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Potential spoilage of extended shelf-life (ESL) milk by *Bacillus subtilis* and *Bacillus velezensis*

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ABSTRACT

We aim to quantify and compare biofilm-induced proteolysis and lipolysis of Bacillus subtilis and Bacillus velezensis with that of the planktonic cells. Planktonic and biofilm cells were enumerated in a flow cytometer after incubation. The concentrations of proteolytic and lipolytic enzymes produced by the submerged biofilm and planktonic cells of the isolates were quantified. There were significant differences ($P < 0.05$) in the log counts of cells within the biofilms as well as concentrations of enzymes within biofilms and planktonic cells in UHT milk. The enumerated cells ranged from 5.6 log CFU/cm 2 for B168 to 7.4 log CFU/cm 2 for B48, respectively while the highest means of proteolysis and lipolysis were observed in B48 (1034 ρL/CFU) and B50 (34.5ρL/CFU± 0.44) respectively. The result of this study indicated that the spoilage potential for both biofilms and planktonic culture are strain-dependent. The implication to the industry is that weak biofilm formers have better spoilage potential than the strong biofilm formers in spore forming bacilli.

1. Introduction

About one-third of processed fluid milk in the United States is lost during the processing with an estimated cost of $6.4 billion (Buzby, Farah-Wells, & Hyman, 2014). Milk is a nutrient-rich diet widely consumed by humans of all age categories. These nutrient compounds also make it a potential medium for microbial contaminants to thrive causing some alterations in the sensorial property of the milk that is usually detrimental. The bacterial contaminants are usually from animal hides, feeds, air, soil, milking equipment (Quigley et al., 2013). Spoilage of milk can also occur as a result of post-pasteurisation contamination (PPC) due to bad hygiene practice or by process biofilms especially around the filler nozzle and other parts of the processing equipment (Alles, Wiedmann, & Martin, 2018). The consequence of these contamination leads to the reduction in the shelf-life of the product because of microbial spoilage which can be humongous on an economic and industrial-scale (González-Rivas, Ripolles-Avila, Fontecha-Umaña, Ríos-Castillo, & Rodríguez-Jerez, 2018; Reich et al., 2017).

We have demonstrated in the previous study the potential of *B. subtilis* and *B. velezensis* to contaminate processing line and form moderately to strongly adherent biofilms on stainless-steel surface (Elegbeleye and Buys, 2020). Bacterial (ComQXP and Rap-Phr) quorum-sensing and its sub-systems contribute significantly to the formation of biofilms and the expression of genes of the microbial cells within the biofilms through the production of chemical signals called autoinducers (Bareia, Pollak, & Eldar, 2018). Signal molecules, such as N-butanoyl-L-homoserine lactone, have been implicated in modulating the production of proteases within biofilms of *Aeromonas hydrophila* (Khajanchi et al., 2009; Yi et al., 2018). Biofilms have been implicated as a reservoir for enzymes produced by actively metabolising cells populating the community thus providing a microenvironment for such enzymatic activity (Khajanchi et al., 2009; Rosche, Li, Hauer, Schmid, & Buehler, 2009). These enzymes are associated with the periplasm of the microbial cells or secreted into the microenvironment either as inducible enzymes or through autolysis of cells populating the biofilms (Z.-W. Wang & Chen, 2009).

Apart from its structural role, the biofilm matrix entraps enzymes as well as other metabolites facilitating the hydrolysis of complex molecules such as lipids and milk protein causing the spoilage and reduction of shelf-life of processed milk (Fysun, Kern, Wilke, & Langowski, 2019). It is generally believed that such production of enzymes (per cell) is commonly higher within biofilms than in the planktonic cells of an isogenic strain. This assumption has been confirmed by a study of a mono-species biofilm of *B. licheniformis* R4 and *Pseudomonas fragi* BC5

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under a submerged condition in milk. The result revealed that proteolysis was greater in biofilms than in the planktonic cells (Teh et al., 2012). In another study, a similar pattern was observed for lipolysis in the mono-species biofilm *Staphylococcus aureus* SF01 (Teh et al., 2013).

Therefore, this study hypothesised that the biofilms of *B. subtilis* and *B. velezensis* will produce proteolysis and lipolysis within their biofilms exceeding that of their planktonic cells. The objective is to quantify the number of cells as well as the concentrations of the spoilage enzymes in the intact biofilms and planktonic cells of the isolates and to determine the proteolysis and lipolysis per cell. This is very significant to the dairy industry because a greater understanding of raw milk contamination and replication or outgrowth of resistant biofilms or endospores during processing will prevent negative impacts on the quality and shelf-life of extended shelf-life (ESL) milk products. Extended shelf-life (ESL) milk products generally have a shelf-life longer than that of high-temperature short-time (HTST) milk but without the burnt flavour commonly associated with ultra-high temperature (UHT) milk.

2. Materials and methods

2.1. Selection of Bacillus subtilis and Bacillus velezensis isolates

Sample collection was done in the ESL milk processing line as previously described by Elegbeleye and Buys (2020). The total number of isolates was 12 and they were obtained from raw milk, pasteurized milk, packaged ESL milk, and ESL milk collected directly from the aseptic filler nozzle from the Gauteng province of South Africa. The reference strains used were obtained from the *Bacillus* Stock Centre of Ohio State University. These reference strains are *B. subtilis* 168 (ATCC 23857) which is a poor biofilm-former and a wild-type *B. subtilis* 3610 (ATCC 6051) which is a moderate biofilm-former.

2.2. Enzymatic screening of Bacillus subtilis and Bacillus velezensis on skim milk agar

The determination of extracellular protease production was done on agar plates by modifying the method used by Vijayaraghavan and Vincent (2013). *B. subtilis* and *B. velezensis* isolates were screened using skim milk agar (2% agar, 10% skim milk, 0.25g yeast extract, 0.1% glucose in water). The media was autoclaved for 5 min at 121 °C after which 0.028% of bromocresol green was added. A 100 μL suspension of an overnight bacterial culture grown in tryptose soy broth (TSB) (Oxoid, Basingstoke, UK) incubated at 30 °C was dispensed on plates containing the skim milk agar to form concentric circles of approximately 1 cm in diameter. The plates were incubated for 24 h and the diameter of the zone of hydrolysis for each bacterial isolate was measured in cm.

