Investigating the impact of different lethality inducing conditions on cells of *Bacillus subtilis* via flow cytometry

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Introduction

Decontamination of surfaces is a vitally important process in industrial settings. *Bacillus subtilis* spores are a good safe alternative to model pathogenic organisms such as *B. cereus* and *Clostridium difficile*.

In this communication a range of novel and commonly used antimicrobials are applied to cells and spores of *B. subtilis*. By looking for alternative antimicrobial agents, this could have far reaching implications for use against antibiotic resistant strains of bacteria. Furthermore, employing natural antimicrobials will have a less detrimental effect on the environment.

Antimicrobials

Common methods of cell killing

- **Heating** 85˚C for 35 minutes is our standard method
- A 50% **Ethanol** (water) treatment is used to kill off vegetative cells (leaving spores unharmed)

Common antimicrobials:

- **Peracetic acid** (PAA)- a strong oxidizing agent thought to be capable of killing spores as well as cells[1].

- **Chlorine** (in the form of sodium hypochlorite)- oxidizing agent commonly used in bleach[2]

Natural Antimicrobial:

- **Green tea extract** –believed to exert an antimicrobial effect due to tea polyphenols[3, 4] in particular Epigallocatechin-3-gallate (EGCG) thought to have anticancer, anti-tumour, and antimicrobial properties

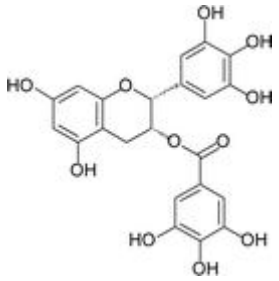
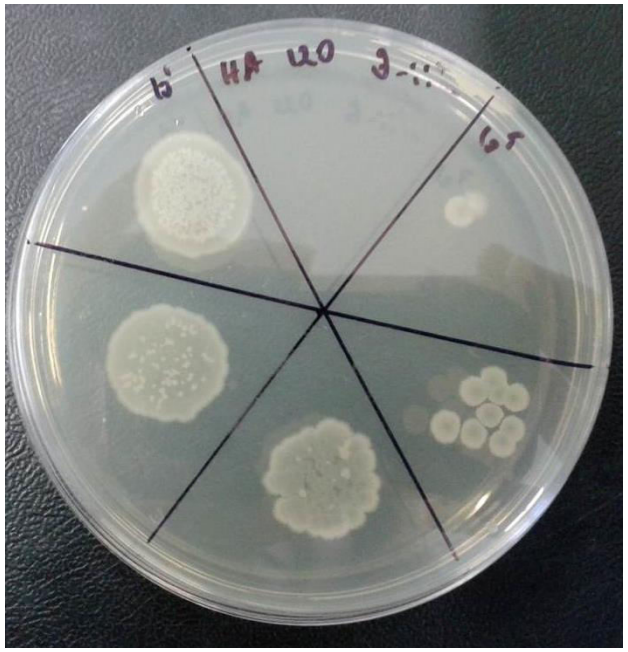


Fig.1. Chemical structure of epigallocatechin gallate.

Assessing viability



Plating

- Serial dilution plating was carried out using LB agar according to the Miles and Misra method[6].

Fig. 4 LB agar plate showing serial dilutions of *B. subtilis*

Nucleic acid dyes

- Syto 16 will penetrate intact bacterial cell membranes, indicating live cells
- Propidium Iodide (PI) fluoresces a bright red. However, it has an additional positive charge meaning it cannot cross intact cell membranes. Used to denote dead cells.

