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Optimising Pre-Incubation Time and Comparing Sterility Methods for the Detection of Thermophilic Bacteria in UHT Dairy Products.

A thesis presented in partial fulfilment of the requirements for the degree of
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Abstract

Globally, UHT milk and UHT dairy products are an important source of nutrition and therefore have the requirement to be safe for consumption. Commercial sterility testing of these products ensures their safety and reduces reputational/financial losses for the dairy companies. However, temperature abuse during storage and transport has been highlighted as a concern due to potential spoilage effects from possible thermophilic contamination. Traditional commercial sterility testing methods, established by governing bodies, are time-consuming and labour-intensive, and they are not within the boundary of rapid food testing.

This study compared two rapid methods: the commercially available Charm Epic ATP bioluminescence method and a flow cytometry method (newly developed as part of this study), with the traditional plate method, for assessing the commercial sterility of UHT dairy products when contaminated with low numbers of obligate thermophiles. Four different UHT product types were investigated (milk, in-house cream, whipping cream and a medical beverage). In addition, the effect of shortening the UHT pack pre-incubation time on method performance was investigated. For instance, pre-incubation times of 6-24 hours were compared with 48 hours.

It was found that a pre-incubation time of 48 hours had only 50% agreement when comparing the ATP bioluminescence method with the plate reference method when measuring UHT milk. Whereas pre-incubation times of 6-24 hours had >95% agreement, thus greatly improving the performance of the ATP bioluminescence method. With UHT milk, the flow cytometry method had >95% agreement with the plate method at all pre-incubation times tested, however overall, the readings tended to be highest at 24 hours, indicating that a 24 hour pre-incubation time would have the lowest chance of a false negative result. Neither the ATP bioluminescence nor the flow cytometry method had acceptable agreement (i.e. ≥95%) with plate counting when measuring the in-house cream or medical beverage products, regardless of the pre-incubation time. Thus, it was concluded that the plate count method would be most appropriate for these matrices. Given these findings, an adjusted approach was applied to improve the performance of the flow cytometry method when analysing the whipping cream product. Firstly, additional sample preparation was used which involved mixing samples with a cation chelator, followed by centrifugation to obtain a bacterial pellet, assisting the extraction of the bacteria from the matrix. Secondly, the gate to capture the live bacterial cells on the flow cytometry plots was positioned further away from the non-bacterial background particles. These two approaches prevented the spillover of non-bacterial particles into the live gate, thereby preventing false positive results, and improving method sensitivity. This resulted in >95% agreement between the flow cytometry method and the plate method, at all pre-incubation times tested with

the whipping cream. Furthermore, this contrasted results with the ATP bioluminescence method, where the highest agreement achieved was 44%, with the whipping cream.

As UHT milk is the most common type of UHT dairy product, the shorter pre-incubation time in commercial sterility testing identified in this study would be highly beneficial to dairy companies, particularly when using methods alternative to the plate method. Not only would a shorter pre-incubation time have potential to improve the performance of the method, but it would also allow for a faster turnaround time. There needs to be recognition of the rapid transition into the death phase of the thermophilic bacterial growth cycle, as this has potential to impact rapid testing methods, such as those that measure ATP. These findings may be adopted into industry standards and regulations in the future.

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List of Acronyms and Abbreviations

ADA: N-(2-Acetamido)iminodiacetic acid

AFU: Active Fluorescence Units

AOAC: Association of Official Analytical Collaboration

ATP: Adenosine Triphosphate

Attune: Attune Nxt Flow cytometry

BL1: Blue Laser 1 Channel

BL2: Blue Laser 2 Channel

BL3: Blue Laser 3 Channel

CFU: Colony-Forming Units

Charm: Charm Epic ATP bioluminescence

CIP: Clean-In-Place

CO_2: Carbon Dioxide

DNA: Deoxyribonucleic Acid

ELISA: Enzyme-Linked Immunosorbent Assay

EPS: Extracellular Polymeric Substances

EU: European Union

FAO: Food and Agriculture Organization of the United Nations

FPR: False Predictive Rate

FSC: Forward Side Scatter

GLP: Good Laboratory Practice

GB: Guobiao Standards (Chinese National Standards)

HACCP: Hazard Analysis and Critical Control Point

IDF: International Dairy Federation

ISO: International Organization for Standardisation

MPI: Ministry of Primary Industries (New Zealand)

MPCA: Milk Plate Count Agar

NPV: Negative Predictive Value

PBS: Phosphate Buffered Saline

PCR: Polymerase Chain Reaction

PMA: Propidium Monoazide

PPV: Positive Predictive Value

RLU: Relative Light Units

ROC: Receiver Operator Characteristics

SSC: Side Scatter

TEM: Transmission Electron Microscopy

TSB: Tryptic Soy Broth

UHT: Ultra-High Temperature

VBNC: Viable But Not Culturable

WMP: Whole Milk Powder

WHO: World Health Organization

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Chapter 1. Literature Review

Introduction

The dairy industry is an important part of the global food economy, providing essential nutrition through a range of products. Among the various processing techniques employed to ensure the safety and longevity of dairy goods, Ultra-High Temperature (UHT) processing stands out as critical for producing shelf-stable milk and dairy products. This review aims to delve into the intricacies of UHT processing, examining its effectiveness in achieving commercial sterility and the challenges associated with maintaining product integrity throughout the product's shelf life.

In New Zealand, dairy products for UHT processing undergo a thermal process, typically between 135°C and 150°C, for a brief duration of 3 to 5 seconds, followed by aseptic dispensing and hermetic sealing into various packaging such as cardboard cartons, plastic containers, and metallic foil sachets (Ministry of Primary Industries.govt.nz, 2022). This process not only extends the shelf life of dairy products by several months while enhancing the bacteriological safety, but it also maintains the nutritional value without the need for refrigeration and limits chemical and organoleptic changes. In New Zealand, as in many other countries, UHT dairy products are subject to food safety regulations and quality control measures, including adherence to the Hazard Analysis and Critical Control Point (HACCP) system and final product sterility testing.

Despite the high microbial inactivation achieved by UHT processing, the presence of thermophilic bacilli capable of surviving extreme temperatures poses a threat to product safety and quality. This review will explore the characteristics of these microorganisms, their impact on UHT dairy products, and the implications of potential sterility failures. Furthermore, the review will consider the role of innovative testing methods in detecting contaminants, assess the optimal pre-incubation for commercial sterility testing, and ensuring the safety and quality of UHT-processed dairy products, as well as the influence of storage conditions, particularly in light of climate change and varying global infrastructures.

As the dairy industry continues to expand its reach and cater to a growing global population, understanding the nuances of UHT processing and the associated food safety and quality considerations is more important than ever. This review will provide an overview of the current state of UHT dairy production, the challenges faced by the industry, and the scientific advancements that support the delivery of safe, high-quality dairy products to consumers around the world.

Importance of UHT milk

The dairy industry is an important part of the global food economy, providing essential nutrition through a range of products. Milk is often touted as the complete food. It is an important part of human nutrition as it contains many macronutrients (protein, carbohydrates (lactose) and fats) and micronutrients (calcium, magnesium, potassium, zinc, vitamins and phosphorus) giving dietary and immunological benefits to the consumer (Górska-Warsewicz et al., 2019; Rizzoli et al., 2014; Mills et al., 2011).

For children during their growth phase, milk consumption assists in dental and bone health by providing essential calcium, minerals, and vitamins. It has been reported that milk consumption increases bone mass density and reduces the risk of fractures in adolescents and osteoporosis in adults. With global reports of deficiencies in calcium and vitamin D, and since milk offers these components in adequate amounts, dairy is recognized and recommended by health authorities as significant part of a balanced and healthy diet (Ratajczak et al., 2021; Herber et al., 2020; Shlisky et al., 2022; Mills et al., 2011).

World milk production was forecast to reach 950 million tonnes in 2023, underlining milk as an important part of global nutrition (International Dairy Federation (IDF), 2024; Food and Agriculture Organization of the United Nations (FAO), 2024). In Europe, 80% of the liquid milk supply is UHT milk and 90% of milk sold in Japan is UHT milk. However, in the USA, Australia, and New Zealand, UHT milk consumption is much lower, around 10% (Khayrullin & Rebezov, 2023; Deeth H., 2010).

Milk is composed of several key components:

1. Water: Approximately 87% of milk is water, which acts as a solvent and transporter for other nutrients.

2. Fat: Milk fat makes up about 3-4% of the total composition, serving as a source of energy and carrying fat-soluble vitamins.

3. Proteins: Proteins such as casein and whey protein account for about 3.3% of milk, playing a crucial role in growth and development.

4. Lactose: The primary carbohydrate in milk is lactose. It totals about 4.8% of its composition, providing energy and aiding in calcium absorption.

5. Minerals: Milk contains essential minerals like calcium, phosphorus, potassium, and magnesium, which contribute to bone health and other physiological functions.

6. Vitamins: It includes vitamins A, D, B2, B12, and others, which are vital for various bodily functions.

7. Enzymes: These proteins accelerate chemical reactions in the body. (dairyprocessinghandbook.tetrapak.com, accessed July 2024; Roy et al., 2020; Spreer et al., 1998; FAO, 2013).

Changes of milk after UHT process and storage

UHT milk is standard milk (made either from raw milk or recombined powdered milk) that has undergone heat treatment to inactivate bacteria and prolong its shelf life. While the composition of UHT milk is like regular milk, the heat treatment does introduce a few differences:

1. Protein Structure: The heat treatment can lead to the denaturation of some proteins, altering their structure without affecting their nutritional value.

2. Vitamin Loss: Certain vitamins, such as vitamin B12 and vitamin C, may experience a slight reduction due to the heat treatment.

3. Taste: The heat treatment can impart a mildly cooked flavour, distinguishing it from the taste of regular milk. (Gaucher et al., 2008; Karlsson et al., 2019; Deeth H, 2020; Coolbear et al., 2021).

Different storage temperatures (4°C, 20°C, and 35°C) of UHT milk stored for 20 weeks affected the taste, where the taste marginally deteriorated as the temperature increased. Another notable difference was observed between UHT milk samples stored at 4°C and 40°C for 24 weeks, with the 4°C sample tasting better (Hansen et al., 1980; Karlsson et al., 2019). Also, the browning colour increased while the pH decreased during storage trials at 40°C compared with storage at 4°C and 20°C (Gaucher et al., 2008). Storing milk for six months at 30°C to 37°C reduces milk lysine by 10% (Khayrullin & Rebezov, 2023).

The UHT Process

Considering the health benefits of milk in general, along with the convenience of storing UHT milk without the need for refrigeration, it becomes crucial to examine the process involved in preparing this nutrient. Pasteurized milk typically remains fresh for 2-3 weeks under refrigeration, UHT milk can last between 3-9 months (sometimes a up to a year) at room temperature or ambient temperatures when unopened (Datta J, 2018; Liem et al., 2016; Kcarcmova et al., 2018).

The high-temperature treatment and aseptic packaging of UHT results in a "commercially sterile" product, free from bacteria likely to grow under normal storage conditions. There are two main types of UHT processes for sterilizing milk and dairy products: direct and indirect (Datta et al., 2002; Burton H, 1988; IDF Bulletin B516, 2022).

Both of these processes involve preheating the product after homogenization. In the direct method, however, steam is mixed with the milk either by injection (injecting steam into a stream of flowing milk) or infusion (spraying milk into a chamber of steam). The indirect method involves heating the milk through conduction and convection, using a barrier between the heating medium and the milk. Heat exchangers used in indirect UHT plants can be of either tubular or plate design. The cooling step can be expansion cooling (direct) or tube or plate exchangers (indirect). All steps are followed by aseptic packaging in hermetically sealed containers (IDF Bulletin B516, 2022; Deeth H, 2010; Burton H, 1988, Dairy processing handbook). The main difference between the two methods is the holding time at the required temperature for sterilisation and the pre-heating and cooling time periods. Indirect heating possibly has more effect on the final product components as it is held at the higher temperature for a longer period (Figure 1).

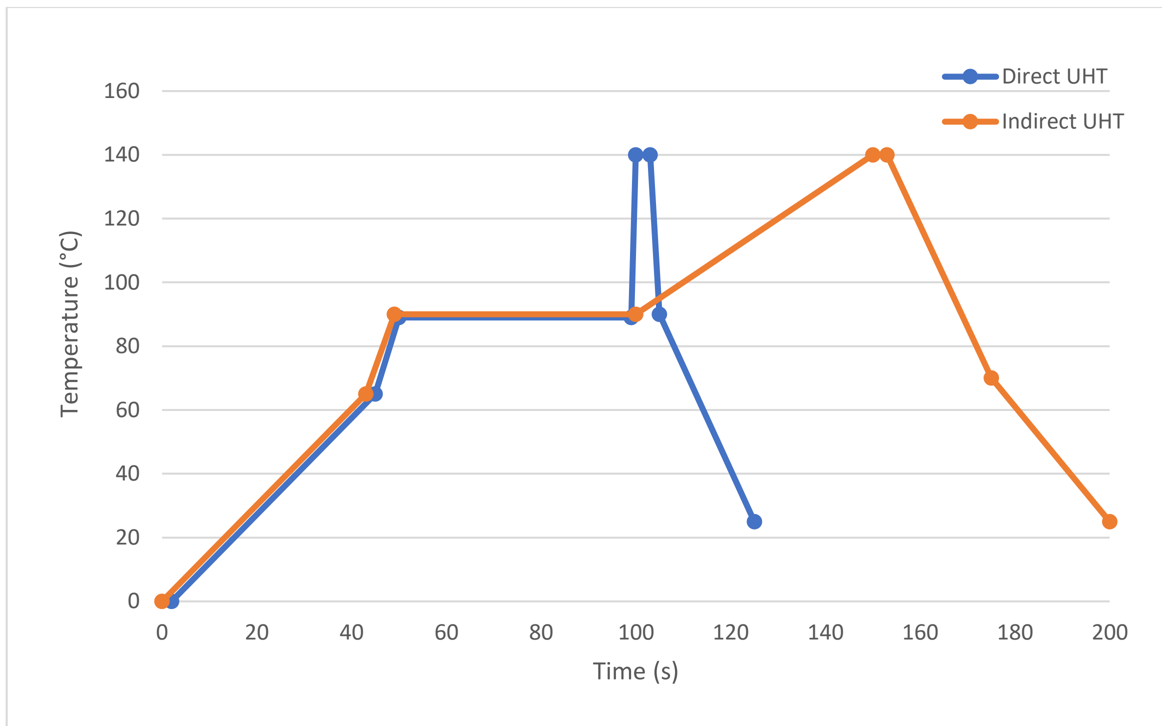

Figure 1. Temperature/time profile of direct UHT process with a preheat hold time and rapid cooling time and indirect process with a longer preheat and cooling time (adapted from IDF B516,2022 with permission).

The model of commercial sterility has conventionally relied on thermal processing techniques to ensure the sterility of canned goods (Rigaux et al., 2014). The established criteria for ascertaining the safety of thermally processed commercially sterile foods is based on the 'Botulinum Cook' method, which requires at least a 12-log reduction in *Clostridium botulinum* spores e.g. by treatment for 3 min at 121°C [or 12 Decimal reduction time (D-value)]. This standard has remained unchanged for nearly a century (Anderson et al., 2011; den Betson et al., 2018) and is based on the theory that the z-value (the change of temperature required to results in a 10-fold change of the D value i.e. change of one unit log D) remains consistent at UHT temperatures, as it does at the 'Botulinum cook' temperature of 121°C.

More recently, the newer B (bactericidal) and C (chemical – thiamine reduction) values have been introduced, which focus on the log reduction of thermophilic spores at higher temperatures (IDF B516, 2022; Deeth, 2010). Since the B and C values are more applicable to higher temperatures of 135°C to 145°C, such as those used in UHT processing, compared with the Botulinum cook temperature of 121°C, and target more heat-resistant thermophilic spores, the B and C value are preferred for UHT processing, (Fig. 2) (IDF B516 2022; Deeth, H., 2010).

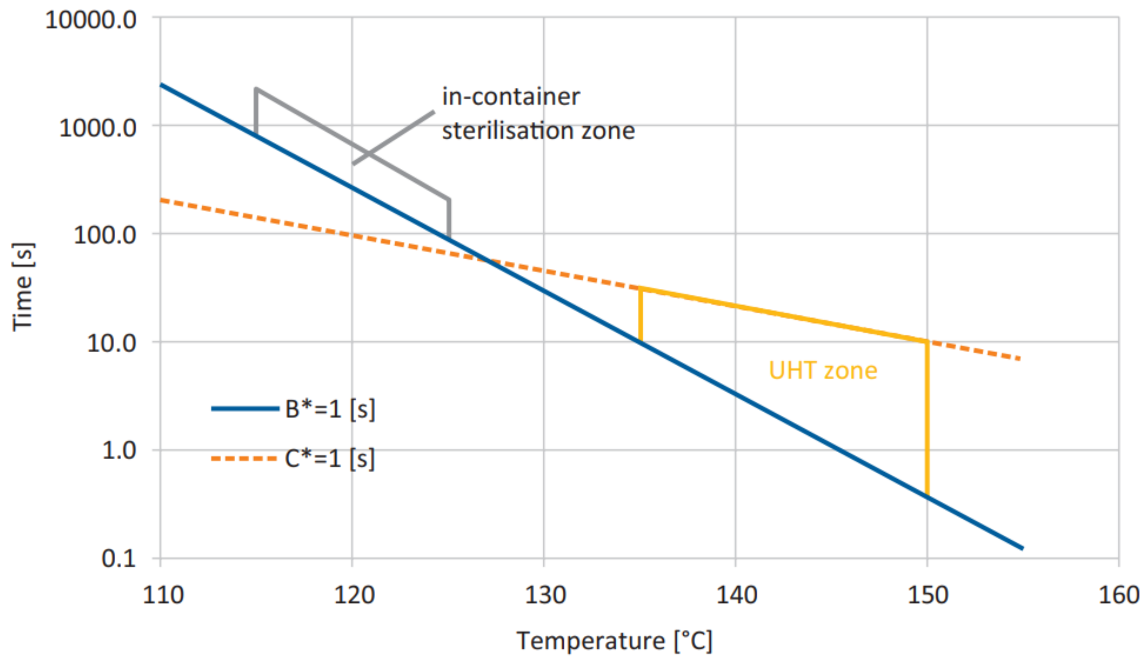

Figure 2. Time/temperature profile showing B and C values at the higher UHT temperatures (IDF B516, 2022, with permission).

To guarantee commercial sterility, dairy producers adhere to protocols outlined by the Hazard Analysis and Critical Control Point (HACCP) system and conduct final product testing (Anderson et al., 2011). Ensuring the quality of these products is crucial for the dairy industry to maintain food safety, prevent product spoilage, and avoid monetary losses and reputation decline (Burgess et al., 2010; Poghossian et al., 2019; Kakgianani et al., 2016; Scott et al., 2007).

Commercial Sterility

The UHT process will usually render the product commercially sterile. The accepted description of commercial sterility is "Commercial sterility of thermally processed food is achieved by:

(1) the application of heat which renders the food free of –

> (a) Microorganisms capable of reproducing in the food under normal nonrefrigerated conditions of storage and distribution; and
>
> (b) Viable microorganisms (including spores) of public health significance;

or

(2) the control of water activity and the application of heat, which renders the food free of microorganisms capable of reproducing in the food under normal nonrefrigerated conditions of storage and distribution." (Food and Drug Administration, 2023; World Health Organisation, 1993; Latimer, G., 2023; Anderson et al., 2011).

Pre-incubation timing

Commercial sterility testing methods of final products are traditionally very slow, taking up to 5-15 days for some culture-based methods. Depending on the reference or method to be applied, the time and temperature of the culture incubation may differ, and the regulations of each country may be different. In New Zealand, MPI (Ministry of Primary Industries) states 'Viable aerobic and anaerobic cells shall be not detected in samples tested following a suitable pre-incubation for the test used, such as 55°C for 7 days or 30°C for 15 days when using a culture method' (Ministry of Primary Industries.govt.nz, 2022).

The Association of Official Analytical Collaboration (AOAC) recommends that containers of low-acid canned foods (to which the UHT belongs) be incubated for a duration of 10 days at temperatures ranging from 21°C to 35°C prior to initiating the culture-based microbiological testing. This pre-incubation is aimed at observation of any potential spoilage or contamination such as detection of abnormal odour, appearance, pH levels, bacterial counts upon microscopic examination, or microbial growth in media from any can, that may not have been evident without the pre-incubation. If such changes are observed, further testing for thermophilic organisms, such as *Geobacillus* species, is advised. These bacteria are known to cause flat sour spoilage without gas production and are of particular concern in the canning industry (Latimer G., 2023; AOAC (www.aoac.org)).

Conversely, the Codex Alimentarius Commission specifies a more narrowly defined temperature range for the incubation test, recommending a period of 10 days at 35°C ± 3.0°C. The Codex's guidelines are internationally recognized and are aimed at ensuring food safety and quality across global food trade (Codex Alimentarius, 2024).

The Chinese Guobiao Standards (GB) method for commercial sterility for low acid canned foods states commercial sterility can be reported if no leakage within the samples, no microbial proliferation is observed in sensory test, pH value determination and smear microscopic examination after samples are held for a heat preservation test of 10 days at 36°C ± 1°C. Thermophilic testing at 55°C for 24-72 hours, is performed if any of the aforementioned has occurred (GB 4789.26 (2013) Food Microbiological Examination Commercial Sterilization Examination).

According to the International Dairy Federation (IDF) Bulletin 516/2022 the pre-incubation of 30°C for 7-15 days and 55°C for 4-7 days (the latter for products stored or sold in hot conditions/climates) for culture-based methods is required. The adenosine triphosphate (ATP) bioluminescence method for measuring sterility of UHT milk requires a pre-incubation at 30°C for 3 days and the European Union Regulation 2021 sates a pre-incubation of 15 days at 30°C and 7 days at 55°C (IDF B516, 2022; Kracmarova et al., 2018; European Union Regulation, 2021).

Furthermore, commercial operating methods, such as Adenosine triphosphate (ATP)-based methods have differing pre-incubation periods. The Charm ATP method has a pre-incubation time of 36 to 48 hours at 30 ± 2°C for mesophilic bacteria, however, there is no reference to pre-incubation conditions for thermophilic bacteria (Charm Operators manual, 2018). A 2 day pre-incubation time at 30°C was stated with the Promicol testing system (Promicol Rapid Microbiology.com, 2024), and 3M does not specify the temperature, but recommends 2-3 days of pre-incubation for enrichment prior to screening to allow growth of low-density populations of microorganisms (3M.com, 2021).

Reinheimer and Demkow showed in 1990 that a reduced preincubation period of three days from 14 days was acceptable for sterility testing of UHT milk with the colourimetric tests that use resazurin.

<u>Testing for Commercial Sterility</u>

Traditional commercial sterility test methods rely on the contaminant organism having the ability to grow in the product, at set temperatures and set incubation times and are subject to interpretation (culture-based or "plating" method and sensory observations).

Newer commercial sterility monitoring methods are becoming more popular, due to their technical advancement and increased acceptance. These methods are typically enzyme-linked immunosorbent

assay (ELISA), ATP bioluminescence, Polymerase chain reaction (PCR), Biochemical i.e. metabolites detected from microorganism growth (O_2, CO_2), impedometric detection, and flow cytometry (Gunasekera et al., 2000; Bottari et al., 2015; Nemati et al., 2016; Deip et al., 2019).

Newer technologies have the ability to shorten the testing turnaround time considerably compared with the traditional plating methods and can have an impact on improved ease of use, requiring less labour and resources (Bottari et al., 2015).

Adenosine triphosphate (ATP) Bioluminescence

The principle of these methods relies on measuring the light emission resulting from the presence of ATP in the microorganisms. Initially, any free non-microbial ATP present in the food item (i.e. somatic cells) is removed by adding an enzyme, followed by another reagent to extract the microbial ATP. The extracted ATP released from the bacteria is then measured as an indicator of the presence of microorganisms.

Examples of products for hygiene monitoring of food contact surfaces and manufacturing plant surfaces with ATP bioluminescence are swab kits such as NovaLUM (Charm Sciences, Inc, Lawrence, MA), Hygiena SystemSure Plus and 3M Neogen Clean trace swabs (Murphy et al., 1998, Vogel et al., 2013, Bottari et al., 2015).

An example of final product testing for ATP detection is the Charm Epic ATP bioluminescence testing method which is based on the firefly (*Photinus pyralis*) ATP luminescent reaction, where the chemical energy contained in the ATP molecule drives the oxidative decarboxylation of luciferin, with the resultant production of light as shown in the reaction.

The ATP derived from contaminant micro-organisms in UHT final product is a limiting factor (i.e. the amount or availability of ATP in the bacteria restricts how much the bioluminescent reaction can proceed) in the bioluminescent reaction. Thus, the reaction can only produce as much light as the available ATP allows. If ATP is low, light output will be low even if everything else (luciferase, luciferin, oxygen, etc.) is plentiful. The light emitted is measured using a luminometer and expressed as relative light units (RLU) (Bottari et al., 2015; Charm Operators manual, 2018; Ziyainaa et al., 2020).

However, reliability of the final product testing based on ATP detection (e.g. Microbial Luminescent Screening MLS system (3M, Minnesota, US), Cellscan Innovate system (Celsis, Chicago, USA),

RapiScreen Dairy 1000 (Celsis Inc., USA), Glomax (Promega) and Promilite III (P III; Promicol, Netherlands) is uncertain due to a limited number of published reports, especially with UHT products (Deip et al.,2019; Cunha et al., 2014; Kracmova et al., 2018; Bottari et al., 2015).

The short time to result of ATP quantification methods is advantageous, and it can detect bacterial cells at concentrations as low as 3.0 to 4.0 $\log_{10}$ CFU/mL (Ziyainaa et al., 2020).

Flow cytometry

Flow cytometry measures the properties of particles/bacteria suspended in fluid as they flow through a flow cell. Laser light is directed at the flow of particles and the light is scattered when particles disrupt the laser light beams. The scattering features of the light are then detected and recorded. The addition of fluorescent dyes enables the detection of particles that have reacted with specific dyes, where the fluorescent dyes are excited by light laser and the emitted light is detected (Gunasekara et al., 2003). A gate is a user-defined boundary drawn on a plot of flow-cytometry parameters (e.g., forward scatter (FSC) vs side scatter (SSC) or fluorescence channels) and is used to determine active fluorescent units (AFU) or the number of bacteria present. Gates are defined using controls: unstained (negative) samples that show instrument/background autofluorescence and positive-control samples known to stain clearly positive (e.g., treated or spiked samples). Gates are applied after selecting the particle population by scatter (FSC/SSC) and excluding aggregates/doublets so that AFU are reported for the intended parent population.

Flow cytometry has been used to determine the total bacterial count in dairy matrices for over 20 years (Gunasekera et al., 2000; O'Grady et al., 2020).

Flow cytometry is a fast method, taking around 1 h (including sample preparation). Besides the fast results turnover, it has another advantage: it can identify the vitality (physiological state) of the organisms and detect viable but not culturable (VBNC) organisms that may not be picked up by traditional culture-based methods like plate counts, or ATP methods (Kell et al., 1998; Wilkinson et al., 2018; Zhao et al., 2017). Furthermore, by using multiparameter dyes, flow cytometry can detect different functions of the bacterial cell and perform cell sorting (Leonard et al., 2016).

Examples of flow cytometry equipment used in dairy settings include the Chemunex D-count (bioMerieux, Marcy-l'Étoile, France), BactoScan™ and BacSomatic™ (Foss, Hillerod, Denmark), BactoCount (Bently Instruments, Chaska, MN, USA), and BactoSense (Sigrist, Ennetbürgen, Switzerland; primarily for water testing) (O'Grady et al., 2020). These devices are mainly employed for raw milk analysis rather than for UHT commercial sterility testing.

Molecular methods

Polymerase Chain Reaction (PCR) is a DNA amplification technique involving repeated cycles of denaturation, primer annealing, and DNA replication using DNA polymerase (Zhao et al., 2014). PCR-based methods, particularly quantitative PCR (qPCR), are widely used for detecting, identifying, and quantifying microbial populations, including pathogens and beneficial microbes i.e. bacteria used in fermentation of foods and relate to flavours of foods (Postollec et al., 2011). Unlike conventional PCR, qPCR allows real-time monitoring of DNA amplification using fluorescent probes, enabling accurate quantification of target DNA. PCR, however, cannot differentiate between DNA from viable and dead cells, potentially leading to overestimation of viable cell numbers. To address this, biological dyes such as ethidium monoazide (EMA) and propidium monoazide (PMA) are used to target DNA from viable cells with intact membranes (Kramer et al., 2009; van Frankenhuyzen et al., 2011; Zeng et al., 2016).

16S rRNA sequencing method is used to identify and compare bacteria in a sample by examining the 16S ribosomal RNA gene, which is highly conserved across bacteria. The 16S rRNA gene is part of the small subunit of the bacterial ribosome, essential for protein synthesis. It is comprised of 1542 base pairs long and contains conserved regions (shared across all bacteria) combined with nine variable regions (unique to specific species) that enable the differentiation of bacterial species.

In PCR-based applications, 16S rRNA sequencing is particularly useful for studying bacterial communities in various environments (Kim & Kim, 2023; de Boer et al., 2015). The method involves amplifying the 16S rRNA gene through PCR and sequencing the resulting DNA fragments. The sequence data is then used to identify bacterial species based on their unique genetic signatures. This approach is integral to understanding microbial diversity and identifying specific bacterial populations in complex samples.

Food product matrices i.e. turbidity, may inhibit the PCR process leading to false negative results. Large dilutions were required to eliminate the food matrices effect, along with very high numbers of contaminating bacteria had to be present before the sterility failure was able to be detected with DNA methods (Gandhi et al., 1999; Scheu et al., 1998). One potential drawback of PCR is DNA persistence, even after bacterial death, which can last from days to weeks or DNA destruction (e.g., due to the thermal processing when the product is made), leading to possible false positive results (Nocker et al., 2006). Moreover, nucleic acid methods often require expensive instruments and extensive training.

Immunodetection methods

Enzyme linked immunosorbent assays (ELISA) is an immunoassay involving highly specific antibody-antigen interactions. ELISA methods rely on the recognition of specific antibodies, however, both live and dead cells can elicit an antigenic response (Hunter & Lim, 2010). Therefore, ELISA assays are an alternative for the detection of specific pathogens to traditional culture methods, however the pathogens viability cannot be assessed. ELISA can also be impaired from cross-reactivity of polyclonal antibodies and the cost of monoclonal antibodies (Singh et al., 2013). Moreover, food product matrices may hinder ELISA assays as they are prone to interference from non-target cells, and other factors e.g. pH, salt concentration, and water activity making them not suitable for sterility testing (Hunter & Lim, 2010). Immunodetection can also be applied to other methodologies such as flow cytometry, molecular and biosensor applications.

Biosensors

Biosensors provide an alternative to traditional pathogen detection methods by identifying specific bio-recognition events through detectable signals and can be both based on nucleic acid hybridisation and antibody-antigen or other types of interaction-based detection. These devices typically feature a probe, which can be a biological material (such as nucleic acids or antibodies), a reporter phage, or an immobilised biomimetic probe such as synthetic catalysts, combinatorial ligands, or imprinted polymers. The probe captures the biological molecule of interest, and this interaction is transduced into a signal that is amplified and detected. This approach offers several advantages, including high sensitivity and specificity, minimal sample preparation, cost-effectiveness, miniaturization, portability for real-time in situ monitoring, and reduced overall detection time (Singh et al., 2013; Zhao et al., 2014). It has been noted that improvement to overcome the product matrices interference with detection remains necessary (Zhao et al., 2014, Nnachi et al., 2022).

Colourimetric, electrochemical and biochemical detection of metabolites

Detection has been reported by turbidity, chromogenic colourimetric assays, by-products detected via conductivity, electrical impedance (measurement of a medium's resistance and reactance to an alternating electrical current that reflects ionic and metabolic changes), and by direct respirometric measurements of O2 consumption and CO2 production (Ziyaina et al., 2020). However, the limitation of using impedance and conductivity measurements to detect spoilage, is the requirement of high

levels of bacterial growth to produce changes in the electrical properties of the test media (6.0–7.0 log_{10} CFU/mL) (Ziyainaa, et al., 2020). An example is the Soleris system, an optical assay that measures microbial growth by monitoring pH and other biochemical reactions (CO_2 production) that generates a colour or fluorescence change as microorganisms in the broth grow and produce CO_2. The 'Soleris Next Generation' system by Neogen for total viable count may be used for commercial sterility and has been validated by ISO standards. This system detects growth of the organisms via the acid produced which is detected by a pH indicator dye resulting in a colour change from green to yellow. This change in colour migrates into a soft agar plug at the base of the vial which is read by the optical sensors in the Soleris system. The vials are read in real time by LED light passing through the agar plug to a photo diode detector with the instrument. (Neogen 2024; Mozola et al., 2013).

Timing of commercial sterility testing

Previous studies have shed light on the exponential and stationary growth phases of bacteria in the UHT dairy products, yet the duration of the stationary phase and the timing of the bacterial death phase remain largely uncharted. Understanding these phases is crucial for refining the testing regimes to accurately assess the viability of bacteria post-UHT processing. Research by Vogel et al. (2014) has shown that ATP levels in mesophilic bacteria decline as they enter the death phase. This is an important observation for ATP bioluminescence detection methods, since if the pre-enrichment stage of the assay was long enough for bacteria to reach death phase this would increase the chance of a false negative result. Moreover, the concept of 'Viable But Not Culturable' (VBNC) organisms, as discussed by Kell et al. (1998), presents a challenge in detecting these bacteria in UHT-treated samples. As the definition of commercial sterility is based on whether the organism is capable of growing; it follows that if a non-culture detection method cannot distinguish between the VBNC organisms and growth-capable ones, it will lead to a false positive due to the presence of the VBNC microorganisms. Current testing methods may or may not differentiate between live bacteria and those that are dead but not yet degraded, or those that have become VBNC due to the UHT process. If the testing method is sensitive to these non-cultivable organisms, this may lead to positive results that do not equate to a culture result. It is also vital to ascertain whether bacteria that cease to be cultivable during the UHT process contribute to product spoilage or remain dormant, potentially resuscitating under favourable conditions later on. The pre-incubation conditions of commercial sterility tests should be developed that will facilitate the detection or culturing of these microorganisms, for improving food safety measures and ensuring the commercial sterility of UHT dairy products.

Miscellaneous features of sterility testing conditions that affect the end results

Food matrices e.g. matrices of varying fat, whey, casein and lactose compositions, may affect the performance of rapid testing methods and require the reduction or removal of their effects to perform accurate testing (O'Grady et al., 2020; Ziyainaa et al., 2020). Clearing of the milk proteins and/or lipids by enzymes or detergents and centrifugation may improve analysis for flow cytometry (Gunasekera et al., 2000; Deip et al., 2019).

Variability of ATP concentration per cell across different bacterial species have been reported to cause discordance between the ATP Relative Light Unit (RLU) results and CFU counts. Furthermore, ATP-based quantification may not be straightforward even for the same species, given that ATP concentration varies between individual cells of the same species, and at varying stages in the bacterial life cycle (Bottari et al., 2015; Kracmarova et al., 2018).

Challenges Associated with UHT Process and Products

The typical tolerable sterility failure rate for aseptic-UHT is one defect per 10,000 units of packaged product (Purjol et al., 2013). Although thermophiles are only occasionally implicated in causing sterility failure of UHT products, their tendency for biofilm formation in dairy plants, fouling of plant equipment seals, resistance to Clean-In-Place (CIP) procedures, and the generation of heat-resistant endospores necessitate special attention, investigation and monitoring (Hill et al., 2012; Burgess et al., 2010; Flint et al., 2020).

Raw milk may contain spores, including heat-resistant spores. During the manufacture of milk powder (and later UHT products), the spore numbers of these organisms may be increased. The milk powder manufacturing process contaminates the product with thermophilic spore formers, ranging from 10^2 to 10^6 cfu/g, primarily due to the production process itself rather than the initial spore concentration in the raw milk (Zhao et al., 2014; Flint et al., 2020). If a failure occurs at any point in a UHT process, or if the packaging is not aseptic, there may be a loss of commercial sterility. It is unlikely that mesophilic organisms will survive the UHT process, but they may contaminate the product downstream of the UHT heat treatment. In contrast, the thermophiles and the thermophilic spores, which are more heat-resistant, may survive UHT heat treatment and cause sterility failures (Andre et al., 2017; Hill et al., 2012; Eijlander et al., 2019; Scott et al., 2007).

Plant fouling and biofilms

Fouling is the accumulation of materials such as whey proteins on the UHT processing plant surfaces, which can disrupt flow or cause blockages. Whey proteins, particularly β-lactoglobulin in milk, can denature and clump together at temperatures above 70°C, such as during short-time pasteurization (72–75°C). Milk foulants also contain calcium phosphate particles, which become more prevalent if the plant's outer wall temperatures exceed 110°C (Table 1; Wedal et al., 2020; Bansal and Chen, 2006).

Table 1. Composition of fouling material at different temperature ranges

Fouling composition/Temperature[a]	75–110°C	Above 110°C
Fat	4%–8%	4%–8%
Proteins	50%–70%	15%–20%
Minerals	30%–40%	70%–80%

[a]*Based on the data from Bansal and Chen (2006).*

These materials can detach from surfaces and enter the product, especially in heated and evaporator areas of the plant. When a community of bacteria sticks to surfaces or gets trapped in these fouling aggregates, it is called a biofilm (Flint et al., 2020).

Wedal et al. (2020) highlighted that milk fouling is a reservoir for thermophilic spores even after CIP as none of their experimental CIP treatments could reduce the spore counts below 3 log in the contaminated fouling.

Biofilm formation happens in several steps. Initially, bacteria attach to a surface, a step that is initially reversible. Over time, the attachment becomes permanent and is followed by the formation of microcolonies and thickening of the biofilm due to the production of extracellular polymeric substances (EPS). The microcolonies grow, creating multiple layers of bacteria within the EPS matrix (Flint et al., 2020).

Resistance to Clean-In-Place (CIP)

CIP is usually a five-stage process: pre-rinsing, alkaline wash, rinse, acid wash, and a final rinse with detergents. Microorganisms present in a biofilm are 100−1000 times more resilient to cleaning chemicals than the released planktonic cells. This resistance can be attributed to the protective nature of EPS matrix, which interferes with the penetration of biocides and limits their interaction with bacterial cells (Bansal and Chen, 2006).

The many layers of the spores in the sporulating bacterial biofilms also contribute to the CIP resistance. The exosporium layer contains proteins and glycoproteins that are heat-resistant. Furthermore, the spore coat contains proteins resistant to lytic enzymes and chemicals. The spore outer membrane contains lipids and proteins that acts as a barrier; the cortex contains peptidoglycan that maintains dormancy, is resistant to core dehydration and is heat resistant; the inner membrane contains lipids and proteins that are a barrier to chemicals; the germ cell wall contains lipids and

proteins that are a barrier to chemicals ; and the core contains chromosomes, enzymes, ribosomes, calcium dipicolinic acid, cations and small acid-soluble spore proteins that are resistant to heat, radiation, UV and gamma oxidants, and biocides (Romero-Rodriguez et al., 2023).

Temperature abuse

UHT dairy products can be produced by recombining ingredient powders, where the powders may contain relatively high numbers of heat resistant thermophile endospores, increasing the risk of sterility failure in final products. In addition, the risk of sterility failure in final product can be increased if UHT product is temperature-abused in storage (Hill et al., 2012; Diep et al., 2019; Flint et al., 2020).

UHT dairy products are often exported to countries with poor storage conditions, where refrigeration is scarce and temperatures can exceed 40°C, including during transportation over the equator, where shipping container temperatures may exceed 37°C (Flint et al., 2020).

One report predicts that even a global warming increase of 2°C may threaten the shelf-life of products and cause a collapse in the food chain (Koutsoumanis et al., 2022). This report proposed a risk mitigation strategy for uninsulated transportation and storage at room temperature ranges under three global warming scenarios, showing that temperatures in uninsulated transportation may reach 45°C to 51°C, thereby increasing the probability of *G. stearothermophilus* causing spoilage defects in shelf-stable products.

Thermophiles of Concern in Dairy Products

In the dairy manufacturing context, thermophilic microorganisms are defined as those growing aerobically in milk and milk products at temperatures typically ranging from 40°C to 65°C, with an optimum of 55°C (Scott et al., 2007; Burgess et al., 2010). Despite the high efficiency of UHT processing, the presence of thermophilic bacilli capable of surviving extreme temperatures poses a threat to product safety and quality. The presence of thermophilic aerobic spore formers in UHT milk has been reported with a detection frequency of 40%. Among the thermophilic strains identified, *Bacillus licheniformis* was the most commonly isolated (24%), followed by *Geobacillus stearothermophilus* (20%) (Khalid et al., 2017). However, this finding was based on a small sample size of only 25. A more recent study involving 100 UHT milk samples found *Geobacillus stearothermophilus* in 25% of the samples (Alonso et al., 2021).

There are two groups of thermophilic bacterial spore-formers of importance within the dairy industry.

1. Facultative thermophiles (e.g. *Bacillus subtilis*, *Bacillus licheniformis* and *Bacillus pumilus*) that can grow at broad range of temperatures, from mesophilic to higher (10°C to 60°C).
2. Obligate thermophiles (*Anoxybacillus* spp. and *Geobacillus* spp.) grow at high temperatures and require minimum temperatures of around 45°C for growth (with optimal temperatures between 55°C and 65°C); (Scott, 2005, Scott et al., 2007, Burgess et al., 2014). However, these obligate thermophiles produce endospores that can survive the UHT process and require a temperature of >37°C for germination and growth within the product after a 7-day incubation (Hill et al., 2012; Koutsoumanis et al., 2022; Eijlander et al., 2019).

Anoxybacillus flavithermus is a Gram-positive, facultatively aerobic bacterium that is motile, rod-shaped and capable of forming spores. It has an optimal growth temperature around 60°C at a pH of 6–9. Strains isolated from the milk powder typically exhibit an optimal growth temperature between 50°C and 65°C (Ronimus et al., 2003).

The *Geobacillus* genus contains Gram-positive, rod-shaped, spore-forming bacteria that have an optimum growth temperature of 55°C to 65°C (Burgess, et al., 2017).

These obligate thermophiles are not pathogenic; however, they are still an indicator of the plant hygiene and process practices (Burgess et al., 2014).

Milk powder is commonly recombined with water to make UHT products. Milk powder has been found to contain *Geobacillus stearothermophilus*, *Anoxybacillus flavithermus*, and *Bacillus*

licheniformis. These three bacteria are the main thermophilic spore formers representing 80% of the bacteria isolated in the powder destined for UHT (Andre et al., 2017).

Growth rate of thermophiles

Past studies have shown that the thermophilic bacteria *Geobacillus* and *Anoxybacillus* can multiply exponentially in a relatively short period of time. These bacteria can thrive across a broad spectrum of temperatures and are characterized by their swift proliferation, with a generation time of roughly 15 to 20 minutes (Burgess, in 2010). Specifically, *Geobacillus* has been previously documented to reaching mid-exponential growth phase between 6 and 10 hours incubation at temperatures of 50-55°C (Burgess et al., 2014) and reach the late exponential or early stationary growth phase within 9 to 10 hours (Somerton et al., 2012, Heinrich et al., 2008).

Heinrich et al. (2008) showed with transmission electron microscopy (TEM) that the cell integrity of *Anoxybacillus flavithermus* and *Geobacillus stearothermophilus* was decreased in 24-hour cultures compared with samples from the exponential growth phase (<24 hours). The *Anoxybacillus* 24-hour cells still maintained their shape and cell walls, but the *Geobacillus* 24-hour cells were almost completely decomposed. Overall, the cell wall thickness and cell length decreased in size as observed with TEM from cells in the exponential growth phase (<24 hours) compared with those after 24 hours of incubation. Additionally, viable bacterial cells of *Anoxybacillus flavithermus* could not be detected after ten hours of incubation at 55°C (Heinrich et al., 2008). Kakagianni et al. (2016) observed that the maximum specific growth rate (µmax) of *Geobacillus* in Tryptic Soy Broth (TSB) increased from 0.293 (±0.016) per hour at 37.5°C to 1.449 (±0.0004) per hour at 64°C. Beyond this temperature, there was a noted gradual reduction in the maximum growth rate. However, the optimal maximum specific growth rate for *Geobacillus* of approximately 2.068 µmax per hour was recorded between 55°C and 65°C (Kakagianni et al., 2016).

Even in 1922, the quick growth rates of thermophilic bacteria were noted by Morrison and Tanner, who wrote: “Most thermophiles grew rapidly, and incubation after 24 hours was unnecessary” (Morrison and Tanner, 1922). It is therefore important that the pre-incubation time used in commercial sterility testing methods is optimised to capture this optimal time-span of the thermophiles’ growth and detect the viable bacteria.

Literature Review Conclusion

UHT processing has become a cornerstone in the dairy industry, enabling the consumer access to dairy products with extended shelf lives and enhanced safety and quality profiles. The rigorous thermal process, coupled with aseptic packaging, effectively ensures commercial sterility, allowing these products to be stored for several months without refrigeration. However, the potential for sterility failure remains a concern, particularly due to the resilience of thermophilic microorganisms that can withstand UHT process conditions, and the challenges posed by inconsistent storage temperatures, which may be exacerbated by the climate change.

The dairy industry continues to rely on the Hazard Analysis and Critical Control Point (HACCP) system and stringent testing protocols to mitigate these risks. The evolution of testing methods, from traditional culturing techniques to rapid detection systems like ELISA, PCR, ATP bioluminescence, and flow cytometry, has improved the industry's ability to monitor and ensure the safety of the UHT dairy products. Nonetheless, the variability of food matrices and the false positives due to the detection of non-culturable organisms highlight the need for ongoing research and adaptation of testing methodologies to maintain the highest standards of food safety. Despite advancements in testing methods, there remains a notable gap in the consensus regarding the thermophile testing of UHT products, with some areas lacking any reference to these organisms in this group of dairy products altogether.

As the demand for UHT dairy products grows globally, particularly in regions with limited refrigeration, the industry must remain vigilant in its efforts to prevent contamination and ensure the integrity of its products. This includes not only adherence to established processing and testing protocols, but also a proactive approach to understanding and addressing the unique challenges presented by the emerging threats and changing environmental conditions. Ultimately, the success of the UHT dairy sector will hinge on its ability to deliver safe, high-quality products that meet the needs of consumers worldwide while navigating an increasingly complex and dynamic food safety landscape.

With an ultimate aim to improve the safety and quality of UHT-processed dairy products, this thesis has explored the effect on duration of pre-incubation timings on accuracy of the commercial sterility testing methods and considered the role of innovative testing methods in detecting microbial contaminants, especially thermophilic bacteria that have rapid life cycle.

As the dairy industry continues to expand its reach and cater to a growing global population, understanding the nuances of UHT processing and the associated food safety and quality considerations is more important than ever.

Hypothesis

1 Decreasing the pre-incubation duration in commercial sterility testing methods of the UHT dairy products can enhance the qualities of testing and accuracy of detecting thermophilic bacteria, while also leading to reducing the time to the test results. Thus, this reduction in pre-incubation time has the potential to improve the successful detection of thermophilic microorganisms in UHT processed dairy products.

2 The rapid growth cycle of thermophilic bacteria in UHT dairy products, characterized by relatively swift transitions through the exponential and stationary phases, suggests that these microorganisms may enter the death phase just as quickly. This rapid onset of the death phase, particularly in thermophiles responsible for UHT sterility failures, implies that they might escape detection by commercial sterility testing methods that use extended pre-incubation periods. Therefore, by the time the sterility tests are conducted, these thermophiles might have already died, leading to a false-negative result where the presence of bacteria is not detected even though they were present in the pack prior to testing, and during earlier pre-incubation times.

3 The Charm Epic Adenosine Triphosphate (ATP) Bioluminescence and Attune Nxt Flow cytometry rapid detection methods offer efficient alternatives to traditional plating for commercial sterility testing by significantly reducing pre-incubation time, simplifying result interpretation, and maintaining the accuracy and reliability of conventional methods. These innovative approaches streamline microbial detection, making them ideal for industries seeking to accelerate testing processes without reducing quality, thereby potentially improving response times to microbial contamination within the UHT process.

Chapter 2. Materials and Methods

Experimental Strategy

Four different UHT dairy products were assessed via three commercial sterility testing methods: traditional culture method, an ATP method, and a flow cytometry method.

Bacterial inoculation (spiking) of the UHT samples (five replicates) was performed at a low level (close to 10 or less cfu/mL) to mimic a real life scenario of a low contamination that may have occurred via the UHT process. Eight bacterial strains of the two main obligate thermophilic bacteria implicated in UHT sterility failures were used (i.e. *Geobacillus* spp. and *Anoxybacillus flavithermus*). One un-inoculated sample was included with the same pre-incubation times, acting as a blank control sample for each pre-incubation period.

Samples that were inoculated and the blank sample were pre-incubated at 55°C for different time periods (6, 12, 24, 48 and 72 hours) to assess the ideal pre-incubation time for the thermophilic bacteria (Fig. 3).

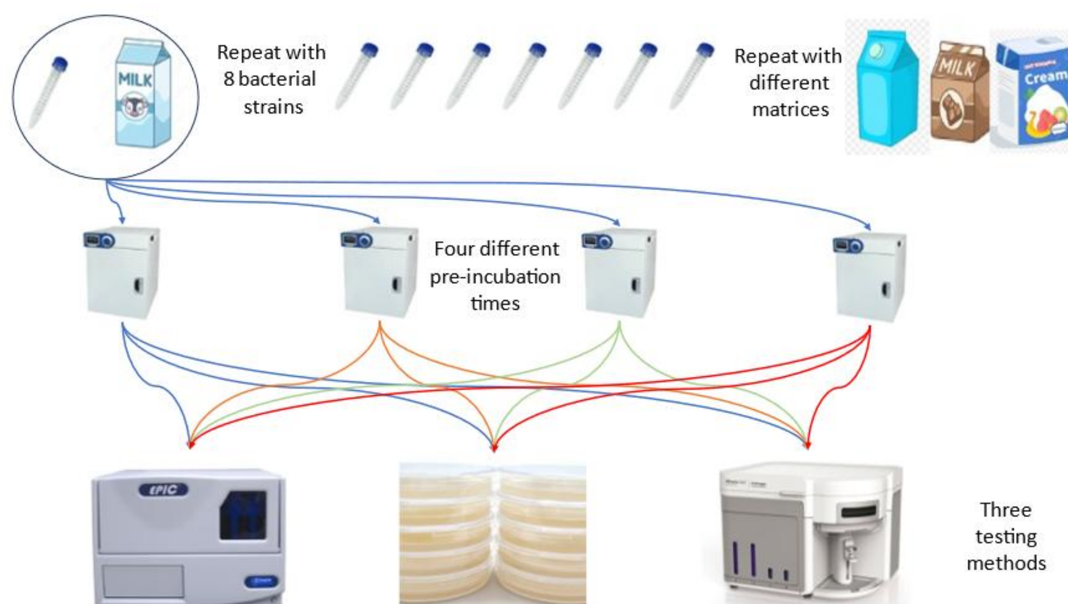

Figure 3. Experimental plan for testing three commercial sterility methods with eight bacterial strains, in four dairy UHT products and differing pre-incubation times at 55°C.

