The impact of spore aggregation on viable and total counts of *Bacillus subtilis*

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Introduction

- In a number of occasions controlling the number of microorganisms on surfaces or in complex liquid environments, from fruit juices and drinks to GIT fluids, is of importance. However in order to do so, it is important that an accurate bacterial enumeration method is in place to avoid over or under-estimating the remaining bacterial counts.

- Traditionally bacterial counts are obtained through plating techniques under the assumption of one colony generated from one cell.
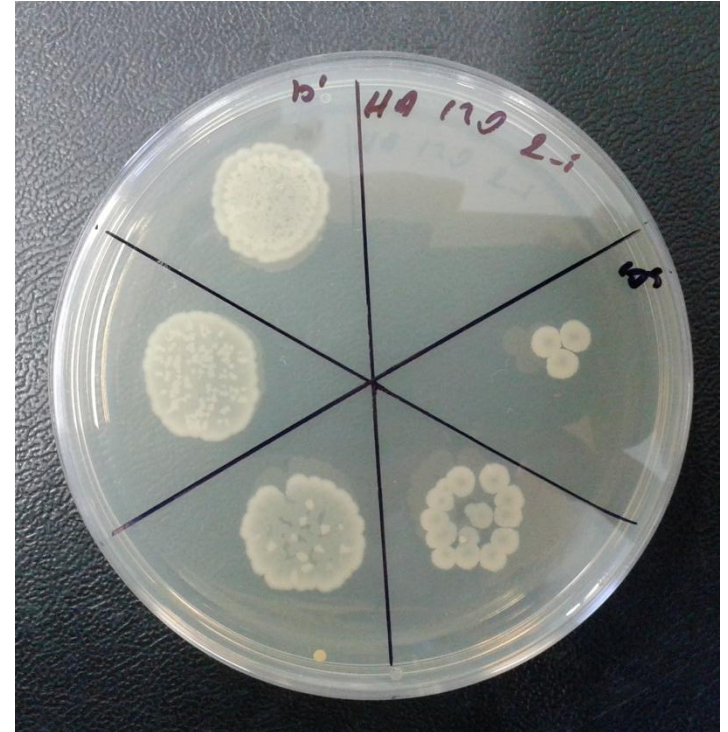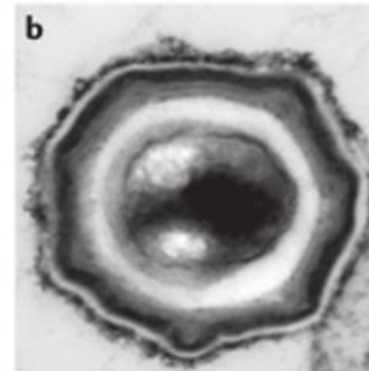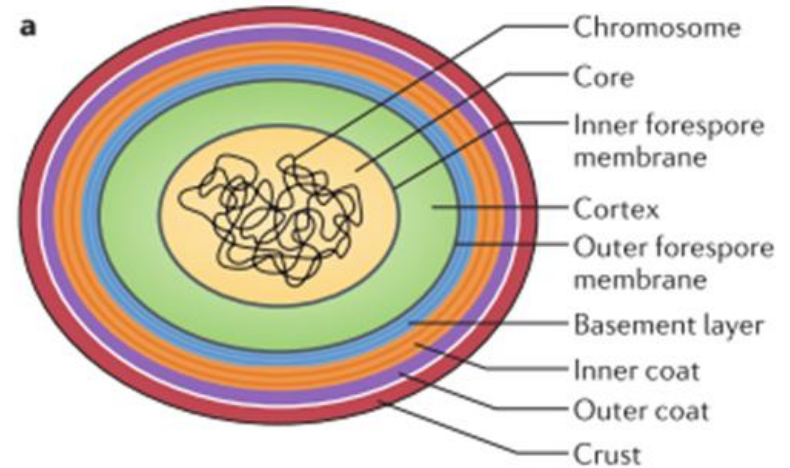
Bacterial aggregation

- Aggregation often occurs in bacterial populations as a result of the physicochemical and biological parameters within the system.

- in the dormant spore form the physicochemical aspects will be the dominating factor

- Attachment of dormant spores, either between themselves or to surfaces, is considered to be a surface-driven phenomenon and can be interpreted using the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory.
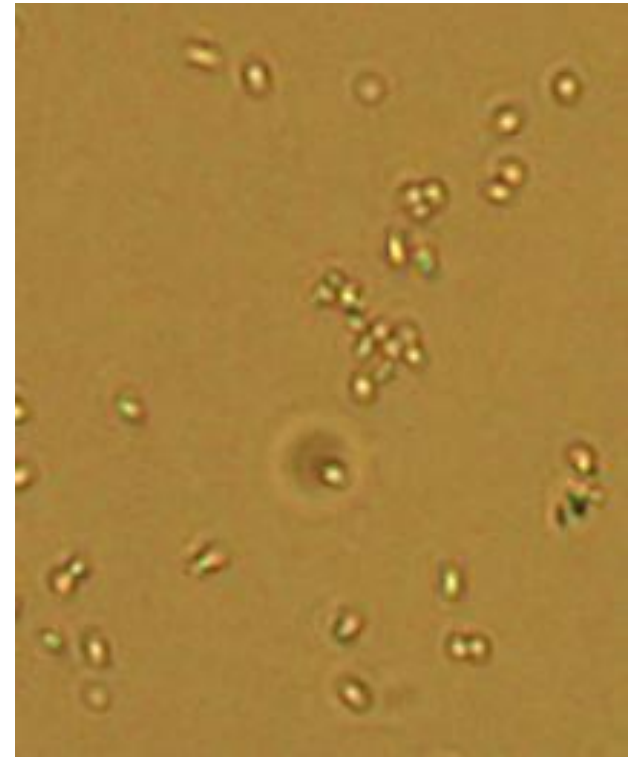
Bacterial aggregation and intermolecular forces

- Based on the DLVO theory the main forces at work causing aggregation are:
  - electrostatic forces
  - permanent dipole - permanent dipole forces
  - Lifshitz-van der Waals forces

a) Diagram showing different layers of the dormant spore, and b) Thin section transmission electron micrograph of a B. subtilis spore stained with ruthenium red.