2.3. Growth of Bacillus subtilis and Bacillus velezensis planktonic cells

The planktonic cells were prepared using 18 h bacterial culture grown in TSB (Oxoid, Basingstoke, UK) incubated at 30 °C. The bacterial culture for each isolate was standardised to 1.0 MacFarland. 0.5 mL of standardised inoculum was pre-incubated in 4.5 mL of UHT milk for 1h for each of the twelve test organisms and the two reference strains. 0.1 mL of the pre-incubated culture was dispensed in another centrifuge tube containing UHT milk and incubated for 24 h at 30 °C after which the total number of cells in the culture was enumerated. The same process was repeated for the proteolytic and lipolytic assays.

2.4. Growth of Bacillus subtilis and Bacillus velezensis biofilms

To grow the submerged biofilms, 0.5 mL of the pre-incubated culture for each isolate was dispensed in a centrifuge tube containing 4.5 mL of the UHT milk. A modified stainless-steel coupon (316L-0.90, 2B PVC; dimension: 50 × 13 mm) was semi-submerged in the centrifuge tube containing UHT milk as a substratum for the biofilm. The length of the semi-submerged coupon was 25 mm within the centrifuge tube containing the milk. The tubes were incubated for 24 h at 30 °C after which the cells within the biofilms were quantitatively determined. An uninoculated coupon in a centrifuge tube was used as a control (Teh et al., 2012).

2.5. Enumeration of cells within biofilms and planktonic culture using flow cytometer

For the enumeration, the stainless-steel coupon containing adhered biofilm was removed from the centrifuge tube containing UHT milk. The coupon was immersed three times in sterile distilled water to wash off unattached cells. The cells within the biofilms were detached according to the method of Wahlen et al. (2018). Each coupon was transferred into a centrifuge tube containing 9 mL phosphate buffer and some pieces of 6 mm glass beads. This was followed by a series of 30 s five steps of vortexing and sonicating in that order. The sonication was done using a sonicator at 40 kHz (Grant Instruments, UK). 2 mL of the samples were stained with SYTO 9 to a concentration of 500 nM and allowed to stand for 15 min and the cells were later enumerated using flow cytometer (Accuri, BD Biosciences, NJ, USA). This was done for the biofilm and planktonic samples. The enumerated cells were expressed as log CFU/cm^2 and log CFU/mL for biofilms and planktonic cells respectively (Z. Wang et al., 2020; Winkelströter, Teixeira, Silva, Alves, & De Martinis, 2014).

2.6. Spoilage potential of biofilms and planktonic cells of Bacillus subtilis and Bacillus velezensis

2.6.1. Proteolysis

The proteolysis assay was performed using the azocasein method of Bussamara et al. (2009). A 3% azocasein solution (Sigma-Aldrich, St. Louis, USA) was prepared in 5 mM phosphate buffer solution, pH 7.5 with 0.1% sodium azide (Sigma-Aldrich, St. Louis, USA) and 0.1 mg chloramphenicol/mL (Sigma-Aldrich). After the incubation of the biofilm and planktonic cells, the stainless-steel coupons were transferred from the centrifuge tube containing UHT milk into 9 mL of the 3% azocasein solution while the planktonic cultures were centrifuged at 10, 000 g for 5 min. 0.1 mL of the supernatant was transferred into 0.9 mL of the 3% azocasein solution in another centrifuge tube which was then incubated at 40 °C for a period of 24 h. After the incubation, 0.4 mL of the azocasein solution was mixed with 0.8 mL of 20% trichloroacetic acid (Merck, Darmstadt, Germany) to stop the reaction process. The mixture was centrifuged at 10,000 g for 5 min. A volume of 0.15 mL of the supernatant was transferred into microtitre plate wells (Thermo Scientific, Massachusetts, US) in six replicates and the absorbance was read at 405 nm (Thermo Scientific, Massachusetts, US). The same thing was done with the control which is the uninoculated centrifuge tube containing UHT milk.

The amount of proteolysis was determined by comparing the absorbance value of the samples with the absorbance value of the standard curve of the proteolysis by *Streptomyces griseus* (3.5 units/mg; Sigma-Aldrich, St. Louis, USA). The approximate concentration of the proteolysis observed was divided by the number of bacterial cells attached to the stainless-steel substratum or in the planktonic cultures. The data were expressed as picolitre of proteolysis per CFU (ρL/CFU) for proteolysis and lipolysis assays.

2.6.2. Lipolysis

The amount of lipolytic enzyme produced by the isolates was determined by using *p*-nitrophenol palmitate (*p*-NPP) assay. This involves the utilization of 0.05% solution of nitrophenol palmitate as described by Teh et al. (2013). This solution was prepared by adding 5 mg of *p*-NPP in 1 mL of isopropanol and 9 mL of 50 mM Tris–HCl solution, pH 8 containing 40 mg of Triton X-100 and 0.2 mg of Arabic gum with 0.1% sodium azide and 0.1 mg chloramphenicol/mL

(Sigma-Aldrich, St. Louis, USA). The stainless-steel coupons containing biofilms were dipped three times in sterile distilled water to wash off unattached cells after incubation. The coupons were then transferred into another set centrifuge tubes containing 9 mL each of the 0.05% *p*-NPP solution while 0.1 mL of the planktonic cell suspension was added to 0.9 mL of the same solution in an Eppendorf tubes. Both the centrifuge tubes containing the coupons and Eppendorf tubes with the cell suspension were further incubated for 8 h for the enzyme-subtrate reaction to occur. After the incubation, 0.6 mL of 96% ethanol was added to 0.6 mL of the 0.05% *p*-NPP solution to halt the reaction. The resulting solution was centrifuged at 10 000 g for 5 min and the absorbance read at 405 nm in a microtitre plate reader (Thermo Scientific). The amount of lipolysis per cell was estimated as described for the proteolysis assay.

