviolet C (UVC) on the spores of Bacillus subtilis and Bacillus velezensis at a molecular and individual level to guide in deciding on the right parameters that must be applied during the processing of liquid foods. The spores were treated with UVC using phosphate buffer saline (PBS) as a suspension medium and their lethality rate was determined for each sample. Purified spore samples of B. velezensis and B. subtilis were treated under one pass in a UVC reactor to inactivate the spores. The resistance pattern of the spores to UVC treatment was determined using dipicolinic acid (Ca-DPA) band of spectral analysis obtained from Raman spectroscopy. Flow cytometry analysis was also done to determine the effect of the UVC treatment on the spore samples at the molecular level. Samples were processed for SEM and the percentage spore surface hydrophobicity was also determined using the Microbial Adhesion to Hydrocarbon (MATH) assay to predict the adhesion strength to a stainless-steel surface. The result shows the maximum lethality rate to be 6.5 for B. subtilis strain SRCM103689 (B47) and highest percentage hydrophobicity was 54.9% from the sample B. velezensis strain LPL-K103 (B44). The surface hydrophobicity for all isolates was statistically significant (P <0.05). Flow cytometry analysis of UVC treated spore suspensions clarifies them further into sub-populations unaccounted for by plate counting on growth media. The Raman spectroscopy identified B4002 as the isolate possessing the highest concentration of Ca-DPA as well as a pattern between Ca-DPA concentration and lethality rate. The study justifies the critical role of Ca-DPA in spore resistance and the possible sub-populations after UVC treatment that may affect product shelf-life and safety. UVC shows a promising application in the inactivation of resistant spores though there is a need to understand the effects at the molecular level to design the best parameters during processing.

Highlights

- Ultraviolet C (UVC) have a considerable lethal action on the spores of spoilage *Bacillus subtilis* and *Bacillus velezensis.*
- There is a morphological heterogeneity in the spores of the isolates in reference to their sizes and shapes.
- Flow cytometry analysis of UVC treated spores reveals different sub-populations.
- Ca-DPA concentration and hydrophobicity of spores showed strain-to-strain variations.

Ultraviolet-C inactivation and hydrophobicity of *Bacillus subtilis* and *Bacillus velezensis* spores isolated from extended shelf-life milk

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ABSTRACT

Bacterial spores are important in food processing due to their ubiquity, resistance to high temperature and chemical inactivation. This work aims to study the effect of ultraviolet C (UVC) on the spores of *Bacillus subtilis* and *Bacillus velezensis* at a molecular and individual level to guide in deciding on the right parameters that must be applied during the processing of liquid foods. The spores were treated with UVC using phosphate buffer saline (PBS) as a suspension medium and their lethality rate was determined for each sample. Purified spore samples of *B. velezensis* and *B. subtilis* were treated under one pass in a UVC reactor to inactivate the spores. The resistance pattern of the spores to UVC treatment was determined using dipicolinic acid (Ca-DPA) band of spectral analysis obtained from Raman spectroscopy. Flow cytometry analysis was also done to determine the effect of the UVC treatment on the spore samples at the molecular level. Samples were processed for SEM and the percentage spore surface hydrophobicity was also determined using the Microbial Adhesion to Hydrocarbon (MATH) assay to predict the adhesion strength to a stainless-steel surface. The result shows the maximum lethality rate to be 6.5 for *B. subtilis* strain SRCM103689 (B47) and highest percentage hydrophobicity was 54.9% from the sample *B. velezensis* strain LPL-K103 (B44). The difference in surface hydrophobicity for all isolates was statistically significant ($P<0.05$). Flow cytometry analysis of UVC treated spore suspensions clarifies them further into sub-populations unaccounted for by plate counting

on growth media. The Raman spectroscopy identified B4002 as the isolate possessing the highest concentration of Ca-DPA. The study justifies the critical role of Ca-DPA in spore resistance and the possible sub-populations after UVC treatment that may affect product shelf-life and safety. UVC shows a promising application in the inactivation of resistant spores though there is a need to understand the effects at the molecular level to design the best parameters during processing.

Keywords : lethality rate, adhesion strength, Raman spectroscopy, dipicolinic acid

INTRODUCTION

Bacterial spores are dormant and adaptive structures able to cope with potential and destructive stresses and to survive harsh environmental conditions. This adaptation enables them to withstand a high-temperature, salt, extreme pH, radiation, low nutrient and desiccation. The germination of contaminating microbial spores in processed food result in spoilage or foodborne illness depending on the type of bacteria (Logan, 2012; Mugadza et al., 2019; Wells-Bennik et al., 2016). A spore is a multi-layer, highly dehydrated and dormant structure encapsulating different chemical compounds that offer resistance against environmental pressure. There is the presence of α/β-type small acid-soluble protein (SASP) and dipicolinic acid (DPA). The former protects the spore against DNA damage while the latter raises the spore wet heat resistance thus protecting core proteins against inactivation (Setlow et al., 2006). The various layers of spores such as the coat are designed to protect against toxic chemicals, lysozyme, desiccation and protozoan ingestion (McKenney et al., 2013). The spread of spores in the food chain is often inevitable thus making them a food safety challenge, especially in the dairy industry.

While the conventional pasteurisation of milk might be sufficient to eradicate most vegetative cells, the application of high temperature such as ultra-high temperature (UHT) is often required to inactivate bacterial spores present in food (Bressuire-Isoard et al., 2018). This results in an undesirable heated

milk flavour due to Maillard reactions (Mehta, 1980). Microfiltration followed by pasteurisation has been observed by Schmidt et al., (2012) to decrease bacterial counts by 5–6 $\log_{10}$ units to lower than 1 CFU/mL but cannot eliminate some spore formers especially in the case of low quality of the raw milk used and post-process contamination (Germain et al., 2013; Huck et al., 2008; Svensson et al., 2000, 1999).

Adhesion of these surviving spores to the wall of the processing equipment poses a big threat to the dairy industry due to the high resistance nature to processing conditions than what vegetative cells are susceptible to. They have been observed to be more hydrophobic with greater adhesive ability to food contact surface such as stainless steel than vegetative cells. The attached spores may then germinate, form biofilms and sporulate therefore contaminating the processing line with the consequential reduction in shelf-life of the processed product (Harimawan et al., 2013). This higher hydrophobicity of spores also, presumably, makes them be of a greater food safety concern than vegetative cells (Seale et al., 2008). Some of these spores are capable of growing at both psychrotrophic and mesophilic temperatures as observed by Mugadza and Buys (2017) thus making their control indispensable during processing. Frequent cleaning of the heat exchanger after the production cycle may reduce the severity of the contamination (Faille et al., 2013).

Non-thermal processing generally preserves the sensorial and nutritional qualities of food maintaining the fresh-like taste that is desirable to the consumers. The application of these emerging technologies to foods have been proven to exceed that of the thermally treated products in terms of quality, safety and shelf-life (Jermann et al., 2015). Such non-thermal processing technologies include ultra-high pressure homogenisation (UHPH) and ultraviolet C (UVC). They have shown a considerable lethal action against the spores of spoilage and pathogenic organisms in liquid foods with no known toxic residue. However, Gram-positive bacteria and their spores, such as *Bacillus* spp., are generally more resistant to UHPH than Gram-negative which can present a setback in its adoption (Smelt, 1998; Wuytack et al., 2002).