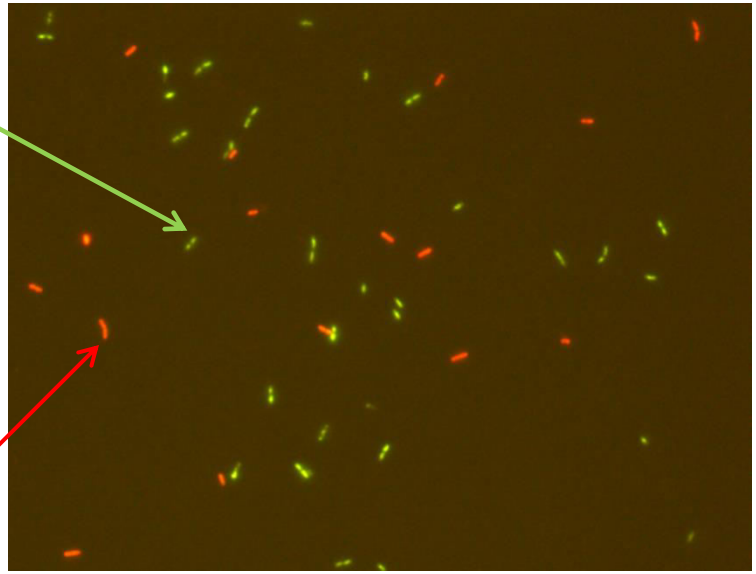
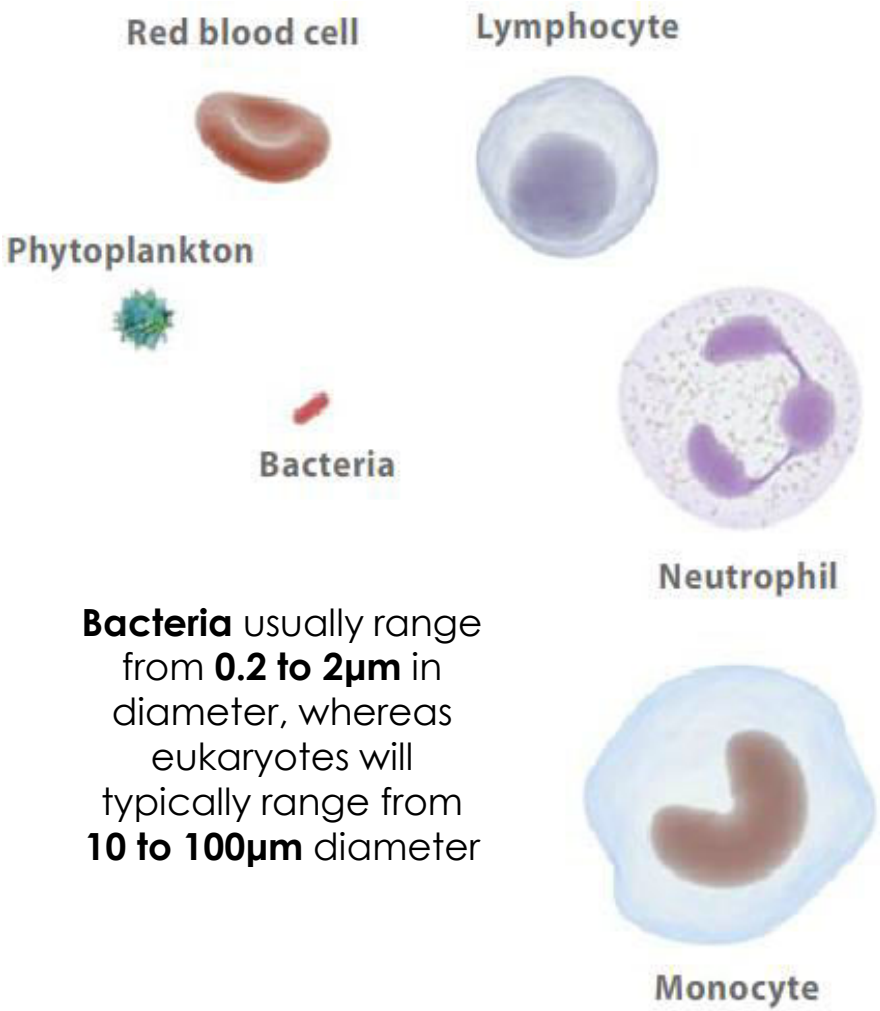


Fig 5. mixture of living *B. subtilis* cells (green) and dead cells (red)

Bacterial Flow cytometry

- Flow cytometry (FCM) was originally used primarily by immunologists to study eukaryotes
- Over the past decade there has been developing interest in using this as a microbial tool[5]
- The main difficulty to overcome in this area is the much smaller size of the bacterial cell



Bacteria usually range from **0.2 to 2µm** in diameter, whereas eukaryotes will typically range from **10 to 100µm** diameter

