Application of flow cytometry for enumerating individual bacterial cultures from a mixed culture system

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Abstract

Cultured dairy products are often made with more than one microbial culture. Yoghurt requires the cultivation of several bacterial species for its production and the level of each is important for different reasons. Differential plate count methods to enumerate the separate species in yoghurt are not ideal because many of the bacteria used have similar growth profiles and plate counts take several days to produce a result. A fast specific method for enumerating each culture would be beneficial because quick results would enable tighter control of processing or experimental conditions and the ability to track individual species amongst a background of similar bacteria. Flow cytometry combined with fluorescent *in-situ* hybridisation (FLOW-FISH) was investigated as a potential solution and successful enumeration was achieved within 1 day for a yoghurt microorganism, *Streptococcus thermophilus* (ST55), grown in M17 medium. This method may be improved to increase the signal-to-noise ratio and to reduce the assay time. The chemical propidium monoazide enabled a closer match to plate counts for flow cytometry results using a total viable count assay and may be useful combined with the FLOW-FISH assay for removing non-viable or viable, but non-culturable, cells from the results. An enzyme and/or detergent pre-treatment may achieve successful FLOW-FISH enumeration of cells grown in reconstituted skim milk – a similar matrix to yoghurt.

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Abbreviations

AC	Aerobic count
BFM	Agar growth medium for *Bifidobacteria* sp.
CFU	Colony forming units
Cy3 and Cy5	Cyanine dyes
DAPI	4',6-Diamidino-2-Phenylindole
DGGE	Denaturing gradient gel electrophoresis
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOPE	Double labeling of oligonucleotide probes
EMA	Ethidium monoazide
FCM	Flow cytometry
FISH	Fluorescent in situ hybridization
FITC	Fluorescein isothiocyanate
FL1 or FL2	Fluorescence level 1 or 2
FLOW-FISH	FISH combined with flow cytometry
LED	Light emitting diode
M17	Agar or broth growth medium for *Streptococcus thermophilus*
MRS	deMan-Rogosa Sharpe growth medium for *Lactobacillus* sp.
N	Sample size
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Propidium iodide
PMA	Propidium monoazide
PMA-FLOW-FISH	PMA treatment of cells combined with a FLOW-FISH assay
PNA	Peptide nucleic acid
PMT	Photomultiplier tube
RB	Raffinose Bifidobacterium: a selective agar medium for *Bifidobacteria*
RCA	Reinforced Clostridial agar
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RSM	Reconstituted skim milk medium
RT-PCR	Real time-polymerase chain reaction
SD	Standard deviation

SEM	Standard error of the mean
Sth	*Streptococcus thermophilus*
ST55	*Streptococcus thermophilus* strain number
SYL	Agar growth medium that allows the growth of both *Streptococcus thermophilus* and *Lactobacillus bulgaricus*
SYTO® 9	Molecular Probes fluorescent stain
T_m	Melting temperature of a duplex DNA-DNA or DNA-RNA molecule
TPPYPB	Tryptone-proteose,peptone-yeast extract with Prussian blue agar growth medium
TVC	Total viable count
UHT	Ultra-heat treated
VBNC	Viable, but non-culturable

1 Introduction

Cultured dairy products (yoghurts, drinking yoghurts, milk-based desserts, cheeses, fermented creams) tend to be manufactured using mixed cultures. Starter inoculation for butters and cheeses will often involve a complex mix of lactic acid bacteria that will affect the formation of the aroma in butter, of eyes in cheese, and other sensory or flavour characteristics (Friedrich and Lenke, 2006; Randazzo et al, 2009). Cheese microbiologists desire to examine the diversity of the microorganisms during cheese manufacture and attempt to correlate the presence of certain bacterial species with specific sensory and flavour characteristics of the product (Randazzo et al, 2009). Another benefit of using a complex mix of starter cultures is better resistance to phage attack that would otherwise stop or hinder commercial production (Friedrich and Lenke, 2006). Yoghurt is another dairy product that requires the cultivation of several bacterial species as part of its production and the level of each species is important for different reasons.

1.1 Yoghurt manufacture

The basic manufacture of yoghurt occurs when lactic acid is produced from the lactose in milk and this is generally accomplished using both of the lactic acid bacteria *Lactobacillus delbrueckii* subspecies *bulgaricus* and *Streptococcus salivarius* subspecies *thermophilus* (Afonso and Maia, 1999). Varying levels of the two bacteria and/or addition of other species may be used to manage the quality of the product. The starter cultures are one of the three main factors that affect yoghurt texture (Sodini et al, 2004). Characteristics of different lactic acid bacteria, such as exopolysaccharide production, are important in fermented dairy products because they affect firmness and creaminess, can increase the viscosity, and may also decrease syneresis (Duboc and Mollet, 2001). For example, such differential characteristics between the bacteria include the type of exopolysaccharide produced and its resulting interaction with the milk proteins (Marshall and Rawson, 1999) or the timing and rate of exopolysaccharide production during the fermentation stage (Gancel and Novel, 1994). A study on the production and composition of exopolysaccharides by yoghurt starter bacteria showed that *S. thermophilus* produced less than *L. bulgaricus* when grown separately in cow's milk. But when grown together, the production of the exopolysaccharides was much greater than the sum of the amounts when grown separately (Frengova et al, 2000).

Another reason for the addition of other bacterial cultures into yoghurt is the perceived health benefits that may occur. Dairy products are promoted as being

excellent to use as carrier vehicles for probiotic bacteria. The reason they are promoted as such is that they generally already contain viable cultures, they (particularly fermented dairy products) tend to have a positive healthy image, and if manufacturers use probiotic bacteria as starter cultures, they cleverly combine these beneficial images (Heller, 2001). Potential health benefits include: the control of intestinal infections, the reduction of lactose intolerance and in serum cholesterol levels, an anti-carcinogenic activity, and perhaps the re-balancing of microbial populations in different gut disorders (Gilliland and Kim, 1984; Gueimonde et al, 2007; Lourens-Hattingh and Viljoen, 2001). There is also a suggestion that exopolysaccharide production plays a role in gut health as prebiotics and so research continues to expand on these new and old areas of fermented milk products (Duboc and Mollet, 2001).

The normal practice is to incorporate the probiotic organisms in with the yoghurt starter cultures. However, some yoghurt starter cultures produce conditions unfavourable to the probiotic organisms, or some species (eg. *Lactobacillus casei*) are better at surviving the production and storage conditions than others (Heller, 2001; Lourens-Hattingh and Viljoen, 2001). Hence, various mixtures of probiotic organisms are incorporated into dairy products in order to maintain sufficient viable cells at the point at which the product is eventually consumed. Products with a combination of probiotic and prebiotic components are labelled as “synbiotic” products. The prebiotic component appears to include oligopolysaccharides and it has been reported that the consumption of products containing both has greater beneficial effects. Lamoureux et al (2002) suggested that it would be useful if the probiotic organisms themselves could supply some of the prebiotic component and found that a mixed culture of *Bifidobacteria* (*B. animalis, B. infantis* or *B. breve*) was needed to achieve both the production of oligosaccarides and the survival of the probiotic organisms in the final product.

Specific knowledge of the different species in yoghurt is often required for marketing, quality assurance or safety purposes (Charteris et al, 1997; Dave and Shah, 1997; Friedrich and Lenke, 2006; Heller, 2001). Timely quantitative information about the composition of starter culture inoculations would be useful in their maintenance or optimization and/or balance of strains in the mixture (Friedrich and Lenke, 2006). Enumeration techniques are required to analyse the balance between aroma- and acid-forming strains in these mixtures to use as a tool for improving production. Research looking for new starter cultures and/or new probiotic organisms would need to be able to enumerate the different strains/species that grow under similar requirements (Salazar et al, 2009).

The minimum level of probiotic organisms needed to promote health benefits is thought to be 10^5 - 10^6 CFU per g or per mL of product (Dave and Shah, 1997; Lourens-Hattingh and Viljoen, 2001). Since studies have shown that probiotic organisms have low viability in market preparations of dairy foods, there is a need for dairy manufacturers to check the levels of probiotic organisms in their products to ensure they can make the health claim in their marketing campaigns (Gueimonde et al, 2004; Lourens-Hattingh and Viljoen, 2001; Shah, 2000). There is ongoing research attempting to increase the viability of probiobic organisms in fermented dairy products by altering production conditions, such as the use of oxygen-impermeable containers or changing additive concentrations, or by protecting the probiotic organisms using different encapsulation methods, or by using acid or bile resistant strains (Dave and Shah, 1997; Krasaekoopt et al, 2003; Lourens-Hattingh and Viljoen, 2001). To assess the effectiveness of these processing changes on the viability of the probiotic organisms would require the use of selective enumeration methods for each species of interest.

1.2 Methods for studying yoghurt populations

1.2.1 General considerations

Methods are needed to study the survival and other characteristics of the yoghurt organisms in the human system, as well as in the food, for optimising probiotic selection and processing conditions (Bunthof and Abee, 2002). Dairy products are employed as delivery agents for the introduction of beneficial probiotic organisms into the human gut and so the survival of probiotic organisms in the gastrointestinal tract of humans after consuming such products is a common area of research in regards to probiotic bacteria (Charteris et al, 1997; Dommels et al, 2009; Gueimonde et al, 2007; Lourens-Hattingh and Viljoen, 2001). Consumers will be attracted to the beneficial health aspects of yoghurt mentioned in Section 1.1 above, so yoghurt manufacturers will also be interested in producing products with the best probiotic capability. Combining information gleaned from both the human and food sides of the research equation can influence processing conditions. For example, pasteurising yoghurt, after it has been manufactured, can increase the shelf life, but leaving viable starters in yoghurt can offer lactose-intolerant people a consumable dairy product (Gilliland and Kim, 1984). The reason is that the viable starters appear to utilise the lactose before it enters the large intestine where it causes problems for these people.

1.2.2 Selective agar techniques

The most widely accepted method for enumerating bacteria is the traditional plate count using different agar media and incubation conditions to target populations of interest. However, differential plate count methods are not ideal, particularly for fermented dairy products, because many of the bacteria have similar growth profiles in addition to the time lag of several days to achieve a result (García-Cayuela et al, 2009; Goncalves et al, 2009).

The long incubation time is often stated as one of the major disadvantages of traditional plate count methods and is generally the same when testing dairy samples. Both Bunthof et al (1999) and Friedrich and Lenke (2006) comment in their introduction that a major drawback of conventional cultivation-based methods is the long incubation times that are needed to obtain a result such as countable colonies when testing samples for dairy applications. Friedrich and Lenke (2006) actually stated that the time length is about 5 days. However this most likely includes the identification time as well as the cultivation time although they do not say as much. Interestingly, looking through the literature related to lactic acid bacteria, this does not appear to be the most important issue.

Many researchers in this area have spent time assessing different agar methods for lactic acid bacteria (Dave and Shah, 1996; De Carvalho Lima et al, 2009). The reason appears to be that these microorganisms have very similar growth profiles, particularly within the same genus, and researchers have attempted to find the right method for enumerating their target organisms within a mixed culture system (Bracquart, 1981). Widely accepted for use with dairy products are the agars M17 for *S. thermophilus* and deMan-Rogosa Sharpe (MRS) for *Lactobacillus* sp, and BFM for *Bifidobacteria* sp (Collado and Hernández, 2007; ISO and IDF, 2003).

Some lactic acid bacteria have been easier than others to accurately enumerate. When there has been only a couple of species present, enumeration of each species is often achieved. Birollo et al (2000) were able to successfully enumerate *Streptococcus thermophilus* and *L. bulgaricus* in different types of yoghurts using skim milk, M17, and MRS agars. There appears to have been no other bacteria present in the samples. Differentiation based on colony morphology was easily achieved on the skim milk agar under aerobic conditions only. The M17 agar grew only *S. thermophilus*. The MRS agar also grew both cultures under both aerobic and

anaerobic conditions but they were only slightly differentiated under anaerobic conditions. All the plates were incubated at 37°C. Lamoureux et al (2002) made minor changes to a single medium (supplemented Columbia agar base medium) in order to differentiate *Bifidobacterium bifidum* from the other species in the *Bifidobacterium* genus using traditional plating methods but the method was only tested on samples containing two *Bifidobacterium* species at a time. Shah (2000) wrote that although some selective media have been proposed for the enumeration of *Lactobacillus acidophilus* and *Bifidobacterium* spp., they were unfortunately based on pure cultures of these bacteria, and claimed that there are only a few reports of selective enumeration of *L. casei* in the presence of yoghurt and other probiotic bacteria. Media and incubation conditions have been characterised to selectively enumerate the different bacterial groups but testing for a specific species is often difficult when there is more than one species present from the same genus (Shah, 2000).

In other cases, only moderate success has been achieved. Hartemink et al (1996) designed a selective medium for *Bifidobacteria* that they designated Raffinose Bifidobacterium (RB) agar. Apparently it was easy to prepare and contained no antibiotics. The actual selective inhibitory agents were propionate and lithium chloride. Raffinose was used as the selective carbon source and casein was used as a protein source. The presence of casein in the medium resulted in a yellow zone of precipitation around the yellow colonies of *Bifidobacteria*. At the time of publication, the authors stated that this medium was the most selective medium available for the detection of *Bifidobacteria* species. However, it was not perfectly selective as five *Bifidobacterium* species either did not grow or did not show the correct defining features. Additionally, a few other species, namely an *Actinomyces* species, a *Clostridium perfringens*, and some animal-derived lactobacilli, were able to grow on this medium. All the cultures were tested as single cultures, so the selectivity of this medium would need to be verified when testing mixed culture systems. In order to be able to specifically enumerate four separate species of probiotic organisms in mixed culture systems, De Carvalho Lima et al (2009) had to evaluate 21 culture media with different incubation conditions to find suitable methods for each species. The problem with their approach is that you would need to know what other species were present in the sample before you could choose the appropriate method for the target species.

All of this research into methodology usually resulted in analysts having to utilise several agar methods on each sample, which is costly in terms of time and money. Perhaps Lee and Lee (2008) have part of the solution to this problem with their bromophenol blue modification to MRS agar. Ten type cultures and 5 commercial yoghurts were tested on this new agar under anaerobic conditions and the results compared to a few other agar media. The modified agar allowed the enumeration, individually, of all 10 species (7 *Lactobacillus* sp, 2 *Bifidobacterium* sp, and 1 *Weissella* sp) when grown in anaerobic conditions at 35-37°C for 48h. This was possible because the colony type for each species was different. A second possible solution was published in 1981 by Bracquart who developed an agar medium (TPPY-eriochrome) that allowed the simultaneous enumeration of *S. thermophilus* and *L. bulgaricus* in just 24 hours. Another advantage in using TPPY-eriochrome was that the lactobacilli colonies were larger than normally achieved on MRS medium and were therefore easier to count. The disadvantage was that the colony numbers of the two species needed to be fairly equal for this medium to be useful. An alternative solution used an agar called tryptone-proteose-peptone-yeast extract with Prussian blue agar (TPPYPB) that allowed the growth and enumeration of all four bacterial types at the same time (Teraguchi et al, 1978; cited by Lourens-Hattingh and Viljoen, 2001). Once again this was because the colonies were visually different to each other. However, in the search for the perfect agar plate count method, the additional ingredients and/or changes made to the methods often created another problem.

The use of these differentiating agar methods often results in reduced cell numbers. Antibiotics, or selective chemicals and method conditions, will often injure the target species to some extent as well as the unwanted species. Dave and Shah (1996) evaluated the suitability of fifteen agar media for the most effective selective enumeration of *S. thermophilus, L. delbrueckii* ssp. *bulgaricus, L. acidophilus,* and *Bifidobacteria* species. However, the methods for *L. bulgaricus* and the *Bifidobacteria* sp. were not accurate as both methods were slightly inhibitory on the growth of the target organisms as well as the non-target organisms. Montoya et al (2009) looked at the impact of putting two probiotic bacteria into a Swiss cheese curd and found that the bacteria appeared to survive and grow with no negative effects on the cheese. One challenge they needed to overcome for their study was to be able to enumerate the two target bacteria without having the results affected by the presence of natural cheese flora. Previous studies by other researchers used

streptomycin for the sole detection of probiotic bacteria so Montoya et al (2009) tested a gradient of streptomycin across an MRS agar plate to determine the concentration of the streptomycin needed to inhibit the natural flora but not the probiotic bacteria. The authors stated that higher concentrations could inhibit the growth of their target probiotic bacteria, particularly if they had previously been subjected to acid stress, although they authors did not publish any data on this.

Finally, to add to the difficulty of accurately enumerating lactic acid bacteria, certain physical attributes will give an apparent reduced cell number, such as chains of cells making one colony, or cells that are still viable but no longer culturable that can be created by the dairy product manufacturing conditions. Bunthof et al (1999) mention that the viable cell count is often underestimated due to cell clumping and chain formation. However, they did not seem to have any referential basis for this comment, unless the referenced IDF methods mention it. Wallner et al (1995) also mention as a minor comment in their discussion that inaccurate plate counts are obtained when activated sludge cells are in chains or flocs. Walker et al (2005) performed direct microscopic counts and showed that a *Bifidobacteria* sp. had the tendency to form clumps during dairy manufacture. The authors suggested that growth of colonies on agar plates from clumps of bacteria rather than from individual cells may give false counts of viable cells and describe how a Most-Probable-Number method gave results 10 times higher than the plate count method for this species. In one study, the levels of 16S ribosomal RNA (rRNA) were compared with culturable and viable-but-non-culturable cell (VBNC) levels of *Bifidobacterium* sp using plate counts and flow cytometry methods (FCM) (Lahtinen et al, 2008). The authors determined that VBNC cells retain levels of 16S rRNA similar to culturable cells, whereas truly non-viable cells have a faster rate of rRNA degradation. The authors concluded that probiotic bacteria stored in fermented products may be unculturable but still have some features that are normal for viable cells. Collado et al (2005) found that plate counts gave a ten-fold lower estimation of viable cells for *Bifidobacterium* species when compared to a FCM total viable count method after exposure to a simulation of the human gastrointestinal tract and surmised that the difference is likely to be due to VBNC cells. Collado et al (2006) also found that plate count results for *Bifidobacterium animalis* were lower than Fluorescent In Situ Hybridisation (FISH) results by 10 – 100 fold, but the FISH method does have the potential to detect the DNA of both viable and non-viable cells.

So in summary, many researchers and manufacturers are interested in knowing the specific level of different bacteria because the information can be used for adjusting the manufacturing conditions or determining the survival of the bacteria that confer a health benefit. Traditional agar methods are time consuming and products such as yoghurt and the probiotic cultures themselves have already been produced long before the agar plate results have been determined. A fast method might allow a manufacturer to monitor the growth of the probiotic organisms or starter cultures and only stop production once the desired level has been achieved. Using an alternative method that specifically detects and enumerates the target organisms would hopefully avoid the issues inherent in the agar plate methods. So, the challenge is to develop a method that would fit this need.

1.2.3 Faster cultivation techniques

The first requirement for this ideal method is to reduce the time taken to achieve a result. Cultivation methods have reduced the incubation time a day or so by using methods such as Petrifilm (Goncalves et al, 2009; Nero et al, 2007) or MicroFoss (Odumeru and Belvedere, 2002).

Petrifilm

Petrifilm has additional benefits in that it is extremely easy to use, requires little in the way of media or glassware preparation, and takes up very little space in the incubator. Goncalves et al (2009) assessed the enumeration of *S. salivarius* subsp. *thermophilus* and *L. delbrueckii* subsp. *bulgaricus* grown in non-fat milk for 4h using dilutions in M17 and acidic MRS broths in association with Petrifilm Aerobic Count (AC) plates incubated in anaerobic conditions. This method took 48h to achieve a result and was compared to results using ISO 9232 methods that take 72h. The acidity of the MRS broth at the moment of inoculation and the acidity of the yoghurt at the end of the incubation appeared to have interfered with results a little by affecting the growth of some of the cultures. The authors showed that Petrifilm AC plates were adequate for enumerating *S. thermophilus* in conjunction with dilutions using M17 broth; whereas the selective enumeration of *L. bulgaricus* using acidified MRS for the dilutions was not achieved. Part of the speed achieved by using Petrifilm lies in the reduction of the 2,3,5-triphenyltetrazolium chlorine compound, present in the Petrifilm agar, that shows up the colonies quickly with a red colour. Unfortunately, many of the lactic acid bacteria associated with milk have a low capacity to reduce this compound to the red form so the colonies do not show up easily (Goncalves et al, 2009; Nero et al, 2007) or they are inhibited by the compound (Nero et al, 2007). It is believed that acidity of

some foods interferes with this method too by inhibiting the growth of some species. Perhaps this is because the food product and the associated dilutions are not diluted when deposited onto the thin Petrifilm layer, whereas they are diluted when mixed with 15-20mL of molten agar in traditional agar plating methods. Even if these issues could be resolved, these methods still have most, if not all, the same problems as the agar plate methods. In particular, it still takes days to obtain a result and it would be difficult to enumerate a specific starter or probiotic culture amongst other lactic acid bacteria.

MicroFoss

Liquid growing methods such as impedance or MicroFoss methods can reduce the time taken to achieve a result to less than 24h. Walker et al (2005) successfully measured a *Bifidobacteria* sp. within 15h at a level of 10^6 CFU/g using an impedance method. This is a significantly faster result than that achieved with the normal plate count method on Reinforced Clostridial Agar (RCA) which takes 3 days. However, this was achieved for samples containing only a single species within the samples. Impedance microbiology has been used to quantify the bacterial content in raw cow's milk and a similar method called the BacT/ALERT 3D system allowed faster monitoring of pasteurised milk for contaminating Gram negative organisms, even when levels of Gram positive organisms were high (Domig et al, 2013; Felice et al, 1999). However, this type of method is limited by the same issue that plagues the traditional plating methods in regards to detection of a single target species within a mixed population.

1.2.4 Alternative methods

Mass spectrometry

Talon et al (2002) obtained a good correlation between plate counts using SYL agar and a turbidimetric/pyrolysis-mass spectrometry method for the enumeration of *S. thermophilus* and *L. bulgaricus.* SYL agar allowed the enumeration of both cultures at the same time because the colonies were different. The authors did not cite the reference for this agar medium, nor was it found after an on-line search, but they did give the recipe for preparing it. For the turbidimetric/pyrolysis-mass spectrometry method, the yoghurt was made transparent by solubilising the fat globules and the casein micelles and then the total population of bacteria was collected using centrifugation. The turbidimetric part of the method estimated the total population and then the relative proportions of each species were determined using the pyrolysis-mass spectrometry method. The benefits of this method appear to be that it

requires little sample treatment, it is rapid (<1h), and can identify species at the same time. However, it was applied to yoghurt samples inoculated with two species only. Additionally, analysis of these results required a lot of mathematical transformations, which would make this a user-*un*friendly method unless computer software is developed that would streamline this part of the method.

PCR methods

Real time (RT) polymerase chain reaction (PCR) methods are an improved way of obtaining information on bacterial populations in samples. The need for viewing the PCR amplified products using gel electrophoresis has been eliminated giving a faster result. Additionally, molecular methods give information on all bacteria present in the sample, whether they can be cultured or not. Furet et al (2004) explain that quantitative PCR is more suitable than other molecular methods, such as quantitative dot blot hybridisation and whole-cell fluorescent in-situ hybridisation methods, when the target population is less than 1% of the total bacterial population in the sample. For dairy samples, quantitative PCR techniques have been utilised to examine starter culture populations (Friedrich and Lenke, 2006), breast and raw milks (Delcenserie et al, 2005; Martín et al, 2009), cheeses (Delcenserie et al, 2005; Serhan et al, 2009), and fermented milk products (Furet et al, 2004; García-Cayuela et al, 2009; Ongol et al, 2009). Friedrich and Lenke (2006) note in their introduction that many studies have developed real time PCR assays but few have gone the extra step of developing quantitative real time PCR assays. This may be due to the extra work needed.

Quantitative PCR methods generally require the production of standard curves, perhaps every time an experiment is performed to take into account experimental variations (Martín et al, 2009). Friedrich and Lenke (2006) themselves mention that the use of absolute standard curves for quantification is laborious and often complicated. Instead they calculated the proportions of their target populations relative to the total 16S rRNA genes but, in order to achieve this result, they had to apply dynamic tube normalisation, slope correction and efficiency value calculations. Delcenserie et al (2005) also advised that the use of a different gene to the 16S rRNA gene for quantifying purposes may be better because many species, such as *Bifidobacteria*, contain multiple copies of the 16S rRNA gene per chromosome. A final point to consider is that DNA is firstly isolated from the all of the cells in the sample before applying the PCR assay, and is often diluted to avoid

complications from PCR inhibitors (Delcenserie et al, 2005), making several hours the overall time needed for this type of method.

1.3 Flow cytometry

Flow cytometry (FCM) could be the fastest solution to enumerating lactic acid bacteria in dairy products. Large numbers of bacterial cells can be evaluated individually and the analysis of an individual sample through a flow cytometer can take as little as a few minutes or perhaps two hours when including additional sample processing such as milk clearing before FCM analysis (Breeuwer and Abee, 2000; Flint et al, 2006). Breeuwer and Abee (2000) stated that highly sensitive fluorescent techniques are nicely matched with FCM for a fast and easy-to-use method. However, this is not a common claim amongst other researchers.

A popular advantage of FCM is the ability to enumerate cells that are dormant or non-culturable, yet still viable. For that reason FCM has been used to assess the levels of *Bifidobacteria* in infant faecal samples (Hong et al, 2009), to determine the extent of the bifidobacterial resistance to bile salt stress (Amor et al, 2002), and to examine the effect of heat and pressure on the viability of *Lactobacillus* species (Ananta and Knorr, 2009) using different staining methods. Dairy applications already developed include sterility testing of Ultra-Heat Treatment (UHT) processes on milk and cream, yeast spoilage of fermented milk products, analysis of probiotic and starter cultures, bacterial quantification in milk, enumeration of total viable organisms and thermophilic bacteria in milk powders and a Gram stain equivalent on milk samples (Bunthof and Abee, 2002; Holm et al, 2004; Flint et al, 2006; Flint et al, 2007; Holm and Jesperson, 2003).

Detection of a target microorganism within a mixed culture sample is possible. Fluorescent antibodies in conjunction with FCM can be used to detect specific pathogens (Comas-Riu and Rius, 2009). However, the use of fluorescent antibodies has limitations such as: changes in target antigen expression due to changing environmental factors; high levels of background fluorescence produced by non-specific binding of the antibodies; and false-positive or false-negative results due to cross-reactivity or a lack of reaction that has occurred (Theron and Cloete, 2000). Detection and/or enumeration of specific bacteria in dairy samples were achieved where *Listeria monocytogenes* was speedily enumerated using fluorescent-in-situ-hybridisation (FISH) combined with FCM, often nicely termed a FLOW-FISH assay. In this study the procedure was performed on a very small testing platform, called a CHIP, in just under 5hrs (Ikeda et al, 2009).

1.3.1 Lactic acid bacteria and flow cytometry

The enumeration of lactic acid bacteria in dairy products using FCM has occurred in the following ways. The BacLight LIVE/DEAD kit in conjunction with FCM was used to assess the levels of *Bifidobacteria* present in Spanish fermented milk products (Collado et al, 2005) and to assess the viability of *L. acidophilus* and *B. animalis* ssp. *lactis* mixed together in a lyophilised product and stored over a period of 3 months (Kramer et al, 2009). Kramer et al (2009) also used a propidium monoazide (PMA)-PCR method that they preferred because it enumerated each species individually, whereas their FCM method gave only a percentage ratio of total live to total dead cells in the total population. A number of FCM viability staining methods were used to assess the viability of and enumerate three *Bifidobacterium* species (two *B. longum* cultures and one *B. animalis* subsp. *lactis* culture) during storage in fermented milk and fermented oat products (Lahtinen et al, 2008). However, each fermented product sample was inoculated with only one of the three species for each trial so the FCM results could be used as enumeration results as well as giving information on the viability state of the culture.

In another study, a FLOW-FISH method was compared to a quantitative PCR method to determine the levels of *Leuconostoc* spp., *Lactococcus lactis* ssp. *cremoris*, and *L. lactis* ssp. *lactis* in mesophilic starter culture mixtures (Friedrich and Lenke, 2006). The quantitative PCR method was apparently more flexible but the benefit of the FLOW-FISH method was the greatly reduced analysis time. The reasons for the greater flexibility of the PCR method was believed to be due to DNA being the target molecule, rather than RNA, and that the bacterial strains could be differentiated at the subspecies level.

1.3.2 Flow cytometry and fluorescent in situ hybridisation (FLOW-FISH)

FCM detects individual fluorescently-labelled cells as they pass through the instrument in a flowing stream of liquid (Shapiro, 2003). The flowing liquid, often called the “sheath fluid”, drags out a very thin line of the sample so that cells are separated from each other. Each cell then passes a laser light that excites the fluorescent label within, or attached to, the cell. Virtually instantaneously, the fluorescent label then emits light of a different wavelength that is captured by a microscope lens and photomultiplier tube detectors. What information is captured depends on the light processing system that is attached to the flow cytometer. Laser light scattered by the

cells (both forward and/or side scattered) can also be captured in addition to that of the fluorescent-label.