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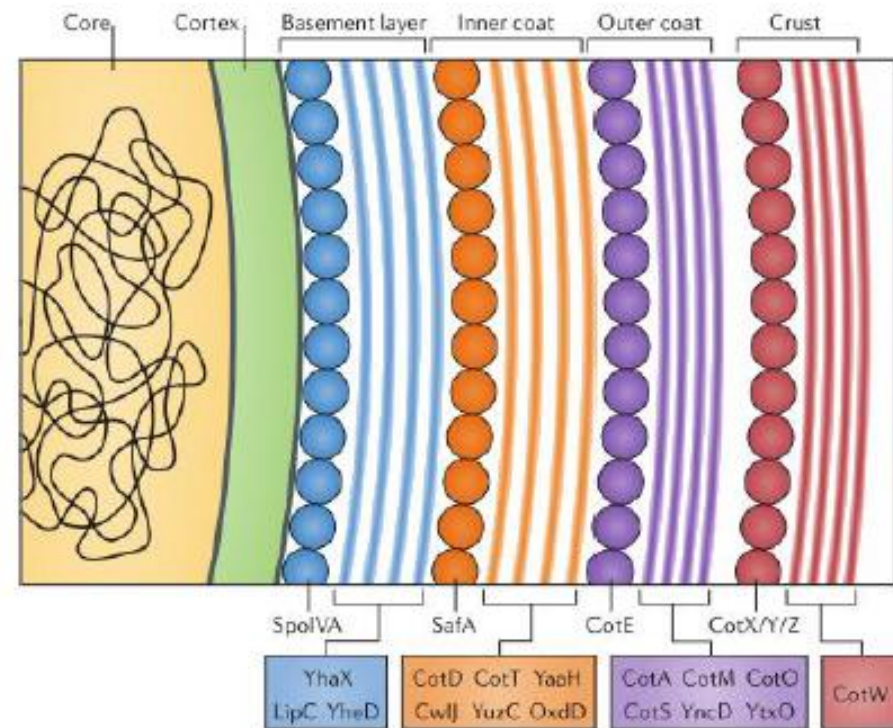 - Lifshitz-van der Waals forces

B. *Subtilis* spore coat and crust proteins

McKenney et al. 2013
Nature Reviews Microbiology 11: 33-44

Aims

In this communication spores of *B. subtilis* are exposed to PBS (100mM) at pH 1, 3 5 and 7 either with or without 1% tween 20 and we aim to investigate the impact of aggregation in the viable and total counts.

- Total counts as well as viable counts were assessed by plating and flow cytometry (FCM).
- Aggregation was assessed by particle size analysis using a Malvern masterziser.
- The electrostatic charge of spores was assessed by Zeta potential measurements using a Malvern zetasizer