Ultraviolet (UV) radiation is a form of non-ionising, electromagnetic radiation occupying the region between visible light and X-ray (Bintsis et al., 2000). There are three different types of UV based on the wavelength of the spectrum with each causing a specific type of injury to the DNA in an overlapping manner. These are ultraviolet A (UVA) with a wavelength of 320nm to 400nm; ultraviolet B (UVB) with a wavelength of 290nm to 320nm and ultraviolet C (UVC) with a wavelength of 100nm to 290nm (Handan Baysal et al., 2013). UVC is approved by the United States Department of Agriculture (USDA) because it can inactivate a wide range of microbial contaminants in liquid foods and contact surfaces such yeasts, viruses, bacteria, moulds etc. (Gunter-Ward et al., 2018; Hu et al., 2017; Jennifer A Crook et al., 2015; Van Impe et al., 2018).

UVC exerts its effects through photons of light penetrating the cells/spores and causing a cross-linking of pyrimidine bases making them unable to replicate which results in the death of vegetative cells (Jennifer A. Crook et al., 2015). In bacterial spores, UVC exposure causes the formation of a spore photoproduct (5-thyminyl-5,6-dihydrothymine), cyclobutene pyrimidine dimers and breakage of single and double strands (Delorme et al., 2020). UVC effectiveness is influenced by several factors such as the type of the microorganisms, retention time, concentration of cells/spores, number of passes of product in the UVC reactor, product characteristics (Fan et al., 2017). Such intrinsic characteristics of the product that may affect UVC efficacy include turbidity, UV absorbance, presence of colour compounds and solid matters.

Although the application of UVC has been validated to be more energy efficient than high-temperature short-time (HTST) processing making it economically and commercially viable, nonetheless, some grey areas need further study before it gain wide acceptance (Rodriguez-Gonzalez et al., 2015). The disadvantage of UVC is that the effect decreases in liquid foods that are opaque or turbid hence the advice to design the UVC reactor to run in thin-film with a capacity for multiple passes (Koutchma, 2009). Besides, extreme UVC treatment may lead to significant changes in some properties of the food such as oxidation of protein and lipid components of processed milk. Hence, the need to apply UVC treatment in moderation during the treatment of liquid foods (Fernández et al., 2014).

This study seeks to determine the effect of ultraviolet C (UVC) on the inactivation of spores of *B. subtilis* and *B. velezensis* in phosphate buffer saline (PBS), the hydrophobicity of the spore surface and resistance pattern to UVC using dipicolinic acid (Ca-DPA) from Raman spectral analysis as chemical features. Also, the different sub-populations of spores will be determined after UVC exposure. This will guide in determining the right parameters that must be applied during the processing of liquid foods. *Bacillus* spp. was used in the study because of their ability of members to perpetuate within the processing environment through sporulation thereby causing enzymatic spoilage of pasteurised milk and illness in some cases (Caplan and Barbano, 2013; Wells-Bennik et al., 2016).

MATERIALS AND METHODS

Selection of *Bacillus* strains

Five *Bacillus* strains isolated from ESL processing plant in the Gauteng province of South Africa were used in the study (Elegbeleye and Buys, 2020). Sample collection was done as using 1 L of raw and packaged ESL milk stored at 5 ^{o}C. All samples were collected from the processing line in a total of four visits and plated out within 4 hours after collection. Matrix-assisted laser desorption ionisation time of flight (MALDI-TOF) mass spectroscopy was used in the preliminary identification of bacterial isolates. The strains were selected based on their ability to form good biofilms on the stainless steel surface and produce proteolytic activity on skim milk agar. The strains are *B. velezensis* strain LPL-K103 (raw milk), *B. velezensis* strain LPL-K103 (ESL milk), *B. subtilis* strain SRCM103689 (raw milk), *B. subtilis* strain ATCC 11774 (ESL milk), *B. velezensis* strain LPL-K103 (raw milk) and a control organism *B. subtilis* CECT 4002 from the Spanish Type Culture Collection (CECT, Universidad de Valencia, Spain). The spore of the control organism *B. subtilis* CECT 4002 is known for its resistance to UVC. The isolates were maintained in peptone broth (Merck, Darmstadt, Germany) containing 20% (v/v) glycerol at −80 °C.

Preparation and purification of spores

Overnight culture from a nutrient agar plate was inoculated into 10 mL volume of tryptone glucose broth (Oxoid Ltd., Hampshire, UK) and incubated for 24 h at 30 ^{o}C ±1. 1mL aliquot from the broth was spread over the surface of Campden Sporulation Agar (60 mL) in a Roux flask. The flasks were then incubated at 30 ^{o}C for 4 weeks. The sporulation process was monitored using phase-contrast microscopy. The spores were harvested after maturation by adding 40 mL of deionized water into each Roux flask. The surface of the agar-containing spores was scratched gently with wire loop with the liquid collected into a centrifuge tube. The centrifuge tube was heated in a water bath at 90 ^{o}C for 30 min and cooled in a cold water bath. The spore suspension was washed three times by centrifuging at 12000 g for 15 min at 4^{o}C. The pellet was resuspended in 20 mL deionised water and centrifuged again for 10 min and standardised to obtain a spore concentration approximately 1×10^{8} (≥ 90% purity) after which it was stored at 4 ^{o}C for later use (López-Pedemonte et al., 2003).

UVC treatment of spores in PBS

The treatment was carried out by running the spore suspension through a UVC reactor as described by (Reverter-Carrión et al., 2018) using 10 mL of inoculum in 1L of PBS. For the control (A_1), the spore suspension was run through the reactor without switching on the UVC lamp while it was switched on for the UVC treated spore suspension (A_2). The UVC parameters consisted of a single lamp of radiance 41 mW/cm^2 with a retention time of 10 seconds which corresponds to a dose in the matrix of 410 mJ/cm^2 or 3.9 J/mL) under 1 pass (20 ± 1 ^{o}C). The other parameters are flow rate (25.2 L/h), length of UVC lamp (765 mm), gap size between lamp and quartz sleeve (2 mm) cold air, gap size between the quartz sleeve and spiral (1 mm) with the volume of the reactor calculated to be 70 mL. Since the matrix was PBS buffer, it is assumed that the absorbance by the PBS is negligible and theoretically all the energy generated by the reactor has a strong effect on the bacterial spores. Treated and untreated samples were

collected aseptically in sterile bottles inside a laminar flow cabinet (Mini-V PCR cabinet Telstar, Terrassa, Spain) fitted with a stainless steel socket connected at the exit of the equipment.

Plate counting of UVC treated and untreated spores samples

The lethality was determined by taking the logarithmic difference between the UVC treated and untreated (control) spore suspension for all the isolates. 1 mL aliquot of the treated and untreated spore suspension was plated in trypticase soy agar (TSA) supplemented with 0.6% glucose (Merck, Darmstadt, Germany) using the pour plate method. The plates were incubated at 30 °C for 24 h to 48 h after which the germinated spores were enumerated. The inactivation of the spore samples by UVC was expressed as a lethality rate using the formula given below:

$$\text{Lethality rate} = \log_{10} \frac{A_1}{A_2}$$

Where A_1 is the number of untreated spores and A_2 the number of UVC-treated spores respectively.