Traditional culture plating methods were used to assess a pass or fail sterility result. The plating method is considered the 'gold standard' method for comparison with the other rapid methods.

Three plate count methods, pour plate, streak and spread plating, were used to complete the plating methods, as the dairy matrices have been known to hinder the observation of colonies on and in the agar plates when observed with the pour plate method, despite it being part of the 'gold standard' method.

Bacterial Isolates

Eight industry isolates were used in this study, four *Geobacillus stearothermophilus* (formerly *Bacillus stearothermophilus*), and four *Anoxybacillus flavithermus* (Table 2). Each bacterial isolate was diluted to approximately 10 CFU/mL in Phosphate Buffered Saline (PBS) (Gibco, Netherlands, 70011-044) and 1 mL of this diluted inoculum was added to 200 mL UHT sample, achieving a target inoculum of approximately 10 CFU per 200 mL product (Table 2). The inocula were verified by plating 1 mL of the final diluted inoculum into approximately 15 mL Milk Plate Count Agar (MPCA) (Oxoid, Basingstoke, Hampshire, England), incubated 2 days at 55°C and colonies were counted. This was performed in duplicate, and the average of the counts recorded.

Five replicate samples were measured at each time point along with a blank sample with no inoculum added. Separate UHT samples were prepared for testing at each time point. The inoculated samples were not re-incubated for further measurement at subsequent time points, as all samples had a comparable inoculum (Fig. 4).

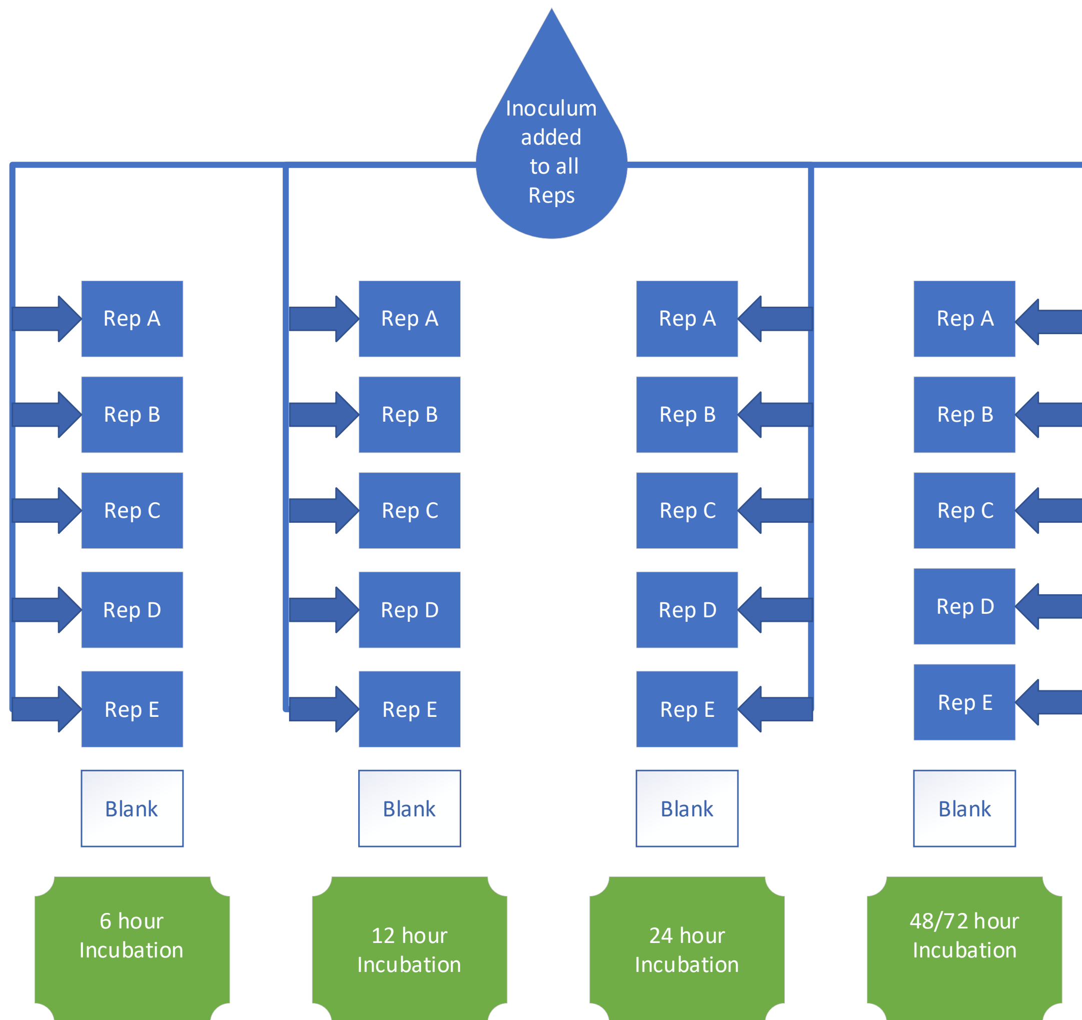

Figure 4. Flow chart indicating the addition of inoculum to the UHT samples prior to incubation.

The samples were incubated at 55°C for 6, 12, 24 and 48 hours (milk, in-house cream, medical beverage); or 12, 24, 48 and 72 hours (whipping cream). After the timed pre-incubation, all samples were tested via plate methods, Charm Epic ATP method, and flow cytometry.

Table 2. Bacterial isolates; verified inoculum size added to samples, and their source.

Strain Identification	Bacterial isolate	Product source	Inoculum added to 200 mL UHT milk sample	Inoculum added to 200 mL UHT cream (in-house) sample	Inoculum added to 200 mL UHT whipping cream sample	Inoculum added to 200 mL UHT medical beverage sample
Geo 1	*Geobacillus stearothermophilus*	WMP	18.5 CFU/mL	5.5 CFU/mL	22.5 CFU/mL	26 CFU/mL
Geo 2	*Geobacillus stearothermophilus*	WMP	8 CFU/mL	5.5 CFU/mL	6 CFU/mL	7 CFU/mL
Geo 3	*Geobacillus stearothermophilus*	WMP	4 CFU/mL	10.5 CFU/mL	1.5 CFU/mL	1.5 CFU/mL
Geo 4	*Geobacillus stearothermophilus*	Unknown	3.5 CFU/mL	3 CFU/mL	6.5 CFU/mL	4.5 CFU/mL
AF 5	*Anoxybacillus flavithermus*	WMP	7 CFU/mL	0.5 CFU/mL	4.5 CFU/mL	0.5 CFU/mL
AF 6	*Anoxybacillus flavithermus*	Unknown	7.5 CFU/mL	1 CFU/mL	1 CFU/mL	2.5 CFU/mL
AF 7	*Anoxybacillus flavithermus*	WMP	7.5 CFU/mL	1 CFU/mL	0.5 CFU/mL	3 CFU/mL
AF 8	*Anoxybacillus flavithermus*	WMP	2 CFU/mL	2 CFU/mL	0.5 CFU/mL	3.5 CFU/mL

Plate Count

Three plating methods were used:

1. Streak - Incubated samples were streaked on to a pre-qualified and solidified Milk Plate Count Agar (MPCA) (Oxoid, Basingstoke, Hampshire, England) plate with a 10 µL loop. (Pre-qualified MPCA plates are incubated with no sample or inoculum at 30°C for 24-48 hours and inspected for no growth before use.)
2. Pour - 1 mL [over three plates (undiluted)], 0.1 mL (10-1 dilution) and 0.01 mL [10-2 dilution (not completed for all test products in this study)] of incubated sample added to approximately 15 mL molten MPCA.
3. Spread - 300 µL of incubated sample was spread over a pre-qualified and solidified MPCA plate (not completed for all test organisms in this study).

For all three methods the plates were incubated at 55°C for 48 hours prior to counting colonies.

Any growth on a 10 µL streak plate was considered a sterility fail for this comparison study. Growth (graded or counted) or no growth was recorded.

With pour plates a count of >10 CFU/mL was regarded a failure of sterility, i.e. more than 10 CFU on a neat plate (NZTM 2.133.01). The 10^{-1} and 10^{-2} dilutions were included to visualise the colonies only. To allow for graphing, the upper value was reported as ≥ 300 CFU/mL, even though the actual counts were often significantly higher and still recorded as a sterility failure.

A count of >3 CFU was regarded as a sterility fail on the spread plate method (i.e. 300 µL is approximately 1/3 of a 1 mL inoculum, hence 3 CFU is approximately 1/3 of 10 CFU).

The streak and spread plate methods were utilised as a consensus check of the pour plating method, as the 1 mL pour plate method acted as the reference method in this study.

Charm Epic ATP bioluminescence

Samples were tested with the Charm Epic system and the method was followed as per manufacturer's instructions (Charm Sciences, Massachusetts, USA). Briefly, a control run was performed and then a sample run, including a reagent blank and a positive control was performed. Pre-incubated sample (50 µL) was added to a sterile microtiter well, before placing in the luminometer analyser. The analyser added 50 µL of EPIC-ASE to each well, and the samples were incubated for 10 minutes. During this step, an ATP-degrading enzyme eliminated non-microbial ATP. EPIC-X (95 µL) was added to the well to be analysed. The EPIC-X reagent extracted microbial ATP for

7 seconds. EPIC-LUM (95 µL) was then added to the well, and after 1 second, the light output was measured and reported in RLU's. As the samples undergo a pre-enrichment during the preincubation period, and 50 µL of the sample is used in the test, the results will be reported as RLU/test.

Interpretation of results was:

Pass = RLU value <150, indicating no detectable microbial ATP.

Suspect = RLU values between 151 and 300 indicated possible microbial contamination by yeast, mould, and/or bacteria and

Fail = RLU value >300, indicating microbial contamination (Charm Operators Manuals 2018, 2011).

Suspect results were not retested in this study.

A pre-treatment trial was conducted with the whipping creams where the sample was diluted after the pre-incubation, ten-fold with Phosphate Buffered Saline (PBS) (Gibco, Netherlands, 70011-044), mixed and then followed the normal the Charm method. Also, the whipping cream underwent a cleaning/chelator step before processing after the pre-incubation. This entailed 0.5 mL of 0.125 mmol/L N-(2-Acetamido)iminodiacetic acid (ADA, Sigma-Aldrich, Merck, Darmstadt, Germany) (cation chelator) added to 1 mL of neat cream sample, inverted 10 times to mix, centrifuged at 10,000 rpm (approximately 6,000 to 8,000 g) for 5 mins, supernatant was aspirated with aspirator (including as much cream fat as possible), then the residual cream inside tube removed with cotton tipped swabs, and the pellet resuspended in 1 mL of PBS. The charm method was then followed.

Attune Nxt Flow Cytometry

Samples were tested by diluting the sample 1:10 µL with staining solution containing Syto24 (Life Technologies Corporation, USA, S7559) and PI (Life Technologies Corporation, USA, P1304MP) dyes in PBS. The dyes were made so the final staining concentration were 1 µmol/L and 2 µmol/L respectively and mixed with PBS before addition of the sample. Sample was incubated with dyes at 37°C for 15 mins.

Light emitted from the Syto24 and PI dyes was captured with a 530/30 and 695/40 bandpass filter, respectively. Two Attune Nxt instruments (Thermo Fisher Scientific, USA, A29001) were used in this study, where both instruments had the 530/30 bandpass filter at the Blue Laser 1 channel (BL1) and one instrument had the 695/40 bandpass filter at the Blue Laser 2 channel (BL2) and the other instrument had the 695/40 bandpass filter at the Blue Laser 3 channel (BL3).

Sample (100µL) was measured with the flow rate set at 200 µL/min, with voltages of 100, 300, 270 and 320 mV for the forward side scatter (FSC), side scatter (SSC), BL1 and BL2/3, respectively, and thresholds of 0.1 x 1000 for all. Live/dead gates were created on BL2/3 (PI, red) versus BL1 (SYTO24, green) density plots. The live population was defined as BL1 high /BL2/3 low (lower-right quadrant), while the dead population was defined as BL2/3 high /BL1 low (upper-left quadrant). The Syto24 dye is membrane-permeant and stains total nucleic acid of the bacterial cell while the PI dye only enters membrane-compromised bacterial cells.

All resulting AFU values were then multiplied by a factor of 100, to account for the 1:10 dilution of the sample with the staining mixture, and the 100 µL sample size measured by the Attune Nxt, to obtain a result of AFU per mL of original sample. Samples with obvious coagulation after the incubation period (usually the 24 and 48 hour samples), Medical Beverages and the in-house creams were further diluted 1:10 with the dye solution before sampling with the Attune Nxt analysers to reduce blockages in the instrument and resulting AFUs were multiplied by an additional ten-fold accordingly.

The whipping cream products underwent a cleaning/chelator step before processing. This involved 0.5 mL of 0.125 mmol/L N-(2-Acetamido)iminodiacetic acid (ADA, Sigma-Aldrich, Merck, Darmstadt, Germany) (cation chelator) added to 1 mL of neat cream sample, inverted 10 times to mix, centrifuged at 10,000 rpm for 5 mins, supernatant was aspirated with aspirator (including as much cream fat as possible), then the residual cream inside tube removed with cotton tipped swabs, and the pellet resuspended in 1 mL of PBS. Then 50 µL of resuspended pellet was added to 450 µL stain solution (1:10 dilution). The results were therefore multiplied by 100 to account for the dilutions.

Statistical Analysis

Statistical analysis was performed using R v4.4.2. (REF R Core Team (2024). R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. (https://www.R-project.org/.)

Chapter 3. Results and Discussion

3.1 UHT Milk

3.1.1. Comparison of pre-incubation times for the detection of thermophiles in UHT milk by the pour plate method

The inoculum levels added to the UHT milk prior to pre-incubation ranged between 2 and 18.5 CFU/unit (Table 2). Thus, inocula were achieved that are representative of low levels of contamination that may occur during manufacture (e.g. of intermittent biofilm shedding or an endospore survived the UHT process and now has favourable conditions to enable growth). Furthermore, this verified that method performance was successfully investigated at low levels of contamination, when there would be the greatest chance of not detecting the contaminant, and thus preventing the over-estimation of method performance.

The number of thermophiles detected by the pour plate method was high (i.e. >300 CFU/mL) at most of the pre-incubation times, indicating successful detection (Fig. 5). This demonstrated the effectiveness of the pour plate method in detecting sterility failure in UHT milk. Thermophiles were detected with the greatest frequency at the 24 and 48 hour time points in the UHT milk matrix (Fig. 5).

Growth by all the *Anoxybacillus* replicates were detected at all pre-incubation times with the pour plate method (Fig. 5). However, only three of the twenty *Geobacillus* replicates (from the *Geobacillus* 2 isolate) were detected at the 6-hour pre-incubation time. Nine of the twenty *Geobacillus* replicates (from three isolates: *Geobacillus* 2,3 & 4) were detected after 12-hours of pre-incubation (Fig. 5). Thirteen *Geobacillus* replicates were detected at 24 hours, and fifteen replicates were detected at 48 hours. At 48 hours, the five replicates not detected all belonged to the *Geobacillus* 4 isolate (Fig. 5).

All the blank samples had not-detected (i.e. pass) results when measured by the pour plate method, as expected. This provided assurance of a low likelihood of obtaining a false positive result due to contamination during this study.

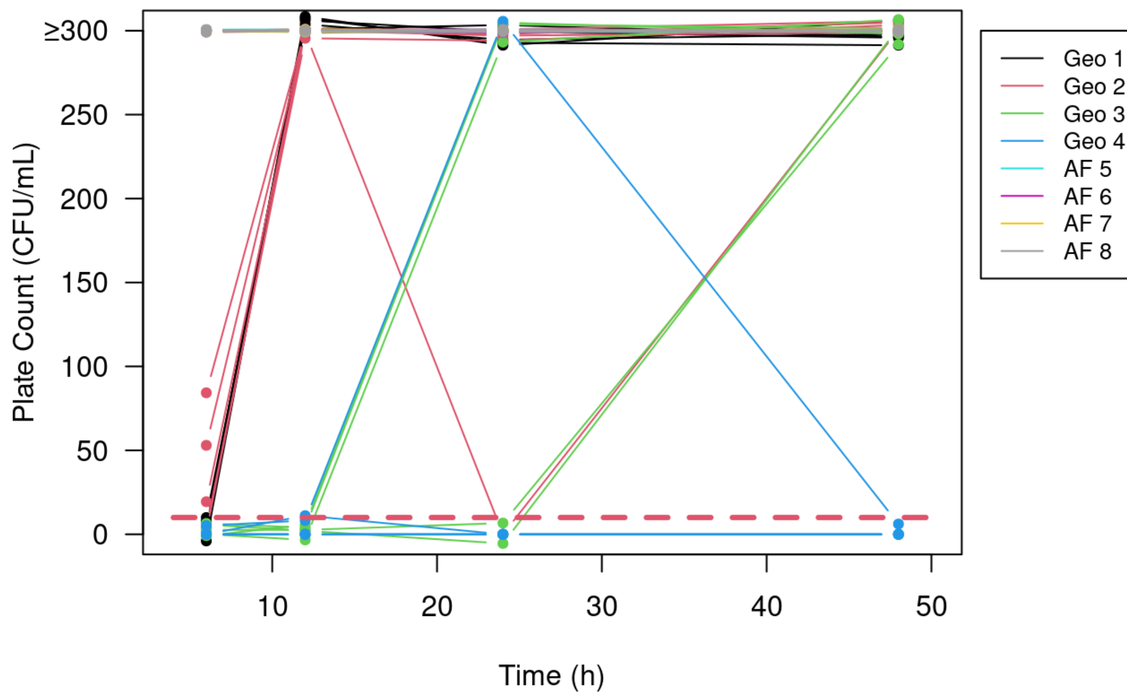

Figure 5. Comparison of the number of thermophiles enumerated (CFU/mL) after different pre-incubation times (6, 12, 24 and 48 hours) in UHT milk, as determined with the pour plate method. Results of individual replicates of all bacterial strains, including four *Geobacillus* sp. and four *Anoxybacillus flavithermus* are shown. The detection threshold was 10 CFU/mL (dotted red line).

3.1.2. Comparison of pre-incubation times for the detection of thermophiles in UHT milk by the Charm ATP method

Growth by all four *Anoxybacillus* isolates was detected at the 6, 12 and 24 hour pre-incubation times (Fig. 6). However, all four *Anoxybacillus* isolates were not detected at the 48 hour time point, despite sterility failure being detected in samples with shorter pre-incubation times, and by the pour plate method at 48 hours.

No sterility failure was detected with the Charm method at the 6 hour time point for the four *Geobacillus* isolates (one was within the 'suspect' threshold range of 2.18-2.48 Log RLU or 150-300 RLU) (Fig. 6). Only ten of the *Geobacillus* replicates were detected at the 12 hour time point (belonging to the *Geobacillus* 1 and 2 isolates). Most (14) of the *Geobacillus* replicates were detected at the 24 hour time point (six were <2.18 Log RLU; belonging to the *Geobacillius* 2, 3 and 4 isolates). Only three *Geobacillus* replicates were detected at the 48 hour time point ie >2.48 Log RLU (Fig. 6).

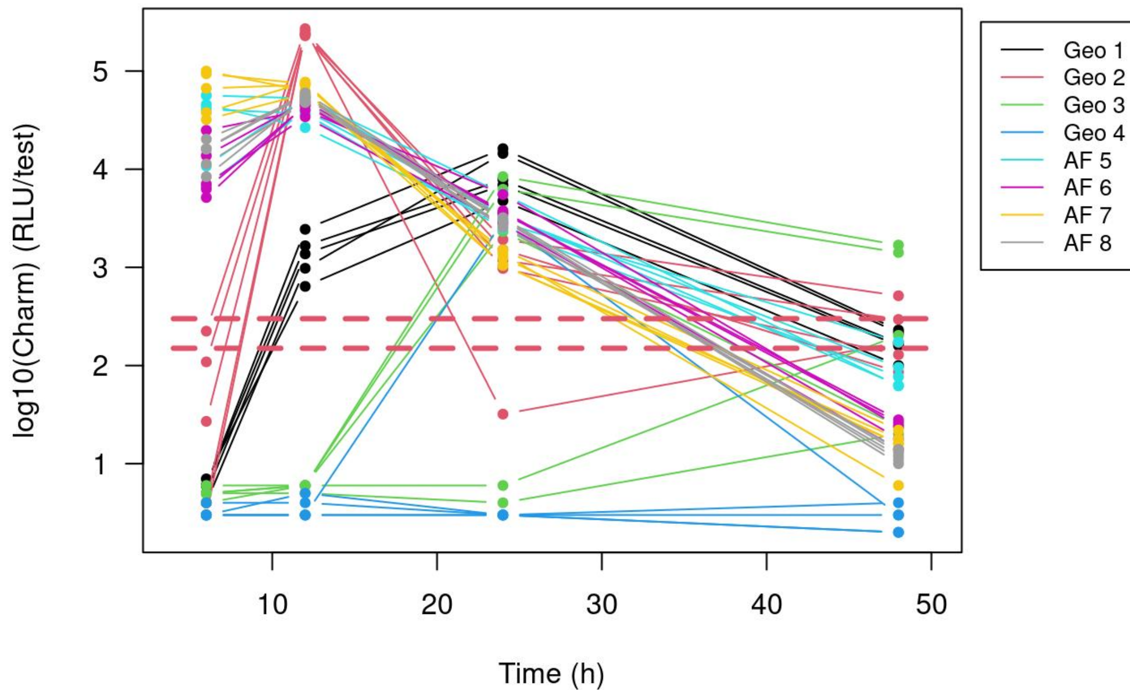

Figure 6. Comparison of pre-incubation time (6, 12, 24 and 48 hours) on the detection of thermophiles in UHT milk by the Charm Epic ATP method (Log RLU/test). Results of individual replicates of all bacterial strains, including four *Geobacillus* sp. and four *Anoxybacillus flavithermus* are shown. The detection threshold was 2.48 Log RLU/test and suspect 2.18 Log RLU/test (dotted red lines).

3.1.3. Comparison of pre-incubation times for the detection of thermophiles in UHT milk by the flow cytometry method

Comparably to the Charm Epic method, the 24 hour pre-incubation duration was optimal for the detection of thermophiles in UHT milk with the Attune Nxt flow cytometry method (Figs. 5-6).

Growth by all twenty *Anoxybacillus* replicates was detected at all time points, when using the Attune Nxt method (Fig. 7).

At the 6 hour pre-incubation time, growth by all *Geobacillus* replicates was not detected, except for two replicates (belonging to the *Geobacillus* 2 isolate) (Fig. 7). Growth by ten of the *Geobacillus* replicates (i.e. all five replicates of the *Geobacillus* 1 and 2 isolates) was detected at the 12 hour time point. thirteen of the *Geobacillus* replicates were detected at the 24 hour time point. Only five *Geobacillus* replicates were not detected at the 48 hour time point (all belonging to the *Geobacillus*

4 isolate) (Fig. 7).

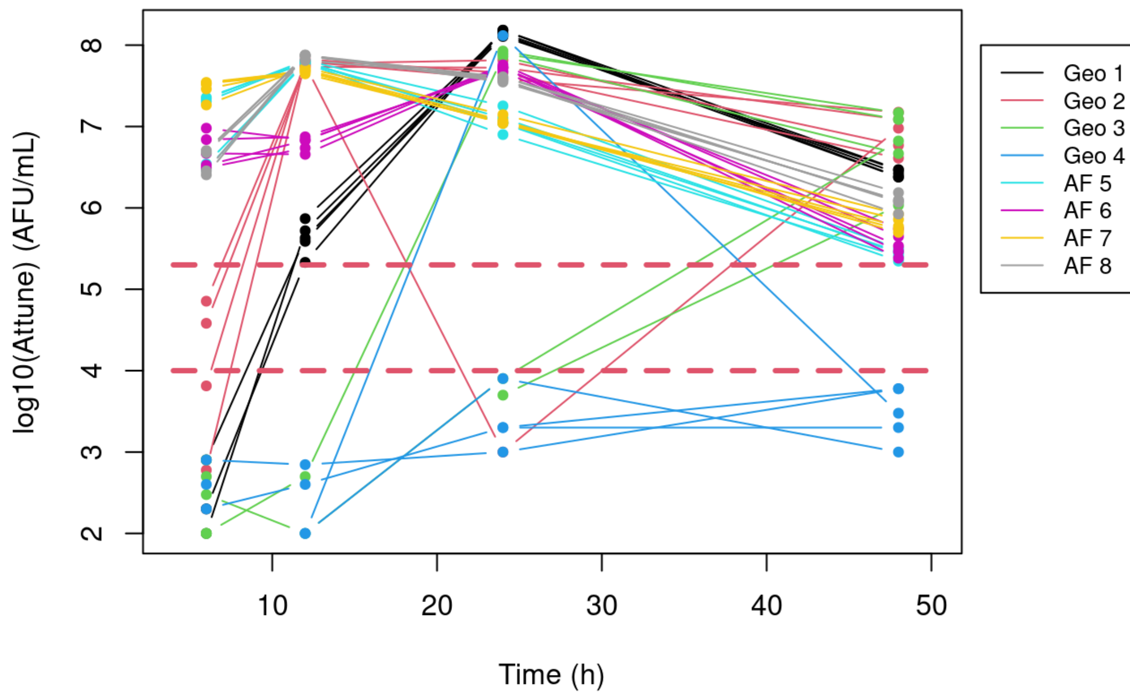

Figure 7. Comparison of pre-incubation time (6, 12, 24 and 48 hours) on the detection of thermophiles in UHT milk by the Attune Nxt flow cytometry method (Log AFU/mL). Results of individual replicates of all bacterial strains including four *Geobacillus* sp. and four *Anoxybacillus flavithermus*) are shown. The detection threshold or cut point was later established at 5.3 Log AFU/mL and the lower threshold of 4.0 Log AFU/mL (dotted red lines).

3.1.4. Comparison of the three methods for the detection of thermophiles in UHT milk

The comparison of the pour plate method with the two alternative methods indicated that all three methods followed similar detection patterns for the bacterial isolates tested (Figs. 5-7), with some notable exceptions. At earlier pre-incubation time points (e.g., 12 and 24 hours), all methods demonstrated comparable results for most strains. However, inconsistencies were observed at the 48-hour pre-incubation time point, particularly with the Charm method.

For the *Geobacillus* strains (1–4), all methods showed similar detection patterns at earlier time points. However, at the 48-hour time point, the Charm method frequently failed to detect bacteria or produced results in the suspect range, even when the other methods successfully detected the same replicates. Some differences included failed detections for certain replicates at the 24-hour time point for *Geobacillus* 2 and *Geobacillus* 3, however these results were consistent across all

methods. *Geobacillus* 4 displayed limited growth, with only one replicate detected at the 24-hour time point, a result that was consistently recorded by all methods.

For the *Anoxybacillus* strains 5–8, detection patterns were largely consistent across all methods at earlier time points. However, at the 48-hour pre-incubation time point, the Charm method consistently failed to detect bacteria due to RLUs falling below the detection threshold, despite the other methods successfully detecting the same replicates (Figs. A1 to A8).

Even when the five replicates of a strain had disagreement with regards to the detected/not detected result, each replicate tended to have a consistent result when comparing among the three methods (Figs. A1-A8). The prevalence can be utilised to indicate the probability of disagreement among replicates of the same strain at the same pre-incubation time, i.e. of when not all five of the replicates matched (Table 3). This discrepancy may be due to a replicate having missed the inoculum added (technician error), or due to an inoculum not containing any cells (as the inoculum was set at low concentrations). The prevalence is calculated using P = positive instances and N = negative instances:

$$(1)\ Prevalence = \frac{P}{P + N}$$

A non-perfect match of the five replicates was evident with the *Geobacillus* 2 at the 24 hour pre-incubation time (Fig. A2), where one of the five replicates was not detected, and the other four replicates were detected. The probability of this occurring in UHT milk is calculated to be 3.125%. The probability of the replicates being matched occurred on 84.375% of occasions, and on 15.625% of occasions they did not match (Table 3).

The probabilities in Table 3 are observed proportions derived from the frequency distribution of five-replicate plate sets. Each five-replicate set is classified by the proportion of plates meeting the positivity threshold (≥10 colonies). The per-set proportions (prevalences) correspond to counts of positive plates in the set of five as follows: prevalence 0.0 = 0/5 positives; 0.2 = 1/5 positives; 0.4 = 2/5 positives; 0.6 = 3/5 positives; 0.8 = 4/5 positives; 1.0 = 5/5 positives. For each prevalence class the observed probability is calculated as

$$(2)\ P\ (class) = \frac{N\ class}{N\ total}$$

Where N class is the number of five-replicate sets in the class and N total is the total number of five-replicate sets (Table 3).

Table 3. Probability of matching replicates in UHT milk by plate method.

Prevalence of Replicate	5 all not detected	4 not detected, 1 detected	3 not detected, 2 detected	2 not detected, 3 detected	1 not detected, 4 detected	5 all detected
Probability	0.15625	0.0625	0	0.0625	0.03125	0.6875

Data includes all pre-incubation times collated. Plate method used as reference for replicate matching in UHT milk.

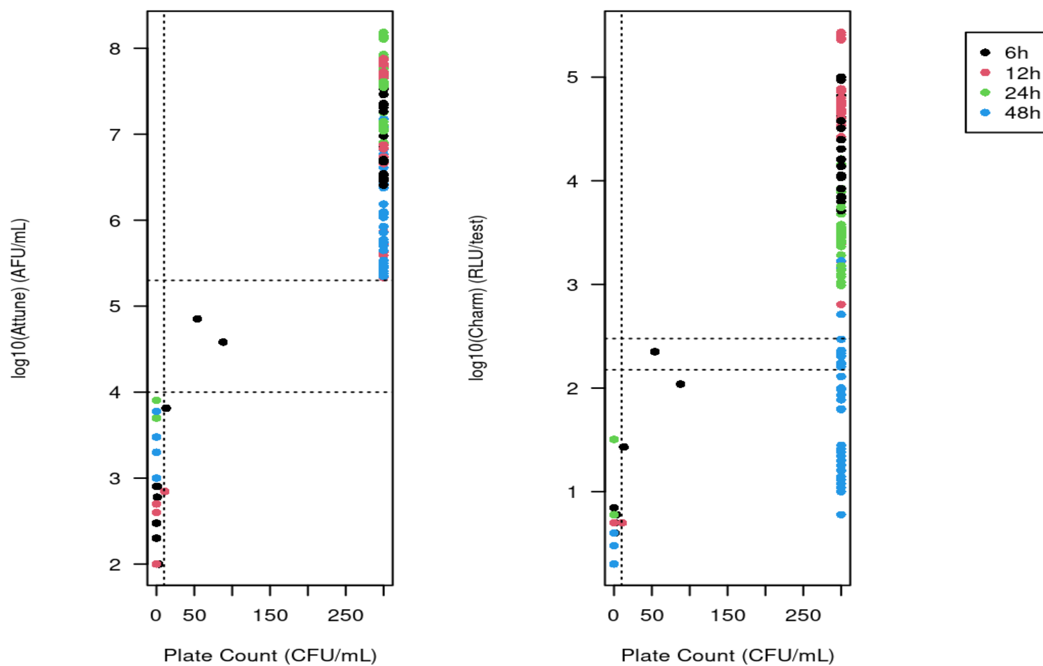

Figure 8. Paired plot analysis of Charm and Attune methods with Plate Count with thresholds highlighted by black dotted Lines.

The paired plot demonstrates when one method yields a positive, the other methods do also (true positives) and similarly when one method is negative, the other methods are negative (true negatives). A similar pattern was found when comparing the Charm and Attune methods with the plate count method, where it was shown that the positives are positive in the upper right part of the paired plot and the negatives are negative in the lower left section of the paired plot (Fig. 8). This

demonstrates consistency between the methods with their ability to detect sterility failure caused by thermophiles in UHT milk. The paired plot also illustrates that the Charm method failing to detect many of the 48 hour samples (blue dots under the threshold line for the Charm, lower right section of plot) compared with the plate method i.e. the false negatives as also seen in previous graphs (Figs. A1-A8).

At the 48 hour pre-incubation time, the Charm method tended to not detect the thermophiles, while the pour plate and Attune methods did detect the thermophiles (Figs. A1-A8). Results from the Charm and Attune methods showed how the number of RLUs (replicate average of 4.8 Log RLU/test at 12 hours compared with a replicate average of 3.5 Log RLU/test at 24 hours) and AFUs (replicate average of 7.7 Log AFU/mL at 24 hours compared with a replicate average of 6.4 Log AFU/mL at 48 hours) appeared to decrease from the 24 to 48 pre-incubation periods. This indicated that the thermophiles tended to die after 24 hours of pre-incubation. The Charm method appeared to have a lower propensity to detect the remaining viable/live bacteria at the 24-48 hour timepoints compared to the pour plate and Attune methods. It is thought that this could be due to the diminishment of ATP after cells lose viability. This finding aligns with other studies where it was found that dairy thermophiles have a rapid growth phase and die off (Burgess et al., 2014). If the bacteria reach the death phase of the growth curve before the 48 hours, it follows the Charm is not able to detect any microbial ATP, as ATP is only present in live bacteria, which caused the false-negative result obtained with the Charm method at 48 hours of pre-incubation. The false negatives from the Charm method at 48 hours are evident with the paired plot (Fig. 8).

The comparison of the three methods highlights that the 12 and 24 hour pre-incubation times were optimal for UHT milk when detecting thermophiles by the Charm and Attune methods, however the 48 hour pre-incubation time was optimal for the pour plate method. The 6 hour pre-incubation time was also agreeable with all three methods, however the plate counts, Charm RLU and Attune AFU counts were mostly higher at the 12 and 24 hour pre-incubation times indicating the growth phase had not peaked at the 6 hour time. Furthermore, results indicated that for some *Geobacillus* strains 6 hours wasn't long enough for growth and detection of viable cells, such that even though there was agreement among the three methods, there was a greater risk of a false negative result being obtained (by all three methods). This is because there was a higher frequency of not detected results at 6 hours (45% not detected) (by all three methods), compared to the 12 and 24 hour pre-incubation times (24% and 18% not detected respectively). For the pour plate method, the frequency of not detected results decreased with increasing pre-incubation time (Fig. 5), thus demonstrating how a longer i.e. 48 hour, pre-incubation time was optimal for the pour plate method.

The pre-incubation time of the standard plate culturing method for assessing commercial sterility when detecting thermophiles at 55°C can vary from 1-7 days, depending on the regulatory body with which the laboratory is associated (7 days, MPI; 1-3 days, GB standards; 4-7 days, IDF) (MPI.govt.nz, 2022; GB 4789.26, 2013; IDF B516, 2022). The optimal 48 hour pre-incubation time found with the pour plate in this study for UHT milk, aligns mostly with the time frame of the GB standards.

3.1.5. Establishing the threshold values for the Attune with UHT milk.

Utilising the plate count method as the reference test to establish threshold values for the Attune method, a selection of possible threshold values was observed to find the best fit for accuracy, sensitivity and specificity across each bacterial strain and each timepoints. The mean data of all bacteria at each pre-incubation time is expressed as True Positive (TP) (when the plate count method is >10 CFU/mL and the Attune method is ≥ threshold value), True Negative (TN) (when the plate count method is ≤ 10 CFU/mL and the Attune method is < threshold value), False Positive (FP) (when the plate count method is ≤ 10 CFU/mL and the Attune method is ≥ threshold value) and False Negative (FN) (when the plate count method is >10 CFU/mL and the Attune method is < threshold value) (Tables E1-4).

The Sensitivity (true positive rate - is the probability of a positive test result, truly being positive), Specificity (true negative rate - is the probability of a negative test result, truly being negative), Accuracy (proportion of correct predictions - both true positives and true negatives), Positive Predictive Value (PPV) (probability that a positive test is positive) and Negative Predictive Value (NPV) (probability that a negative test is negative) are calculated by the following equations (Altman, D. and Bland, J. (1994).):

$$(3)\ Sensitivity = \frac{True\ Positive}{True\ Positive + False\ Negative}$$

$$(4)\ Specificity = \frac{True\ Negative}{True\ Negative + False\ Positive}$$

$$(5)\ Accuracy = \frac{True\ Positive + True\ Negative}{Positive + Negative}$$

$$(6)\ Positive\ Predictive\ Value = \frac{True\ Positive}{Predicted\ Postive\ (TP + FP)}$$

$$(7)\ Negative\ Predictive\ Value = \frac{True\ Negative}{Predicted\ Negative\ (TN + FN)}$$

The PPV and NPV also measures the performance of a test and an ideal value, with a perfect PPV test i.e. no false positives, is 1.0 (100%) whilst the worst possible value is 0 and with a perfect NPV test, one which returns no false negatives, the value of the NPV is 1.0 (100%). Ideally, a perfect comparative test has accuracy, sensitivity and specificity all equalling 1.0, as well as the PPV and NPV. However, this happens very rarely, and this study found when the sensitivity was good, the specificity was poor at the lower end of the threshold range i.e. an increased likelihood of false positives for UHT milk. Inversely at the higher end of the threshold range the specificity was good, and the sensitivity was poor i.e. more prone to have false negative results (Table E1-E4, Fig. B1). For reputational purposes of the dairy company, the result of a product with false negative being released to consumers (i.e. the product was actually contaminated, with the test result negative) would not be ideal. Therefore, NPV of 1.0 is more beneficial than a false positive (PPV=1.0). A false positive would have inconvenience of having to investigate the positive result, however product would not be released to consumers and would not result in reputational losses.

The ideal detection thresholds for the Attune method varied across time points and bacterial isolates to achieve an accuracy of 1.0.

- At 6 hours, thresholds ranged from 3.0–3.8 Log AFU/mL, limited by *Geobacillus* 4 (>3.0 Log AFU/mL) and *Geobacillus* 2 (<3.8 Log AFU/mL), while the *Anoxybacillus* isolates required higher thresholds (<6.5 Log AFU/mL). The negative predictive value (NPV) was 1.0 at thresholds <3.8 Log AFU/mL, with higher thresholds increasing false negatives.
- At 12 hours, the threshold range narrowed to 2.7–2.8 Log AFU/mL, with *Geobacillus* 4 requiring a precise range for accuracy, while other isolates maintained accuracy at thresholds <5.3 Log AFU/mL; NPV remained 1.0 at thresholds <2.8 Log AFU/mL.
- By 24 hours, the threshold range expanded to 4.0–6.9 Log AFU/mL, with *Geobacillus* 4 requiring >4.0 Log AFU/mL and *Anoxybacillus* 5 needing <6.9 Log AFU/mL, while all isolates retained NPV of 1.0 within 3.0–6.9 Log AFU/mL.
- At 48 hours, thresholds ranged from 3.8–5.3 Log AFU/mL, with *Geobacillus* 4 requiring >3.8 Log AFU/mL and *Anoxybacillus* 5 and 6 needing <5.3 Log AFU/mL, maintaining NPV of 1.0 within this range but increasing false negatives outside it (Fig. B1, Tables E1-E4).

Table 4. Optimal threshold values for each bacterial strain and time point in UHT milk (Attune method).

Bacteria/Time	**6h**	**12h**	**24h**	**48h**
Geo 1	<=3.0	<=5.3	all	<=6.3
Geo 2	>=2.8 and <=3.8	<=7.7	>=3.0 and <=7.5	<=6.6
Geo 3	>=3.0	>=2.7	>=4.0 and <=7.8	<=6.0
Geo 4	>=3.0	<=2.8 and >=2.7	>=4.0	>=3.8
AF 5	<=6.4	<=7.7	<=6.9	<=5.3
AF 6	<=6.4	<=6.6	<=7.6	<=5.3
AF 7	<=7.2	<=7.6	<=7.0	<=5.6
AF 8	<=6.4	<=7.8	<=7.5	<=5.9

Threshold (log_{10}) values determined to maximize accuracy. (Accuracy of 1 was not always attainable.)

The best threshold for accuracy for the Attune method at all time points was 2.7 Log AFU/mL to approximately 5.3 Log AFU/mL. This indicates that the Attune method losses accuracy above 5.3 Log AFU/mL (the sensitivity is lost) and below 2.7 Log AFU/mL (the specificity is lost), at all-time points for UHT milk (Table 4).

The Receiver Operator Characteristics (ROC) curve is another indication of the performance of a test and the ideal discriminator, where the best performance is indicated, is the when the curve is closest to the top left corner of the graph. The best performance of the mean of all the bacterial strains is at 24 and 48 hour time points (Fig. C1 (c) and (d)).

Therefore, the overall optimal threshold chosen in this study was 4.0 Log AFU/mL to 5.3 Log AFU/mL, as this accommodated the best of all performance characteristics at all four time points with the Attune method for UHT milk i.e. the best fit for the accuracy, sensitivity, specificity, ROC and NPV (Figs. B1, C1).

As the milk coagulated at the 24 hour and 48 hour marks, an extra dilution factor was applied (Fig. 10). The threshold range values could also be interpreted by the gate, equating to 10 AFU/gate to 200 AFU/gate over the 24 and 48 hour time points (without the dilution factors). However, the dilution factor caused the threshold range per mL to differ from the per gate range across all time points, as the 6 and 12 hour dilution factor varied from the 24 and 48 hour dilution factor. Thus, in this study an overall per gate range of 10 to 63 AFU/gate (1.0-1.8 Log AFU/mL threshold) could be selected to accommodate all four time points (Table 5).

Table 5. Optimal threshold values for each bacterial strain and time point in UHT milk (Attune method).

Bacteria/Time	6h	12h	24h	48h
Geo 1	>=1.0	<=3.3	<=5.1	<=3.3
Geo 2	>=0.8 and <=1.8	<=5.7	<=4.5	<=3.6
Geo 3	>=1.0	>=0.7	>=1.0 and <=4.8	<=3.0
Geo 4	>=1.0	>=0.7 and <=0.8	>=1.0 and <=5.1	>=0.8
AF 5	<=4.4	<=5.7	<=3.9	<=2.3
AF 6	<=4.4	<=4.6	<=4.6	<=2.3
AF 7	<=5.2	<=5.6	<=4.0	<=2.6
AF 8	<=4.4	<=5.8	<=4.5	<=2.9

Threshold ($\log_{10}$) values determined to maximize accuracy. (Accuracy of 1 was not always attainable.). Data shown without dilution calculations (per gate).

The average number of background particles in an uninoculated sample was 5 AFU/gate (excluding one outlier due to technician error in not rinsing lines before sampling) for the 6 and 12 hour time points, and 2.4 AFU/gate for the 24 and 48 hour time points. This background noise from the product needs to be considered when establishing the detection threshold. This equates to 2.7 Log AFU/mL for the 6 and 12-hour time points and 3.4 Log AFU/mL for the 24 and 48-hour time points. These values are below the chosen optimal threshold established with accuracy, sensitivity, specificity and NPV calculations at the respective time points, providing confidence in the set thresholds outlined above, thus supporting the detection thresholds determined in this study of 4.0 Log AFU/mL to 5.3 Log AFU/mL (Tables E1-E4 and 4-5, Figs. B1, C1).

3.1.6. Optimising the threshold values for the Charm method with UHT milk

The Charm has manufacturer set thresholds of 150 RLU/test to 300 RLU/test. The same criteria as above for the Attune was used the same way to establish possible thresholds for the Charm with UHT milk. This was done to compare the manufacturer set thresholds, with thresholds determined with data in this study.

The alternate detection thresholds with the Charm method for UHT milk in terms of Log RLU/test varied across time points.

- At 6 hours, the threshold range was 0.9–1.4 Log RLU/test.
- At 12 hours, the range expanded to 0.8–2.8 Log RLU/test, however accuracy of 1.0 was never obtained with *Geobacillus* 4 acting as the limiting factor.
- At 24 hours, the threshold was narrow at 1.6–2.8 Log RLU/test.

- By 48 hours, only a single threshold of 0.7 Log RLU/test was applicable, constrained by both *Geobacillus* 4 and *Anoxybacillus* 7 (Fig. B2).

The detection threshold found in this study for the Charm was 40-794 RLU/test (1.6 to 2.9 Log RLU/test). Thus, the manufacturers threshold range is within the determined range of this study for UHT milk (Table 6, Fig. 19). If the alternative threshold determined in this study was applied, there would be a greater likelihood of obtaining detect results, and thus many of false negative results obtained at the 48 hour pre-incubation time in UHT milk with the Charm would actully be recorded as true positives (Figs. A1-A8, Table 6).

Table 6. Optimal threshold values for each bacterial strain and time point in UHT milk (Charm method).

Bacteria/Time	6h	12h	24h	48h
Geo 1	>=0.9	<=2.8	<=3.6	<=1.9
Geo 2	>0.7 and <=1.4	<=5.3	>1.6 and <=2.9	<=1.9
Geo 3	>0.8	>0.8	>0.8 and <=3.3	<=1.3
Geo 4	>0.7	NA (max 0.8)	>0.5 and <=3.4	>0.7
AF 5	<=3.8	<=4.4	<=3.3	<=1.7
AF 6	<=3.7	<=4.5	<=3.4	<=1.1
AF 7	<=4.5	<=4.7	<=3.0	<=0.7
AF 8	<=3.9	<=4.6	<=3.4	<=0.9

Threshold (log_{10}) values determined to maximize accuracy. (Accuracy of 1 was not always attainable.)

3.1.7. Difficulties with pour plating method when measuring UHT milk

Colonies in the pour plates were difficult to interpret as the plates appeared grainy (neat plates appeared quite milky/grainy looking), and the colonies looked like product matrix dispersed throughout the agar (Fig. 9). Also, it was particularly difficult to decipher colonies from product matrix in samples that had obvious coagulation after the incubation as the coagulated particles of product mimicked typical colony morphologies (Figs. 11-12). The colonies were easier to see on the 10^{-1} dilution and 10^{-2} dilution plates, especially with the thicker products and supported the theory that the grainy particles on neat plates were colonies (Figs. 11-12). Gram staining confirmed this graininess was actual colonies.

The total plate count that is used for commercial sterility plate method is open to interpretation from the technician, requiring skill and good laboratory practice. It is a semi-qualitative test performed on what is usually a quantitative test method i.e. only determining if there are colonies present on the plate (and therefore in the product) and if the number of colonies that are present

are over the threshold of 10 cfu/mL. However, the sensitivity of the pour plate method is high as the counts can be determined as low as 1 cfu/mL, although the specificity may be hindered as product debris and coagulated particles may be counted accidentally (which may be countered with Gram staining). The dilution of the product, to be able to visualise the colonies easier, as was performed in this study, may introduce laboratory contamination from the diluent and the extra handling of the sample. This may then cause false positive result and as the Charm and Attune methods do not require more incubation after the pre-incubation period, where the plating method does, they are not susceptible to this possible contamination.

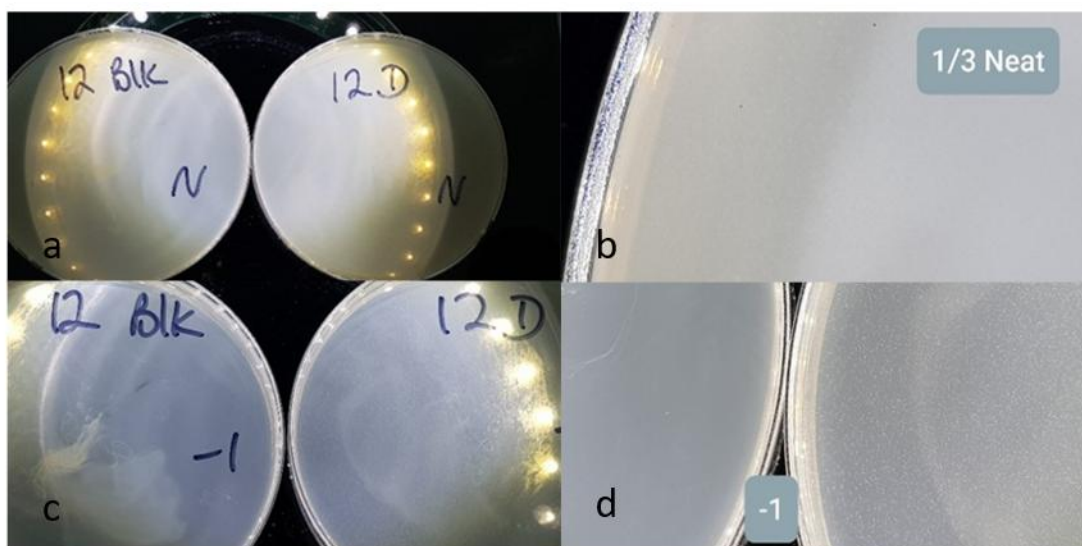

Figure 9. Pour plates of UHT milk samples. (a) neat plates, (b) close up of a neat plate, (c) 10-1 diluted plates and (d) close up of 10-1 diluted plates, left: blank and right: sample replicate.

Figure 10. Inoculated samples after 48 hours pre-incubation (a and b) and after 24 hours pre-incubation (c) showing thickening and coagulation of the product.

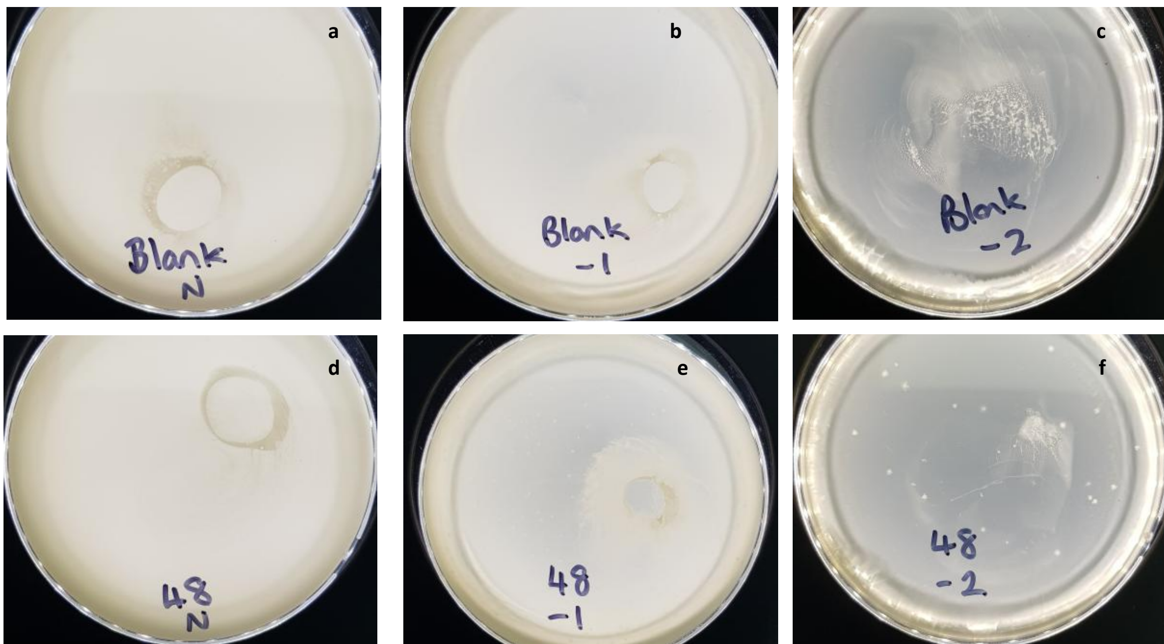

Figure 11. Pour plates of uninoculated samples and inoculated samples highlighting the difficulties of seeing the colonies with the product masking the view (a b c showing serial dilution of an uninoculated sample and d e f showing serial dilution of a replicate inoculated sample).