2.7. Statistical analysis

All experiments were carried out independently and repeated at least three times. Analysis of variance (ANOVA) was done using GraphPad Prism (version 8.0.2) and the Dunnett multiple comparison hypothesis testing was used to determine significant differences between the isolates and concentration of enzymes produced per cell for the biofilm and planktonic cells respectively with a probability of $P \leq 0.05$. FlowJo (version 10 CL) software was used in analysing the flow cytometry data. The result of the enzyme assay was expressed as picolitre of proteolysis/lipolysis per CFU (ρL/CFU) for both biofilm and planktonic cells.

3. Results

3.1. Extracellular protease production on skim milk agar

All the bacterial isolates produced zone of proteolysis on skim milk agar in varying degrees. This was observed as a transparent clearing in the middle of the bluish cloud of the skim milk agar. The 0.028% of bromocresol green dye made the media blue which turned greenish with increasing incubation time. The dye allowed for a marked distinction of the clearing of the milk proteins from the surrounding medium which made the measurement easier. In all, there were significant differences in the extracellular production of protease among the isolates ($P < 0.05$) with $F_{(13,112)} = 48.82$, after the incubation time of 24 h on skim milk agar at 30 °C. The characteristic cloudiness of milk within the media disappeared to give rise to a dark blue clearing. From the result, B50 has the highest mean zone of hydrolysis of 2.10 ± 0.11 while the negative control, B168, has the lowest with 1.27 ± 0.06 (see Fig. 1).

3.2. Enumeration of biofilms and planktonic cells

Enumeration of all the cells, regardless of their physiological and morphological states within the biofilms and planktonic cells, is given as density plots (see Fig. 8). All *Bacillus* isolates used in this study produced biofilms *in vitro* with stainless-steel coupons as the substratum. All isolates exhibited different biofilm-forming potentials at 30 °C after the incubation time of 24 h with UHT milk as the growth medium. There was an overall significant difference at $P < 0.05$ in the log counts of cells within the biofilms on the stainless-steel surface $F_{(13,14)} = 23.4$, $P < 0.0001$. From Fig. 2, the total number of colonising bacterial cells retrieved from the coupon surface from the stainless-steel surfaces enumerated using a flow cytometer ranged from 5.6 log CFU/cm^2 ($\sigma = \pm 0.08$) for B168 to 7.4 log CFU/cm^2 ($\sigma = \pm 0.08$) for B48, respectively.

For the planktonic samples, the enumeration of the cells was expressed as log CFU/mL. There was no significant difference in the total number of cells at $P < 0.05$, $F_{(13,14)} = 44$, $P = 0.45$. The highest population mean for the planktonic culture was $\log_{10}$ 7.6 CFU/mL (B52) and the lowest was $\log_{10}$ 7.2 CFU/mL (B48) (see Fig. 3).

3.3. Proteolysis in biofilm and planktonic cells

All the *Bacillus* isolates produced some degrees of proteolysis in the biofilms and planktonic cells with both results expressed as picolitre of proteolysis per CFU (ρL/CFU). For the planktonic cells, the result of the proteolytic assay showed that there was an overall significant difference at $P < 0.05$ level in the proteolysis per planktonic cell $F_{(13,14)} = 4001$. From Fig. 4, the observed highest and lowest means of proteolysis among isolates range from 1034 ρL/CFU± 11.37 in B48 and 178 ρL/CFU± 0.45 in B52, respectively.

Unlike in the planktonic culture, there is an observable little amount of proteolysis produced within the biofilms of the isolates. There was an overall significant difference at $P < 0.05$ level in the proteolysis per cell within the biofilms $F_{(13,14)} = 306.1$, $P < 0.0001$. From Fig. 5, the biofilm of the B168 produced the highest amount of proteolytic enzymes per cell which is conspicuously higher than in its planktonic form. Overall, the planktonic cell produced significantly more proteolysis per cell than cells within the biofilms under a condition of nutrient abundance for most of the isolates except for B168 and B3610 which are the negative and positive control strains.

3.4. Lipolysis in biofilm and planktonic states

All the isolates produced lipolysis but in smaller amount when

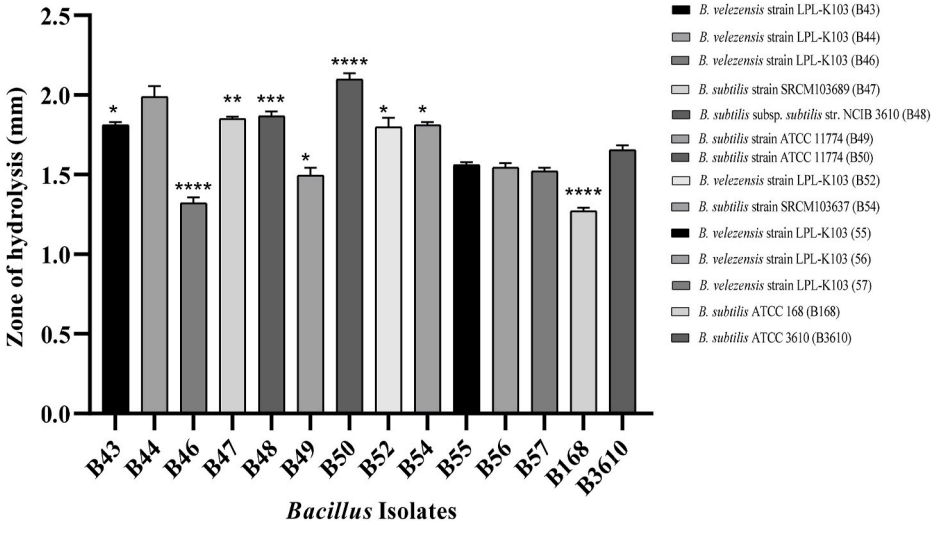

Fig. 1. The zone of hydrolysis in millimetres originating from 100 μL overnight bacterial culture standardised to 0.5 MacFarland (1 cm in diameter) on skim milk agar. Samples with asterisks (*) indicate significant differences among means, $P \leq 0.05$ (Dunnett hypothesis testing). The asterisk (*) means that * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$ and **** = $P \leq 0.0001$. All of the experiments were done in duplicates with at least three independent experiments. Error bars show standard error of means (SEM) of the bacterial population.