FISH is simply one way of fluorescently-labelling cells. Often this is used to detect specific cells of interest using a microscope but counting cells in this manner is laborious. Combining a FISH method with a FCM method eliminates the tedious labour component (Charteris et al, 1997). To label cells in this manner, they will generally need to be fixed and perhaps the cell membrane permeabilised to allow entry of the fluorescently-labelled oligonucleotide probe into the cell (Schleifer et al, 1995). Once inside the cell, the oligonucleotide probe must find its target sequence and hybridise to it. The cells are then washed to remove unbound oligonucleotide probe and are resuspended into a buffer of the correct pH for the fluorescent-label. The sample with the fluorescently-labelled cells can then be entered directly into the flow cytometer for analysis. The detection limit for FISH methods is often reported to be close to 10^3 cells/mL (Justé et al, 2008). Not an ideal detection limit for many applications, but for the enumeration of lactic acid bacteria in yoghurts where the levels are as high as 10^8 – 10^9 cells/mL, this would not present a problem.

FISH assays also have the potential to simultaneously detect two or more species with the use of differently-coloured DNA probes (Alagappan et al, 2009). However, this application is only useful in a FLOW-FISH assay if the flow cytometer has the capability to detect more than one fluorescent product. For example, Hong et al (2009) found that FLOW-FISH was not as flexible for their studies because there was a limited number of fluorophores that worked in the UV range that could be differentiated from one another by their emission spectra.

1.3.3 Dealing with non-viable cells

The ability of FISH assays to detect all cells, from those that can be cultured to those that cannot, is both a benefit and a limitation of the method because all the non-viable cells will be detected as well. The same double-sided characteristic is true of PCR assays but this has been solved by pre-treating cells with propidium monoazide (PMA). Ethidium monoazide (EMA) was originally used in PCR applications to remove the non-viable cells (Nogva et al, 2003) but was superseded by PMA in 2006 as EMA had been found to enter the viable cells of some species (Nocker et al, 2006).

Propidium monoazaide (PMA) is a shortened name for phenanthridium, 3-amino-8-azido-5-[3-(diethylmethylammonio) propyl]-6-phenyl dichloride. PMA is a chemical identical to propidium iodide (PI), widely used to determine non-viable cell populations for different methods, except that it contains an azide group that can cross-link to the DNA just as EMA does (Nocker et al, 2006). One dye molecule of PI binds between the bases of DNA at every 4^{th} to 5^{th} base. It is assumed that PMA binds similarly and it appears that the addition of the azide group has not affected the action of the chemical. PMA has a higher charge than EMA, two positive charges instead of one, and this higher charge is thought to be the reason why it does not enter through the membrane of viable cells like EMA does for some bacterial species (Amor et al, 2002; Flekna et al, 2007; Nocker et al; 2006; Shapiro and Nebe-von-Caron, 2004). Sometimes PI is unable to stain non-viable cells and this is thought to be due to maintenance of the integrity of the cell membrane (Breeuwer & Abee, 2000).

This additional step to PCR assays appears to remove the non-viable cells from the population and give results comparable to plate count methods. The main reason for the inhibition of PCR amplification for non-viable cells appears to arise from the loss of the EMA- or PMA-bound DNA template during the DNA extraction step (Nocker and Camper, 2006; Soejima et al, 2007). The use of EMA or PMA has also been explored in other assays such as Denaturing Gradient Gel Electrophoresis (DGGE) analysis (Nocker et al, 2006; Nocker et al, 2007), microarrays (Nocker et al, 2009), and FISH (Regan et al, 2003) with promising results. Many of these methods would utilise a DNA extraction step except for the FISH assays. Since a PMA treatment can successfully remove signals from non-viable cells in a FISH assay, it is highly likely to do the same for a FLOW-FISH assay. Nocker and Camper (2006) also mention that amplification of the EMA-bound DNA template itself does not occur, although it appeared to a minor factor in reducing the PCR signal from non-viable cells.

Interestingly, earlier, Riedy et al (1991) had already combined EMA with flow cytometry to distinguish non-viable cells from viable cells for eukaryotic cells. They were using fluorescent antibodies to detect human peripheral blood cells and they found that antibodies had the same limitation as PCR and FISH assays of detecting non-viable cells in addition to viable cells. Riedy et al (1991) explained that the advantage of EMA staining, before their specific immuno-staining method, was that it was irreversible and not affected by fixation and washing steps. Additionally, although the EMA bound to the DNA was excited

by laser light at 488nm, the light emitted was different to that given by fluorescein and the phycoerythin labelled antibodies. Hence they were able to detect the viable portion of their specific cells of interest. In regards to bacterial cells, Soejima et al (2009) also combined EMA with FCM for pure cultures of *Mycobacterium tuberculosis* to remove injured cells from their FCM assay.

While PMA or EMA has been linked to FISH or to FCM, it appears that all three methods have not yet been linked together. What is clear from many studies (Delgado-Viscogliosi et al, 2009; Soejima et al, 2007) is that optimisation of the PMA or EMA concentration is needed to avoid harming/affecting the viable cells in the population.

1.4 Concluding remarks

In conclusion, a successful FLOW-FISH method would be able to rapidly enumerate a specific lactic acid bacterial species within a mixed population of other lactic acid bacteria. As there are already robust FISH methods available for enumerating *S. thermophilus* in dairy product samples it makes it the target of choice for a first approach to a FLOW-FISH method. Set yoghurt is one type of preparation of yoghurt that is fermented in the retail container. Detection of *S. thermophilus* grown in reconstituted skim milk (RSM) overnight could be used as the model system for set yoghurt. Interestingly, although PMA (or EMA) has been applied to both FISH and FCM separately, it appears that it has not been used in combination with both methods together as a PMA-FLOW-FISH method. A PMA treatment could be applied to the *S. thermophilus* cells before the successfully operating FLOW-FISH method as a new way of rapidly enumerating specific viable cells in a mixed population.

1.5 Objectives of this study

- Adapt a previously designed FISH assay into a FLOW-FISH assay for *S. thermophilus* grown in RSM
- Investigate the potential of a PMA treatment for eliminating non-viable *S. thermophilus* cells

2 Materials and methods

2.1 Summary

S. thermophilus (strain ST55) was grown in reconstituted skim milk as a model for set yoghurt products. A fluorescent in-situ hybridisation method for *S. thermophilus* utilised by Flint (1998) was adapted for use in a FLOW-FISH assay. Two additional flow cytometry assays were used to give further information for several experiments. The first enumerates the total viable cells in a sample (Flint et al, 2006) and the second differentiates between viable and non-viable cells (Molecular Probes, 2004). A propidium monoazide treatment successfully used for real-time PCR assays on various lactic acid bacteria was explored for the potential of removing non-viable cells from the FLOW-FISH assay (García-Cayuela et al, 2009).

2.2 Culture methods

2.2.1 Liquid culture

The yoghurt starter bacterium *S. thermophilus* (strain ST55) was obtained from the Microfermentation Group at the Fonterra Research Centre in Palmerston North, New Zealand. It was grown in 10mL of autoclaved 10% reconstituted skim milk (RSM) medium overnight at 37°C under aerobic conditions in an incubator.

2.2.2 Plate count enumeration

Samples were serially diluted ten-fold using 0.1% peptone (Fort Richard). Duplicate 1mL aliquots of each dilution were pipetted into petri dishes and 15-20mL of molten M17 agar (cooled to approximately 45°C) was poured on top, mixed well, and left to set. Once set, the plates were inverted and incubated in bags in a 37°C incubator for 48h. Colonies are counted on the appropriate dilution plate (with 30-300 colonies present) and adjusted to give the number of colony forming units (CFU)/mL after taking any dilution factors into account.

2.3 Yoghurt emulation and treatment

There are many varieties of yoghurt but there are two common types based on the physical state in the retail container: set and stirred yoghurts (Afonso and Maia, 1999). For set yoghurt, the milk is inoculated with starter culture, poured into the retail container, and the fermentation stage occurs in the retail container, with no stirring, through incubation at a set temperature for a number of hours. Set yoghurt was emulated by using a single starter culture (*S. thermophilus* [ST55]) grown in 10%

reconstituted skim milk (RSM) at 37°C overnight with no movement. RSM was used as it is sterile so there would be no other bacteria present to interfere with the experiments.

It is common to treat dairy samples with chemicals or centrifugation procedures to remove the dairy protein or lipid fractions (García-Cayuela et al, 2009; Flint et al, 2007) as they tend to interfere with many assays. According to a personal communication from the manufacturer, the accurate enumeration range of the flow cytometer used in this study is thought to be between 10^3 - 10^6 cells/mL. Cell numbers of cultures in yoghurt generally reach large numbers outside the accurate range and so samples will require diluting to bring the numbers down to be within the accurate range for the flow cytometer. It was assumed that this dilution procedure would also greatly reduce the amount of dairy components present in the assays and therefore avoid having to pre-treat the samples. Hence, samples were diluted in a phosphate-buffered saline (PBS; 130mM sodium chloride, 10mM sodium phosphate buffer, pH7.2) or 0.1% peptone, usually to 10^{-4} - 10^{-5}.

2.4 Microscopy

A drop, approximately 10µL, of sample liquid was placed onto a glass microscope slide and covered with a coverslip. This sample was then examined using epifluorescent microscopy with a Zeiss axioscop 2 plus microscope. After excitation via a mercury bulb, the image was captured using a filter set appropriate for fluorescein isothiocyanate (FITC) and acridine orange stains (Excitation Band Pass filter: 450-490, Emission Long Pass: 515).

2.5 Flow cytometry (FCM) methods

2.5.1 Flow cytometer

A BactiFlow™ flow cytometer (Figure 1) manufactured by AES Chemunex (Paris, France) was used for all of the FCM assays. It has a 20mW solid state excitation laser light operating at 488nm (blue wavelength) and photomultiplier tubes (PMT) that catch the emitted light in the red and green light ranges after passing through different filter sets. The FL1 (probably stands for Fluorescence Level 1 but is not defined as such by the manufacturer) PMT collects light after it has passed through an EM515 filter, which allows only green light through. The FL2 PMT collects light after it has passed through a DM540 filter and this lets the majority of red light through with a small amount of green and blue.

The machine was calibrated at the beginning of each day using the fluorescent beads (Standard G) supplied by the manufacturer.

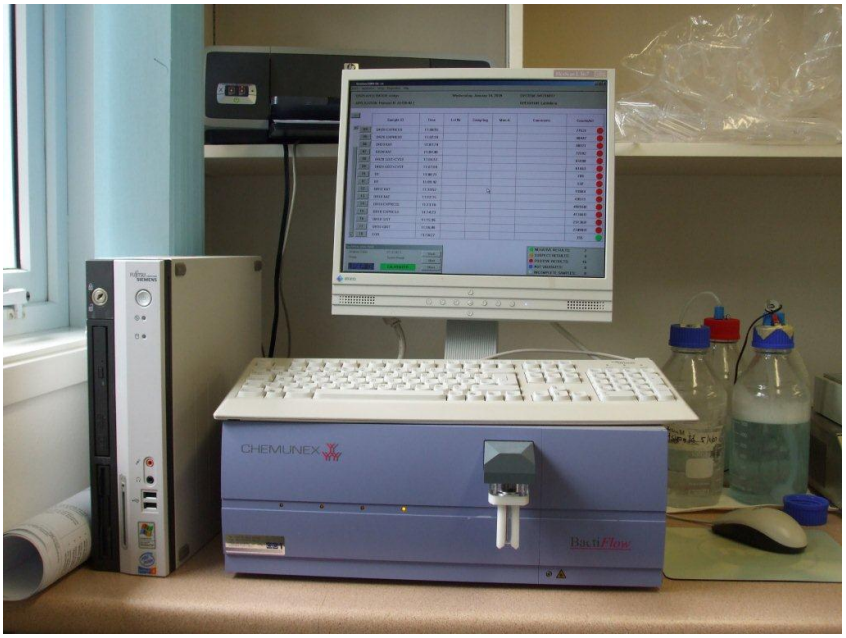

Figure 1 - BactiFlow™ flow cytometer. A single sample unit produced by AES Chemunex (Paris, France).

2.5.2 Total Viable Cell (TVC) assay

A slightly modified (combined) version of the total viable cell assay as used by Flint et al (2006) and Flint et al (2007) was used as an alternative method to plate count enumeration to determine the number of viable cells in samples. Due to the speed of this method, it was also very useful as an initial check of cell numbers before proceeding with an experiment.

This method utilises a non-fluorescent substrate that is modified by an internal esterase enzymatic reaction within each bacterial cell to a product that fluoresces upon exposure to the light produced by the 488nm laser. Light emitted by the fluorescent product is in the green light wavelength range (515nm). Viable cells will have larger numbers of the enzyme and also have an intact cell membrane that will retain the majority of the fluorescent end product within the cell for detection by the flow cytometer.

Duplicate or triplicate samples (0.4mL) were placed into test tubes. The following AES Chemunex (Paris, France) reagents were added in this order:

3mL of ChemSol B26/1 (buffer solution), 115µL of CSV (0.1% blue dye counterstain), and 30µL of V23 (non-fluorescent substrate). The test tubes were capped and incubated in a 30°C heating block for 20 min. A small spatula of CSR powder (background reducing reagent) was added and mixed quickly using 3 short bursts on a vortex mixer. This step helps to reduce background fluorescence. A 0.6mL aliquot was placed into a small sample tube that was then inserted into the sample tube holder on the flow cytometer. Of this aliquot, only 100µL was analysed by the flow cytometer, provided the number of fluorescent events did not exceed the maximum value and then only approximately 6.5µL would be analysed.

The analysis of the samples used software provided by AES Chemunex (Paris, France). The protocol is set so that fluorescent events are only recorded when the amount of the green fluorescence emitted by a particle crosses a threshold value. The fluorescent events are classified as viable cells when the amount of the emitted green fluorescence roughly matches the amount of emitted red fluorescence (auto-fluorescence of a particle). A box is placed around these events, the number within recorded and defined as total viable cells (TVC)/mL after taking dilution factors into account.

A reagent control (containing 3.4mL of ChemSol B26/1, 115µL of CSV, and 30µL V23) was always placed at the beginning and end of each batch of test samples to check that the reagents were not contaminated.

2.5.3 Flow cytometry and fluorescent in-situ hybridisation (FLOW-FISH)

As explained previously, the theory behind the FLOW-FISH assay is that specific target cells are fluorescently labeled using a fluorescently-labelled oligonucleotide probe that binds only to the target cells and these fluorescent cells are then enumerated using FCM.

Origin of the species specific oligonucleotide probe for the detection of S. thermophilus.

Ehrmann et al (1992) designed an oligonucleotide for the specific identification of *S. thermophilus*. The design of the probe was based on a region of the 23S rRNA and was found to be specific for *S. thermophilus* after being tested on *S. thermophilus* and *S. salivarius* as well as other lactic acid bacteria. This probe was then used successfully in subsequent studies (Beimfohr et al, 1993; Flint, 1998; Schleifer et al, 1995). However, it should be noted that the sequence of the probe used in these later studies was slightly different to that originally published by Ehrmann et al (1992). It has been surmised that there were

mistakes printed in the original paper. At least two of the authors of the original paper were co-authors on the papers written by Beimfohr et al (1993) and Schleifer et al (1995). The revised DNA probe that was used by these papers was purchased for this study, with a fluorescent label, using the online ordering system for OligosEtc, Inc. (Wilsonville, OR 97070, United States). The synthesis conditions were: 0.2µM scale, modification with 5'6-FAM (a fluorescein derivative used for labelling oligonucleotides), and purification using High Performance Liquid Chromatography to ≥90%. The sequence of the probe was 5' – CAT GCC TTC GCT TAC GCT – 3' and the primer to target melting temperature (T_m) of the probe was 67.6°C according to OligosEtc, Inc. The labelled DNA probe was reconstituted to a concentration of 134µg/mL using sterile DNAse-free water and aliquoted into 100µL amounts in Eppendorf vials. A working vial was kept wrapped in tin-foil at 4°C and the remainder of the vials were stored at -20°C.

Method adaptation

Flint (1998) performed a FISH assay using this probe to detect *S. thermophilus* cells adhered to stainless steel using fluorescence microscopy. For this study, adaptation of that FISH assay for a FLOW-FISH assay was based upon the method reported by Fuchs et al (1998) because it was one of the few studies that reported a FLOW-FISH method using a fluorescein-labelled probe and a 488nm laser-equipped flow cytometer. The hybridisation temperature (37°C) was kept the same as that reported by Flint (1998) as this is generally probe specific.

Cell fixation

Samples diluted in PBS (pH7.2) as explained above were put through a fixation step prior to the FISH step. The cells in the sample were fixed using 4% formaldehyde by adding 1.5mL of a 38% formaldehyde solution (BDH, Thermo Fisher Scientific) to 18.5mL of diluted sample. The minimum contact time was 10min before proceeding with the hybridisation step. Fixed cells were stored at 4°C.

FLOW-FISH assay

The fixed sample (5µL) was hybridised with 100µL pre-warmed hybridisation solution (0.9M sodium chloride, 0.1% SDS, 20mM TrisHCl, pH7.2) and 1.2µL fluorescent DNA probe (equivalent to 1.5ng/µL in the hybridisation reaction), and incubated at 37°C overnight. After incubation, cells were centrifuged at 10,000g for 5 min and re-suspended in 100µL hybridisation solution with no probe added. This was incubated again at 37°C for 30 min as a wash step. At the end, 500µL of a PBS solution with a higher pH (pH8.4) was added to this

mixture and the sample was analysed within 3hrs using the flow cytometer. According to Fuchs *et al* (1998), this pH is supposed to be the optimum for fluorescein fluorescence and they also used this PBS buffer (pH8.4) as the sheath fluid running through the flow cytometer.

The analysis of the samples also used software provided by AES Chemunex (Paris, France) for the flow cytometer. The basic protocol used for capturing the esterase-derived fluorescent events in the TVC assay was modified by adjusting the box to capture the DNA probe-derived fluorescent population and the dilution factor used for calculating the events/mL. The protocol was still set so that fluorescent events were only recorded when the amount of the green fluorescence emitted by a particle crossed a threshold value. The number of fluorescent events within the box was recorded and defined as *S. thermophilus* cells (Sth)/mL after taking dilution factors into account.

An outline of the FLOW-FISH assay from the culture stage through to the flow cytometer is shown in Figure 2.

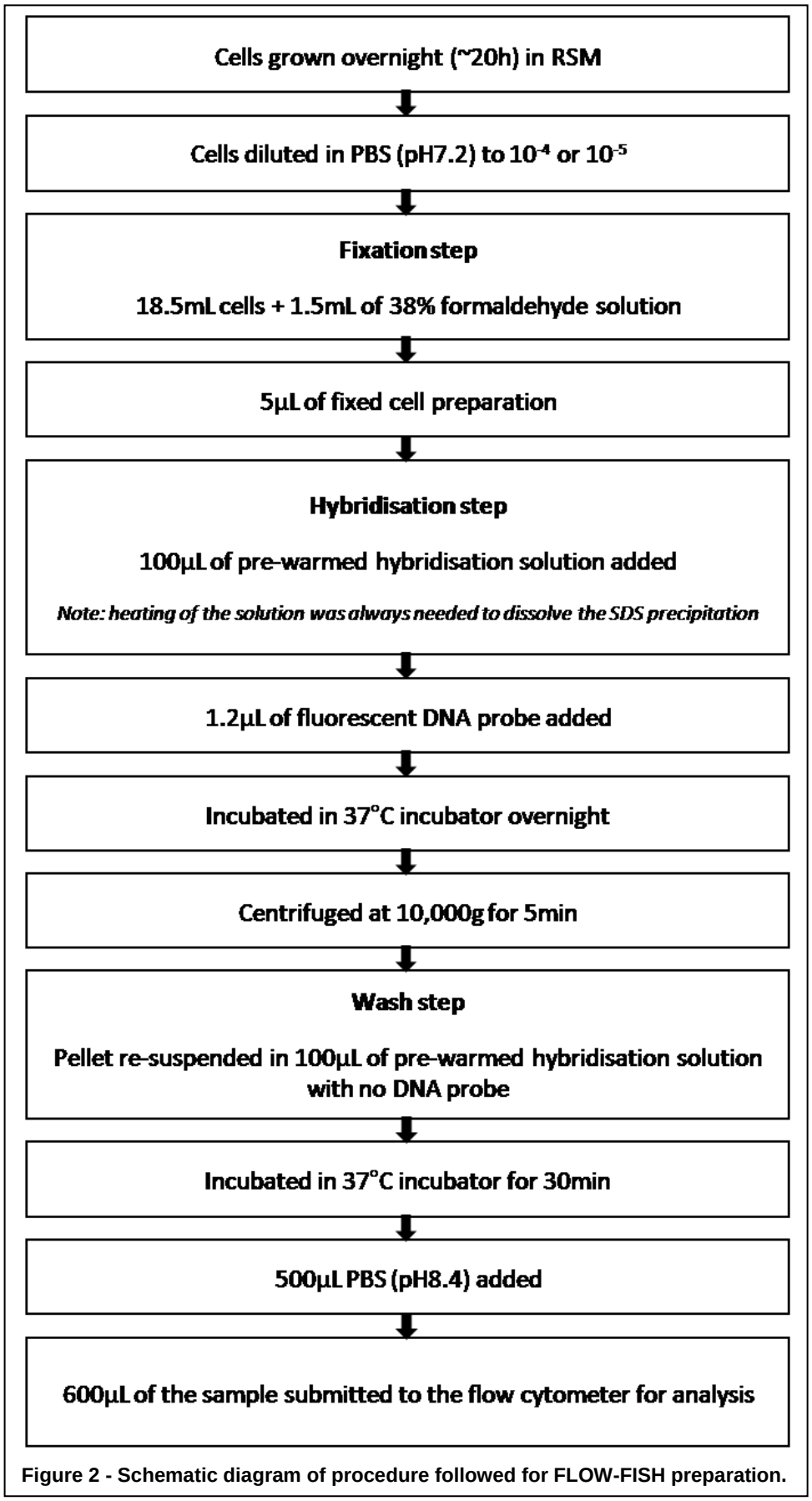

Figure 2 - Schematic diagram of procedure followed for FLOW-FISH preparation.

2.5.4 LIVE/DEAD® BacLight™ Bacterial Viability staining

The use of the LIVE/DEAD® BacLight™ Bacterial Viability staining kit (Molecular Probes, Leiden, The Netherlands, 2004) was employed as an alternative fluorescent staining technique for examining a number of issues. It is designed to separate viable from non-viable cell populations using a two-colour fluorescence staining of cells. All cells will generally stain with the green fluorescent stain, SYTO® 9, whereas cells with damaged membranes will stain red because they also allow the entry of the red stain, PI.

Equal amounts (1.5µL) of each stain, SYTO® 9 and PI, were added to 987µL of sterile saline (0.85% NaCl) before adding 10µL of the sample. The total volume was always 1mL, so if any reagent was omitted, then the volume was made up using sterile saline. Incubation of the sample occurred at room temperature in the dark for 15min.

The sample was then viewed using epifluorescent microscopy as described in Section 2.4 or analysed by the flow cytometer.

2.6 Propidium monoazide (PMA) treatment

As discussed previously, the basis of this treatment is that non-viable cells allow the PMA reagent to enter and bind to the DNA, whereas viable cells tend to remain impermeable. The connection between the PMA reagent and the DNA of non-viable cells becomes a permanent covalent bond after exposure to light. Any PMA reagent that is left un-bound is also converted to a non-reactive form after exposure to light. Downstream processing such as fixation and permeabilisation of the cells are possible because of the permanent nature of the bond and that no un-bound PMA is available to bind to the cells that were viable. Hence this allows differentiation of non-viable from viable cells.

Many studies mention the need for optimising the PMA concentration for PMA-PCR assays. PMA-PCR assays have already been applied to lactic acid bacteria successfully (García-Cayuela et al, 2009; Kramer et al, 2009) so utilising the same PMA concentration would be sensible to avoid this extra work. The method published by

García-Cayuela et al (2009) was chosen as they worked with a *S. thermophilus* culture in addition to other lactic acid bacterial species whereas Kramer et al (2009) did not.

2.6.1 PMA stock solution

Propidium monoazide was purchased from Biotium, Inc. (Hayward, USA). A stock solution was prepared by adding 20% dimethyl sulfoxide (DMSO, Sigma-Aldrich) to provide a 20mM concentration. This stock solution was stored at -20°C in the dark.

2.6.2 PMA treatment

Replicate diluted samples (0.5mL) were placed into 1.5mL eppendorf vials. A volume (1.25µL) of the stock PMA solution was added to each vial to give a final reaction concentration of PMA of 50µM and mixed using a vortex mixer. All vials were placed in the dark for 5min, with a brief mix again half-way through this step to allow the PMA to enter non-viable cells. Then the vials were placed on their side on ice (to prevent heating) beneath a 500W halogen light source situated 20cm above the vials for 15min. This step causes the PMA within any cells to cross-link with the DNA in a covalent, and permanent, manner. It also serves to inactivate any unbound PMA in the solution. Following this the samples were pelleted using 10,000g for 10min and washed, first in saline (0.85% NaCl) and then in sterile MilliQ water, to remove the inactivated PMA before analysis.

Following PMA treatment, samples were tested using the plate count, TVC, or FLOW-FISH assay methods.

See Figure 3 for a diagrammatic representation of the PMA treatment method.

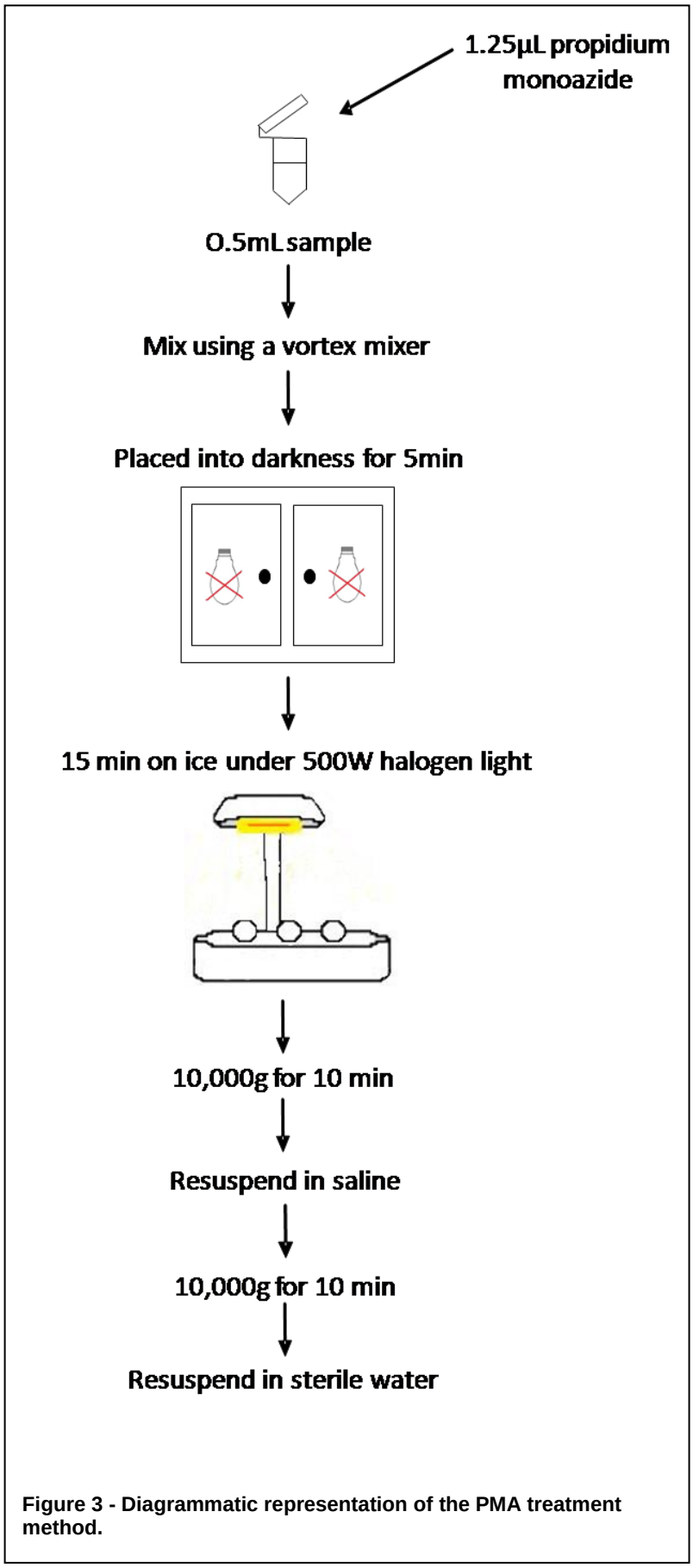

Figure 3 - Diagrammatic representation of the PMA treatment method.