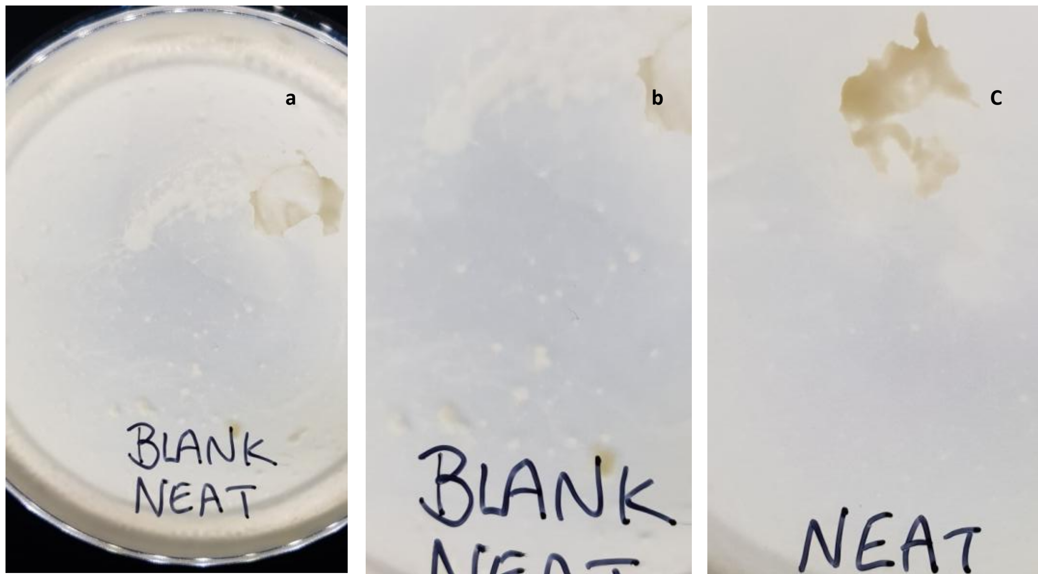

Figure 12. (a and b) Uninoculated blank sample showing product particles/plaques on neat pour plates. (c) Sample replicate showing some particular matter.

3.1.8. Growth phase populations determined by the Charm and Attune methods

The correlation among all three methodologies was generally consistent for all pre-incubation timepoint measurements, except for the 48-hour pre-incubation time, where it was found that the Charm method tended to have false negative results. High levels of growth were observed all points with the plate method. However, the peak was detected with the plate method at 48 hours, and at 12 hours with the Charm method and 24 hour time point with the Attune. The rapid growth of the thermophiles and transition from live to dead was indicated by the reduction of ATP with the Charm method at the 48 hour timepoint and was also evident and supported by the Attune density plots over the timepoints (Fig. 24).

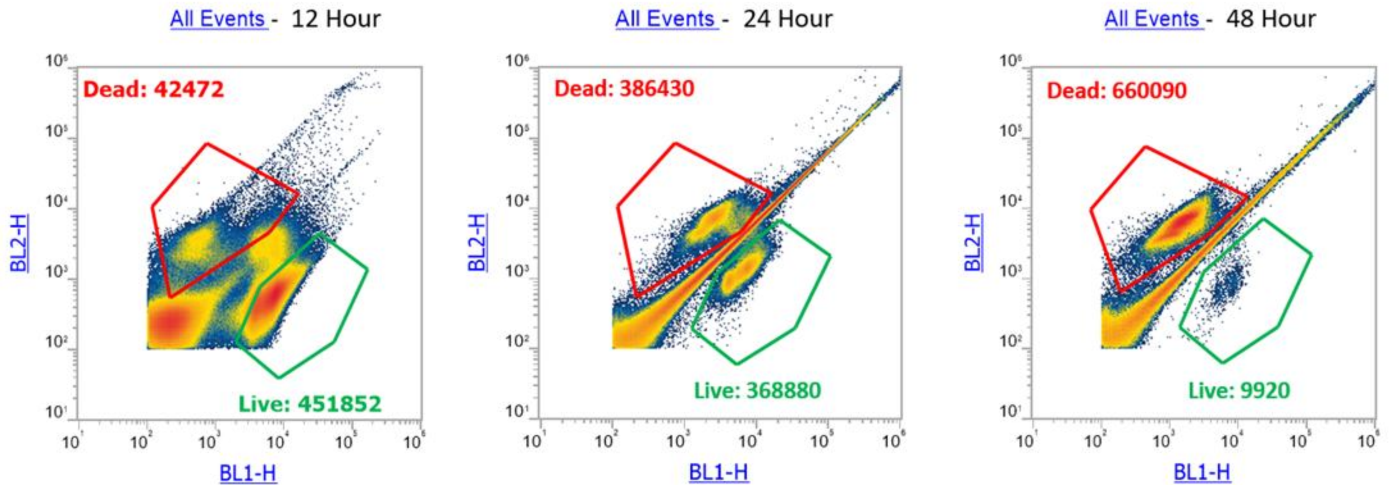

Figure 13. Density scatter plots from the Attune with live (green) and dead (red) thermophile cell gates, with examples from the 12, 24 and 48 hour pre-incubation times.

Samples that were pre-incubated for 48 hours may still have some vitality or dormancy of the organisms persisting and when supplied with nutrient agar and other favourable conditions the organism will grow with culturing methods, as also noted by Kell et al., 1998; Wilkinson et al., 2018; Zhao et al., 2017. This study highlighted this, where the plate counts were high, usually over 300 CFU/mL and stayed high at the later timepoints (Figs. A1-A8). Whereas, these favourable growth conditions were not supplied during the Charm method or Attune method. Kracmarova et al. (2018) also found that the detection of mesophilic microorganisms reduced from the 24 hour time point to the 48 hour time point with an ATP method while the plating method had an increase in counts.

3.1.9. Ease of use, optimal pre-incubation time for detecting thermophiles in UHT milk and ideal automated method

The plate method is more time consuming and labour intensive, where the Charm and Attune are easier to use after initial set up. However, the Attune requires expertise to interpret and align the density population gates, therefore making the Charm the easiest to use of the two automated methods.

The three methods indicated that for UHT milk contaminated with thermophiles, the 12, 24 and 48 hour pre-incubation times are the most agreeable and had the highest frequency of detect results.

The plate method was ideal at all pre-incubation times except the 6 hours, as the growth of the thermophiles was at the early stages of the exponential growth phase and not all the isolates rendered detectable results until the later times and the 48 hour time had the most replicates as

detected than the other times (Fig. 5). This aligns closer to the standard requirement set by governing bodies, where the pre-incubation is between 5 -15 days.

Once the threshold for the Attune method was established, a success criterion of agreement between the methods can be applied to all replicates, including the blank (uninoculated) replicates. The lower threshold was used to determine the percentage of agreement as this reduced the false negatives. When applying typical Good Laboratory Practice (GLP), the success criterion for agreement percentages is typically set at 95% or higher (± 2 standard deviation of a population), i.e. when the Attune method aligns with the plate method 95% or more times. (Table 7). Similarly, the percentage of agreement can also be applied to the Charm method using the manufacturer's pre-determined threshold and the Charm with the alternate thresholds applied (Table 7). The 48 hour pre-incubation time was not ideal with the Charm method, as the decline in population numbers rendered false non-detect results. Applying the possible alternate threshold, slightly improved the Charm method to some extent at 48 hour time point but would not eliminate all the false negatives (Table 6 & 7). The Attune, with the applied optimal threshold, indicated that all pre-incubation times were all ideal (Figs. 17). Among these, the 48 hour pre-incubation time rendered the best performance characteristics with the optimal threshold applied (Table 4 & 7, Fig. 17). Additionally, the 6 and 12 hour pre-incubation times are not ideal as they require staffing resources at multiple periods during the day, necessitating 24 hour working shifts.

Therefore, the 24 hour pre-incubation time is optimal overall with all two automated methods and the 48 hour pre-incubation time with the plate method for detecting thermophiles in UHT milk.

Table 7. Percentage agreement of alternate methods with plate method in UHT milk.

Time (hours)	Charm Epic (% agreement)	Attune NxT (% agreement)	Alternate threshold for Charm Epic (% agreement)
6	95.8	97.9	95.8
12	97.9	97.9	97.9
24	100.0	97.9	100.0
48	50.0	100.0	64.6

3.2 UHT Cream (in-house)

3.2.1. Comparison of pre-incubation times for the detection of thermophiles in UHT in-house cream by the pour plate method

The inoculum levels added to the UHT in-house creams prior to pre-incubation were sufficiently low, ranging between 0.5- 10.5 CFU/unit (Table 2).

As with the UHT milk, the UHT in-house cream also had high numbers of thermophilic bacteria detected by pour plate at most of the pre-incubation times, and similarly with the UHT milk the highest levels of detection were at the 48 hour timepoint (Fig. 14).

Growth by the *Anoxybacillus* isolates were mainly detected at the 6, 12 ,24 and 48 hour pre-incubation times (except for one *Anoxybacillus* 5 replicate at 6 and 12 hours and three replicates at 24 hours). The *Geobacillus* isolates were mainly detected at the 12, 24 and 48 hour pre-incubation times (except for *Geobacillus* 4, where detection was only at 48 hour time, by four of the five replicates)(Fig. 14).

All the blank samples had not-detected (i.e. pass) results when measured by the pour plate method, as expected. This provided assurance of a low likelihood of obtaining a false positive result due to contamination during this study.

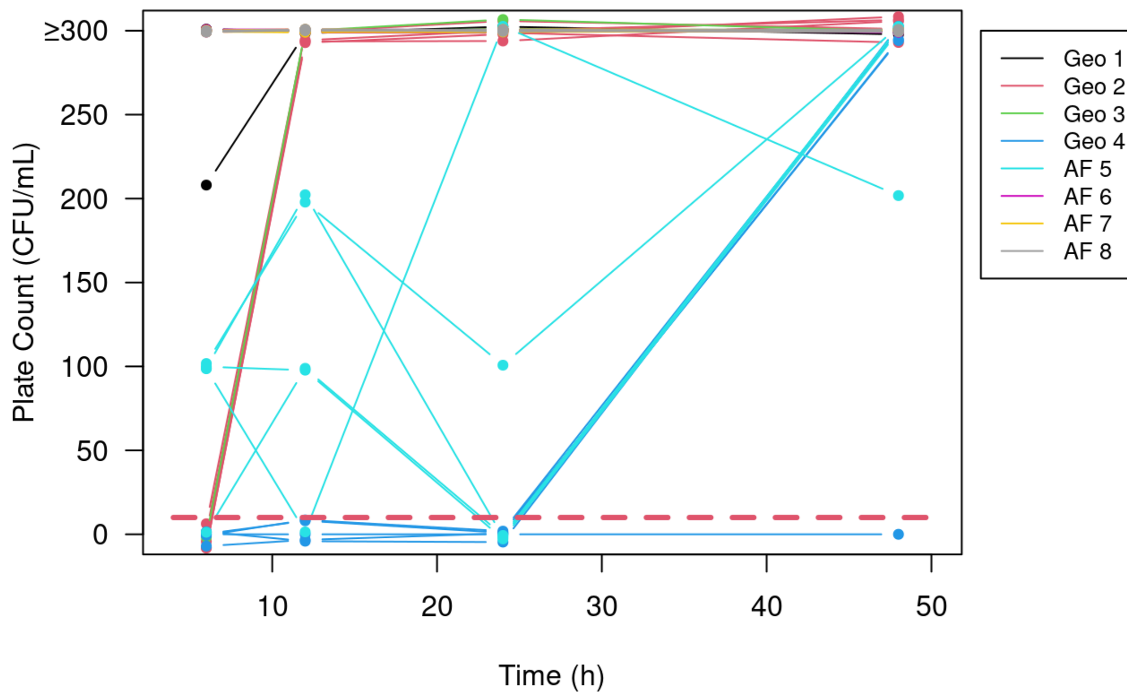

Figure 14. Comparison of the number of thermophiles enumerated (CFU/mL) after different pre-incubation times (6, 12, 24 and 48 hours) in UHT in-house cream, as determined with the pour plate method. Results of individual replicates of all bacterial strains, including four *Geobacillus* sp. and four *Anoxybacillus flavithermus* are shown. The detection threshold was 10 CFU/mL (dotted red line).

3.2.2. Comparison of pre-incubation times for the detection of thermophiles in UHT in-house cream by the Charm ATP method

No growth was detected by any of the isolates with the Charm method at 6 and 12 hour pre-incubation times. Only one replicate of *Geobacillus* 2 was detected at the 24 hour pre-incubation time (in the suspect range). Four replicates of *Geobacillus* isolates were detected at the 48 hour time, including two replicates of *Geobacillus* 1, one replicate of *Geobacillus* 2 and one replicate of *Geobacillus* 3. None of the *Anoxybacillus* isolates were detected at any of the pre-incubation times, despite fourteen of the fifteen *Anoxybacillus* replicates being detected with the pour plate method at 6, 12 and 24 hour time points and five of five replicates detected at the 48 hour time point (Fig. 15).

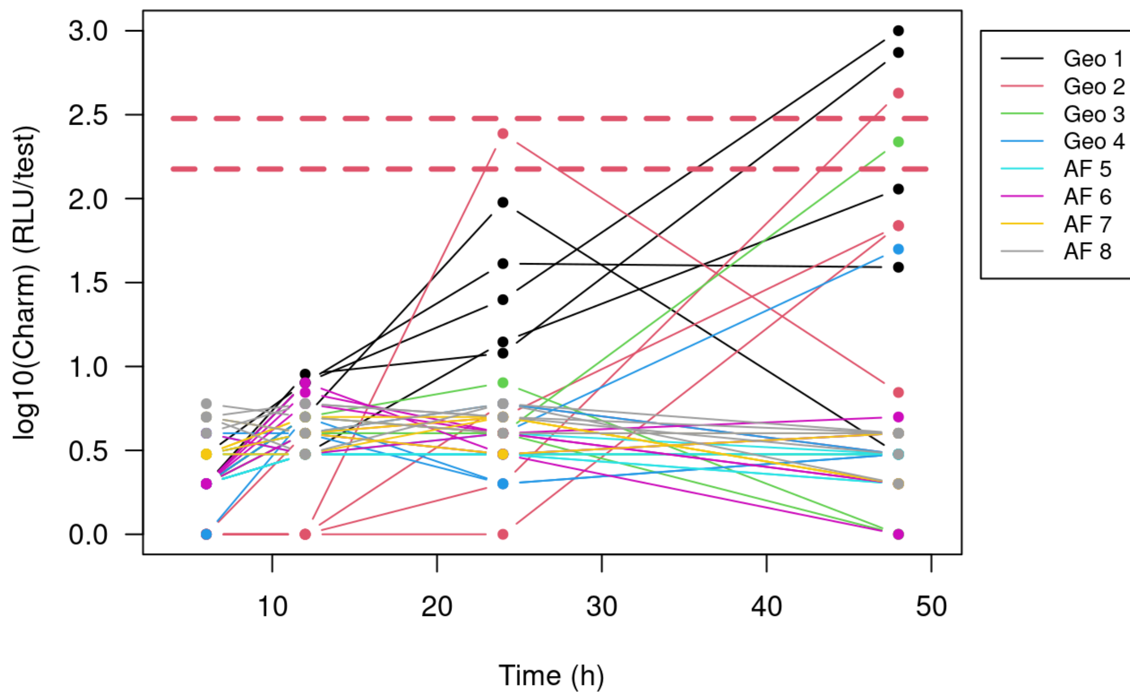

Figure 15. Comparison of pre-incubation time (6, 12, 24 and 48 hours) on the detection of thermophiles in UHT in-house cream by the Charm ATP method (Log RLU/test). Results of individual replicates of all bacterial strains, including four *Geobacillus* sp. and four *Anoxybacillus flavithermus*) are shown. The detection threshold was 2.48 Log RLU/test and suspect 2.18 Log RLU/test (dotted red lines).

3.2.3. Comparison of pre-incubation times for the detection of thermophiles in UHT in-house cream by the Attune flow cytometry method

After the pre-incubation time periods, the in-house cream thickened, thus it required a dilution to be analysed with the Attune analyser. A 1:10 dilution with PBS was performed and then the normal staining protocol was then followed before analysis with the Attune.

Geobacillus isolates were mostly detected at the 12, 24 and 48 hour times and *Anoxybacillus* isolates were mostly detected at all times. One replicate of *Anoxybacillus* 5 was not detected at the 12 hour time. Interestingly, one replicate of *Anoxybacillus* 5 peaked at the 12 hour time point and had lower detection levels at the other times, and had the lowest inoculum level, indicating that very small levels of contamination can elicit growth with high numbers in the sample. (Fig. 16).

All *Geobacillus* 1 replicates were not detected at the 6 hour time, along with three *Geobacillus* 2 replicates, and one *Geobacillus* 3 replicate (Fig. A19-A20).

Similar to the plate method (and to some extent the Charm method), the Attune method showed higher detection levels at the 48-hour pre-incubation period.

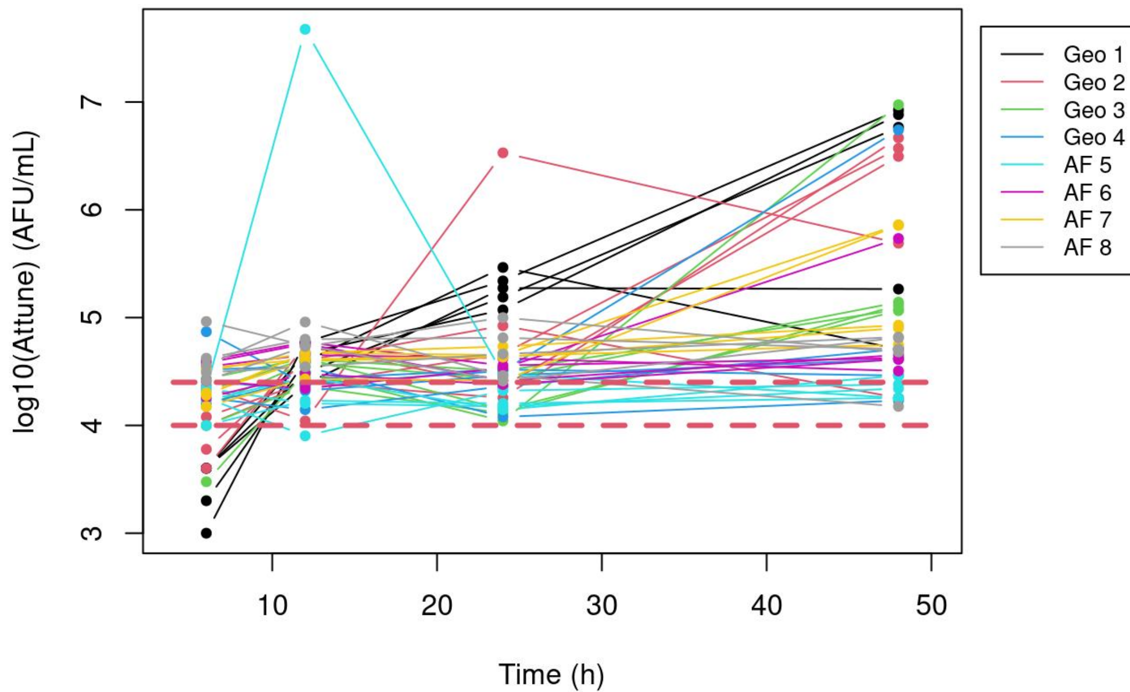

Figure 16. Comparison of pre-incubation time (6, 12, 24 and 48 hours) on the detection of thermophiles in UHT in-house cream by the Attune Nxt flow cytometry method (Log AFU/mL). Results of individual replicates of all bacterial strains including four *Geobacillus* sp. and four *Anoxybacillus flavithermus* are shown. The detection threshold or cut point was later established at 4.4 Log AFU/mL and the lower threshold of 4.0 Log AFU/mL (dotted red lines).

3.2.4. Comparison of the three methods for the detection of thermophiles in UHT in-house cream

The comparison of the pour plate method with the Charm and Attune methods highlighted significant differences in detection patterns, with the Charm method notably under-detecting multiple bacteria isolates and multiple time points.

With *Geobacillus* 1, the Charm method failed to detect any replicates at the 6, 12, and 24-hour pre-incubation times, detecting only two replicates at 48 hours, while the plate and Attune methods detected all replicates at 12, 24, and 48 hours; only the plate method detected replicates at 6 hours. Similarly, for *Geobacillus* 2, the Charm method detected just one replicate at 24 and 48 hours, while the plate and Attune methods detected all replicates at 12, 24, and 48 hours, with the plate method

also detecting all replicates at 6 hours. For *Geobacillus* 3, the Charm method detected only one replicate at 48 hours, while the plate and Attune methods showed agreement in detecting all replicates at 12, 24, and 48 hours, with one replicate missed at 6 hours. The Charm method also failed to detect *Geobacillus* 4 replicates at all time points, while the Attune method detected all replicates across all times, and the plate method detected four replicates at 48 hours.

For *Anoxybacillus* 5, the Charm method failed to detect any replicates at all times, while the plate and Attune methods detected most replicates, with both achieving detection of all replicates at 48 hours. For *Anoxybacillus* 6, 7, and 8, the plate and Attune methods consistently detected bacteria in all replicates across all time points, whereas the Charm method failed to detect any replicates at any time. These results highlight the better detection consistency of the plate and Attune methods compared to the Charm method, which showed significant limitations in bacterial detection across isolates and time points.

The prevalence of replicates not matching was calculated in the same way as for the UHT milks. Similarly, for the UHT in-house creams, there was an 84.375% probability of the replicates matching and a 15.625% probability of them not matching, indicating a low amount of disagreement and good consistency (Table 8).

Table 8. Probability of matching replicates in UHT in-house cream by plate method.

Prevalence of Replicates	5 all not detected	4 not detected, 1 detected	3 not detected, 2 detected	2 not detected, 3 detected	1 not detected, 4 detected	5 all detected
Probability	0.12500	0	0.03125	0	0.12500	0.71875

Data include all pre-incubation times collated. Plate method used as reference for replicate matching.

The paired plot illustrates that the Charm method fails to detect bacteria in many of the contaminated samples (bottom right corner of the Charm paired plot) at all times, compared with the plate method i.e. false negatives as also seen appendix A graphs (Fig. A9-A16) and the Attune method indicates some false negatives at the 6 hour time point and false positives at the 6, 12 and 24 hour times (upper left section)(Fig. 17). Although the Attune has a few false results when scrutinised alongside the Charm with the paired plots, the Attune has many more true positives

(mainly at 12, 24 and 48 hours) than the Charm, and the Charm has more true negatives than the Attune (mainly at 6 and 24 hours) (Fig. 17).

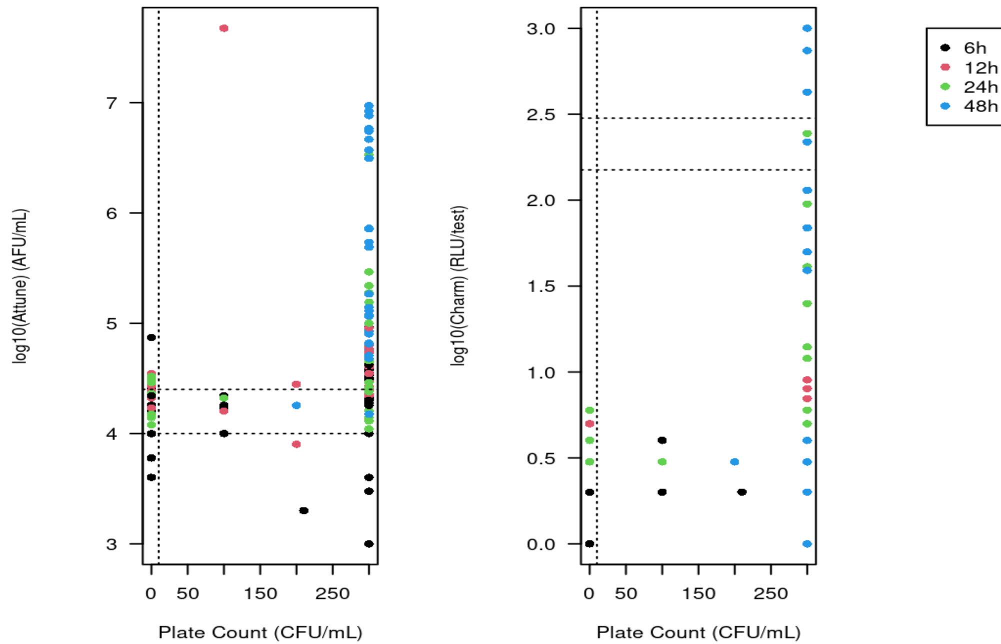

Figure 17. Paired plots of Charm method and Attune method vs plate counts with the set threshold in black dotted lines for UHT in-house cream.

3.2.5. Establishing the threshold values for the Attune with UHT in-house cream.

As with the UHT milk data, the plate count method was used as the reference test to establish threshold values for the Attune method, a selection of possible threshold values was observed to find the best fit for accuracy, sensitivity and specificity across each bacterial strain and at all times. The ideal detection thresholds for the Attune method varied across time points and bacterial isolates to achieve an accuracy of 1.0.

- At 6 hours, perfect accuracy (1.0) was unattainable for the Attune Nxt flow cytometry method, as *Geobacillus* 1 and 3, along with *Anoxybacillus* 5, only achieved an accuracy of 0.8. Specificity of 1.0 was observed for *Geobacillus* 4 at >4.9 Log AFU/mL; however, no ideal threshold values emerged due to the inability to achieve an accuracy of 1.0 as the sensitvity of *Anoxybacillus* 7 was at a lower threshold range of <4.1 Log AFU/mL.
- At 12 hours, sensitivity of 1.0 remained out of reach, with *Anoxybacillus* 5 limiting accuracy to 0.8. Specificity of 1.0 was observed for *Geobacillus* 4 at >4.6 Log AFU.mL, but the

sensitivity of other isolates were lower at <4.0 Log AFU/mL which ensured no perfect accuracy across all bacterial isolates.

- At 24 hours, sensitivity of 1.0 was still not achieved, as *Anoxybacillus* 5 further reduced accuracy to 0.4. Specificity of 1.0 was again observed for *Geobacillus* 4, but no perfect accuracy for all isolates within the threshold range of >4.6–<4.0 Log AFU/mL was achieved as the sensitivity of one isolate was overlapped by the specificity of another isolate.
- By 48 hours, *Geobacillus* 4 demonstrated good accuracy at thresholds of 4.3–4.4 Log AFU/mL, while all other isolates required thresholds of <4.1 Log AFU/mL to achieve acceptable accuracy. However, no single threshold value achieved perfect accuracy (1.0) across all bacterial isolates at this time point, as *Geobacillus* 4 obtained an accuracy of 0.8 at the threshold values <4.1 Log AFU/mL (Fig. B3).

Some of the individual bacteria did have perfect accuracy (data not shown). The best possible threshold values of 4.0 to 4.4 Log AFU/mL accomadates all time points, for the Attune method (Table 9).

Table 9. Optimal threshold values for each bacterial strain and time point in UHT in-house cream (Attune method).

Bacteria/Time	**6h**	**12h**	**24h**	**48h**
Geo 1	NA (max 0.8)	<=4.3	<=5.0	<=4.7
Geo 2	>=4.4	<=4.0	<=4.2	<=4.2
Geo 3	NA (max 0.8)	<=4.3	<=4.0	<=5.0
Geo 4	>=4.9	>=4.6	>=4.6	>=4.3 and <=4.4
AF 5	NA (max 0.8)	NA (max (0.8)	NA (max 0.8)	<=4.2
AF 6	<=4.2	<=4.3	<=4.3	<=4.5
AF 7	<=4.1	<=4.4	<=4.4	<=4.7
AF 8	<=4.4	<=4.5	<=4.4	<=4.1

Threshold (log_{10}) values determined to maximize accuracy. (Accuracy of 1 was not always attainable.)

The ROC curve indicated the best performance of the mean of all the bacterial strains is at 48 hour time point for UHT in-house cream analysed with the Attune (Fig. C2(d)).

The ideal threshold for the Attune can also be interpreted as per gate i.e. 10-25 AFU/gate. The uninoculated samples ranged from 0 to 27 AFU/gate, with an average of 14 AFU/gate and ideally the negative control samples would be below the threshold limit not within it, indicating the Attune

would not be successful in the detection of thermophiles with UHT in-house creams and did not provide confidence at that set threshold.

3.2.6. Optimising the threshold values for the Charm method with UHT in-house cream

The Charm method was not successful at detecting the bacteria in the in-house cream samples, with only five samples over the lower manufacturing threshold of 2.17 Log RLU/test (150 RLU/test), across all pre-incubation times (Fig. 15), indicating many false negatives.

As with the UHT milk and the Attune results a possible alternate threshold that balances sensitivity and accuracy across all time points can be determined for the UHT in-house cream with the Charm method. An alternate threshold of 2 to 5 RLU/test (0.3 to 0.7 Log RLU/test) was identified, which is well below the manufacturer's threshold. However, this range is not ideal, as uninoculated samples fell within 1-7 RLU/test, averaging 3 RLU/test. This suggests that the alternate threshold would result in a high rate of false negatives, compromising sterility detection. Accuracy of 1.0 was inconsistent across time points as the sensitivity of one isolate overlapped with the specificity of another isolate (Fig. B4).

- At 6 hours, thresholds of <0.3 to >0.7 Log RLU/test failed to achieve perfect accuracy.
- Similarly, at 12 a threshold of <0.4 to >0.7 Log RLU/test failed to achieve perfect accuracy
- 24 hours, thresholds of <0.4 to >0.8 Log RLU/test, unable to achieve perfect accuracy.
- By 48 hours, most isolates achieved an accuracy of 1.0 at thresholds <0.3 Log RLU/test, except for *Anoxybacillus* 6 and *Geobacillus* 3, which still fell short, reaching accuracy of 0.8 and 0.6 respectively.

These findings indicate that achieving reliable sterility detection with the Charm method for UHT in-house creams is highly dependent on time and isolate-specific performance. The optimal threshold would have to be set very low to successfully detect a sterility failure and reduce the number of false negatives with the Charm method for UHT in-house creams (Table 10, Fig. B4).

Table 10. Optimal threshold values for each bacterial strain and time point in UHT in-house cream (Charm method).

Bacteria/Time	**6h**	**12h**	**24h**	**48h**
Geo 1	<=0.3	<=0.45	<=1.05	<=0.45
Geo 2	all	NA (max 0.2)	NA (max 0.8)	<=0.45
Geo 3	NA (max 0.8)	<=0.45	<=1.0	NA (max 0.6)
Geo 4	>=0.65	>=0.7	>=0.8	NA (max 0.8)
AF5	NA (max 0.8)	NA (max 0.8)	NA (max 0.6)	<=0.3
AF KAB	<=0.3	<=0.45	<=0.45	NA (max 0.8)
AF 7	<=0.45	<=0.45	<=0.45	<=0.3
AF 8	<=0.6	<=0.45	<=0.6	<=0.3

Threshold (log_{10}) values determined to maximize accuracy. (Accuracy of 1 was not always attainable.)

The high incidence of false negatives with the Charm ATP method may be due to variability in microbial levels (and therefore the levels of ATP present in each cell), dependant on the sample matrix i.e. the viscosity of the in-house cream and the ability of the methods reagents to release the intrinsic ATP. After the pre-incubation at 55°C, the cream samples were noticeable thickened similar with the milks (Fig. 10). It has been noted that the ATP luminescence reaction may be affected by colour and turbidity and that dilution to reduce the background slows the rate of the bioluminescence reaction resulting in low results (Baker et al., 1992). Filtration methods, to decrease the background effect with bioluminescence methods have been investigated, to assist in detection of low numbers of bacteria in UHT tea beverages. (Shinozaki et al., 2016). Other thick UHT products e.g. soymilk beverages have high background noise, inhibiting the detection of the intracellular ATP. The removal of the extracellular ATP (soy proteins, fibres etc) and lessening of the background noise with pretreatments from enzymes, detergent buffers and chelators in soymilk beverages improved ATP detection as demonstrated by Shinozaki et al. (2013). This study did not indicate high background levels (the RLU numbers were very low); however, the thickness of the product may have contributed to low results and false negatives due to inhibition of the ATP bioluminescence reaction.

Another factor that may have caused the high incidence of false negatives with the Charm ATP method may have been due to its limited sensitivity. The lower limit of detection for an ATP method has previously been stated to be 10^3 -10^4 CFU/mL in milk and apple juice when detecting pathogens *Salmonella* and *E. coli* (Hunter and Lim, 2010), and 10^3 -10^7 CFU/mL spoilage organism was required for detection by the MLS (3M, Minnesota, US) and the Cellscan system (Celsis, Chicago, US) ATP methods (Deip et al., 2019). In this study the contaminant thermophiles may not have reached this lower limit of detection for the Charm ATP method and therefore caused the false negatives.

3.2.7. Difficulties with pour plating method with UHT in-house cream

Similar to the UHT milks, the colonies on the culture plates of the in-house creams were difficult to see, especially due to the thickness of the product. The colonies were easier to see on the 10^{-1} dilution and 10^{-2} dilution plates and Gram staining confirmed colonies that mimicked fat particles and coagulation particles. The spread plate proved useful with the in-house creams in distinguishing colonies as they were very clear to visualise on these plates as opposed to the standard pour plate method (Fig. 18).

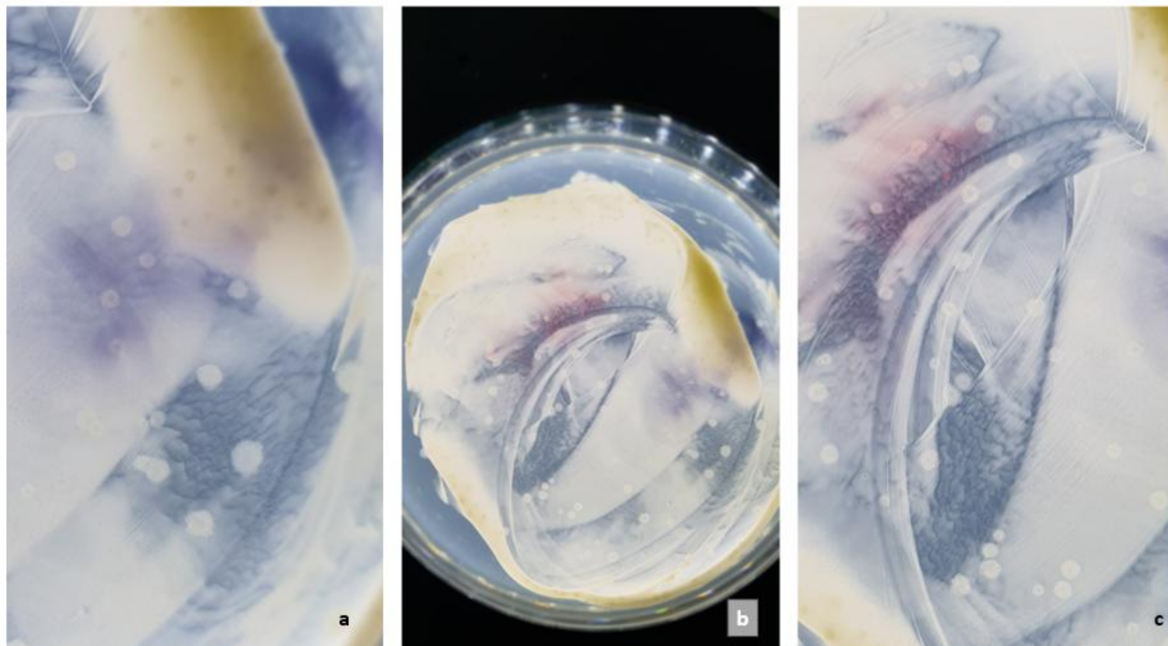

Figure 18. Spread plates examples of UHT in-house cream samples showing the thermophile colonies well.

3.2.8. Growth phase populations determined by the Charm and Attune methods

The UHT in-house creams supported growth of thermophiles which was more evident at the later pre-incubation times of 12, 24 and 48 hours. It appears that the peak of the exponential growth phase may not have been reached as there was a positive trend at the 48 hour time point (Figs. A9-A16) and as the highest growth levels were detected at the 48 hour time point for all methods (Fig. D1). Interestingly, the average growth peak was overall lower at the 24 hour time point, than the 12 and 48 hour time points, when tested with the Attune method compared with a steady exponential growth curve for the time points with the plate and Charm methods.

3.2.9. Optimal pre-incubation time for detecting thermophiles in UHT in-house creams and ideal automated method

Overall, the three commercially sterile methods indicated that for UHT in-house cream contaminated with thermophiles, the 48 hours pre-incubation was optimal (Figs. A9-A16). Once the threshold for the Attune method is established, success criteria can be applied to all replicates, including the blank (uninoculated) replicates (Table 11). Of the two alternative methods the Attune reached better accuracy levels with the established threshold at the 48 hour pre-incubation time and also indicated by the ROC graph and the percentage of agreement (Table 11, Fig. C2). However, the Attune also had some false positive results, as found at the earlier pre-incubation times of 6, 12 and 24 hours (Fig. 17). Applying the lower alternate threshold for the Charm method did improve the overall agreement with the plate method. However, this threshold seemed too low as many of the blanks were higher than the threshold. Overall, the Charm and Attune methods were not ideal as the Charm failed to detect many samples, while the Attune had a tendency to obtain false positive results. Thus, for the UHT in-house cream matrix, it would be recommended to use the pour plate method.

Table 11. Percentage agreement of alternate methods with plate method in UHT in-house cream.

Time (hours)	Charm Epic (% agreement)	Attune NxT (% agreement)	Alternate threshold for Charm Epic (% agreement)
6	41.7	52.1	47.9
12	29.2	72.9	62.5
24	33.3	70.8	62.5
48	27.1	83.3	60.4

3.3 UHT Whipping Cream

3.3.1. Comparison of pre-incubation times for the detection of thermophiles in UHT whipping cream by the pour plate method

The inoculum levels added to the UHT whipping creams prior to pre-incubation ranged between 0.5-22.5 CFU/unit (Table 2).

It was noted in the previous section, that the in-house creams showed an increase of growth at the 48 hour time point, and negligible growth at the 6 -12 hour times. Therefore, the pre-incubation times for the whipping creams were extended to 72 hours and the 6 hour time removed, to investigate if growth would continue beyond 48 hours, at 72 hours.

Growth of all the *Anoxybacillus* isolates were detected by the pour plate method at the 12, 24, 48 and 72 hour pre-incubation times. The *Geobacillus* isolates were mainly detected at the 48 and 72 hour pre-incubation times, with thirteen *Geobacillus* replicates not detected with the pour plate method (one *Geobacillus* 3 replicate and four *Geobacillus* 4 replicates were not detected at 12 hours; four *Geobacillus* 4 replicates were not detected at 24 hours; two *Geobacillus* 4 replicates were not detected at 48 hours and two *Geobacillus* 4 replicates were not detected at 72 hours) (Fig. 19).

All the blank samples had not detected (i.e. pass) results when measured by the pour plate method, as expected. This provided assurance of a low likelihood of obtaining a false positive result due to contamination during this study.

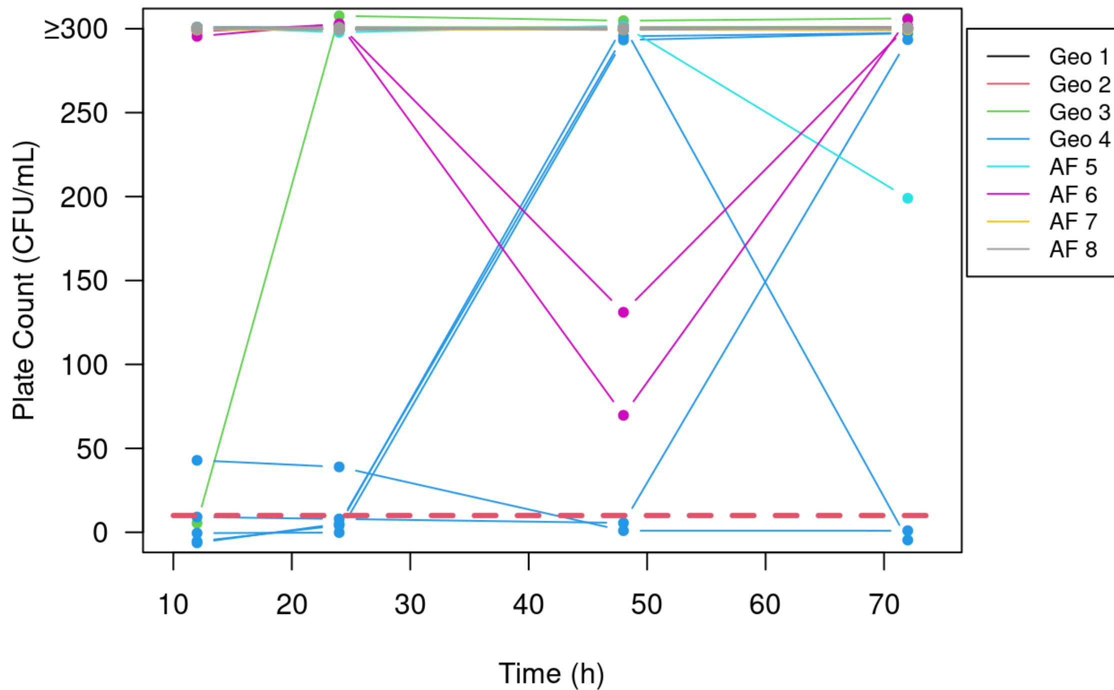

Figure 19. Comparison of the number of thermophiles enumerated (CFU/mL) after different pre-incubation times (12, 24, 48 and 72 hours) in UHT whipping cream, as determined with the pour plate method. Results of individual replicates of all bacterial strains, including four *Geobacillus* sp. and four *Anoxybacillus flavithermus* are shown. The detection threshold was 10 CFU/mL (dotted red line).

3.3.2. Comparison of pre-incubation times for the detection of thermophiles in UHT whipping cream using the Charm method

Many of the whipping cream samples resulted in a non-detection for bacteria using the Charm method, as they were below the manufacturers lower suspect threshold level. Six replicates were detected at the 12 hour time (*Geobacillus* 1, 2 and 3) and nine were detected at the 24 hour time (*Geobacillus* 1, 2and one *Anoxybacillus* 7). Two *Geobacillus* 4 replicates were detected at the 48 hour timepoint along with one *Anoxybacillus* 6 replicate and one *Anoxybacillus* 7 replicate. Two *Anoxybacillus* 6 replicates and one *Anoxybacillus* 8 replicate were detected at the 72 hour time point (Fig. 20).

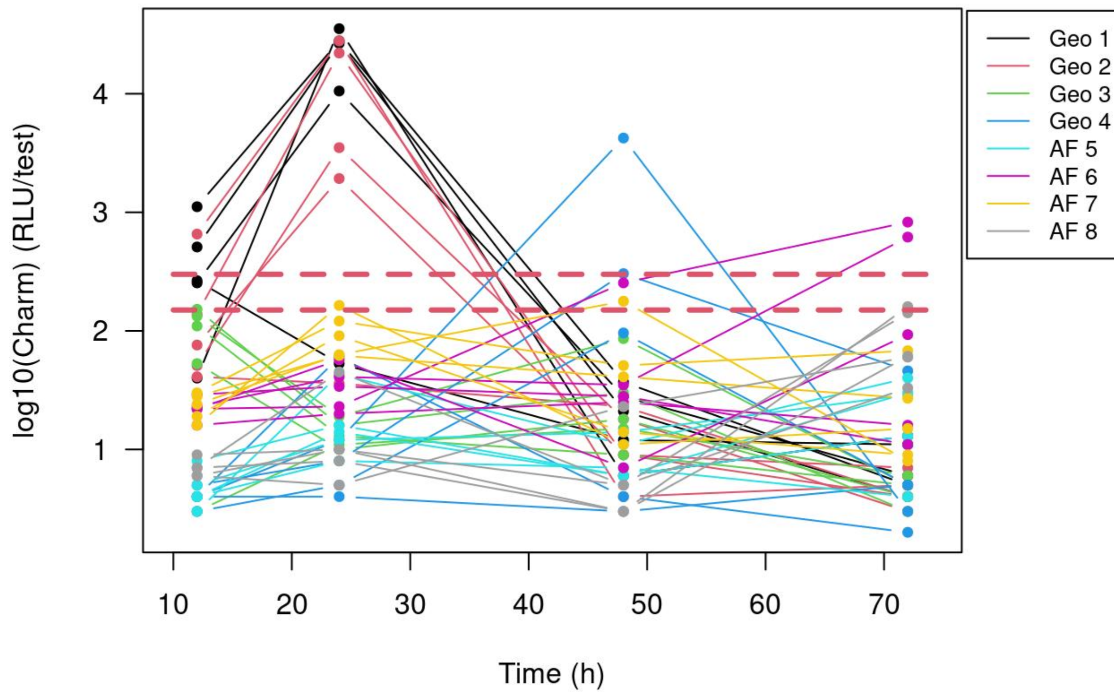

Figure 20. Comparison of pre-incubation time (12, 24, 48 and 72 hours) on the detection of thermophiles in UHT whipping cream by the Charm method (Log RLU/test). Results of individual replicates of all bacterial strains, including four *Geobacillus* sp. and four *Anoxybacillus flavithermus* are shown. The detection threshold was 2.48 Log RLU/test and suspect 2.18 Log RLU/test (dotted red lines).

3.3.3. Comparison of pre-incubation times for the detection of thermophiles in UHT whipping cream using the Attune method

Comparable with the plate method the Attune had more detection across all the times than with the Charm method. Five *Geobacillus* (one *Geobacillus* 3 and four *Geobacillus* 4) and two *Anoxybacillus* were not detected at the 12 hour time point, one *Geobacillus* at 24, 48 and 72 hour time point with the Attune method. All other isolates were detected at all times (Fig. 21).

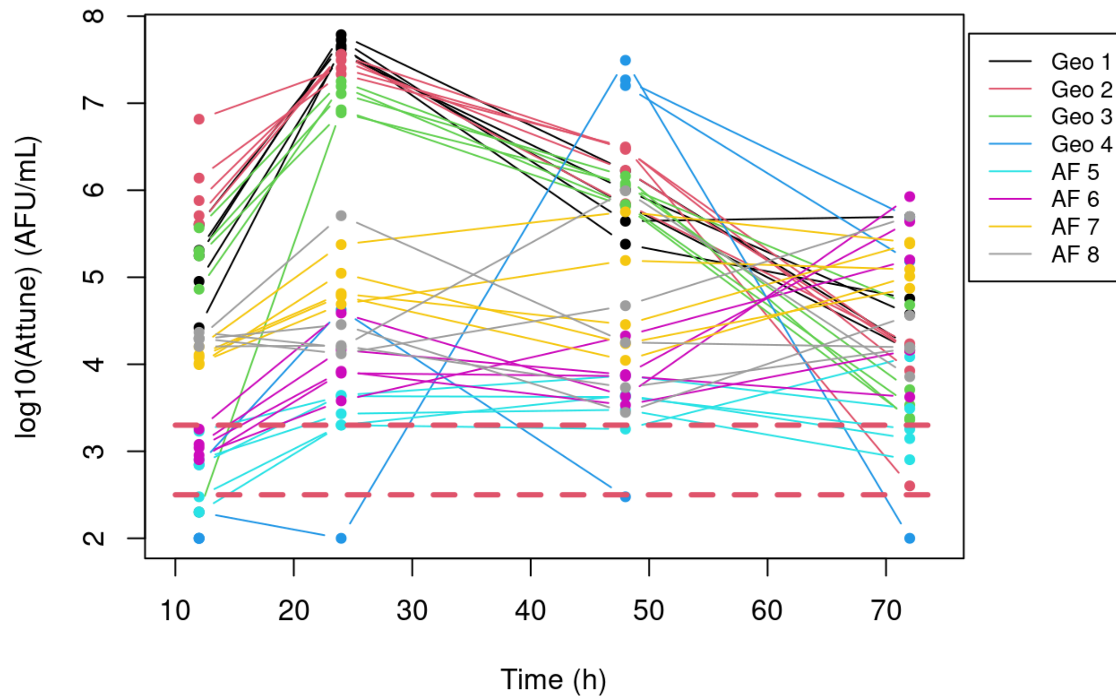

Figure 21. Comparison of pre-incubation time (12, 24, 48 and 72 hours) on the detection of thermophiles in UHT whipping cream by the Attune Nxt flow cytometry method (Log AFU/mL). Results of individual replicates of all bacterial strains including four *Geobacillus* sp. and four *Anoxybacillus flavithermus* are shown. The detection threshold or cut point was later established at 3.3 Log AFU/mL and the lower threshold of 2.5 Log AFU/mL (dotted red lines).

3.3.4. Comparison of the three methods for the detection of thermophiles in UHT whipping cream

The comparison of the pour plate method with the two alternative methods revealed that the plate and Attune methods consistently indicated detection across all time points, except for *Geobacillus* 4. While the Charm method frequently failed to detect many sterility failures, resulting in false negatives, unlike the other two methods, which successfully identified these failures (Figs. 19-21).

As with the previous UHT products, the whipping cream also had occasions of not all of the five replicates matched with each other, and the prevalence can be determined to show the probability of this discrepancy. For example, it was noted with *Geobacillus* 4 at the 12 and 24 hour timepoints that one replicate was detected when the other four were not detected. The probability of this type of scenario occurring was 6.250%. Similar with the previous UHT products the probability of the replicates matching was 84.375% and not matching 15.625% (Table 12).

Table 12. Probability of matching replicates in UHT whipping cream by plate method.

Prevalence of Replicates	5 all not detected	4 not detected, 1 detected	3 not detected, 2 detected	2 not detected, 3 detected	1 not detected, 4 detected	5 all detected
Probability	0	0.06250	0	0.06250	0.03125	0.84375

Data include all pre-incubation times collated. Plate method used as reference for replicate matching.