# Total counts from FCM showing spores immersed in PBS
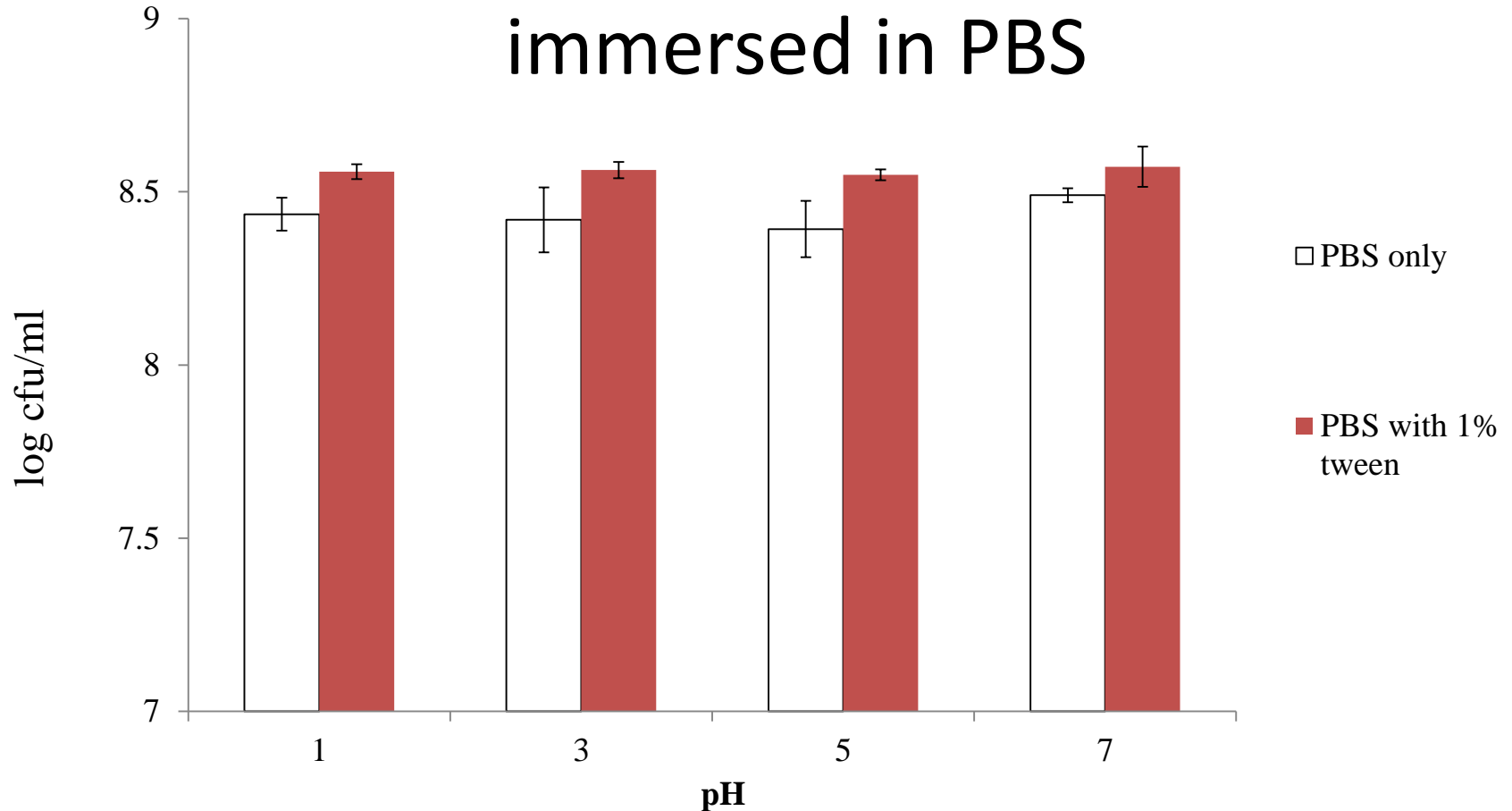
Comparison of total spore FCM counts in PBS over a range of pHs. White bars indicate spores in PBS only and red bars show spores in PBS supplemented with 1% tween. Values are based on the total number of cells and spores according to FCM. Error bars represent the 95% CI.