2.7 Analysis of results

The standard deviation (SD) of a population shows the variability or scatter of the data in that population. Another way of describing this is that the SD shows how much the values vary from one another. According to Motulsky (2007), the standard deviation computed from duplicate data (sample size [N] = 2) are a valid assessment of the variability, or scatter, of the data.

Often the standard error of the mean (SEM) is the preferred value to use and covers both the SD and the sample size (N). Since a data set is usually a sub-set of a larger population, this value shows how close the mean of the sub-set is to the mean of the larger population.

In this study, the number of data points produced for each experiment was small because each individual experiment was exploratory in nature as part of the refinement of the method. The SD was chosen because the samples were not part of a larger population and because the variability of results given by different methods was of interest. Hence duplicate results were averaged to find the mean and then the SD was calculated.

3 FLOW-FISH experimental work

3.1 Summary

Varying levels of *S. thermophilus* (ST55) cells were analysed with the FLOW-FISH protocol. Fluorescent labelling of the cells was validated using a microscope. The next objective was to find the fluorescent population on the graph outputs of the flow cytometer so that a box could be positioned to capture the number of fluorescent events corresponding to the cells. Once that had been established, the fluorescent event results were compared against plate count results. A large discrepancy was found between the results where the FLOW-FISH results were much higher than the plate count results. Two areas of possible interference were investigated. Chains of cells can give single colonies on plates whereas flow cytometers are supposed to detect each single cell resulting in a higher count. In this case, it was found that the flow cytometer was not separating out the cells for individual enumeration. Another check of cell fluorescence using the microscope suggested the cells were exhibiting auto-fluorescence and that the background fluorescence was also high. Extra washing steps in the FLOW-FISH protocol were used to reduce the background fluorescence. Successful enumeration was achieved with cells grown in M17 growth medium but not in RSM.

3.2 Visual confirmation of the fluorescent labelling of the *S. thermophilus* (ST55) cells

Fluorescent labelling of the cells was validated using a microscope. Visual confirmation of fluorescently labelled cells was difficult. A small drop of cells, labelled using the FLOW-FISH protocol (see Methods section 2.5.3), was placed on a microscope slide, covered with a coverslip, and examined using fluorescence microscopy. Initially the number of cells was too low to be seen under the microscope so the fluorescently labelled sample was centrifuged, supernatant removed, and resuspended in the small amount of leftover liquid. A further complication was that the fluorescence of the cells would fade during examination to become non-visible. By the time the view was in focus for the camera, the fluorescent cells had disappeared from the view. Hence a “rapid jump to new position and take a picture” method was adopted in an attempt to capture the appropriate images.

Chains of fluorescent cells were seen and eventually captured by the camera showing that the FLOW-FISH method was successful in fluorescently labelling the cells and should be detected using a flow cytometer (Figure 4). The background was brighter in the sample with fluorescently-labelled cells present.

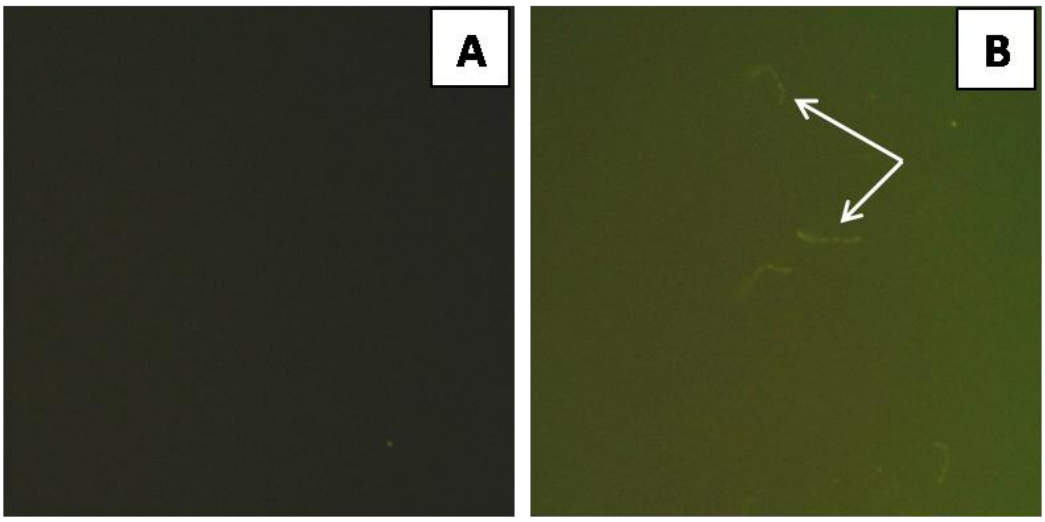

Figure 4 - Epifluorescent microscopy images of FLOW-FISH stained samples. Image A shows a control sample with no fluorescently-labelled cells present. In image B, arrows show chains of *Streptococcus thermophilus* (ST55) cells fluorescently labelled using the FLOW-FISH protocol.

3.3 Capturing the fluorescent population of the *S. thermophilus* (ST55) cells with flow cytometry

The next objective was to find the fluorescent population on the graph outputs of the flow cytometer so that a box could be positioned to capture the number of fluorescent events corresponding to the fluorescently-labelled cells.

In this experiment, *S. thermophilus* (ST55) was grown overnight at 37°C in RSM and then diluted to 10^{-4} to a final volume of 30mL. A plate count and a total viable count (see Method sections 2.2.2 and 2.5.2 respectively) were performed on this sample before the cells were fixed. Then 18.5mL of the sample was fixed using 1.5mL of 38% formaldehyde.

Two different scenarios were tested for their effect on the end result of achieving a discrete population of fluorescent events. The first scenario looked at whether

hybridising was better in a 37°C waterbath than in a 37°C incubator. The second scenario looked at whether the fixed cells needed a wash step before performing the hybridisation or a simple method of diluting a small amount of sample (5µL) with the FLOW-FISH reagents would suffice. All samples were tested in duplicate.

A reagent control was run through the flow cytometer that consisted of the same volumes of hybridisation solution and PBS (pH8.4) as the samples contained at the end of the FLOW-FISH procedure.

One of the output forms of the flow cytometer are dot plot graphs (Figure 5) that depict the fluorescent events of particles passing through the flow cytometer detection platform and are plotted on the basis of red fluorescence (Y axis [D4:FL2]) against green fluorescence [D3:FL1]). According to the manufacturer of the instrument many particles, including bacteria, exhibit red fluorescence in this system. Bacteria tend to be differentiated on the basis of a fluorescent label that exhibits green fluorescence.

A population of fluorescent events was seen in the non-washed cell samples, using either an incubator (Figure 5, Image C) or a waterbath (Figure 5, Image E) for the hybridisation incubation step, which was not present in either the reagent control or the washed cell samples. Since the only difference between the reagent control and the non-washed cell samples was the presence of the fluorescently-labelled *S. thermophilus* (ST55) cells, it was assumed that this extra population being detected must be the fluorescently-labelled cells and a white “counting box” was placed around it. Table 1 shows the settings for this “counting box” used for the results in this experiment.

Table 1 - Initial settings for the "counting box" position.

“Counting box” classifier	Minimum	Maximum
FL1	0	300
FL2	100	600
FL2/FL1	2	12

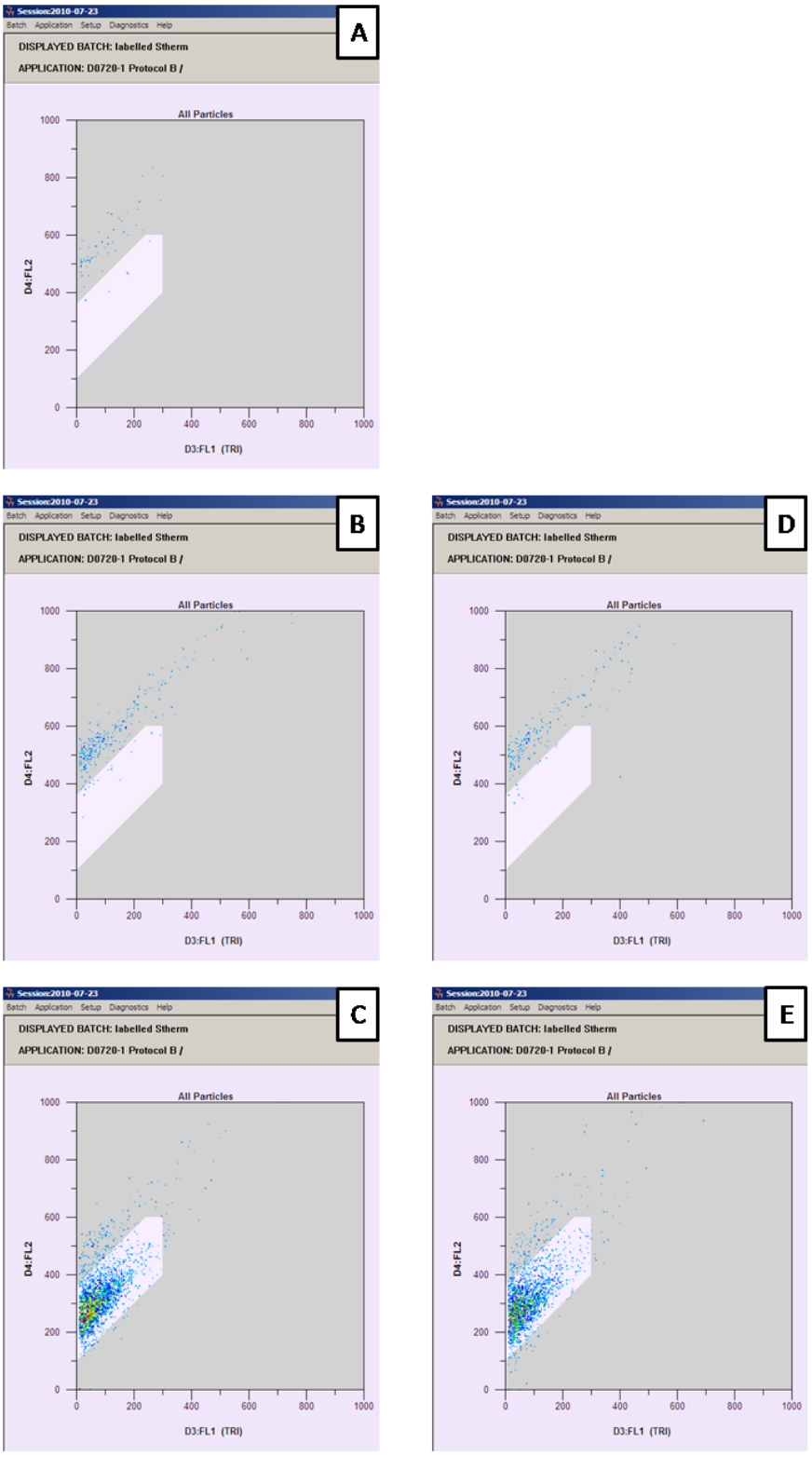

Figure 5 - Fluorescent population position of FLOW-FISH labelled *Streptococcus thermophilus* (ST55) cells. These dot plot pictures are one output form of the flow cytometer and depict the fluorescent events plotted on the basis of red fluorescence (Y axis [D4:FL2]) against green fluorescence [D3:FL1]). Image A: Reagent control (100µL hybridisation solution and 500µL of PBS (pH8.4)). Image B: Fixed cells (500µL) that were washed once before the FLOW-FISH procedure and an incubator was used for the hybridisation step. Image C: Fixed cells (5µL) that were not washed and an incubator was used for the hybridisation incubation step. Image D: Fixed cells (500µL) that were washed once before the FLOW-FISH procedure and a waterbath was used for the hybridisation incubation step. Image E: Fixed cells (5µL) that were not washed and a waterbath was also used for the hybridisation step.

3.4 Comparison of FLOW-FISH results with plate count results

Once the position of the fluorescently labelled cells had been established, the FLOW-FISH results of the samples were compared against the plate count and total viable count results of the samples before the cells were fixed. Results are shown below in Figure 6. The total viable count result of the sample before the fixation step gave an equivalent result to the plate count result. None of the FLOW-FISH results matched the plate count. The FLOW-FISH results for the non-washed fixed cell samples were greater than the plate count result by more than 1 Log_{10}/mL, whereas the FLOW-FISH results for the washed fixed cell samples were lower than the plate count by greater than 2 Log_{10}/mL. It did not appear to matter where the hybridisation incubation took place.

A possible reason for the low result obtained using washed cells is that the cells may have been lost during the washing manipulations. An alternative reason could be that the probe concentration was not enough to fluorescently-label the larger concentration of cells in these samples. It was decided to use 5µL of un-washed fixed cell samples in the FLOW-FISH protocol for future experiments since this method produced a population of fluorescently-labelled cells detected by the flow cytometer.

There was also an issue of low fluorescence intensity. This can be seen above, in Figure 5, where the fluorescent population sat in the lower left quadrant of the dot plot graph and did not appear as a complete population. This means that the fluorescent signal was weak and also that the FLOW-FISH count result could be higher still if the entire population was captured.

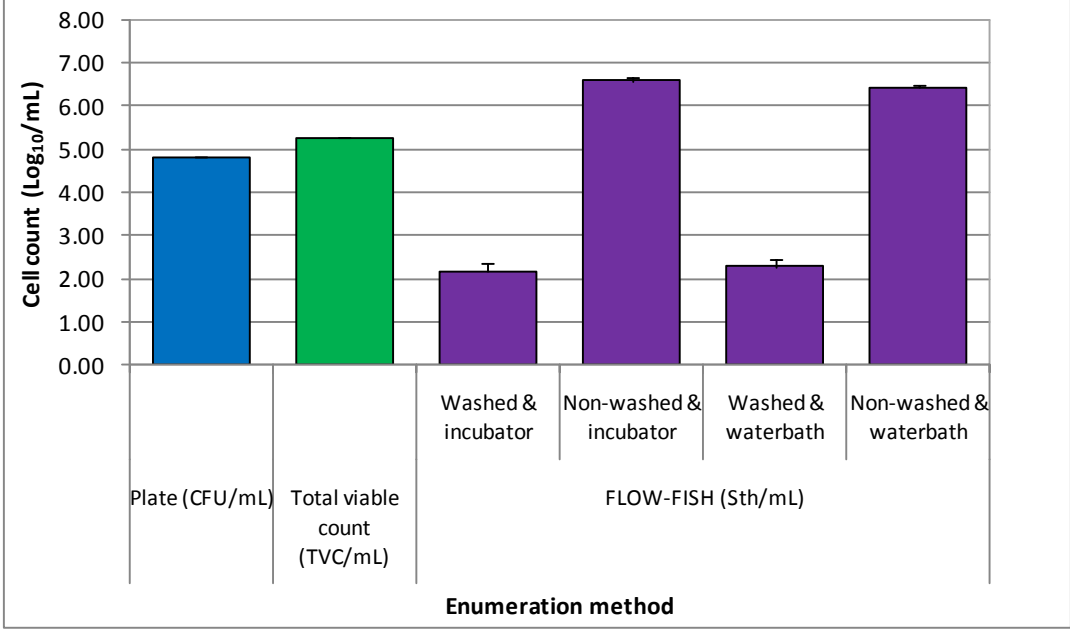

Figure 6 - Comparison of FLOW-FISH results against plate and total viable count (flow cytometry) methods for *Streptococcus thermophilus* (ST55). The plate and total viable counts of the sample were performed before the fixation step. There were four different FLOW-FISH methods tested that examined the need for washing the fixed cell samples before the introduction of the DNA probe and whether the DNA probe hybridisation step should be incubated in a waterbath or an incubator. Results shown are the mean of duplicate results and the standard deviation.

3.5 Cells in chains

Manufacturers of flow cytometers aim to utilise hydrodynamic focusing in the fluid part of the instruments so that cells pass through one at a time (Shapiro, 2003). Hence, one possible reason for the large difference seen between the FLOW-FISH results of the un-washed fixed cells and the plate count results could be that chains of cells would give single colonies on plates, whereas a flow cytometer would detect each single cell. The objective of this experiment was to examine fluorescently labelled cells before and after going through the flow cytometer.

S. thermophilus (ST55) was grown in M17 broth overnight at 37°C. Two 1mL aliquots were washed twice using filter-sterile saline and centrifugation at 10,000g for 5min. The final resuspension was also in filter-sterile saline and the two aliquots were combined to make a total of 2mL. A plate and total viable count were performed as a standard check on the number of cells present. The same sample (10µL) was stained using the BacLight LIVE/DEAD kit (see Methods section 2.5.4). This fluorescent staining method was used because the fluorescent output was much stronger than that of the FLOW-FISH method and was seen more successfully using epifluorescence microscopy. The total volume of the sample, after addition of the BacLight reagents was 1mL. One

part of the sample (0.4mL) was centrifuged at 10,000g for 5min and resuspended in a small amount of leftover liquid once the supernatant was removed. This sample was the "BEFORE" sample and was examined using epifluorescence microscopy. The other part of the sample (0.6mL) was submitted to the flow cytometer and both the sample and the washing liquids were collected as they exited the flow cytometer. All of this collected fluid (~7mL) was also centrifuged and resuspended in a small amount of leftover liquid. This sample was the "AFTER" sample and was then examined using epifluorescence microscopy.

Fluorescently labelled cells were present in chains both before and after going through the flow cytometer (Figure 7). Hence, the flow cytometer did not separate out the cells as expected. One further observation noted in this experiment is that each chain can be a different mixture of viable and non-viable cells in different configurations. Both of these observations may have implications in the successful enumeration of *Streptococcus* species using flow cytometry. A flow cytometer detects each fluorescently-labelled cell as a discrete event of light passing by detectors, with the light being detected in a bell-curve manner. It has a start, finish, and a middle section of sampling with the most light detected. Cells in chains may only have a start and finish to the light being detected at the start and finish of the chain. The use of the BacLight LIVE/DEAD kit may be complicated for cells in chains since the tail end of the fluorescent light detected from each cell may cross over into that of another cell. Or perhaps a viable cell situated in a chain of predominately non-viable cells may not be detected as viable.

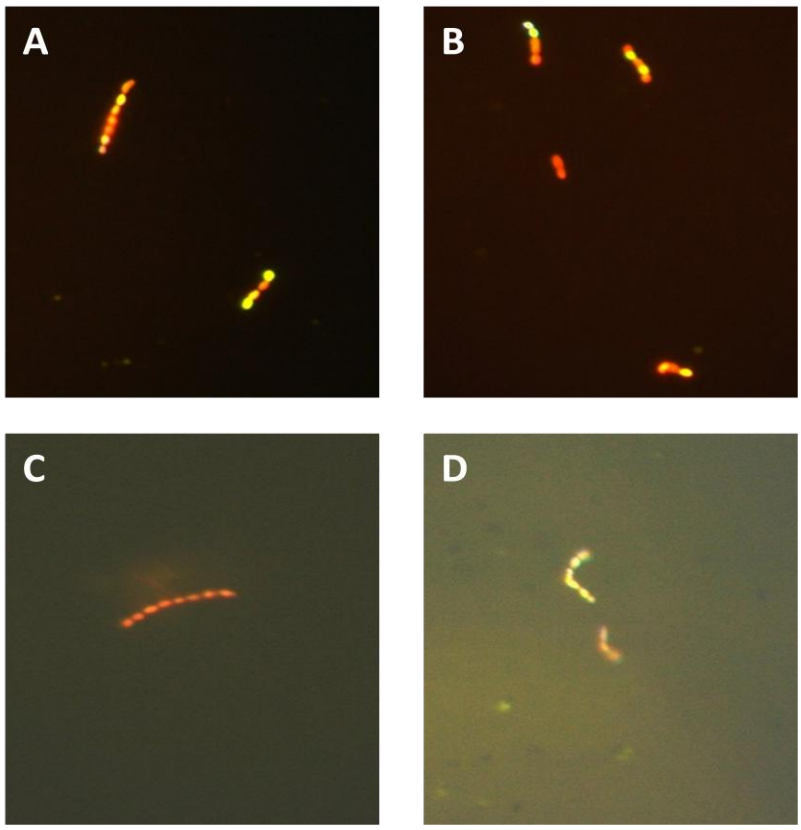

Figure 7 - BacLight LIVE/DEAD labelled *Streptococcus thermophilus* (ST55) cells before and after passing through a flow cytometer. Images A and B show cells before going through the flow cytometer. Images C and D shows cells from the same sample that have exited the flow cytometer.

3.6 Probe deterioration

At one point in the investigations, the experiments showed weak or negative results with *S. thermophilus* (ST55) cell samples (results not shown). Deterioration of the probe was a possible problem. Two fixed cell samples were tested using the FLOW-FISH method and compared against their respective plate count results obtained before fixation.

The first sample was 5µL of a sample that was fixed at the beginning of the experiments using the FLOW-FISH method in June 2010 that gave a strongly positive result. The second sample was 5µL a freshly fixed cell sample containing more cells prepared in June 2011 with the objective of getting a stronger population for analysis. The negative control was 5µL of a mixture of PBS (18.5mL, pH 7.2) and formaldehyde (1.5mL, 38% stock solution) put through the FLOW-FISH method the same as the samples.

Figure 8 shows that, on this occasion, there was a positive result for both samples. However, the result for each sample was the same despite there being higher numbers in the freshly fixed cells. And also the results were not much different to that obtained from the negative control. In retrospection, a plate count should have been done on the negative control to ensure that there were no *S. thermophilus* (ST55) cells present. However, the high result of the negative control is most likely due to background fluorescence events that are emphasised once the dilution factors have been applied to the results and this means that the cell results are not different to the negative control.

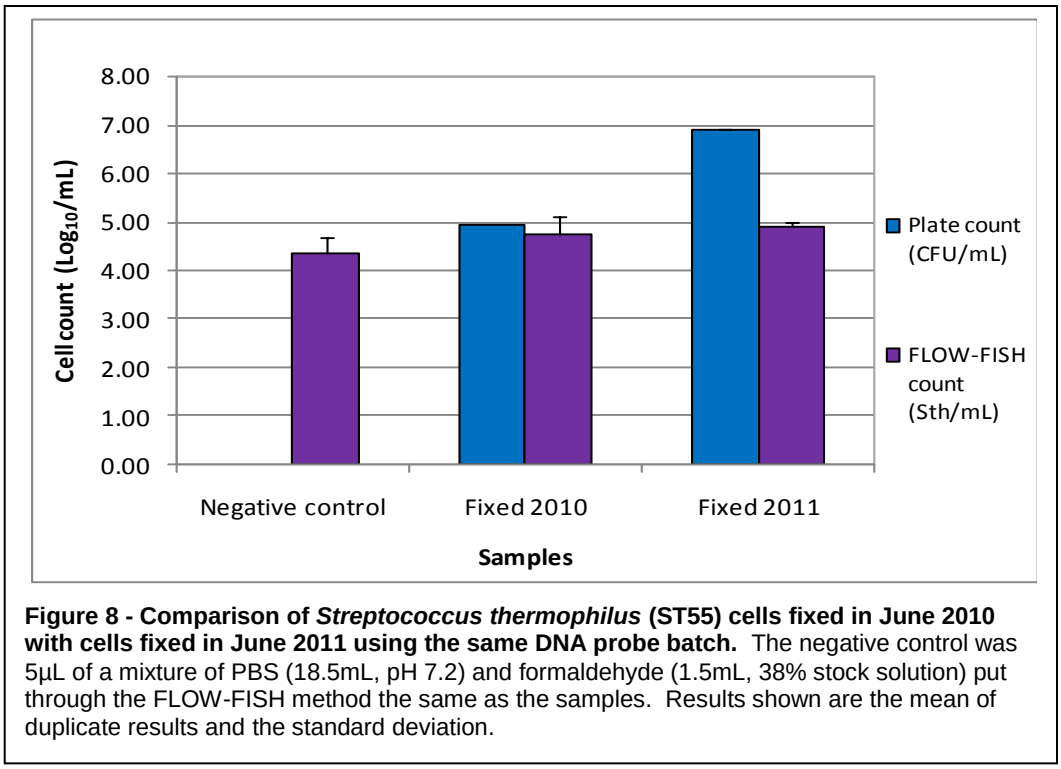

Figure 8 - Comparison of *Streptococcus thermophilus* (ST55) cells fixed in June 2010 with cells fixed in June 2011 using the same DNA probe batch. The negative control was 5µL of a mixture of PBS (18.5mL, pH 7.2) and formaldehyde (1.5mL, 38% stock solution) put through the FLOW-FISH method the same as the samples. Results shown are the mean of duplicate results and the standard deviation.

Comparison of the results of the cell preparation fixed in June 2010 tested using the FLOW-FISH method in both June 2010 and in June 2011 showed some reduction in FLOW-FISH results (Figure 9).

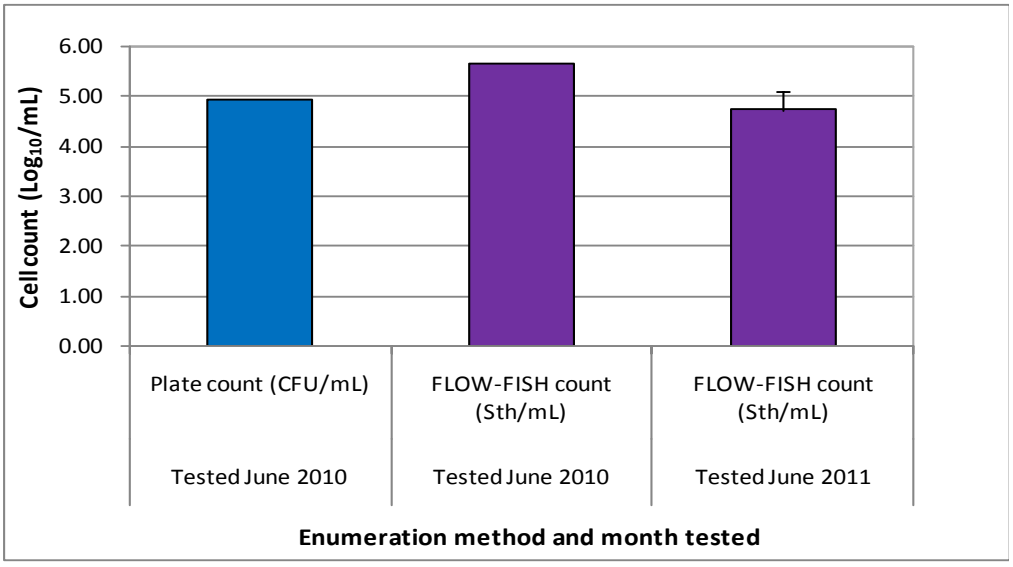

Figure 9 - Results of *Streptococcus thermophilus* cells fixed in June 2010, tested in June of 2010 and 2011. Results shown are the mean of duplicate results and the standard deviation except for the singular FLOW-FISH result of the cells tested in June 2010.

The DNA probe was unable to be stored as recommended and this may have been contributing to the conflicting results so a new batch of DNA probe was obtained and used immediately. The same fixed cell samples that were used previously (Figure 8) were tested with both the new batch and the old batch of DNA probe (Figure 10). A new batch of DNA probe did not improve the FLOW-FISH results.

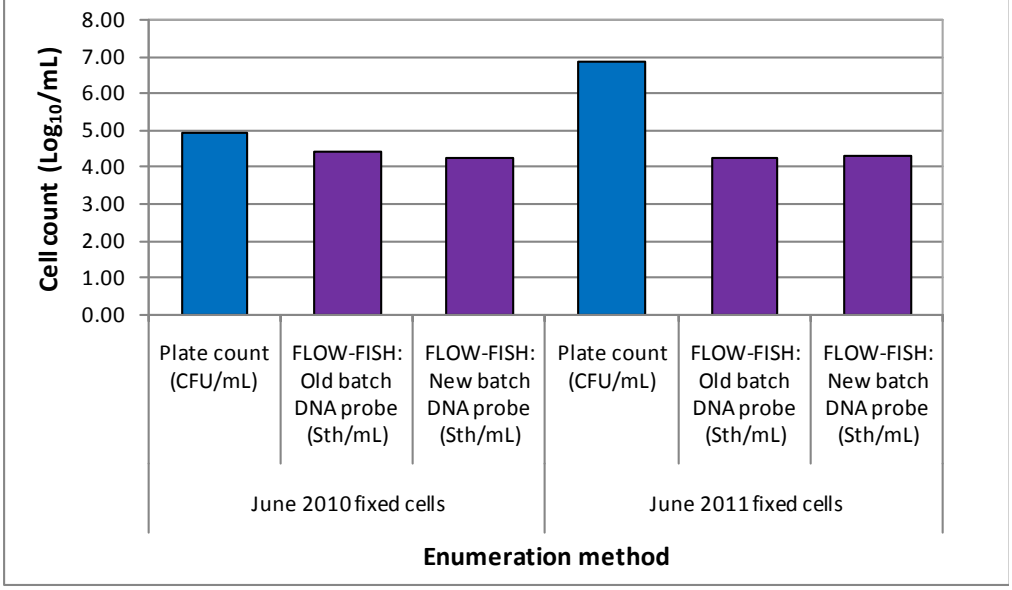

Figure 10 - Comparison of two DNA probe batches on two sets of fixed *Streptococcus thermophilus* (ST55) cells. Cells fixed in June 2010 were tested using the FLOW-FISH method using two different batches of probe, one older batch that had been stored incorrectly and a newer batch that was used immediately upon arrival. A second preparation of cells fixed in June 2011 with higher cell numbers was also tested with both batches of DNA probe. Results are singular results for this experiment.