The detection patterns for *the* individual bacterial isolates varied across the three methods.

For *Geobacillus* 1, the plate and Attune methods consistently detected bacteria at all time points, while the Charm method failed to detect one replicate at 12 and 24 hours and all replicates at 48 and 72 hours, with peak detection occurring at 24 hours for both Charm and Attune. Similarly, *Geobacillus* 2 showed consistent detection with the plate and Attune methods across all time points, but the Charm method missed four replicates at 12 hours, one replicate at 24 hours, and all replicates at 48 and 72 hours, again with peak detection at 24 hours for Charm and Attune. For *Geobacillus* 3, the plate and Attune methods detected bacteria across most time points, except for one replicate at 12 hours, while the Charm method detected only one replicate at 12 hours and none at other time points, with peak detection at 24 hours for the Attune method. *Geobacillus* 4 showed limited detection across all methods, with sporadic detection at 12 and 24 hours and improved detection at 48 and 72 hours for the plate and Attune methods, while the Charm method remained inconsistent.

For the *Anoxybacillus* strains, detection patterns were more consistent across the plate and Attune methods, while the Charm method struggled to detect bacteria reliably. *Anoxybacillus* 5 showed consistent detection with the plate and Attune methods across all time points, except for two replicates missed by the Attune method at 12 hours, while the Charm method failed to detect bacteria at any time point. *Anoxybacillus* 6 was detected consistently by the plate and Attune methods, but the Charm method detected no bacteria at 12 and 24 hours, one replicate at 48 hours, and two replicates at 72 hours. For *Anoxybacillus* 7, the plate and Attune methods detected bacteria at all time points, while the Charm method detected only one replicate at 24 and 48 hours. Similarly, *Anoxybacillus* 8 was consistently detected by the plate and Attune methods, with the Charm method

detecting only one replicate at 72 hours (Figs. A17-A24).

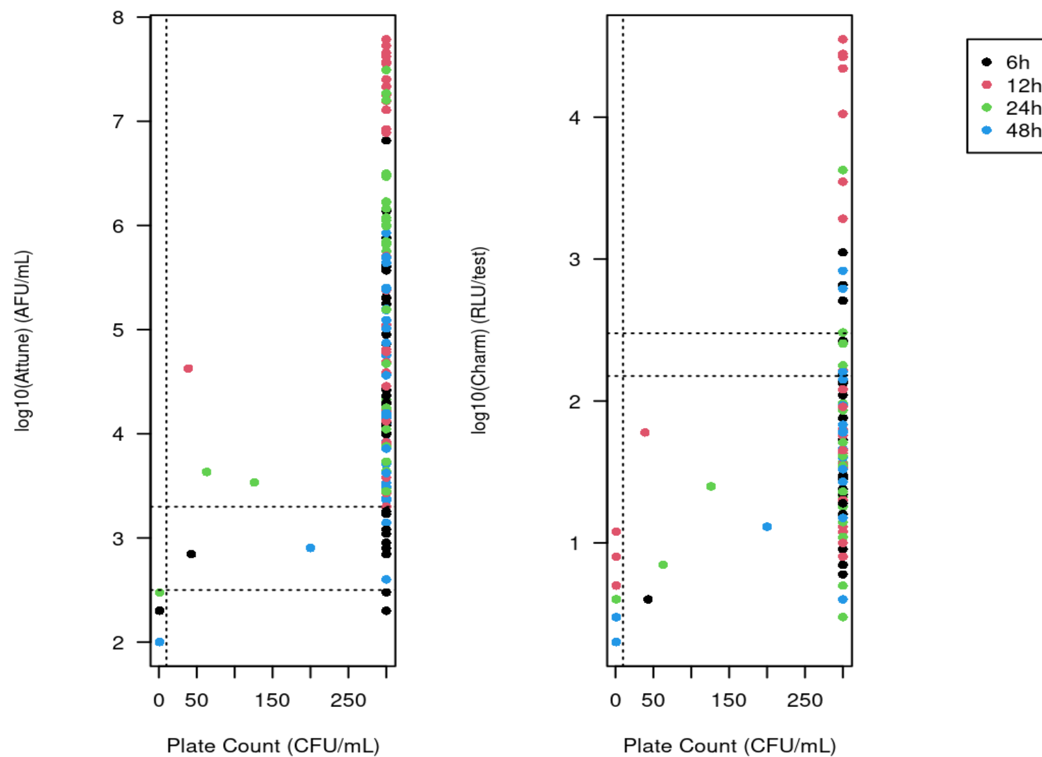

Figure 22. Paired plots of Charm method and Attune method vs plate counts with the set threshold in black dotted lines for UHT whipping cream.

Resembling the UHT milk and the in-house cream the paired plot illustrates that the Charm method fails to detect bacteria in many of the contaminated samples (bottom right corner of Charm plot), compared with the plate method i.e. false negatives. The paired plot highlights only two false negatives with the Attune method, both at the 6 hour time point, indicating a better detection rate with the Attune method. Both methods indicate no false positives with the paired plot (Fig. 22).

3.3.5. Establishing the detection threshold values for the Attune with UHT whipping cream.

As with the UHT milk data, the plate count method was used as the reference test to establish threshold values for the Attune method, a selection of possible threshold values was observed to find the best fit for accuracy, sensitivity, and specificity across each bacterial strain and over all times (Section 1.5).

The ideal detection thresholds for the Attune Nxt flow cytometry method varied across time points, with accuracy of 1.0 dependent on specific bacterial isolates.

- At 12 hours, *Geobacillus* 4 required a threshold of 2.4–2.8 Log AFU/mL, and *Anoxybacillus* 5 required <2.3 Log AFU/mL to achieve perfect accuracy. However, overall accuracy of 1.0 was not attained, though the NPV was 1.0 at thresholds <2.3 Log AFU/mL, with higher thresholds increasing the likelihood of false negatives.
- By 24 hours, a threshold of <3.3 Log AFU/mL enabled all isolates to achieve sensitivity, specificity, accuracy, and NPV of 1.0, with *Anoxybacillus* 5 being the limiting isolate.
- At 48 hours, thresholds of 2.5–3.2 Log AFU/mL achieved accuracy of 1.0, with *Geobacillus* 4 and *Anoxybacillus* 5 were limiting factors.
- At 72 hours, a threshold of <2.6 Log AFU/mL achieved accuracy of 1.0, with *Geobacillus* 2 as the limiting factor.

Across all time points, results below the respective thresholds carried an increased risk of false negatives (Table 13, Fig. B5).

Table 13. Optimal threshold values for each bacterial strain and time point in UHT whipping cream (Attune method).

Bacteria/Time	**12h**	**24h**	**48h**	**72h**
Geo 1	<=4.4	<=7.5	<=5.3	<=4.1
Geo 2	<=5.6	<=7.3	<=5.8	<=2.6
Geo 3	>=2.4 and <=4.8	<=6.8	<=5.8	<=3.3
Geo 4	>=2.5 and <=2.8	<=4.6	>=2.5 and <=7.2	<=5.1
AF 5	<=2.3	<=3.3	<=3.2	<=2.9
AF 6	<=2.9	<=3.5	<=3.5	<=3.6
AF 7	<=3.9	<=4.6	<=4.0	<=4.9
AF 8	<=4.2	<=4.1	<=3.4	<=3.8

Threshold (log_{10}) values determined to maximize accuracy. (Accuracy of 1 was not always attainable.)

The optimal threshold for accuracy for the Attune at all time points was <3.3 Log AFU/mL for the 24, 48 and 72 hour time points. This indicates that the Attune method losses accuracy above 3.3 Log AFU/mL (the sensitivity is lost) at these time points and the sensitivity is lost above 2.3 Log AFU/mL at 12 hours for UHT whipping cream (Tables E9-E12).

The Receiver Operator Characteristics (ROC) curve indicates the best performance of the mean of all the bacterial strains is at 12 and 48 hour times for UHT whipping cream when analysed with the Attune (Fig. C3(a, c)). The absence of horizontal movement with the ROC curve for UHT whipping cream indicates the discriminator never misclassified any negative samples as positive (i.e. no false

positives), regardless of the threshold, however, it did separate the positive and negative samples at 24 and 72 hour time points (Fig. C3(b, d)).

The overall best performance characteristics at all four time points with the Attune were at the threshold <3.3 Log AFU/mL, as this accommodated the best fit for the accuracy, sensitivity, specificity, ROC and NPV in UHT whipping cream (Tables E9-E12, 13, Figs. B5, C3).

The uninoculated samples tested with the Attune method ranged from 0 to 2.5 Log AFU/mL with an average of 1.7 Log AFU/mL, which is below the established threshold values and indicates confidence that the detect results are true positive and the non-detect results are true negatives as also shown by the paired plot (Fig. 22).

3.3.6. Optimising the threshold values for the Charm method with whipping cream

The same criteria as above for the Attune and previous products was applied to establish possible thresholds for the Charm method when testing UHT whipping creams as an alternate to the manufacturers (2.17-2.48 Log RLU/test) threshold (Table 14).

Table 14. Optimal threshold values for each bacterial strain and time point in UHT whipping cream (Charm method).

Bacteria/Time	12h	24h	48h	72h
Geo 1	<=1.6	<=1.7	<=0.7	<=0.6
Geo 2	<=1.6	<=1.5	<=0.6	<=0.4
Geo 3	>=0.5 and <=1.7	<=0.9	<=0.9	<=0.4
Geo 4	NA (max 0.8)	>=1.1 and <=1.7	>=0.7 and <=1.9	>=0.5 and <=0.6
AF 5	<=0.4	<=0.9	<=0.7	<=0.6
AF 6	<=1.2	<=1.3	<=0.8	<=1.0
AF 7	<=1.2	<=1.7	<=1.0	<=0.9
AF 8	<=0.7	<=0.6	<=0.4	<=1.5

Threshold (log_{10}) values determined to maximize accuracy. (Accuracy of 1 was not always attainable.)

The Charm detection thresholds for accuracy varied across time points.

- At 12 hours, thresholds of 0.4–0.5 Log RLU/test were identified.
- By 24 hours, the threshold range expanded to 0.6–1.1 Log RLU/test.
- At 48 hours, thresholds narrowed to 0.4–0.7 Log RLU/test.

- At 72 hours, the threshold values returned to 0.4–0.5 Log RLU/test.

The Charm did not achieve accuracy of 1.0 at all the time points, as there was overlap of the individual bacteria isolates sensitivity and specificity, therefore limiting the overall ability to reach an accuracy of 1.0 (Fig.B6).

The Charm method has an alternate optimal threshold value of 0.4 to 1.1 Log RLU/test of all the times with the greater range at 24 hours. This is considerably lower than the manufacturers threshold value of 2.18 Log RLU/test. This has the advantage of eliminating many of the false negative results. However, the uninoculated samples resulted in a range of 0.3 to 1.0 Log RLU/test with an average of 0.6 Log RLU/test, which is very close to the alternate threshold values. This indicates that the alternate threshold is not ideal, if the alternative threshold was applied, the Charm would likely produce some false positives. (Table 14).

The Charm method was less effective in detecting sterility failures in UHT packs of whipping cream compared to the Attune and plate methods. Similarly, with the in-house cream, there was little or no effect from background noise, as the RLU results were low, rather than high. Product viscosity might have been a contributing factor; however, a preliminary trial aimed at improving the Charm method involved testing different sample preparation techniques before analysis. These techniques included diluting the sample in PBS at a 1:10 ratio and removing background fat from the sample using centrifugation and a chelator (ADA). The pre-treatment trial showed that dilution to reduce the amount of thickening before testing did not support this hypothesis, nor did the removal of cream fat alter the RLU results (Figs. 23-24). This raises the question of whether the bacterial cells have died and are not viable, or if they have simply not reached the limit of detection of 10^3 CFU/mL for the ATP method (which is not supported by the plate and Attune methods, given the high frequency of detect results, and the high numbers of AFUs enumerated by the Attune method). Additionally, quenching of emitted light, possibly from the product matrix itself, is another consideration that may adversely affect microbial ATP determination (Bottari et al., 2015).

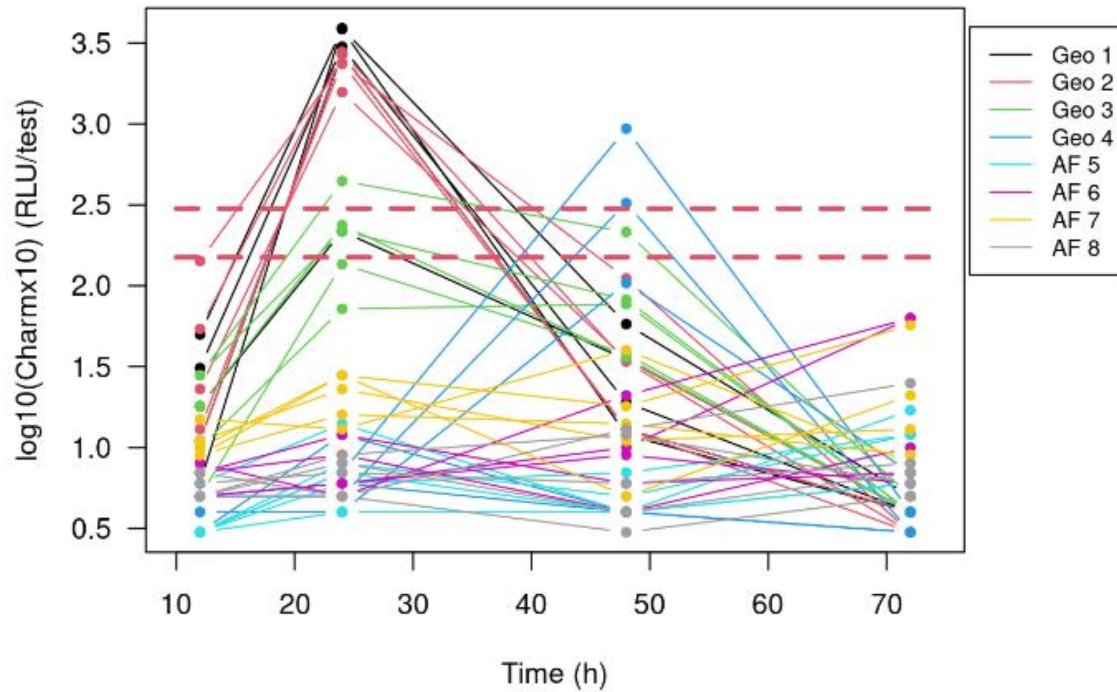

Figure 23. Comparison of pre-incubation time (12, 24, 48 and 72 hours) on the detection of thermophiles in UHT whipping cream by the Charm method with pre-treatment of 10 fold dilution (Log RLU/test). Results of individual replicates of all bacterial strains, including four *Geobacillus* sp. and four *Anoxybacillus flavithermus* are shown with the manufacturers set threshold at 2.48 Log RLU/test and suspect threshold at 2.18 Log RLU/test (dotted red lines).

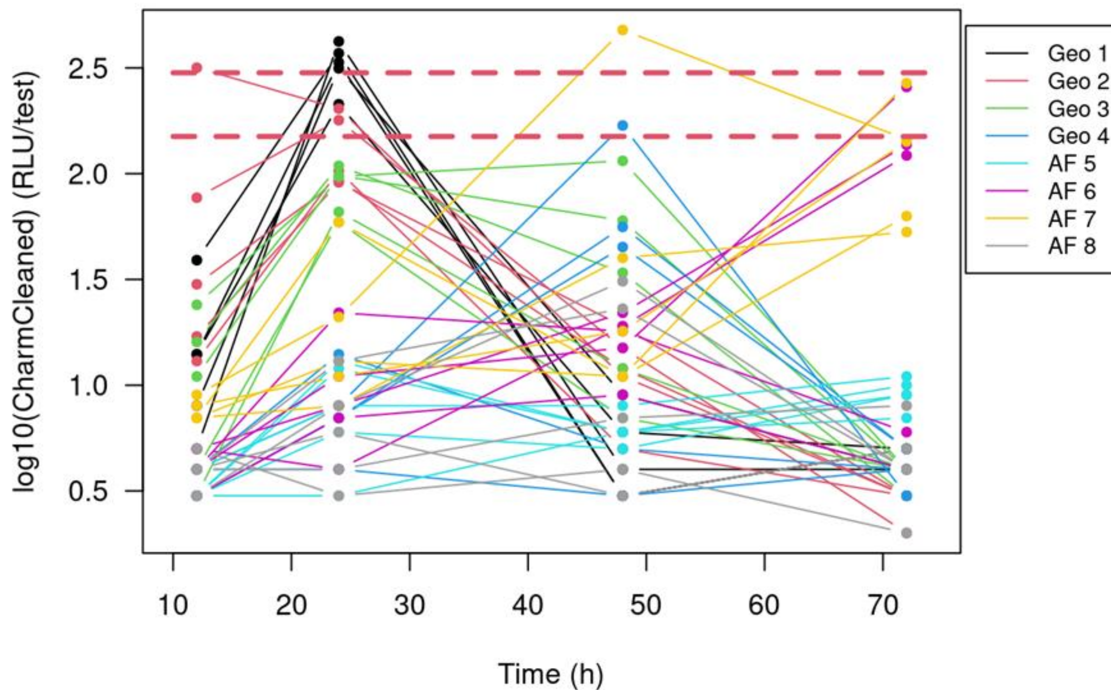

Figure 24. Comparison of pre-incubation time (12, 24, 48 and 72 hours) on the detection of thermophiles in UHT whipping cream by the Charm method with pre-treatment of centrifugation and chelator (cleaned). Results of individual replicates of all bacterial strains, including four *Geobacillus* sp. and four *Anoxybacillus flavithermus* are shown with the manufacturers set threshold at 2.48 Log RLU/test and suspect threshold at 2.18 Log RLU/test (dotted red lines).

The alternate pre-treatments for the Charm method, including dilution and centrifugation with a chelator, produced more false negatives and fewer true positives compared to the standard protocol, making them unacceptable (Figs. 23–25). The standard Charm method also detected more replicates, further showing the ineffectiveness of these alternate approaches.

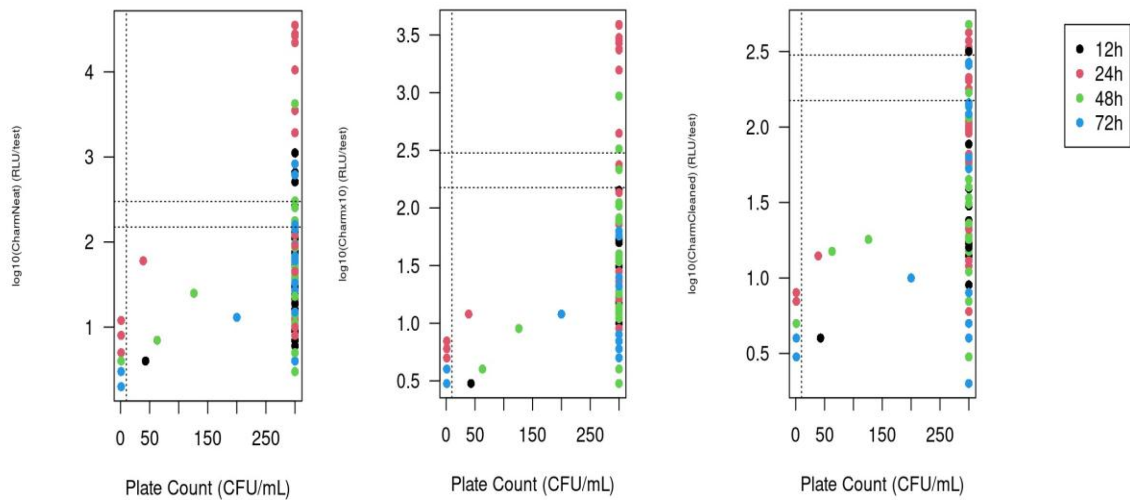

Figure 25. Paired plot of Charm methods vs plate counts with the set thresholds in black dotted lines for UHT whipping cream. Charm method with the 10 fold dilution and the centrifugation and chelator steps (cleaned).

3.3.7. Peak growth phase populations

Overall, the three commercial sterility methods indicated that for UHT whipping cream contaminated with thermophiles, the ideal pre-incubation times are 24 and 48 hours pre-incubation (although the Charm failed to detect many samples at the 48 hour timepoint). High levels of growth were observed all points with the plate method. However, the growth peak was detected with the plate method at 48 hours, and at 24 hours with the Charm method the Attune method.

As before with the UHT milk, the transition of live to dead populations was evident with the Attune density plots and the live population numbers peaking at the 24 hour time. The whipping cream had the pre-treatment applied (centrifugation and chelator steps) and additionally the gate setting for the UHT whipping cream was adjusted to detect the live population better, by removing the effects of the background included in the gates and therefore reducing the false positives (Fig. D2).

3.3.8. Optimal pre-incubation time for detecting thermophiles in UHT whipping cream and ideal automated method

The three methods indicated that for UHT whipping cream contaminated with thermophiles, the 24 hour pre-incubation time is the most agreeable. The plate method was ideal at all pre-incubation times. The 24 hour pre-incubation time was most ideal with the Charm method, as the population

numbers were highest (Fig. 20). Applying the possible alternate threshold calculated improved the Charm method, however this would not eliminate all the false negatives and is too low as the blank replicates were higher on many occasions, and therefore caution needs to be applied when applying as low a threshold (Table 14 & 15). The Attune, with the applied optimal threshold, indicated that the 24, 48 and 72 hour pre-incubation times were ideal. Among these, the 24 hour pre-incubation time rendered the best performance characteristics with the optimal threshold applied with the Attune method (Table 13, Fig. B5). The success criteria can also be applied to all replicates, including the blank (uninoculated) replicates. The criteria of percentage of agreement occurs when the Attune method aligns with the plate method. Similarly, the percentage of agreement can also be applied to the Charm method using the manufacturer's pre-determined threshold and an alternate threshold. (Table 15).

Table 15. Percentage agreement of alternate methods with plate method in UHT whipping cream.

Time (hours)	Charm Epic (% agreement)	Attune NxT (% agreement)	Alternate threshold for Charm Epic (% agreement)
12	39.6	95.8	72.9
24	43.8	100.0	75.0
48	29.2	100.0	81.3
72	27.1	100.0	85.4

3.4 UHT Medical Beverage

3.4.1. Comparison of pre-incubation time for the detection of thermophiles in UHT medical beverage with the pour plate method

The inoculum levels added to the UHT medical beverages prior to pre-incubation ranged between 0.5-26 CFU/unit (Table 2).

The plate count method was successful at detecting the contaminant thermophiles mostly at 24 time point. Only one *Geobacillus* 1 replicate was detected and six of *Anoxybacillus* replicates (*Anoxybacillus* 6 and 8) were detected at the 6 hour time. Ten *Geobacillus* (*Geobacillus* 1 and 3) replicates were detected and eleven of *Anoxybacillus* replicates (*Anoxybacillus* 5, 6 and 8) were detected at the 12 hour time. Only one *Geobacillus* 2 replicate was not detected at the 24 hour time and all other replicates were detected. At the 48 hour pre-incubation time all sample had a detection except for three *Geobacillus* 4 (Fig. 26).

All the blank samples had not detected (i.e. pass) results when measured by the pour plate method, as expected. This provided assurance of a low likelihood of obtaining a false positive result due to contamination during this study.

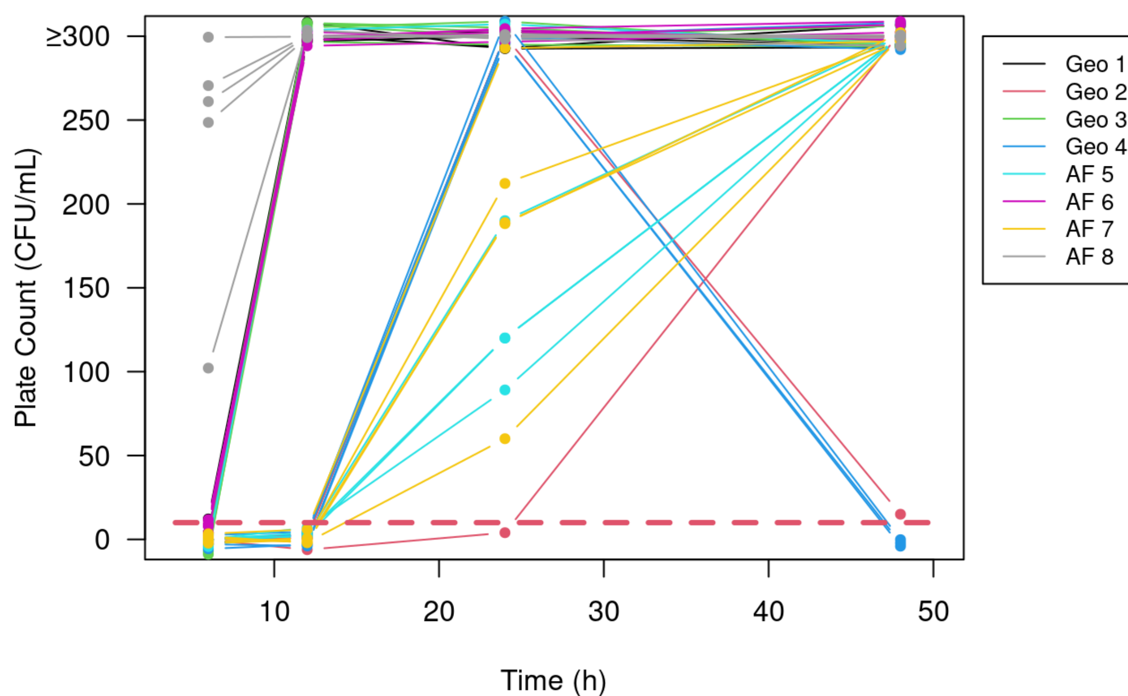

Figure 26. Comparison of the number of thermophiles enumerated (CFU/mL) after different pre-incubation times (6, 12, 24 and 48 hours) in UHT medical beverage, as determined with the pour plate method. Results of individual replicates of all bacterial strains, including four *Geobacillus* sp. and four *Anoxybacillus flavithermus* are shown. The detection threshold was 10 CFU/mL (dotted red line).

3.4.2. Time point detection of UHT medical beverage sterility failure by thermophiles using the Charm method

No sample contamination was detected at the 6 hour timepoint. Five *Anoxybacillus* 8 replicates were successfully detected at the 12 hour timepoint with the Charm method. One *Geobacillus* 4, four *Anoxybacillus* 5, one *Anoxybacillus* 6 and all five *Anoxybacillus* 7 replicates were not detected at the 24 hour time. Interestingly with *Anoxybacillus* 5, only one samples was detected at the 24 hour timepoint and all were detected at the 48 hour timepoint indicating slower growth in the medical beverage of that isolate. The higher RLU values were noted at the 24 hour time (Fig. 27).

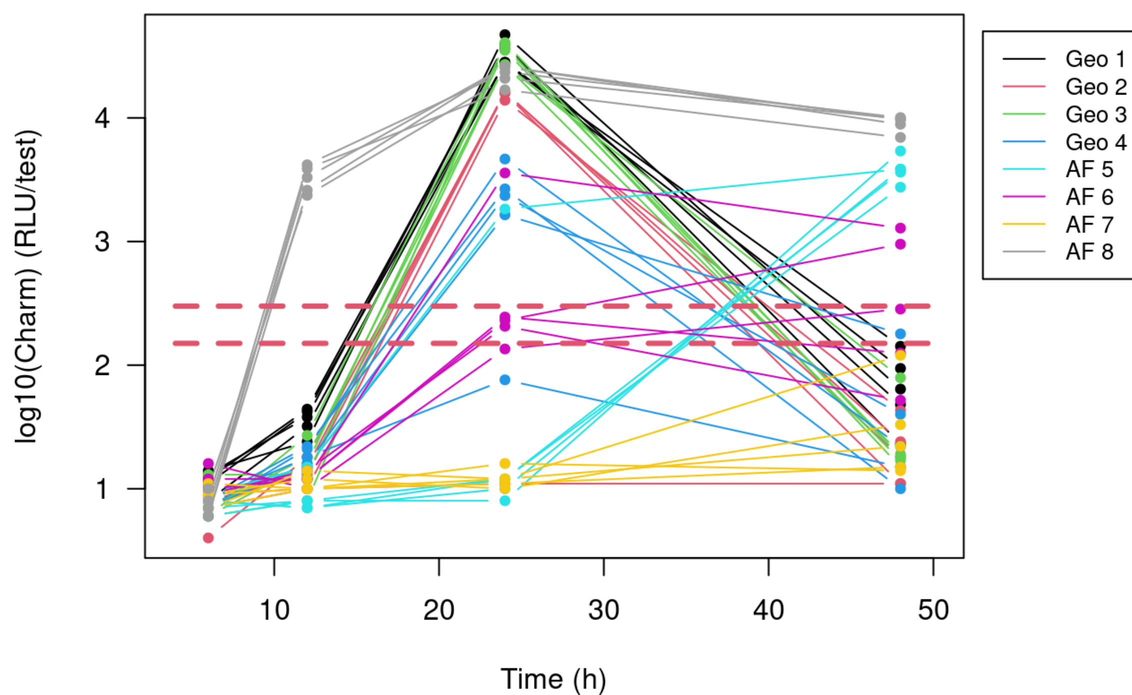

Figure 27. Comparison of pre-incubation time (6, 12, 24 and 48 hours) on the detection of thermophiles in UHT medical beverage by the Charm method (Log RLU/test). Results of individual replicates of all bacterial strains, including four *Geobacillus* sp. and four *Anoxybacillus flavithermus* are shown. The detection threshold was 2.48 Log RLU/test and suspect 2.18 Log RLU/test (dotted red lines).

3.4.3. Time point detection of UHT medical beverage sterility failure by thermophiles using the Attune method

The Attune method was successful at detecting 3 *Geobacillus* and 1 *Anoxybacillus* replicates at 6 hours, 10 *Geobacillus* and 2 *Anoxybacillus* at 12 hours, 19 *Geobacillus* and 7 *Anoxybacillus* at 24 hours and all *Geobacillus* replicates and 15 of the twenty *Anoxybacillus* replicates at 48 hour time

point (Fig. 28).

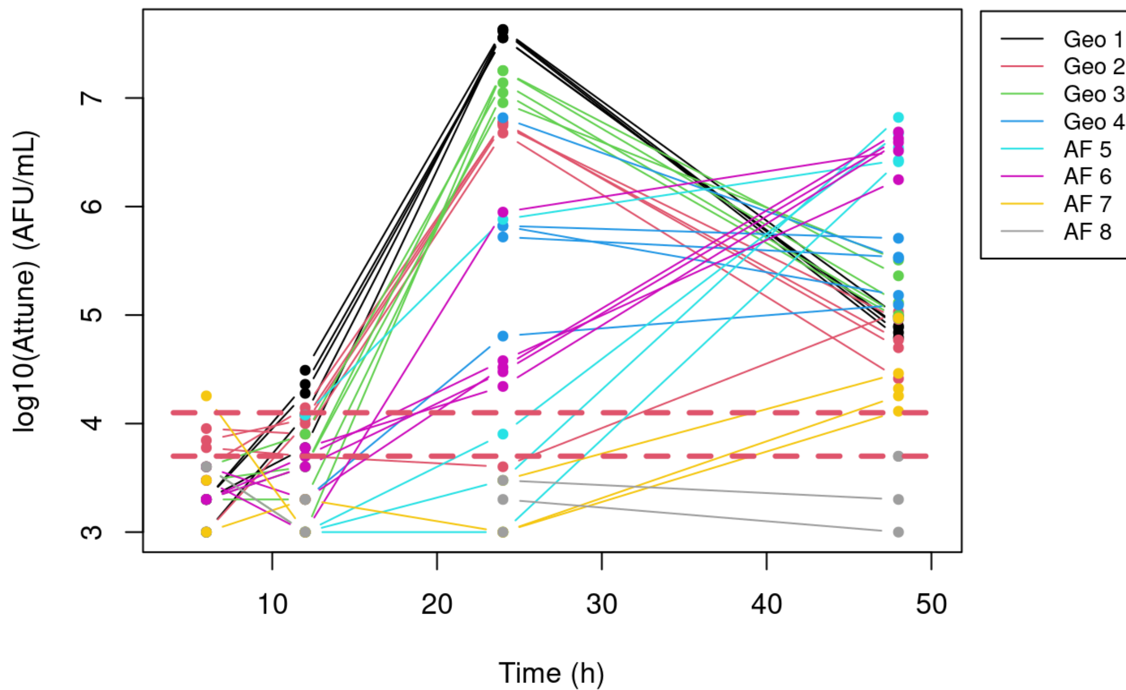

Figure 28. Comparison of pre-incubation time (6, 12, 24 and 48 hours) on the detection of thermophiles in UHT medical beverage by the Attune method (Log AFU/mL). Results of individual replicates of all bacterial strains including four *Geobacillus* sp. and four *Anoxybacillus flavithermus* are shown. The detection threshold or cut point was later established at 4.1 Log AFU/mL and the lower threshold of 3.7 Log AFU/mL (dotted red lines).

3.4.4. Comparison of the three methods

The comparison of the plate pour method with the two alternate methods indicated that all methods mostly followed similar patterns of detection (Figs. 62-64).

As with the previous UHT products, with the UHT medical beverage, not all of the five replicates matched with each other, and the prevalence can be determined to show the probability of this discrepancy. However, compared with the other products the probability of the replicates matching was higher at 87.5% and not matching was lower at 12.5% (Table 16).

Table 16. Probability of matching replicates in UHT medical beverage by plate method.

Prevalence of Replicates	5 all not detected	4 not detected, 1 detected	3 not detected, 2 detected	2 not detected, 3 detected	1 not detected, 4 detected	5 all detected
Probability	0.28125	0.0625	0.03125	0	0.03125	0.59375

Data include all pre-incubation times collated. Plate method used as reference for replicate matching.

For the individual bacterial isolates, detection patterns varied across methods and time points.

Geobacillus 1 was detected by both the Attune and plate methods at 12, 24, and 48 hours, but the Charm method detected contamination only at 24 hours and in one replicate at 48 hours. For *Geobacillus* 2, the plate and Charm methods failed to detect contamination at 6 and 12 hours, while the Attune detected false positives at these time points. At 24 hours, all methods detected four replicates, and at 48 hours, only the plate and Attune methods detected all replicates, while the Charm method failed. *Geobacillus* 3 showed similar trends, with no detection by any method at 6 hours. At 12 hours, the plate method detected all replicates, the Attune detected one, and the Charm failed. At 24 hours, all methods were successful, but at 48 hours, only the plate and Attune methods detected bacteria, while the Charm method failed. *Geobacillus* 4 followed a similar pattern, with no detection at 6 and 12 hours across all methods. At 24 hours, the Charm method missed one replicate, while all methods detected bacteria at 48 hours, with the Attune method producing false positives.

For the *Anoxybacillus* strains, detection patterns were broadly similar across methods, with differences in sensitivity. At 6 hours, no method detected *Anoxybacillus* 5 or 6, but at 12 hours, the plate method detected one replicate for *Anoxybacillus* 5 and all replicates for *Anoxybacillus* 6, while the Attune detected one replicate for each strain, and the Charm failed. At 24 hours, all methods detected bacteria for both strains, except the Charm method, which missed some replicates. At 48 hours, all methods detected all replicates for *Anoxybacillus* 5, while the Charm method missed two replicates for *Anoxybacillus* 6. For *Anoxybacillus* 7, no replicates were detected at 6 or 12 hours, except for one false positive with the Attune at 6 hours. At 24 hours, only the plate method detected bacteria, and at 48 hours, both the plate and Attune methods detected all replicates, while the

Charm failed. *Anoxybacillus* 8 was detected only by the plate method at 6 hours, while at 12, 24, and 48 hours, the plate and Charm methods detected all replicates, but the Attune failed entirely (Figs. A25-A32).

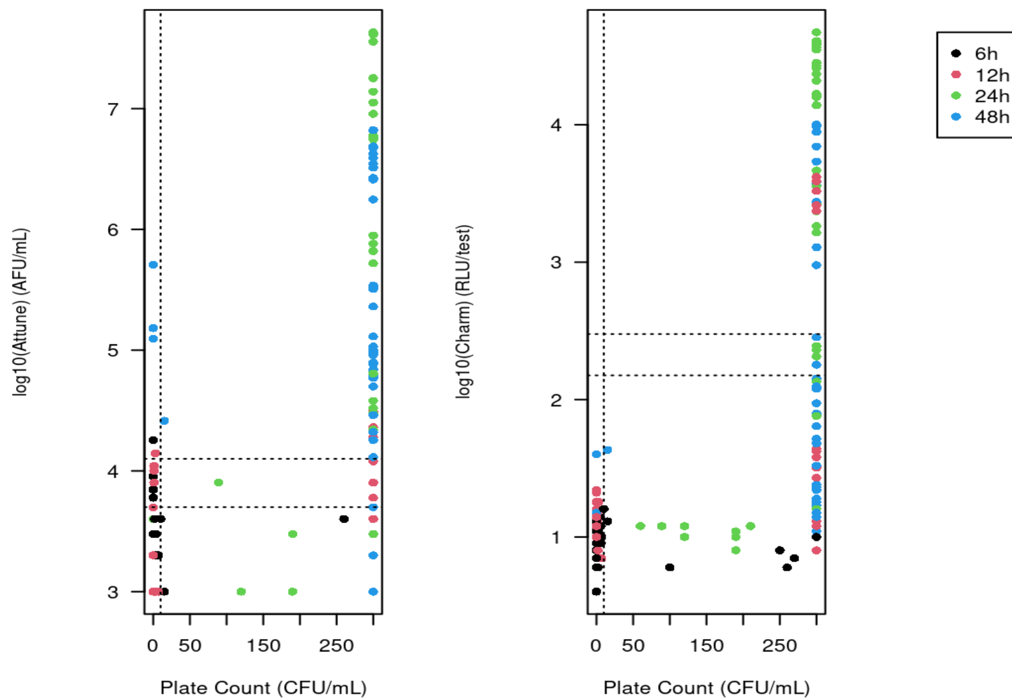

Figure 29. Paired plots of Charm method and Attune method vs plate counts with the set threshold in black dotted lines for UHT medical beverage.

The pair plot comparison indicates nine false negatives with the Attune and many false negatives with the Charm. The plot also indicates false positives with the Attune method (top left region of the plot): one at the 6 hour, one at the 12 hour and three at the 48 hour time points. Similar to the previous products, the paired plot illustrates that the Charm method fails to detect bacteria in many of the contaminated samples (bottom right corner for the Charm) at all times, compared with the plate method i.e. false negatives as also seen in previous graphs (Fig. 29).

3.4.5. Establishing the threshold values of detect and non-detect for the Attune with UHT medical beverage

As with the previous product data, the plate count method was used as the reference test to establish threshold values for the Attune method, a selection of possible threshold values was

observed to find the best fit for accuracy, sensitivity and specificity across each bacterial strain and at all times. See section 1.5 for the equations used.

- 6 Hours: Perfect accuracy (1.0) was achieved for *Geobacillus* 4 and *Anoxybacillus* 5 across all thresholds due to no bacterial growth; other isolates achieved accuracy of 1.0 within 3.7–4.3 Log AFU/mL, except *Geobacillus* 1 and *Anoxybacillus* 8, which did not achieve perfect accuracy.
- 12 Hours: Accuracy of 1.0 was observed for some isolates within 3.7–4.2 Log AFU/mL; *Geobacillus* 1 achieved it at ⩽3.7 Log AFU/mL, *Geobacillus* 2 at ⩾4.2 Log AFU/mL, while *Geobacillus* 3, *Anoxybacillus* 6, and *Anoxybacillus* 8 failed to achieve perfect accuracy.
- 24 Hours: Thresholds of 3.7–4.3 Log AFU/mL resulted in perfect accuracy of 1.0 for some isolates; however, *Anoxybacillus* 5, *Anoxybacillus* 7, and *Anoxybacillus* 8 did not achieve perfect accuracy, with thresholds limited by *Geobacillus* 2 at 3.7 Log AFU/mL and *Anoxybacillus* 6 at 4.3 Log AFU/mL.
- 48 Hours: A threshold of <4.1 Log AFU/mL yielded perfect accuracy for some isolates, but *Anoxybacillus* 7 was the limiting factor, and *Geobacillus* 4 and *Anoxybacillus* 8 did not achieve perfect accuracy at any threshold (Fig. B7).

Table 17. Optimal threshold values for each bacterial strain and time point in UHT medical beverage (Attune method).

Bacteria/Time	**6h**	**12h**	**24h**	**48h**
Geo 1	NA (max 0.8)	<=3.7	<=7.5	<=4.7
Geo 2	>=4.0	>=4.2	>=3.7 and <=6.6	<=4.4
Geo 3	>=3.7	NA (max 0.8)	<=6.9	<=4.9
Geo 4	ALL OK	>=3.4	<=4.8	NA (max 0.8)
AF 5	ALL OK	<=4.0	NA (max 0.6)	<=6.4
AF 6	>=3.7	NA (max 0.8)	<=4.3	<=6.2
AF 7	>=4.3	>=3.4	NA (max 0.2)	<=4.1
AF 8	NA (max 0.2)	NA (max 0.2)	NA (max 0.4)	NA (max 0.6)

Threshold (log_{10}) values determined to maximize accuracy. (Accuracy of 1 was not always attainable.)

Perfect accuracy from the mean data of all bacteria at each timepoint for the Attune method with UHT medical beverages was not achieved (Fig. B7 and Tables 15, E13-E16). However, individual bacteria did achieve perfect accuracy (data not shown). The optimal threshold between 3.7 to 4.1 Log AFU/mL is achieved for *Geobacillus* 1, 2, 3, 4 and *Anoxybacillus* 6 and 8 at 24 hour time and all

except *Anoxybacillus* 8 at 48 hours. It is also compatible with *Geobacillus* 2 at 12, 24 and 48 hour times and *Geobacillus* 4 at 6, 24 and 48 hour times (Table 17).

The ROC graphs indicated suboptimal performance across all times for the UHT medical beverage, except at 24 hours (Fig. C4(c)), which shows the most ideal performance with the Attune method. At this time point, with a threshold of 3.7 to 4.1 Log AFU/mL the NPV only reaches 0.25, however the sensitivity is the highest at 0.67, PPV =1.0 and Specificity =1.0 with this range. As the top left corner has not been reached with the ROC curve indicating that the detect and not-detect isolates have not been perfectly separated and that as the false positive rate increases, the true positive rate remains roughly constant, indicating the test is failing to accurately identify true positives (Fig. C4). The false positives were also highlighted (Fig. 29).

3.4.6. Optimising the threshold values for the Charm method with medical beverage

The optimal threshold values for the Charm method have been calculated as the Attune above and previous products (see section 1.5).

Table 18. Optimal threshold values for each bacterial strain and time point in UHT medical beverage (Charm method).

Bacteria/Time	6h	12h	24h	48h
Geo 1	NA (max 0.8)	<=1.3	<=4.4	<=1.3
Geo 2	>=1.0	>=1.3	>=1.1 and <=4.1	<=1.0
Geo 3	1.2-4.7	<=0.9	<=4.4	<=1.1
Geo 4	>=1.0	>=4.0	<=1.8	NA (max 0.8)
AF 5	>=1.0	>=1.0 and <=1.1	<=0.9	<=3.4
AF 6	>=1.3	<=0.9	<=2.1	<=1.7
AF 7	>=1.1	>=1.2	<=0.9	<=1.1
AF 8	<=0.7	<=3.3	<=4.2	<=3.8

Threshold (log_{10}) values determined to maximize accuracy. (Accuracy of 1 was not always attainable.)

- At 6 hours, accuracy of 1.0 was achieved for some isolates within the threshold range of 0.7–1.3 Log RLU/test. *Geobacillus* 1 only reached an accuracy of 0.8 Log RLU/test, while all other isolates achieved accuracy at >1.3 Log RLU/test, except for *Anoxybacillus* 8, which required <0.7 Log RLU/test.

- By 12 hours, thresholds of 1.0–1.4 Log RLU/test yielded accuracy of 1.0 for some isolates. *Anoxybacillus* 6 required <0.9 Log RLU/test, while *Anoxybacillus* 5 had a narrow range of 1.0–1.1 Log RLU/test, and *Geobacillus* 4 achieved accuracy at >1.4 Log RLU/test.
- At 24 hours, accuracy of 1.0 was observed for some isolates within the range of 0.9–1.1 Log RLU/test. *Anoxybacillus* 5 and 7 required <0.9 Log RLU/test, while *Geobacillus* 2 achieved accuracy between 1.1 and 4.1 Log RLU/test.
- By 48 hours, accuracy of 1.0 was attained for most isolates at thresholds <1.0 Log RLU/test, except for *Geobacillus* 4, which did not achieve perfect accuracy at any threshold (Fig. B8).

Therefore, the Charm method has a best threshold value of 1.0-1.1 Log RLU/test of all the times with the greater range at 24 hours (Table 18). This appears to be a low threshold as the manufacturers lower threshold value is 2.18 Log RLU/test (150 RLU/test) and the uninoculated samples resulted in a range of 0.7 to 1.2 Log RLU/test with an average of 1 Log RLU/test. The best performance for the Charm method is at 24 hours as seen with the ROC curves also.

3.4.7. Peak growth phase, optimal pre-incubation time for detecting thermophiles in UHT medical beverages and ideal automated method

The plate count method had increasing levels of bacteria detected as the time points increased, while the peak growth was detected at the 24 hour time point for both the Charm and Attune methods. The Charm and Attune methods both showed a decreased in bacterial load levels at the 48 hour time point compared with the 24 hour time point (Fig. D3). High levels of growth were observed all points with the plate method (Fig. 26).

Similar to the other UHT products the most agreeable pre-incubation period is the 24 hour time point for UHT medical beverages contaminated with thermophiles.

The plate method was ideal at all pre-incubation times; however, the growth was not detected at the 6 hour time point with all the isolates, indicating the early stages of the exponential growth phase. The 24 hour pre-incubation time was most ideal with the Charm method and Attune, as the population numbers were highest (Figs. 27 & 28). Once the threshold for the Attune method is established, success criteria can be applied to all replicates, including the blank (uninoculated) replicates. The criteria of percentage of agreement occurs when the Attune method aligns with the plate method. Similarly, the percentage of agreement can also be applied to the Charm method using the manufacturer’s pre-determined threshold and the alternate threshold. The highest agreement of the Charm method with the plate method is at 24 hour time point, while the best agreement for the Attune method compared with the plate method is at the 48 hour time point

(Table 19). The alternate threshold improved the agreement of the Charm results when compared with the plate method. However, this is a low threshold, and the blank samples were actually above the alternate threshold, making it not ideal (Table 19).

Therefore, the 24 hour pre-incubation time is optimal with the Charm method and 48 hour pre-incubation for the Attune method for detecting thermophiles in UHT medical beverage.

Table 19. Percentage agreement of alternate methods with plate method in UHT medical beverage.

Time (hours)	Charm Epic (% agreement)	Attune NxT (% agreement)	Alternate threshold for Charm Epic (% agreement)
6	85.4	75.0	64.6
12	66.7	60.4	54.2
24	77.1	70.8	83.3
48	52.1	85.4	81.3

The detection of thermophilic bacteria in UHT dairy products was shown to have a high incidence of false negatives in this study. These false negatives may be attributed not only to ATP degradation with the Charm method but also to other factors that inhibit the growth of thermophiles in UHT products. For example, *Geobacillus* 4 consistently struggled to grow in the UHT medical beverage and the other UHT products, where most of the other thermophilic strains reached ideal population levels, even with low initial inoculum levels. *Geobacillus* 4 also exhibited limited detection across various methods and was reliably detected only after extended incubation—at 24 hours in UHT milk and the medical beverage, and at 48 hours in the two cream products. Detection was generally more reliable at longer incubation times, with the plate and Attune methods proving most sensitive.

Other strains also demonstrated slow or inhibited growth under certain conditions. *Geobacillus* 2 was also slower in growing with the UHT medical beverage, as was *Geobacillus* 3 with UHT milk. *Anoxybacillus* 5 did not grow well in the UHT in-house cream or the UHT medical beverage and with the three methods, it was not detected until the 48 and 24 hour times respectively. Another slow grower was *Anoxybacillus* 7 in the UHT medical beverages.

The limited growth of *Geobacillus* 4 and the other slow growing isolates may be attributed to the challenging characteristics of the dairy products themselves i.e. pH, viscosity, nutrients. After UHT processing, products are packaged in sterile, airtight containers, which create an inhibitory environment. The combination of reduced oxygen levels, nutrient depletion (protein denaturation

and heat-labile vitamin degradation), and unsuitable growth conditions may prevent surviving thermophiles from thriving. This is consistent with studies showing that the casein fraction of milk proteins, as well as the presence of free cations—specifically, high sodium and low levels of calcium and magnesium—can inhibit growth of and biofilm formation by *Geobacillus* in milk products (Ashton & Busta, 1968; Somerton, B. et al., 2015).

3.5 Overall Summary

Along with other considerations, like the ease of use, the cost per test needs to be examined before implementation into a laboratory for regular use (Table 20).

Table 20. Summary of method performance, cost, and ease of use by product type.

	Parameter	Plate	Charm	Attune	Charm with alternate threshold
UHT MILK	Best pre-incubation time to achieve the greatest % agreement	N/A	24*	All*	24
	Peak bacterial levels detected at what pre-incubation time	48	12	24	N/A
	False negative or false positives		FN++		
UHT in-house cream	Best pre-incubation time to achieve the greatest % agreement	N/A	24	48	24
	Peak bacterial levels detected at what pre-incubation time	48*	48	48	N/A
	False negative or false positives		FN++	FP+ FN+	
UHT whipping cream	Best pre-incubation time to achieve the greatest % agreement	N/A	24	24-72*	24
	Peak bacterial levels detected at what pre-incubation time	72	24	24	N/A
	False negative or false positives		FN+++	FN+	
UHT medical beverage	Best pre-incubation time to achieve the greatest % agreement	N/A	24	48	24
	Peak bacterial levels detected at what pre-incubation time	24*	24	24	N/A
	False negative or false positives		FN+++	FP+ FN+	
Cost per test		≈$7.50 (Including labour)	≈$3.80 to $6.80 (Including labour) + Initial Luminometer cost.	≈$3.13 to $6.13 (Including labour) + Initial Flow cytometer cost.	N/A

Ease of use	Manual testing and time consuming.	Yes, very automated.	Yes, after initial set up and dye preparation, easy to use. Gate setting requires expertise.	N/A

**The greatest or best of condition indicated.*
FN = false negatives, FP = false positives, + to +++ extent of occurrence.

Chapter 4. Conclusion

This study has indicated the optimal pre-incubation timing for the commercial sterility methods for detecting thermophilic bacteria in UHT dairy products. This assessment also measures the effectiveness of the two alternate proprietary methods; Charm Epic ATP bioluminescence and Attune Nxt flow cytometry across four UHT products (milk, in-house cream, whipping cream, and medical beverage) for the reliability of the alternate rapid detection method compared to the traditional plate count method, which served as the reference standard.