Plating counts on spores immersed in PBS
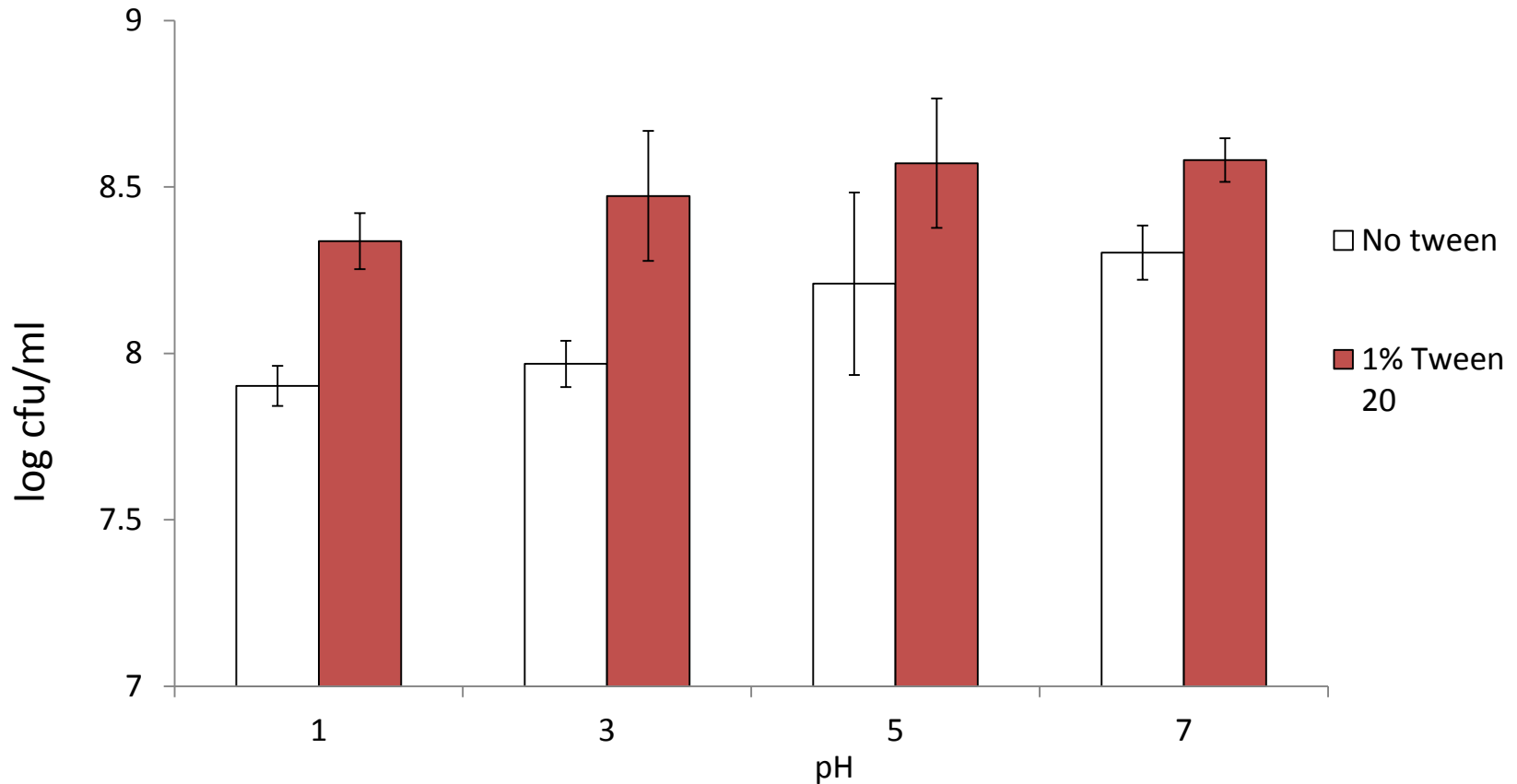
Comparison of plating counts for PBS alone and PBS with 1% tween 20. White bars indicate spores immersed in PBS without tween 20 and shaded bars represent spores immersed in PBS with 1% tween 20. Error bars represent the 95% CI.