The FLOW-FISH method takes more than 24hrs from start to finish and perhaps the efficiency of the DNA probe is compromised during the FLOW-FISH procedure. This possibility was investigated using a spectrophotometer to scan the fluorescent light emitted by the DNA probe after being subjected to variations in the FLOW-FISH protocol. The DNA probe was diluted to 1/10 using the hybridisation buffer and divided into eight 1mL aliquots. Duplicate aliquots were subjected to variations of the FLOW-FISH protocol conditions (incubation time, incubation temperature, and washing manipulations). See Figure 11 for a schematic diagram of the experiment. All samples were then scanned using a spectrophotometer of wavelengths of approximately 300nm to 700nm to ascertain any change in fluorescence. A 1/100 dilution of the DNA probe was initially trialled as this concentration is similar to what was being used in the FLOW-FISH protocol. However, the peak resulting from this concentration was too small to see changes so the stronger concentration was used for this experiment. An eppendorf vial containing 1mL of hybridisation buffer was put through each scenario and used as the blank liquid for the spectrophotometer.

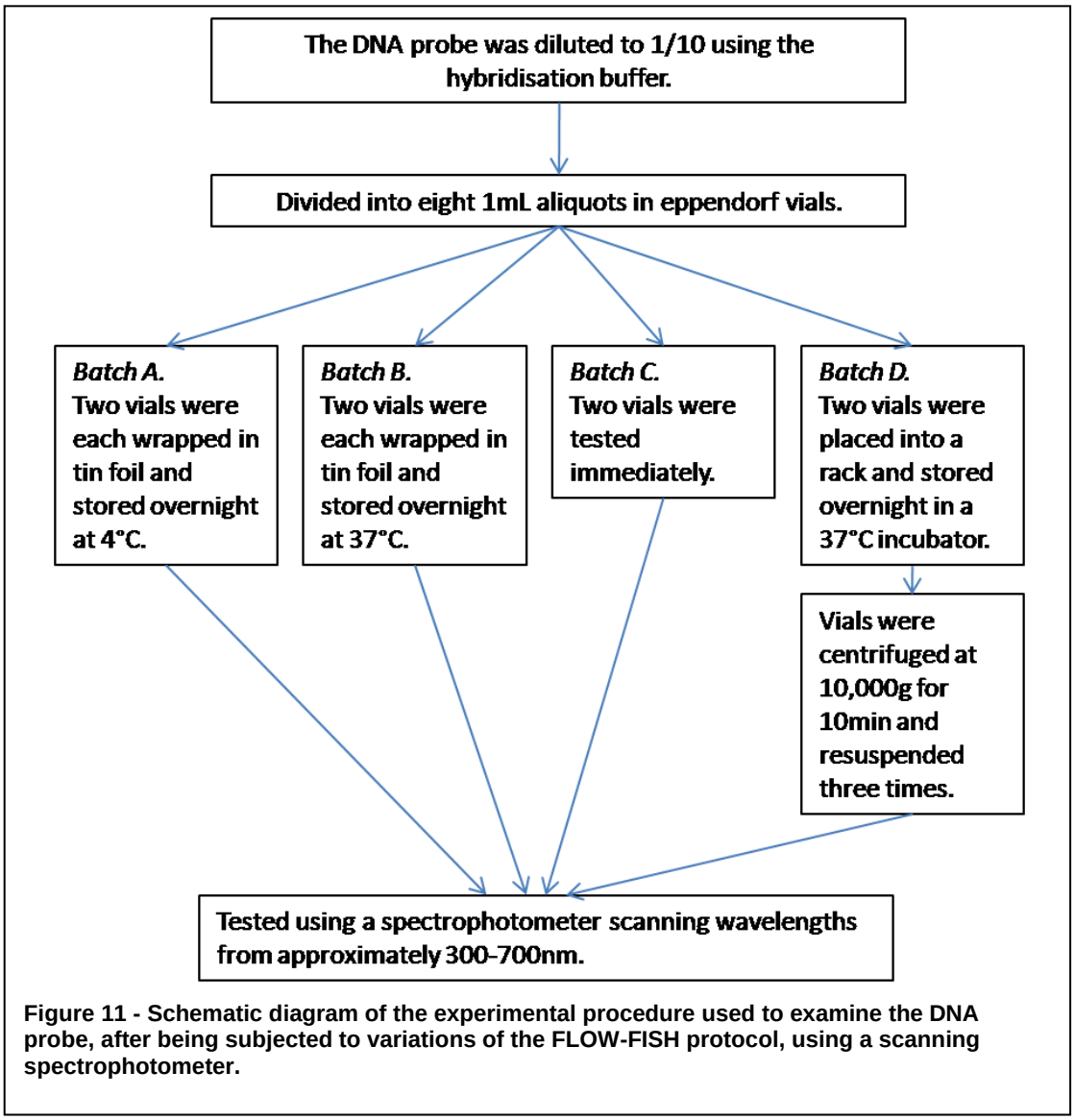

Figure 11 - Schematic diagram of the experimental procedure used to examine the DNA probe, after being subjected to variations of the FLOW-FISH protocol, using a scanning spectrophotometer.

Each sample was scanned three times by the spectrophotometer. Many of the samples had precipitated during the experiment, particularly those incubated at 4°C. These samples were warmed gently before testing in the spectrophotometer. However precipitation occurred in the spectrophotometer cuvette as well and as a result high baselines were observed for some samples, particularly for the second and third scans.

A strong peak was observed at 500nm that was believed to be produced by the DNA probe (Figure 12). There was no difference in the peak for the samples and this indicated that the incubation conditions and the centrifugation manipulations did not affect the DNA probe.

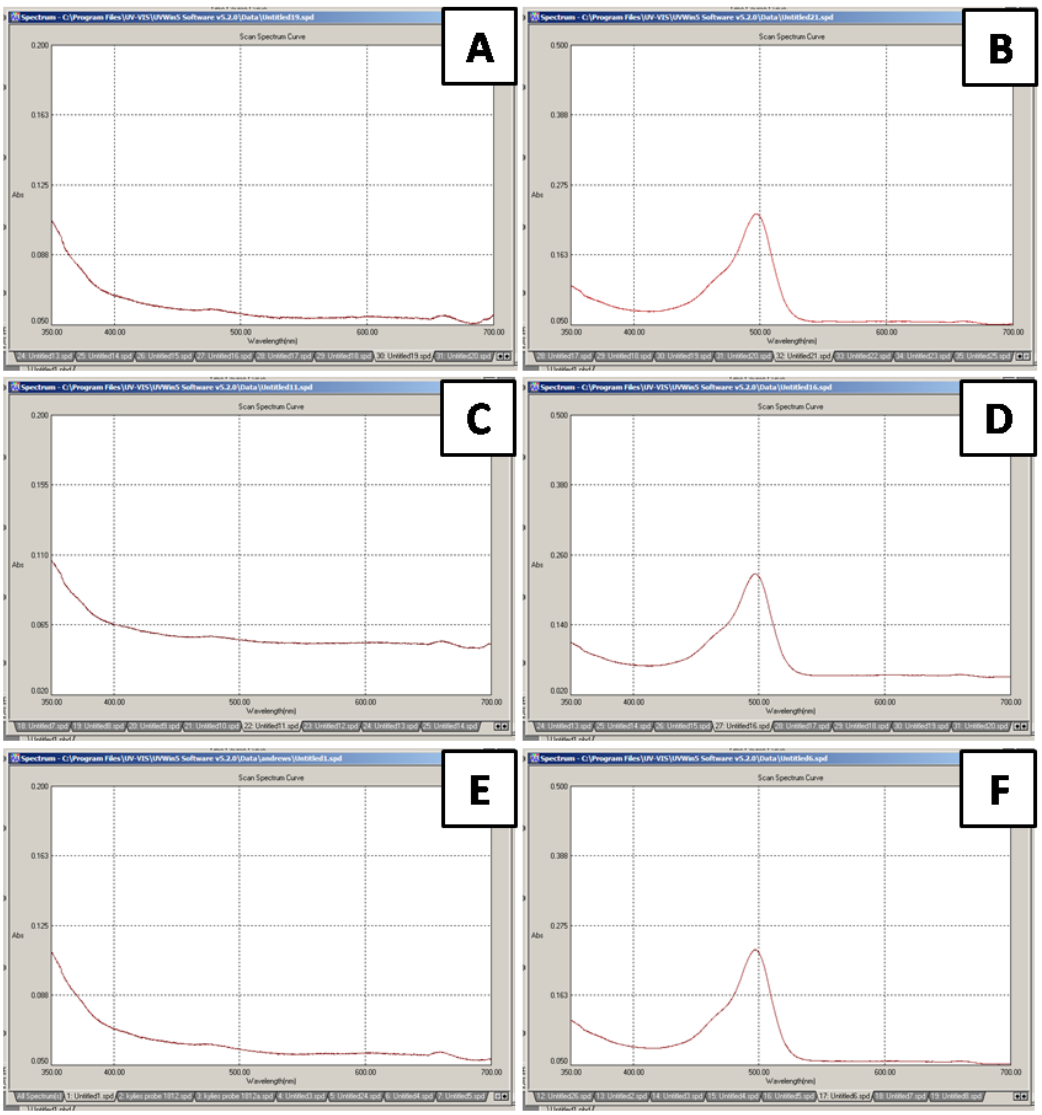

Figure 12 - Wavelength scans (approximately 300nm to 700nm) of 1/10 dilution of DNA probe after being subjected to different variations of the FLOW-FISH protocol. Images A, C, and E were aliquots of hybridisation solution used as controls. Images B, D, and F were samples of DNA probe diluted to 1/10 using hybridisation solution. Images A and B were samples that were wrapped in tin foil and stored overnight at 4°C. Images C and D were samples that were wrapped in tin foil and stored overnight at 37°C. Images E and F were samples that were placed into a rack, stored overnight at 37°C, and then washed three times.

3.7 Low fluorescent signal intensity

Ideally, in flow cytometry, a fluorescent population needs to be a discrete population that can be detected and evaluated. Three areas (length of hybridisation incubation, probe concentration and PMT gain settings) were investigated for their effect on the low signal of the population that was being detected using the FLOW-FISH method.

3.7.1 Length of hybridisation incubation

The length of the hybridisation incubation step was approximately 21h. In order to gain the most benefit of using a flow cytometer that enumerates cells rapidly, it would be ideal to reduce the time needed for fluorescently labelling the cells. However, reducing the time might have a negative effect of the strength of the fluorescent signal.

Duplicate samples of fixed *S. thermophilus* (ST55) cells that contained 4.93 Log_{10}CFU/mL were fluorescently-labelled using the FLOW-FISH method for 3, 5, and 21h. The fluorescent population grew more numerous as the incubation length increased but, while many cells may have had a stronger fluorescent signal, overall the population did not have a stronger fluorescent signal (Figure 13). Additionally when the cells were labelled for only 3 and 5h, the position of the fluorescently-labelled cell populations were not clear and not identifiably separate from the background fluorescent events.

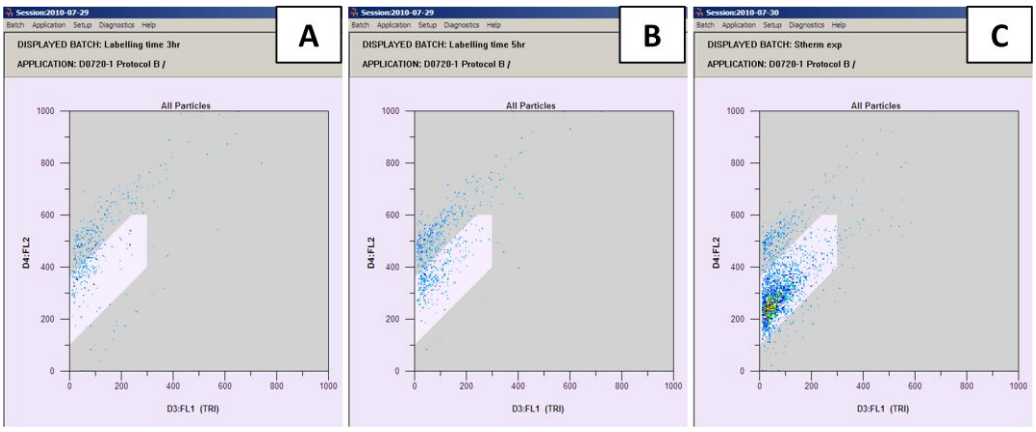

Figure 13 - Varying length of the FISH-FLOW hybridisation step for *Streptococcus thermophilus* (ST55) cells. Images A, B, and C show the effect of increasing the length of the hybridisation incubation from 3h, to 5h, and to 21h respectively.

Comparison of the counts obtained with the FLOW-FISH method using different lengths of hybridisation time with both the plate and total viable counts showed that the FLOW-FISH method still gave larger numbers (Figure 14). However, the 3h result was only greater by 0.39 Log_{10}/mL.

One reason for why the FLOW-FISH results are higher could be the method is detecting non-viable cells as well as the viable cells detected by the plate and total viable count methods. Another reason could be that non-specific staining is occurring.

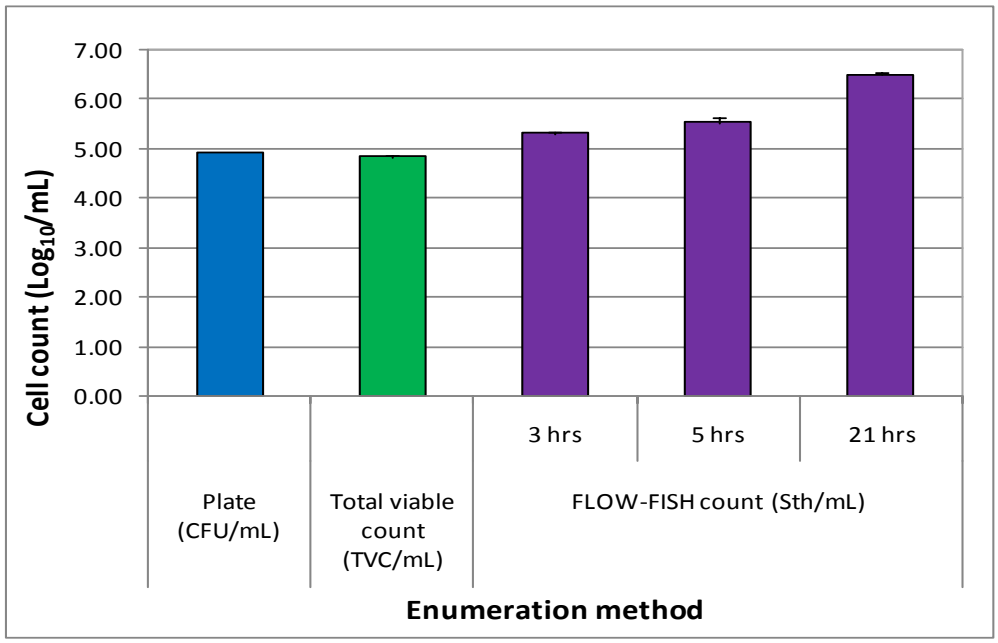

Figure 14 - Enumeration comparison with different lengths of hybridisation time for the FLOW-FISH method. *Streptococcus thermophilus* (ST55) was enumerated using the plate count, the total viable count, and the FLOW-FISH methods using 3, 5, and 21h hybridisation incubations. Results shown are the mean of duplicate results and the standard deviation.

3.7.2 Probe concentration

A *S. thermophilus* (ST55) culture grown in M17 growth medium was washed twice in filter-sterile PBS (pH7.2) and resuspended a third time in filter-sterile PBS (pH7.2). A plate and total viable count were performed to determine the cell numbers before the fixation step. There was no dilution of the culture at this point. The FLOW-FISH method was then applied on duplicate samples with varying amounts of DNA probe to see if this might increase the fluorescent signal of the population.

Normally 100µL of sample is analysed by the flow cytometer, for which the result then needs a multiplication factor of 10 to reach a result per mL. In this experiment, only 6.6µL of the samples were tested by the flow cytometer when 63.8ng/µL DNA probe was used in the hybridisation mix. The reason for this is that the number of fluorescent events detected by the flow cytometer exceeded the maximum limit. This situation should be avoided as it lowers the accuracy of the results. Despite this, it was easier to see chains of fluorescent cells as more DNA probe was used (Figure 15). However, the background also grew brighter as a result.

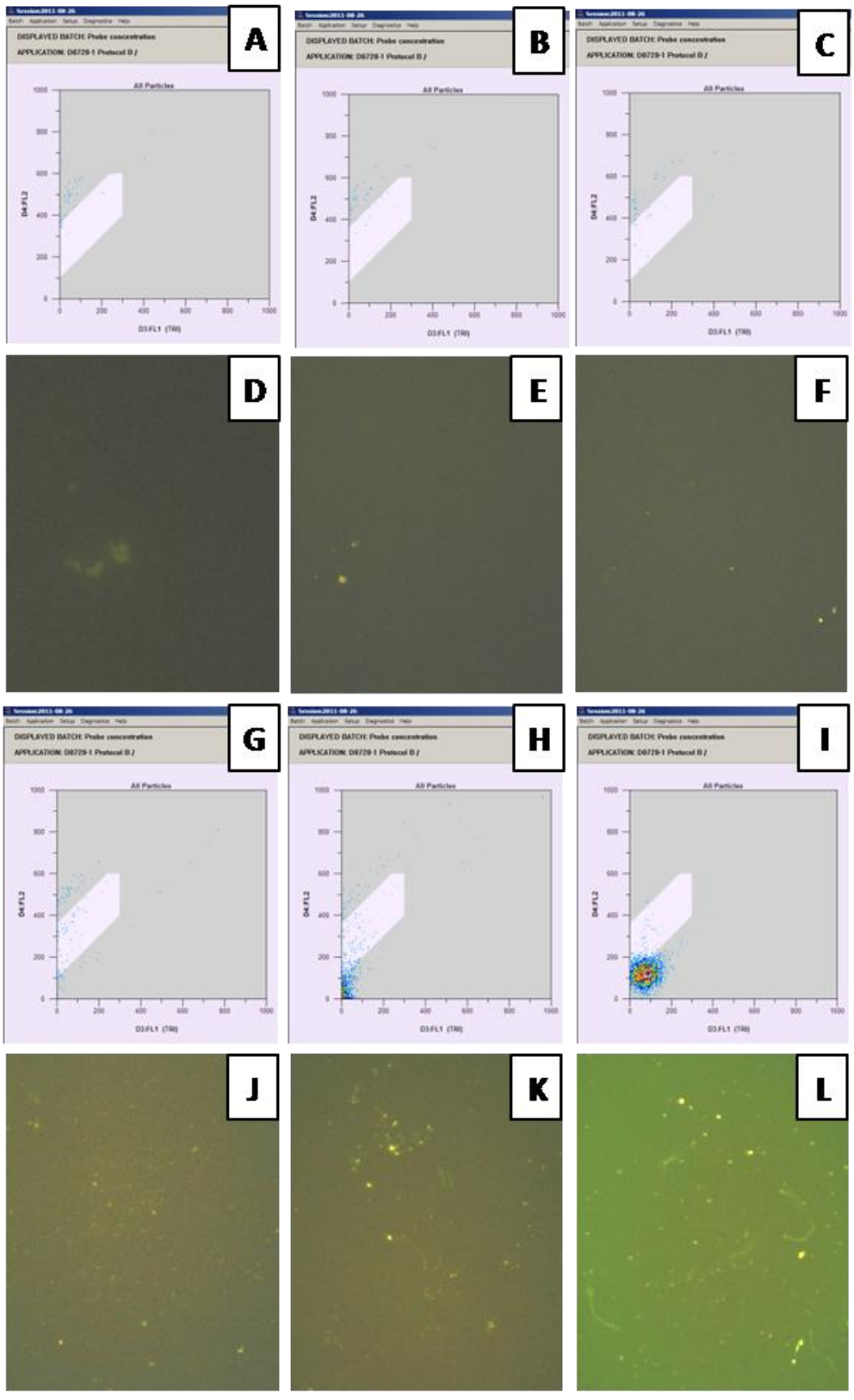

Figure 15 - FLOW-FISH results for *Streptococcus thermophilus* (ST55) with increasing DNA probe concentration. Images A to C and G to I show the dot plot outputs from the flow cytometer. Images A and B are controls 1 (hybridisation buffer) and 2 (hybridisation buffer and cells). Images C, and G to I are the FLOW-FISH analysis of the same bacterial sample with increasing concentrations of DNA probe (1.5ng/µL, 6.4ng/µL, 12.8ng/µL and 63.8ng/µL respectively). Images D to F and J to L are the corresponding epifluorescent microscope photographs.

An additional observation is that there were fluorescent particles of varying shape and size present in all samples, even in the samples that had no DNA probe added. The number of particles grew more numerous and brighter as more DNA probe was added to the sample.

Using the "counting box" settings as detailed in Table 1, the FLOW-FISH results were compared to the plate and total viable counts as the concentration of the DNA probe was increased (Figure 16).

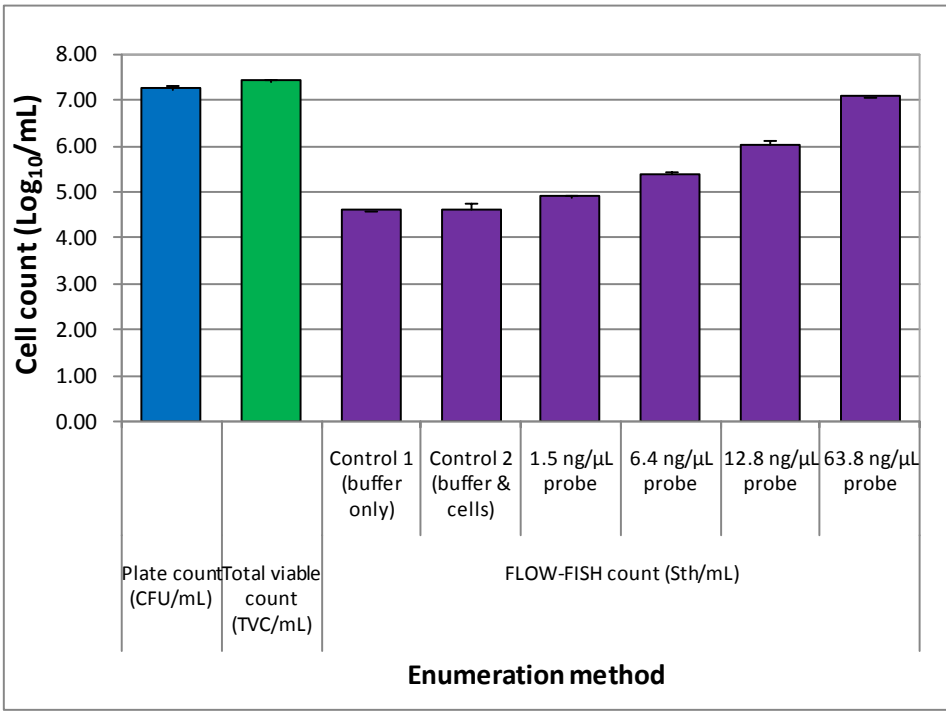

Figure 16 - FLOW-FISH with increasing DNA probe concentration compared to plate and total viable counts of *Streptococcus thermophilus* (ST55). Results shown are the mean of duplicate results and the standard deviation.

However, a strong population appeared in a different position to the normal counting window. Modifying the counting window (see Table 2) to accommodate the new strong population gave the following results (Figure 17).

Table 2 - Modified "counting box" settings.

"Counting box" classifier	Minimum	Maximum
FL1	0	300
FL2	0	300
FL2/FL1	Not used	Not used

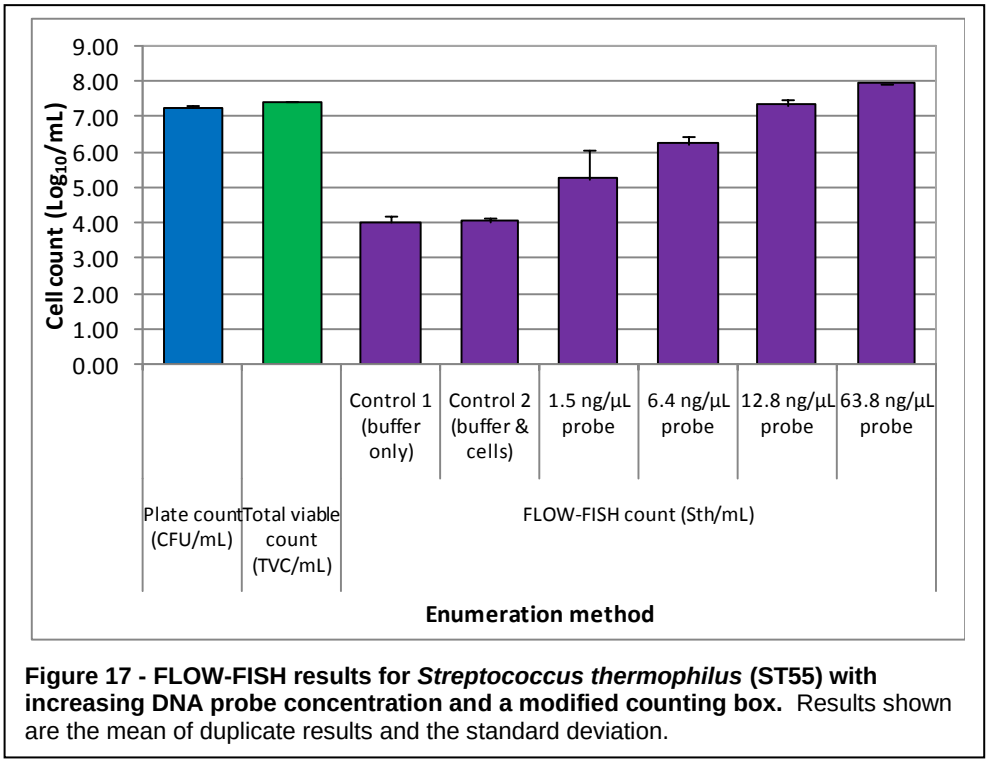

Figure 17 - FLOW-FISH results for *Streptococcus thermophilus* (ST55) with increasing DNA probe concentration and a modified counting box. Results shown are the mean of duplicate results and the standard deviation.

3.7.3 PMT gain adjustment

The gains on the PMT detectors can be altered on the flow cytometer and has the effect of raising or lowering the fluorescence detected for each particle. On this machine it is occasionally used for ensuring that the detection of the calibration beads is optimised for the day of use. Then the bacterial enumeration protocols are set to the same gain levels.

The same culture sample prepared for trialling different probe concentrations was also subjected to FLOW-FISH analysis with 1.5ng/µL DNA probe and tested with four different PMT gain settings relative to the settings for the calibration beads: lower by 50 points, equivalent, and higher by 20 and 40 points (Figure 18). A similar fluorescent population appeared that was seen in the probe concentration experiment.