With UHT milk the 24-hour pre-incubation period emerged as the most effective for all three methods. While the plate count method detected thermophiles reliably at the 48-hour mark, the Charm and Attune methods were optimal at 24 hours, with the Charm method showing false negatives at 48 hours due to bacterial death and ATP degradation, along with the possible interference of aspects of the matrix i.e. thickening, at the longer pre-incubation times. This finding supports the hypothesis posed that the pre-incubation times may need to be reduced for standard regulatory methods, particularly if they were to include methods alternative to plate methods.

The 48-hour pre-incubation period was optimal for the plate count method, as it captured the highest levels of bacterial growth with the UHT in-house cream. However, the Attune method showed better performance at 24 hours, while the Charm method consistently underperformed across all time points, likely due to the complexity of the product nature and interference with ATP detection. This indicated that the two alternate methods were not ideal and comparable to the traditional plate count method for UHT in-house cream.

The 24-hour pre-incubation time was determined to be the optimal for all three methods, with the Attune method demonstrating the best performance at this time point for UHT whipping cream. The Charm method failed to detect many sterility failures, particularly at later time points, and was less effective overall compared to the other methods.

Again, the 24-hour pre-incubation time was again the most effective for all three methods with UHT medical beverages. The Attune method performed well at both 24 and 48 hours, while the Charm method showed limited sensitivity and produced false negatives, particularly at shorter pre-incubation times.

The two alternate methods were compared with plate method, where the plate method consistently detected thermophiles across all products and time points, particularly at longer pre-incubation

periods (48 hours). The plate method was labour-intensive, prone to interpretation errors due to product matrices interference, and requiring extended incubation times.

The Charm method struggled with sensitivity, particularly at longer pre-incubation times, often failing to detect thermophiles due to ATP degradation in dying cells. While it was most effective at the 24-hour time point, its reliability was limited for highly complex products (e.g., in-house cream and whipping cream). Adjusting the detection threshold below the manufacturer's set level or mitigating the effects of product thickening and coagulation may improve performance and reduce false negatives. However, the method remains less reliable for detecting low levels of contamination or in products with highly complex matrix and should be used with caution.

The Attune Nxt flow cytometry method demonstrated the highest overall accuracy and sensitivity across all products and time points, particularly at 24 and 48 hours. It was able to detect viable but non-culturable (VBNC) cells and showed fewer false negatives compared to the Charm method, with a faster turnaround time than the plate count method. However, it required expertise for data interpretation, gate setting and was affected by product matrices, particularly in thicker products like cream. Despite these challenges, the method is recommended for its high sensitivity and ability to detect VBNC cells.

The study concurred, that thermophilic bacteria, particularly *Geobacillus* and *Anoxybacillus* species, exhibited rapid growth phases, reaching peak detection around 24 hours. Also, it indicated the quick growth phase was followed by rapid decline of the population, leading to potential false negatives in methods reliant on live cell detection (e.g. Charm ATP).

The plate count method remained effective at later time points (48 hours), as it could detect bacterial colonies even after ATP degradation, as it provided a nurturing environment on agar (i.e. after pre-incubation). However, the Attune method provided a more accurate representation of bacterial viability during earlier growth phases. This indicated that the Attune method was the more comparable of the two alternate methods with the plate count method.

The other considerations that remain important are the viscosity of the dairy products, where additional preparation steps, and along with the detection threshold adjustment to improve the sensitivity, specificity and accuracy to reduce the false positive and false negative results. However, these thresholds must account for the product-specific characteristics and background noise.

Going forward the assessment of other dairy products, for example chocolate and coffee UHT drinks, with the same testing protocol, will provide a more thorough evaluation of these findings. Also, the inclusion of *Bacillus licheniformis*, as it has been associated with UHT spoilage previously, would

complement this study of the obligate thermophiles. As this study targeted obligate thermophiles that grow at 55°C and to mimic potential storage temperature abuse, the temperature could be lowered to 50°C, in addition to 55°C, for the study with *B. licheniformis,* which may be a more realistic temperature that products may reach during transport and storage. The study could also be enhanced by including spores, at differing inoculum levels, into the products to explore the optimal pre-incubation period for spore-contaminated samples, providing further insights into spoilage under temperature abuse conditions.

Finally, this study highlights the importance of selecting sterility testing methods and pre-incubation times according to product type and microbial growth characteristics. The Attune Nxt flow cytometry method is recommended for routine sterility screening of UHT milk, whipping cream and medical beverage using a 24-hour pre-incubation; the Charm Epic ATP method may be acceptable for routine screening of UHT milk at 24 hours but is not recommended for more complex, high-viscosity products. While rapid detection methods like Attune flow cytometry offer significant advantages in terms of speed and sensitivity, the traditional plate count method remains key for comprehensive sterility testing. A 24-hour pre-incubation period strikes the best balance between accuracy, sensitivity, and practicality, ensuring reliable detection of thermophilic bacteria in some UHT dairy products.

No single rapid method evaluated in this study was suitable for all product types and all pre-incubation time points. Therefore, the standard plate count method should remain the reference. The tested rapid methods from this study may be adopted on a product and pre-incubation time point specific basis only, and after individual laboratory validation. Ongoing optimisation of rapid methods and threshold values will further enhance the dairy industry's ability to ensure the safety and quality of UHT-processed dairy products.

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Appendix A

<u>UHT milk A1-A8</u>

Comparison of plate, Charm and Attune methods at different pre-incubation times (6, 12, 24 and 48 hours). The detection threshold for plate count was 10 CFU/mL (black dotted line), for Charm was 2.18-2.48 Log RLU/test (blue dotted lines), and for Attune was 4-5.3 Log AFU/mL (red dotted lines).

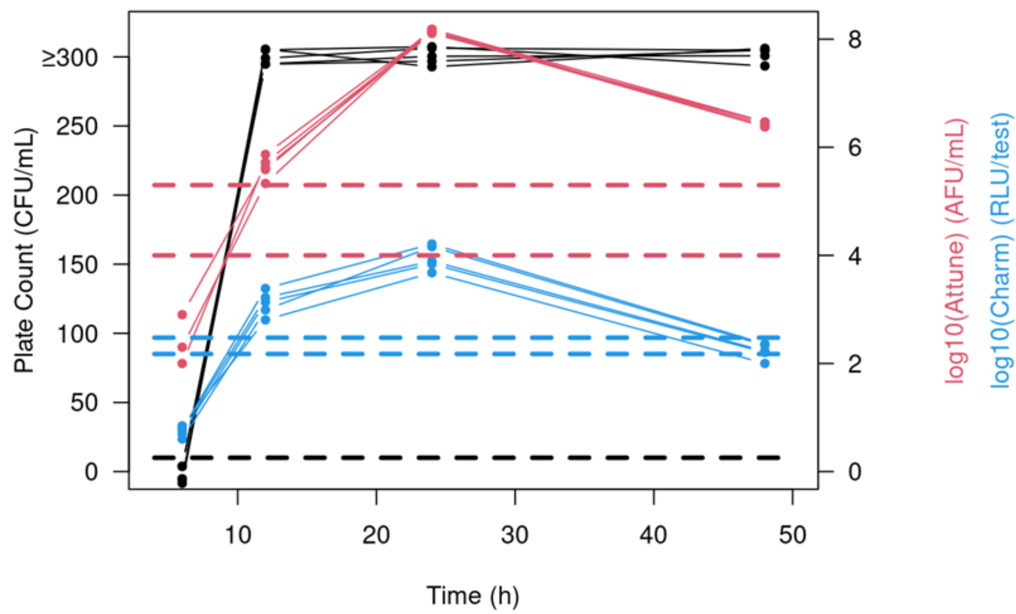

Figure A1. *Geobacillus* 1.

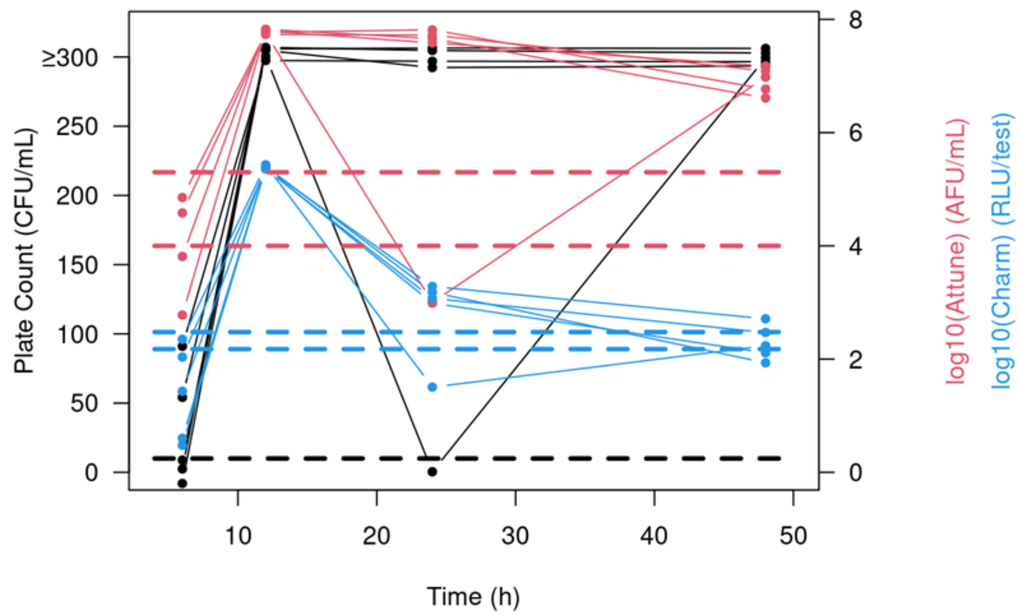

Figure A2. *Geobacillus* 2.

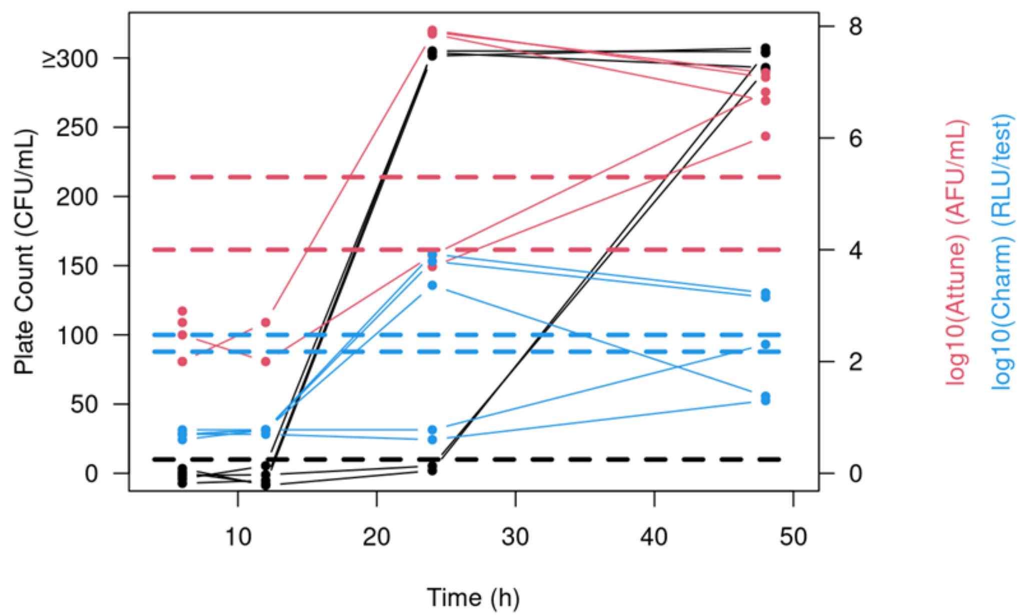

Figure A3. *Geobacillus* 3.

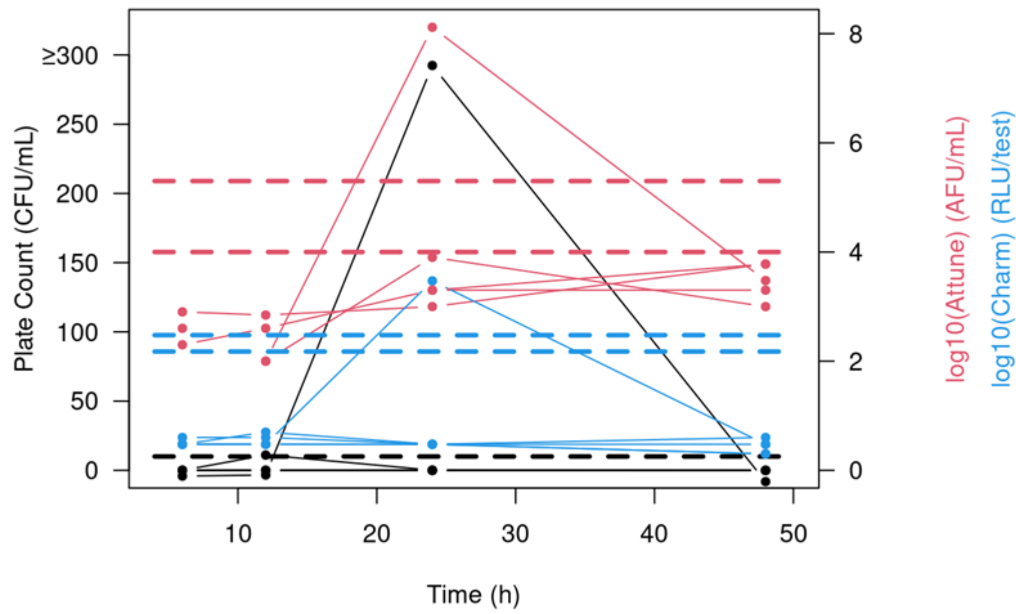

Figure A4. *Geobacillus* 4.

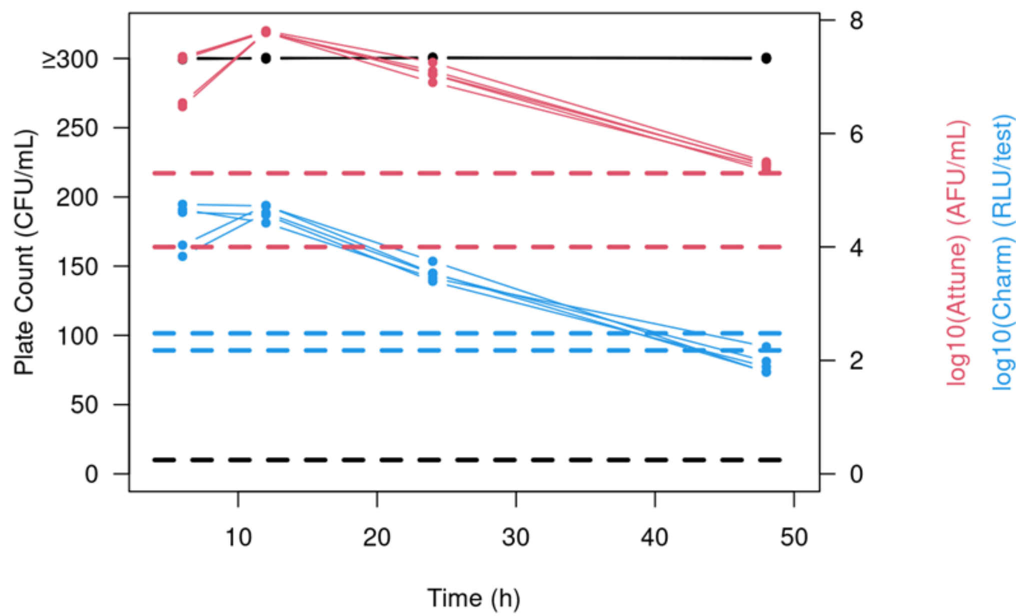

Figure A5. *Anoxybacillus* 5.

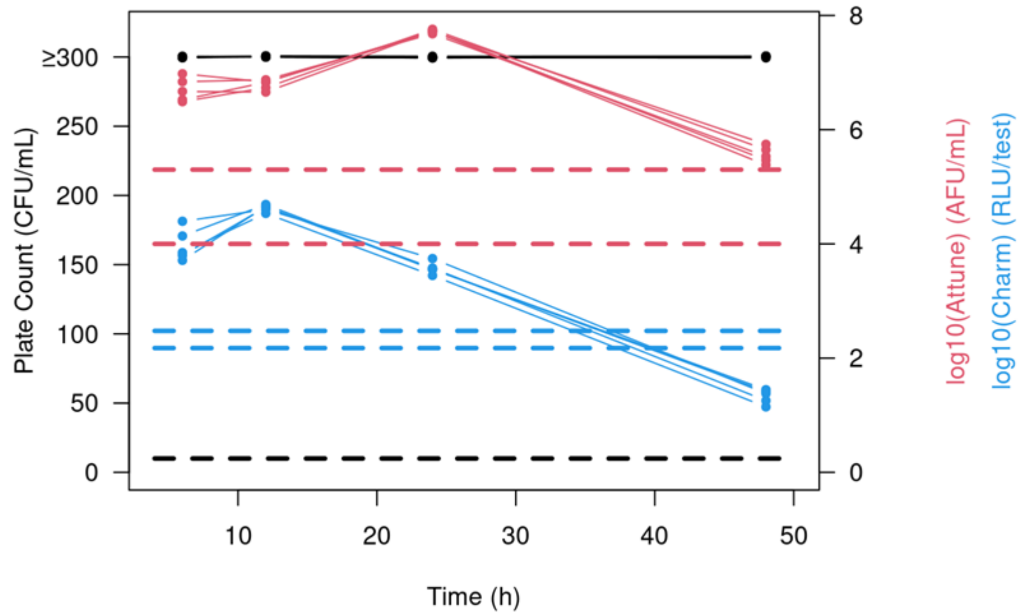

Figure A6. *Anoxybacillus 6.*

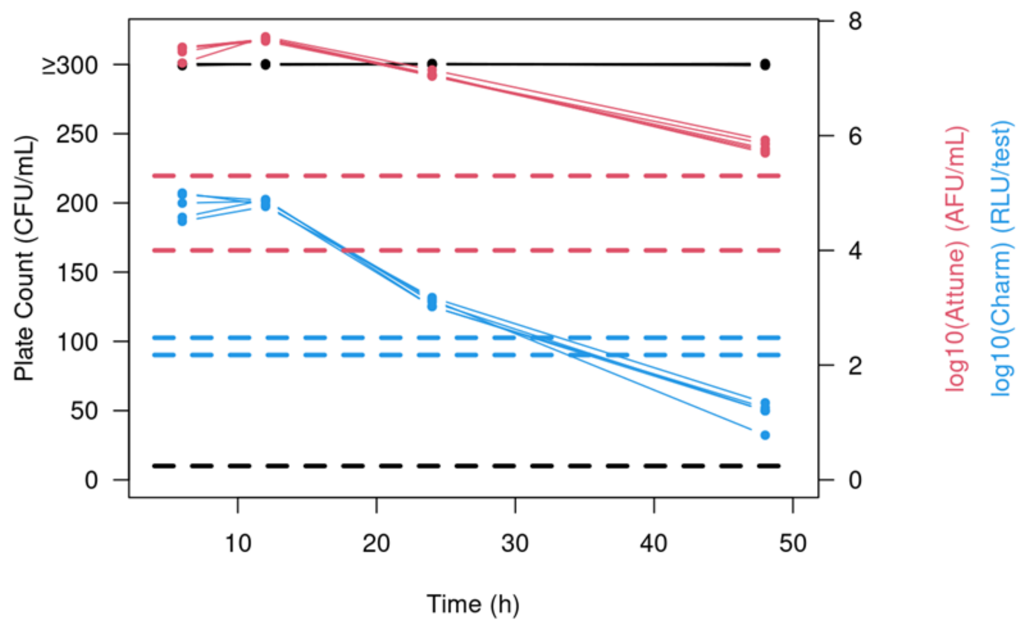

Figure A7. *Anoxybacillus* 7.

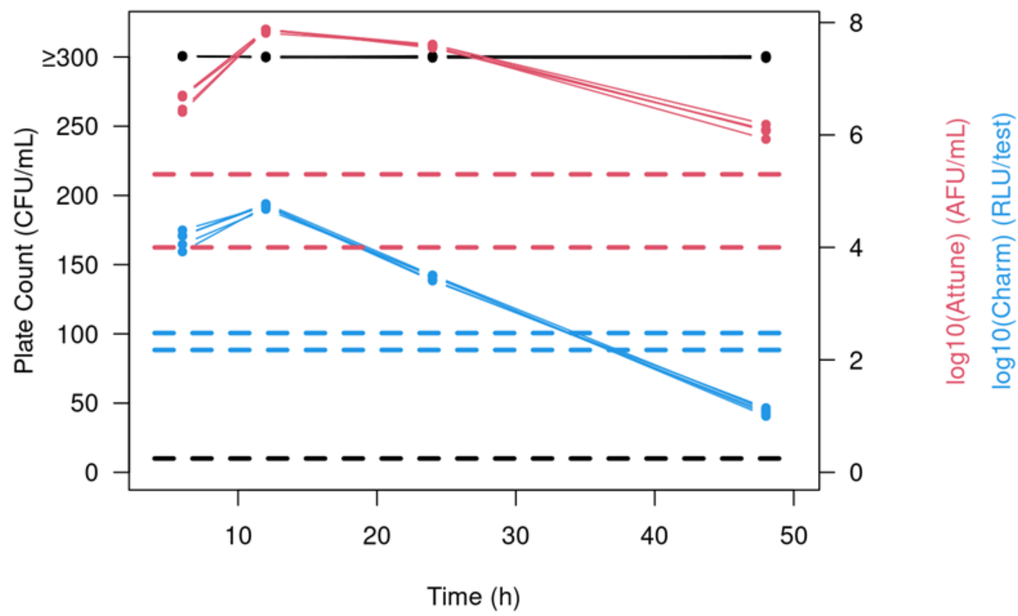

Figure A8. *Anoxybacillus* 8.

UHT in-house cream

Comparison of plate, Charm and Attune methods at different pre-incubation times (6, 12, 24 and 48 hours). The detection threshold for plate count was 10 CFU/mL (black dotted line), for Charm was 2.18-2.48 Log RLU/test (blue dotted lines), and for Attune was 4-4.4 Log AFU/mL (red dotted lines).

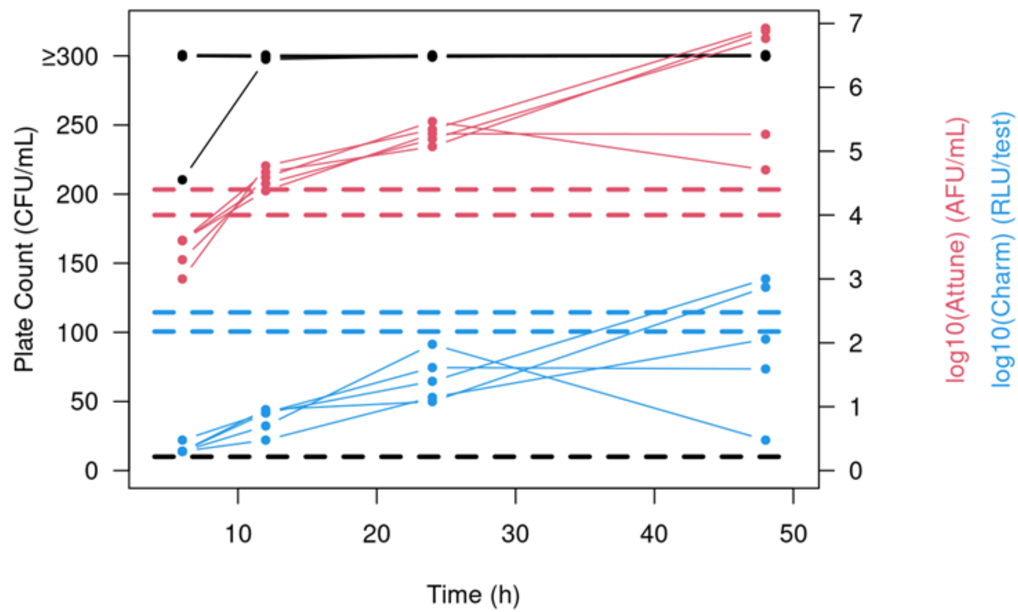

Figure A9. *Geobacillus* 1.

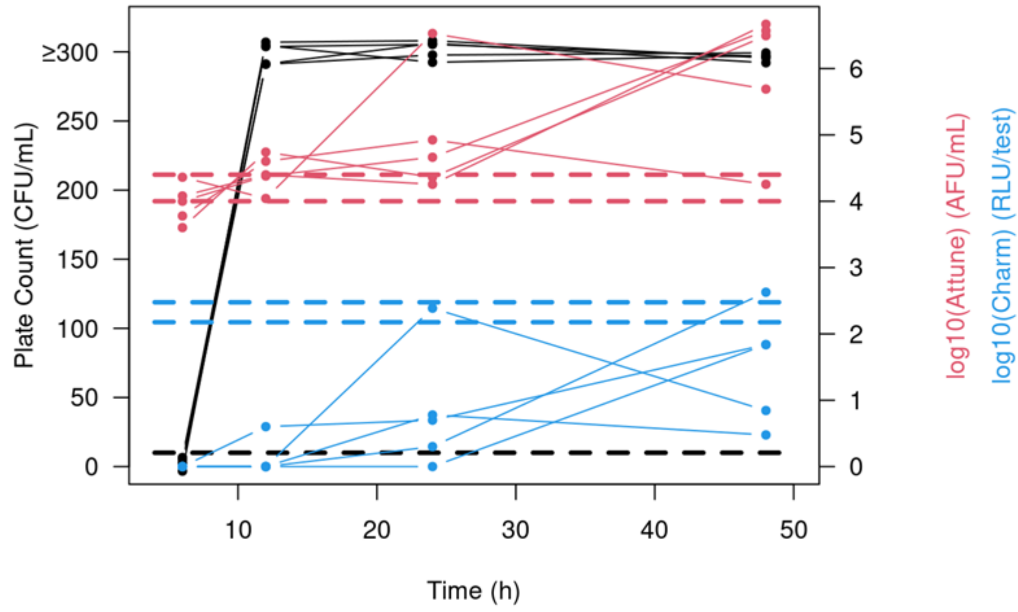

Figure A10. *Geobacillus* 2.

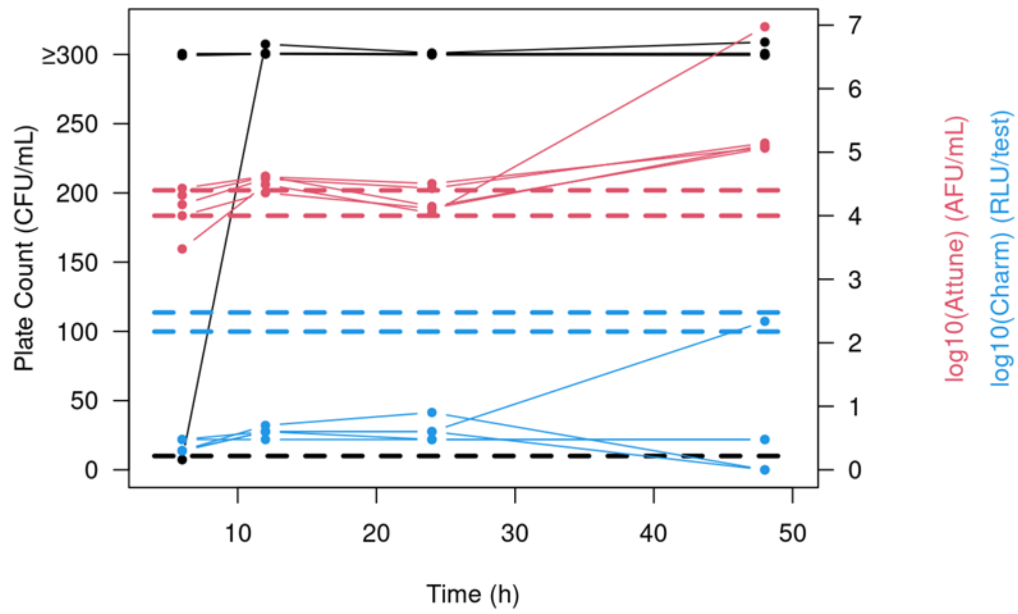

Figure A11. *Geobacillus* 3.

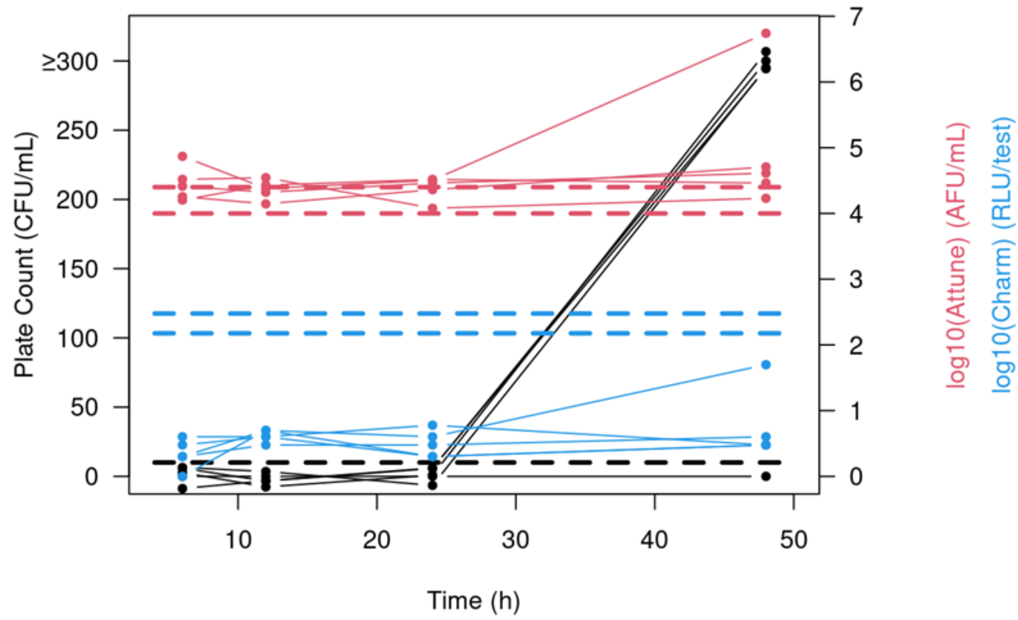

Figure A12. *Geobacillus* 4.

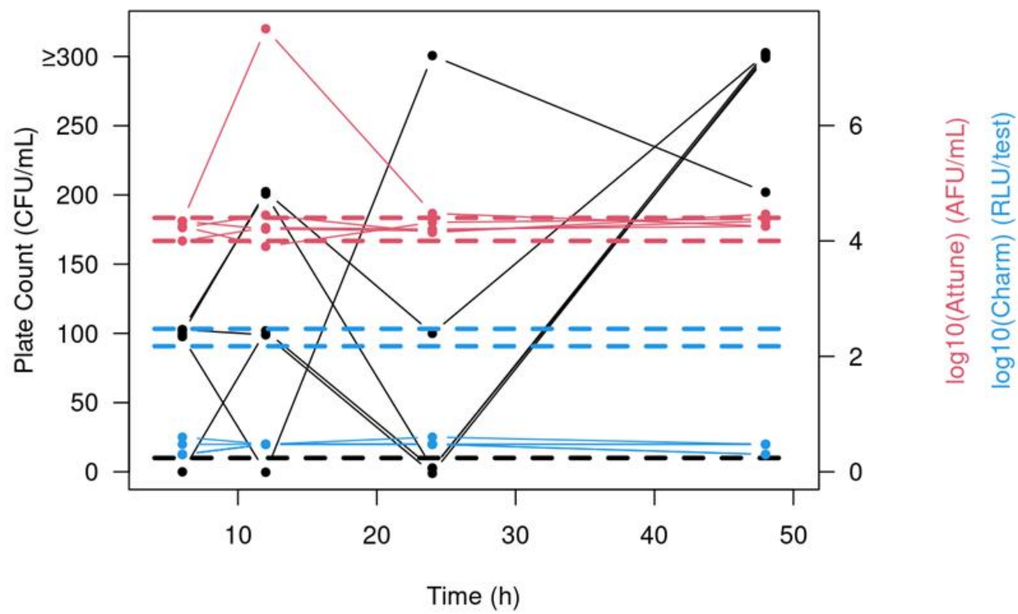

Figure A13. *Anoxybacillus* 5.

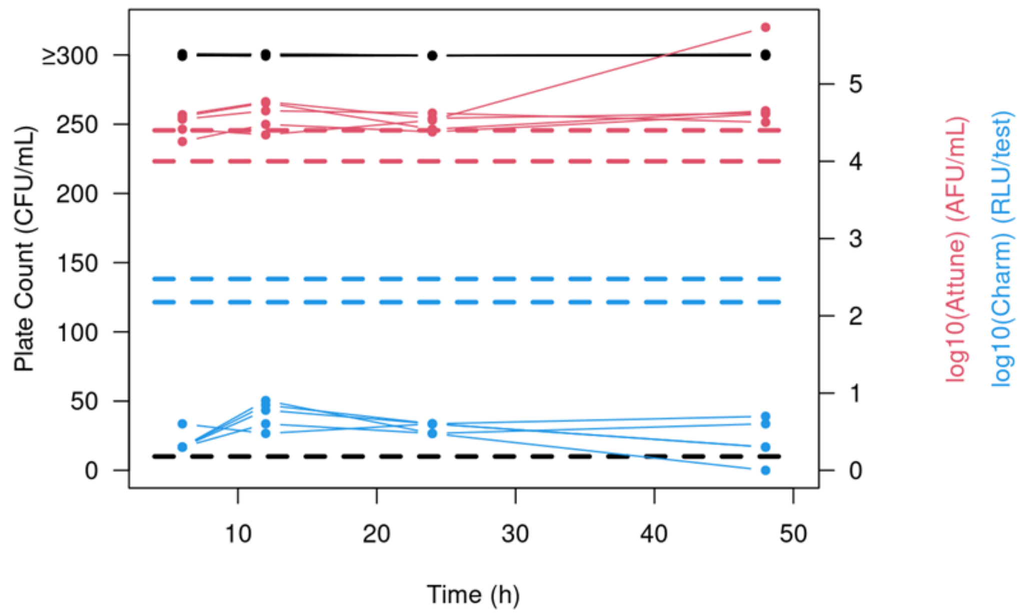

Figure A14. *Anoxybacillus* 6.

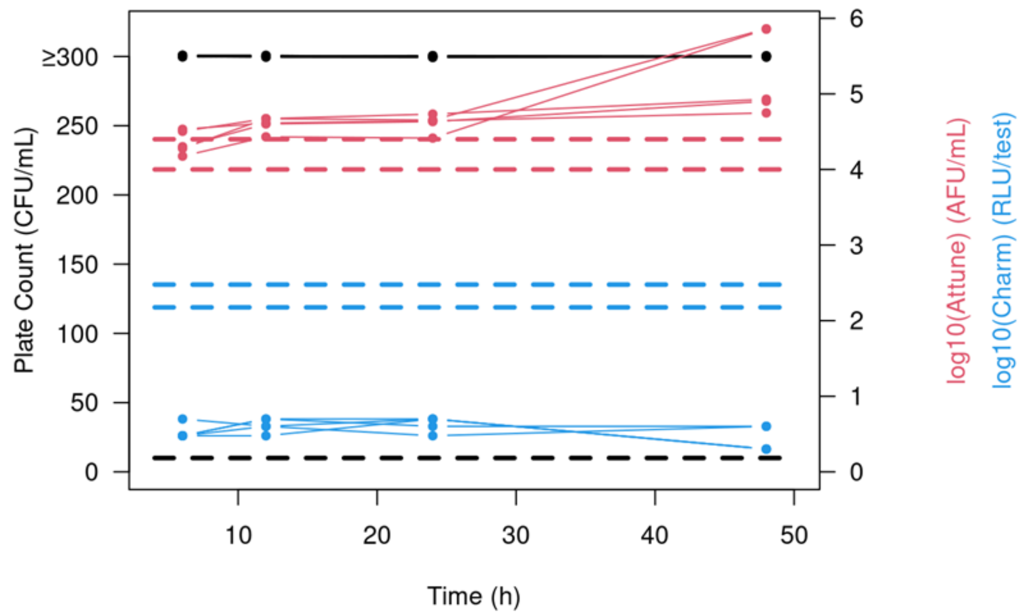

Figure A15. *Anoxybacillus* 7.

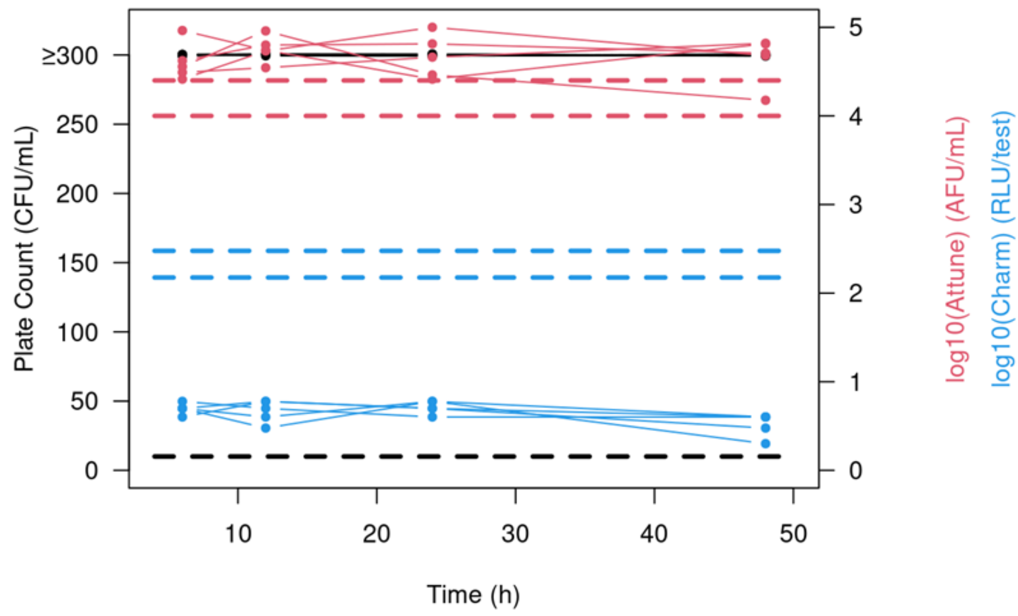

Figure A16. *Anoxybacillus* 8.

UHT whipping cream

Comparison of plate, Charm and Attune methods at different pre-incubation times (12, 24, 48 and 72 hours). The detection threshold for plate count was 10 CFU/mL (black dotted line), for Charm was 2.18-2.48 Log RLU/test (blue dotted lines), and for Attune was 2.5-3.3 Log AFU/mL (red dotted lines).

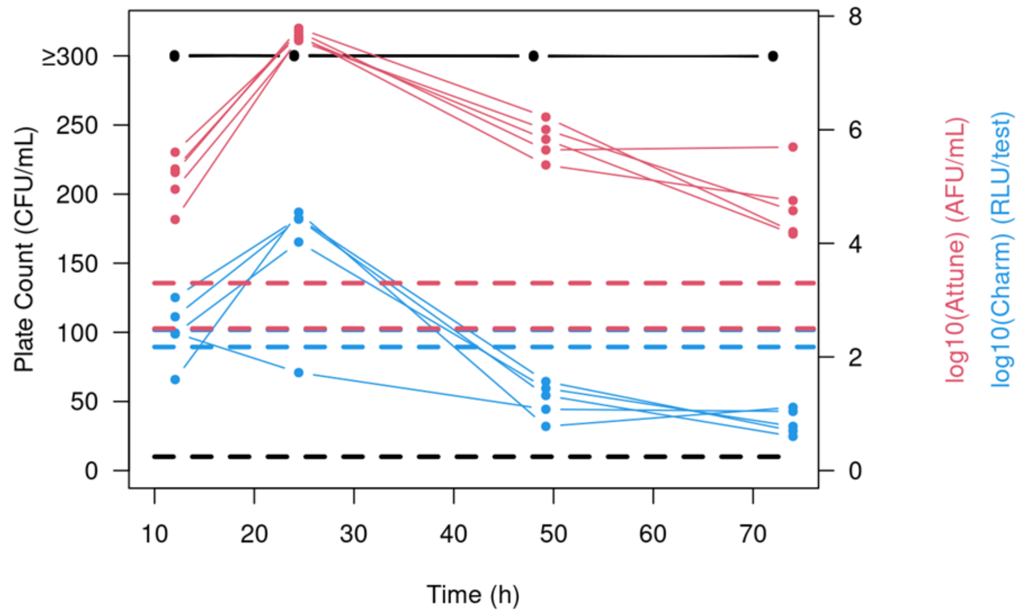

Figure A17. *Geobacillus* 1.

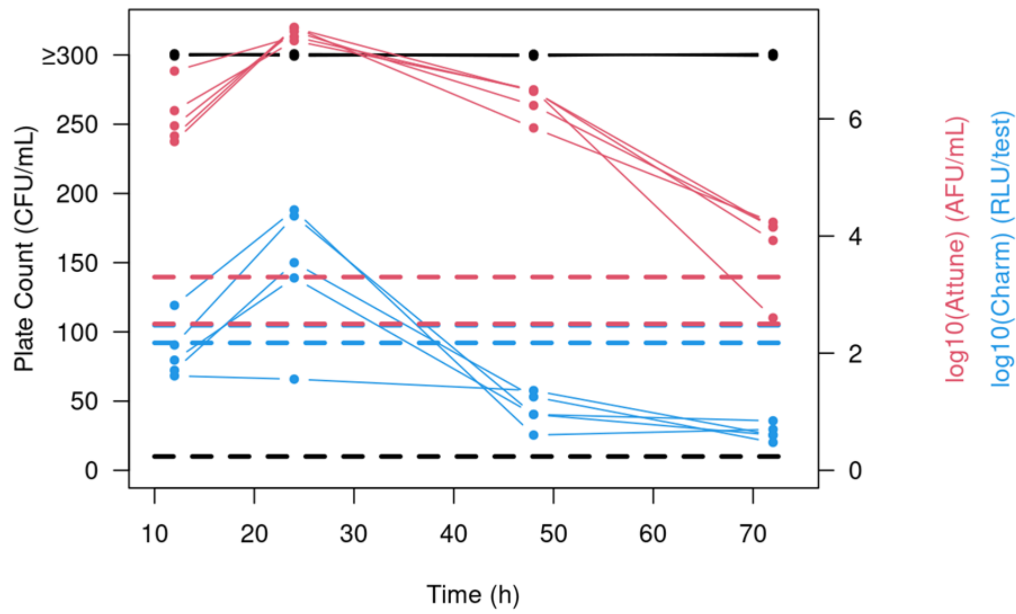

Figure A18. *Geobacillus* 2.

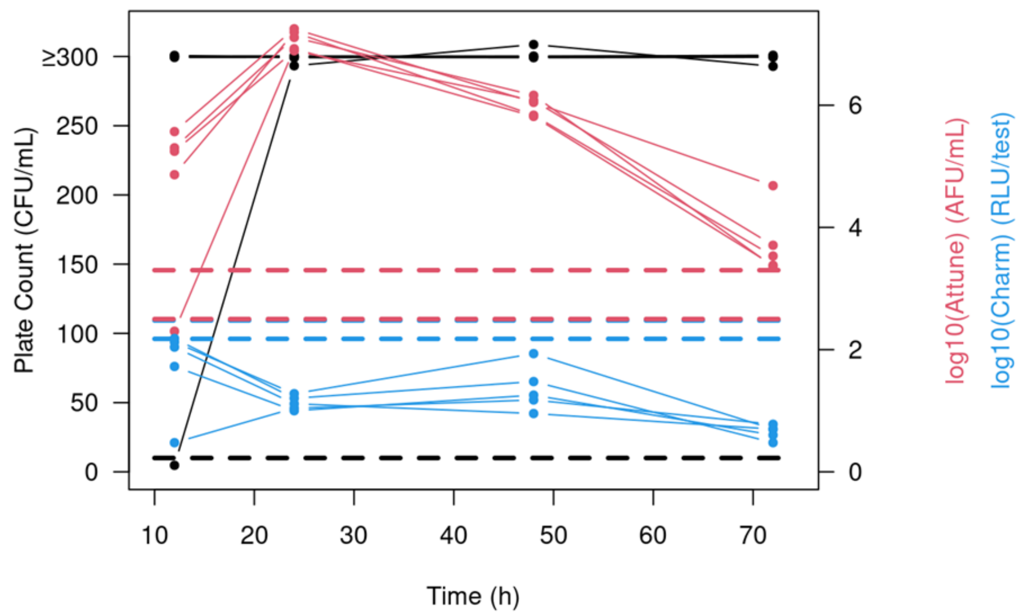

Figure A19. *Geobacillus* 3.

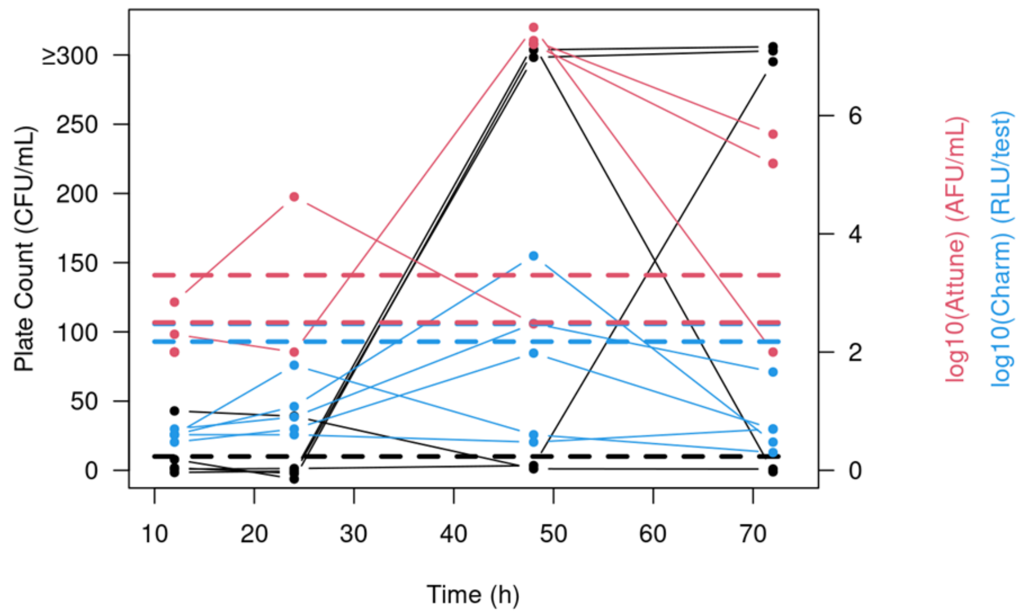

Figure A20. *Geobacillus* 4.

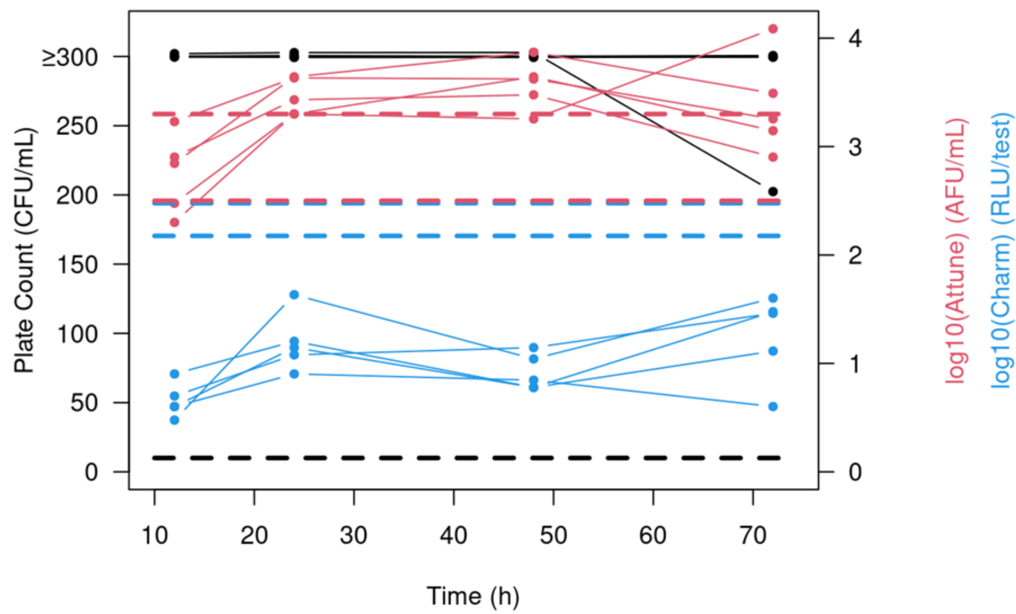

Figure A21. *Anoxybacillus* 5.

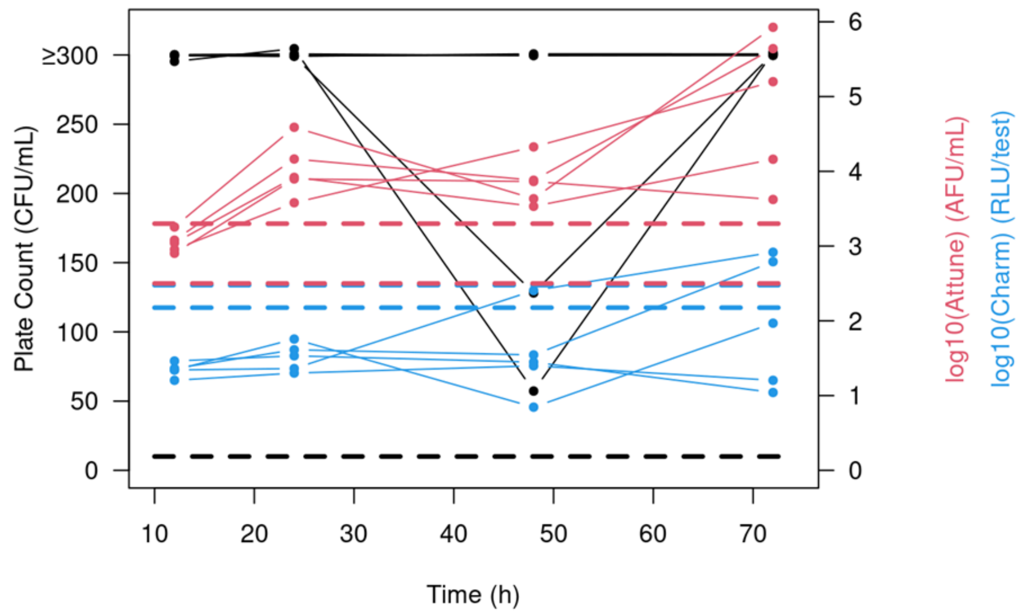

Figure A22. *Anoxybacillus* 6.