Fig 2. Comparison of eukaryotic and prokaryotic cell sizes

Image from Invitrogen

Flow Cytometry Principles

- **Forwards Scatter** is indicative of cell **size**
- **Side Scatter** is relative to cell **granularity**
- FL1 (Green Fluorescence) in this case indicates living cells stained strongly with Syto 16
- FL3 (Red Fluorescence) depicts dead cells stained with PI

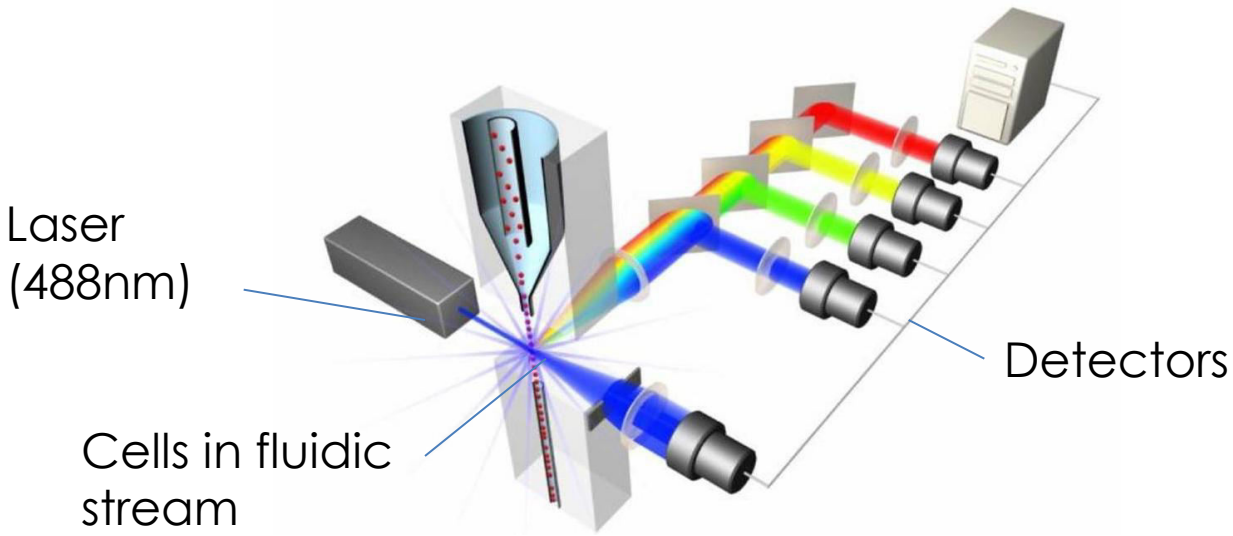


Fig 3. Schematic of inner flow cytometer system.

Image from Invitrogen

Aims and Objectives

Aim:

- Assess the **reliability** of FCM as a tool for enumeration of microbial cells and spores.

- To assess the efficacy of both common and novel antimicrobials as bactericidal and sporicidal agents

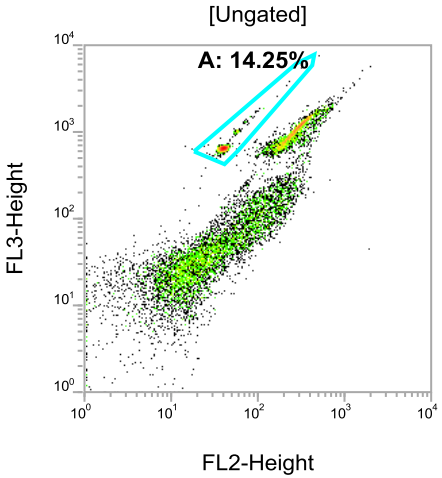
Objectives:

- **1.** Test these antimicrobials on cells of *Bacillus subtilis*, comparing the results of the **FCM analysis** with **serial dilution plating** and **Petroff-Hausser Haemocytometer counts**

- **2.** Ensure we can **differentiate** between cells (living/dead) and spores in the FCM