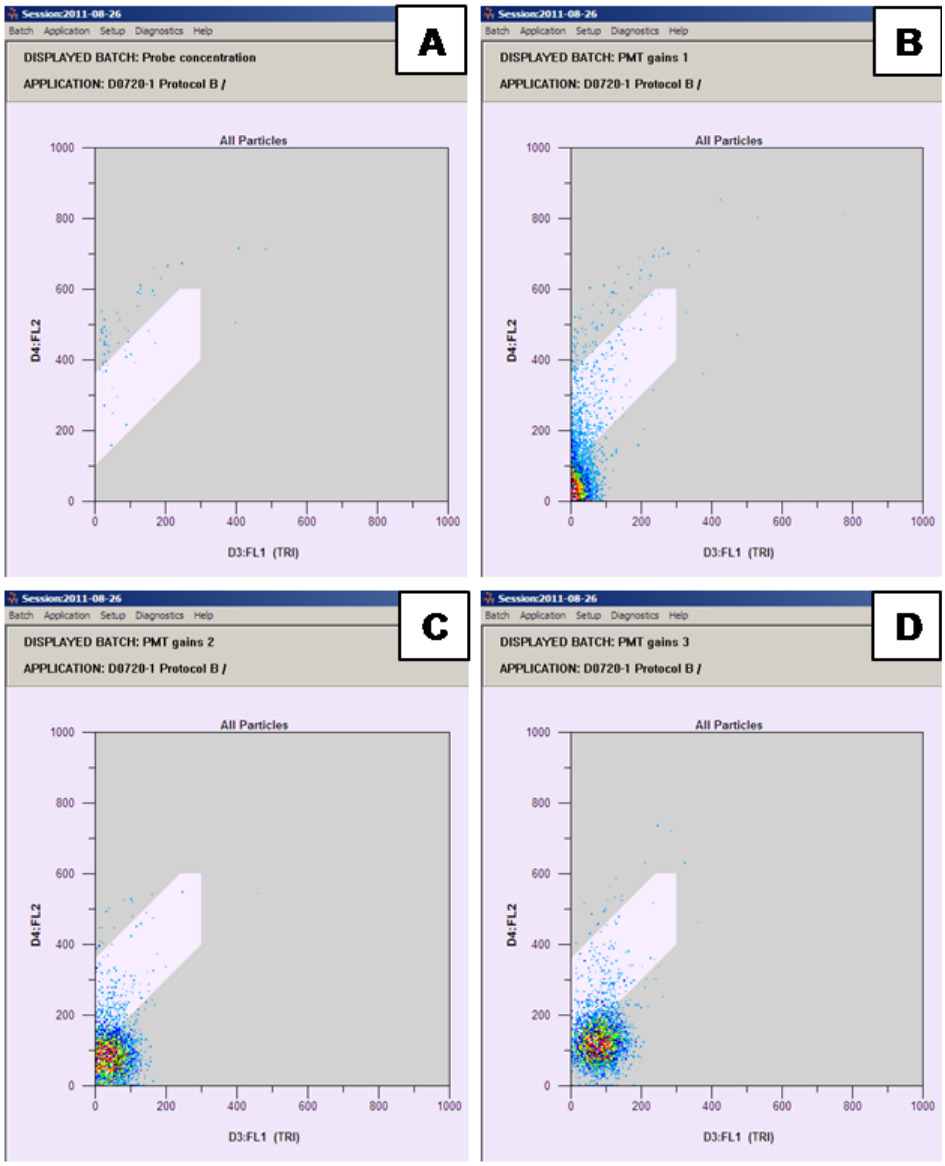

Figure 18 - Effect of changing PMT gain settings on FLOW-FISH results for *Streptococcus thermophilus* (ST55). The same sample was subjected to FLOW-FISH analysis with different PMT gain settings relative to that used for the calibration beads. Image A shows the dot plot output when the gain settings were lower than the calibration bead settings by 50 points. Image B shows the result when the settings were the equivalent to the calibration bead settings. And images C and D show the dot plot outputs when the settings were raised higher than that for the calibration beads by 20 and 40 points respectively.

3.8 Check of cell fluorescence (autofluorescence, background fluorescence)

Another check of cell fluorescence using the microscope suggested the cells are exhibiting auto-fluorescence and that the background fluorescence is high.

A *S. thermophilus* (ST55) culture was plated onto a M17 agar plate. The cells were harvested using a sterile cotton swab and suspended in PBS (pH7.2). A plate count was performed on this sample and 0.5mL was also taken for the FLOW-FISH staining procedure. This sample was centrifuged at 10,000g for 10min, the supernatant removed, and the pellet was resuspended in a small amount of leftover liquid. Hybridisation buffer (100µL) was added to each sample plus, or minus, the DNA probe (1.5ng/µL) and the samples were incubated at 37°C overnight. There was a single wash step after the hybridisation step where the samples were centrifuged at 10,000g for 10min, the pellet was then resuspended in minimal leftover liquid and 100µL of hybridisation minus the DNA probe was added. The samples were incubated for 30min at 37°C before 500µL of PBS (pH8.5) was added. Once again the samples were centrifuged at 10,000g for 10min and resuspended in the minimal leftover liquid before being viewed using epifluorescent microscopy.

The epifluorescent images are shown in Figure 19.

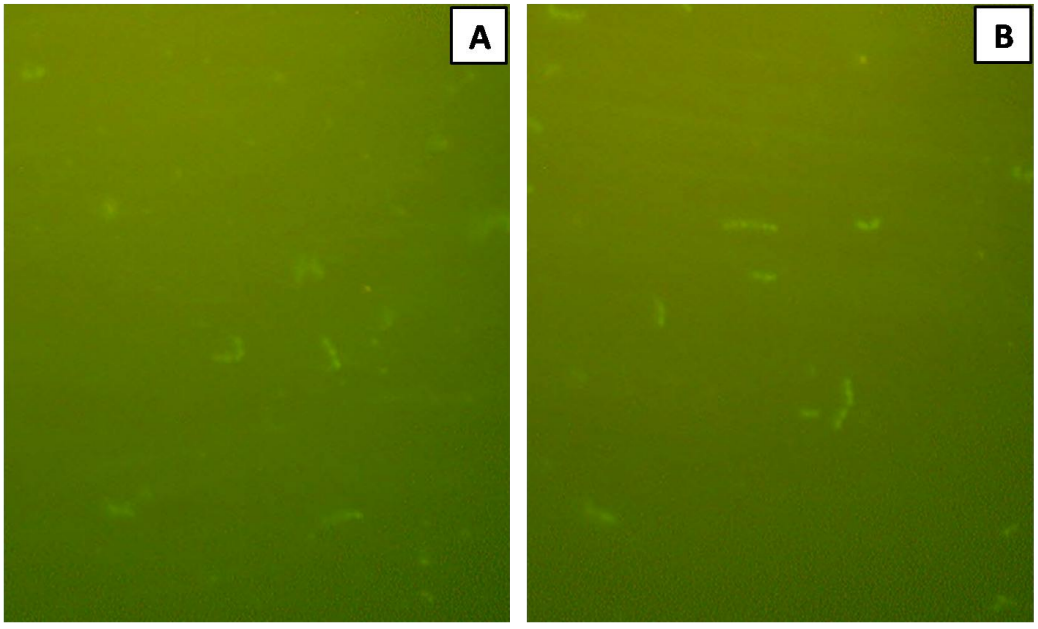

Figure 19 - Cell autofluorescence of *Streptococcus thermophilus* (ST55). Image A shows cells treated with the FLOW-FISH protocol with 1.5ng/µL DNA probe. Image B shows cells treated with the FLOW-FISH protocol minus the DNA probe.

In a repeat experiment (Figure 20), it was noted that the epifluorescent photograph of the cells that were incubated without the DNA probe (Image C) needed a little more contrast and were made a little brighter in order to visualise them. This observation suggests that, although they have autofluorescence, the cells are brighter when incubated with the DNA probe.

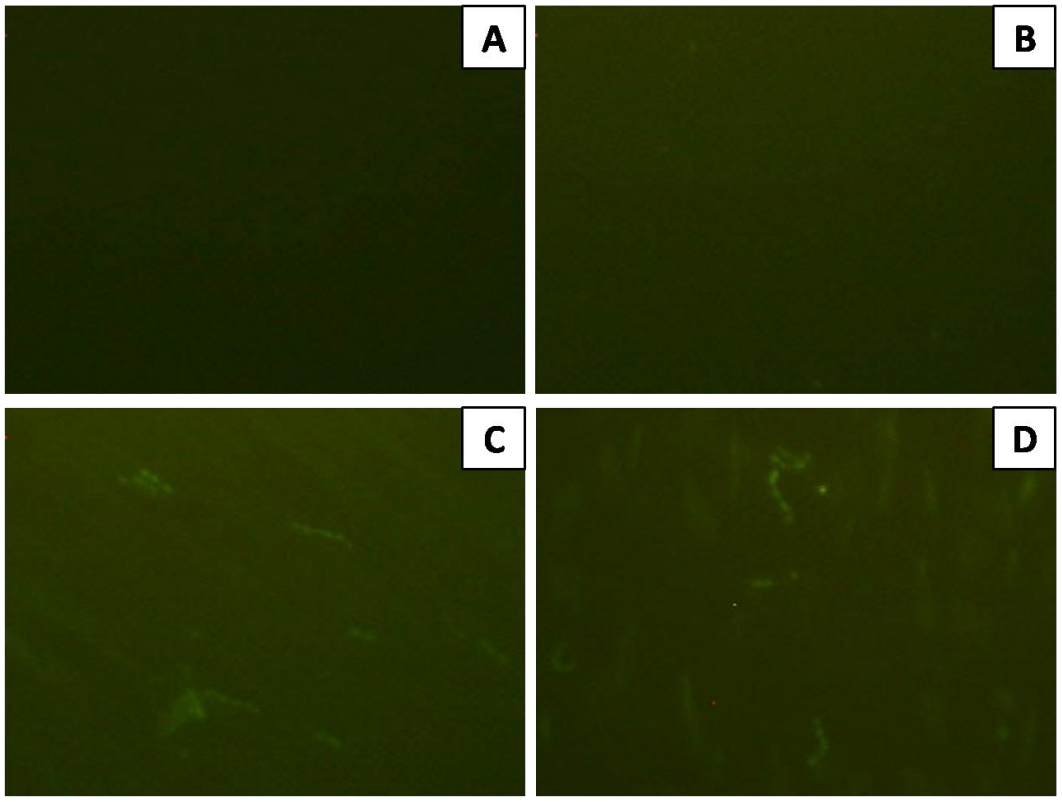

Figure 20 - Cell autofluorescence repeat experiment. Samples treated with FLOW-FISH protocol and then examined using epifluorescent microscopy. Images A and B show PBS (pH7.2) minus and plus DNA probe respectively. Images C and D show *Streptococcus thermophilus* (ST55) cells minus and plus DNA probe respectively.

A difference was observed also in the FCM results (Figure 21) but the count results obtained were much lower than the plate count by approximately 5 Log_{10} cells/mL. The count window was modified as per Table 3 to help eliminate the autofluorescent cells from the count results.

Table 3 - Settings for the "counting box" classifiers to eliminate autofluorescent cells

"Counting box" classifier	Minimum	Maximum
FL1	50	300
FL2	0	600
FL2/FL1	0.5	5.0

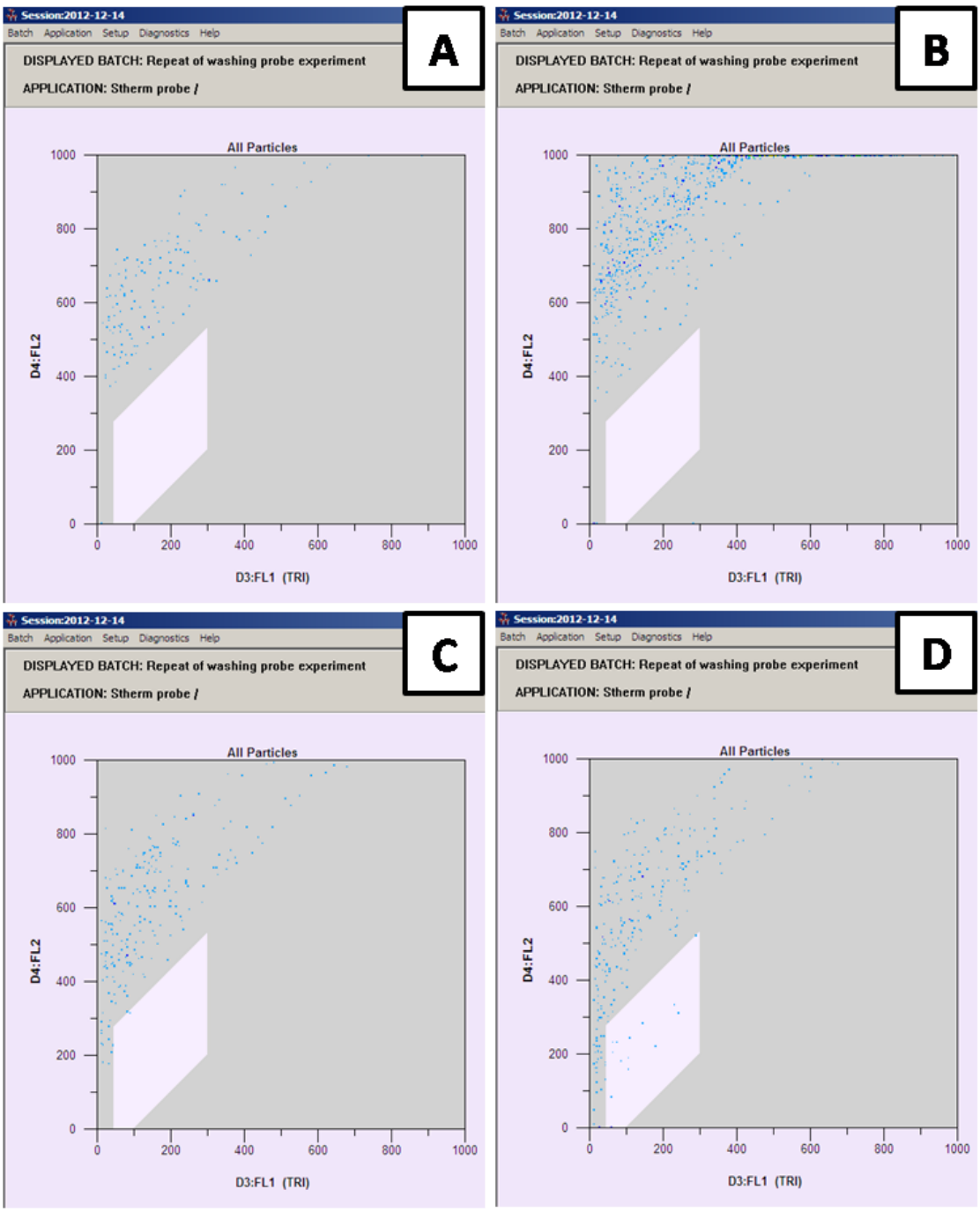

Figure 21 - Removal of autofluorescent cells from FLOW-FISH results. Images A and B show PBS (pH7.2) minus and plus DNA probe respectively. Images C and D show *Streptococcus thermophilus* cells minus and plus DNA probe respectively.

3.9 Extra washing steps

An accidentally added extra step in one experiment showed the benefit of adding extra washing steps to the FLOW-FISH method.

3.9.1 FLOW-FISH analysis of *S. thermophilus* (ST55) grown in M17 medium

The original objective was to use the FLOW-FISH method to enumerate *S. thermophilus* in yoghurt with RSM being used as the model for a milk-based product. However, milk products can have a lot of background fluorescence in flow cytometry methods and this can falsely increase the number of fluorescence events (Flint et al, 2006). Trialling the FLOW-FISH method on a culture grown in a non-milk-based medium might give a clearer idea where the cell population is placed on the dot plot outputs for the flow cytometer. The following experiment did not show the position of the cell population, but instead showed the possible benefit of an extra washing step.

A *S. thermophilus* (ST55) culture was grown in M17 medium and then diluted to 10^{-4} using peptone. At this point a plate count was performed as well as the FLOW-FISH method on both fixed and unfixed cells using a higher concentration of DNA probe (38.3ng/µL). The use of a higher concentration of DNA probe was based on the earlier experiments that showed a more obvious population of fluorescent events when the concentration was increased. The gain settings were left at the same level as that for the calibration beads. A number of different negative controls were also set up to go through the FLOW-FISH method (see Table 4).

All the samples were prepared, incubated plus/minus 38.3ng/µL DNA probe, and then washed once with 100µL of hybridisation buffer before the addition of the PBS (pH8.4) and analysed with the flow cytometer. However, sample 1 (marked with an asterix) had an accidental extra step after the incubation with the DNA probe. The PBS (pH8.4, 500µL) was added instead of the 100µL hybridisation buffer so this sample was centrifuged down again, resuspended in the 100µL hybridisation buffer and the rest of the FLOW-FISH method was performed as for the rest of the samples.

Note that sample 6 had a shorter incubation with the DNA probe. The reason for this reduced incubation time was that the shorter incubation had looked promising in Section 3.7.1 and had not yet been tested with a higher DNA probe concentration.

Table 4 - Experimental sample constitution for FLOW-FISH analysis of a *Streptococcus thermophilus* (ST55) culture grown in M17 medium and various controls.

Sample number	Unfixed cells (µL)	Fixed cells (µL)	Peptone (µL)	Hybridisation buffer (µL)	DNA probe (µL)	Total volume (µL)	Length incubation (hr)
1 *	5			100	30	135	18
2		5		100	30	135	18
3				105	30	135	18
4			5	100	30	135	18
5			5	130		135	18
6	5			100	30	135	2

***This sample had an extra (accidental) step added to the FLOW-FISH method. PBS (pH8.4, 500µL) was added after the hybridisation step and before the wash step with hybridisation buffer.

Strong fluorescent populations were apparent in all samples that had DNA probe added (Figure 22, images 2, 3, 4, and 6), with the exception of the sample that had an extra (accidental) wash step (Figure 22, image 1). The only other sample that did not have the same strong fluorescent population was one of the negative controls that had no DNA probe added (Figure 22, image 5). This strong fluorescent population is not from the *S. thermophilus* (ST55) culture as this was not added to two of the negative controls that showed this population. These results showed that the DNA probe can easily contribute to the background fluorescence. The sample with the extra (accidental) step (sample 1) showed that wash steps may be useful in reducing this interference because the strong fluorescent population was not present even though DNA probe had been added to this sample.

Fluorescently labelled cells were not visualised on the results containing cells (samples 1, 2, and 6) and this may be due to the fact that, once dilution factors were calculated, only about 1 - 2 cells would have been tested by the flow cytometer of both the unfixed and fixed cell samples. The plate count result for the sample before fixation and testing with the FLOW-FISH method was 3.30 Log_{10} CFU/mL so this would be close to the lowest value that could be detected with this method.

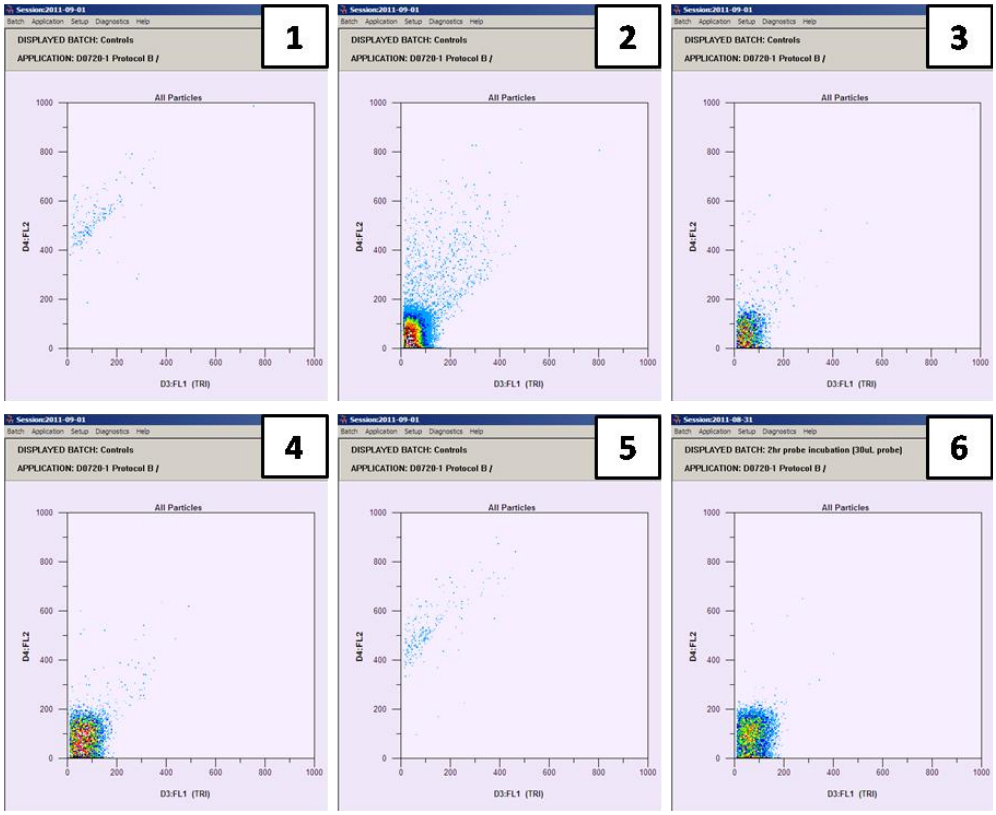

Figure 22 - Dot plot outputs from the flow cytometer for a *Streptococcus thermophilus* (ST55) culture grown in M17 medium and tested with the FLOW-FISH method along with a number of negative controls. The FLOW-FISH method was performed using 38.3ng/µL DNA probe in the hybridisation reaction mix. Image 1 shows the result of unfixed *S. thermophilus* (ST55) cells that had an extra (accidental) wash step added to the FLOW-FISH method. Image 2 shows the result of fixed *S. thermophilus* (ST55) cells. Images 3, 4 and 5 shows the results of three different negative controls: hybridisation buffer plus DNA probe; peptone with hybridisation buffer plus DNA probe; and peptone with hybridisation buffer minus DNA probe respectively. Image 6 shows the result of unfixed *S. thermophilus* (ST55) cells that were incubated with the DNA probe for only 2h.

3.9.2 Addition of extra wash steps

Extra washing steps in the FLOW-FISH protocol both before and after the hybridisation incubation were trialled to reduce the background fluorescence for *S. thermophilus* (ST55) cultured in M17 and RSM media. Additionally, the amount of DNA probe was reduced to 12.8ng/µL for two reasons. Increasing the concentration of the DNA probe in the reaction mix had previously contributed to higher background fluorescence. Although extra wash steps appeared to be successful in section 3.9.1, it is desirable to attempt to avoid the issue occurring rather than have to reduce the problem. Additionally, 38.3ng/µL appeared to be excessive compared to the concentrations that Flint (1998) and Beimfohr et al (1993) used, which were 2.5ng/µL and 6.25ng/µL respectively.

Both of the cultures (M17 and RSM) were diluted in peptone to 10^{-2} in peptone and were then fixed. A negative control was prepared that contained peptone with the same amount of formaldehyde as the fixed samples but no cells. Duplicate samples (1mL each) were then washed twice, hybridised overnight, and then washed twice again before being resuspended in 600µL PBS (pH8.4) and analysed by the flow cytometer. The pre-hybridisation washes were with 1mL of PBS (pH7.2) each time. The post-hybridisation washes were with 100µL hybridisation buffer minus the DNA probe with the second one incubated at 37°C for 30min before being centrifuged to remove the wash liquid. The M17 and RSM cultures were also tested by plate and total viable counts before being fixed to determine the cell numbers.

The extra wash steps before the hybridisation enabled the analysis of a larger amount of sample because 1mL of sample containing cells was tested instead of 5µL.

It was assumed that the common fluorescent population seen in both samples B and C (Figure 23) were the fluorescently-labelled cells as this would be the common factor in these samples. This population was also not present, as expected, in the control sample A.

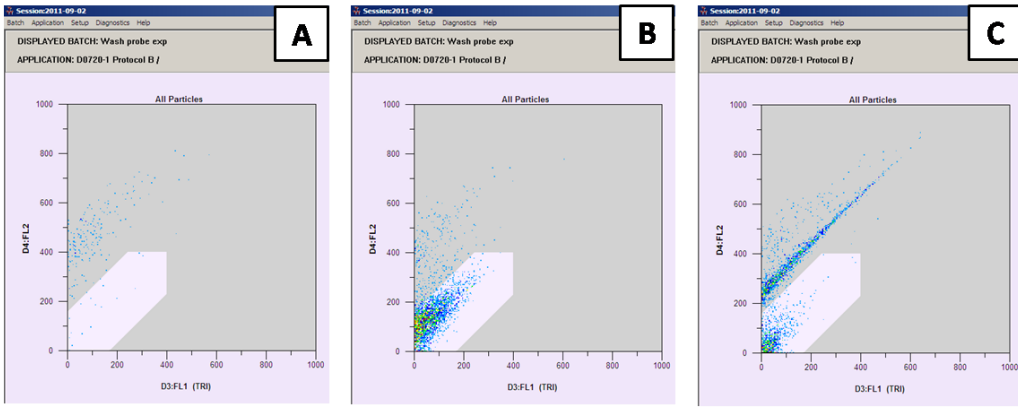

Figure 23 - FLOW-FISH dot plot results from the flow cytometer with more sample and extra wash steps pre- and post-hybridisation. Image A shows the FLOW-FISH result for the control sample that contained the same concentration of formaldehyde and peptone as the samples did. Image B shows the FLOW-FISH result for *Streptococcus thermophilus* (ST55) grown in M17 medium and diluted 10^{-2} in peptone before being tested. Image C shows the FLOW-FISH result for *Streptococcus thermophilus* (ST55) grown in RSM medium and diluted 10^{-2} in peptone before being tested.

A counting box was placed around this population as per Table 5 (Figure 23) and the results were compared against the plate and total viable count results

(Figure 24). Once again, the population sits on the axes and this means that the count obtained may not be accurate. There was also an unexpected extra population in the RSM sample (sample C) and this suggests that a 10^{-2} dilution is not enough to remove the background fluorescence of milk product constituents.

Table 5 - "Counting box" settings for the FLOW-FISH method with extra wash steps.

"Counting box" classifier	Minimum	Maximum
FL1	0	400
FL2	0	400
FL2/FL1	0.3	3.0

The results of the three enumeration methods for the culture grown in M17 medium appear similar. The RSM culture has given a different picture. Over the same period of time, the RSM culture has grown a higher number of cells but this is not reflected in the FLOW-FISH results. The FLOW-FISH results show a much lower value.

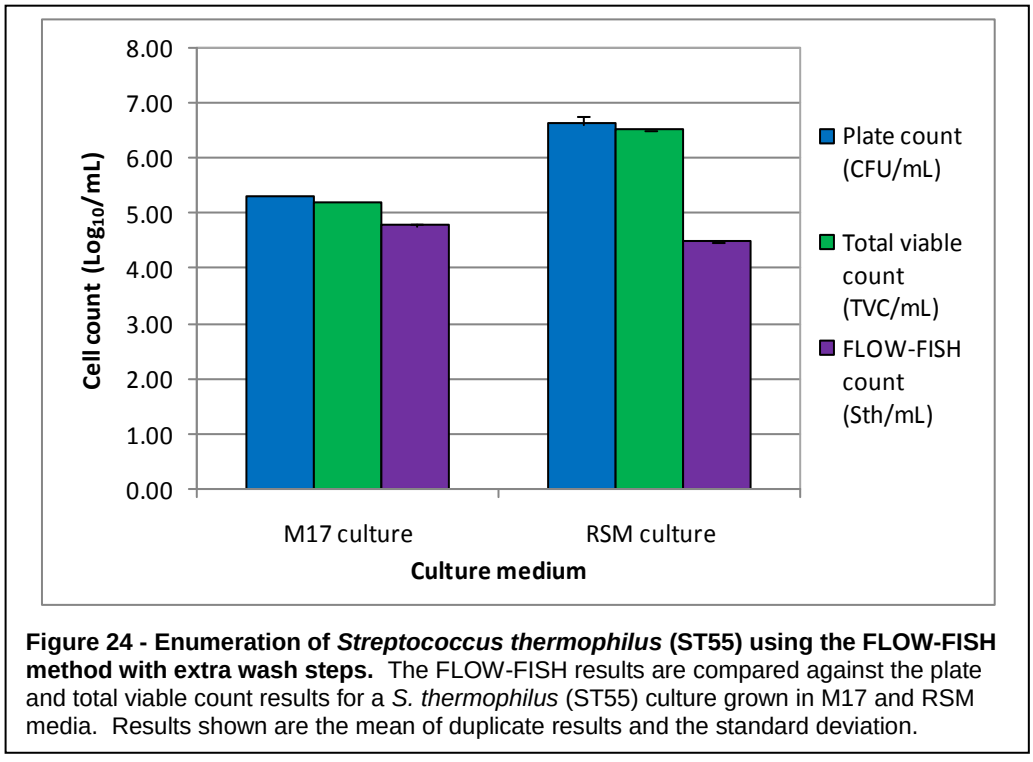

Figure 24 - Enumeration of *Streptococcus thermophilus* (ST55) using the FLOW-FISH method with extra wash steps. The FLOW-FISH results are compared against the plate and total viable count results for a *S. thermophilus* (ST55) culture grown in M17 and RSM media. Results shown are the mean of duplicate results and the standard deviation.

The results were similar when cultures were grown for short periods of 2 and 4 hours only (Figure 25). Cultures were prepared by adding 100µL of an overnight culture of either a M17 culture or a RSM culture into 10mL of either M17 or RSM respectively so it was the same medium used for each culture step. The M17 culture was sampled at 2 and 4 hours and diluted in 0.1% peptone to 10^{-1} and 10^{-3} respectively based on the total viable count results to keep the FLOW-FISH results within the best range for the flow cytometer. The RSM culture had a higher number of cells and the 2 and 4 hour samples were diluted to 10^{-2} and 10^{-5} respectively. The control sample consisted of 0.1% peptone and was put through the entire FLOW-FISH method from the fixation step to the end. The same counting box (see Table 5) was placed on the graph outputs of the flow cytometer (Figure 25) and the results compared to plate and total viable counts (Figure 26).