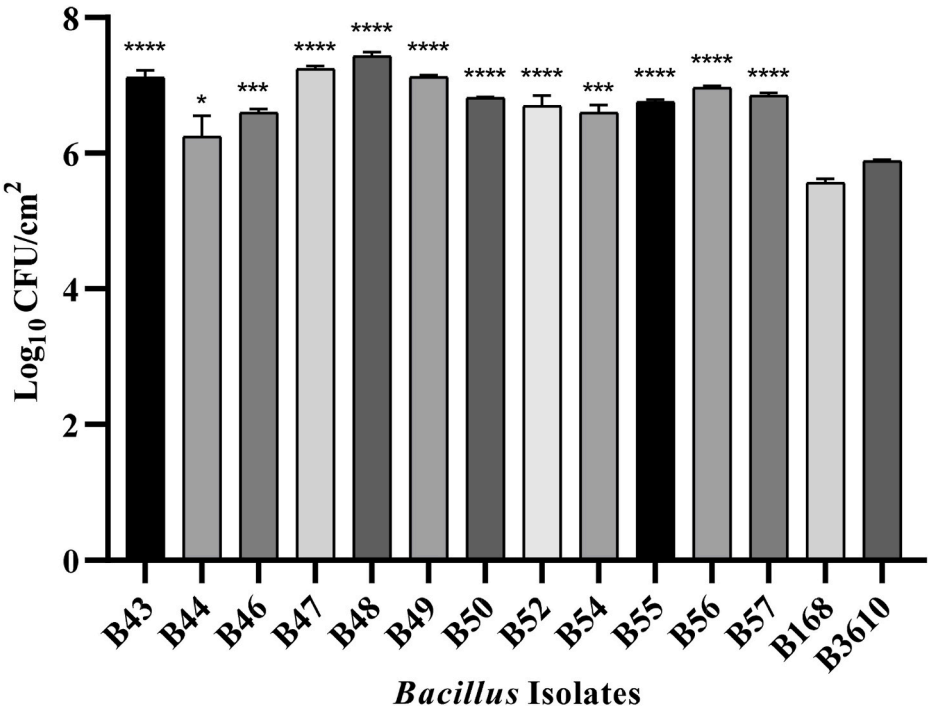

Fig. 2. The number of cells within *in vitro* biofilms formed on stainless steels semi-submerged in UHT milk grown at 30 °C. The highest population means for the biofilm model Log_{10} 7.4 CFU/cm^2 for B48, and the minimum was 5.6 log CFU/cm^2 for B168. Post-hoc analysis was done using Fisher's LSD test with a critical probability of $P \leq 0.05$. The asterisk. Samples with asterisks (*) indicate significant differences among means, $P \leq 0.05$ (*) means that * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$ and *** = $P \leq 0.0001$. All experiments were done in duplicates with at least three independent experiments. Error bars show standard error of means (SEM) of the bacterial population within the biofilm.

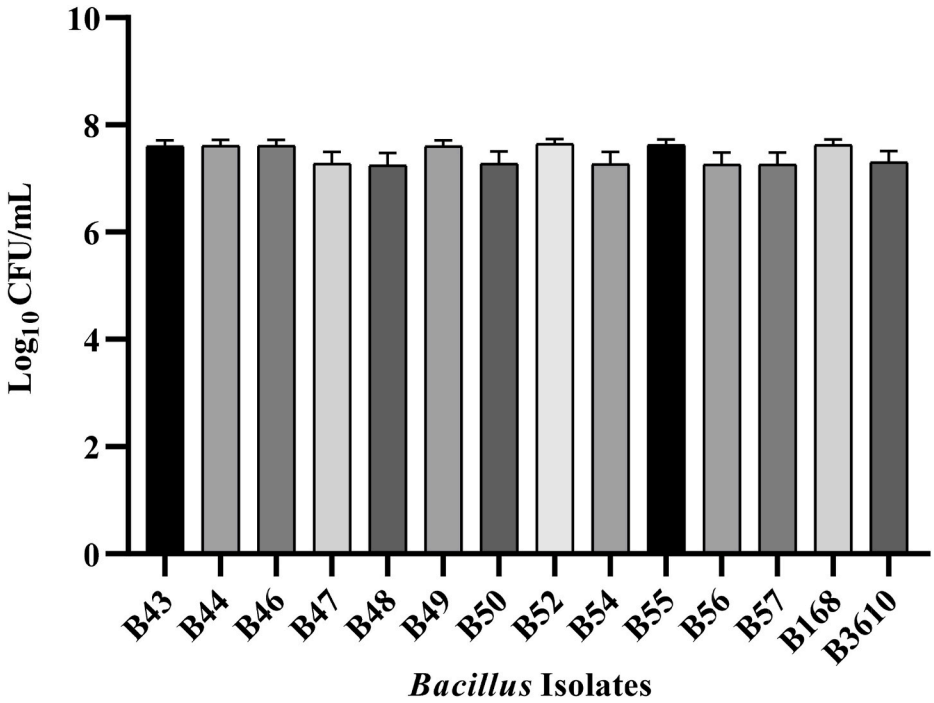

Fig. 3. The number of cells within planktonic cultures grown in UHT milk grown at 30 °C. The highest population mean for the planktonic culture was log_{10} 7.6 CFU/mL (B52), and the lowest was log_{10} 7.2 CFU/mL (B48). Post-hoc analysis was done using Fisher's LSD test with a critical probability of $P \leq 0.05$. All experiments were done in duplicates with at least three independent experiments. Error bars indicate standard error of the means (SEM) of the planktonic culture.

compared with the proteolysis. The result shows there were significant differences between the lipolysis produced within the biofilms and the planktonic cells except in some isolates such as B47, B48, B49 and B56. In the planktonic cells, there was an overall significant difference at $P < 0.05$ level in the lipolysis per cell $F_{(13,14)} = 7001$, $P < 0.0001$ with the observed highest and lowest mean of lipolysis being samples B168 (1.13 ρL/CFU± 0.002) and B50 (34.5ρL/CFU± 0.44) as shown in Fig. 6.