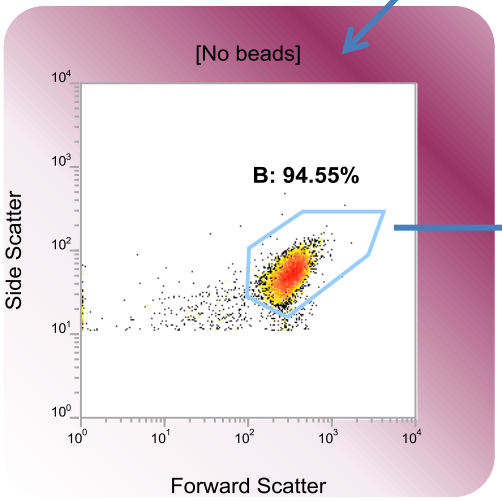
Enumerating sub-population using FCM

Cells immersed in filtered (0.22µm) PBS

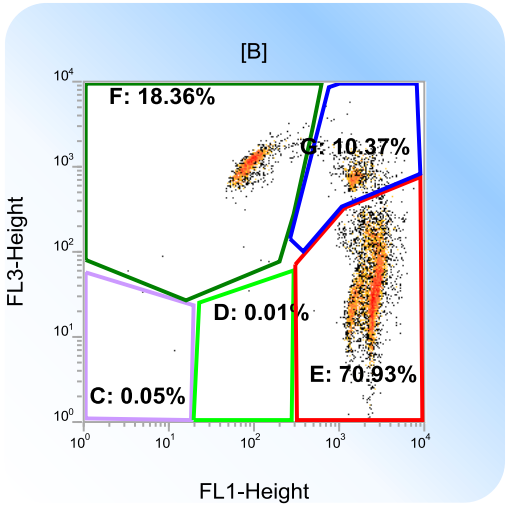


a) Region drawn around beads

Remove beads from plot using equation NOT A



b) Region drawn around cells (main population)



c) Assignment of sub-population regions

Fig 6. FCM enumeration procedure

Enumeration of sub-populations

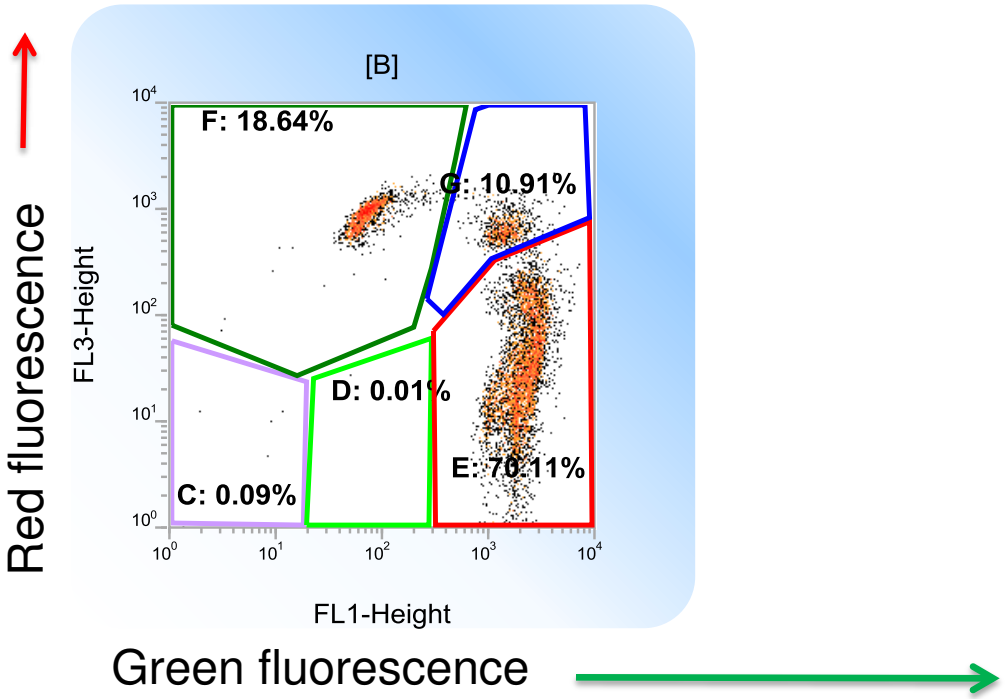


Fig 7. Green (FL1) fluorescence against red (FL3) fluorescence density plot, showing cells immersed in GTE at time 0. Region C: dormant spores, Region D: germinating spores, **E: Live cells**, **F: Dead cells**, Region G: double stained cells