Adhesion of spore to hydrocarbon (hexadecane)

The surface hydrophobicity of the spore samples was determined by adapting the microbial adhesion to hydrocarbon (MATH) assay as developed by (Rosenberg et al., 1980; Wiencek et al., 1990). Bacterial spore suspension (in distilled water) was adjusted to an optical density between 1.2 to 1.6 at 600 nm (OD_{600nm}). 3 mL each of hexadecane and suspension of spores was added together followed by vigorous mixing on a vortex mixer at room temperature for 60 s and then incubated at 30°C for 10 min. After 10 min of incubation, the suspension was agitated on a vortex mixer for 2 min and allowed to stand for 20 min at ambient temperature. The optical density of the aqueous layer was measured at OD_{600nm} using a spectrophotometer (Thermo Scientific, MA, US). The experiment was repeated three times in

duplicates (*n=6*). The percentage of cell surface hydrophobicity was calculated using the formula as provided:

$$\mathrm{RH} = \frac{OD\mathrm{initial} - ODresidual}{ODinitial} \times 100\%$$

Flow cytometry analysis of UVC-treated spore suspension

Flow cytometry was done on the UVC-treated spore suspension using 500 µL of treated and control samples with a cell concentration of approximately10^6 cfu/mL. The analysis was done using FACS Calibur flow cytometer (Franklin Lakes, NJ, USA) equipped with a 15 mW air-cooled 488 nm argon-ion laser and a 633 nm red diode laser. Side-angle-scatter (SSC-H), green fluorescence (FL1-H detector, 530/30 filter) and red autofluorescence (FL3-H detector, 670LP filter) were used to quantify the spores into different sub-populations. Double-distilled water was used as the sheath fluid (Coulter Corporation, Miami, FL, USA). Propidium iodide and SYTO 9 (Invitrogen, Mount Waverley, Australia) were added to 500 µL of the spore suspension to make a final concentration of 15 µM and 500 nM respectively (Park et al., 2013; Smelt et al., 2008). The stained suspensions were mixed and incubated in the dark at room temperature for 15 min before analysis. Data acquisition was done on a logarithmic scale set at a nominal flow rate of 1000 events s^{-1}. To discriminate the spores, the side scatter (SSC) threshold level was adjusted manually to 307 mV. The green emission from SYTO 9 (indicative of cortex hydrolysis) was collected through a 530 nm band-pass filter, and the red fluorescence from propidium iodide-staining (indicative of damage to the inner membrane) was collected through a 585 nm band-pass filter. The detected signals were amplified logarithmically and a gate generated in the dot plot of the forward scatter versus side scatter to differentiate spores from artifacts. (Borch-Pedersen et al., 2017; Mathys et al., 2007; Stiefel et al., 2015). Gates were drawn using purified spore sample that was untreated and heat treated sample at 121 ˚C for 1 h and 121 ˚C for 15 min. This was done in triplicate with either stained with SYTO 9, PI or SYTO 9+ PI. The operating software was BD CellQuest Pro (version 4.0.2; BD) (Mathys et al., 2007).

Morphology of spores using scanning electron microscopy

To visualise the morphology of purified spore, two preparations were made, and these are spores attached to a contact surface (stainless steel) and spores suspended in distilled water. The first preparation was done by adding the spore samples to centrifuge tubes containing PBS with stainless-steel (316L-0.90mm-2B PVC; dimension: 50 mm x 13mm) semi-submerged in the liquid. The tubes were then incubated at 30 ˚C for 24 h. After incubation, the stainless coupons were dipped three times to remove unattached spores and prepare for microscopy. The second preparation was done by adding some spore suspension into Eppendorf tubes which was then washed in 0.075M phosphate buffer. The buffer was removed, and 2.5% glutaraldehyde/formaldehyde solution was used to pre-fix the samples for 1 h (Reineke et al., 2013). The fixative was then removed after which the samples were washed 3 times in buffer. 1% osmium tetraoxide solution was later added to the samples and post-fixed for 1 h. Samples were washed 3 times for 15 min then centrifuged to get a pellet from each step. After the removal of the wash buffer, the spore samples were dehydrated using a graded series of ethanol (30%, 50%, 70%, 90% and 3x100%) for 15 min each. Samples were left in the last 100% ethanol for 30 min. Coupons were left in a 50:50 mixture of hexamethyldisilazane (HDMS) and 100% ethanol for 1 h. The same process was repeated for HDMS only for another 1 h. After the addition of an aliquot of fresh HDMS, coupons were left to dry. The coupons were coated with carbon before mounting on the Scanning Electron Microscope (Asahi et al., 2015; Jing et al., 2019; Rozali et al., 2017).

Raman spectroscopy

This analysis is a non-destructive and label-free imaging technique. 100 µL of purified spore suspension in distilled water was dispensed on microscopic coverslips. The samples were dried under vacuum (Thermo Scientific, USA). The spatially isolated analytes (spores) were then exposed to the excitation from the laser beam. The Raman spectrum was determined by using a laser with a power of 1.5 mW

and wavelength of 532 nm and at ×100 magnification. The acquisition time of 13 min was used to obtain a high-quality vibrational spectrum appropriate for the analysis. The spectral acquisition was done using a confocal Raman spectroscopy (WITec alpha300 R, Germany) and the method of Kong et al. (2012). The processing of the data derived from the analysis, comprising of the smoothing, averaging and peak heights, was performed with the WITec software.

Statistics

All experiments were performed three times in duplicate ($n=6$). Analysis of variance (ANOVA) was done using GraphPad Prism (version 8.0.2) using Dunnette multiple comparison hypothesis testing to determine significant differences between the treatments ($P \leq 0.05$). FlowJo (version 10 CL) software was used in analysing the flow cytometry data. Further analysis of the Raman spectra was done using the software OriginPro 2019 (version 9.6).

RESULTS AND DISCUSSION

Lethality rate of spore samples after UVC treatment

From our results, we confirmed the ability of UVC to either inactivate or injure *B. subtilis* spores as proven by other observations (Martinez-Garcia et al., 2019; Thi Tuyet Nhung et al., 2012; Zhang et al., 2014). The UVC treatment showed that the difference between the spore samples with a highest lethality rate of 6.5 and the lowest lethality rate of 3.4 is statistically significant (Figure 1). There was an overall significant difference at the $P < 0.05$ level in the lethality rate of spores F (5,6) = 44, P = 0.0001 with mean lethality rate and standard deviations for samples given as follows: B43 (M = 4.6, SD = 0.1), B44 (M = 4.2, SD =0.1), B47 (M =6.5, SD = 0.5), B50 (M = 3.4, SD =0.3), B52 (M =5.0, SD =0.1) and B4002 (M = 3.7, SD =0.1). Three of the samples have (B43, B47 and B52) had significant higher lethality rate when compared with the positive control whereas the rest of the samples exhibited

no difference. The spores of B50, from observation, proved to be the most resistant among the samples with the lowest lethality rate of 3.4 in PBS followed by the positive control (B4002) with 3.7. Lethality of spores generally increases with increasing dose of UVC applied during processing and decreased with the presence and type of pigmented compounds in spores or the suspending medium (Khaneja et al., 2009).

The absorbance coefficient of a medium such as PBS is quite low with a high penetration unlike in food matrices such as milk or fruit juices, therefore, the effect of the UVC is expected to be greater in PBS. Factors responsible for the low penetration and high absorption coefficient include organic matter, solutes and colour complexes present in the food which is absent in this present study (Sauceda-Gálvez et al., 2019; Guerrero-Beltrán and Barbosa-Cánovas, 2004). Consequently, to obtain treated products that possess the intended shelf-life under prolonged storage condition will require the processing of the product with UVC not as a stand-alone technique but in combination with other novel technologies. Another way to ensure the total inactivation of spores is to apply the UVC treatment at a dosage that guarantees maximum effect without alteration to the physicochemical and sensorial properties of the processed food. Besides, the UVC reactor can be equipped with an arrangement that creates a turbulent flow of product to ensure homogenous distribution creating a maximum exposure of the spores to the lethal effect of UVC during processing. Although UVC treatment is effective against the spores of diverse strains of *B. subtilis* complex group used, there is a variation in the effectiveness. What accounts for the differences in resistance or lethality may be the relative amount of Ca-DPA present in each spore which is a factor of genetics and other environmental factors such as growth medium used for the cultivation of the spores.