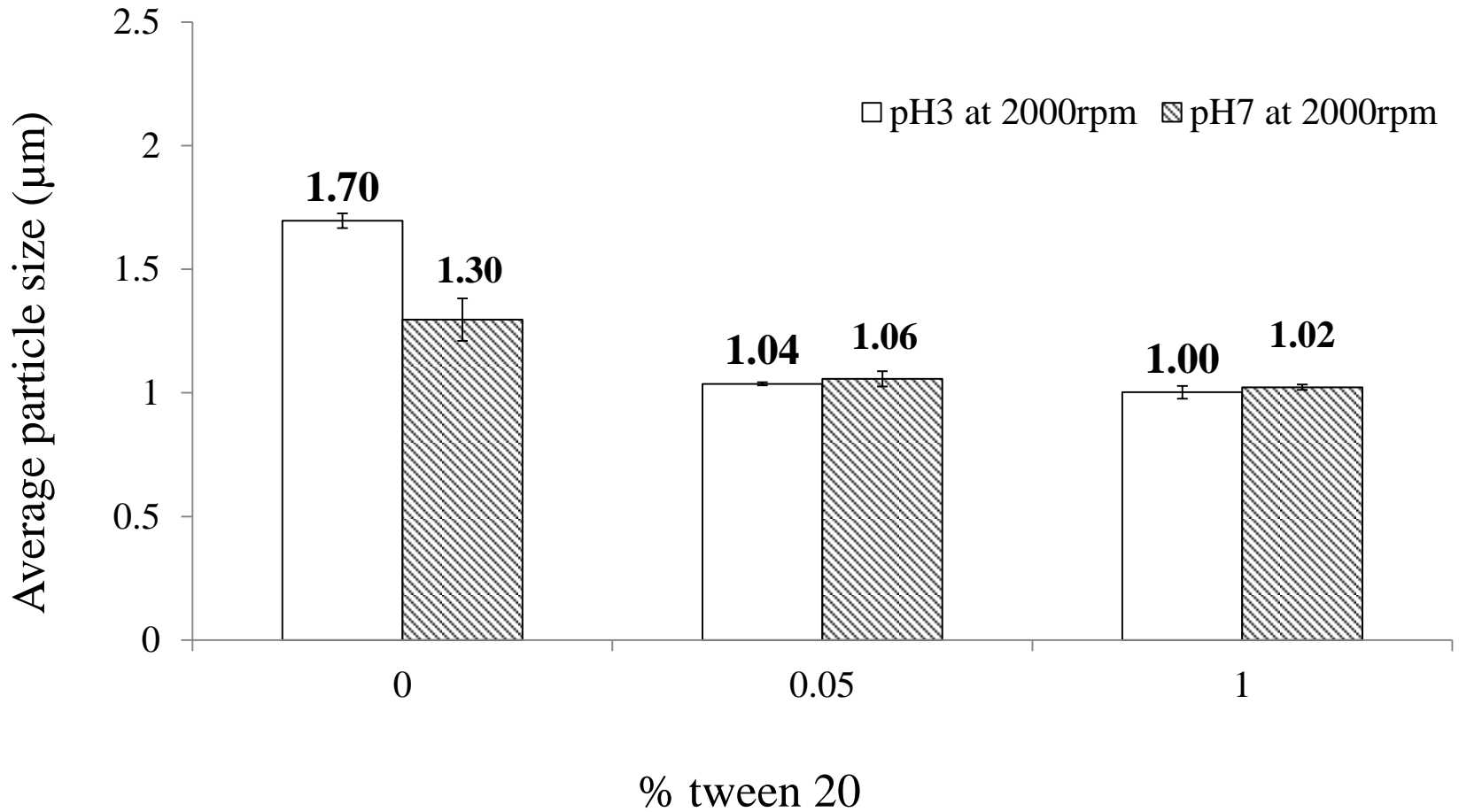
Spore particle size analysis
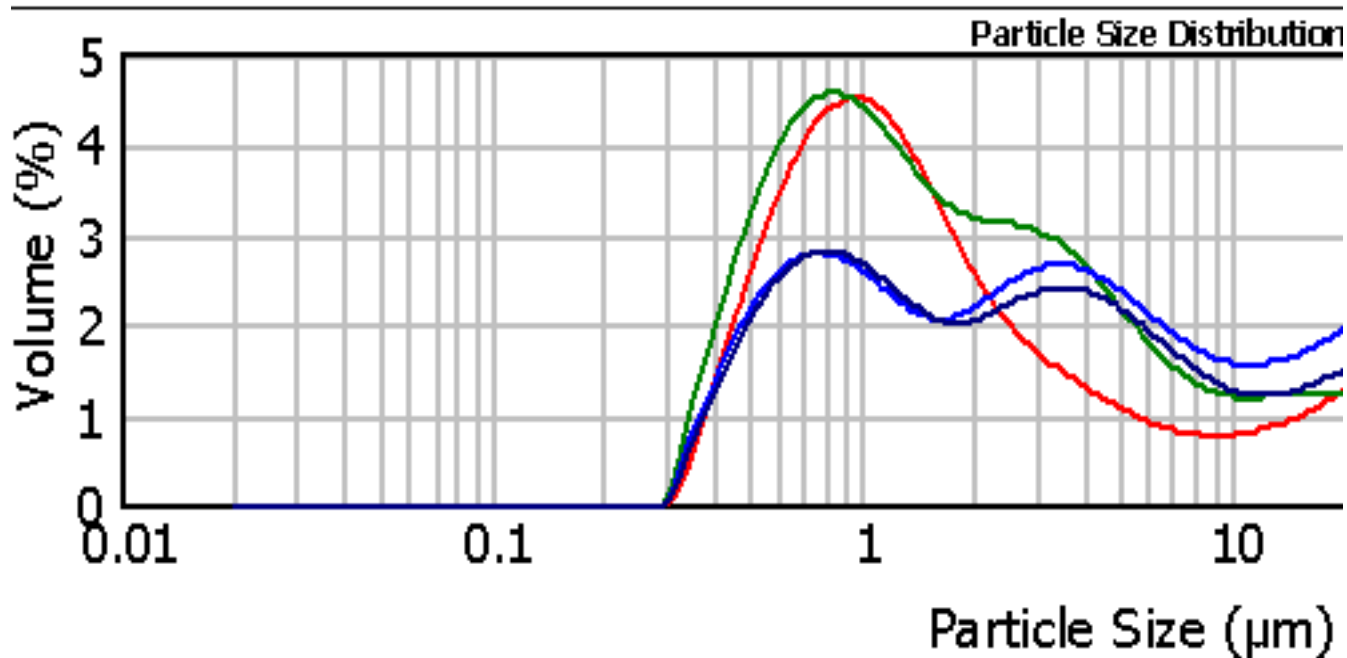
Particle size distribution of spores in PBS (100mM) at different pH levels.
Lines represent the average of three samples.
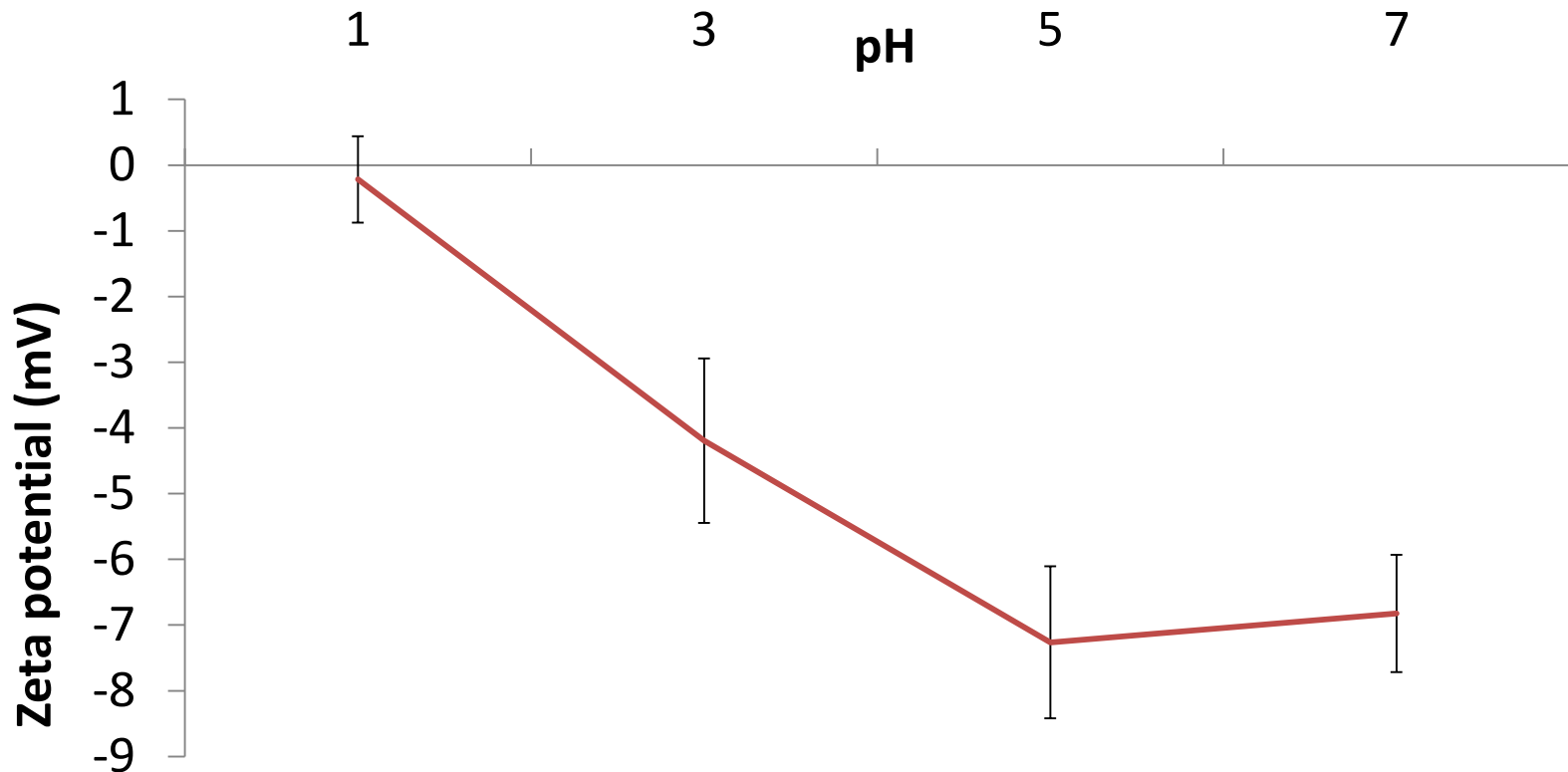
pH 7 - Red line