The events from each region can be converted to cell counts/ml using counting beads in the following equation[7]:

$$\frac{\#\text{events in cell region}}{\#\text{events in bead region}} x \frac{\#\text{ beads/test}}{\text{test volume}} x \text{ dilution factor}$$

Petroff-Hausser counting chamber

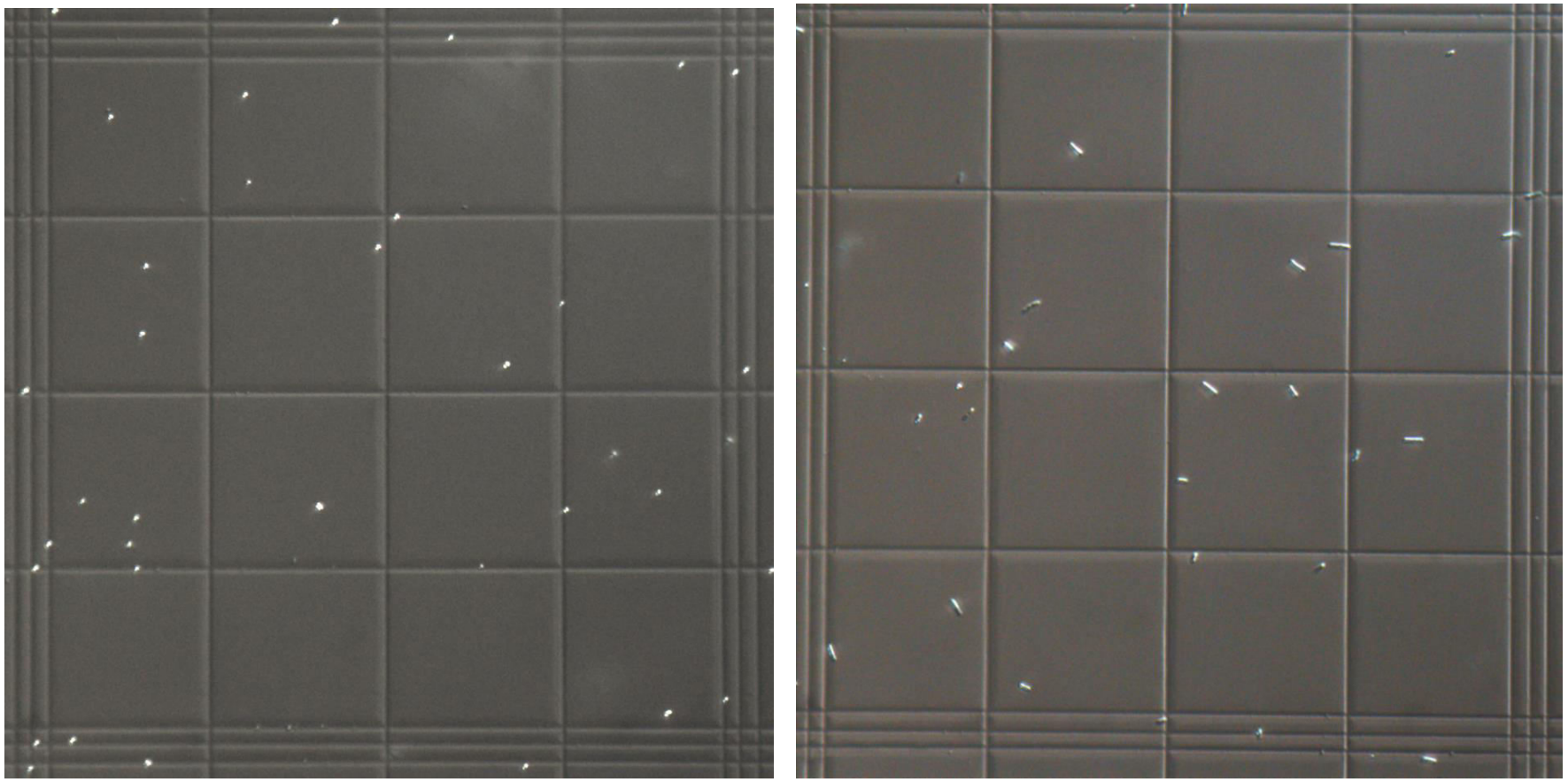


Fig 8. DIC images of shallow depth (10µm) Petroff-Hauser (PH) counting chamber haemocytometer, for counting spores (left) and cells (right)