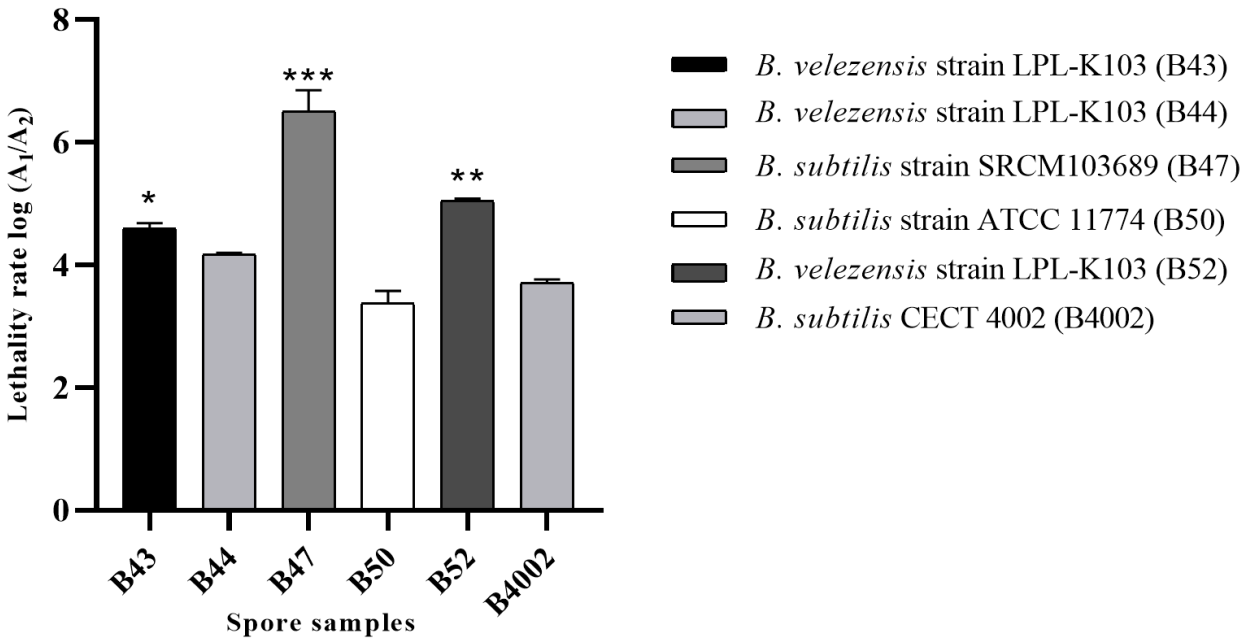

Figure 1: The lethality rate of *B. subtilis* and *B. velezensis* spores with phosphate buffer (PBS) as a suspending medium after treatment with UVC radiation (410 mJ/cm^2) for 10s at 20 ˚C. The treatment was performed three times and plated out in triplicate *(n=9)*. Differences in the lethality rate were determined using one-way ANOVA (*P*< 0.05). Asterisks (*) indicate significant difference among means (*=*P*≤ 0.05, **= *P* ≤ 0.01, ***= P≤ 0.001).

It may be practically challenging for UVC processing either as a stand-alone or in a combinatorial system to completely inactivate all spores present in food during processing based on the complexity of the food and presence of pigments. Though the present study was conducted in PBS, which is a clear medium without much interference to the inactivating effect of UVC, presumably, the result reflects what may be obtainable in liquid foods with little or no turbidity. A desirable level of commercial sterility may be achieved that is in tandem with all regulatory standards without compromising safety. The sterilization value is usually set above a margin that is considered safe while taking into consideration the history of contamination in the factory and the initial spore/bacterial load (Membré and van Zuijlen, 2011). This then could be done for a pathogenic *Bacillus* spp. such as *B. cereus* following their approach.

Previous study using the same strains besides the control organism showed their ability to grow at 6 ˚C although slowly (Elegbeleye and Buys, 2020). Some of the spores, though sub-lethally injured after exposure to UVC radiation, may have the possibility of germinating in the post-processed food such as

extended shelf-life (ESL) milk thereby compromising the quality of such product. This may occur especially under long storage or in any part of the food chain resulting in spoilage. This potentially dormant spore sub-population is not likely to be picked up using the traditional plate counting. Hence, the application of flow cytometric technique to account for the different sub-populations of the post-treated spores thus enabling the visualisation of the physiological diversity of the samples.

Flow cytometry analysis provided better clarity into the heterogeneity of spore sub-populations at single-cell levels. Dual-parameter (FL3 versus FL1) dot plots was constructed by plotting the parameters from the red autofluorescence detector (FL3-H/PI) against the green fluorescence detector (FL1-H/SYTO 9). Five distinct sub-populations were observed in the spores post-UVC treatment based on their uptake of the stains (PI/SYTO 9) and staining characteristics. The different heterogeneous sub-populations are presumably inactivated spores, live spores, dormant, sub-lethally injured spores and an unknown group whose physiological state cannot be readily deciphered.

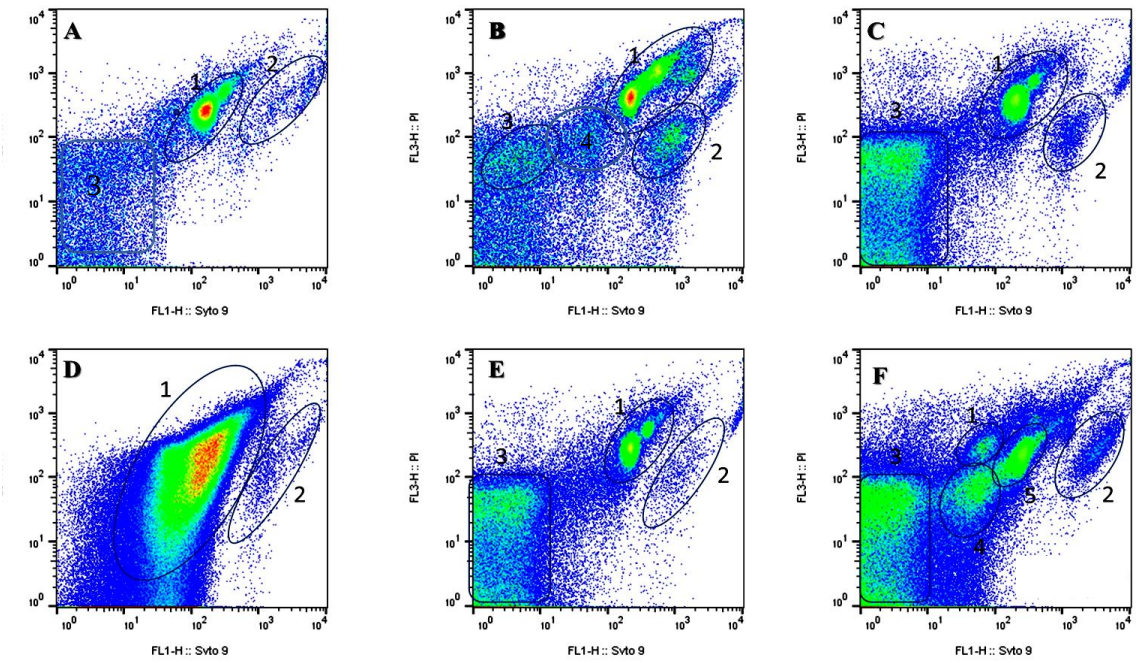

Figure 2: Flow cytometric analysis of UVC-treated *B. subtilis* and *B. velezensis* spores. All isolates are from raw milk except B50 and B44 which were isolated from packaged ESL milk stored at refrigeration temperature. Note the different clusters indicating the heterogeneity of the sub-populations after UVC treatments using density plots. (A) *B. velezensis* strain LPL-K103 (B43), (B) *B. velezensis* strain LPL-K103 (B44), (C) *B. subtilis* strain SRCM103689 (B47), (D) *B. subtilis* strain ATCC 11774 (B50), (E) *B. velezensis* strain LPL-K103 (B52), (F) *B.*

subtilis strain CECT 4002 (B4002). The numbers represent different spore sub-populations 1. inactivated spores 2. live spores 3. dormant 4. unknown 5. sub-lethally injured spores. The live sub-population may be because of induced germination by UVC.