The extra population seen in image C in Figure 25 is likely to be interference from the RSM medium. The most likely reason why it is not seen in the 4h sampling of the culture is that this sample had higher cell numbers and was diluted more before the FLOW-FISH testing.

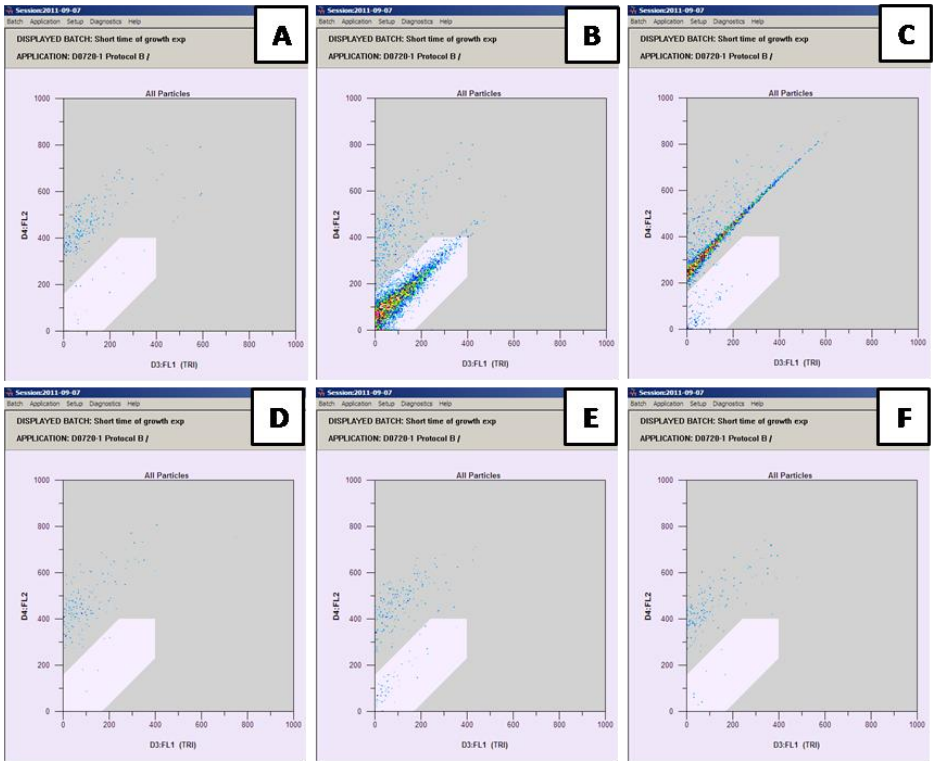

Figure 25 - Dot plot graphs from the flow cytometer showing FLOW-FISH results of 2 and 4 hr M17 and RSM cultures of *Streptococcus thermophilus* (ST55).
Images A to C are the results for the 2h cultures (A: 0.1% peptone control, B: *S. thermophilus* (ST55) grown in M17 medium, C: *S. thermophilus* (ST55) grown in RSM medium). Images D to F are the results for the 4h cultures (D: 0.1% peptone control, E: *S. thermophilus* (ST55) grown in M17 medium, F: *S. thermophilus* (ST55) grown in RSM medium).

The FLOW-FISH results for the M17 culture at 2 and 4 hours appeared to match the plate and total viable counts nicely, whereas both of the RSM samples gave a lower result with the FLOW-FISH method greater than 1 Log_{10} (Figure 26). Testing RSM samples clearly had an effect on the FLOW-FISH results and, although dilution helped to clear the dot plot graph, it didn't help the comparison with the plate and total viable counts.

Peptone (0.1%) had been used as the negative control because the samples are diluted in peptone before the FLOW-FISH testing and it was assumed there would be little growth medium present. However upon reflection, more accurate negative controls would have been M17 or RSM diluted in 0.1% peptone to the same extent as each sample.

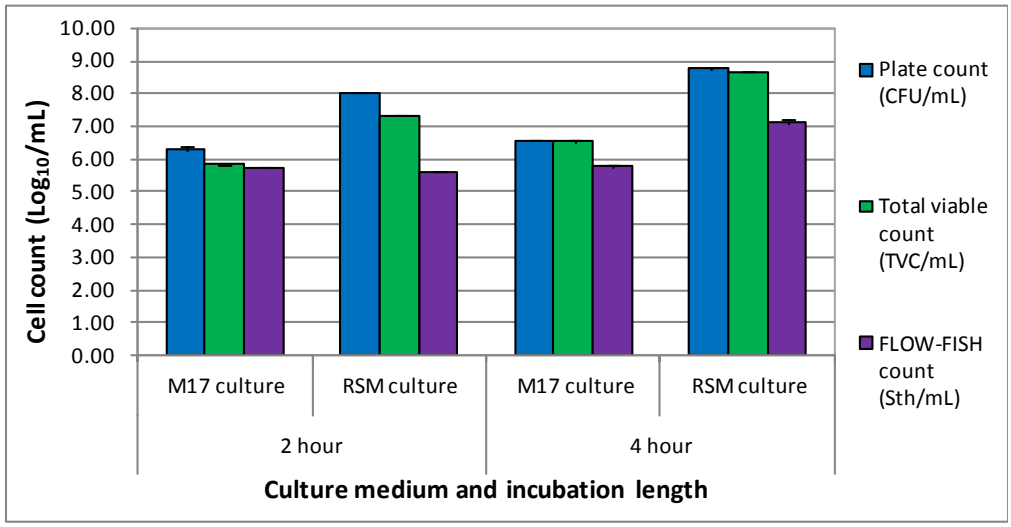

Figure 26 - Comparison of FLOW-FISH counts of *Streptococcus thermophilus* (ST55) grown for 2 and 4h in M17 and RSM media compared to plate and total viable counts. Results shown are the mean of duplicate results and the standard deviation.

A more accurate control for a sample containing M17 medium with *S. thermophilus* (ST55) cells was tested using M17 medium and was compared to a peptone control and a sample of M17 medium containing cells. All three samples were tested exactly the same with a 10^{-1} dilution using peptone (0.1%), fixation with formaldehyde (total volume 1mL), and analysis with the FLOW-FISH method. The FLOW-FISH method contained two 1mL washes before reconstitution in 100µL hybridisation buffer and 12.8ng/µL DNA probe and incubation overnight at 37°C. The next day this was followed by two washes with 100µL hybridisation buffer and final reconstitution in 600µL PBS (pH8.4).

The dot plot graphs show low background fluorescence from the control samples (Figure 27, images A and B) and a strong population of fluorescent events from the sample containing *S. thermophilus* (ST55) cells (Image C). A counting box was placed on the dot plot graph outputs of the flow cytometer using the settings outlined in Table 5.

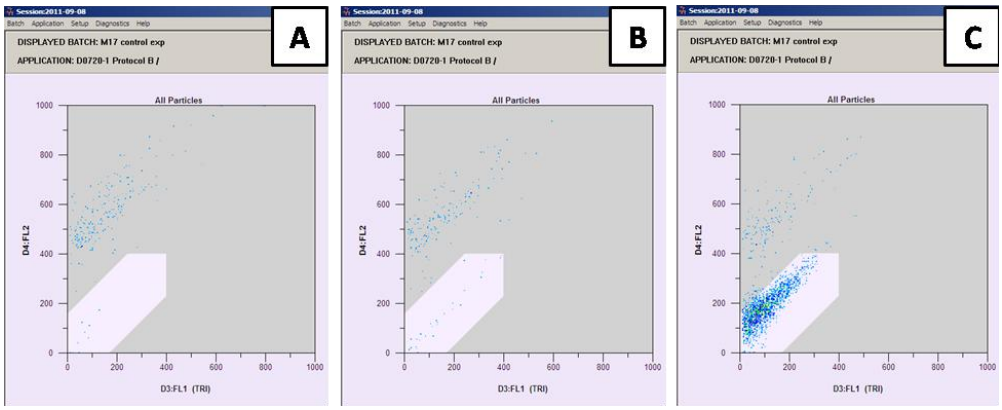

Figure 27 - Dot plot graphs of the FLOW-FISH result of two control samples compared with a sample containing *Streptococcus thermophilus* (ST55) cells. Image A shows the peptone (0.1%) control sample and image B shows the M17 medium control sample. Image C shows M17 medium containing *S. thermophilus* (ST55) cells that was analysed in the same manner as the control samples.

There was little difference between the two control samples (Figure 28). While the control results look large, if the M17 medium control results were used as a "blank" for the FLOW-FISH method by averaging the window counts and taking this value off the sample results, it would result in a FLOW-FISH count reduction for the sample of only 0.01 Log_{10} for this experiment.

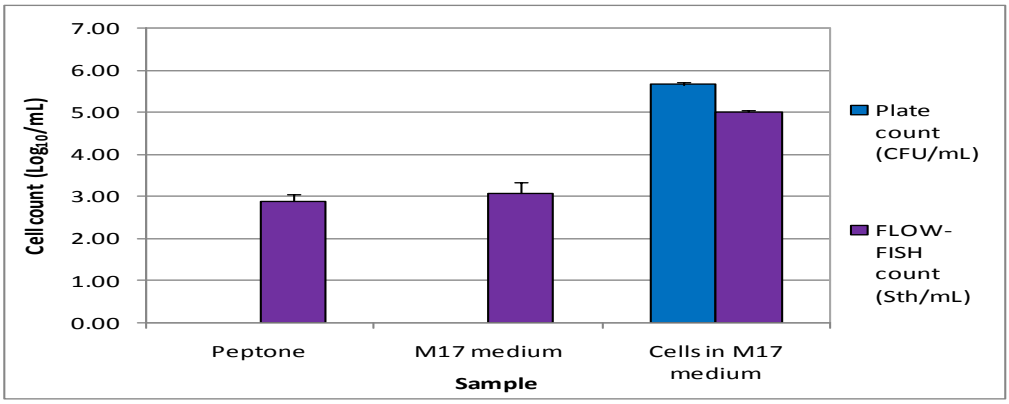

Figure 28 - Peptone (0.1%) and M17 medium used as controls compared to M17 medium containing *Streptococcus thermophilus* (ST55) cells. Peptone (0.1%) and M17 medium were tested exactly the same as M17 medium containing cells with a 10^{-1} dilution in peptone (0.1%), fixation with formaldehyde, and FLOW-FISH analysis. Results shown are the mean of duplicate results and the standard deviation.

4 PMA treatment

4.1 Summary

A PMA treatment previously used successfully by García-Cayuela et al (2009) to remove non-viable cells of various lactic acid bacteria from real-time PCR assays was explored for the potential of removing non-viable *S. thermophilus* (ST55) cells from the FLOW-FISH assay. The initial part of the investigation was to determine what effect the PMA treatment might have on the detection of the cells using the flow cytometer. The PMA treatment was found to be toxic to the viable cells at the levels, and the experimental conditions used, giving rise to no growth on agar plate count assays. Variations in halogen light exposure, PMA concentration, and PMA reagent preparation were trialled in an attempt to optimise the PMA treatment. Although the plate count results of the cells before and after PMA treatment did not match, the total viable count results of the cells *after* the PMA treatment, as measured by flow cytometry, were similar to the plate count results *before* the PMA treatment was applied. This suggested that the viable and culturable cells had become viable but non-culturable after the PMA treatment. Mixtures of viable and non-viable cells were tested to see if the total viable cell method, after PMA treatment, would still give the expected result for the reduced number of viable cells in the presence of non-viable cells and it was found that it did. A preliminary trial combining the PMA treatment with the FLOW-FISH assay was not successful partly because the cell numbers were too low for detection.

4.2 Effect of PMA treatment on the detection of *S. thermophilus* (ST55) cells using the flow cytometer

The PMA treatment was used in conjunction with the total viable cell assay to see what effect would occur to the staining or fluorescent events recorded by the flow cytometer. The total viable cell assay was used because it was an established working method for fluorescently labelling cells for detection with the flow cytometer (Flint et al, 2006). Additionally, it served as a positive control for the ability of the flow cytometer to detect fluorescently labelled cells. An overnight culture of *S. thermophilus* (ST55) grown in RSM was diluted to 10^{-5} using 0.1% peptone. Both plate and total viable counts were performed on this sample. Then 0.5mL samples of the culture were put through the PMA treatment method, plus and minus the PMA reagent, and were then enumerated using both the plate and total viable count methods. The samples were put through the full treatment minus the PMA reagent to

see if the DMSO diluent (for the PMA reagent) or the incubation step under the 500W halogen light would have any effect on the results. The purpose of the halogen light in this PMA treatment method covalently, permanently, binds the PMA reagent to the DNA of non-viable cells. The effect of the halogen light without PMA present may be harmful.

There was an additional sample set that was put through the PMA treatment and then tested directly with the flow cytometer to see if there was any fluorescence created by the PMA reagent. To do this, four 0.5mL aliquots were treated with PMA, and then two were combined to form 1mL aliquots. The reason for this is that the flow cytometer needs 0.6mL in the sample tube to begin testing even though it actually only tests 100µL. This means that these samples had a higher concentration of cells introduced to the flow cytometer for testing.

Plate count and total viable cell counts agreed that just over 3.5 Log_{10} cells/mL of viable cells were present in the sample before any treatment was applied (Figure 29). The results of the full treatment minus the PMA reagent showed that there was no effect by the rest of the method for either the plate or the total viable counts. However, PMA treated samples that were tested with the two methods of enumeration did not agree. There was no growth (<1Log) on the agar plates, whereas the total viable count showed the same number of viable cells present as in the untreated control sample. If the PMA treatment prevented the cells growing on the M17 agar plates but did not prevent the total viable cell method from giving a total viable count, it suggests that the PMA treatment results in viable, but non-culturable cells. The PMA fluorescence samples showed no fluorescence caused by the cells being treated solely with the PMA treatment.

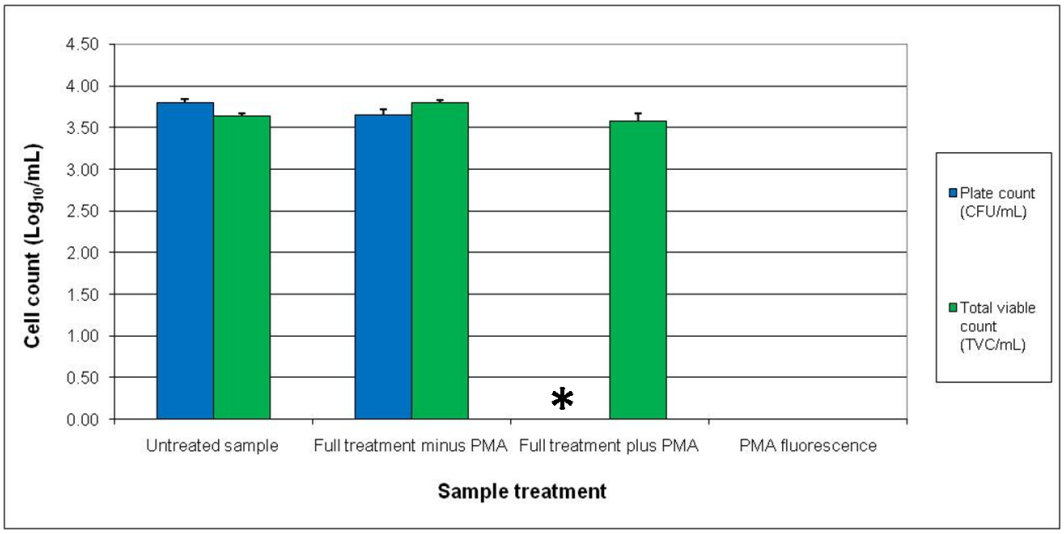

Figure 29 - Effect of propidium monoazide (PMA) treatment on the enumeration of viable *Streptococcus thermophilus* (ST55) cells. * Note that the plate count result for the samples that had full treatment with PMA should read <1 Log. The "PMA fluorescence" samples were treated with the full PMA treatment but put through the flow cytometer with no total viable cell reagents added. No plate counts were done on "PMA fluorescence" samples. Results shown are the mean of duplicate results and the standard deviation.

4.3 PMA treatment optimisation

The PMA treatment was modified in an attempt to reduce the toxic effect on viable and culturable cells. Variations in halogen light exposure, PMA concentration, and PMA reagent preparation were trialled in an attempt to optimise the PMA treatment.

4.3.1 Halogen light exposure

S. thermophilus (ST55) was grown in M17 medium overnight and diluted to 10^{-5} using 0.1% peptone to a final volume of 20mL. A plate count was performed on this diluted sample and then 0.5mL aliquots of the remainder were subjected to the PMA treatment. The amount of time that the samples were exposed to the 500W halogen light source (15, 10, 5, and 0 min) was tested in two separate experiments.

The only time cells were able to be cultured after the PMA treatment was when there was no exposure (0 min) to the 500W halogen light source (Figure 30). However, there was still a toxic effect from the PMA treatment as the plate count was lower than before the PMA treatment by nearly 2 Logs.

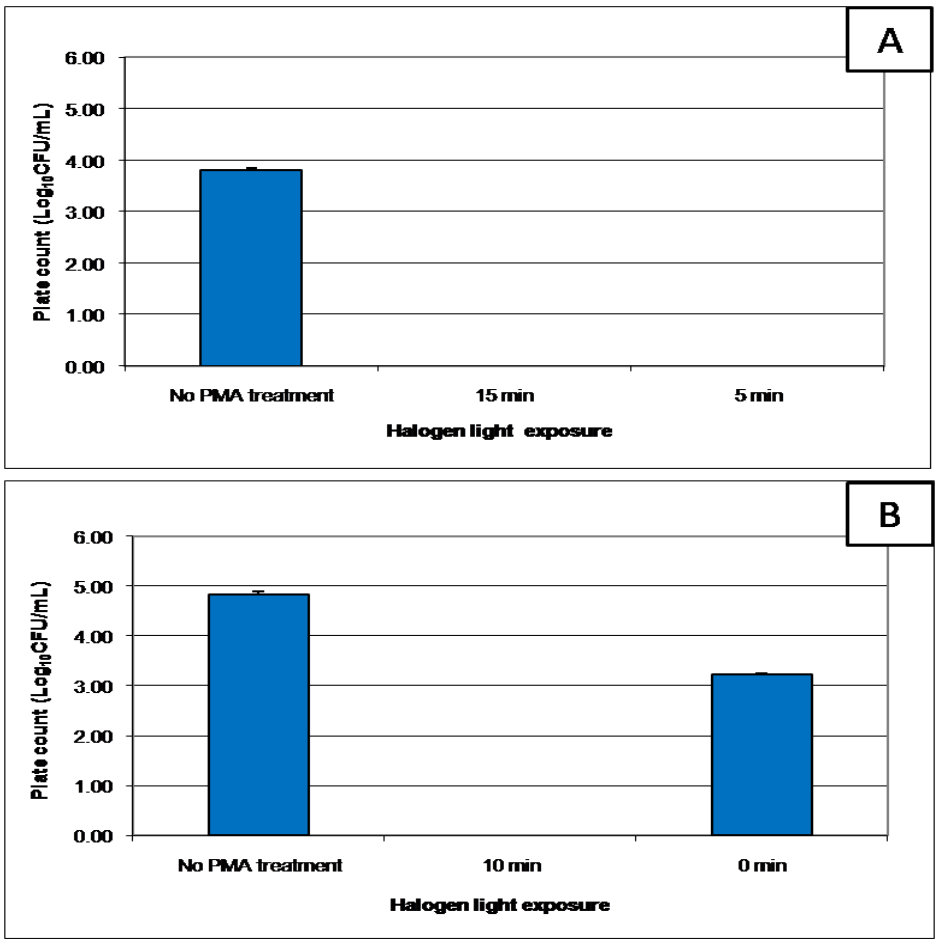

Figure 30 - Effect of reducing sample exposure to 500W halogen light source. Samples containing *Streptococcus thermophilus* (ST55) were treated with propidium monoazide using different lengths of exposure to halogen light and plated onto M17 agar, incubated at 37°C for 48h. The "No PMA treatment" results for both experiments show the amount of viable and culturable cells present before the PMA treatments. PMA concentration for all treatments was fixed at 50µM. **Graph A**: Sample exposure was reduced from the original 15 min to 5 min. **Graph B:** Sample exposure of 10 min and 0 min. Results shown are the mean of duplicate results and the standard deviation.

4.3.2 PMA concentration

As the PMA reagent appeared to also have a detrimental effect on the plate count result of the cells, the effect of reducing the PMA concentration from 50µM to 30µM and 10µM in the treatment reaction mix containing the sample was also examined. A culture of *S. thermophilus* (ST55) grown in M17 medium was again diluted to 10^{-5} using 0.1% peptone. A plate count was performed to ascertain the number of cells in the sample and then 0.5mL aliquots were treated with different levels of PMA reagent by adding 1.25µL of different concentrations of PMA stock solutions. The 500W halogen light source exposure time for all treatments was fixed at 15min so that the only

parameter that was changed from the original method was the PMA concentration.

Both of the lower concentrations of PMA reagent enabled the growth of some viable and culturable cells as shown by the plate count results (Figure 31), but still both sets of results were much lower than what was present before the PMA treatment.

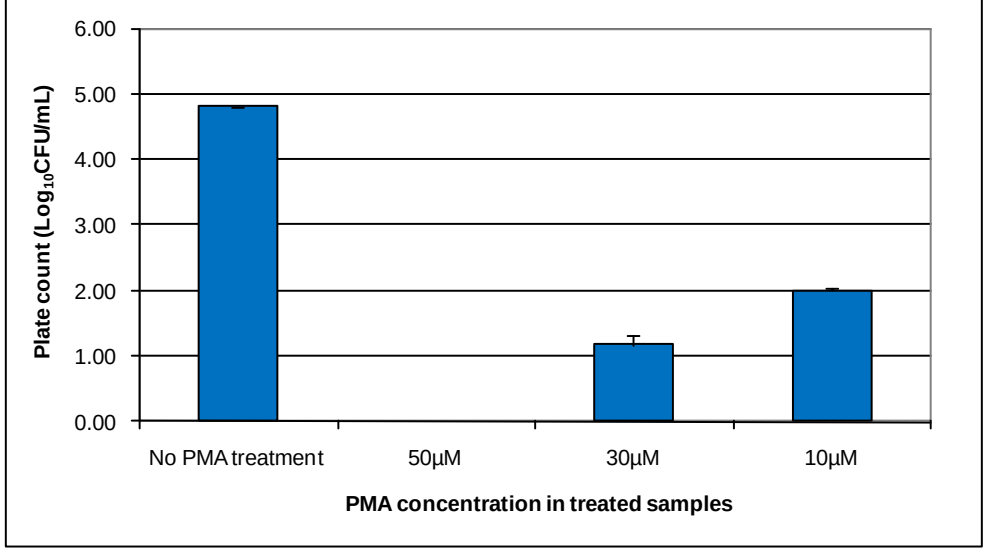

Figure 31 - Reduction of propidium monoazide (PMA) concentration in sample treatments. Samples containing *Streptococcus thermophilus* (ST55) treated with different concentrations of PMA were plated onto M17 agar and incubated at 37°C for 48h. The "No PMA treatment" result shows the amount of viable and culturable cells present before the PMA treatments. The 500W halogen light source exposure time for all treatments was fixed at 15 min. Results shown are the mean of duplicate results and the standard deviation.

A combination of a lower PMA concentration with a reduced exposure time may allow the cells to resist the toxic action of the PMA. However, this further optimisation was not investigated due to time constraints.

4.3.3 PMA reagent preparation

The PMA reagent was sourced from the company Biotium Inc. (Hayward, CA. USA) in a powder form with the recommendation to reconstitute the powder using DMSO. During these studies Biotium Inc. began to market a new, less-toxic, PMA preparation that was already dissolved in water to the same 20mM concentration. Despite the results shown in Figure 29 where DMSO was seen

to have no toxic effect, this new preparation was trialled twice in two repeat experiments to see if it would be more successful for these studies.

For this set of experiments, a new vial of PMA reagent, obtained from the same source company, was dissolved in DMSO to replenish the stock of this reagent. A *S. thermophilus* (ST55) culture grown in M17 medium was diluted to 10^{-3} using 0.1% peptone. A plate and a total viable count were performed on the diluted culture sample. Aliquots of the diluted culture sample (0.5mL) were treated with 50µM of either the DMSO or the water preparation of the PMA reagent. A plate and a total viable count were also performed on these samples after the PMA treatment.

Despite keeping the PMA treatment in the original format, plate counts were achieved with both of the PMA preparations, although they were still well below the result for the sample before the PMA treatment (Figure 32). A possible reason for this result being different to that seen previously may be that a new vial of the PMA reagent diluted in DMSO was prepared and used for these experiments. It was noted at the time of the first comparison experiment that this PMA preparation was a weaker orange colour than the previous vial and also that the water preparation was a stronger orange colour. All vials of PMA were thought to be the same concentration but the colour may indicate some difference in concentration. The comparison studies showed that the new water preparation gave lower results than the DMSO preparation, prepared in-house, for both methods of enumeration. Where the total viable count for the DMSO preparation showed promise by generally being equivalent to the number of culturable cells present in the samples before treatment, the water preparation showed a reduction and so was less equivalent.

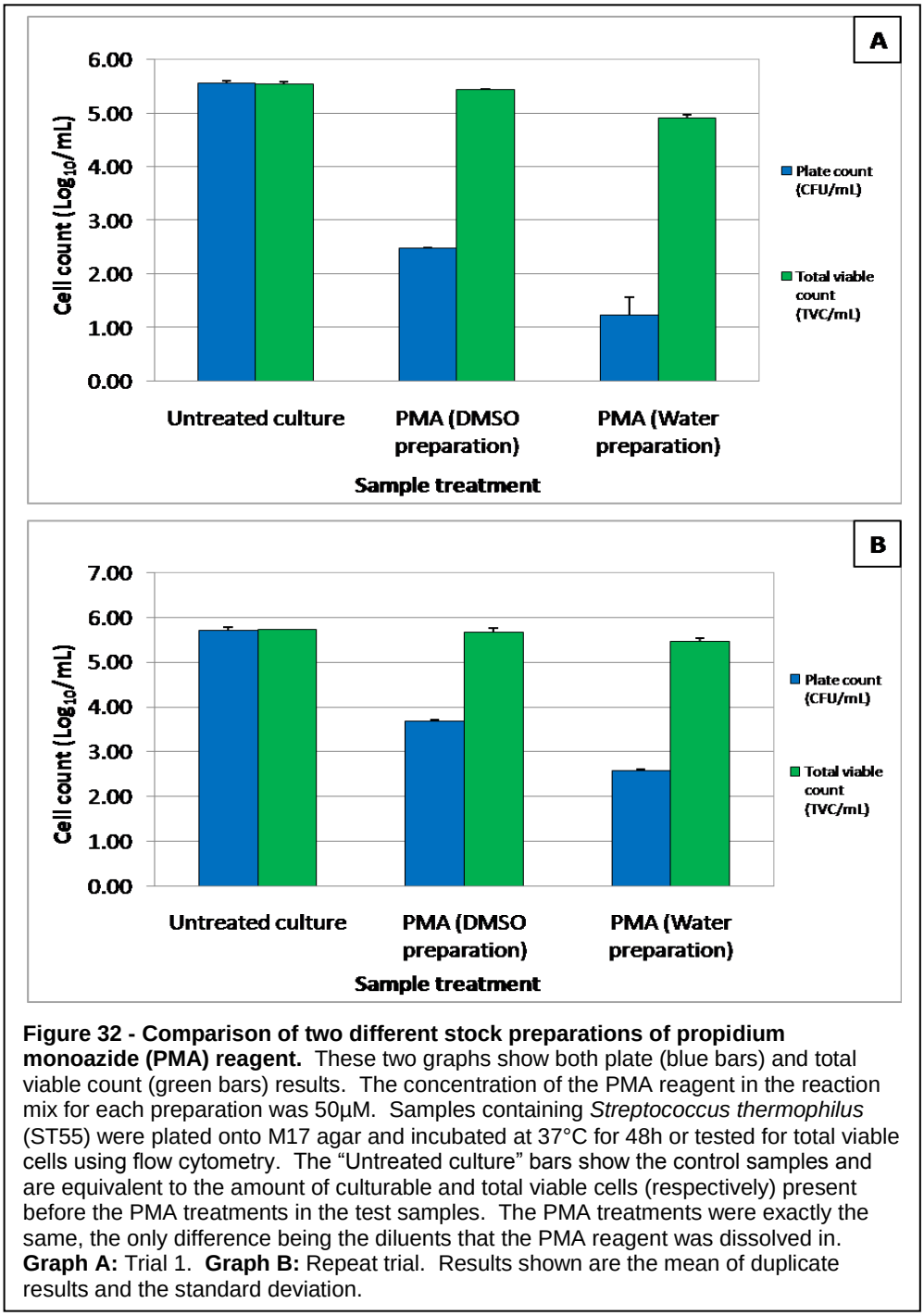

Figure 32 - Comparison of two different stock preparations of propidium monoazide (PMA) reagent. These two graphs show both plate (blue bars) and total viable count (green bars) results. The concentration of the PMA reagent in the reaction mix for each preparation was 50µM. Samples containing *Streptococcus thermophilus* (ST55) were plated onto M17 agar and incubated at 37°C for 48h or tested for total viable cells using flow cytometry. The "Untreated culture" bars show the control samples and are equivalent to the amount of culturable and total viable cells (respectively) present before the PMA treatments in the test samples. The PMA treatments were exactly the same, the only difference being the diluents that the PMA reagent was dissolved in. **Graph A:** Trial 1. **Graph B:** Repeat trial. Results shown are the mean of duplicate results and the standard deviation.