Hydrolysis of the *p*-NPP substrate was greater within the biofilms compared to the planktonic cultures in some of the isolates. An example is found in isolate B168 with a quantifiable lipolysis within its biofilms that is more than hundred and fifty times greater than its planktonic cell as observed in Fig. 7.

4. Discussion

This study confirms the ability of *B. subtilis* and *B. velezensis* to secrete

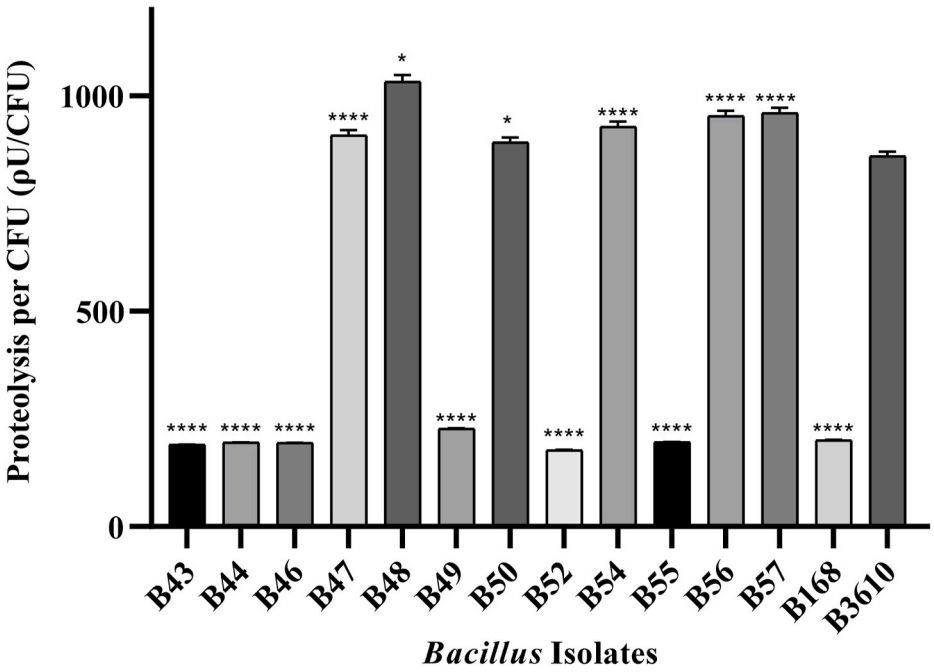

Fig. 4. The proteolysis of planktonic culture in UHT milk grown at 30 °C as picolitre of proteolysis per CFU (ρL/CFU). The highest and lowest mean proteolysis was observed in B47 (1034 ρL/CFU) and B52 (178 ρL/CFU) respectively. Post-hoc analysis was done Dunnett multiple comparisons with a critical probability of $P \leq 0.05$. Error bars indicate the standard deviation of the means (SD). Asterisks (*) indicate significant difference among means (* = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$ and **** = $P \leq 0.0001$).

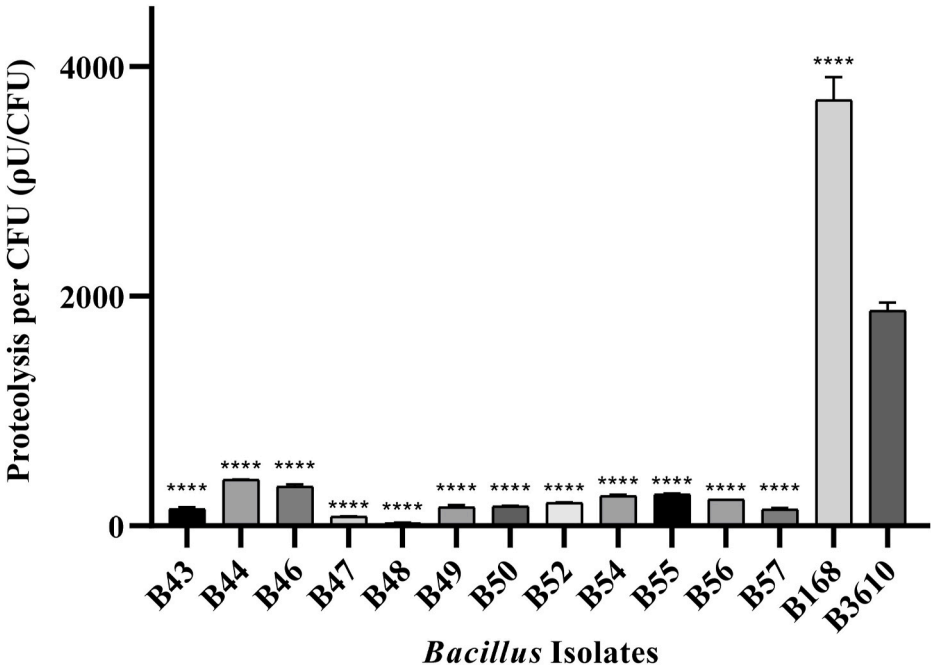

Fig. 5. The proteolysis within the biofilms on stainless-steel substratum in UHT milk grown at 30 °C expressed as picolitre of proteolysis per CFU (ρL/CFU). The highest and lowest mean proteolysis was observed in B168 (3706 ρL/CFU) and B48 (27 ρL/CFU) respectively. Post-hoc analysis was done Dunnett multiple comparisons with a critical probability of $P \leq 0.05$. Error bars indicate the standard deviation of the means (SD). Asterisks (*) indicate significant difference among means (* = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$ and **** = $P \leq 0.0001$).