Observable responses of the spore samples to the UVC dosage appeared to be strain-specific and varies from sample to sample as shown in Figure 2. The gating strategy was done using controls stained with either PI or SYTO 9 or the two at the same time on untreated and wet-heat inactivated spore samples as previously described.

The red fluorescent stain, PI, only gains entry into the spore in the case of damage while SYTO 9 can permeate the inner membrane (IM) of the spore in the absence of any damage or rupture of the spore. The degree of the fluorescence intensity to either one or both of the stains was used to classify the spores into the sub-populations. The intensity of the cytometric analysis using the dual-staining method of the spores reflected the degree of damage inflicted by the UVC radiation on the spore cortex as well as the outer membrane. This made all observations or sub-populations to be between PI positive or SYTO 9 positive. All spore samples have sub-populations that are inactivated, live and dormant in various degrees except sample B50 which is dominated by the inactivated and live sub-populations. Meanwhile, B44 had an unknown sub-population whose state cannot be clearly defined besides the inactivated and live sub-populations. The live sub-population is possibly a group of spores with UVC-induced germination. There is a no evidence of UVC-induced germination of spores but there are evidences of high pressure-induced germination of *Bacillus* spores which can happen with or without the presence of nutrients (Reineke et al., 2012; Shigeta et al., 2007; Wuytack et al., 2002).

In all, the flow cytometry analysis accounted for sub-lethally injured, inactivated, live, dormant and an unknown sub-population. The dormant and sub-lethally injured spore sub-populations are the potential risks in food because they are likely to germinate and contaminate post-processed product under storage and favourable growth conditions. In addition to this, both sub-populations are mostly undetected by the traditional enumeration method hence complicating spore quantification during sterilization test (Zhang and Mathys, 2019). Although, sample B50, which is the most resistant because it has the lowest

lethality rate, may likely exert its potential spoilage faster than B47 with a large percentage of dormant spores.

The sub-lethally injured sub-population presumably has it cortex damaged by UVC but within its intact inner membrane is an undamaged DNA allowing for a partial displacement of the stain SYTO 9 by PI. A probable explanation for the unknown sub-population according to Mathys et al., (2007) is that there was indeed an uptake of both PI and SYTO 9 through the UVC-permeabilised membrane though the spores are generally non-viable unlike in the case of the injured spores. The simultaneous uptake of the two dyes (PI and SYTO) within a spore or cell is known as fluorescence energy transfer (FRET) (Manoil et al., 2014).

Analysis of Ca-DPA domain by Raman spectroscopy

Raman scattering plays a role in revealing compositional variability and relative concentrations of functional groups that promote resistance of the spores to UVC treatment. A spectral range of 0 to 2000 cm^{-1} was used as observed in figure 3. The background noise was modelled and subtracted during the spectral analysis. Cauchy-Lorentzian distribution of the data identified the following vibrational features dominating the analytes. Table 1 summarises the Raman shifts and band assignments from major studies on the endospores of some microorganisms to be 661, 824, 1017,1395, 1555 and 1582 cm^{-1}.

The observed spectra are 216, 284, 394,495, 599, 659, 817 and 1300 cm^{-1} with som differences in samples B43, B44 and B4002. B44 lacks the 817 cm^{-1} and 659 cm^{-1} spectra that are common in the other samples besides B47. A comparison of the peak heights of the vibrational spectra of each analyte reveals that B4002 has a unique spectral signature with the maximum intensity among the spore samples which is an indication of the abundance of functional groups, especially of the Ca-DPA bands. This means that the relative concentrations of the compounds in B4002 (positive control), well known for its resistance to UVC, is more than the rest of the samples. Calcium dipicolinic acid (Ca-DPA) is one of

the notable compounds within a spore structure. It is a pyridine-2,6-dicarboxylic ring chelated to a divalent Calcium cation in 1:1 ratio (Magge et al., 2008). The characteristic Raman bands for the Ca-DPA domain are in the range of 824, 1017, 1395, 1446 and 1572 cm^{-1} in some *B. subtilis* and 662, 824, 1017, 1395, 1450, and 1572 cm^{-1} in some dormant spore of *B. cereus* as observed by Kong et al. (2017).

The intensities (*a.u.*) of the Ca-DPA bands of samples B50 and the control B4002 are higher than the average observed in other spores. Comparing the peak intensities of the Ca-DPA bands of samples B43 and B4002 reveals that there are relatively no significant differences in most of the bands except in the 1395 cm^{-1} spectrum with an intensity of 2984 a.u. and 5964 a.u. in B43 and B4002 respectively. However, there seem to be other contributors to the resistance of spores besides the Ca-DPA signature when a comparison is made between the samples B43 and B44 especially in the 200 to 700 cm^{-1} spectra. The other contributors adding to the peak intensities are likely compounds such as the amino acids e.g. tyrosine and phenylalanine. Juxtaposing the observations of samples B43 and B44 in figures 1 and 3 easily reveals this supposition. Despite B43 having a slight intensity in its Ca-DPA band especially in the 1395 cm^{-1} spectrum with an intensity of 2984 a.u., sample B44 has a lower intensity with 2074 a.u. but with significant higher intensities. At positions 218 cm^{-1}, 310 cm^{-1}, 400 cm^{-1} and 615 cm^{-1}, the intensities of B43 were 1732, 1882, 1712 and 1327 respectively whereas for at the same positions B44 has 2980, 3000, 2035 and 1617as the peak intensities.

B47 has its highest intensity as 1666 a.u which appeared to be the lowest intensity of Ca-DPA among all the samples. The position of the Ca-DPA in B47 is also different from the rest of the samples. While the rest of the samples have the highest concentration at wave number 1395 cm^{-1} B47 has its highest concentration at 1582 cm^{-1}. The differences in the band positions of Ca-DPA observed in the strains is linked to the physicochemical forms the compound takes within the spore structure (Jamroskovic et al., 2016). Besides Ca-DPA concentration, another unique thing about B47 is the relatively abundance of phenylalanine at wave number 992 cm^{-1}. Other peaks found in the sample besides the ones mentioned are 65 cm^{-1}, 123 cm^{-1}, 512 $cm^{-1}$and 670 cm^{-1} which mostly compounds such as amino acids present in different forms within the spore.

From all indications, we can conclude that the relative concentration of Ca-DPA in the spore core is a possible indicator of the level of resistivity exhibited by a specific spore to the deleterious effects of processing conditions such as UVC. Apparently, besides the presence of pigmentation in the spore and presence of other compounds, the concentration of Ca-DPA plays the most important role in the lethality or susceptibility of a spore to UVC exposure (Khaneja et al., 2009).