pH 5 - Green line

pH 3 - Blue line

pH 1 - Black line

Zeta-potential of B *Subtilis* spores in PBS (100mM)

Zeta potential for spores immersed in PBS at pH 1, 3, 5 and 7. Data represent the average of 9 measurements and error bars represent the 95% CI

Conclusions

- Across the investigated pH range 1-7 in PBS, tween increased plate counts between 50-75%. This highlights the level of error in count one could expect as a result of the aggregation from standard enumeration techniques

- Data from the Malvern mastersizer strongly support the theory that the average particle size increases as the pH lowers, denoting a higher number of aggregate in the system.

- Data from the Malvern Zetasizer strongly support the theory that the aggregation is enhanced as the pH lowers as a result of the reduction of spore zeta potential.

THANK YOU!

Acknowledgements

Back-up slides

Total FCM counts on spores immersed in LB
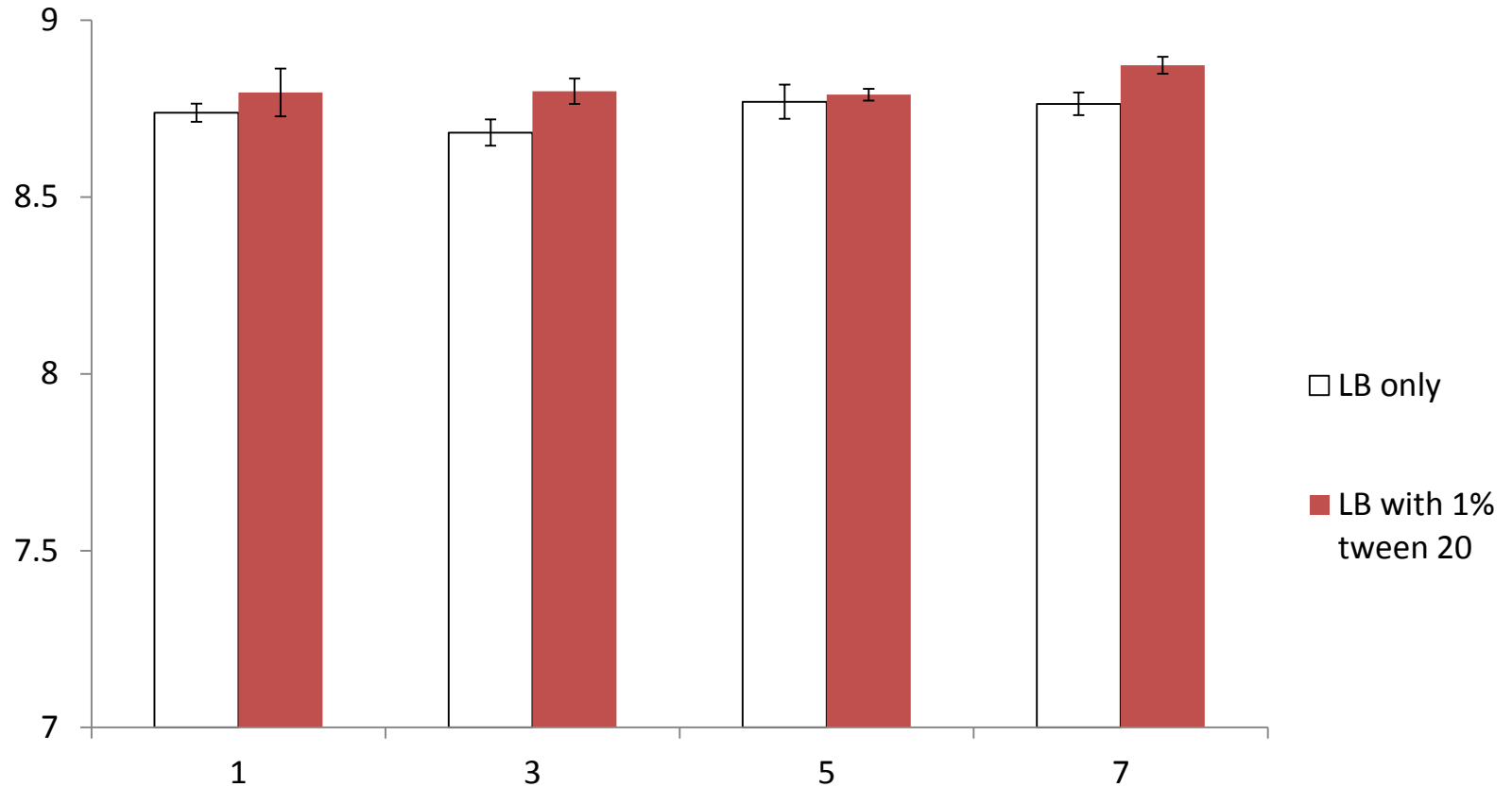
White bars indicate spores in LB broth only and red bars show spores in LB broth supplemented with 1% tween. Values are based on the number of events in region B. Error bars represent the 95% CI .