4.4 Samples containing a mixture of viable and non-viable cells.

As shown previously, the plate count results of the cells before and after the PMA treatment did not match, but the total viable count results of the cells *after* the PMA treatment were similar to the plate count results *before* the PMA treatment was applied. This suggested that the viable and culturable cells on agar plates had become viable but non-culturable. Mixtures of viable and non-viable cells were tested to see if

the PMA treatment, in conjunction with the total viable cell method, would still give the expected result for the number of viable cells in the presence of a large number of non-viable cells.

A culture of *S. thermophilus* (ST55) was diluted to 10^{-5} using 0.1% peptone. Samples were then treated separately with two sanitising chemicals, 12.5% Janola (Pental) and 1% Firstsinald (JohnsonDiversey), for 10 and 15 minutes respectively. These samples were washed twice with 0.1% peptone before being reconstituted a third time in 0.1% peptone. A mixture of 10% viable cells to 90% non-viable cells for each sanitiser was created by adding 1mL of the diluted viable (non-sanitised) culture with 9mL of the sanitised culture. The diluted viable culture (assumed to be 100% viable cells) and these two mixtures (10% viable cells each) were enumerated using plate and total viable counts before PMA treatment (50µM). A second total viable count was performed after the PMA treatment.

The diluted viable culture contained 4.8 Log_{10} cells/mL, agreed by both the plate and the total viable cell counts (Figure 33). The total viable cells counts after the PMA treatment of the viable culture sample showed a reduction of 0.7 Log_{10} cells/mL. Previously the total viable count after the PMA treatment had been equivalent to that performed before the PMA treatment.

According to the plate count, there were 10% viable cells present in the Janola-disinfected sample as planned and the total viable count agreed (Figure 33). The viable count, after PMA treatment, also showed that there were 10% viable cells present, although a slightly reduced number by approximately 0.2 Log_{10} cells/mL. The presence of large numbers of non-viable cells did not increase the results.

The aim was to have 10% viable cells in the Firstsinald-disinfected sample as well. However, the plate count (performed before the PMA treatment) showed a reduction of over 3 Log_{10} cells/mL, instead of 1 Log_{10} cells/mL, which suggested that only 0.01% viable cells were present instead (Figure 33). A residual action of the Firstsinald chemical may account for this reduced result even though the treated cells were washed before the viable cells were added. Interestingly the total viable count, *before* PMA treatment, showed the desired 10% viable cell value. This indicates that the Firstsinald sanitiser had created viable, but non-culturable cells when the viable cell portion was added because the culturable results (plate count results) did not match and were much reduced in number. The viable count (*after* PMA treatment) gave a similar result to the plate count for this sample.

These results confirmed that the total viable counts *after* PMA treatment follow the plate count results *before* treatment. Plate counts show viable cells that can be cultured. Total viable counts will show both these cells and those that are viable but non-culturable. The PMA treatment may cause damage to healthy cells giving viable, but non-culturable cells, and causes further damage to already damaged cells, killing them completely. Therefore when a total viable count is performed *after* PMA treatment, it now shows the same value as the plate count because the previously unculturable cells have been taken out of the equation. Or to explain this in another way, this work has shown the potential benefit of using the PMA treatment to produce a flow cytometry result that matches the plate count result.

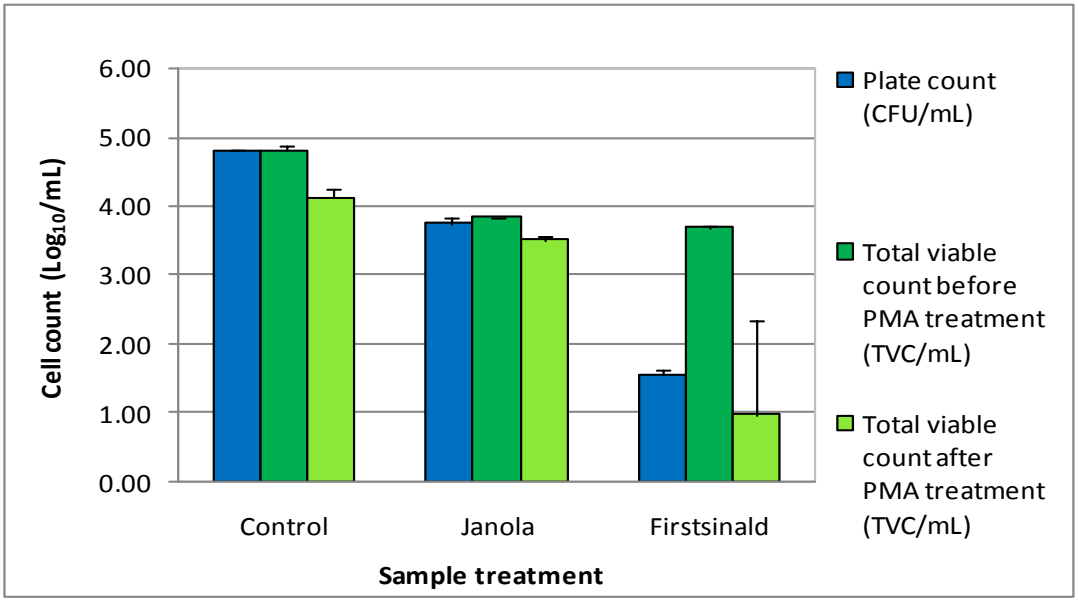

Figure 33 - The total viable count after propidium monoazide (PMA) treatment of viable cells in the presence of non-viable cells. Two 10% viable to 90% non-viable *Streptococcus thermophilus* (ST55) culture samples were prepared by treating portions of a viable culture with two disinfectants separately (12.5% Janola and 1% Firstsinald [JohnsonDiversey]) and mixing 9mL of this treated sample with 1mL non-treated sample. A plate count and a flow cytometry total viable count were performed before the two samples were treated with PMA. A second total viable count was performed after the PMA treatment. The "Control" bars show the amount of cells present in the original culture before preparing the mixtures (ie. 100% viable cells). Results shown are the mean of duplicate results and the standard deviation.

4.5 PMA-FLOW-FISH combination

Combining the PMA treatment with the FLOW-FISH assay was the final objective of this study. Only one preliminary trial of the PMA-FLOW-FISH combination was achieved to observe what might happen.

A new vial of PMA diluted in DMSO was prepared at the beginning of this experiment. A *S. thermophilus* (ST55) culture grown in M17 medium was diluted to 10^{-4} using 0.1% peptone. Aliquots (0.5mL) of the diluted culture were treated with PMA before being recombined to give 1mL samples. The 1mL samples were fixed and hybridised with 12.8ng/µL of the DNA probe as per section 3.9.2 in the FLOW-FISH chapter. The control sample was M17 medium put through the entire method procedure (PMA treatment, fixation, and FLOW-FISH assay).

Using the settings outlined in Table 5, there appeared to be no difference between the control sample and the cell sample for the PMA-FLOW-FISH results (Figure 34).

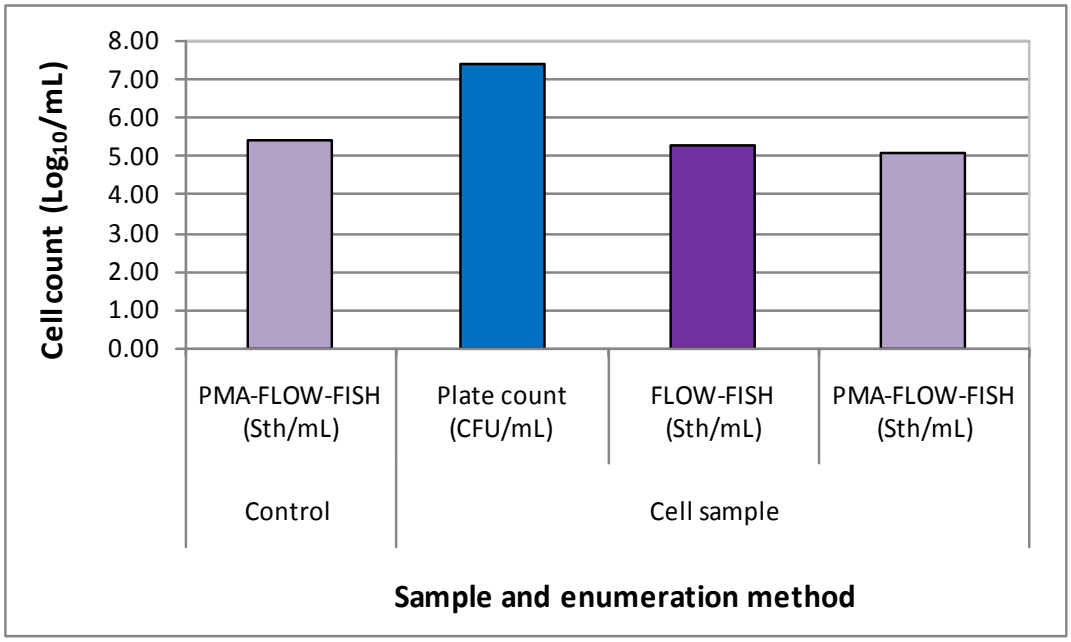

Figure 34 - Preliminary trial of the propidium monoazide (PMA) treatment combined with the FLOW-FISH assay for enumeration of *Streptococcus thermophilus* (ST55) cells. The plate count for the cell sample shows the mean of a duplicate set of samples. All other samples were single results only.

Looking at the dot plot graph outputs of the flow cytometer, it was not clear where the fluorescent cell population was positioned (Figure 35).

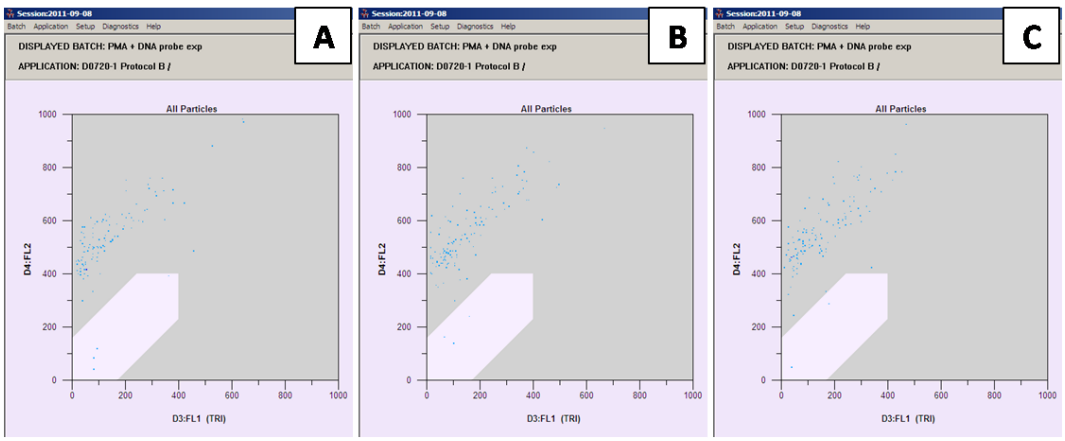

Figure 35 - Dot plot outputs of the PMA-FLOW-FISH preliminary trial for enumeration of *Streptococcus thermophilus* (ST55). Image A is the Control sample (M17 medium) with no cells tested with the entire PMA-FLOW-FISH protocol. Image B shows the Cell sample using the FLOW-FISH count method alone. Image C shows the Cell sample using the entire PMA-FLOW-FISH protocol.

However, the raw data of absolute values of fluorescent events recorded by the flow cytometer showed a difference between the control and the cell samples (Figure 36).

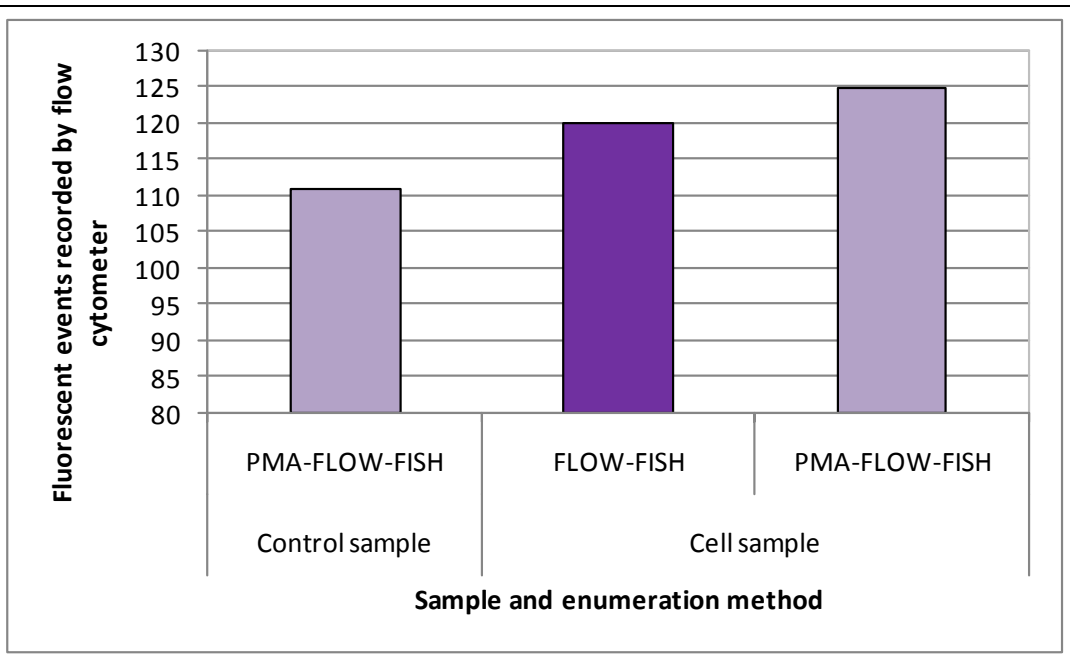

Figure 36 - Absolute fluorescent event values recorded by the flow cytometer. The control sample was M17 medium put through the entire PMA-FLOW-FISH protocol.

Altering the settings in the flow cytometer software to that in Table 1 appeared to capture most of the fluorescent events that were not in the control sample. This enabled a slightly closer match of the two flow cytometry methods (FLOW-FISH and

PMA-FLOW-FISH) to the plate count result (Figure 37) but did also raise the level of the control sample result.

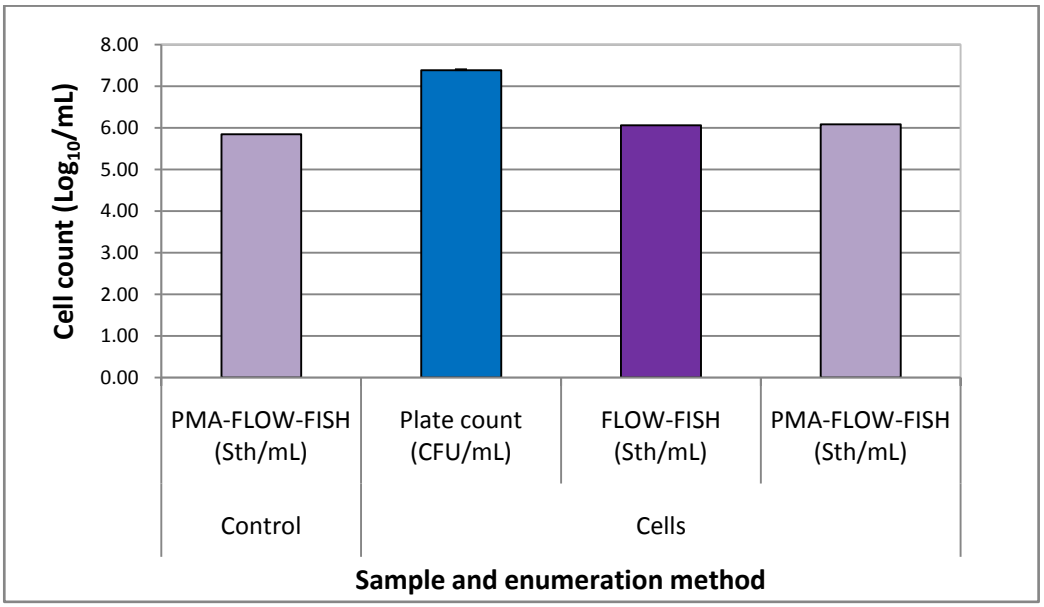

Figure 37 - Preliminary trial of PMA-FLOW-FISH method for enumerating *Streptococcus thermophilus* (ST55). The plate count for the cell sample shows the mean of duplicate results and the standard deviation. All other samples were single results only.

Because the difference between the control and the cell sample fluorescent events was small, it was difficult to know where to place the count box on the flow cytometer software to capture the fluorescent cell population. The dilution factor for these samples was large (64,800x) due mostly to the initial dilution of the culture in 0.1% peptone to 10^{-4}. Unfortunately this dilution factor must be applied to control sample results as well and this enhances the background fluorescence value. In addition, diluting the culture to that extent meant that only 370 fluorescent cells, or 370 extra fluorescent events on top of the background events, were introduced to the flow cytometer for detection. The difference seen between the control and cell samples was only 10-15 extra fluorescent events for the cell samples so there may be something else happening to the sample.

The cell numbers were probably too low for detection and replicate results may have given more information. This preliminary experiment was not successful in achieving an equivalent cell count to the plate count using the PMA-FLOW-FISH protocol for the cell sample but that does not mean it is not possible.

5 General Discussion

5.1 Summary

The overall aim of this study was to apply flow cytometry to enumerate individual cultures in a mixed culture system. Enumeration of specific lactic acid bacteria in dairy products is one area that would benefit from this study. The resulting objectives of this study were to develop a FLOW-FISH method for enumerating *S. thermophilus* (ST55) grown in RSM as a model for a set yoghurt dairy product and to evaluate the use of a PMA treatment as a potential additional step to the FLOW-FISH method to selectively enumerate viable cells. The first part of the discussion looks at the FLOW-FISH method by itself and the second part focuses on the application of the PMA treatment. The third discussion section examines an additional flow cytometry issue that could apply to any flow cytometry method. The final conclusion section ties the sections together and recommends areas for future studies.

5.2 FLOW-FISH enumeration of *S. thermophilus* (ST55)

Fluorescently-labelled cells of *S. thermophilus* (ST55) using a FLOW-FISH method were successfully captured by epifluorescent microscopy and flow cytometry in this study. However, this success was best achieved with cells grown in a M17 culture medium. The FLOW-FISH results for cells grown in RSM, used as a model for a set yoghurt dairy product, were always lower than the plate and total viable counts by more than 1 Log_{10} cells/mL and had an additional fluorescent population present.

Despite the success of the FLOW-FISH method using cells grown in M17, there was one trend seen in all experiments where the fluorescence of the cells labelled using the DNA probe was low. This caused difficulty in visualisation using epifluorescent microscopy and also in achieving a discrete fluorescent population on the dot plot graph outputs of the flow cytometer away from the axes of the graphs. Attempts to raise the position of this population by increasing the probe concentration or adjusting the gain settings of the detectors were not successful and simply caused background fluorescent levels to rise.

Although not useful for increasing the position of the fluorescent population, this optimisation work did highlight the need for extra washing steps to remove unbound DNA probe from the samples. The following discussion will explore possible reasons for the observations seen in this study.

5.2.1 Background fluorescence

Flow cytometers detect pulses of light as they pass the detector assembly (Shapiro, 2003; p16). If the background is high, this may make the signal-to-noise ratio smaller and the flow cytometer may miss many target signals as a result. Fluorescein isothiocyanate (FITC) is a small molecule so it is useful for intracellular staining but its negative charge can lead to increased nonspecific binding (Holmes et al, 2003). A probe should be added at saturation concentrations and it is the stringent conditions that ensure that the probe only binds to the target sequence (Pernthaler et al, 2001).

Veal et al (2000) commented that the FISH technique often doesn't produce a strong enough signal to discriminate bacteria from background fluorescence in many sample types. Time-resolved fluorescence, where the target is labelled with fluorochromes that have a long decay time, can help to discriminate fluorescently-labelled targets from the fluorescent background noise. However, it is not clear from this reference source whether such labelling technology could be used in conjunction with a FISH technique.

The success of the FLOW-FISH method on *S. thermophilus* (ST55) cells cultured in M17 medium appears to be due to two alterations to the original method used in the beginning of this study. These alterations were an increase in the concentration of the DNA probe in the hybridisation reaction and the addition of numerous washing steps, both before and after the hybridisation step. It was noted in one experiment that as the concentration of the DNA probe increased, the entire background grew brighter in the epifluorescent microscopy images. A second observation was the presence of fluorescent particles of varying size and shape in the same epifluorescent images, including samples without DNA probe or bacterial cells added. Interestingly, the fluorescent particles increased in number and grew brighter as more DNA probe was added to the sample. A final point to note was that the last sample containing the highest concentration of DNA probe was not entirely sampled by the flow cytometer because the number of fluorescent events crossed a maximum threshold value. All of these observations suggest that increasing the DNA probe concentration and autofluorescent particles can contribute to the background fluorescence.

The numerous washing steps eventually employed helped to reduce the background fluorescence but unfortunately also created an unwieldy number of steps in the protocol. Also, although wash steps were useful in reducing the

background fluorescence, the procedure should be approached with some care. Lenaerts et al (2007) advise against cell washing, particularly when the ultimate objective is to enumerate the cells, because the process encourages clumping and cell loss.

Reviewing earlier studies using the same probe as the one used in this study, the following observations can be made about wash conditions. Erhmann et al (1992) used a washing temperature 14°C higher than the hybridisation temperature of 40°C in dot blot hybridisation assays but did not mention the number of wash steps used and formamide did not appear to be used in the hybridisation or post-hybridisation wash solutions. Flint (1998) used the probe in a FISH assay on *S. thermophilus* cells adhered to stainless steel. Following the hybridisation step in the FISH assay, the samples were washed three times with hybridisation solution minus probe and then a fourth time with a washing solution containing Tris (1M, pH7.2) and NaCl (5M). The hybridisation conditions in this particular study included 40% formamide in the hybridisation solution and a hybridisation temperature of 37°C. It was not clear what temperature was used for the wash step. Washing is supposed to remove unbound probe and the use of a higher temperature or the presence of formamide is beneficial for tightening the stringency of the hybridisation reaction. Perhaps this higher temperature encourages DNA probe bound to incorrect structures or sequences to be released and washed away. Pernthaler et al (2001) performed the post-hybridisation wash step at a higher temperature and their objective of this step was to remove excess probe molecules using conditions that inhibit unspecific binding.

The rate of the hybridisation reaction depends on temperature in a bell-shaped curve manner (Anderson and Young, 1985). At low temperatures the reaction is slow. The rate increases as the temperature increases until an optimum is reached. At higher temperatures, the duplex of probe and target molecule will begin to dissociate and the rate of hybridisation slows down again. The actual temperature values are dependent on the melting temperature (T_m) of the duplex molecule. Ideally the incubation temperature of the hybridisation reaction should be 20-25°C below the T_m and, in practice, is often around 68°C. The presence of formamide in the hybridisation reaction lowers the T_m and means that the incubation temperature can be lowered. Or the incubation temperature can be kept constant and the percentage of formamide can be altered to modify the stringency of the hybridisation reaction. Note; for the hybridisation to RNA, formamide is a necessity to lower the incubation temperature of the reaction because RNA is extremely fragile

(Williams and Mason, 1985). Additionally, the hybridisation incubation should be kept at the minimum length to protect the integrity of the RNA molecules.

Almeida et al (2009) found that they had to optimise their FISH method using a Peptide nucleic acid probe (PNA-FISH) to get a stronger signal-to-noise ratio for both a slide-based and a solution-based hybridisation. Inclusion of a 10min paraformaldehyde immersion step performed first before an ethanol fixation appeared to result in reduced sample autofluorescence and was important in obtaining a good signal-to-noise ratio. However, it is not clear whether it is autofluorescence of the bacterial cells, or the sample matrix (powdered infant formula), or the background that they are referring to. Trialling different ethanol concentrations (50 and 80%) did not change the signal intensity. Hybridisation and washing temperatures were modified to find the best one for their assay to give the highest signal-to-noise ratio. Almeida et al (2009) also found that different hybridisation times (30, 45, 60 and 90 min) were found to be equally efficient for their PNA-FISH assay, but the autofluorescence seemed to get worse as the hybridisation time was increased. So, the authors reduced the hybridisation time of their assay to 30min.

5.2.2 Cell autofluorescence

Part way through this study, it was found that the *S. thermophilus* (ST55) cells portrayed fluorescence without the DNA probe present. These autofluorescent cells were positioned in the far bottom left area on the flow cytometer dot plot outputs. Fluorescent events sitting in this position are ones that have low fluorescence in the green spectrum. Autofluorescence, or natural fluorescence, is attributed to flavin nucleotides, pyridine and photosynthetic pigments within or on the cell (Veal et al, 2000) and that the green emission spectrum of cellular autofluorescence is in the same range as that of FITC (Holmes et al, 2003).

Moter and Göbel (2000) describe how it is important to check for autofluorescence of the target cells, and also of the surrounding tissue/medium, because it can lower the signal-to-noise ratio. Interestingly, *Streptococcus* species were not mentioned as a common culprit for this issue, although this may be simply because it hadn't been noted prior to their review of the area.

In this study, the results showed that the autofluorescence was lower than that of cells labelled with the FLOW-FISH method when only 1.5ng/µL DNA probe was used. The autofluorescent population of cells in that particular experiment were excluded from the FLOW-FISH enumeration result by re-positioning the count box. However, this work should be repeated with the higher concentration of DNA probe (12.8ng/µL) that was settled on at the end of the study. Ideally the higher concentration of DNA probe would raise the level of fluorescence in the green spectrum and enable further delineation of the FLOW-FISH population from the autofluorescent population.

5.2.3 Effect on hybridisation by cell pre-treatment

Many studies report that Gram-positive bacteria generally require extensive preparation for FISH type assays including lysozyme and proteinase K digestions, although such procedures should be used with some care as overdigestion may occur resulting in loss of cells, or alteration of light-scattering properties that could interfere with epifluorescent microscopy or FCM (Brehm-Stecher et al, 2005; Cerqueira et al, 2008).

Looking back at the reference used as a basis for the FLOW-FISH method used in this study (Fuchs et al 1998), it was designed for *Escherichia coli*, a Gram-negative microorganism. However, accessibility of the target molecule for the DNA probe did not appear to be a problem in the final experiments in this study because the number of fluorescently-labelled cells was equivalent to the number of cells ascertained by the plate count method.

However, it appears that extra pre-treatment of the cell samples, or alternative fixation procedures, would have been beneficial. Formaldehyde fixatives are not used for DNA hybridisations because of an effect on DNA denaturation, but they are often good for RNA hybridisations (Pardue, 1985). Moter and Göbel (2000) describe that both fixation and permeabilization is crucial for FISH methods to allow entry of the probe and to protect RNA from RNAses. They also mention that, although 3-4% formaldehyde or paraformaldehyde is sufficient for most Gram-negative organisms, heat-treatment or ethanol or ethanol/formalin treatments are recommended for Gram-positive organisms. And Gram-positive organisms may still require further treatments before a FISH method can be applied. For example lysozyme or lysostaphin may be needed to open up the peptidoglycan layer. Brehm-Stecher et al (2005) reported that ethanol-based fixation gave brighter and more homogeneous hybridisations than did formalin-based fixation based

on both microscopy and FCM results for *Listeria* species. Schleifer et al (1995) also found a lysozyme and paraformaldehyde pretreatment step was needed to give a uniform and strong probe-conferred signal with certain rRNA-targeted oligonucleotides directed at lactic acid bacteria.

Formaldehyde used alone only fixes cells by cross-linking proteins and so needs detergents to help solubilise the membranes afterwards, whereas alcohols simultaneously fix and permeabilize cells by coagulating cellular proteins and solubilising lipids (Holmes et al, 2003). Although Flint (1998) had used an ethanol dehydration series for the FISH method for the same DNA probe as used in this study, it had been assumed that this step was needed for drying out the samples prepared on slides for FISH staining and analysis using epifluorescent microscopy as the step is often termed a dehydration step. This step was not present in the FLOW-FISH protocol reported by Fuchs et al (1998) and therefore was omitted in adaptation of the original FISH method (Flint, 1998) for a FLOW-FISH assay, in part because it made the method simpler to perform and also because a dehydration step seemed irrelevant to a FLOW-FISH procedure. Reviewing these considerations has shown that further pre-treatment of the cells may have resulted in a stronger and more uniform signal from the FLOW-FISH labelled cells. However, it should be noted that Bunthof and Abee (2002) found that 50% of the starter cultures were membrane-permeabilized cells and that this occurred during the fermentation process.