proteolytic and lipolytic enzymes in both biofilm and planktonic states in a manner that is strain-dependent in ESL milk. This observation is consistent with previous works on the ability of planktonic cells as well as biofilms to produce lipolysis and proteolysis which may impact on the quality of processed milk (Ribeiro Júnior et al., 2017; Schmidt, Kaufmann, Kulozik, Scherer, & Wenning, 2012; Teh et al, 2012, 2013, 2014). However, the results, under the assay conditions, showed that proteolysis and lipolysis in the planktonic and biofilms states may be strain-dependent besides their biofilm-forming potential. We compared the quantity of proteolysis and lipolysis within intact biofilms and planktonic cells of *B. subtilis* and *B. velezensis* contaminants of milk at 30 °C. Previous studies mostly evaluated the crude enzymes that were produced within the intact biofilms and planktonic in a temperature-dependent manner using the plate count method to enumerate the number of microbial cells (Glück et al., 2016; Teh et al., 2012; 2013). This study employed the use of flow cytometer to enumerate the cells within the biofilm and planktonic culture to prevent the challenge of under-enumeration that is often associated with the plate count method. This ensures that all possible sub-populations of cells within the biofilms are enumerated using the flow cytometer. Such subpopulations may include viable but putatively non-culturable (VPNC) cells such as persister cells and dormant spores which may not be able to grow and enumerated on the conventional growth media (Z. Wang et al., 2020). This approach allows for the enumeration of cells that may be damaged during the detachment of cells within the biofilms using vortexing (with beads) and mild sonication. The DNA binding

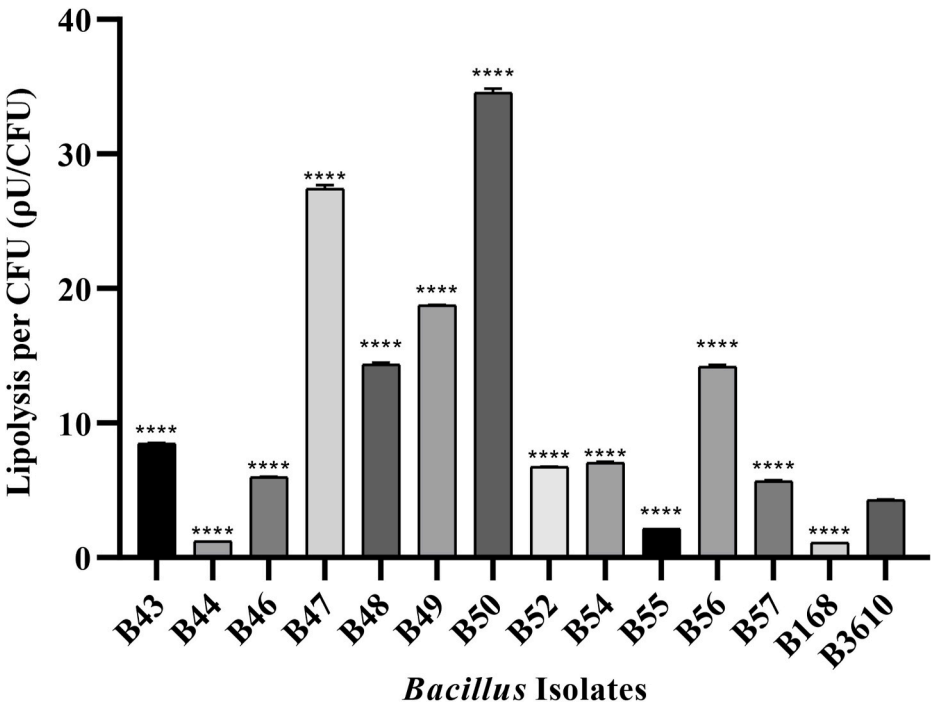

Fig. 6. The lipolysis of planktonic culture in UHT milk grown at 30 °C expressed as picolitre of proteolysis per CFU (ρL/CFU). The highest and lowest mean proteolysis was observed in B50 (34.54 ρL/CFU) and B168 (1.13 ρL/CFU) respectively. Post-hoc analysis was done Dunnett multiple comparisons with a critical probability of $P \leq 0.05$. Error bars indicate the standard deviation of the means (SD). Asterisks (*) indicate significant difference among means (* = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$ and **** = $P \leq 0.0001$).

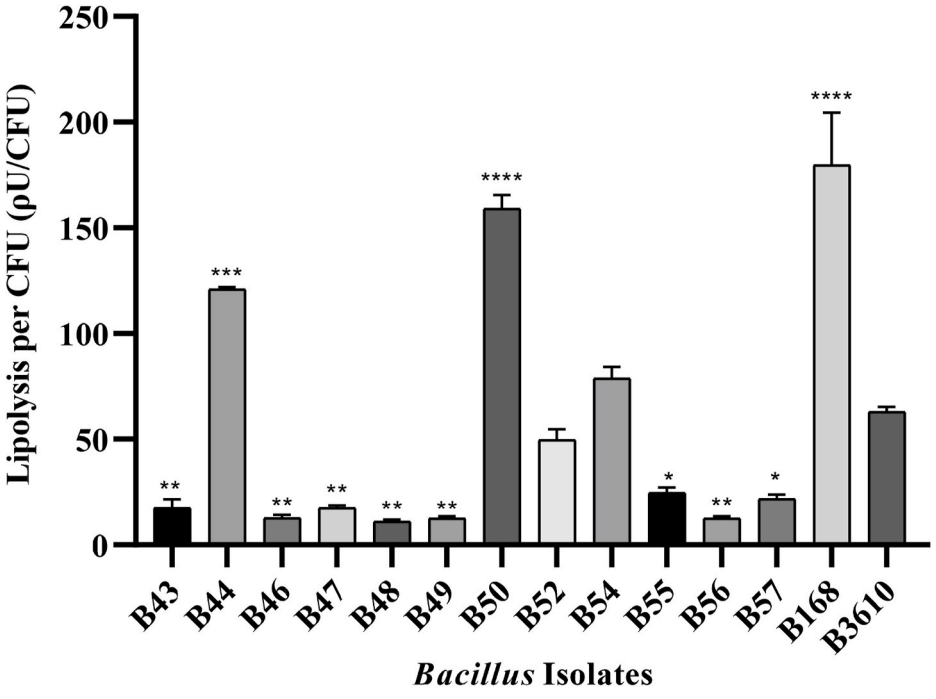

Fig. 7. The lipolysis within the biofilms on stainless-steel substratum in UHT milk grown at 30 °C expressed as picolitre of proteolysis per CFU (ρL/CFU). The highest and lowest mean proteolysis was observed in B168 (179.70 ρL/CFU) and B48 (11.07 ρL/CFU) respectively. Post-hoc analysis was done Dunnett multiple comparisons with a critical probability of $P \leq 0.05$. Error bars indicate the standard deviation of the means (SD). Asterisks (*) indicate significant difference among means (* = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$ and **** = $P \leq 0.0001$).