Table 1. Viable counts of cells and spores subject to different treatment

Data set description	Enumeration Data			
	FCM		Plating	
	cfu/ml	%CV	cfu/ml	%CV
Spores no treatment (RUN 1)	4.08×10^7	4.2	4.70×10^7	14.0
Spores no treatment (RUN 2)	2.03×10^7	12.3	2.72×10^7	9.0
No treatment on cells grown for 24 hour in LB broth at 35°C	1.27×10^9	5.8	5.08×10^8	17.0
Peracetic acid 50 ppm contact with cells for 5 minutes at 4°C.	0.00	N/A	0.00	N/A
Chlorine 100 ppm contact with cells for 5 minutes at 4°C.	0.00	N/A	0.00	N/A
Cells heated for 85°C for 35 minutes	0.00	N/A	0.00	N/A
Cells and spores mix	2.32×10^8	17.7	1.61×10^8	8.0
Spores heated for 85°C for 35 minutes	2.67×10^8	5.5	1.67×10^8	6.0
Green tea extract 40 ppm for 5 minutes at 4°C	9.63×10^7	7.3	7.20×10^7	5.0
Cells and spores heated at 85°C for 20 minutes	1.01×10^8	0.6	1.61×10^8	8.0

Results of plating and FCM

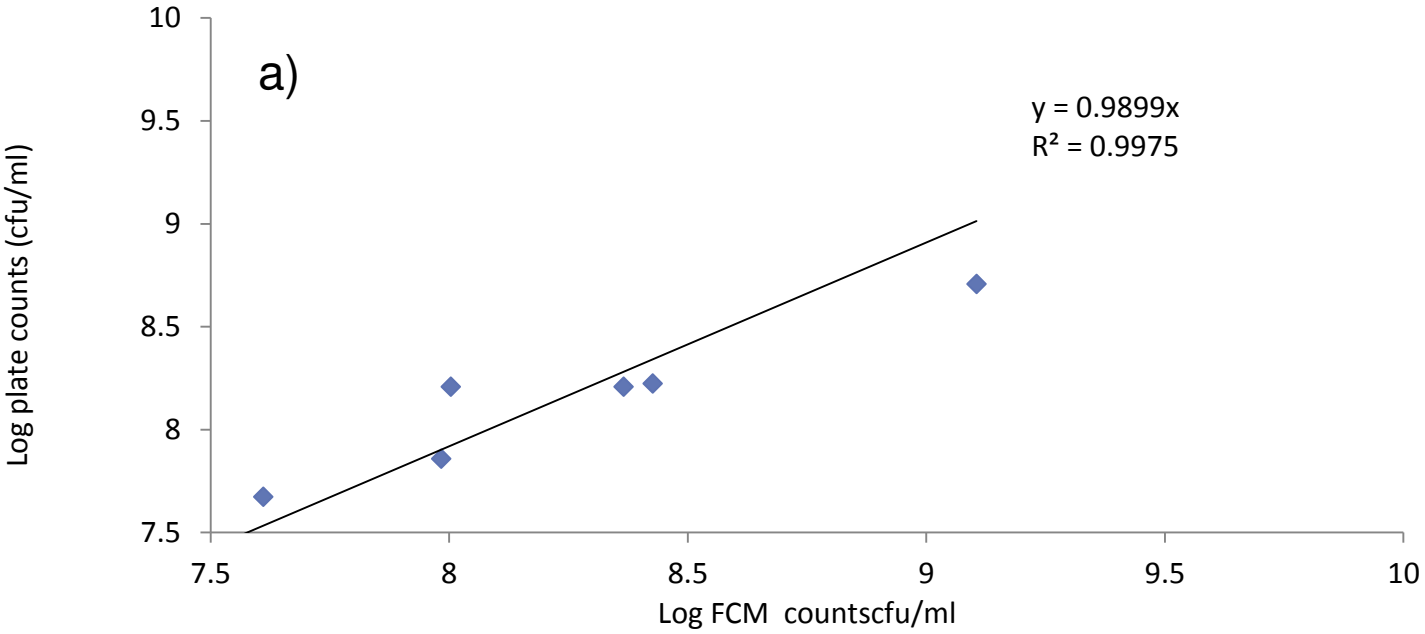


Figure 9. Log counts of FCM vs plating from samples in table 1. The r^2 value of 0.998 indicates a strong agreement between the two techniques.

Sample	Plates	FCM 4°C	FCM 28°C	PH
EtOH	1.79 x 10^7	1.99 x 10^7	8.60 x 10^6	1.90 x 10^7
HA*EtOH	1.43 x 10^7	1.55 x 10^7	8.54 x 10^7	1.20 x 10^7
Spores	2.37 x 10^7	9.32 x 10^7	2.02 x 10^7	3.05 x 10^7

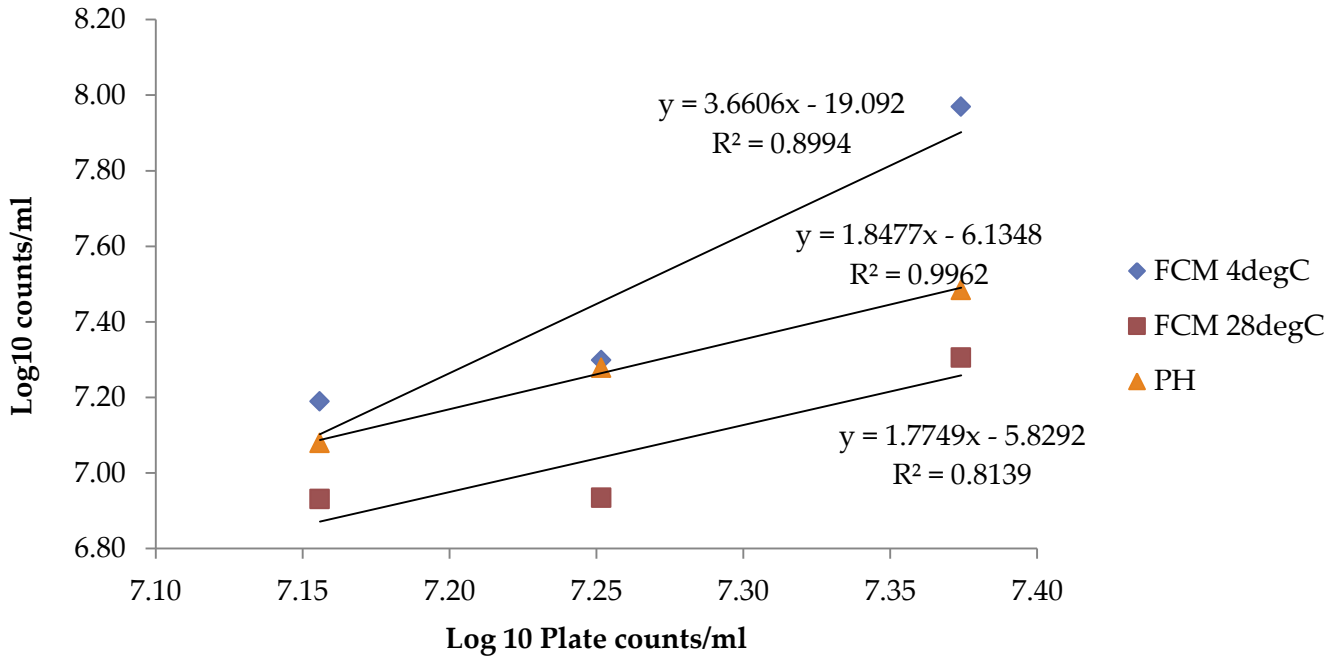


Figure 10. Plate count in comparison with FCM and PH counts. indicates PH counts are in a very strong accordance with plating results, and FCM shows a good level of agreement with plating given the r^2 value of 0.814 to 0.899.