Plate counts from spores immersed in LB broth
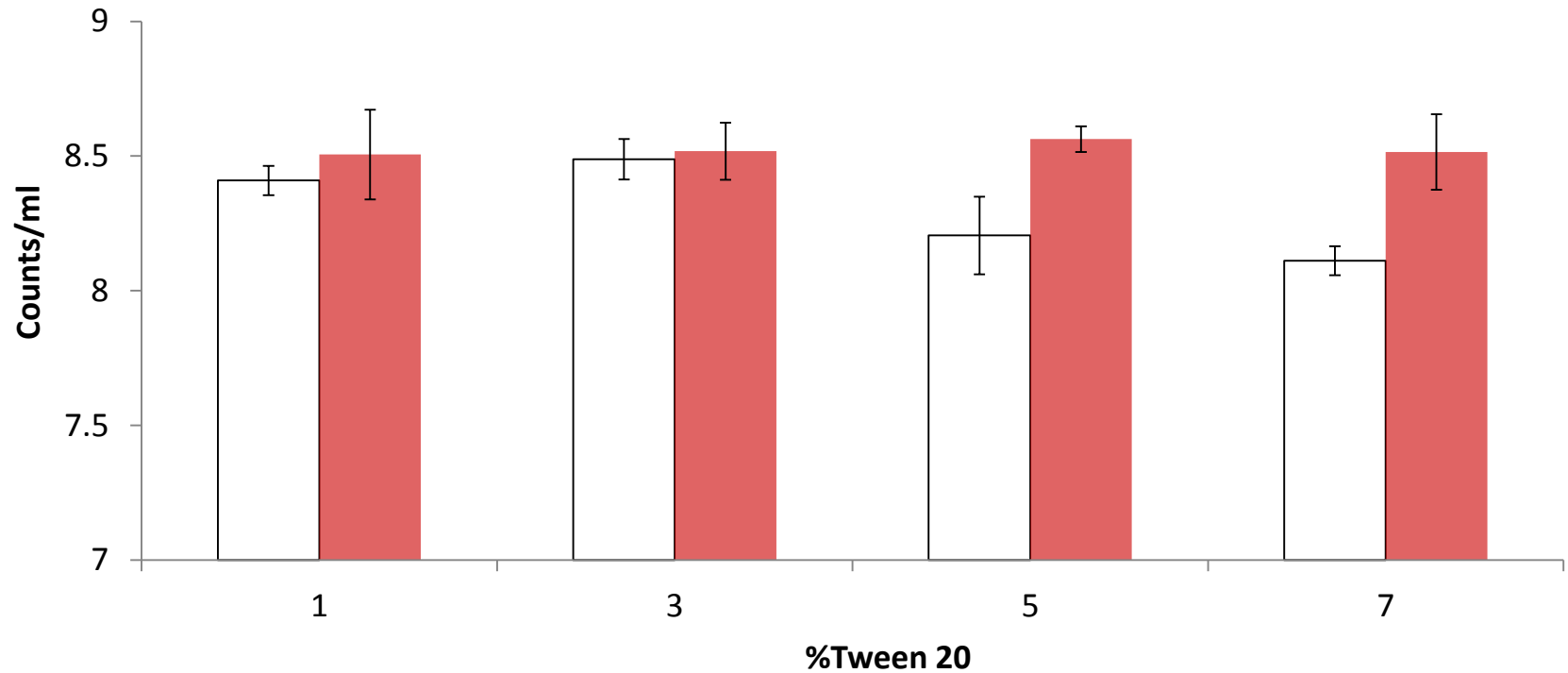
Plate counts of spores immersed in acidified LB broth. Error bars represent the 95% CI.

Methods

- Bacterial flow cytometry (FCM) was implemented to measure total counts of spores. Viability was also analysed by plating.

- FCM event were converted to counts/ml using the following equation

$$\frac{no.\ of\ events\ in\ cell\ region}{no.\ of\ events\ in\ bead\ region} \times \frac{no.\ of\ beads\ per\ test}{test\ volume} \times dilution\ factor$$
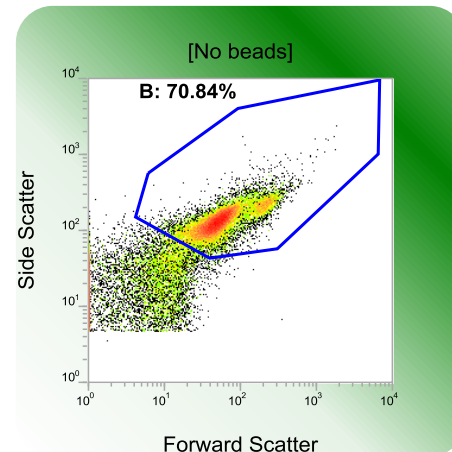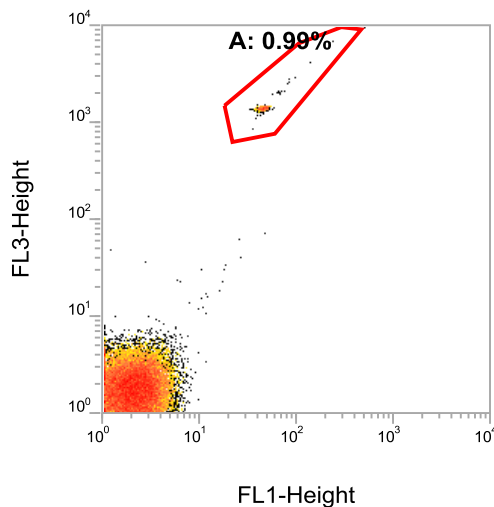
Enumeration