Table 1: Putative band assignments of the Raman bands in the spectrum of *B. subtilis and B. velezensis* spores

Raman shift (cm^{-1})	Band assignments
527	S-S stretch (cysteine in spore coat)
622	Proteins (phenylalanine)
638	C-S stretch (cysteine in spore coat)
661	Ca-DPA
725	DNA
780	DNA
824	Ca-DPA
864	C–C stretch (Proteins)
1004	Phenylalanine
1017	Ca-DPA (pyridine ring vibrations)
1155	Proteins (C–N, C–C) Carotenoids
1200–1244	Protein amide III
1336	DNA
1395	Ca-DPA(Carboxyl group: O–C–O symmetrical stretching)
1448	Proteins, Lipids and Ca-DPA (pyridine ring vibrations)
1485	DNA
1555	Ca-DPA, Proteins (alanine, glycine), Lipids, Amide II
1582	Ca-DPA, Proteins (phenylalanine, tyrosine)
1602	Lipids
1616-1624	Proteins (phenylalanine, tyrosine)
1645–1674	Protein amide I, Lipids

*Band assignments are from previous studies (De Gelder et al., 2007; Nelson et al., 2004; Noothalapati et al., 2016; Romano et al., 2018)

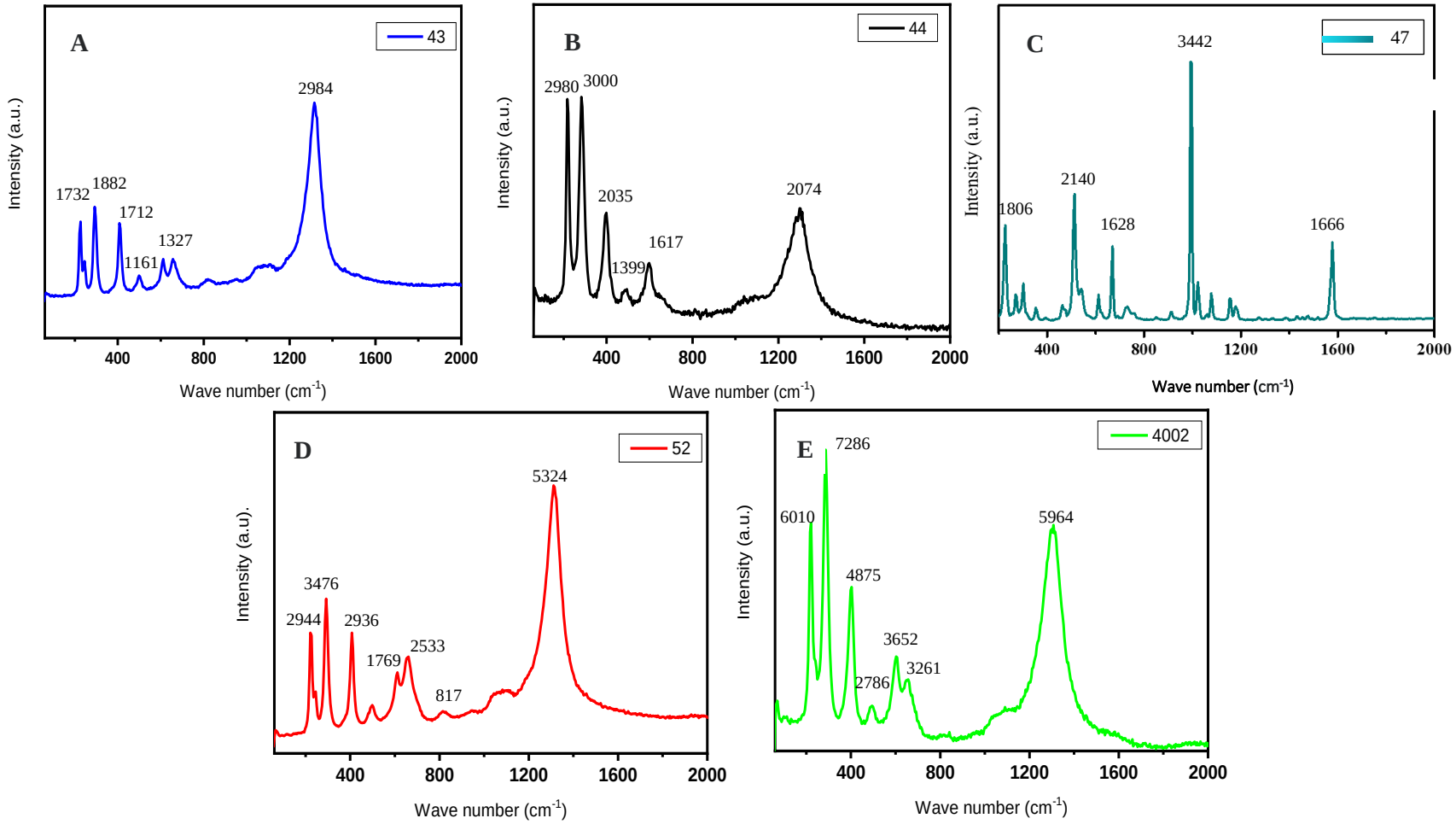

Figure 3: Raman spectroscopic analysis of vacuum-dried *B. subtilis* and *B. velezensis* spores with various peaks and intensities. The peak intensities indicate the relative abundance of some chemical compounds or signatures within the spore. (A) *B. velezensis* strain LPL-K103 (B43), (B) *B. velezensis* strain LPL-K103 (B44), (C) *B. subtilis* strain SRCM103689 (B47), (D) *B. subtilis* strain ATCC 11774 (B50), (E) *B. velezensis* strain LPL-K103 (B52), (F) *B. subtilis* strain CECT 4002 (B4002). Note the height of the peaks in samples B52 and B4002 which corresponds in a level to the result of their lethality rate post-UVC treatment.

Adhesion potential of spore on a food contact surface

A one-way between-groups analysis of variance was conducted to determine the differences in the percentage (%) hydrophobicity of the spores by comparing the control strain with the test samples. There was a statistically significant difference at $P < 0.05$ in the % hydrophobicity of spores F (5, 40) = 105, P = 0.0001. Post-hoc analysis was done using the Dunnette multiple comparison hypothesis testing. The mean % relative hydrophobicity for the spore samples are given as follows: B43 (M = 15.43 %, SD = 2.72), B44 (M = 54.86 %, SD = 1.68), B47 (M = 47.26 %, SD = 2.20), B50 (M = 35.20 %, SD =2.16), B52 (M = 27.3 %, SD =0.93) and B4002 (M = 38.11 %, SD =9.53). All isolates significantly differ in their % hydrophobicity when compared with the control (B4002) except sample B50 (see Figure 4). B44 has the highest % hydrophobicity with B43 having the lowest. The result implies that B44 is capable of greater adhesiveness to food contact surfaces such as stainless steel. Though both B43 and B44 are typed and identified to be *B. velezensis* strain LPL-K103, we observed that surface hydrophobicity is type-specific just like in the case of UVC treatment. Microbial spores are generally more hydrophobic with a greater zeta potential than the vegetative counterpart and thus adheres better to food contact surfaces. Since spores are generally more resistant to processing, such as thermal or high-pressure processing, their attachment on surfaces can readily initiate the development of biofilm consequently contaminating the downstream processing (Sadiq et al., 2018).

Apart from being a hydrophobicity assay, MATH also takes into consideration other interactions at play such as the van der Waals and electrostatic forces. These spore surface properties of adhesion and hydrophobicity are influenced by certain morphogenic protein components of the spore crust (Seale et al., 2008; Shuster et al., 2019). Processing conditions such as thermal processing are reported to increase surface hydrophobicity of surviving spores and hence promotes adhesion to food contact surface such as stainless-steel (Wiencek et al., 1990). It has also been reported that the variation in hydrophobicity of the stainless-steel substratum is also affected by prior chemical treatment (Boulange-Petermann et al., 1993). Though there are no reported cases of UVC increasing the hydrophobicity of

surviving spores after processing, the rule of the thumb is to apply UVC in combination with other novel processing technologies such as high pressure or with inhibitory compounds such as nisin.