Results of cells and spores subject to antimicrobial treatment

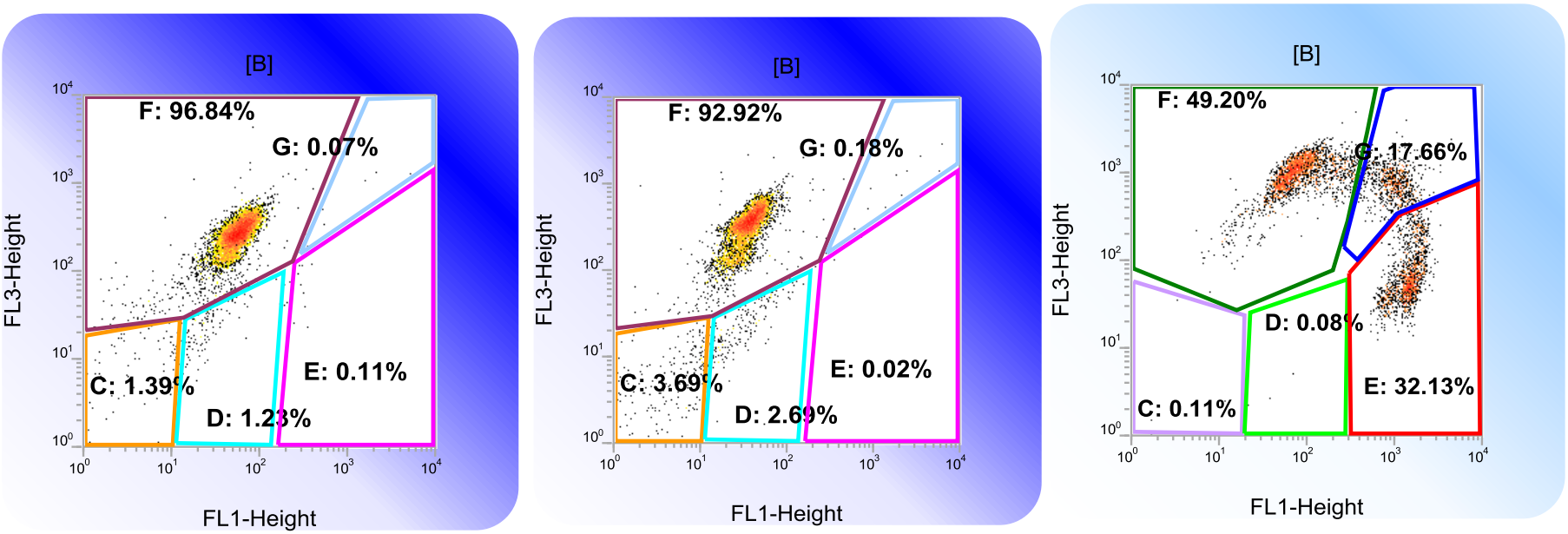


Fig 11. Green fluorescence (FL1-Height) x axis, against red fluorescence (FL3-Height) y axis density plot of a) PAA treated cells, b) Chlorine treated cells, and c) Green tea treated cells.

PAA and chlorine (Fig 11. a and b) cause cells to become highly stained with PI, whereas green tea gave rise to double staining (Fig 11. c)

Conclusions

- **FCM is a good method to enumerate sub-populations, based on a strong correlation with plate counts**

- Antimicrobials **PAA** and **Chlorine** both **have high bactericidal effects**, with PAA being the most effective antimicrobial. Previous research indicates this has the potential to kill spores as well as cells[1].

- **Green tea extract** also has an impact on viability, with around a **1log reduction in cell number**.

- **Green tea** caused more cells to become **damaged** or mildly **membrane permeabilised** as opposed to completely killed. Demonstrated by a strong double staining with PI and Syto 16.

- **Such insights are only possible by FCM multi-parametric analysis.**

i.e. Findings such as these highlight the significance of FCM as a descriptive tool, as plating or fluorescent microscopy would not give us information as to the numbers of damaged cells. It is also highly significant when one considers the lack of FCM enumeration data available.

Thank you for listening

Any Questions?

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Images

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