stain (SYTO 9) used for the flow cytometry analysis can bind the vegetative and damaged cells as well the spores making it more sensitive than plate counting used in another study (Elegbeleye & Buys, 2020).

All isolates were able to grow and attached to the stainless-steel substratum under a submerged condition producing biofilms with variations in the total number of cells enumerated. There was no significant difference in the log count of the isolates in the planktonic culture unlike in the biofilms. This shows that the growth rate across the isolates for the planktonic culture is somewhat the same without much variation. In contrast, there were significant differences in the number of cells recovered from the biofilms of all the isolates. The result also implies that B168 has a weak biofilm forming potential and presumably the highest spread on the stainless-steel surface. This potentially makes the biofilms structurally and architecturally less complicated than in other isolates. They are suspected to be an aggregation of attached cells than a community of microorganisms often associated with a mature biofilm (Kragh et al., 2016).

We observed that the spoilage potential (proteolysis and lipolysis) of both biofilms and planktonic cells are strain-dependent at the conditions applied in this study. There seems to be a relationship between the strength or complexity of the biofilms and spoilage potential of the isolates as observed in B168 which is a reference strain of *B. subtilis* with

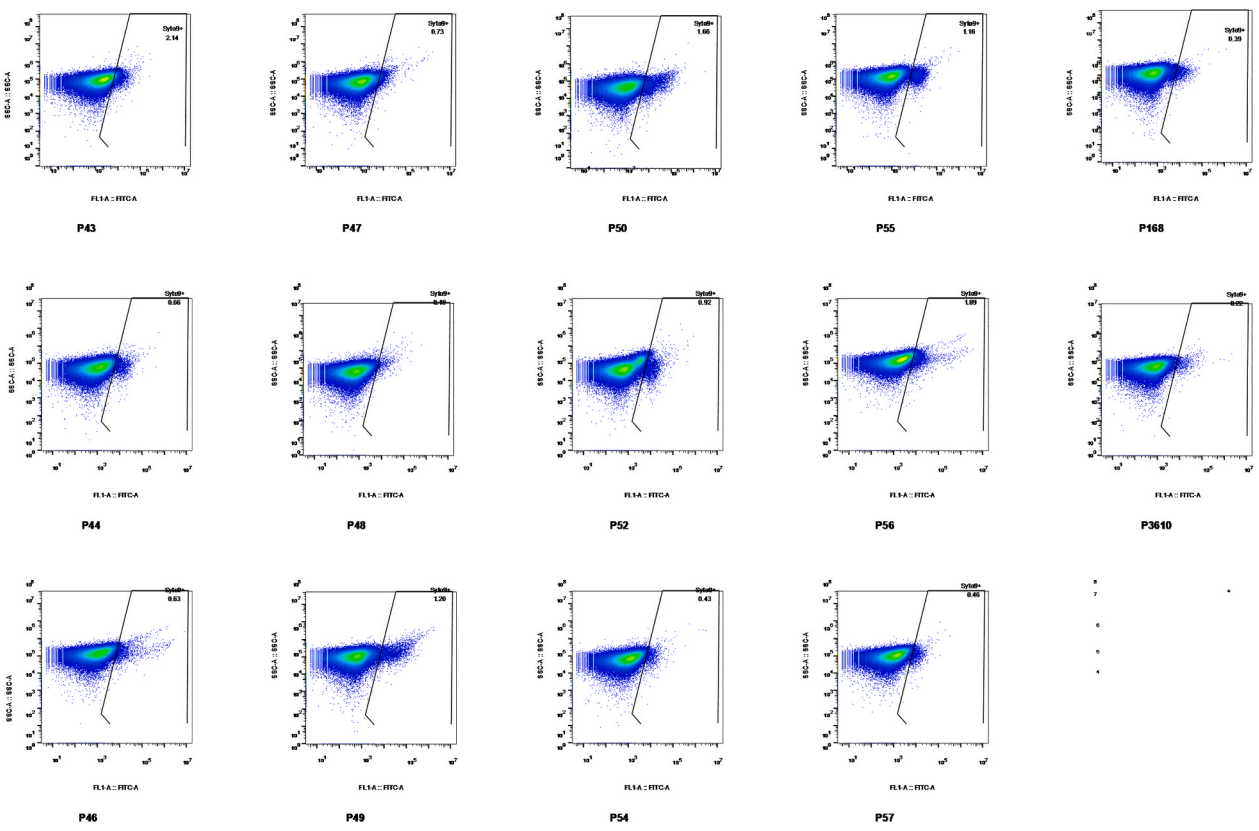

Fig. 8. Flow cytometric analysis of planktonic cells grown in sterile UHT milk. Total bacterial count for all of the strains was done by staining cells in milk with SYTO 9 for both dead and living cells to be counted. SYTO 9 can bind the DNA of both living and dead bacterial by entering the cell through the semi-permeable membrane. The highest count was $\log_{10}$ 7.6 CFU/mL (B52), and the lowest was $\log_{10}$ 7.2 CFU/mL (B48).

a very weak biofilm forming potential on the stainless-steel as stated in our previous study (Elegbeleye and Buys, 2020). We observed that sample B168, in its biofilm state, has the highest mean proteolysis and lipolysis per cell. The sample also has the lowest number of cells enumerated within its biofilm. Thus, one can safely deduce that under the assay condition, the less complicated the biofilm is, the less complicated the structure is on a substratum, and the higher its spoilage potential. The reason for this can be adduced to the fact that the stronger a biofilm is, the more the complicated the structure becomes. The complex structure allows for the different gradient of nutrients and oxygen, resulting in the formation of different microenvironments within the biofilm matrix. These gradients lead to the heterogeneity of cells within the biofilms and such a situation may lead to the formation of dormant cell forms like persister cells and spores in the case of sporulating bacteria like *Bacillus* spp. Both persister cells and spores are metabolically inactive which implies that they are not secreting enzymes like the metabolically active cells which may account for the low lipolytic and proteolytic enzymes in some of the cases (Sultana, Call, & Beyenal, 2016; Whitehead & Verran, 2015).