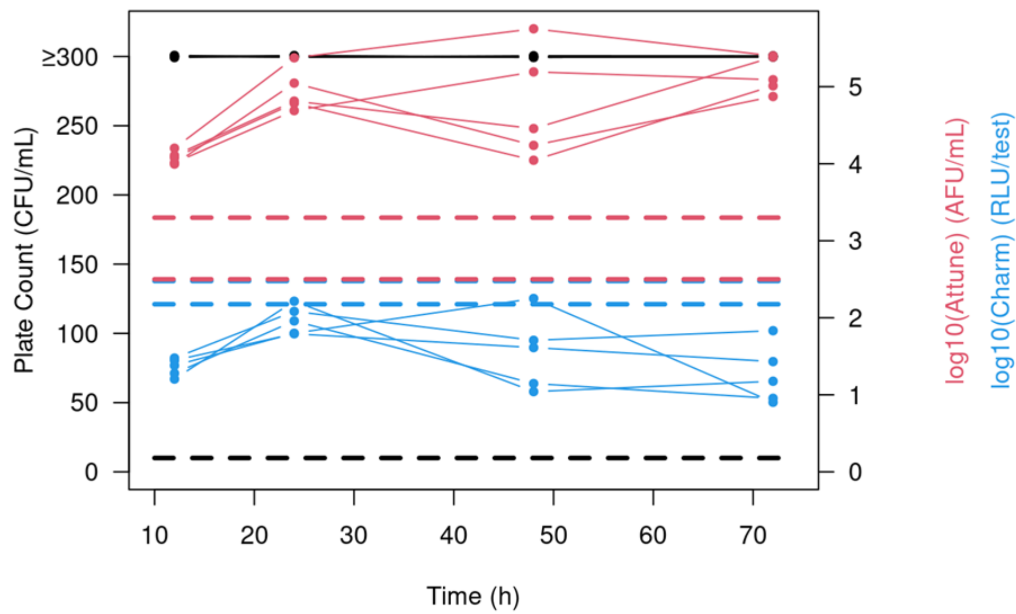

Figure A23. *Anoxybacillus* 7

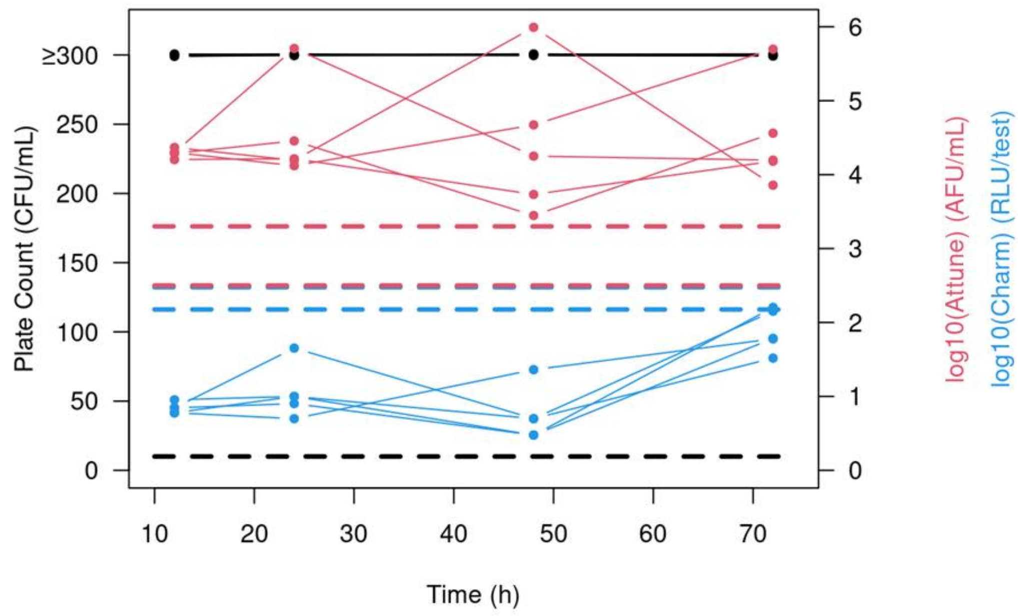

Figure A24. *Anoxybacillus* 8

UHT medical beverage

Comparison of plate, Charm and Attune methods vs time (6, 12, 24 and 48 hours). The detect/not detect threshold for - plate count is 10 CFU/mL (black dotted line), Charm is 2.18-2.48 Log RLU/test (blue dotted lines) and Attune is 3.7-4.1Log AFU/mL (red dotted lines).

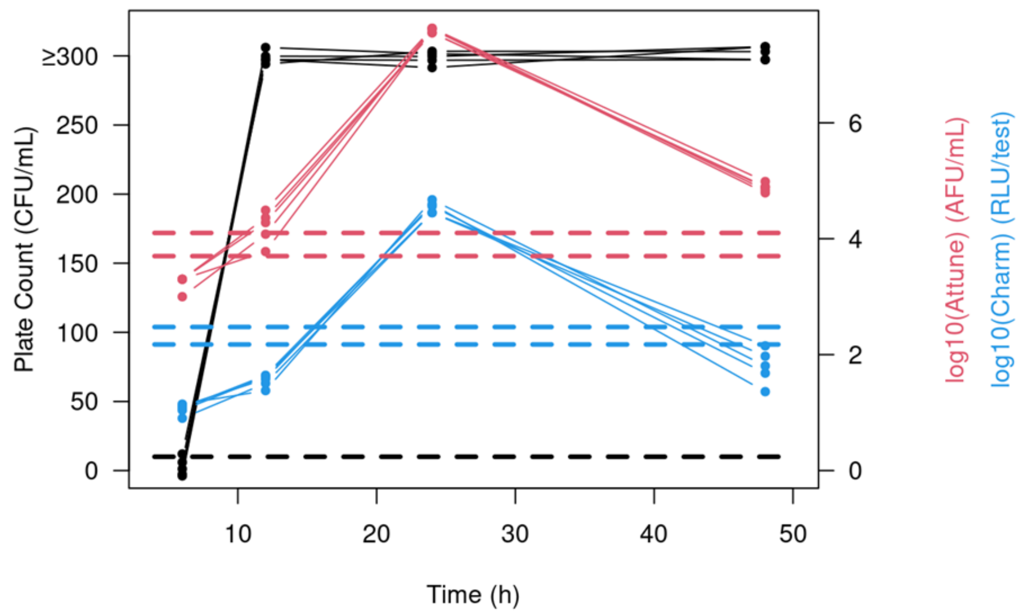

Figure A25. *Geobacillus* 1.

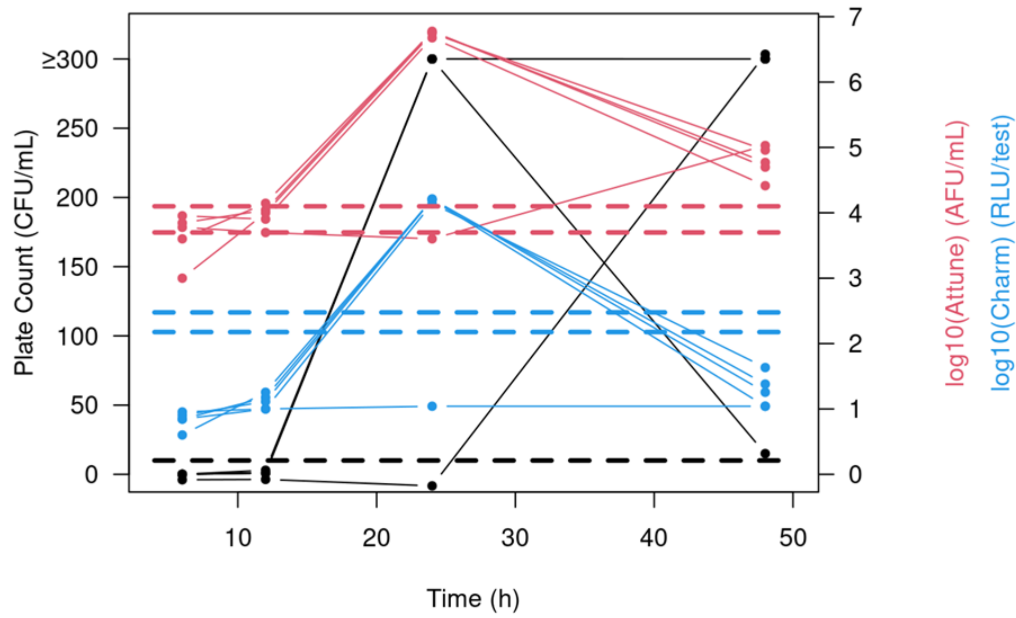

Figure A26. *Geobacillus* 2.

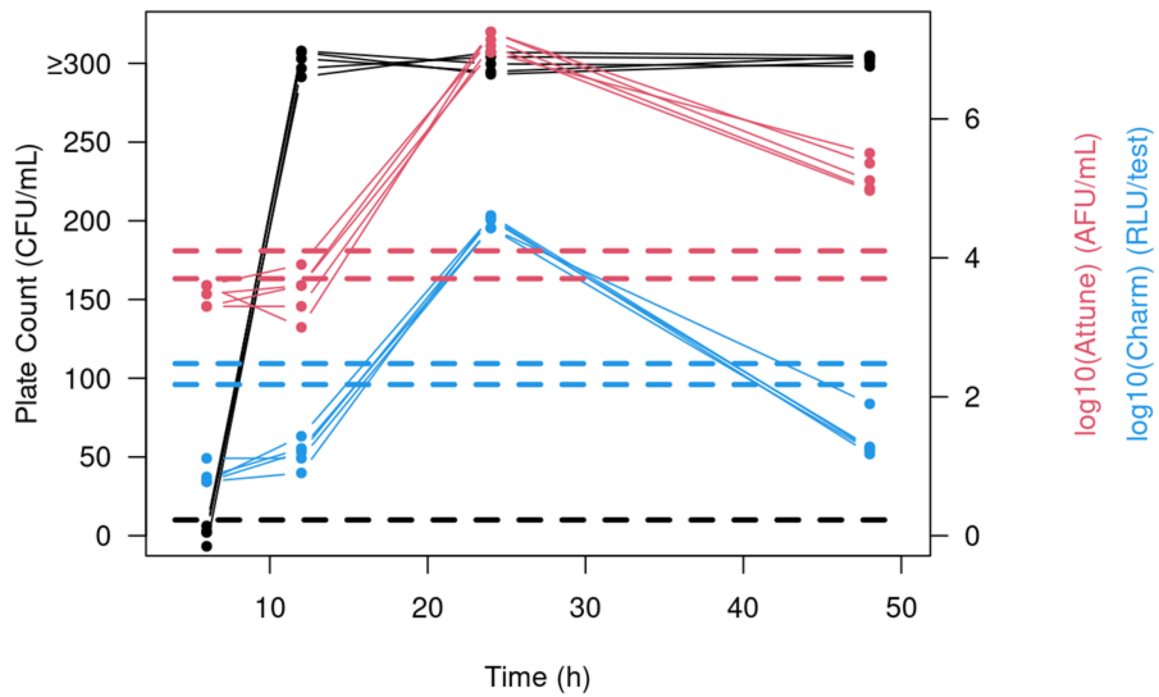

Figure A27. *Geobacillus* 3.

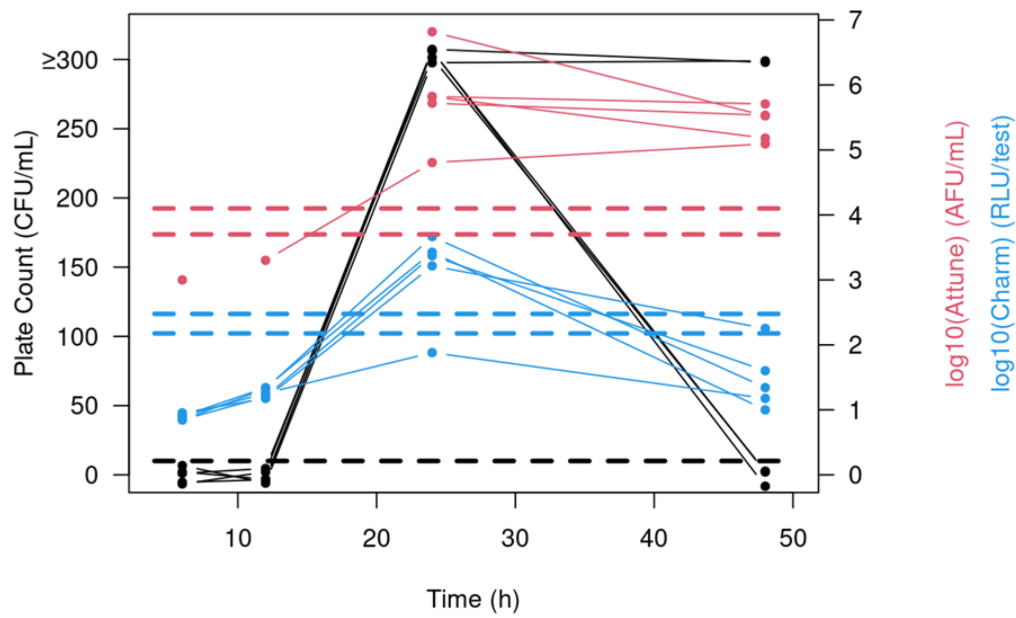

Figure A28. *Geobacillus* 4.

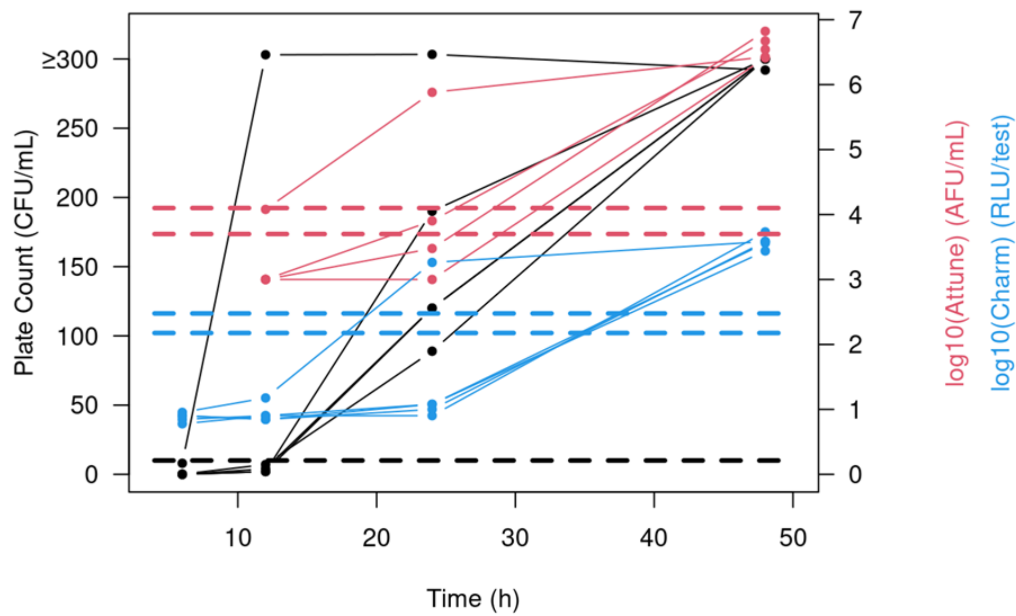

Figure A29. *Anoxybacillus* 5.

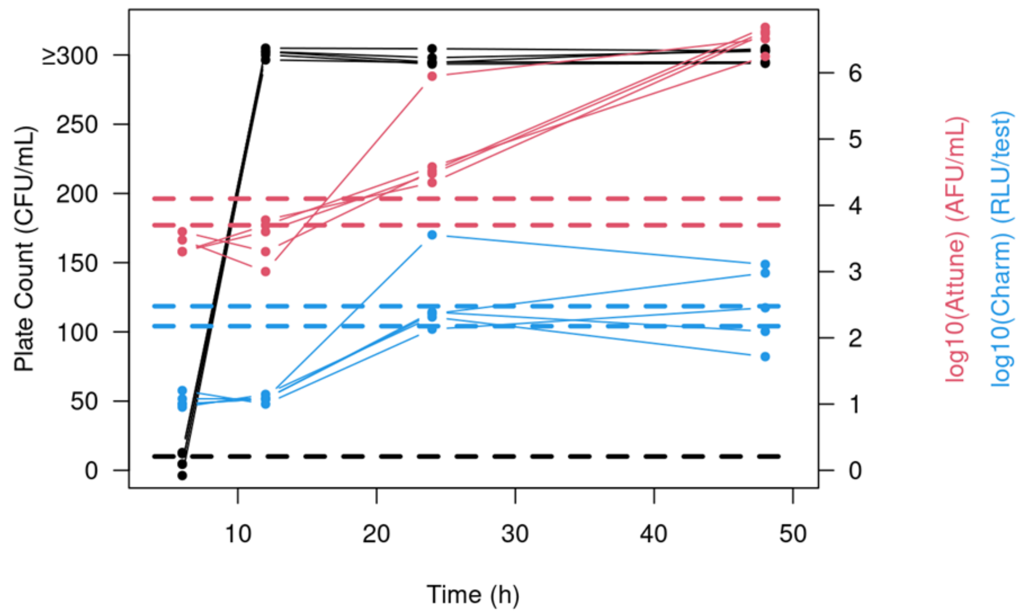

Figure A30. *Anoxybacillus* 6.

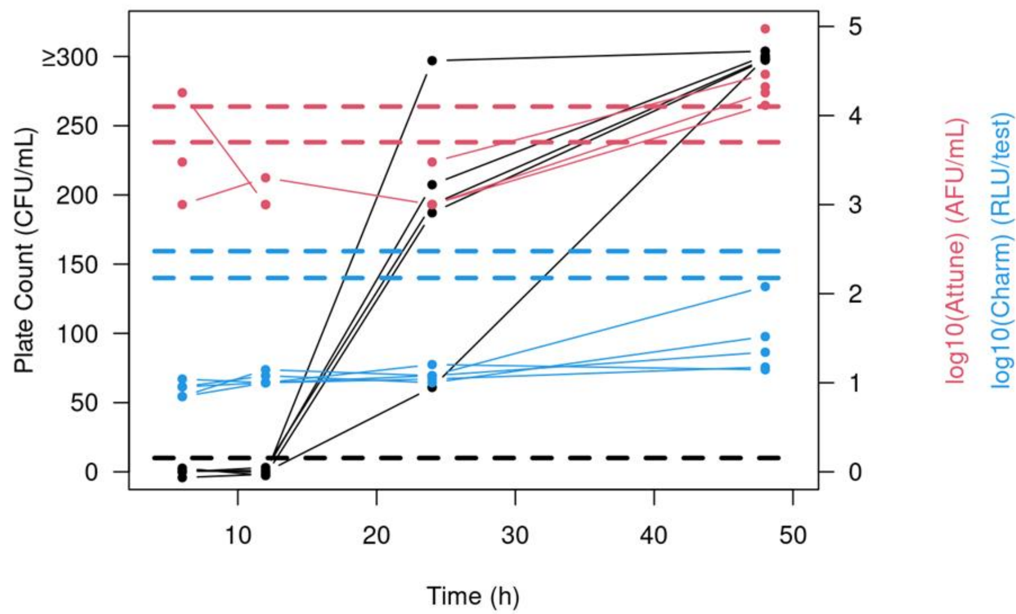

Figure A31. *Anoxybacillus* 7.

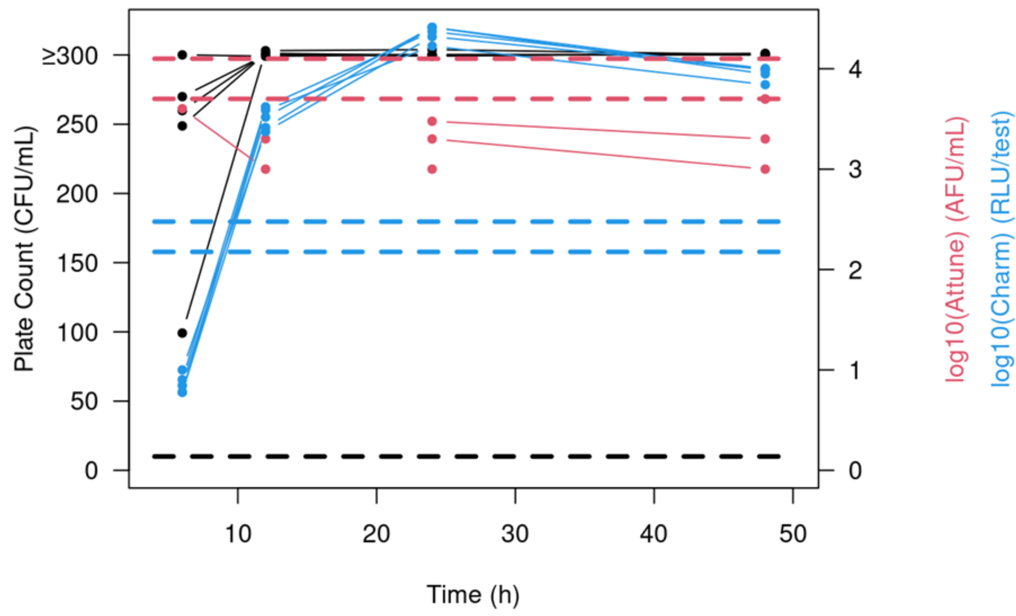

Figure A32. *Anoxybacillus* 8.

Appendix B

<u>UHT milk accuracy</u>

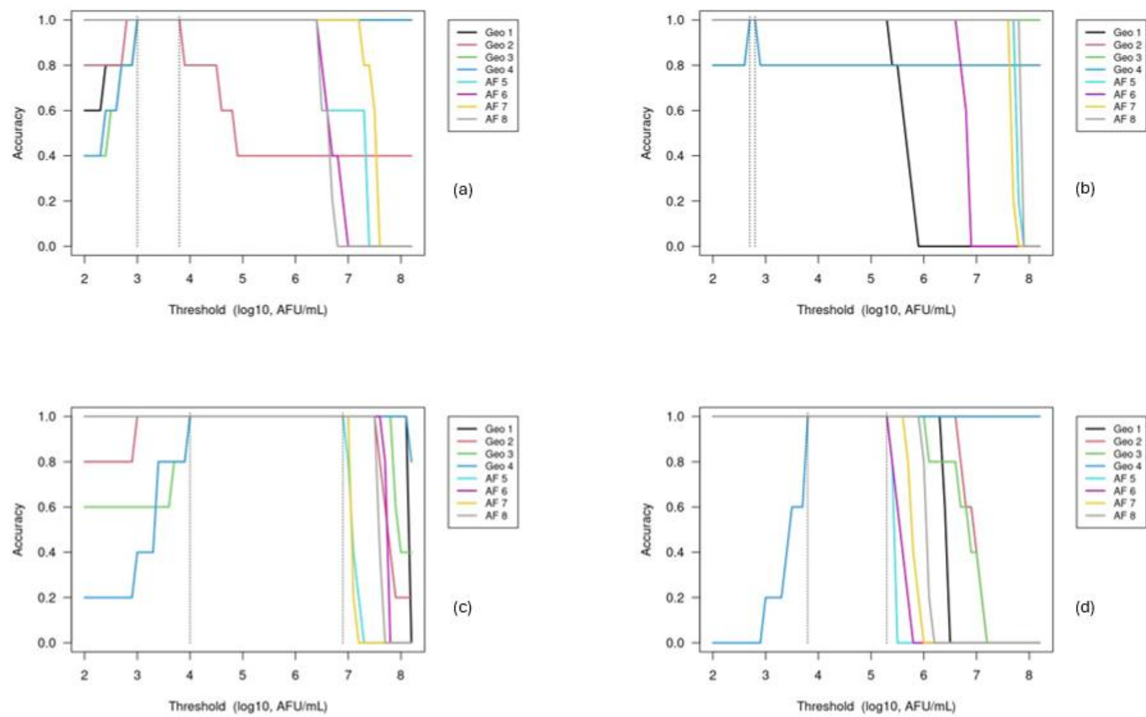

Figure B1. Relationship of accuracy with Attune Nxt flow cytometry detection threshold for all eight bacterial isolates at 6 (a), 12 (b), 24 (c) and 48 (d) hours. The dotted lines depict threshold values of where accuracy was 1.0.

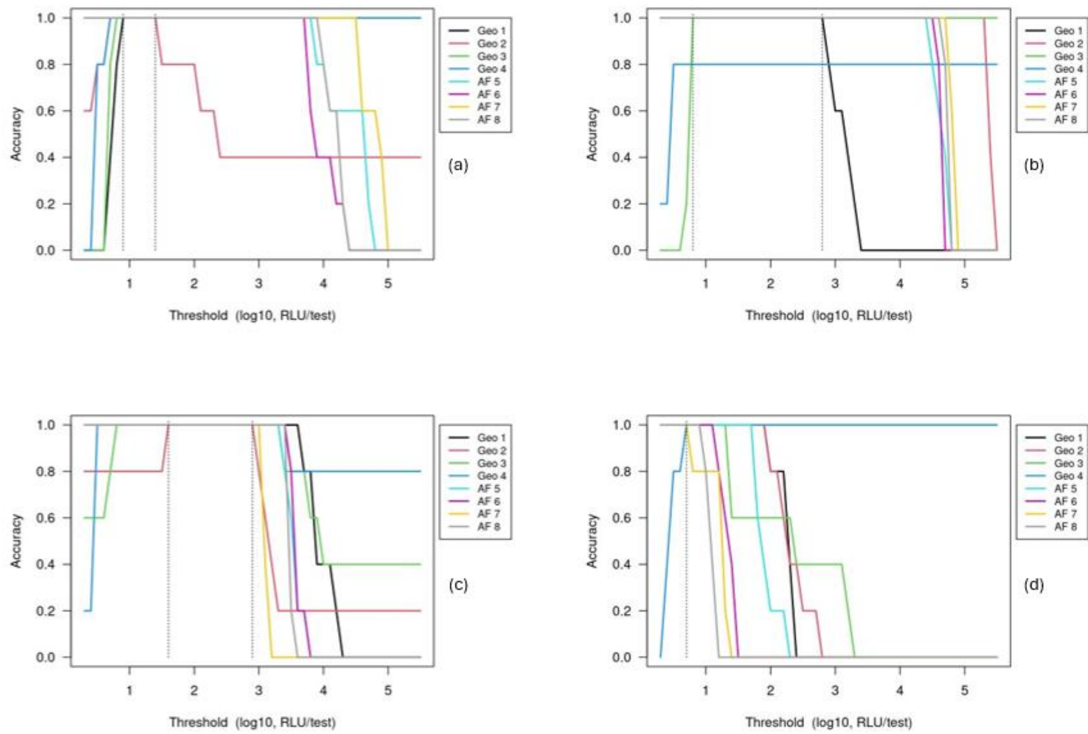

Figure B2. Relationship of accuracy with Charm method detection threshold for all eight bacterial isolates at 6 (a), 12 (b), 24 (c) and 48 (d) hours. The dotted lines depict threshold values of where accuracy was 1.0.

UHT in-house cream

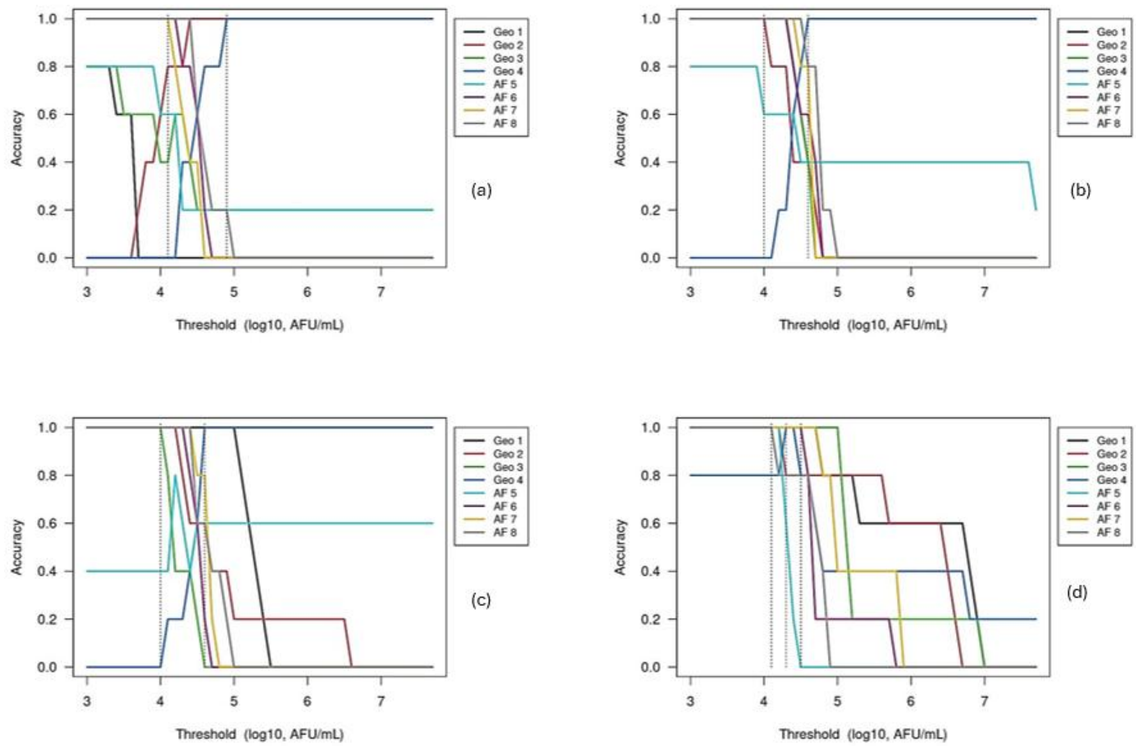

Figure B3. Relationship of accuracy with Attune Nxt flow cytometry detection threshold for all eight bacterial isolates at 6 hours. The dotted lines depict threshold where accuracy was unatainable at 1.0.

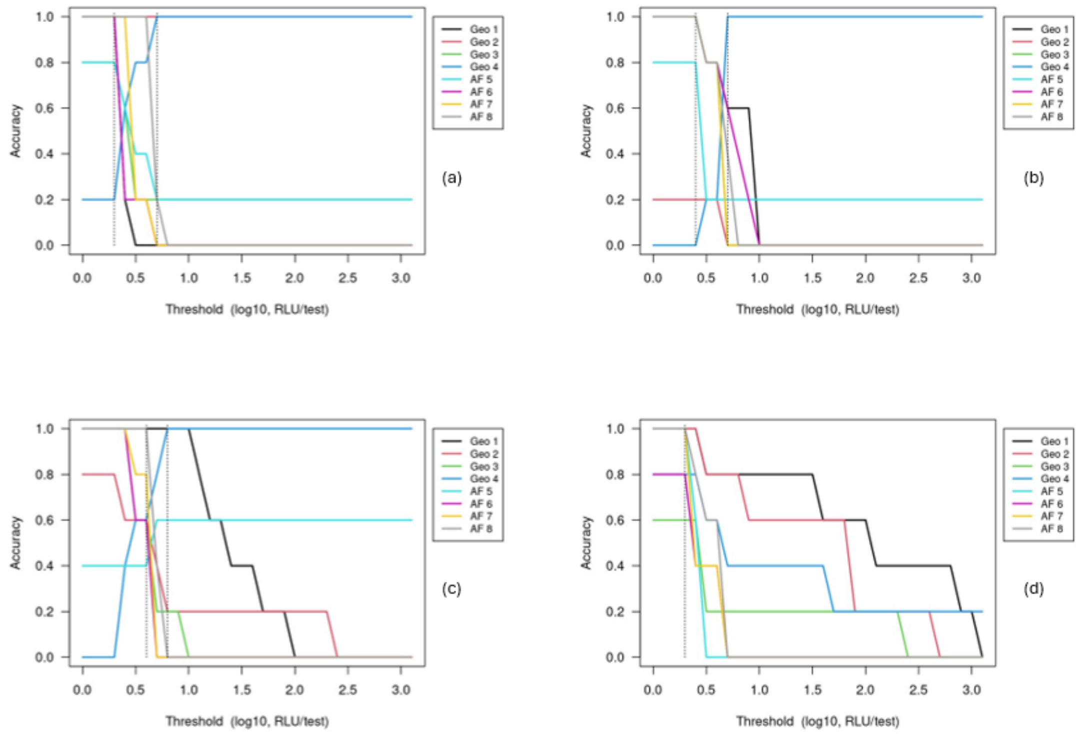

Figure B4. Relationship of accuracy with Charm detection threshold for all eight bacterial isolates at 6 (a), 12 (b), 24 (c) and 48 (d) hours. The dotted lines depict threshold values where accuracy of 1.0 was unattainable.

UHT whipping cream

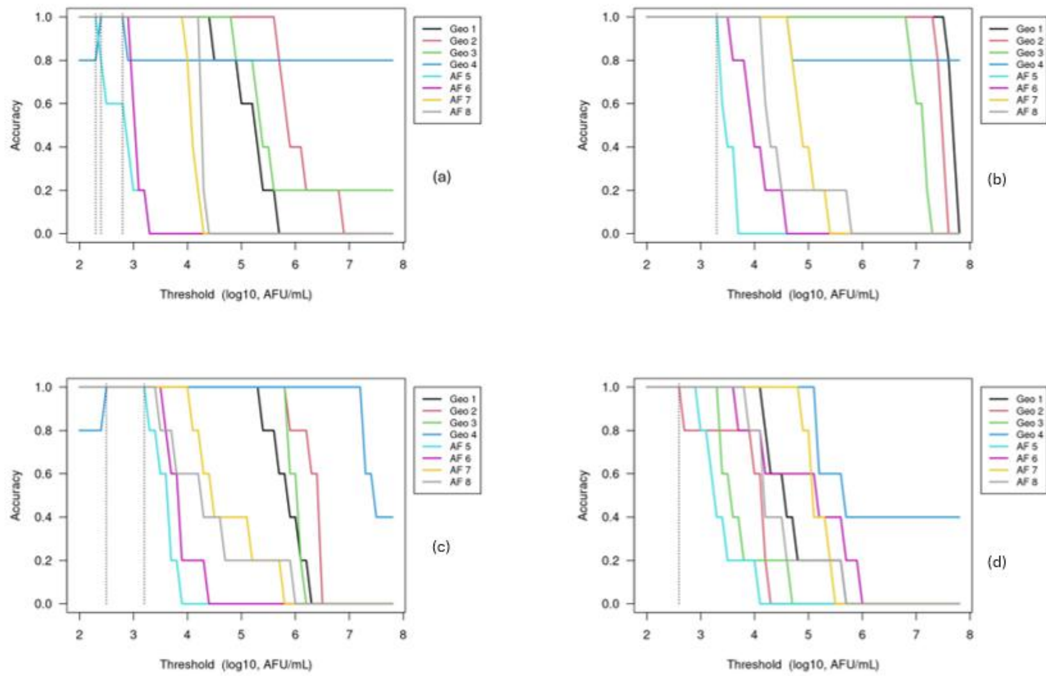

Figure B5. Relationship of accuracy with Attune Nxt flow cytometry detection threshold for all eight bacterial isolates where accuracy of 1.0 was attained.

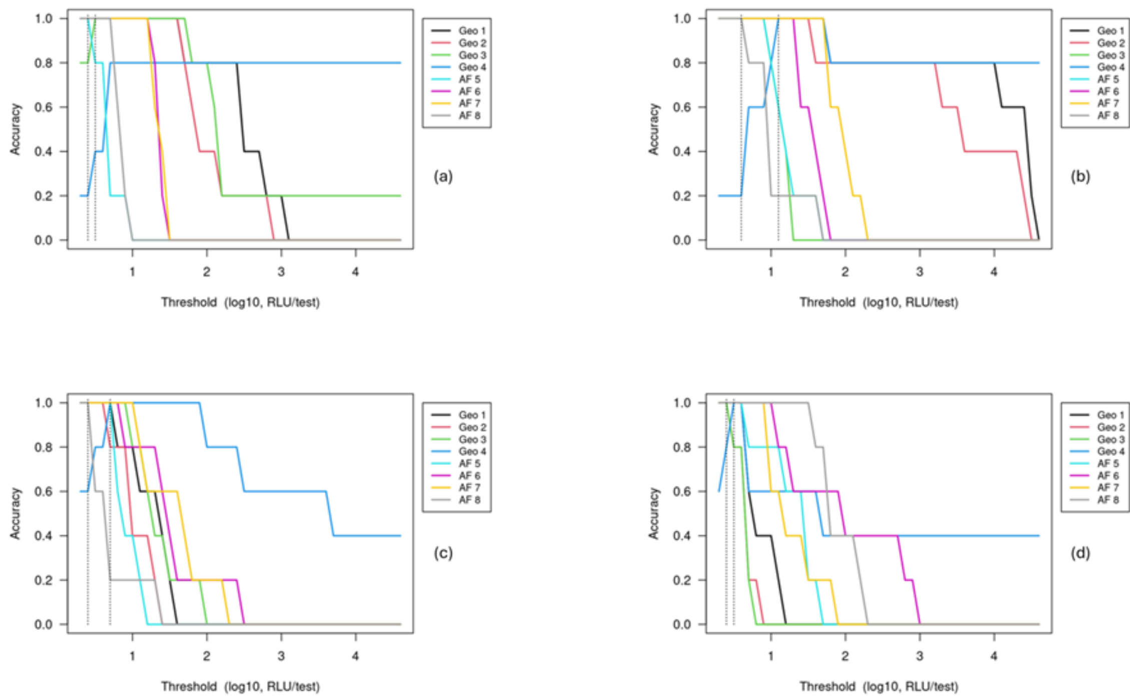

Figure B6. Relationship of accuracy with Charm detection threshold for all eight bacterial isolates at 12 (a), 24 (b), 48 (c) and 72 (d) hours.

UHT medical beverage

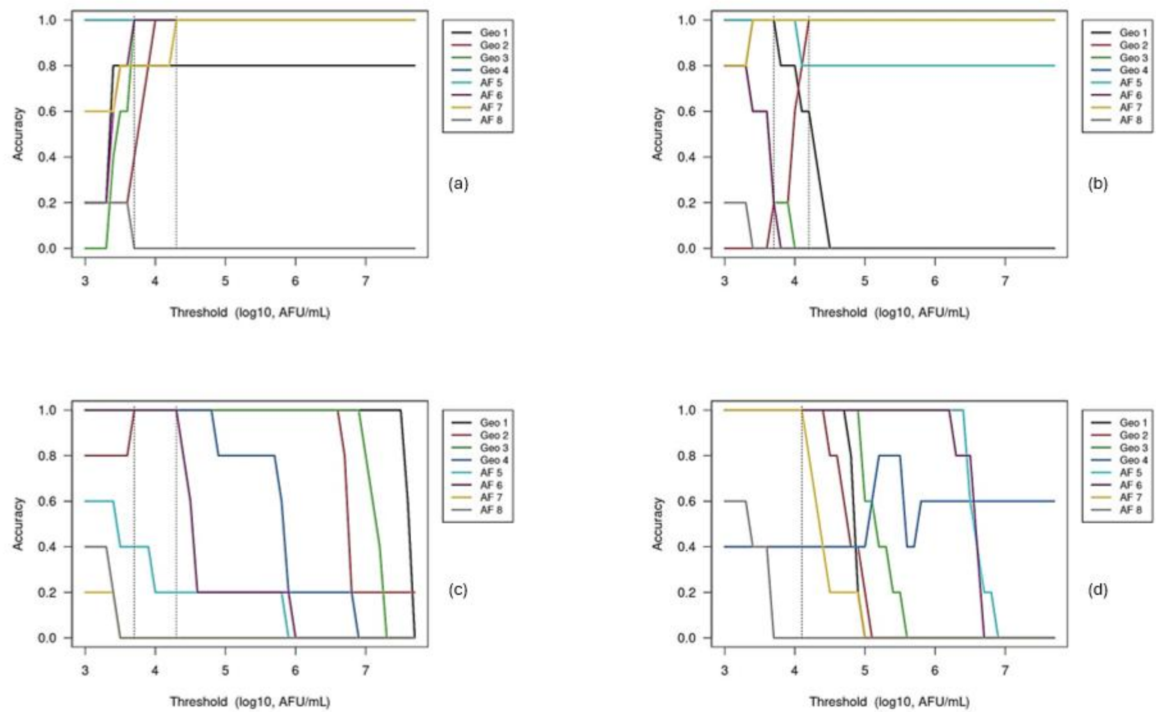

Figure B7. Relationship of accuracy with Attune Nxt flow cytometry detection threshold for all eight bacterial isolates at 6 (a), 12 (b), 24 (c), 48 (d) hours. The dotted lines depict threshold values where accuracy was 1.0 for some of the isolates.

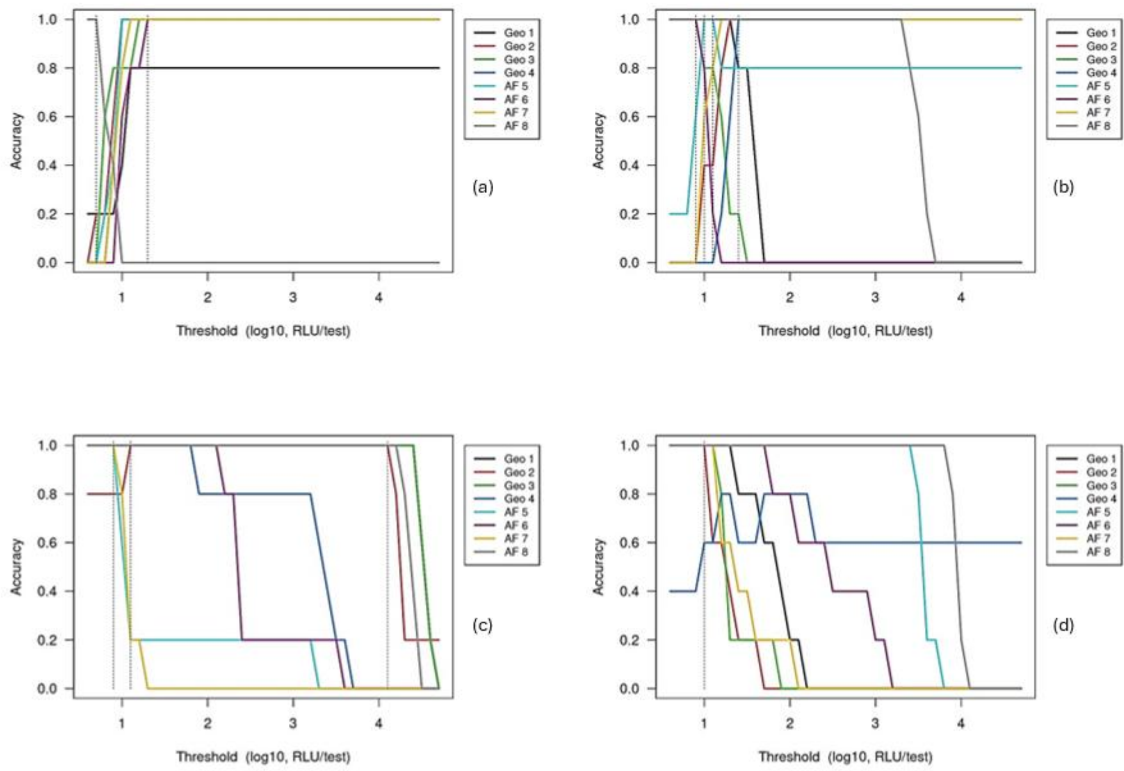

Figure B8. (a) Relationship of accuracy with Charm detection threshold for all eight bacterial isolates at 6 hours. The dotted lines depict threshold value where the best accuracy was achieved.

Appendix C

<u>UHT milk</u>

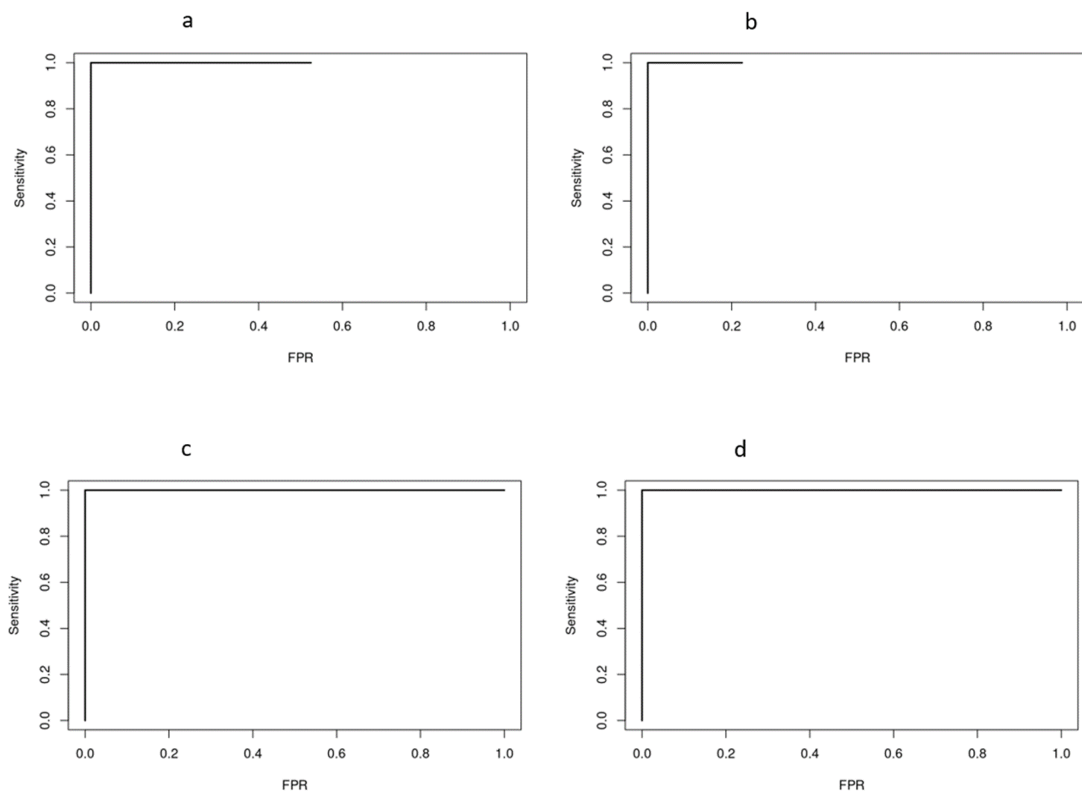

Figure C1. ROC curves of the mean of all bacterial strains for 6(a), 12(b), 24(c) and 48(d) hours with Attune method.

UHT in house cream

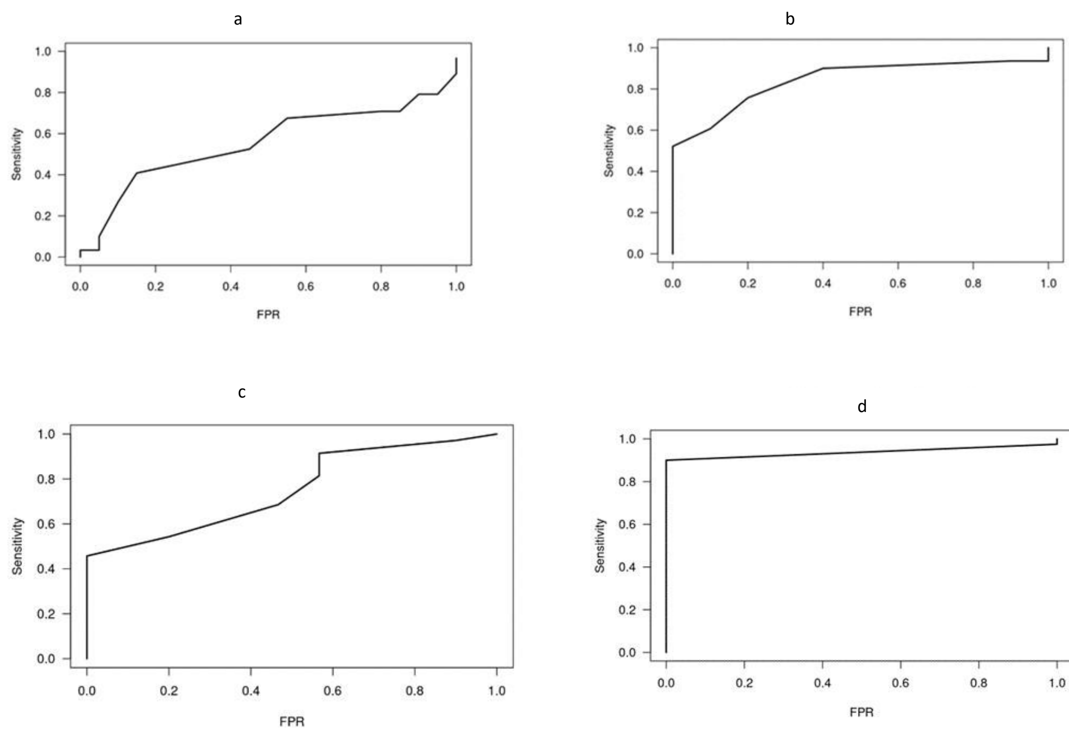

Figure C2. ROC curves of the mean of all bacterial strains for 6(a), 12(b), 24(c) and 48(d) hours with the Attune.

UHT whipping cream

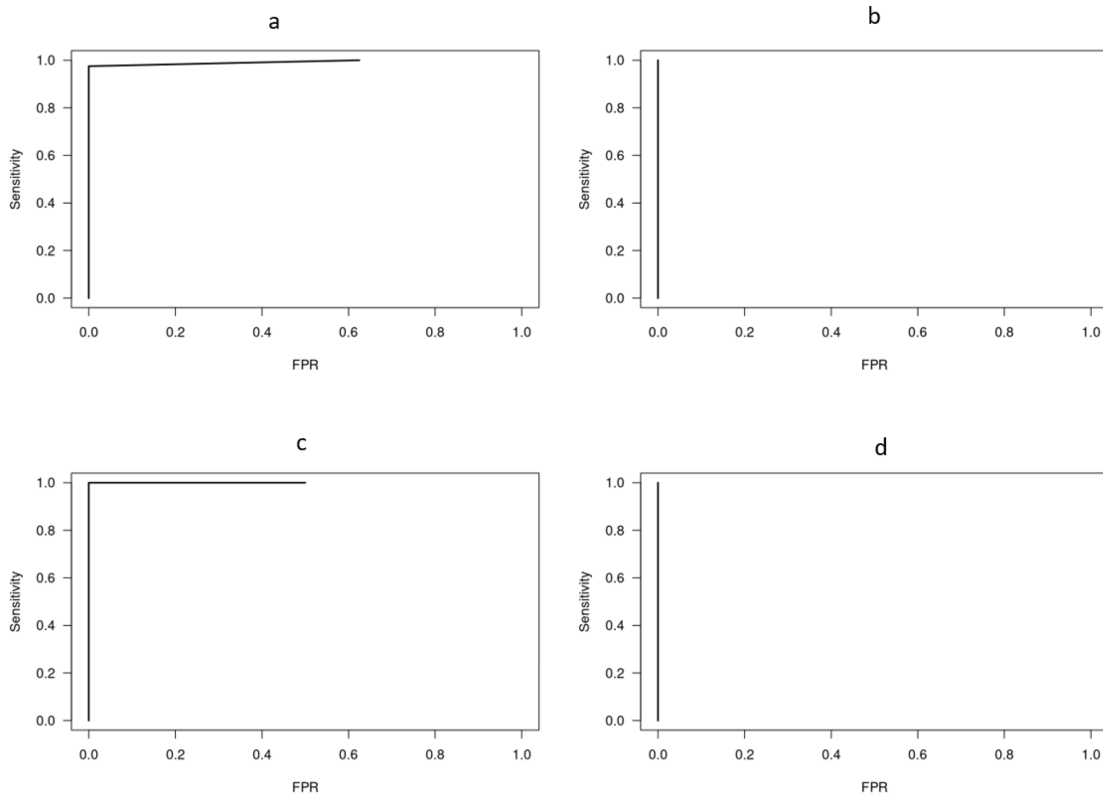

Figure C3. ROC curves of the mean of all bacterial strains for each timepoint; 12(a), 24(b), 48(c) and 72(d) hours with the Attune.