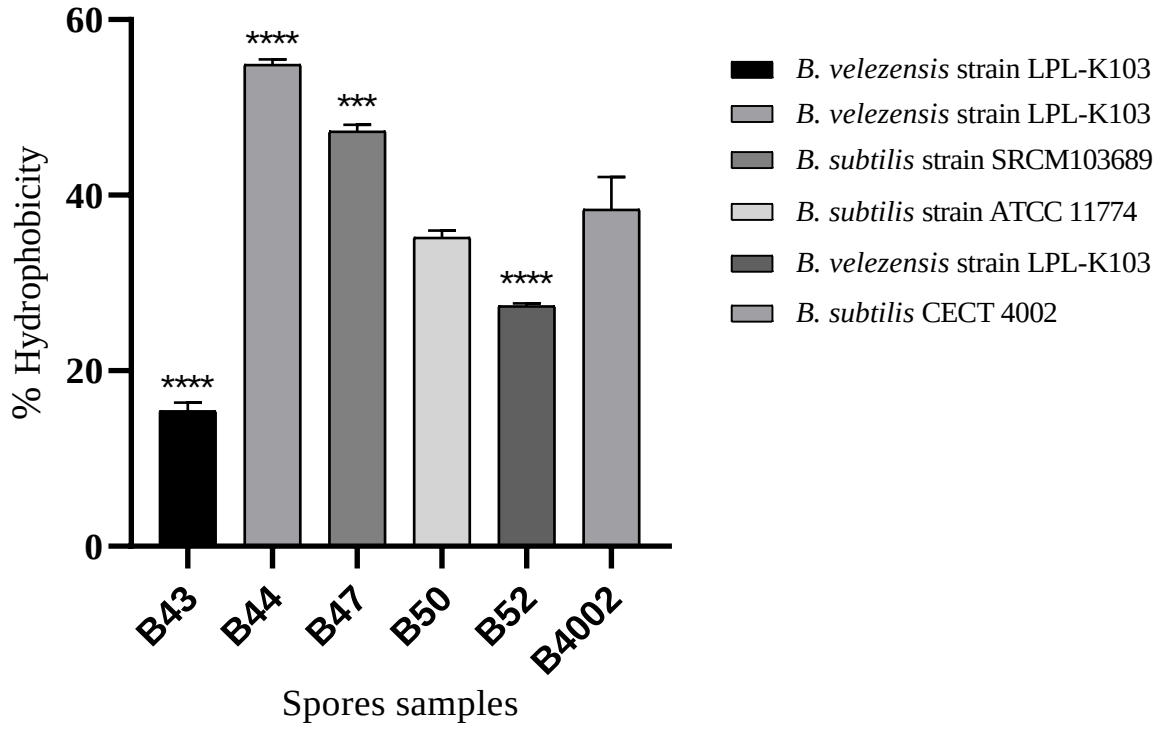

Figure 4. The adhesion of *B. subtilis* and *B. velezensis* spore samples to hexadecane (hydrocarbon) shown as % hydrophobicity. All isolates are from raw milk except B50 and B44 which were isolated from packaged ESL milk stored under refrigeration temperature. Statistical differences were done using Dunnett multiple comparisons for the post-hoc test ($P < 0.05$) by comparing all the spore samples with the positive control (*B. subtilis* CECT 4002). *B. velezensis* strain LPL-K103 (B44) has the highest % hydrophobicity with 54.9% and *B. velezensis* strain LPL-K103 (B43) with 15.4%. Samples with asterisks (*) indicate significant difference among means (*=$P \leq 0.05$, **= $P \leq 0.01$, ***= $P \leq 0.001$). Error bars indicate standard error of means of the population (SEM).

Figure 5 reveals the morphological heterogeneity among the strains in reference to their sizes and shapes under an electron microscope at a magnification of 2 µm in length. Though the differences in size are not conclusive, all samples have approximately the same size and a common elliptical shape associated with most bacilli except B50 (*B. subtilis* strain ATCC 11774) which is spherical-shaped with a bunch of grapes in appearance. Apart from variation in shape, sample B50 also has the smallest size with an average size of 1 µm in length in comparison with other samples except for the reference control which is slightly larger than sample B50. The rest of the samples are larger than the average spore size which

is typically 1.2 µm in length and 0.8 µm in width with none of the samples possessing an exosporium (Chada et al., 2003). The limitation of previous work is the failure carry out genetic typing of the sporulating organisms used in UVC study. The present work indicates that the response of bacterial spores to UVC and adhesion to food contact surface are likely strain-dependent. Nonetheless, the result of UVC-induced inactivation of *Bacillus* spores is consistent with other studies despite the application of different parameters.

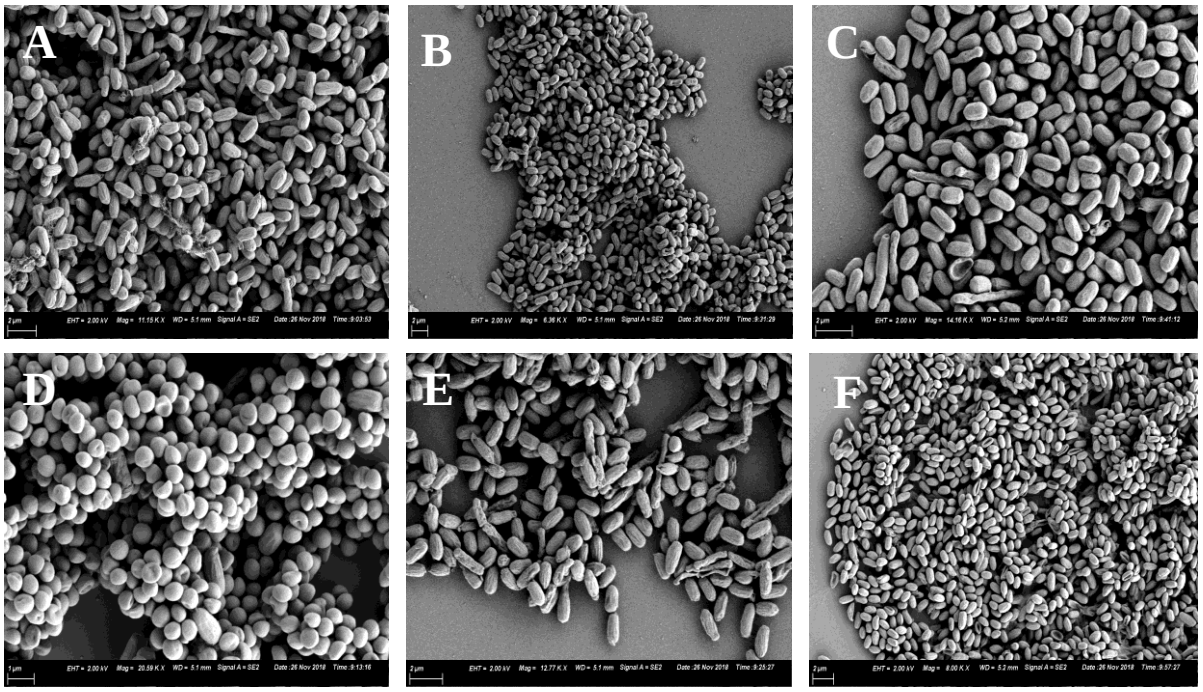

Figure 5: Scanning electron micrographs (SEM) images of strains of *Bacillus subtilis* and *Bacillus velezensis* on (glass) coverslips. Note the heterogeneity in spores morphology A (*B. velezensis* strain LPL-K103 B43), B (*B. velezensis* strain LPL-K103 B44), C (*B. subtilis* strain SRCM103689 B47), D (*B. subtilis* strain ATCC 11774 B50), E (*B. velezensis* strain LPL-K103 B52) and (f) *B. subtilis* strain CECT 4002**.** Note the spherical shape of sample D and its bunch of grape appearance which is different compared to other samples including the control. All samples (A, B, C, E and F) have the characteristic shape of a rod except sample D which has the shape of a bunch of grapes. All isolates are from raw milk except B50 and B44 which were isolated from packaged ESL milk stored at 5 ºC.