5.2.4 RNA as the target molecule

The FLOW-FISH assay in this study utilised a fluorescently-labelled DNA oligonucleotide probe based on a 23S rRNA sequence.

There are three advantages to using RNA as the target molecule for a FLOW-FISH assay. The first advantage arises from the fact that 16S and 23S rRNA sequences have been reported for a great number of bacterial species and these are useful for developing methods that can identify bacterial groups or species (Charteris et al, 1997; Schleifer et al, 1995; Theron and Cloete, 2000). For this reason, a method can be developed for a target microorganism without having to cultivate it in a preliminary investigation (Amann et al, 1990a; Theron and Cloete, 2000). The second advantage is that there is more than one opportunity for the same fluorescent probe to bind in each individual cell. Each ribosome in a cell, each containing the three rRNA molecules, can be stained by one probe molecule and that the high number of ribosomes within a cell will give a naturally amplified FISH signal, enabling visualisation of single

bacterial cells (Moter and Göbel, 2000; Pernthaler et al, 2001; Wallner et al, 1995). The number of rRNA can range from more than 10^3 in a cell (Theron and Cloete, 2000) to 10^5 (Amann et al, 1990a). The third advantage is around stability and access for a DNA probe targeted at rRNA. The single-stranded state of RNA allows better access for probes and that DNA-rRNA hybrids are more stable than DNA-DNA hybrids (Hertel et al, 1991). DNA or RNA can be used as the probe but DNA is used because it is more stable and easier to synthesize (Theron and Cloete, 2000).

The number of ribosomes appears to be related to metabolic activity. The sensitivity of an assay is increased considerably when rRNA-targeted probes are used in samples containing exponentially growing cells because these active cells have higher numbers of ribosomes (Charteris et al, 1997; Fuchs et al, 1998; Schleifer et al, 1995). However, since the rRNA content varies according to the physiological state of the cells, low physiological activity can result in a low signal or give false negative results. Low ribosome content is often associated with naturally occurring populations, whose growth would be slower because nutrients would be limited (Lenaerts et al, 2007; Theron and Cloete, 2000). This means that any FISH signal obtained from the target cells will be low and difficult to distinguish between debris and non-target cells.

Research suggests that it may be possible to utilise the brightness of an rRNA hybridisation signal as an indicator of viability. Wallner et al (1995) write that high DNA and rRNA contents should at least be regarded as good indicators for viability because highly metabolically active cells and fast growing populations have larger cell volumes, more DNA and rRNA content, and this means highly active cells will give bright hybridisation signals. As mentioned previously, Lahtinen et al (2008) found that truly non-viable *Bifidobacteria* cells had a faster rate of rRNA degradation and that VBNC cells retained levels of 16S rRNA similar to that of culturable cells. Matte-Talliez et al (2001) reported that bacteriophage attacks on starter cultures gave bad or no hybridisation signals giving another example of how the viability status of bacterial cells can affect the FISH result presumably through the level of RNA or DNA.

The cells used in this study often had grown past the logarithmic stage so it is possible that the ribosome numbers had dropped and hence a reason why the fluorescent signal was low. But this wasn't always the case as some of the experiments had short incubation periods. Additionally, the cells appeared to remain viable even after passing the logarithmic stage of growth. If VBNC cells of *Streptococcus* species retain similar rRNA levels as viable cells the same as

the *Bifidobacteria* cells did in the study of Lahtinen et al (2008), then it is likely that this is not the reason for the low FLOW-FISH signal. In the original, and subsequent, references there was no mention of which target (DNA and/or RNA) that this *S. thermophilus* probe binds to inside the bacterial cell.

At the beginning of this study, it had been assumed that the target for the DNA oligonucleotide probe would be the DNA component of the cell. Upon further reading, it seems that many probes can bind to the RNA component and this can give the advantage of increasing the fluorescent signal due to the larger copy numbers. However, from this information, it is not clear whether DNA oligonucleotide probes bind only to one type of target, the DNA or the RNA, or bind to both at the same time. Additionally, it is not clear what the target is for the probe used in this study. Examining the original references regarding this query did not give the answer.

5.2.5 Probe considerations

The following section approaches solving the issue of the low fluorescent signal from a probe-based angle. Often accessibility of target site for the probe can be a problem due to the way the target molecule is folded which may prevent access to the target site (Theron and Cloete, 2000) but that is normally a consideration when a newly designed probe is being trialled. This study used a previously designed and successfully used probe so this is assumed not to be the problem.

There are ways of making a fluorescent probe more successful. Elongation of the DNA probes has been shown to increase the affinity and hence the brightness of the probes (Cerqueira et al, 2008). Double-labelling of oligonucleotide probes (DOPE) – FISH utilises the simplicity of the conventional FISH method but has approximately double the signal intensity (Behnam et al, 2012). Single probes can be double-labelled with the same or different fluorophores. Although Behnam et al (2012) mention briefly the use of the same fluorescent label for double-labelling, it seems they were more interested in double-labelling with different probes. They then had to be careful because some probes would quench the partnering probe. Moter and Göbel (2000) also mention the use of double-labelling of probes in their FISH review but also mention that putting a spacer of 18 carbon units in between the probe and FITC increased the signal intensity compared to FITC directly labelling the probe.

The use of a different fluorescent label may produce a stronger fluorescent signal. Moter and Göbel (2000) state that a better and more sensitive signal design is the use of polyribonucluotide probes (internally labeled with digoxygenin) combined with a tyramide signal amplification system. Cyanine dyes (Cy3 and Cy5) are superior to rhodamine and fluorescein based fluorescent labels because they are brighter and very stable to photo-bleaching. However, only Cy3 may be of use in the flow cytometer used in this study since the colour emitted is in the orange/red range, which is one of the colour ranges that this flow cytometer detects. Molecular Probes also offer three green dyes that are better than fluorescein (BODIPY FL, Oregon Green, and Alexa 488) but are not popular. Shapiro (2003) believes this is because of familiarity with fluorescein despite the well-known problems.

More than one fluorescent probe can be used to target the bacterial cell. Amann et al (1990a) used a FLOW-FISH method for the analysis of mixed microbial populations of *Escherichia coli*, *Desulfovibrio gigas*, and *Desulfobacter hydrogenophilus*. The intensity of fluorescence was increased by using a combination of two or three fluorescent probes for different regions of the 16S rRNA. Amann et al (1990b) also did the same for their target bacterial species (*Fibrobacter intestinalis*) and achieved approximately double the signal intensity.

An alternative type of probe could be used. Replacing the standard linear oligonucleotide probe with a molecular beacon in FISH assays gives better discriminatory power and a more accurate enumeration (Justé et al, 2008). The increase in the signal-to-noise ratio for molecular beacons may be due to the molecular beacons being in a “dark” mode when closed and not bound to the DNA target, whereas linear DNA probes have a constant fluorescence whether they are bound to the DNA target or still in solution (Lenaerts et al, 2007). Other benefits from using molecular beacons include removal of washing steps and reduction of cell clumping and cell loss. Peptide nucleic acids (PNAs) are another type of probe used in FISH assays but they are expensive, give low signal-to-noise-ratios, and need long hybridization times (Justé et al, 2008).

5.2.6 Reducing the time for the FISH experiment

The length of the hybridisation step in this study was around 18h. Attempts to produce stronger FLOW-FISH signals had suggested that the long incubation with the DNA probe was needed. The reason for this may be that the probe

needed this time to fully access the target molecules. A shorter incubation would be great for reducing the entire length of the FLOW-FISH procedure and it appears that it is possible as many studies have successfully used shorter hybridisation times (Almeida et al, 2009; Olsen et al, 2007). As noted earlier, Almeida et al (2009) found that a shorter hybridisation time of 30min was just as efficient as the longer periods of 45, 60 and 90 min and was also beneficial in reducing the autofluorescence in their samples.

5.2.7 Reconstituted milk samples

Samples containing milk product posed an additional problem for this FLOW-FISH assay. While the samples grown in M17 growth medium had FLOW-FISH results that matched the plate and total viable counts, the same was not true for samples grown in RSM. Additionally, an extra population of fluorescent events showed on the dot plot output that had the potential to interfere with the results. This is not uncommon for fluorescent-based methods. Milk contains a high level of particles that are the same size as bacteria (Bunthof and Abee, 2002). Almeida et al (2009) showed that a 10% reconstituted powdered infant formula autofluoresced in the green channel. Gunasekera et al (2000) reported that both the lipids and the proteins in their milk samples had to be removed or modified with treatment before the fluorescently-labelled bacterial cells could be detected with FCM. They used either proteinase K or savinase enzymes to treat ultra-heat treated milk samples, and included the detergent Triton X-100 for raw milk samples. Centrifugation then enabled the removal of the lipids and digested proteins, leaving the bacteria in the pellet. The same researchers utilised the same milk-clearing methods in combination with a FISH assay a few years later (Gunasekera et al, 2003).

However, the use of savinase may require some caution. Smith et al (2003) queried the use of savinase for clearing milk as they found it to be bactericidal for *Staphylococcus aureus* in milk samples. Hence the use of savinase may depend on the desired outcome. Using savinase may be useful if the end result is a total enumeration of the cells in the milk sample, but not when the desired result is for viable cell enumeration. Gunasekera et al (2002) recommend a lower concentration of savinase enzyme to clear milk for viability assays**.**

Alternative successful treatments have also been reported. Matte-Tailliez et al (2001) mixed a destabilising solution (0.5% Triton X-100 and 1.32 g/L of trypsin in 0.16x PBS; 800µL) with the milk sample containing bacterial cells (100µL) in

an Eppendorf tube. The tube was incubated at 50°C for 10 min in a waterbath. Talon et al (2002) cleared milk and yoghurt samples by using a ten-fold dilution of the sample in a glycine buffer (0.5 mM, pH 10.0) containing ethylenediaminetetraacetic acid (EDTA; 10mM), 2-butanone (9.1% v/v), and Triton X-100 (9.1% v/v). This apparently solubilised the fat globules and the casein micelles. The samples were then centrifuged and the resulting pellet was washed before using a pyrolysis-mass spectrometry assay. Flint et al (2007) added 0.8% EDTA to dissociate the casein micelles in reconstituted milk samples to avoid them being labelled by their FCM method. Cassoli et al (2007) incubated milk samples at 50°C in the presence of ethidium bromide and proteolytic enzymes for 12min but didn't explain why they used the enzymes or which enzymes they used. They also used sonication to eliminate somatic cells.

There are commercial reagents available that may be simpler to use. Holm et al (2004) used a ten-fold dilution of the milk sample in a reagent marketed by FOSS Analytical A/S that was designed to clear the sample. This mixture was incubated at 40°C for 10min before staining occurred for FCM analysis. Bunthof and Abee (2002) used a commercially available milk-clearing reagent marketed by Promega (Leiden, The Netherlands). The Promega reagent is a reagent provided with the Enliten milk assay kit for total viable organisms by ATP measurement and contains a chelating agent, a non-ionic detergent, and polystyrene beads. Apparently this clearing reagent causes the milk micelles to flocculate, coalesce, and increase in size, which encourages them to move readily to the surface during centrifugation. In addition, the somatic (mammalian) cells were lysed. The polystyrene beads interfered with the FCM analysis so they were removed by filtration and extra milk cream left at the end of the procedure was removed using a cotton-tipped stick. It should be noted that this milk-clearing reagent worked best on milk samples, and was not so successful on yoghurt or probiotic products. In fact, it increased the number of cells with damaged membranes.

In this study, the extra fluorescing population was most likely due to components in the RSM medium as it was eliminated with samples that required extra ten-fold dilutions before testing. However, removing this population of fluorescent events did not achieve a closer match with the plate and total viable counts. The problem is unlikely to be the amount of DNA probe in the reaction mixes because the samples were diluted to similar levels before the FLOW-FISH method was applied.

The same reasoning applies to the possibility of the DNA probe binding non-specifically to the RSM components and leaving little remaining to bind to the target cells as the heavily diluted sample still did not match, nor did it become closer to, the plate and total viable counts. It is possible that the DNA probe has difficulty in accessing cells that have grown in RSM because the cells may align with, or be coated in, the RSM components. Although, it might be argued that the total viable cell assay should have a similar issue since it also requires a reagent to enter the cell. However, the cells in the total viable assay are still active and may help in the process by either clearing the RSM components from the surface or allowing the reagent to enter across the cell membrane and wall.

5.2.8 FLOW-FISH summary

Moderate success was achieved with the FLOW-FISH method using M17 culture samples. However**,** the fluorescence of the FLOW-FISH labelled cells was low in all the experiments so this is an area that needs improvement. Alternative probes or modifications to the current DNA probe could be utilised but there are a number of areas that could be improved in the current FLOW-FISH method that may solve the problem. The *S. thermophilus* (ST55) cells showed the capacity to autofluoresce and clearly increasing the FLOW-FISH signal would help to differentiate the fluorescent signals. Certainly increasing the concentration of the DNA probe wasn't the exact answer as it had the tendency to increase background fluorescence.

The stringency of the hybridisation procedure could be tighter by using formamide and/or a higher washing temperature. It had been assumed that this consideration wasn't needed because only the target species was present in the samples. However, the non-specific binding of the probe may have included other sequences within the target organism or background particles.

This FLOW-FISH method had been based on a FLOW-FISH method published that utilised a similar type of DNA probe with the same fluorescent label. However, the pre-treatment of the cells in the original FLOW-FISH method (used on a Gram-negative microorganism) was probably not suitable for *S. thermophilus* (ST55) cells. In this study, the *S. thermophilus* (ST55) cells were fixed but not permeabilised. Gram-positive microorganisms often need to be membrane-permeabilised and hence it is likely that the accessibility of the fluorescent DNA probe to the target rRNA was the inhibiting factor.

Enumeration of *S. thermophilus* (ST55) in RSM using this FLOW-FISH assay proved to have additional issues. Combining a permeabilisation step with a milk-clearing step may be worth investigating to enable successful enumeration of *S. thermophilus* (ST55) cells in RSM samples.

5.3 Potential of the PMA treatment

In theory, a FLOW-FISH assay detects all cells, whether they are viable, injured, or non-viable. As a result, a FLOW-FISH enumeration of a sample that contains a mixture of cells in these three physiological states should give a higher result than that of a plate count method. Indeed Harmsen et al (1999) found this to be the case for total anaerobic, bifidobacteria, lactobacilli, and bacteroides microorganisms. The same situation occurs for PCR protocols but many studies have successfully employed a PMA treatment to remove the non-viable cells from the PCR assays. Hence PMA treatment was investigated as a possibility for modifying the FLOW-FISH assay to enable enumeration of viable cells only.

5.3.1 Effect on FCM results

The first part of this section of the study was to see what, if any, effect there was on the FCM results when using the PMA treatment on *S. thermophilus* (ST55) cells.

The product information sheet supplied with the PMA reagent used in this study described the excitation wavelength as being approximately 510nm once the reagent has been covalently attached to DNA or RNA (Biotium, 2010). This 510nm excitation value is higher than the 488nm excitation laser light in the flow cytometer but this is highly likely to be the wavelength at which the reagent is maximally excited. The related reagents, EMA and PI can be excited by a laser at 488nm (Riedy et al, 1991) and 490nm (Molecular Probes, 2004) respectively. The emission wavelength of PMA is approximately 610nm (Biotium, 2010), which is in the red spectrum and should be detected by the second channel of this flow cytometer. Hence a fluorescent signal from cells treated with PMA could reasonably be expected.

The PMA reagent and the actual process of the treatment did not change the FCM assay results for total viable cells. Cells treated solely with the PMA treatment did not produce detectable fluorescent events. Hence, it appears

that even if the cells were stained by the PMA treatment, it did not affect the results from the flow cytometer used in this study. However, the PMA treatment did have a strong effect on the plate count results because no culturable cells were observed. This effect appeared to be caused by the PMA reagent as the treatment minus the PMA reagent did not have an effect.

5.3.2 PMA treatment optimisation

Attempts to modify the PMA treatment method were only partially successful in achieving a culturable cell result on agar plates. Future investigations might find a combination of a lower PMA concentration with a reduced exposure time that will allow the culturable cells of *S. thermophilus* (ST55) to resist the toxic action of the PMA reagent. A low-dose double treatment as Minami et al (2010) reported for EMA might be more successful in allowing the detection of viable cells. Further optimisation was not investigated in this study due to time constraints.

A water preparation of the PMA reagent was also trialled even though the DMSO solvent used for the original preparations of PMA reagent had shown no detrimental effects on the culturable cell numbers. Unexpectedly the water preparation of the PMA reagent proved to be harsher on the culturable cells. Additionally, a difference was seen between batches of DMSO preparations based on both culturable cell results and colour of the PMA reagents. The supplier had not heard of this issue before although they did admit that they had only used the PMA treatment on samples to be tested with a real-time PCR method. However, it seems that other groups have had a similar issue. Riedy et al (1991) found that each new lot of EMA had to be titered to find the optimal concentration and that it could range from 1-5 pg/ml.

Another area for optimisation is the size and type of lamp used for the photoactivation step. A 500W halogen bulb was used in this study and by García-Cayuela et al (2009). Kramer et al (2009) used the same concentration of PMA for their assays on lactic acid bacteria as was used in this study and by García-Cayuela et al (2009), but used a larger wattage bulb (650W) for cross-linking the PMA to the DNA. Hellein et al (2012) describe the use of 460nm LED bulbs as an alternative because the heat from the high wattage bulbs may cause viable cells to become membrane-compromised. This would then allow the entry of PMA into the cells.

5.3.3 Matching PMA-FCM counts to plate counts

Many authors that have successfully used the PMA treatment for their real-time PCR assays have either not tested the PMA treatment on their culturable cells or not reported this information. The first situation is most likely as their objective was to compare the real-time-PCR result after PMA treatment to the un-treated culturable cell result. Hence, the same comparison could occur in this study and further optimisation of the PMA treatment may not be needed.

In this study, the total viable count results of the cells *after* PMA treatment were similar to the plate count results *before* PMA treatment. It appeared that the viable and culturable cells on agar plates had become viable but non-culturable because, *after* PMA treatment, the samples did not grow on plates but there were still viable cells present according to the total viable count FCM method. Deliberate mixtures of 10 % viable cells mixed with 90% non-viable cells were tested to see if the same result was achieved and these results confirmed that the total viable FCM count *after* PMA treatment followed the plate count results *before* PMA treatment. This study suggests that PMA treatment causes damage to healthy cells giving viable, but non-culturable cells, and causes further damage to already damaged cells, killing them completely. This means that the previously unculturable cells were removed from the total viable count result when performed *after* a PMA treatment and the result then showed the same value as the plate count.

It appears that the action of the PMA chemical is not fully understood since many researchers use it in the belief that it does not affect viable cells like EMA had shown. Clearly PMA does affect viable cells, but how? The following points may provide clues to the answer.

Since PMA is a chemical identical to PI, except for the presence of the azide group, it has been assumed that PMA binds similarly by attaching one dye molecule between every 4^{th} to 5^{th} DNA base (Nocker et al, 2006). According to the PMA product information page, PMA binds preferentially to double-stranded DNA with high affinity (Biotium, 2010). But Shapiro (2003) mentions that neither ethidium, nor propidium, binds preferentially to DNA and generally samples are treated with RNase enzyme to remove double-stranded RNA if the intended target molecule is DNA (p307).

Many studies agree that the main reason for the inhibition of PCR amplification for non-viable cells is from the loss of the EMA or PMA bound

DNA template during the DNA extraction step. However, one research group believes that this occurs because the EMA-bound DNA becomes insoluble and is pelleted with all the other cell debris during the DNA extraction step (Nocker and Camper, 2006; Nocker et al, 2006). This group suggested that it may be due to linkages of EMA-DNA material with the cell debris, although this had not been investigated. Another group looked at the effect of EMA treatment after the DNA was extracted and using electron microscopy found that the resulting loss of chromosomal DNA after EMA treatment was due to direct and random cleavage of the double-stranded DNA, in various forms (Soejima et al, 2007). The actual mechanism of the cleavage was not defined.

And yet there is clearly more to the story than the loss of the DNA template after PMA or EMA treatment. Apparently EMA-bound DNA template can itself inhibit PCR amplification and the suggestion of EMA-bound DNA template possibly being linked to cell debris is interesting (Nocker and Camper, 2006). Flekna et al (2007) attempted to cultivate their target species (*Listeria monocytogenes* and *Campylobacter jejuni*) after EMA treatment and found neither bacterial species grew. They also looked at the treated cells using epifluorescent microscopy and found that the viable cells had stained with EMA. Additionally, they found no statistical difference between the number of EMA-stained cells and the number of viable cells but there was a statistical difference between the number of EMA-stained cells and the number of non-viable cells. They suggested that future research should focus on the influx and efflux of EMA and the efficiency of the cross-linking reaction to DNA via photoactivation in different bacterial species and strains. There was one interesting comment that might also need investigation. Apparently metabolic pathways need to be blocked with sodium azide before sufficient staining occurs with stains such as ethidium bromide to overcome the expulsion of the stain by viable cells (Nebe-von Caron et al, 1998).

There is more information needed before we can understand how the PMA treatment works, especially how it provides a notable difference between culturable, and VBNC cells. Can it be as simple as damaging culturable cells so that they become VBNC cells, and damaging the already damaged cells further so that they become non-viable and/or non-detectable? The discussion and results of this study above suggest that PMA binds irreversibly to the DNA of culturable cells preventing DNA replication and as a result, preventing growth on agar plates. Yet, the cells keep the ability to process and retain the substrate that forms the basis of the total viable count, and therefore are detected as viable cells that can no longer be cultured. How does this scenario

then fit the VBNC cells? Perhaps the PMA treatment goes that step further in these cells and doesn't just bind to the DNA, it cleaves it as Soejima et al (2007) described. And perhaps the reason why this doesn't occur in the culturable cells is that the repair systems may still be functional or there is an inhibitor present. Or perhaps the concentration of PMA within previously culturable cells is kept low by pumping mechanisms still active in the cell membrane. However, this does not explain why the VBNC cells are no longer detectable by the total viable count method, which relies on an enzymatic reaction to cleave the substrate and an intact cell membrane and wall to produce the detectable fluorescent signal. What has the PMA treatment done that interferes with this enzymatic reaction that forms the basis of the total viable count?

5.3.4 Addition of PMA treatment to the FLOW-FISH assay

At the beginning of this study, there were no reports of PMA treatment being combined with a FISH assay and only one using EMA, so this combination of methods is relatively new and unknown. In 2003, Regan et al described, what appears to be, the very first use of an EMA treatment with a FISH assay for both planktonic and biofilm cells. They were of the same opinion as Riedy et al (1991) on how EMA is one of the few viability reagents that are able to be used with FISH assays because of the permanent bond formed between EMA and the DNA and RNA. Other viability reagents will generally diffuse in and out of the cells once the fixation and permeabilisation steps of the FISH protocol occur.

Since then, Mohapatra and La Duc (2012) reported the first use of a PMA treatment combined with a FISH method that was partially successful in enumerating viable *Bacillus pumilus* spores. Viable and non-viable spores were treated with PMA first before being permeabilised, and then stained using a FISH protocol. They did find that they needed to optimise the PMA treatment to reduce the effect on the viable spores. They also detected spores from the non-viable samples at low levels. The non-viable samples were produced by heat-treating viable spores. In both cases of a PMA or EMA treatment being combined with a FISH assay, the detection of the fluorescently-labelled cells was achieved using epifluorescent microscopy, not a flow cytometer.

The attempt to combine a PMA treatment to the FLOW-FISH assay in this study was not successful. Unfortunately, only one experiment was performed and so little information was obtained. Additionally, it did not help that the

cell numbers were too low for detection. Despite this result, and considering the success of the two studies above, designing a PMA-FLOW-FISH assay is still a worthwhile goal.

Some experimental considerations may need some thought. Wallner et al (1995) felt that propidium iodide and ethidium bromide should not be combined with fluorescein-labelled probes because they quenched the fluorescein emission by energy transfer. Yet Shapiro (2003) stated that PI is suitable for use with fluorescein (p96) and Riedy et al (1991) reported that EMA fluorescence was distinguishable from fluorescein fluorescence. And indeed the two studies mentioned that used EMA or PMA in conjunction with a FISH assay did not use a fluorescein label. Regan et al (2003) used Cy5-labelled probes in conjunction with EMA and Mohapatra and La Duc (2012) used Alexa Fluor® 488 to label the FISH probes. However, it seems that the reasoning behind the Cy5 label choice made by Regan et al (2003) was based on the need to have a different emission wavelength to that of EMA fluorescence and also that of a third stain (4',6-Diamidino-2-Phenylindole [DAPI]) used for total cell counts. Quenching issues were not mentioned.

5.3.5 PMA treatment summary

Cells treated with PMA alone were not detected using the flow cytometer used in this study. The PMA treatment appears to be useful in conjunction with the total viable assay as a way of tightening the correlation between the plate and FCM enumeration methods. Optimisation of the PMA treatment may always be needed to account for different batches of PMA reagent. A PMA-FLOW-FISH assay is still a reasonable objective but was not achieved in this study.

5.4 An additional issue for FCM testing

The training and reference manual material supplied by the manufacturer of the flow cytometer used in this study did not recommend any extra sample pre-treatment to break up chains or groups of cells. In fact they stated that only single cells would pass through due to the way the fluidic system was designed. However, this study found that chains of cells were still intact after passing through the flow cytometer. The idea that the flow cytometer can still detect signals from individual cells in a chain is unlikely. Shapiro (2003) says that a doublet will give a wider pulse but still be recorded as a single pulse (p17). And Michelsen et al (2007) found that chains of *Lactococcus lactis* gave broad distributions of fluorescence unless treated with vigorous shaking before analysis but even then only 70% were present as single cells afterwards.

Wallner et al (1995) also found inaccurate FCM counts when cells were present in chains or flocs.

Dispersal of chains or groups of bacteria, often for flow cytometry, is not a new phenomenon. Sonication and homogenisation are often used with sonication as probably the best technique for retaining viability (Cassoli et al, 2007; Falcioni et al, 2006). Papadimitriou et al (2006) tested a closely related species, *Streptococcus macedonicus*, for the best sonication procedure and determined that the optimum disaggregation occurred after 3 min of sonication. Brehm-Stecher (2006) compared the use of a Pulsifier® (Microgen Bioproducts, Ltd) to the typical approach of "stomaching" samples for the purpose of releasing bacteria from pork products. The Pulsifier® was found to be quicker, caused less sample destruction, and was equally efficient at releasing bacteria from the pork products. The minimisation of the amount of particulate matter in the sample means that clearer samples, with less interference, are produced for analysis using flow cytometry.

5.5 Conclusions

The overall aim of this study was to apply flow cytometry to enumerate individual cultures in a mixed culture system. Enumeration of specific lactic acid bacteria in dairy products was the area targeted for this study. The objectives were to develop a FLOW-FISH method for enumerating *S. thermophilus* (ST55) grown in RSM as a model for a set yoghurt dairy product and to evaluate the use of a PMA treatment as a potential additional step to the FLOW-FISH method to selectively enumerate viable cells. The objectives were not achieved so the method cannot be used by the dairy industry or other interested research groups at this point. However, there were some successful lines of investigation and the points below outline these and the suggested avenues for future work.

- Enumeration of *S. thermophilus* (ST55) in M17 medium using the FLOW-FISH method was successful but the following improvements should be trialled:
    - Tighten the stringency of the hybridisation reaction to ensure the probe binds to target molecules only
    - Determine the position of autofluorescent cells in the flow cytometer graph records in relation to cells stained with 12.8ng/µL DNA probe
    - Increase permeabilisation of the cells to allow more fluorescent probe to bind

- Modifications to the FLOW-FISH method that might be useful include:
    - The use of a molecular beacon as the fluorescent probe

- The use of the FLOW-FISH method may be successfully applied to reconstituted milk samples once improvements have been made to enhance the fluorescence of the labelled cells.

- Enzymatic or detergent treatments should be trialled on reconstituted milk samples if the autofluorescence of the milk components interferes with the FLOW-FISH results.

- The PMA treatment of *S. thermophilus* (ST55) cells combined with the total viable count method provided a closer match to plate counts by eliminating viable, but non-culturable cells from the results.

- There is still potential for the PMA-FLOW-FISH method to work. This study only briefly trialled the combination of methods with low cell numbers. Although the experiment was not successful, there was no indication that it would not work with higher cell numbers and also the PMA treatment had shown previous success when combined with the other flow cytometry method (the total viable count assay) used in this study.

- Samples containing *S. thermophilus* (ST55) should be treated with sonication or Pulsifier® to ensure single cells are detected by the FLOW-FISH assay

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