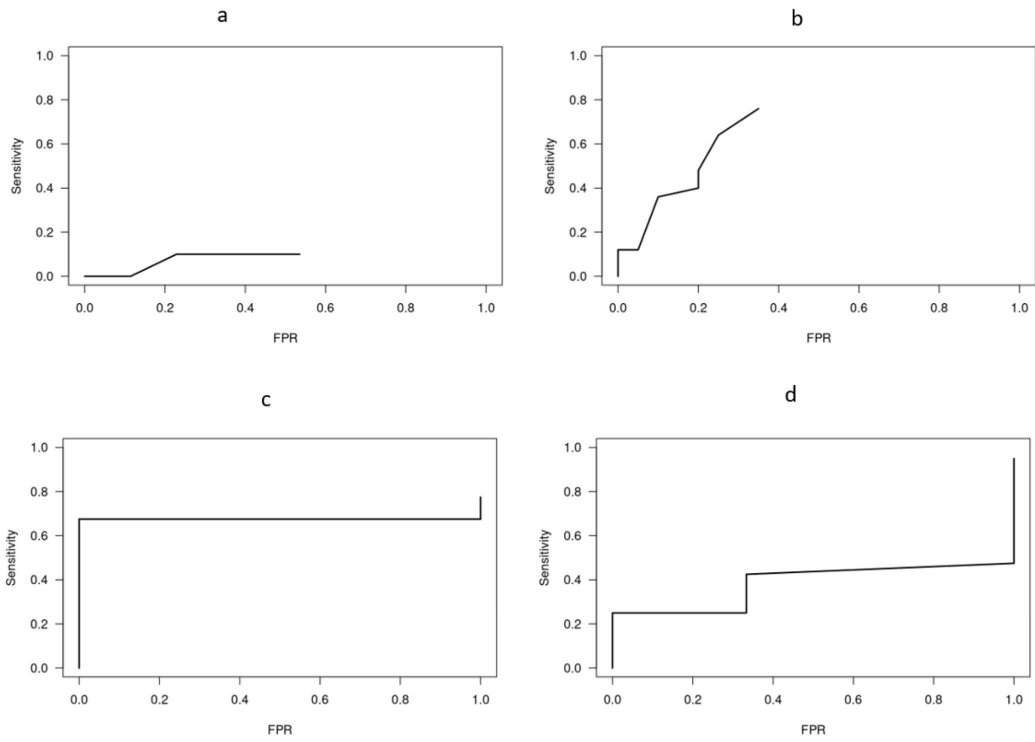

Figure C4. ROC curves of the mean of all bacterial strains for each timepoint; 6(a), 12 (b), 24(c) and 48(d) hours with the Attune method.

Appendix D

UHT in-house cream

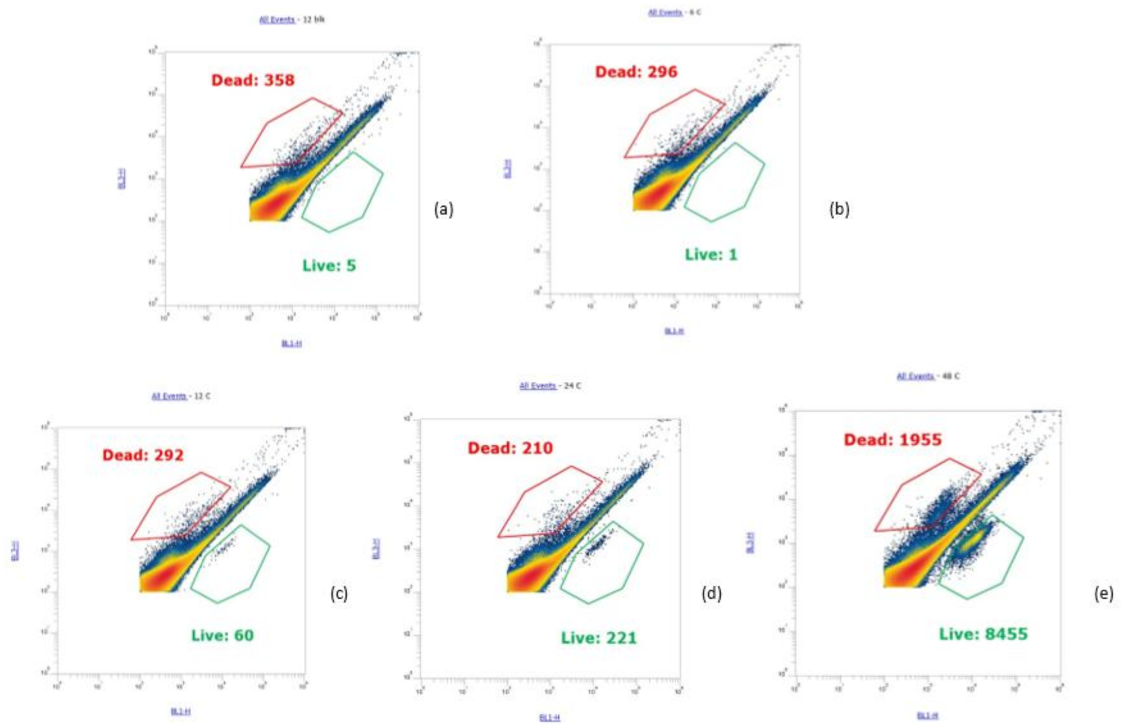

Figure D1. Density scatter plots from the Attune with live (green) and dead (red) thermophile cell gates, with examples from the Blank (a), 6 (b), 12 (c), 24 (d) and 48 (e) hour pre-incubation times.

UHT whipping cream

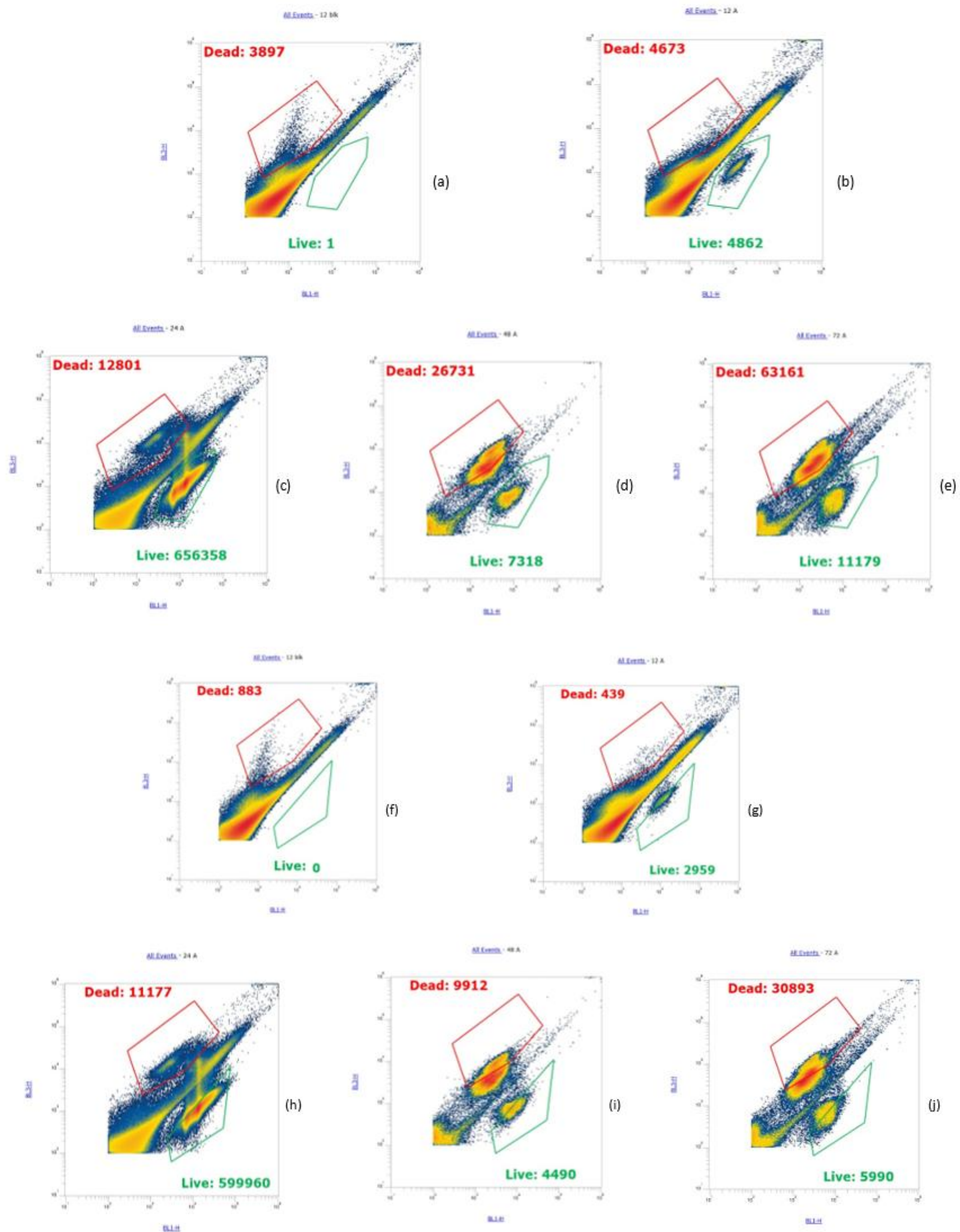

Figure D2. Density scatter plots from the Attune with live (green) and dead (red) thermophile cell gates, with examples from the Blank (a), 12 (b), 24 (c), 48 (d), 72 (e) hour pre-incubation times before gate adjustment and Blank (f), 12 (g), 24 (h), 48 (i), 72 (j) hour pre-incubation times after gate adjustment.

UHT medical beverage

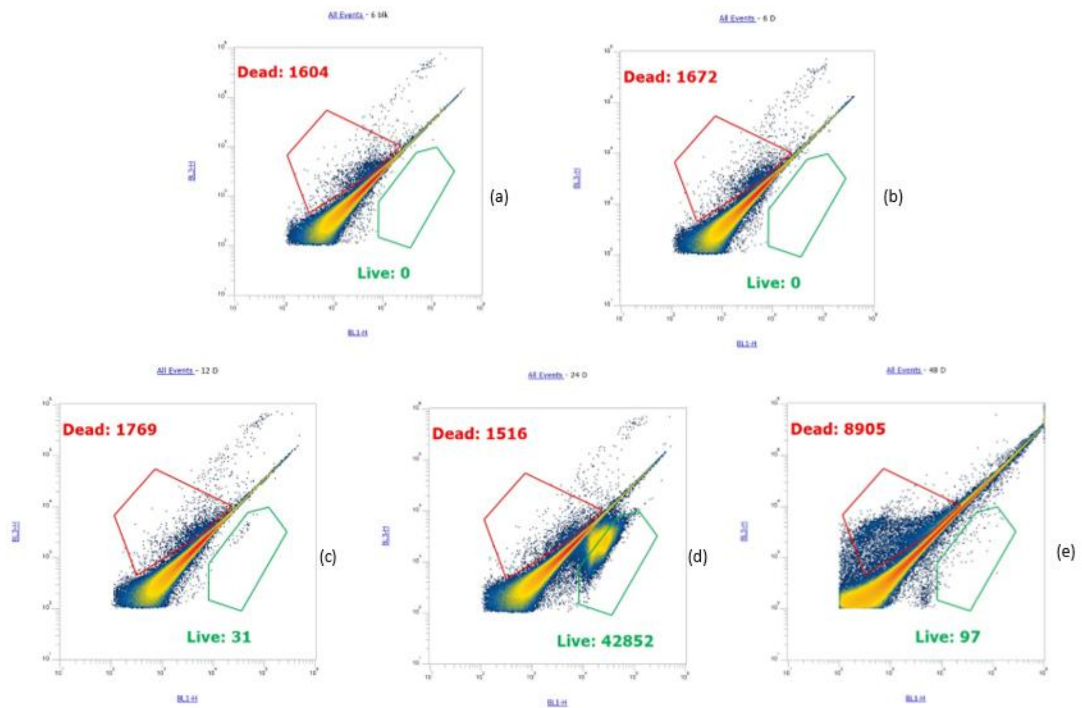

Figure D3. Density scatter plots from the Attune with live (green) and dead (red) thermophile cell gates, with examples from the Blank (a), 6 (b), 12 (c), 24 (d), 48 (e) hour pre-incubation times with medical beverage.

Appendix E

UHT milk Mean of all bacterial strains (four *Geobacillus* sp. and four *Anoxybacillus flavithermus*) expressed as threshold index for the Attune at different time points including True Positive (TP), False Positive (FN), True Negative (TN), False Negative (FN), Sensitivity, Specificity, Positive Predictive value (PPV), Negative Predictive Value (NPV), False Predictive Rate (FPR), Prevalence and Accuracy.

Table E1. 6 hours

Index	TP	FP	FN	TN	Sen	Spe	PPV	NPV	FPR	Prevalence	Accuracy
2.0	2.88	1.13	0.00	1.00	1.00	0.48	0.59	1.00	0.53	0.58	0.78
2.1	2.88	1.13	0.00	1.00	1.00	0.48	0.59	1.00	0.53	0.58	0.78
2.2	2.88	1.13	0.00	1.00	1.00	0.48	0.59	1.00	0.53	0.58	0.78
2.3	2.88	1.13	0.00	1.00	1.00	0.48	0.59	1.00	0.53	0.58	0.78
2.4	2.88	0.88	0.00	1.25	1.00	0.58	0.59	1.00	0.43	0.58	0.83
2.5	2.88	0.75	0.00	1.38	1.00	0.63	0.59	1.00	0.38	0.58	0.85
2.6	2.88	0.75	0.00	1.38	1.00	0.63	0.59	1.00	0.38	0.58	0.85
2.7	2.88	0.50	0.00	1.63	1.00	0.73	0.59	1.00	0.28	0.58	0.90
2.8	2.88	0.38	0.00	1.75	1.00	0.85	0.63	1.00	0.15	0.58	0.93
2.9	2.88	0.38	0.00	1.75	1.00	0.85	0.63	1.00	0.15	0.58	0.93
3.0	2.88	0.00	0.00	2.13	1.00	1.00	1.00	1.00	0.00	0.58	1.00
3.1	2.88	0.00	0.00	2.13	1.00	1.00	1.00	1.00	0.00	0.58	1.00
3.2	2.88	0.00	0.00	2.13	1.00	1.00	1.00	1.00	0.00	0.58	1.00
3.3	2.88	0.00	0.00	2.13	1.00	1.00	1.00	1.00	0.00	0.58	1.00
3.4	2.88	0.00	0.00	2.13	1.00	1.00	1.00	1.00	0.00	0.58	1.00
3.5	2.88	0.00	0.00	2.13	1.00	1.00	1.00	1.00	0.00	0.58	1.00
3.6	2.88	0.00	0.00	2.13	1.00	1.00	1.00	1.00	0.00	0.58	1.00
3.7	2.88	0.00	0.00	2.13	1.00	1.00	1.00	1.00	0.00	0.58	1.00
3.8	2.88	0.00	0.00	2.13	1.00	1.00	1.00	1.00	0.00	0.58	1.00
3.9	2.75	0.00	0.13	2.13	0.93	1.00	1.00	0.92	0.00	0.58	0.98
4.0	2.75	0.00	0.13	2.13	0.93	1.00	1.00	0.92	0.00	0.58	0.98
4.1	2.75	0.00	0.13	2.13	0.93	1.00	1.00	0.92	0.00	0.58	0.98
4.2	2.75	0.00	0.13	2.13	0.93	1.00	1.00	0.92	0.00	0.58	0.98
4.3	2.75	0.00	0.13	2.13	0.93	1.00	1.00	0.92	0.00	0.58	0.98
4.4	2.75	0.00	0.13	2.13	0.93	1.00	1.00	0.92	0.00	0.58	0.98
4.5	2.75	0.00	0.13	2.13	0.93	1.00	1.00	0.92	0.00	0.58	0.98
4.6	2.63	0.00	0.25	2.13	0.87	1.00	1.00	0.88	0.00	0.58	0.95
4.7	2.63	0.00	0.25	2.13	0.87	1.00	1.00	0.88	0.00	0.58	0.95
4.8	2.63	0.00	0.25	2.13	0.87	1.00	1.00	0.88	0.00	0.58	0.95
4.9	2.50	0.00	0.38	2.13	0.80	1.00	1.00	0.85	0.00	0.58	0.93
5.0	2.50	0.00	0.38	2.13	0.80	1.00	1.00	0.85	0.00	0.58	0.93
5.1	2.50	0.00	0.38	2.13	0.80	1.00	1.00	0.85	0.00	0.58	0.93
5.2	2.50	0.00	0.38	2.13	0.80	1.00	1.00	0.85	0.00	0.58	0.93
5.3	2.50	0.00	0.38	2.13	0.80	1.00	1.00	0.85	0.00	0.58	0.93
5.4	2.50	0.00	0.38	2.13	0.80	1.00	1.00	0.85	0.00	0.58	0.93
5.5	2.50	0.00	0.38	2.13	0.80	1.00	1.00	0.85	0.00	0.58	0.93
5.6	2.50	0.00	0.38	2.13	0.80	1.00	1.00	0.85	0.00	0.58	0.93
5.7	2.50	0.00	0.38	2.13	0.80	1.00	1.00	0.85	0.00	0.58	0.93
5.8	2.50	0.00	0.38	2.13	0.80	1.00	1.00	0.85	0.00	0.58	0.93
5.9	2.50	0.00	0.38	2.13	0.80	1.00	1.00	0.85	0.00	0.58	0.93
6.0	2.50	0.00	0.38	2.13	0.80	1.00	1.00	0.85	0.00	0.58	0.93
6.1	2.50	0.00	0.38	2.13	0.80	1.00	1.00	0.85	0.00	0.58	0.93
6.2	2.50	0.00	0.38	2.13	0.80	1.00	1.00	0.85	0.00	0.58	0.93
6.3	2.50	0.00	0.38	2.13	0.80	1.00	1.00	0.85	0.00	0.58	0.93
6.4	2.50	0.00	0.38	2.13	0.80	1.00	1.00	0.85	0.00	0.58	0.93
6.5	2.00	0.00	0.88	2.13	0.64	1.00	1.00	0.49	0.00	0.58	0.83
6.6	1.75	0.00	1.13	2.13	0.56	1.00	1.00	0.49	0.00	0.58	0.78
6.7	1.38	0.00	1.50	2.13	0.44	1.00	1.00	0.49	0.00	0.58	0.70
6.8	1.25	0.00	1.63	2.13	0.40	1.00	1.00	0.49	0.00	0.58	0.68
6.9	1.13	0.00	1.75	2.13	0.36	1.00	1.00	0.49	0.00	0.58	0.65
7.0	1.00	0.00	1.88	2.13	0.32	1.00	1.00	0.49	0.00	0.58	0.63
7.1	1.00	0.00	1.88	2.13	0.32	1.00	1.00	0.49	0.00	0.58	0.63
7.2	1.00	0.00	1.88	2.13	0.32	1.00	1.00	0.49	0.00	0.58	0.63
7.3	0.88	0.00	2.00	2.13	0.28	1.00	1.00	0.43	0.00	0.58	0.60
7.4	0.50	0.00	2.38	2.13	0.16	1.00	1.00	0.43	0.00	0.58	0.53
7.5	0.38	0.00	2.50	2.13	0.12	1.00	1.00	0.43	0.00	0.58	0.50
7.6	0.00	0.00	2.88	2.13	0.00	1.00	NaN	0.43	0.00	0.58	0.43
7.7	0.00	0.00	2.88	2.13	0.00	1.00	NaN	0.43	0.00	0.58	0.43
7.8	0.00	0.00	2.88	2.13	0.00	1.00	NaN	0.43	0.00	0.58	0.43
7.9	0.00	0.00	2.88	2.13	0.00	1.00	NaN	0.43	0.00	0.58	0.43
8.0	0.00	0.00	2.88	2.13	0.00	1.00	NaN	0.43	0.00	0.58	0.43
8.1	0.00	0.00	2.88	2.13	0.00	1.00	NaN	0.43	0.00	0.58	0.43
8.2	0.00	0.00	2.88	2.13	0.00	1.00	NaN	0.43	0.00	0.58	0.43

Table E2. 12 hours

Index	TP	FP	FN	TN	Sen	Spe	PPV	NPV	FPR	Prevalence	Accuracy
2.0	3.88	0.25	0.00	0.88	1.00	0.78	0.81	1.00	0.23	0.78	0.95
2.1	3.88	0.25	0.00	0.88	1.00	0.78	0.81	1.00	0.23	0.78	0.95
2.2	3.88	0.25	0.00	0.88	1.00	0.78	0.81	1.00	0.23	0.78	0.95
2.3	3.88	0.25	0.00	0.88	1.00	0.78	0.81	1.00	0.23	0.78	0.95
2.4	3.88	0.25	0.00	0.88	1.00	0.78	0.81	1.00	0.23	0.78	0.95
2.5	3.88	0.25	0.00	0.88	1.00	0.78	0.81	1.00	0.23	0.78	0.95
2.6	3.88	0.25	0.00	0.88	1.00	0.78	0.81	1.00	0.23	0.78	0.95
2.7	3.88	0.00	0.00	1.13	1.00	1.00	1.00	1.00	0.00	0.78	1.00
2.8	3.88	0.00	0.00	1.13	1.00	1.00	1.00	1.00	0.00	0.78	1.00
2.9	3.75	0.00	0.13	1.13	0.86	1.00	1.00	0.90	0.00	0.78	0.98
3.0	3.75	0.00	0.13	1.13	0.86	1.00	1.00	0.90	0.00	0.78	0.98
3.1	3.75	0.00	0.13	1.13	0.86	1.00	1.00	0.90	0.00	0.78	0.98
3.2	3.75	0.00	0.13	1.13	0.86	1.00	1.00	0.90	0.00	0.78	0.98
3.3	3.75	0.00	0.13	1.13	0.86	1.00	1.00	0.90	0.00	0.78	0.98
3.4	3.75	0.00	0.13	1.13	0.86	1.00	1.00	0.90	0.00	0.78	0.98
3.5	3.75	0.00	0.13	1.13	0.86	1.00	1.00	0.90	0.00	0.78	0.98
3.6	3.75	0.00	0.13	1.13	0.86	1.00	1.00	0.90	0.00	0.78	0.98
3.7	3.75	0.00	0.13	1.13	0.86	1.00	1.00	0.90	0.00	0.78	0.98
3.8	3.75	0.00	0.13	1.13	0.86	1.00	1.00	0.90	0.00	0.78	0.98
3.9	3.75	0.00	0.13	1.13	0.86	1.00	1.00	0.90	0.00	0.78	0.98
4.0	3.75	0.00	0.13	1.13	0.86	1.00	1.00	0.90	0.00	0.78	0.98
4.1	3.75	0.00	0.13	1.13	0.86	1.00	1.00	0.90	0.00	0.78	0.98
4.2	3.75	0.00	0.13	1.13	0.86	1.00	1.00	0.90	0.00	0.78	0.98
4.3	3.75	0.00	0.13	1.13	0.86	1.00	1.00	0.90	0.00	0.78	0.98
4.4	3.75	0.00	0.13	1.13	0.86	1.00	1.00	0.90	0.00	0.78	0.98
4.5	3.75	0.00	0.13	1.13	0.86	1.00	1.00	0.90	0.00	0.78	0.98
4.6	3.75	0.00	0.13	1.13	0.86	1.00	1.00	0.90	0.00	0.78	0.98
4.7	3.75	0.00	0.13	1.13	0.86	1.00	1.00	0.90	0.00	0.78	0.98
4.8	3.75	0.00	0.13	1.13	0.86	1.00	1.00	0.90	0.00	0.78	0.98
4.9	3.75	0.00	0.13	1.13	0.86	1.00	1.00	0.90	0.00	0.78	0.98
5.0	3.75	0.00	0.13	1.13	0.86	1.00	1.00	0.90	0.00	0.78	0.98
5.1	3.75	0.00	0.13	1.13	0.86	1.00	1.00	0.90	0.00	0.78	0.98
5.2	3.75	0.00	0.13	1.13	0.86	1.00	1.00	0.90	0.00	0.78	0.98
5.3	3.75	0.00	0.13	1.13	0.86	1.00	1.00	0.90	0.00	0.78	0.98
5.4	3.63	0.00	0.25	1.13	0.83	1.00	1.00	0.60	0.00	0.78	0.95
5.5	3.63	0.00	0.25	1.13	0.83	1.00	1.00	0.60	0.00	0.78	0.95
5.6	3.50	0.00	0.38	1.13	0.80	1.00	1.00	0.60	0.00	0.78	0.93
5.7	3.38	0.00	0.50	1.13	0.77	1.00	1.00	0.60	0.00	0.78	0.90
5.8	3.25	0.00	0.63	1.13	0.74	1.00	1.00	0.60	0.00	0.78	0.88
5.9	3.13	0.00	0.75	1.13	0.71	1.00	1.00	0.60	0.00	0.78	0.85
6.0	3.13	0.00	0.75	1.13	0.71	1.00	1.00	0.60	0.00	0.78	0.85
6.1	3.13	0.00	0.75	1.13	0.71	1.00	1.00	0.60	0.00	0.78	0.85
6.2	3.13	0.00	0.75	1.13	0.71	1.00	1.00	0.60	0.00	0.78	0.85
6.3	3.13	0.00	0.75	1.13	0.71	1.00	1.00	0.60	0.00	0.78	0.85
6.4	3.13	0.00	0.75	1.13	0.71	1.00	1.00	0.60	0.00	0.78	0.85
6.5	3.13	0.00	0.75	1.13	0.71	1.00	1.00	0.60	0.00	0.78	0.85
6.6	3.13	0.00	0.75	1.13	0.71	1.00	1.00	0.60	0.00	0.78	0.85
6.7	3.00	0.00	0.88	1.13	0.69	1.00	1.00	0.45	0.00	0.78	0.83
6.8	2.88	0.00	1.00	1.13	0.66	1.00	1.00	0.45	0.00	0.78	0.80
6.9	2.50	0.00	1.38	1.13	0.57	1.00	1.00	0.45	0.00	0.78	0.73
7.0	2.50	0.00	1.38	1.13	0.57	1.00	1.00	0.45	0.00	0.78	0.73
7.1	2.50	0.00	1.38	1.13	0.57	1.00	1.00	0.45	0.00	0.78	0.73
7.2	2.50	0.00	1.38	1.13	0.57	1.00	1.00	0.45	0.00	0.78	0.73
7.3	2.50	0.00	1.38	1.13	0.57	1.00	1.00	0.45	0.00	0.78	0.73
7.4	2.50	0.00	1.38	1.13	0.57	1.00	1.00	0.45	0.00	0.78	0.73
7.5	2.50	0.00	1.38	1.13	0.57	1.00	1.00	0.45	0.00	0.78	0.73
7.6	2.50	0.00	1.38	1.13	0.57	1.00	1.00	0.45	0.00	0.78	0.73
7.7	2.00	0.00	1.88	1.13	0.46	1.00	1.00	0.36	0.00	0.78	0.63
7.8	0.88	0.00	3.00	1.13	0.20	1.00	1.00	0.26	0.00	0.78	0.40
7.9	0.00	0.00	3.88	1.13	0.00	1.00	NaN	0.23	0.00	0.78	0.23
8.0	0.00	0.00	3.88	1.13	0.00	1.00	NaN	0.23	0.00	0.78	0.23
8.1	0.00	0.00	3.88	1.13	0.00	1.00	NaN	0.23	0.00	0.78	0.23
8.2	0.00	0.00	3.88	1.13	0.00	1.00	NaN	0.23	0.00	0.78	0.23

Table E3. 24 hours

Index	TP	FP	FN	TN	Sen	Spe	PPV	NPV	FPR	Prevalence	Accuracy
2.0	4.13	0.88	0.00	0.00	1.00	0.00	0.83	NaN	1.00	0.83	0.83
2.1	4.13	0.88	0.00	0.00	1.00	0.00	0.83	NaN	1.00	0.83	0.83
2.2	4.13	0.88	0.00	0.00	1.00	0.00	0.83	NaN	1.00	0.83	0.83
2.3	4.13	0.88	0.00	0.00	1.00	0.00	0.83	NaN	1.00	0.83	0.83
2.4	4.13	0.88	0.00	0.00	1.00	0.00	0.83	NaN	1.00	0.83	0.83
2.5	4.13	0.88	0.00	0.00	1.00	0.00	0.83	NaN	1.00	0.83	0.83
2.6	4.13	0.88	0.00	0.00	1.00	0.00	0.83	NaN	1.00	0.83	0.83
2.7	4.13	0.88	0.00	0.00	1.00	0.00	0.83	NaN	1.00	0.83	0.83
2.8	4.13	0.88	0.00	0.00	1.00	0.00	0.83	NaN	1.00	0.83	0.83
2.9	4.13	0.88	0.00	0.00	1.00	0.00	0.83	NaN	1.00	0.83	0.83
3.0	4.13	0.63	0.00	0.25	1.00	0.42	0.86	1.00	0.58	0.83	0.88
3.1	4.13	0.63	0.00	0.25	1.00	0.42	0.86	1.00	0.58	0.83	0.88
3.2	4.13	0.63	0.00	0.25	1.00	0.42	0.86	1.00	0.58	0.83	0.88
3.3	4.13	0.63	0.00	0.25	1.00	0.42	0.86	1.00	0.58	0.83	0.88
3.4	4.13	0.38	0.00	0.50	1.00	0.58	0.89	1.00	0.42	0.83	0.93
3.5	4.13	0.38	0.00	0.50	1.00	0.58	0.89	1.00	0.42	0.83	0.93
3.6	4.13	0.38	0.00	0.50	1.00	0.58	0.89	1.00	0.42	0.83	0.93
3.7	4.13	0.25	0.00	0.63	1.00	0.75	0.91	1.00	0.25	0.83	0.95
3.8	4.13	0.25	0.00	0.63	1.00	0.75	0.91	1.00	0.25	0.83	0.95
3.9	4.13	0.25	0.00	0.63	1.00	0.75	0.91	1.00	0.25	0.83	0.95
4.0	4.13	0.00	0.00	0.88	1.00	1.00	1.00	1.00	0.00	0.83	1.00
4.1	4.13	0.00	0.00	0.88	1.00	1.00	1.00	1.00	0.00	0.83	1.00
4.2	4.13	0.00	0.00	0.88	1.00	1.00	1.00	1.00	0.00	0.83	1.00
4.3	4.13	0.00	0.00	0.88	1.00	1.00	1.00	1.00	0.00	0.83	1.00
4.4	4.13	0.00	0.00	0.88	1.00	1.00	1.00	1.00	0.00	0.83	1.00
4.5	4.13	0.00	0.00	0.88	1.00	1.00	1.00	1.00	0.00	0.83	1.00
4.6	4.13	0.00	0.00	0.88	1.00	1.00	1.00	1.00	0.00	0.83	1.00
4.7	4.13	0.00	0.00	0.88	1.00	1.00	1.00	1.00	0.00	0.83	1.00
4.8	4.13	0.00	0.00	0.88	1.00	1.00	1.00	1.00	0.00	0.83	1.00
4.9	4.13	0.00	0.00	0.88	1.00	1.00	1.00	1.00	0.00	0.83	1.00
5.0	4.13	0.00	0.00	0.88	1.00	1.00	1.00	1.00	0.00	0.83	1.00
5.1	4.13	0.00	0.00	0.88	1.00	1.00	1.00	1.00	0.00	0.83	1.00
5.2	4.13	0.00	0.00	0.88	1.00	1.00	1.00	1.00	0.00	0.83	1.00
5.3	4.13	0.00	0.00	0.88	1.00	1.00	1.00	1.00	0.00	0.83	1.00
5.4	4.13	0.00	0.00	0.88	1.00	1.00	1.00	1.00	0.00	0.83	1.00
5.5	4.13	0.00	0.00	0.88	1.00	1.00	1.00	1.00	0.00	0.83	1.00
5.6	4.13	0.00	0.00	0.88	1.00	1.00	1.00	1.00	0.00	0.83	1.00
5.7	4.13	0.00	0.00	0.88	1.00	1.00	1.00	1.00	0.00	0.83	1.00
5.8	4.13	0.00	0.00	0.88	1.00	1.00	1.00	1.00	0.00	0.83	1.00
5.9	4.13	0.00	0.00	0.88	1.00	1.00	1.00	1.00	0.00	0.83	1.00
6.0	4.13	0.00	0.00	0.88	1.00	1.00	1.00	1.00	0.00	0.83	1.00
6.1	4.13	0.00	0.00	0.88	1.00	1.00	1.00	1.00	0.00	0.83	1.00
6.2	4.13	0.00	0.00	0.88	1.00	1.00	1.00	1.00	0.00	0.83	1.00
6.3	4.13	0.00	0.00	0.88	1.00	1.00	1.00	1.00	0.00	0.83	1.00
6.4	4.13	0.00	0.00	0.88	1.00	1.00	1.00	1.00	0.00	0.83	1.00
6.5	4.13	0.00	0.00	0.88	1.00	1.00	1.00	1.00	0.00	0.83	1.00
6.6	4.13	0.00	0.00	0.88	1.00	1.00	1.00	1.00	0.00	0.83	1.00
6.7	4.13	0.00	0.00	0.88	1.00	1.00	1.00	1.00	0.00	0.83	1.00
6.8	4.13	0.00	0.00	0.88	1.00	1.00	1.00	1.00	0.00	0.83	1.00
6.9	4.13	0.00	0.00	0.88	1.00	1.00	1.00	1.00	0.00	0.83	1.00
7.0	4.00	0.00	0.13	0.88	0.98	1.00	1.00	0.75	0.00	0.83	0.98
7.1	3.25	0.00	0.88	0.88	0.83	1.00	1.00	0.60	0.00	0.83	0.83
7.2	3.00	0.00	1.13	0.88	0.78	1.00	1.00	0.60	0.00	0.83	0.78
7.3	2.88	0.00	1.25	0.88	0.75	1.00	1.00	0.60	0.00	0.83	0.75
7.4	2.88	0.00	1.25	0.88	0.75	1.00	1.00	0.60	0.00	0.83	0.75
7.5	2.88	0.00	1.25	0.88	0.75	1.00	1.00	0.60	0.00	0.83	0.75
7.6	2.38	0.00	1.75	0.88	0.64	1.00	1.00	0.42	0.00	0.83	0.65
7.7	1.88	0.00	2.25	0.88	0.54	1.00	1.00	0.33	0.00	0.83	0.55
7.8	1.25	0.00	2.88	0.88	0.41	1.00	1.00	0.32	0.00	0.83	0.43
7.9	0.88	0.00	3.25	0.88	0.29	1.00	1.00	0.24	0.00	0.83	0.35
8.0	0.75	0.00	3.38	0.88	0.25	1.00	1.00	0.23	0.00	0.83	0.33
8.1	0.75	0.00	3.38	0.88	0.25	1.00	1.00	0.23	0.00	0.83	0.33
8.2	0.00	0.00	4.13	0.88	0.00	1.00	1.00	0.18	0.00	0.83	0.18

Table E4. 48 hours

Index	TP	FP	FN	TN	Sen	Spe	PPV	NPV	FPR	Prevalence	Accuracy
2.0	4.38	0.63	0.00	0.00	1.00	0.00	0.88	NaN	1.00	0.88	0.88
2.1	4.38	0.63	0.00	0.00	1.00	0.00	0.88	NaN	1.00	0.88	0.88
2.2	4.38	0.63	0.00	0.00	1.00	0.00	0.88	NaN	1.00	0.88	0.88
2.3	4.38	0.63	0.00	0.00	1.00	0.00	0.88	NaN	1.00	0.88	0.88
2.4	4.38	0.63	0.00	0.00	1.00	0.00	0.88	NaN	1.00	0.88	0.88
2.5	4.38	0.63	0.00	0.00	1.00	0.00	0.88	NaN	1.00	0.88	0.88
2.6	4.38	0.63	0.00	0.00	1.00	0.00	0.88	NaN	1.00	0.88	0.88
2.7	4.38	0.63	0.00	0.00	1.00	0.00	0.88	NaN	1.00	0.88	0.88
2.8	4.38	0.63	0.00	0.00	1.00	0.00	0.88	NaN	1.00	0.88	0.88
2.9	4.38	0.63	0.00	0.00	1.00	0.00	0.88	NaN	1.00	0.88	0.88
3.0	4.38	0.50	0.00	0.13	1.00	0.20	0.88	1.00	0.80	0.88	0.90
3.1	4.38	0.50	0.00	0.13	1.00	0.20	0.88	1.00	0.80	0.88	0.90
3.2	4.38	0.50	0.00	0.13	1.00	0.20	0.88	1.00	0.80	0.88	0.90
3.3	4.38	0.50	0.00	0.13	1.00	0.20	0.88	1.00	0.80	0.88	0.90
3.4	4.38	0.38	0.00	0.25	1.00	0.40	0.88	1.00	0.60	0.88	0.93
3.5	4.38	0.25	0.00	0.38	1.00	0.60	0.88	1.00	0.40	0.88	0.95
3.6	4.38	0.25	0.00	0.38	1.00	0.60	0.88	1.00	0.40	0.88	0.95
3.7	4.38	0.25	0.00	0.38	1.00	0.60	0.88	1.00	0.40	0.88	0.95
3.8	4.38	0.00	0.00	0.63	1.00	1.00	1.00	1.00	0.00	0.88	1.00
3.9	4.38	0.00	0.00	0.63	1.00	1.00	1.00	1.00	0.00	0.88	1.00
4.0	4.38	0.00	0.00	0.63	1.00	1.00	1.00	1.00	0.00	0.88	1.00
4.1	4.38	0.00	0.00	0.63	1.00	1.00	1.00	1.00	0.00	0.88	1.00
4.2	4.38	0.00	0.00	0.63	1.00	1.00	1.00	1.00	0.00	0.88	1.00
4.3	4.38	0.00	0.00	0.63	1.00	1.00	1.00	1.00	0.00	0.88	1.00
4.4	4.38	0.00	0.00	0.63	1.00	1.00	1.00	1.00	0.00	0.88	1.00
4.5	4.38	0.00	0.00	0.63	1.00	1.00	1.00	1.00	0.00	0.88	1.00
4.6	4.38	0.00	0.00	0.63	1.00	1.00	1.00	1.00	0.00	0.88	1.00
4.7	4.38	0.00	0.00	0.63	1.00	1.00	1.00	1.00	0.00	0.88	1.00
4.8	4.38	0.00	0.00	0.63	1.00	1.00	1.00	1.00	0.00	0.88	1.00
4.9	4.38	0.00	0.00	0.63	1.00	1.00	1.00	1.00	0.00	0.88	1.00
5.0	4.38	0.00	0.00	0.63	1.00	1.00	1.00	1.00	0.00	0.88	1.00
5.1	4.38	0.00	0.00	0.63	1.00	1.00	1.00	1.00	0.00	0.88	1.00
5.2	4.38	0.00	0.00	0.63	1.00	1.00	1.00	1.00	0.00	0.88	1.00
5.3	4.38	0.00	0.00	0.63	1.00	1.00	1.00	1.00	0.00	0.88	1.00
5.4	4.13	0.00	0.25	0.63	0.94	1.00	1.00	0.33	0.00	0.88	0.95
5.5	3.50	0.00	0.88	0.63	0.80	1.00	1.00	0.33	0.00	0.88	0.83
5.6	3.38	0.00	1.00	0.63	0.77	1.00	1.00	0.33	0.00	0.88	0.80
5.7	3.13	0.00	1.25	0.63	0.71	1.00	1.00	0.25	0.00	0.88	0.75
5.8	2.75	0.00	1.63	0.63	0.63	1.00	1.00	0.25	0.00	0.88	0.68
5.9	2.63	0.00	1.75	0.63	0.60	1.00	1.00	0.25	0.00	0.88	0.65
6.0	2.38	0.00	2.00	0.63	0.54	1.00	1.00	0.20	0.00	0.88	0.60
6.1	1.88	0.00	2.50	0.63	0.43	1.00	1.00	0.17	0.00	0.88	0.50
6.2	1.75	0.00	2.63	0.63	0.40	1.00	1.00	0.17	0.00	0.88	0.48
6.3	1.75	0.00	2.63	0.63	0.40	1.00	1.00	0.17	0.00	0.88	0.48
6.4	1.50	0.00	2.88	0.63	0.34	1.00	1.00	0.14	0.00	0.88	0.43
6.5	1.13	0.00	3.25	0.63	0.26	1.00	1.00	0.14	0.00	0.88	0.35
6.6	1.13	0.00	3.25	0.63	0.26	1.00	1.00	0.14	0.00	0.88	0.35
6.7	0.88	0.00	3.50	0.63	0.20	1.00	1.00	0.13	0.00	0.88	0.30
6.8	0.75	0.00	3.63	0.63	0.17	1.00	1.00	0.13	0.00	0.88	0.28
6.9	0.63	0.00	3.75	0.63	0.14	1.00	1.00	0.13	0.00	0.88	0.25
7.0	0.50	0.00	3.88	0.63	0.11	1.00	1.00	0.13	0.00	0.88	0.23
7.1	0.25	0.00	4.13	0.63	0.06	1.00	1.00	0.13	0.00	0.88	0.18
7.2	0.00	0.00	4.38	0.63	0.00	1.00	NaN	0.13	0.00	0.88	0.13
7.3	0.00	0.00	4.38	0.63	0.00	1.00	NaN	0.13	0.00	0.88	0.13
7.4	0.00	0.00	4.38	0.63	0.00	1.00	NaN	0.13	0.00	0.88	0.13
7.5	0.00	0.00	4.38	0.63	0.00	1.00	NaN	0.13	0.00	0.88	0.13
7.6	0.00	0.00	4.38	0.63	0.00	1.00	NaN	0.13	0.00	0.88	0.13
7.7	0.00	0.00	4.38	0.63	0.00	1.00	NaN	0.13	0.00	0.88	0.13
7.8	0.00	0.00	4.38	0.63	0.00	1.00	NaN	0.13	0.00	0.88	0.13
7.9	0.00	0.00	4.38	0.63	0.00	1.00	NaN	0.13	0.00	0.88	0.13
8.0	0.00	0.00	4.38	0.63	0.00	1.00	NaN	0.13	0.00	0.88	0.13
8.1	0.00	0.00	4.38	0.63	0.00	1.00	NaN	0.13	0.00	0.88	0.13
8.2	0.00	0.00	4.38	0.63	0.00	1.00	NaN	0.13	0.00	0.88	0.13

UHT in-house cream Mean of all bacterial strains (four *Geobacillus* sp. and four *Anoxybacillus flavithermus*) expressed as threshold index for the Attune at different time points including True Positive (TP), False Positive (FN), True Negative (TN), False Negative (FN), Sensitivity, Specificity, Positive Predictive value (PPV), Negative Predictive Value (NPV), False Predictive Rate (FPR), Prevalence and Accuracy

Table E5. 6 hours

Index	TP	FP	FN	TN	Sen	Spe	PPV	NPV	FPR	Prevalence	Accuracy
3.0	3.38	1.50	0.13	0.00	0.97	0.00	0.70	0.00	1.00	0.70	0.68
3.1	3.38	1.50	0.13	0.00	0.97	0.00	0.70	0.00	1.00	0.70	0.68
3.2	3.38	1.50	0.13	0.00	0.97	0.00	0.70	0.00	1.00	0.70	0.68
3.3	3.38	1.50	0.13	0.00	0.97	0.00	0.70	0.00	1.00	0.70	0.68
3.4	3.25	1.50	0.25	0.00	0.93	0.00	0.70	0.00	1.00	0.70	0.65
3.5	3.13	1.50	0.38	0.00	0.89	0.00	0.69	0.00	1.00	0.70	0.63
3.6	3.13	1.50	0.38	0.00	0.89	0.00	0.69	0.00	1.00	0.70	0.63
3.7	2.75	1.38	0.75	0.13	0.79	0.05	0.65	0.33	0.95	0.70	0.58
3.8	2.75	1.25	0.75	0.25	0.79	0.10	0.65	0.33	0.90	0.70	0.60
3.9	2.75	1.25	0.75	0.25	0.79	0.10	0.65	0.33	0.90	0.70	0.60
4.0	2.50	1.13	1.00	0.38	0.71	0.15	0.63	0.25	0.85	0.70	0.58
4.1	2.50	1.00	1.00	0.50	0.71	0.20	0.63	0.25	0.80	0.70	0.60
4.2	2.38	0.88	1.13	0.63	0.68	0.45	0.68	0.27	0.55	0.70	0.60
4.3	1.88	0.63	1.63	0.88	0.53	0.55	0.64	0.33	0.45	0.70	0.55
4.4	1.50	0.38	2.00	1.13	0.41	0.85	0.80	0.35	0.15	0.70	0.53
4.5	1.00	0.25	2.50	1.25	0.27	0.90	0.75	0.30	0.10	0.70	0.45
4.6	0.38	0.13	3.13	1.38	0.10	0.95	0.67	0.30	0.05	0.70	0.35
4.7	0.13	0.13	3.38	1.38	0.03	0.95	0.50	0.30	0.05	0.70	0.30
4.8	0.13	0.13	3.38	1.38	0.03	0.95	0.50	0.30	0.05	0.70	0.30
4.9	0.13	0.00	3.38	1.50	0.03	1.00	1.00	0.30	0.00	0.70	0.33
5.0	0.00	0.00	3.50	1.50	0.00	1.00	NaN	0.30	0.00	0.70	0.30
5.1	0.00	0.00	3.50	1.50	0.00	1.00	NaN	0.30	0.00	0.70	0.30
5.2	0.00	0.00	3.50	1.50	0.00	1.00	NaN	0.30	0.00	0.70	0.30
5.3	0.00	0.00	3.50	1.50	0.00	1.00	NaN	0.30	0.00	0.70	0.30
5.4	0.00	0.00	3.50	1.50	0.00	1.00	NaN	0.30	0.00	0.70	0.30
5.5	0.00	0.00	3.50	1.50	0.00	1.00	NaN	0.30	0.00	0.70	0.30
5.6	0.00	0.00	3.50	1.50	0.00	1.00	NaN	0.30	0.00	0.70	0.30
5.7	0.00	0.00	3.50	1.50	0.00	1.00	NaN	0.30	0.00	0.70	0.30
5.8	0.00	0.00	3.50	1.50	0.00	1.00	NaN	0.30	0.00	0.70	0.30
5.9	0.00	0.00	3.50	1.50	0.00	1.00	NaN	0.30	0.00	0.70	0.30
6.0	0.00	0.00	3.50	1.50	0.00	1.00	NaN	0.30	0.00	0.70	0.30
6.1	0.00	0.00	3.50	1.50	0.00	1.00	NaN	0.30	0.00	0.70	0.30
6.2	0.00	0.00	3.50	1.50	0.00	1.00	NaN	0.30	0.00	0.70	0.30
6.3	0.00	0.00	3.50	1.50	0.00	1.00	NaN	0.30	0.00	0.70	0.30
6.4	0.00	0.00	3.50	1.50	0.00	1.00	NaN	0.30	0.00	0.70	0.30
6.5	0.00	0.00	3.50	1.50	0.00	1.00	NaN	0.30	0.00	0.70	0.30
6.6	0.00	0.00	3.50	1.50	0.00	1.00	NaN	0.30	0.00	0.70	0.30
6.7	0.00	0.00	3.50	1.50	0.00	1.00	NaN	0.30	0.00	0.70	0.30
6.8	0.00	0.00	3.50	1.50	0.00	1.00	NaN	0.30	0.00	0.70	0.30
6.9	0.00	0.00	3.50	1.50	0.00	1.00	NaN	0.30	0.00	0.70	0.30
7.0	0.00	0.00	3.50	1.50	0.00	1.00	NaN	0.30	0.00	0.70	0.30
7.1	0.00	0.00	3.50	1.50	0.00	1.00	NaN	0.30	0.00	0.70	0.30
7.2	0.00	0.00	3.50	1.50	0.00	1.00	NaN	0.30	0.00	0.70	0.30
7.3	0.00	0.00	3.50	1.50	0.00	1.00	NaN	0.30	0.00	0.70	0.30
7.4	0.00	0.00	3.50	1.50	0.00	1.00	NaN	0.30	0.00	0.70	0.30
7.5	0.00	0.00	3.50	1.50	0.00	1.00	NaN	0.30	0.00	0.70	0.30
7.6	0.00	0.00	3.50	1.50	0.00	1.00	NaN	0.30	0.00	0.70	0.30
7.7	0.00	0.00	3.50	1.50	0.00	1.00	NaN	0.30	0.00	0.70	0.30