- FCM samples were stained with 48µM PI and 1.5µM Syto 16 to obtain viable counts. A microtube was filled with 540µl of this staining mixture and following the experimental 10µl of the relevant sample was added to this. Before FCM analysis, 490µl of this mixture was pipette into an FCM tube and 10µl of Spherotech beads were added to enable conversion of events to counts/ml.

- Plate counts were undertaken according to the Miles and Misra method (Miles *et al.* 1938). Briefly, serial dilutions (10^{-1} to 10^{-6}) were plated out on an LB agar plate by pipetting 20µl of the respective dilution onto the labelled segment of the plate. The dilutions were performed in triplicate. After allowing time to be absorbed, the plates were inverted and incubated at 37°C overnight. The drop method reduces the number of bacteria lost by spreading (Pope *et al.* 2008).

- **Particle size analysis:** To gain a better understanding of the level of aggregation, the spores were also subject to particle size analysis using a Mastersizer 2000. Spores were immersed in PBS (100mM) at pH 1, 3, 5 and 7 or in the PBS (100mM) at pH 1, 3, 5 and 7 with 1 % tween 20.

- **Zeta Potential:** Measurement were conducted in the National Centre for Macromolecular Hydrodynamics at Nottingham University led by Prof Harding. A Malvern zetasizer was used to assess the zeta potential of spores at 7×10^7/ml (OD_{580} = 0.45) in accordance with the previous study by Harding and Johnson (1984) in 100mM PBS at pH 1, 3 5 and 7 with and without 1% tween 20.

FCM settings

- The FCM model used in these studies is the BD FACSCalibur, equipped with a 15 mW, 488 nm air-cooled argon ion laser. BD CellQuest Pro software was used for setting and acquiring data.

- All sample analysis was collected using the cytometer 'low' setting, with data acquisition set to 20,000 relevant events or 300 seconds. Two data acquisitions were performed, once using the FSC trigger, with threshold set to 190 and a second acquisition using the Side Scatter (SSC 390). The E01 voltage setting was selected for the Forward Scatter (FSC) channel. The Photomultiplier detector (PMT) voltage settings for each of the three fluorescent detector channels used in the studies were: a.) Green Fluorescent channel 1 (FL1) 595, b.) Red Fluorescent channel 2 (FL2) 634 and c.) Far Red Fluorescent Channel 3 (FL3) 683. Under these settings, unstained cells were kept in the first decade of the FL1 versus FL3 density plots. Compensation settings were FL2 to FL 1 30.6%, FL3-FL2 12.7%. These were checked monthly, using single stained cells and spores. The 'low' flow rate setting was selected corresponding with a flow of 12μL/min (Picot *et al.* 2012)

- To prevent the build-up of non-specific noise, both a daily and a monthly cleaning was carried out on the sheath lines and sample tubes. The monthly cleaning consisted of a 10% sodium hypochlorite solution followed by a ddH$_2$O cleaning of the system (Cronin 2012).

Spore Production

Spores of *Bacillus subtilis* DSM 23778 (German Collection of Microorganisms, Germany) were prepared on AK sporulating agar (BD, UK). Each plate was supplemented with 200µl of 3.4mM $MnCl_2$ spread onto the surface with a sterile glass rod. This was then allowed to be absorbed and the plates were inoculated with 200µl of living cells, (grown overnight in LB broth). Plates were left an additional 30 minutes, then inverted and incubated for seven days at 37°C.

After this, the spores were harvested by flooding the plate with cold (4°C) sterile deionised water and poured into sterile (15ml) falcon tubes. Based on the method of Zhao *et al.* (2008) these suspensions were washed twice at 10,000rpm at 4°C (Sorvall RC 5B plus centrifuge, Thermo Scientific, UK), and then re-suspended in 0.2µm filtered 50% EtOH (with H_2O). This mixture was left for 18 hours at 6°C, and then washed three times more in ddH_2O This method was modified slightly by leaving spores for two more days in pure water to burst any remaining vegetative cells, and washed again before being stored either at 6°C or -20°C (Kong *et al.* 2010).

References

- Ampatzoglou, A., Schurr, B., Deepika, G., Baipong, S. and Charalampopoulos, D. (2010). "Influence of fermentation on the acid tolerance and freeze drying survival of *Lactobacillus rhamnosus* GG." Biochemical Engineering Journal **52**(1): 65-70.

- McKenney, P. T., Driks, A. and Eichenberger, P. (2013). "The *Bacillus subtilis* endospore: assembly and functions of the multilayered coat." Nature Reviews Microbiology **11**(1): 33-44. doi: 10.1038/nrmicro2921. Epub 2012 Dec 1033.

- Shapiro, H. M. (2005). Practical Flow Cytometry, Wiley.

- Wilson, W. W., Wade, M. M., Holman, S. C. and Champlin, F. R. (2001). "Status of methods for assessing bacterial cell surface charge properties based on zeta potential measurements." Journal of Microbiological Methods **43**(3): 153-164.