The formation of biofilms and spores has been observed to occur simultaneously in sporulating bacteria with spore population as high as 10–50% within the 8 h of attachment to the substratum according to the study by Burgess, Brooks, Rakonjac, Walker, and Flint (2009). Since B168 is a weak biofilm former, it implies that the biofilm lacks the three-dimensional complexity of a model biofilm with no gradient in oxygen or nutrient that allows for the formation of metabolically dormant structures. This typically means there will be more metabolically active cells present in a sample with low biofilm-forming potential to secrete enzymes that will, in turn, act on the substrates in the medium (Kearns, 2008).

The reverse scenario is also true in the case of moderate or strong biofilm former with a more considerable heterogeneity, complexity and likelihood of producing spores within its biofilm. The implication of this to the industry is that the biofilms of the isolates used in the study pose a challenge during the processing and storage of milk (Faille et al., 2014). From the result, it seems as the more complex the biofilm stricture is, the more disposition it is for the biofilm to form dormant structures such as spores and persister cells. While the weak biofilm formers have better spoilage potential per CFU than the strong biofilm formers, the strong biofilm formers are hot-beds to produce heat resistant spores which can withstand the processing condition and further contaminate the downstream process as well as the ESL milk. The phospholipase enzymes produced by these *Bacillus* spp. break down the milk fat globules thus making the milk more prone to further action of other hydrolytic enzymes (Mehta, Metzger, Hassan, Nelson, & Patel, 2019).

Generally, the metabolites produced within mature biofilms are several-folds higher than the ones produced within the planktonic cells of the same strain. A similar study to the current one by Teh et al. (2013) corroborated the assertion that lipolysis was higher by ten-fold in biofilms than the planktonic cells of *Staphylococcus aureus*, *Streptococcus uberis, Pseudomonas fluorescens* and *Serratia liquefaciens* isolated from milk. The same trend was observed in the result of our study of the lipolysis observed within the biofilms.

From the result of the planktonic cells, B48 has the highest mean proteolysis with 1033.6 ρL/CFU while B50 has mean proteolysis of 892.0 ρL/CFU the highest mean lipolysis of 34.6 ρL/CFU which is about thirty times higher than the mean lipolysis of the reference strain B168 with 1.13 ρL/CFU. This implies that in its planktonic state, B50 poses the greatest risk to the milk since it requires shorter time to cause the development of rancidity as well as a breakdown of casein micelles resulting in deterioration of the product and change in its textural and sensorial properties (Malmgren et al., 2017). The presence of biofilms in dairy processing plant has been observed to serve as a reservoir where planktonic cells are liberated or sloughed-off from the biofilm structure

thereby leading to the spoilage of finished. This makes the assessment of dairy processing plant for the presence of biofilms an indispensable parameter during the development of any HACCP strategy (Kim & Lee, 2016; Sharma & Anand, 2002).

All the isolates produced more of the proteolytic than lipolytic enzymes in both the planktonic and biofilm states in no specific order under a submerged condition. The submerged method best simulates the situation of planktonic culture than the semi-quantitative agar diffusion method. Though the agar diffusion method is faster in generating a result, it can only be applied in screening isolates for their ability to secrete extracellular enzymes on an agar surface containing the appropriate substrate. Just as in the planktonic cells, B50 has the maximum mean proteolysis of 2.10 ± 0.11 while the negative control, B168, has the lowest with 1.27 ± 0.06 as zones of hydrolysis. This suggests a relationship between the two methods and the ease at which the agar assay lends itself as the first-in-line profiling of potential spoilage microorganisms of milk.

The limitation in the present study is that it did not take into consideration the presence of enzymes that may be present in the milk before the introduction of the test microorganisms. The effect is thought to be usually negligible and was also mitigated by using the same UHT milk sample for all experiments. Some bacterial lipases even possess the ability to survive pasteurisation even in the absence of the bacteria producing them and effect the hydrolysis of lipids in the milk sample (Salgado, Baglinière, & Vanetti, 2020). It is therefore advisable to measure the initial proteolytic and lipolytic enzymes that may be present in the milk sample before any experiment is done. This is highly recommended in a situation where the presence of heat-resistant enzymes is suspected. Otherwise, it is necessary to set-up controls, as we did in this study, containing only the milk samples with distilled water replacing the microbial inoculum instead of the test organisms. The assays used in this study can also be complemented with other methods such as chromatography and other chemometric strategies that can be optimised and automated on an industrial scale (Kamal & Karoui, 2015; Stuknytė et al., 2016).

5. Conclusion

Although, our study confirmed the ability of the isolates to produce spoilage enzymes in both biofilm and planktonic states, however, the result, under the assay conditions revealed that proteolysis and lipolysis in the planktonic and biofilms states is dependent on the strains. This is in contrast with the initial hypothesis that the concentrations of the enzymes will be higher within the biofilms than in the planktonic cultures. These enzymes degrade the protein and lipid components in milk which can potentially alter the sensorial and nutritional properties of the milk, especially under storage condition. An innovative strategy to mitigate the contamination of milk by thermophilic *Bacillus* spp. either as biofilms or planktonic cells cannot be over-emphasised.

CRediT authorship contribution statement

James A. Elegbeleye: Conducting research, writing document, Formal analysis. **Elna M. Buys:** PI, Conceptualization, Funding acquisition, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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