The spores can be seen under an electron microscope to be firmly attached to stainless-steel surface pre-treated by increasing the surface roughness as shown in figure 6. The attached spores can be differentiated from the vegetative cells and maturing biofilms as in figures 6A and 6C. The adhesion

was initiated by dispensing aqueous suspension of the spores into centrifuge containing 4.5 mL PBS and stainless-steel coupons (316L-0.90mm-2B PVC; dimension: 50 mm x 13mm) semi-submerged and incubated vertically at 30 °C for 24 h. Germination of the attached spores was induced by some residual milk on the surface of the coupons though in a limiting way to allows for differentiation of vegetative cells and their biofilms from the attached spores.

Adhesion of spores or their vegetative cells to an abiotic surface such as stainless steel or glass is a complex process with diverse interwoven environmental factors playing pivotal roles in the process such as surface roughness, surface charge, strain-type and other environmental factors (Bohinc et al., 2016). Although the type of organism has been linked to the attachment strength of bacterial spores, there is no evidence of the shape and size of a bacterial spore as a contributor to its attachment on substratum especially food contact surface (Faille et al., 2002).

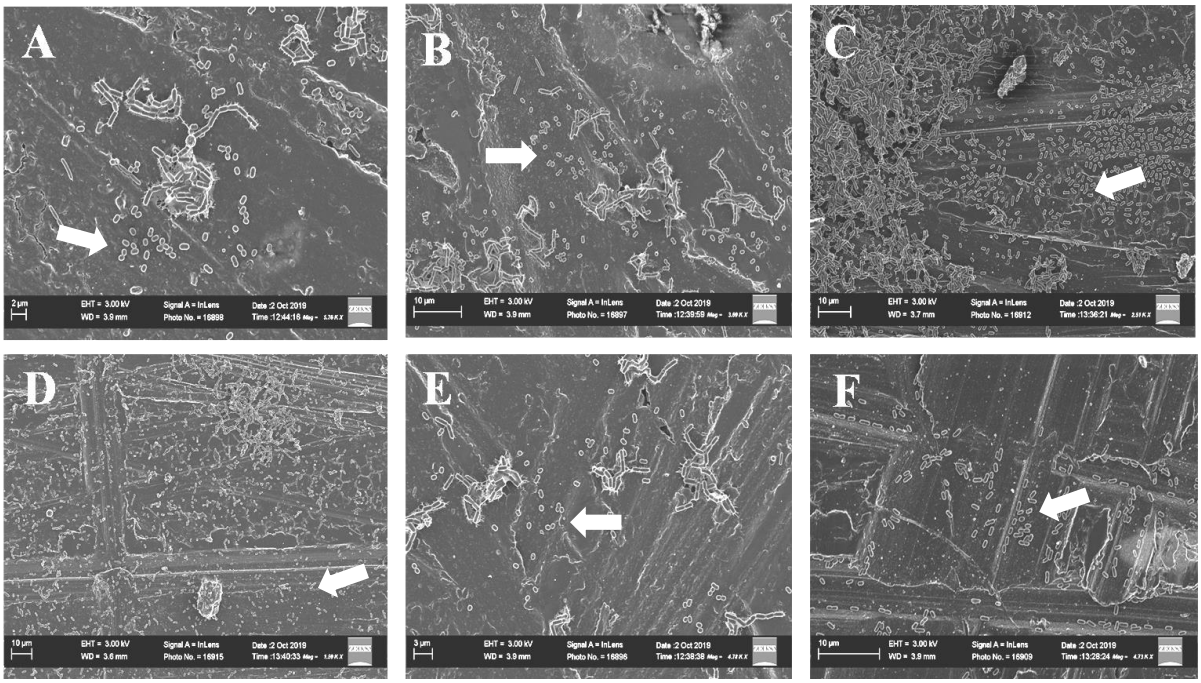

Figure 6: Scanning electron micrographs (SEM) of *B. subtilis* and *B. velezensis* spores attached to stainless-steel (grade 316L, 2B finish). Attachment of spores to the food contact surface is mainly influenced by factors such as hydrophobicity of the spore and nanotopography of the food contact surface. A (*B. velezensis* strain LPL-K103 B43), B (*B. velezensis* strain LPL-K103 B44), C (*B. subtilis* strain SRCM103689 B47), D (*B. subtilis* strain ATCC 11774 B50), E (*B. velezensis* strain LPL-K103 B52) and (f) *B. subtilis* strain CECT 4002**.** Arrows point to the attached spores as differentiated from vegetative cells whose germination was induced by residual nutrient and conditioning film.

Though the resistance of spores to UVC is presumably multifactorial, there is a trend that can be observed between the lethality rate and the result of the Raman spectral analysis. Lethality rate seems to increase with increasing intensity or concentration of Ca-DPA. For the lethality rate, the samples followed this order from the most resistance to the least B50, B4002, B44, B43, B47 (excluding B52) and for Ca-DPA concentrations the order is given from the most concentrated to the least as B50, B4002, B44, B43, B47. This observation is supported by a similar study in which *B. subtilis* spores were exposed to UV from solar radiation (Slieman and Nicholson, 2001). The heterogeneity in the content may be attributed to the size of the spore than the shape. It is safe to assume that the bigger the spore the more the concentration of Ca-DPA and hence the more the resistance to UVC (Huang et al., 2007).

Overall, B50 and B44 hypothetically have the greatest threat during processing. Specifically, B50 is the most resistant with a high concentration of Ca-DPA and a relatively good % hydrophobicity among all samples. This means more of the spores of B50 can potentially survive UVC exposure under the same conditions used in this study with good adhesion to the food contact surface. The implication of this is that the spores may germinate in the processed food thereby causing its spoilage during storage. The adhered spores may also form biofilms on food contact surface which may contaminate the processed food if their development is not properly handled using effective cleaning-in-place (CIP) regime. Sample B44 also has the potential of causing the same damage though with less severity compared to B50.

Despite the observation in the resistance of the spore samples, their presence can be mitigated and further inactivated by either increasing the retention time in the UVC reactor or having another run of the suspending medium. These are highly recommended especially in the case the food to be processed is milk due to the high turbidity and low penetration of the UV radiation in such medium. Besides the increased retention time and a second run of the processed food in the reactor, a mild agitation may also be incorporated in the design of the reactor to facilitate effective mixing and exposure of contaminating microflora to the lethal power of UVC. There is a need to further investigate the recovery ability of the dormant and injured sub-populations after UVC exposure, the process parameters that can potentially

inactivate all vegetative cells and spores without any alteration to the nutritional and sensory properties of different liquid foods with which UVC processing may find application.

Conclusion

Although UVC may have a promising application in the inactivation of spores in the processing of liquid foods, there is a need to understand the effects and responses at the molecular and individual level. The outcome of this study suggests that the adhesion of spore to hydrocarbon, UVC treatment and Ca-DPA spectral from the Raman analysis are mostly heterogeneous with some strain-to-strain variations as observed from the spectral characteristics. This factor must be taken into consideration when designing a UVC regime. UVC regime must be designed in such a way that almost all the spores and vegetative cells are inactivated without undesirable impact or alteration to the food. Apart from the interference of pigments to UVC penetration of a spore, the concentration of Ca-DPA in the spore possibly plays an important role in the lethality rate or resistance of spores to UVC. This assumption, though supported by the high concentration or intensity of Ca-DPA band in the positive control, is not conclusive. There is a necessity to further study the roles of Ca-DPA in the resistance of a bacterial spore to UVC. We proposed the utilisation of other methods (e.g. flow cytometry) besides plate counting on agar plates to analyse the response of spores to UVC. The information from the study will guide in deciding on the right parameters that can be applied during the processing of liquid foods. The study justifies the critical role of Ca-DPA in spore resistance and the possible sub-populations after UVC treatment that may compromise product shelf-life and safety.

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