Table E6. 12 hours

Index	TP	FP	FN	TN	Sen	Spe	PPV	NPV	FPR	Prevalence	Accuracy
3.0	4.25	0.75	0.00	0.00	1.00	0.00	0.85	NaN	1.00	0.85	0.85
3.1	4.25	0.75	0.00	0.00	1.00	0.00	0.85	NaN	1.00	0.85	0.85
3.2	4.25	0.75	0.00	0.00	1.00	0.00	0.85	NaN	1.00	0.85	0.85
3.3	4.25	0.75	0.00	0.00	1.00	0.00	0.85	NaN	1.00	0.85	0.85
3.4	4.25	0.75	0.00	0.00	1.00	0.00	0.85	NaN	1.00	0.85	0.85
3.5	4.25	0.75	0.00	0.00	1.00	0.00	0.85	NaN	1.00	0.85	0.85
3.6	4.25	0.75	0.00	0.00	1.00	0.00	0.85	NaN	1.00	0.85	0.85
3.7	4.25	0.75	0.00	0.00	1.00	0.00	0.85	NaN	1.00	0.85	0.85
3.8	4.25	0.75	0.00	0.00	1.00	0.00	0.85	NaN	1.00	0.85	0.85
3.9	4.25	0.75	0.00	0.00	1.00	0.00	0.85	NaN	1.00	0.85	0.85
4.0	4.13	0.75	0.13	0.00	0.96	0.00	0.84	0.00	1.00	0.85	0.83
4.1	4.00	0.75	0.25	0.00	0.94	0.00	0.84	0.00	1.00	0.85	0.80
4.2	4.00	0.63	0.25	0.13	0.94	0.10	0.84	0.33	0.90	0.85	0.83
4.3	3.88	0.50	0.38	0.25	0.90	0.60	0.88	0.44	0.40	0.85	0.83
4.4	3.25	0.25	1.00	0.50	0.76	0.80	0.88	0.22	0.20	0.85	0.75
4.5	2.63	0.13	1.63	0.63	0.61	0.90	0.88	0.18	0.10	0.85	0.65
4.6	2.25	0.00	2.00	0.75	0.52	1.00	1.00	0.16	0.00	0.85	0.60
4.7	1.13	0.00	3.13	0.75	0.26	1.00	1.00	0.16	0.00	0.85	0.38
4.8	0.25	0.00	4.00	0.75	0.06	1.00	1.00	0.16	0.00	0.85	0.20
4.9	0.25	0.00	4.00	0.75	0.06	1.00	1.00	0.16	0.00	0.85	0.20
5.0	0.13	0.00	4.13	0.75	0.04	1.00	1.00	0.16	0.00	0.85	0.18
5.1	0.13	0.00	4.13	0.75	0.04	1.00	1.00	0.16	0.00	0.85	0.18
5.2	0.13	0.00	4.13	0.75	0.04	1.00	1.00	0.16	0.00	0.85	0.18
5.3	0.13	0.00	4.13	0.75	0.04	1.00	1.00	0.16	0.00	0.85	0.18
5.4	0.13	0.00	4.13	0.75	0.04	1.00	1.00	0.16	0.00	0.85	0.18
5.5	0.13	0.00	4.13	0.75	0.04	1.00	1.00	0.16	0.00	0.85	0.18
5.6	0.13	0.00	4.13	0.75	0.04	1.00	1.00	0.16	0.00	0.85	0.18
5.7	0.13	0.00	4.13	0.75	0.04	1.00	1.00	0.16	0.00	0.85	0.18
5.8	0.13	0.00	4.13	0.75	0.04	1.00	1.00	0.16	0.00	0.85	0.18
5.9	0.13	0.00	4.13	0.75	0.04	1.00	1.00	0.16	0.00	0.85	0.18
6.0	0.13	0.00	4.13	0.75	0.04	1.00	1.00	0.16	0.00	0.85	0.18
6.1	0.13	0.00	4.13	0.75	0.04	1.00	1.00	0.16	0.00	0.85	0.18
6.2	0.13	0.00	4.13	0.75	0.04	1.00	1.00	0.16	0.00	0.85	0.18
6.3	0.13	0.00	4.13	0.75	0.04	1.00	1.00	0.16	0.00	0.85	0.18
6.4	0.13	0.00	4.13	0.75	0.04	1.00	1.00	0.16	0.00	0.85	0.18
6.5	0.13	0.00	4.13	0.75	0.04	1.00	1.00	0.16	0.00	0.85	0.18
6.6	0.13	0.00	4.13	0.75	0.04	1.00	1.00	0.16	0.00	0.85	0.18
6.7	0.13	0.00	4.13	0.75	0.04	1.00	1.00	0.16	0.00	0.85	0.18
6.8	0.13	0.00	4.13	0.75	0.04	1.00	1.00	0.16	0.00	0.85	0.18
6.9	0.13	0.00	4.13	0.75	0.04	1.00	1.00	0.16	0.00	0.85	0.18
7.0	0.13	0.00	4.13	0.75	0.04	1.00	1.00	0.16	0.00	0.85	0.18
7.1	0.13	0.00	4.13	0.75	0.04	1.00	1.00	0.16	0.00	0.85	0.18
7.2	0.13	0.00	4.13	0.75	0.04	1.00	1.00	0.16	0.00	0.85	0.18
7.3	0.13	0.00	4.13	0.75	0.04	1.00	1.00	0.16	0.00	0.85	0.18
7.4	0.13	0.00	4.13	0.75	0.04	1.00	1.00	0.16	0.00	0.85	0.18
7.5	0.13	0.00	4.13	0.75	0.04	1.00	1.00	0.16	0.00	0.85	0.18
7.6	0.13	0.00	4.13	0.75	0.04	1.00	1.00	0.16	0.00	0.85	0.18
7.7	0.00	0.00	4.25	0.75	0.00	1.00	NaN	0.15	0.00	0.85	0.15

Table E7. 24 hours

Index	TP	FP	FN	TN	Sen	Spe	PPV	NPV	FPR	Prevalence	Accuracy
3.0	4.00	1.00	0.00	0.00	1.00	0.00	0.80	NaN	1.00	0.80	0.80
3.1	4.00	1.00	0.00	0.00	1.00	0.00	0.80	NaN	1.00	0.80	0.80
3.2	4.00	1.00	0.00	0.00	1.00	0.00	0.80	NaN	1.00	0.80	0.80
3.3	4.00	1.00	0.00	0.00	1.00	0.00	0.80	NaN	1.00	0.80	0.80
3.4	4.00	1.00	0.00	0.00	1.00	0.00	0.80	NaN	1.00	0.80	0.80
3.5	4.00	1.00	0.00	0.00	1.00	0.00	0.80	NaN	1.00	0.80	0.80
3.6	4.00	1.00	0.00	0.00	1.00	0.00	0.80	NaN	1.00	0.80	0.80
3.7	4.00	1.00	0.00	0.00	1.00	0.00	0.80	NaN	1.00	0.80	0.80
3.8	4.00	1.00	0.00	0.00	1.00	0.00	0.80	NaN	1.00	0.80	0.80
3.9	4.00	1.00	0.00	0.00	1.00	0.00	0.80	NaN	1.00	0.80	0.80
4.0	4.00	1.00	0.00	0.00	1.00	0.00	0.80	NaN	1.00	0.80	0.80
4.1	3.88	0.88	0.13	0.13	0.97	0.10	0.80	0.50	0.90	0.80	0.80
4.2	3.63	0.63	0.38	0.38	0.91	0.43	0.83	0.67	0.57	0.80	0.80
4.3	3.38	0.63	0.63	0.38	0.81	0.43	0.81	0.42	0.57	0.80	0.75
4.4	3.00	0.50	1.00	0.50	0.69	0.53	0.75	0.30	0.47	0.80	0.70
4.5	2.38	0.25	1.63	0.75	0.54	0.80	0.86	0.23	0.20	0.80	0.63
4.6	2.00	0.00	2.00	1.00	0.46	1.00	1.00	0.23	0.00	0.80	0.60
4.7	1.25	0.00	2.75	1.00	0.29	1.00	1.00	0.23	0.00	0.80	0.45
4.8	1.13	0.00	2.88	1.00	0.26	1.00	1.00	0.23	0.00	0.80	0.43
4.9	1.00	0.00	3.00	1.00	0.23	1.00	1.00	0.23	0.00	0.80	0.40
5.0	0.75	0.00	3.25	1.00	0.17	1.00	1.00	0.23	0.00	0.80	0.35
5.1	0.63	0.00	3.38	1.00	0.14	1.00	1.00	0.20	0.00	0.80	0.33
5.2	0.50	0.00	3.50	1.00	0.11	1.00	1.00	0.20	0.00	0.80	0.30
5.3	0.38	0.00	3.63	1.00	0.09	1.00	1.00	0.20	0.00	0.80	0.28
5.4	0.25	0.00	3.75	1.00	0.06	1.00	1.00	0.20	0.00	0.80	0.25
5.5	0.13	0.00	3.88	1.00	0.03	1.00	1.00	0.20	0.00	0.80	0.23
5.6	0.13	0.00	3.88	1.00	0.03	1.00	1.00	0.20	0.00	0.80	0.23
5.7	0.13	0.00	3.88	1.00	0.03	1.00	1.00	0.20	0.00	0.80	0.23
5.8	0.13	0.00	3.88	1.00	0.03	1.00	1.00	0.20	0.00	0.80	0.23
5.9	0.13	0.00	3.88	1.00	0.03	1.00	1.00	0.20	0.00	0.80	0.23
6.0	0.13	0.00	3.88	1.00	0.03	1.00	1.00	0.20	0.00	0.80	0.23
6.1	0.13	0.00	3.88	1.00	0.03	1.00	1.00	0.20	0.00	0.80	0.23
6.2	0.13	0.00	3.88	1.00	0.03	1.00	1.00	0.20	0.00	0.80	0.23
6.3	0.13	0.00	3.88	1.00	0.03	1.00	1.00	0.20	0.00	0.80	0.23
6.4	0.13	0.00	3.88	1.00	0.03	1.00	1.00	0.20	0.00	0.80	0.23
6.5	0.13	0.00	3.88	1.00	0.03	1.00	1.00	0.20	0.00	0.80	0.23
6.6	0.00	0.00	4.00	1.00	0.00	1.00	NaN	0.20	0.00	0.80	0.20
6.7	0.00	0.00	4.00	1.00	0.00	1.00	NaN	0.20	0.00	0.80	0.20
6.8	0.00	0.00	4.00	1.00	0.00	1.00	NaN	0.20	0.00	0.80	0.20
6.9	0.00	0.00	4.00	1.00	0.00	1.00	NaN	0.20	0.00	0.80	0.20
7.0	0.00	0.00	4.00	1.00	0.00	1.00	NaN	0.20	0.00	0.80	0.20
7.1	0.00	0.00	4.00	1.00	0.00	1.00	NaN	0.20	0.00	0.80	0.20
7.2	0.00	0.00	4.00	1.00	0.00	1.00	NaN	0.20	0.00	0.80	0.20
7.3	0.00	0.00	4.00	1.00	0.00	1.00	NaN	0.20	0.00	0.80	0.20
7.4	0.00	0.00	4.00	1.00	0.00	1.00	NaN	0.20	0.00	0.80	0.20
7.5	0.00	0.00	4.00	1.00	0.00	1.00	NaN	0.20	0.00	0.80	0.20
7.6	0.00	0.00	4.00	1.00	0.00	1.00	NaN	0.20	0.00	0.80	0.20
7.7	0.00	0.00	4.00	1.00	0.00	1.00	NaN	0.20	0.00	0.80	0.20

Table E8. 48 hours

Index	TP	FP	FN	TN	Sen	Spe	PPV	NPV	FPR	Prevalence	Accuracy
3.0	4.88	0.13	0.00	0.00	1.00	0.00	0.98	NaN	1.00	0.98	0.98
3.1	4.88	0.13	0.00	0.00	1.00	0.00	0.98	NaN	1.00	0.98	0.98
3.2	4.88	0.13	0.00	0.00	1.00	0.00	0.98	NaN	1.00	0.98	0.98
3.3	4.88	0.13	0.00	0.00	1.00	0.00	0.98	NaN	1.00	0.98	0.98
3.4	4.88	0.13	0.00	0.00	1.00	0.00	0.98	NaN	1.00	0.98	0.98
3.5	4.88	0.13	0.00	0.00	1.00	0.00	0.98	NaN	1.00	0.98	0.98
3.6	4.88	0.13	0.00	0.00	1.00	0.00	0.98	NaN	1.00	0.98	0.98
3.7	4.88	0.13	0.00	0.00	1.00	0.00	0.98	NaN	1.00	0.98	0.98
3.8	4.88	0.13	0.00	0.00	1.00	0.00	0.98	NaN	1.00	0.98	0.98
3.9	4.88	0.13	0.00	0.00	1.00	0.00	0.98	NaN	1.00	0.98	0.98
4.0	4.88	0.13	0.00	0.00	1.00	0.00	0.98	NaN	1.00	0.98	0.98
4.1	4.88	0.13	0.00	0.00	1.00	0.00	0.98	NaN	1.00	0.98	0.98
4.2	4.75	0.13	0.13	0.00	0.98	0.00	0.98	0.00	1.00	0.98	0.95
4.3	4.38	0.00	0.50	0.13	0.90	1.00	1.00	0.25	0.00	0.98	0.90
4.4	4.13	0.00	0.75	0.13	0.85	1.00	1.00	0.25	0.00	0.98	0.85
4.5	3.88	0.00	1.00	0.13	0.79	1.00	1.00	0.13	0.00	0.98	0.80
4.6	3.75	0.00	1.13	0.13	0.77	1.00	1.00	0.10	0.00	0.98	0.78
4.7	3.13	0.00	1.75	0.13	0.64	1.00	1.00	0.07	0.00	0.98	0.65
4.8	2.63	0.00	2.25	0.13	0.53	1.00	1.00	0.04	0.00	0.98	0.55
4.9	2.38	0.00	2.50	0.13	0.48	1.00	1.00	0.04	0.00	0.98	0.50
5.0	2.13	0.00	2.75	0.13	0.43	1.00	1.00	0.04	0.00	0.98	0.45
5.1	1.88	0.00	3.00	0.13	0.38	1.00	1.00	0.03	0.00	0.98	0.40
5.2	1.63	0.00	3.25	0.13	0.33	1.00	1.00	0.03	0.00	0.98	0.35
5.3	1.50	0.00	3.38	0.13	0.31	1.00	1.00	0.03	0.00	0.98	0.33
5.4	1.50	0.00	3.38	0.13	0.31	1.00	1.00	0.03	0.00	0.98	0.33
5.5	1.50	0.00	3.38	0.13	0.31	1.00	1.00	0.03	0.00	0.98	0.33
5.6	1.50	0.00	3.38	0.13	0.31	1.00	1.00	0.03	0.00	0.98	0.33
5.7	1.38	0.00	3.50	0.13	0.28	1.00	1.00	0.03	0.00	0.98	0.30
5.8	1.25	0.00	3.63	0.13	0.26	1.00	1.00	0.03	0.00	0.98	0.28
5.9	1.00	0.00	3.88	0.13	0.21	1.00	1.00	0.03	0.00	0.98	0.23
6.0	1.00	0.00	3.88	0.13	0.21	1.00	1.00	0.03	0.00	0.98	0.23
6.1	1.00	0.00	3.88	0.13	0.21	1.00	1.00	0.03	0.00	0.98	0.23
6.2	1.00	0.00	3.88	0.13	0.21	1.00	1.00	0.03	0.00	0.98	0.23
6.3	1.00	0.00	3.88	0.13	0.21	1.00	1.00	0.03	0.00	0.98	0.23
6.4	1.00	0.00	3.88	0.13	0.21	1.00	1.00	0.03	0.00	0.98	0.23
6.5	0.88	0.00	4.00	0.13	0.18	1.00	1.00	0.03	0.00	0.98	0.20
6.6	0.75	0.00	4.13	0.13	0.16	1.00	1.00	0.03	0.00	0.98	0.18
6.7	0.63	0.00	4.25	0.13	0.13	1.00	1.00	0.03	0.00	0.98	0.15
6.8	0.38	0.00	4.50	0.13	0.08	1.00	1.00	0.03	0.00	0.98	0.10
6.9	0.25	0.00	4.63	0.13	0.05	1.00	1.00	0.03	0.00	0.98	0.08
7.0	0.00	0.00	4.88	0.13	0.00	1.00	NaN	0.03	0.00	0.98	0.03
7.1	0.00	0.00	4.88	0.13	0.00	1.00	NaN	0.03	0.00	0.98	0.03
7.2	0.00	0.00	4.88	0.13	0.00	1.00	NaN	0.03	0.00	0.98	0.03
7.3	0.00	0.00	4.88	0.13	0.00	1.00	NaN	0.03	0.00	0.98	0.03
7.4	0.00	0.00	4.88	0.13	0.00	1.00	NaN	0.03	0.00	0.98	0.03
7.5	0.00	0.00	4.88	0.13	0.00	1.00	NaN	0.03	0.00	0.98	0.03
7.6	0.00	0.00	4.88	0.13	0.00	1.00	NaN	0.03	0.00	0.98	0.03
7.7	0.00	0.00	4.88	0.13	0.00	1.00	NaN	0.03	0.00	0.98	0.03

UHT whipping cream Mean of all bacterial strains (four *Geobacillus* sp. and four *Anoxybacillus flavithermus*) expressed as threshold index for the Attune at different time points including True Positive (TP), False Positive (FN), True Negative (TN), False Negative (FN), Sensitivity, Specificity, Positive Predictive value (PPV), Negative Predictive Value (NPV), False Predictive Rate (FPR), Prevalence and Accuracy

Table E9. 12 hours

Index	TP	FP	FN	TN	Sen	Spe	PPV	NPV	FPR	Prevalenc	Accuracy
2.0	4.38	0.25	0.00	0.38	1.00	0.38	0.91	1.00	0.63	0.88	0.95
2.1	4.38	0.25	0.00	0.38	1.00	0.38	0.91	1.00	0.63	0.88	0.95
2.2	4.38	0.25	0.00	0.38	1.00	0.38	0.91	1.00	0.63	0.88	0.95
2.3	4.38	0.25	0.00	0.38	1.00	0.38	0.91	1.00	0.63	0.88	0.95
2.4	4.25	0.00	0.13	0.63	0.98	1.00	1.00	0.67	0.00	0.88	0.98
2.5	4.13	0.00	0.25	0.63	0.95	1.00	1.00	0.67	0.00	0.88	0.95
2.6	4.13	0.00	0.25	0.63	0.95	1.00	1.00	0.67	0.00	0.88	0.95
2.7	4.13	0.00	0.25	0.63	0.95	1.00	1.00	0.67	0.00	0.88	0.95
2.8	4.13	0.00	0.25	0.63	0.95	1.00	1.00	0.67	0.00	0.88	0.95
2.9	3.88	0.00	0.50	0.63	0.80	1.00	1.00	0.60	0.00	0.88	0.90
3.0	3.50	0.00	0.88	0.63	0.73	1.00	1.00	0.45	0.00	0.88	0.83
3.1	3.25	0.00	1.13	0.63	0.68	1.00	1.00	0.45	0.00	0.88	0.78
3.2	3.25	0.00	1.13	0.63	0.68	1.00	1.00	0.45	0.00	0.88	0.78
3.3	3.00	0.00	1.38	0.63	0.63	1.00	1.00	0.45	0.00	0.88	0.73
3.4	3.00	0.00	1.38	0.63	0.63	1.00	1.00	0.45	0.00	0.88	0.73
3.5	3.00	0.00	1.38	0.63	0.63	1.00	1.00	0.45	0.00	0.88	0.73
3.6	3.00	0.00	1.38	0.63	0.63	1.00	1.00	0.45	0.00	0.88	0.73
3.7	3.00	0.00	1.38	0.63	0.63	1.00	1.00	0.45	0.00	0.88	0.73
3.8	3.00	0.00	1.38	0.63	0.63	1.00	1.00	0.45	0.00	0.88	0.73
3.9	3.00	0.00	1.38	0.63	0.63	1.00	1.00	0.45	0.00	0.88	0.73
4.0	2.88	0.00	1.50	0.63	0.60	1.00	1.00	0.36	0.00	0.88	0.70
4.1	2.63	0.00	1.75	0.63	0.55	1.00	1.00	0.36	0.00	0.88	0.65
4.2	2.50	0.00	1.88	0.63	0.53	1.00	1.00	0.36	0.00	0.88	0.63
4.3	1.88	0.00	2.50	0.63	0.40	1.00	1.00	0.30	0.00	0.88	0.50
4.4	1.75	0.00	2.63	0.63	0.38	1.00	1.00	0.30	0.00	0.88	0.48
4.5	1.63	0.00	2.75	0.63	0.35	1.00	1.00	0.26	0.00	0.88	0.45
4.6	1.63	0.00	2.75	0.63	0.35	1.00	1.00	0.26	0.00	0.88	0.45
4.7	1.63	0.00	2.75	0.63	0.35	1.00	1.00	0.26	0.00	0.88	0.45
4.8	1.63	0.00	2.75	0.63	0.35	1.00	1.00	0.26	0.00	0.88	0.40
4.9	1.50	0.00	2.88	0.63	0.32	1.00	1.00	0.19	0.00	0.88	0.43
5.0	1.38	0.00	3.00	0.63	0.29	1.00	1.00	0.19	0.00	0.88	0.40
5.1	1.38	0.00	3.00	0.63	0.29	1.00	1.00	0.19	0.00	0.88	0.40
5.2	1.38	0.00	3.00	0.63	0.29	1.00	1.00	0.19	0.00	0.88	0.40
5.3	1.13	0.00	3.25	0.63	0.24	1.00	1.00	0.16	0.00	0.88	0.35
5.4	0.88	0.00	3.50	0.63	0.18	1.00	1.00	0.15	0.00	0.88	0.30
5.5	0.88	0.00	3.50	0.63	0.18	1.00	1.00	0.15	0.00	0.88	0.30
5.6	0.75	0.00	3.63	0.63	0.15	1.00	1.00	0.14	0.00	0.88	0.28
5.7	0.50	0.00	3.88	0.63	0.10	1.00	1.00	0.13	0.00	0.88	0.23
5.8	0.38	0.00	4.00	0.63	0.08	1.00	1.00	0.13	0.00	0.88	0.20
5.9	0.25	0.00	4.13	0.63	0.05	1.00	1.00	0.13	0.00	0.88	0.18
6.0	0.25	0.00	4.13	0.63	0.05	1.00	1.00	0.13	0.00	0.88	0.18
6.1	0.25	0.00	4.13	0.63	0.05	1.00	1.00	0.13	0.00	0.88	0.18
6.2	0.13	0.00	4.25	0.63	0.03	1.00	1.00	0.13	0.00	0.88	0.15
6.3	0.13	0.00	4.25	0.63	0.03	1.00	1.00	0.13	0.00	0.88	0.15
6.4	0.13	0.00	4.25	0.63	0.03	1.00	1.00	0.13	0.00	0.88	0.15
6.5	0.13	0.00	4.25	0.63	0.03	1.00	1.00	0.13	0.00	0.88	0.15
6.6	0.13	0.00	4.25	0.63	0.03	1.00	1.00	0.13	0.00	0.88	0.15
6.7	0.13	0.00	4.25	0.63	0.03	1.00	1.00	0.13	0.00	0.88	0.15
6.8	0.13	0.00	4.25	0.63	0.03	1.00	1.00	0.13	0.00	0.88	0.15
6.9	0.00	0.00	4.38	0.63	0.00	1.00	NaN	0.13	0.00	0.88	0.13
7.0	0.00	0.00	4.38	0.63	0.00	1.00	NaN	0.13	0.00	0.88	0.13
7.1	0.00	0.00	4.38	0.63	0.00	1.00	NaN	0.13	0.00	0.88	0.13
7.2	0.00	0.00	4.38	0.63	0.00	1.00	NaN	0.13	0.00	0.88	0.13
7.3	0.00	0.00	4.38	0.63	0.00	1.00	NaN	0.13	0.00	0.88	0.13
7.4	0.00	0.00	4.38	0.63	0.00	1.00	NaN	0.13	0.00	0.88	0.13
7.5	0.00	0.00	4.38	0.63	0.00	1.00	NaN	0.13	0.00	0.88	0.13
7.6	0.00	0.00	4.38	0.63	0.00	1.00	NaN	0.13	0.00	0.88	0.13
7.7	0.00	0.00	4.38	0.63	0.00	1.00	NaN	0.13	0.00	0.88	0.13
7.8	0.00	0.00	4.38	0.63	0.00	1.00	NaN	0.13	0.00	0.88	0.13

Table E10. 24 hours

Index	TP	FP	FN	TN	Sen	Spe	PPV	NPV	FPR	Prevalenc	Accuracy
2.0	4.50	0.00	0.00	0.50	1.00	1.00	1.00	1.00	0.00	0.90	1.00
2.1	4.50	0.00	0.00	0.50	1.00	1.00	1.00	1.00	0.00	0.90	1.00
2.2	4.50	0.00	0.00	0.50	1.00	1.00	1.00	1.00	0.00	0.90	1.00
2.3	4.50	0.00	0.00	0.50	1.00	1.00	1.00	1.00	0.00	0.90	1.00
2.4	4.50	0.00	0.00	0.50	1.00	1.00	1.00	1.00	0.00	0.90	1.00
2.5	4.50	0.00	0.00	0.50	1.00	1.00	1.00	1.00	0.00	0.90	1.00
2.6	4.50	0.00	0.00	0.50	1.00	1.00	1.00	1.00	0.00	0.90	1.00
2.7	4.50	0.00	0.00	0.50	1.00	1.00	1.00	1.00	0.00	0.90	1.00
2.8	4.50	0.00	0.00	0.50	1.00	1.00	1.00	1.00	0.00	0.90	1.00
2.9	4.50	0.00	0.00	0.50	1.00	1.00	1.00	1.00	0.00	0.90	1.00
3.0	4.50	0.00	0.00	0.50	1.00	1.00	1.00	1.00	0.00	0.90	1.00
3.1	4.50	0.00	0.00	0.50	1.00	1.00	1.00	1.00	0.00	0.90	1.00
3.2	4.50	0.00	0.00	0.50	1.00	1.00	1.00	1.00	0.00	0.90	1.00
3.3	4.50	0.00	0.00	0.50	1.00	1.00	1.00	1.00	0.00	0.90	1.00
3.4	4.25	0.00	0.25	0.50	0.95	1.00	1.00	0.50	0.00	0.90	0.95
3.5	4.13	0.00	0.38	0.50	0.93	1.00	1.00	0.50	0.00	0.90	0.93
3.6	4.00	0.00	0.50	0.50	0.90	1.00	1.00	0.33	0.00	0.90	0.90
3.7	3.75	0.00	0.75	0.50	0.85	1.00	1.00	0.33	0.00	0.90	0.85
3.8	3.75	0.00	0.75	0.50	0.85	1.00	1.00	0.33	0.00	0.90	0.85
3.9	3.63	0.00	0.88	0.50	0.83	1.00	1.00	0.33	0.00	0.90	0.83
4.0	3.50	0.00	1.00	0.50	0.80	1.00	1.00	0.33	0.00	0.90	0.80
4.1	3.50	0.00	1.00	0.50	0.80	1.00	1.00	0.33	0.00	0.90	0.80
4.2	3.13	0.00	1.38	0.50	0.73	1.00	1.00	0.25	0.00	0.90	0.73
4.3	3.00	0.00	1.50	0.50	0.70	1.00	1.00	0.25	0.00	0.90	0.70
4.4	3.00	0.00	1.50	0.50	0.70	1.00	1.00	0.25	0.00	0.90	0.70
4.5	2.88	0.00	1.63	0.50	0.68	1.00	1.00	0.25	0.00	0.90	0.68
4.6	2.75	0.00	1.75	0.50	0.65	1.00	1.00	0.25	0.00	0.90	0.65
4.7	2.50	0.00	2.00	0.50	0.50	1.00	1.00	0.16	0.00	0.90	0.60
4.8	2.38	0.00	2.13	0.50	0.48	1.00	1.00	0.16	0.00	0.90	0.58
4.9	2.25	0.00	2.25	0.50	0.45	1.00	1.00	0.16	0.00	0.90	0.55
5.0	2.25	0.00	2.25	0.50	0.45	1.00	1.00	0.16	0.00	0.90	0.55
5.1	2.13	0.00	2.38	0.50	0.43	1.00	1.00	0.16	0.00	0.90	0.53
5.2	2.13	0.00	2.38	0.50	0.43	1.00	1.00	0.16	0.00	0.90	0.53
5.3	2.13	0.00	2.38	0.50	0.43	1.00	1.00	0.16	0.00	0.90	0.53
5.4	2.00	0.00	2.50	0.50	0.40	1.00	1.00	0.16	0.00	0.90	0.50
5.5	2.00	0.00	2.50	0.50	0.40	1.00	1.00	0.16	0.00	0.90	0.50
5.6	2.00	0.00	2.50	0.50	0.40	1.00	1.00	0.16	0.00	0.90	0.50
5.7	2.00	0.00	2.50	0.50	0.40	1.00	1.00	0.16	0.00	0.90	0.50
5.8	1.88	0.00	2.63	0.50	0.38	1.00	1.00	0.16	0.00	0.90	0.48
5.9	1.88	0.00	2.63	0.50	0.38	1.00	1.00	0.16	0.00	0.90	0.48
6.0	1.88	0.00	2.63	0.50	0.38	1.00	1.00	0.16	0.00	0.90	0.48
6.1	1.88	0.00	2.63	0.50	0.38	1.00	1.00	0.16	0.00	0.90	0.48
6.2	1.88	0.00	2.63	0.50	0.38	1.00	1.00	0.16	0.00	0.90	0.48
6.3	1.88	0.00	2.63	0.50	0.38	1.00	1.00	0.16	0.00	0.90	0.48
6.4	1.88	0.00	2.63	0.50	0.38	1.00	1.00	0.16	0.00	0.90	0.48
6.5	1.88	0.00	2.63	0.50	0.38	1.00	1.00	0.16	0.00	0.90	0.48
6.6	1.88	0.00	2.63	0.50	0.38	1.00	1.00	0.16	0.00	0.90	0.48
6.7	1.88	0.00	2.63	0.50	0.38	1.00	1.00	0.16	0.00	0.90	0.48
6.8	1.88	0.00	2.63	0.50	0.38	1.00	1.00	0.16	0.00	0.90	0.48
6.9	1.75	0.00	2.75	0.50	0.35	1.00	1.00	0.13	0.00	0.90	0.45
7.0	1.63	0.00	2.88	0.50	0.33	1.00	1.00	0.13	0.00	0.90	0.43
7.1	1.63	0.00	2.88	0.50	0.33	1.00	1.00	0.13	0.00	0.90	0.43
7.2	1.38	0.00	3.13	0.50	0.28	1.00	1.00	0.13	0.00	0.90	0.38
7.3	1.25	0.00	3.25	0.50	0.25	1.00	1.00	0.13	0.00	0.90	0.35
7.4	1.13	0.00	3.38	0.50	0.23	1.00	1.00	0.11	0.00	0.90	0.33
7.5	0.88	0.00	3.63	0.50	0.18	1.00	1.00	0.11	0.00	0.90	0.28
7.6	0.50	0.00	4.00	0.50	0.10	1.00	1.00	0.10	0.00	0.90	0.20
7.7	0.25	0.00	4.25	0.50	0.05	1.00	1.00	0.10	0.00	0.90	0.15
7.8	0.00	0.00	4.50	0.50	0.00	1.00	NaN	0.10	0.00	0.90	0.10

Table E11. 48 hours

Index	TP	FP	FN	TN	Sen	Spe	PPV	NPV	FPR	Prevalenc	Accuracy
2.0	4.75	0.13	0.00	0.13	1.00	0.50	0.97	1.00	0.50	0.95	0.98
2.1	4.75	0.13	0.00	0.13	1.00	0.50	0.97	1.00	0.50	0.95	0.98
2.2	4.75	0.13	0.00	0.13	1.00	0.50	0.97	1.00	0.50	0.95	0.98
2.3	4.75	0.13	0.00	0.13	1.00	0.50	0.97	1.00	0.50	0.95	0.98
2.4	4.75	0.13	0.00	0.13	1.00	0.50	0.97	1.00	0.50	0.95	0.98
2.5	4.75	0.00	0.00	0.25	1.00	1.00	1.00	1.00	0.00	0.95	1.00
2.6	4.75	0.00	0.00	0.25	1.00	1.00	1.00	1.00	0.00	0.95	1.00
2.7	4.75	0.00	0.00	0.25	1.00	1.00	1.00	1.00	0.00	0.95	1.00
2.8	4.75	0.00	0.00	0.25	1.00	1.00	1.00	1.00	0.00	0.95	1.00
2.9	4.75	0.00	0.00	0.25	1.00	1.00	1.00	1.00	0.00	0.95	1.00
3.0	4.75	0.00	0.00	0.25	1.00	1.00	1.00	1.00	0.00	0.95	1.00
3.1	4.75	0.00	0.00	0.25	1.00	1.00	1.00	1.00	0.00	0.95	1.00
3.2	4.75	0.00	0.00	0.25	1.00	1.00	1.00	1.00	0.00	0.95	1.00
3.3	4.63	0.00	0.13	0.25	0.98	1.00	1.00	0.50	0.00	0.95	0.98
3.4	4.63	0.00	0.13	0.25	0.98	1.00	1.00	0.50	0.00	0.95	0.98
3.5	4.38	0.00	0.38	0.25	0.93	1.00	1.00	0.33	0.00	0.95	0.93
3.6	4.25	0.00	0.50	0.25	0.90	1.00	1.00	0.25	0.00	0.95	0.90
3.7	3.88	0.00	0.88	0.25	0.83	1.00	1.00	0.25	0.00	0.95	0.83
3.8	3.75	0.00	1.00	0.25	0.80	1.00	1.00	0.25	0.00	0.95	0.80
3.9	3.38	0.00	1.38	0.25	0.73	1.00	1.00	0.25	0.00	0.95	0.73
4.0	3.38	0.00	1.38	0.25	0.73	1.00	1.00	0.25	0.00	0.95	0.73
4.1	3.25	0.00	1.50	0.25	0.70	1.00	1.00	0.20	0.00	0.95	0.70
4.2	3.25	0.00	1.50	0.25	0.70	1.00	1.00	0.20	0.00	0.95	0.70
4.3	3.00	0.00	1.75	0.25	0.65	1.00	1.00	0.20	0.00	0.95	0.65
4.4	2.88	0.00	1.88	0.25	0.63	1.00	1.00	0.20	0.00	0.95	0.63
4.5	2.75	0.00	2.00	0.25	0.60	1.00	1.00	0.20	0.00	0.95	0.60
4.6	2.75	0.00	2.00	0.25	0.60	1.00	1.00	0.20	0.00	0.95	0.60
4.7	2.63	0.00	2.13	0.25	0.58	1.00	1.00	0.20	0.00	0.95	0.58
4.8	2.63	0.00	2.13	0.25	0.58	1.00	1.00	0.20	0.00	0.95	0.58
4.9	2.63	0.00	2.13	0.25	0.58	1.00	1.00	0.20	0.00	0.95	0.58
5.0	2.63	0.00	2.13	0.25	0.58	1.00	1.00	0.20	0.00	0.95	0.58
5.1	2.63	0.00	2.13	0.25	0.58	1.00	1.00	0.20	0.00	0.95	0.58
5.2	2.50	0.00	2.25	0.25	0.55	1.00	1.00	0.20	0.00	0.95	0.55
5.3	2.50	0.00	2.25	0.25	0.55	1.00	1.00	0.20	0.00	0.95	0.55
5.4	2.38	0.00	2.38	0.25	0.53	1.00	1.00	0.17	0.00	0.95	0.53
5.5	2.38	0.00	2.38	0.25	0.53	1.00	1.00	0.17	0.00	0.95	0.53
5.6	2.38	0.00	2.38	0.25	0.53	1.00	1.00	0.17	0.00	0.95	0.53
5.7	2.25	0.00	2.50	0.25	0.50	1.00	1.00	0.17	0.00	0.95	0.50
5.8	2.13	0.00	2.63	0.25	0.48	1.00	1.00	0.17	0.00	0.95	0.48
5.9	1.63	0.00	3.13	0.25	0.38	1.00	1.00	0.13	0.00	0.95	0.38
6.0	1.50	0.00	3.25	0.25	0.35	1.00	1.00	0.13	0.00	0.95	0.35
6.1	1.13	0.00	3.63	0.25	0.28	1.00	1.00	0.13	0.00	0.95	0.28
6.2	1.00	0.00	3.75	0.25	0.25	1.00	1.00	0.13	0.00	0.95	0.25
6.3	0.75	0.00	4.00	0.25	0.20	1.00	1.00	0.13	0.00	0.95	0.20
6.4	0.75	0.00	4.00	0.25	0.20	1.00	1.00	0.13	0.00	0.95	0.20
6.5	0.38	0.00	4.38	0.25	0.13	1.00	1.00	0.13	0.00	0.95	0.13
6.6	0.38	0.00	4.38	0.25	0.13	1.00	1.00	0.13	0.00	0.95	0.13
6.7	0.38	0.00	4.38	0.25	0.13	1.00	1.00	0.13	0.00	0.95	0.13
6.8	0.38	0.00	4.38	0.25	0.13	1.00	1.00	0.13	0.00	0.95	0.13
6.9	0.38	0.00	4.38	0.25	0.13	1.00	1.00	0.13	0.00	0.95	0.13
7.0	0.38	0.00	4.38	0.25	0.13	1.00	1.00	0.13	0.00	0.95	0.13
7.1	0.38	0.00	4.38	0.25	0.13	1.00	1.00	0.13	0.00	0.95	0.13
7.2	0.38	0.00	4.38	0.25	0.13	1.00	1.00	0.13	0.00	0.95	0.13
7.3	0.13	0.00	4.63	0.25	0.04	1.00	1.00	0.06	0.00	0.95	0.08
7.4	0.13	0.00	4.63	0.25	0.04	1.00	1.00	0.06	0.00	0.95	0.08
7.5	0.00	0.00	4.75	0.25	0.00	1.00	NaN	0.05	0.00	0.95	0.05
7.6	0.00	0.00	4.75	0.25	0.00	1.00	NaN	0.05	0.00	0.95	0.05
7.7	0.00	0.00	4.75	0.25	0.00	1.00	NaN	0.05	0.00	0.95	0.05
7.8	0.00	0.00	4.75	0.25	0.00	1.00	NaN	0.05	0.00	0.95	0.05

Table E12. 72 hours

Index	TP	FP	FN	TN	Sen	Spe	PPV	NPV	FPR	Prevalenc	Accuracy
2.0	4.75	0.00	0.00	0.25	1.00	1.00	1.00	1.00	0.00	0.95	1.00
2.1	4.75	0.00	0.00	0.25	1.00	1.00	1.00	1.00	0.00	0.95	1.00
2.2	4.75	0.00	0.00	0.25	1.00	1.00	1.00	1.00	0.00	0.95	1.00
2.3	4.75	0.00	0.00	0.25	1.00	1.00	1.00	1.00	0.00	0.95	1.00
2.4	4.75	0.00	0.00	0.25	1.00	1.00	1.00	1.00	0.00	0.95	1.00
2.5	4.75	0.00	0.00	0.25	1.00	1.00	1.00	1.00	0.00	0.95	1.00
2.6	4.75	0.00	0.00	0.25	1.00	1.00	1.00	1.00	0.00	0.95	1.00
2.7	4.63	0.00	0.13	0.25	0.98	1.00	1.00	0.50	0.00	0.95	0.98
2.8	4.63	0.00	0.13	0.25	0.98	1.00	1.00	0.50	0.00	0.95	0.98
2.9	4.63	0.00	0.13	0.25	0.98	1.00	1.00	0.50	0.00	0.95	0.98
3.0	4.50	0.00	0.25	0.25	0.95	1.00	1.00	0.33	0.00	0.95	0.95
3.1	4.50	0.00	0.25	0.25	0.95	1.00	1.00	0.33	0.00	0.95	0.95
3.2	4.38	0.00	0.38	0.25	0.93	1.00	1.00	0.33	0.00	0.95	0.93
3.3	4.25	0.00	0.50	0.25	0.90	1.00	1.00	0.33	0.00	0.95	0.90
3.4	4.00	0.00	0.75	0.25	0.85	1.00	1.00	0.25	0.00	0.95	0.85
3.5	3.88	0.00	0.88	0.25	0.83	1.00	1.00	0.25	0.00	0.95	0.83
3.6	3.75	0.00	1.00	0.25	0.80	1.00	1.00	0.25	0.00	0.95	0.80
3.7	3.63	0.00	1.13	0.25	0.78	1.00	1.00	0.20	0.00	0.95	0.78
3.8	3.50	0.00	1.25	0.25	0.75	1.00	1.00	0.20	0.00	0.95	0.75
3.9	3.38	0.00	1.38	0.25	0.73	1.00	1.00	0.17	0.00	0.95	0.73
4.0	3.25	0.00	1.50	0.25	0.70	1.00	1.00	0.17	0.00	0.95	0.70
4.1	3.13	0.00	1.63	0.25	0.68	1.00	1.00	0.17	0.00	0.95	0.68
4.2	2.38	0.00	2.38	0.25	0.53	1.00	1.00	0.14	0.00	0.95	0.53
4.3	2.13	0.00	2.63	0.25	0.48	1.00	1.00	0.14	0.00	0.95	0.48
4.4	2.13	0.00	2.63	0.25	0.48	1.00	1.00	0.14	0.00	0.95	0.48
4.5	2.13	0.00	2.63	0.25	0.48	1.00	1.00	0.14	0.00	0.95	0.48
4.6	1.88	0.00	2.88	0.25	0.43	1.00	1.00	0.14	0.00	0.95	0.43
4.7	1.75	0.00	3.00	0.25	0.40	1.00	1.00	0.14	0.00	0.95	0.40
4.8	1.63	0.00	3.13	0.25	0.38	1.00	1.00	0.14	0.00	0.95	0.38
4.9	1.50	0.00	3.25	0.25	0.35	1.00	1.00	0.13	0.00	0.95	0.35
5.0	1.50	0.00	3.25	0.25	0.35	1.00	1.00	0.13	0.00	0.95	0.35
5.1	1.25	0.00	3.50	0.25	0.30	1.00	1.00	0.13	0.00	0.95	0.30
5.2	0.88	0.00	3.88	0.25	0.19	1.00	1.00	0.06	0.00	0.95	0.23
5.3	0.88	0.00	3.88	0.25	0.19	1.00	1.00	0.06	0.00	0.95	0.23
5.4	0.75	0.00	4.00	0.25	0.17	1.00	1.00	0.06	0.00	0.95	0.20
5.5	0.63	0.00	4.13	0.25	0.14	1.00	1.00	0.06	0.00	0.95	0.18
5.6	0.63	0.00	4.13	0.25	0.14	1.00	1.00	0.06	0.00	0.95	0.18
5.7	0.13	0.00	4.63	0.25	0.03	1.00	1.00	0.05	0.00	0.95	0.08
5.8	0.13	0.00	4.63	0.25	0.03	1.00	1.00	0.05	0.00	0.95	0.08
5.9	0.13	0.00	4.63	0.25	0.03	1.00	1.00	0.05	0.00	0.95	0.08
6.0	0.00	0.00	4.75	0.25	0.00	1.00	NaN	0.05	0.00	0.95	0.05
6.1	0.00	0.00	4.75	0.25	0.00	1.00	NaN	0.05	0.00	0.95	0.05
6.2	0.00	0.00	4.75	0.25	0.00	1.00	NaN	0.05	0.00	0.95	0.05
6.3	0.00	0.00	4.75	0.25	0.00	1.00	NaN	0.05	0.00	0.95	0.05
6.4	0.00	0.00	4.75	0.25	0.00	1.00	NaN	0.05	0.00	0.95	0.05
6.5	0.00	0.00	4.75	0.25	0.00	1.00	NaN	0.05	0.00	0.95	0.05
6.6	0.00	0.00	4.75	0.25	0.00	1.00	NaN	0.05	0.00	0.95	0.05
6.7	0.00	0.00	4.75	0.25	0.00	1.00	NaN	0.05	0.00	0.95	0.05
6.8	0.00	0.00	4.75	0.25	0.00	1.00	NaN	0.05	0.00	0.95	0.05
6.9	0.00	0.00	4.75	0.25	0.00	1.00	NaN	0.05	0.00	0.95	0.05
7.0	0.00	0.00	4.75	0.25	0.00	1.00	NaN	0.05	0.00	0.95	0.05
7.1	0.00	0.00	4.75	0.25	0.00	1.00	NaN	0.05	0.00	0.95	0.05
7.2	0.00	0.00	4.75	0.25	0.00	1.00	NaN	0.05	0.00	0.95	0.05
7.3	0.00	0.00	4.75	0.25	0.00	1.00	NaN	0.05	0.00	0.95	0.05
7.4	0.00	0.00	4.75	0.25	0.00	1.00	NaN	0.05	0.00	0.95	0.05
7.5	0.00	0.00	4.75	0.25	0.00	1.00	NaN	0.05	0.00	0.95	0.05
7.6	0.00	0.00	4.75	0.25	0.00	1.00	NaN	0.05	0.00	0.95	0.05
7.7	0.00	0.00	4.75	0.25	0.00	1.00	NaN	0.05	0.00	0.95	0.05
7.8	0.00	0.00	4.75	0.25	0.00	1.00	NaN	0.05	0.00	0.95	0.05

UHT medical beverage Mean of all bacterial strains (four *Geobacillus* sp. and four *Anoxybacillus flavithermus*) expressed as threshold index for the Attune at different time points including True Positive (TP), False Positive (FN), True Negative (TN), False Negative (FN), Sensitivity, Specificity, Positive Predictive value (PPV), Negative Predictive Value (NPV), False Predictive Rate (FPR), Prevalence and Accuracy.

Table E13. 6 hours

Index	TP	FP	FN	TN	Sen	Spec	PPV	NPV	FPR	Prevalence	Accuracy
3.0	0.13	2.25	0.63	2.00	0.10	0.46	0.17	0.79	0.54	0.15	0.43
3.1	0.13	2.25	0.63	2.00	0.10	0.46	0.17	0.79	0.54	0.15	0.43
3.2	0.13	2.25	0.63	2.00	0.10	0.46	0.17	0.79	0.54	0.15	0.43
3.3	0.13	2.25	0.63	2.00	0.10	0.46	0.17	0.79	0.54	0.15	0.43
3.4	0.13	1.38	0.63	2.88	0.10	0.69	0.20	0.85	0.31	0.15	0.60
3.5	0.13	1.00	0.63	3.25	0.10	0.77	0.20	0.85	0.23	0.15	0.68
3.6	0.13	1.00	0.63	3.25	0.10	0.77	0.20	0.85	0.23	0.15	0.68
3.7	0.00	0.50	0.75	3.75	0.00	0.89	0.00	0.85	0.11	0.15	0.75
3.8	0.00	0.38	0.75	3.88	0.00	0.91	0.00	0.85	0.09	0.15	0.78
3.9	0.00	0.25	0.75	4.00	0.00	0.94	0.00	0.85	0.06	0.15	0.80
4.0	0.00	0.13	0.75	4.13	0.00	0.97	0.00	0.85	0.03	0.15	0.83
4.1	0.00	0.13	0.75	4.13	0.00	0.97	0.00	0.85	0.03	0.15	0.83
4.2	0.00	0.13	0.75	4.13	0.00	0.97	0.00	0.85	0.03	0.15	0.83
4.3	0.00	0.00	0.75	4.25	0.00	1.00	NaN	0.85	0.00	0.15	0.85
4.4	0.00	0.00	0.75	4.25	0.00	1.00	NaN	0.85	0.00	0.15	0.85
4.5	0.00	0.00	0.75	4.25	0.00	1.00	NaN	0.85	0.00	0.15	0.85
4.6	0.00	0.00	0.75	4.25	0.00	1.00	NaN	0.85	0.00	0.15	0.85
4.7	0.00	0.00	0.75	4.25	0.00	1.00	NaN	0.85	0.00	0.15	0.85
4.8	0.00	0.00	0.75	4.25	0.00	1.00	NaN	0.85	0.00	0.15	0.85
4.9	0.00	0.00	0.75	4.25	0.00	1.00	NaN	0.85	0.00	0.15	0.85
5.0	0.00	0.00	0.75	4.25	0.00	1.00	NaN	0.85	0.00	0.15	0.85
5.1	0.00	0.00	0.75	4.25	0.00	1.00	NaN	0.85	0.00	0.15	0.85
5.2	0.00	0.00	0.75	4.25	0.00	1.00	NaN	0.85	0.00	0.15	0.85
5.3	0.00	0.00	0.75	4.25	0.00	1.00	NaN	0.85	0.00	0.15	0.85
5.4	0.00	0.00	0.75	4.25	0.00	1.00	NaN	0.85	0.00	0.15	0.85
5.5	0.00	0.00	0.75	4.25	0.00	1.00	NaN	0.85	0.00	0.15	0.85
5.6	0.00	0.00	0.75	4.25	0.00	1.00	NaN	0.85	0.00	0.15	0.85
5.7	0.00	0.00	0.75	4.25	0.00	1.00	NaN	0.85	0.00	0.15	0.85
5.8	0.00	0.00	0.75	4.25	0.00	1.00	NaN	0.85	0.00	0.15	0.85
5.9	0.00	0.00	0.75	4.25	0.00	1.00	NaN	0.85	0.00	0.15	0.85
6.0	0.00	0.00	0.75	4.25	0.00	1.00	NaN	0.85	0.00	0.15	0.85
6.1	0.00	0.00	0.75	4.25	0.00	1.00	NaN	0.85	0.00	0.15	0.85
6.2	0.00	0.00	0.75	4.25	0.00	1.00	NaN	0.85	0.00	0.15	0.85
6.3	0.00	0.00	0.75	4.25	0.00	1.00	NaN	0.85	0.00	0.15	0.85
6.4	0.00	0.00	0.75	4.25	0.00	1.00	NaN	0.85	0.00	0.15	0.85
6.5	0.00	0.00	0.75	4.25	0.00	1.00	NaN	0.85	0.00	0.15	0.85
6.6	0.00	0.00	0.75	4.25	0.00	1.00	NaN	0.85	0.00	0.15	0.85
6.7	0.00	0.00	0.75	4.25	0.00	1.00	NaN	0.85	0.00	0.15	0.85
6.8	0.00	0.00	0.75	4.25	0.00	1.00	NaN	0.85	0.00	0.15	0.85
6.9	0.00	0.00	0.75	4.25	0.00	1.00	NaN	0.85	0.00	0.15	0.85
7.0	0.00	0.00	0.75	4.25	0.00	1.00	NaN	0.85	0.00	0.15	0.85
7.1	0.00	0.00	0.75	4.25	0.00	1.00	NaN	0.85	0.00	0.15	0.85
7.2	0.00	0.00	0.75	4.25	0.00	1.00	NaN	0.85	0.00	0.15	0.85
7.3	0.00	0.00	0.75	4.25	0.00	1.00	NaN	0.85	0.00	0.15	0.85
7.4	0.00	0.00	0.75	4.25	0.00	1.00	NaN	0.85	0.00	0.15	0.85
7.5	0.00	0.00	0.75	4.25	0.00	1.00	NaN	0.85	0.00	0.15	0.85
7.6	0.00	0.00	0.75	4.25	0.00	1.00	NaN	0.85	0.00	0.15	0.85
7.7	0.00	0.00	0.75	4.25	0.00	1.00	NaN	0.85	0.00	0.15	0.85

Table E14. 12 hours

Index	TP	FP	FN	TN	Sen	Spec	PPV	NPV	FPR	Prevalence	Accuracy
3.0	1.88	0.88	0.75	1.50	0.76	0.65	0.63	0.50	0.35	0.53	0.68
3.1	1.88	0.88	0.75	1.50	0.76	0.65	0.63	0.50	0.35	0.53	0.68
3.2	1.88	0.88	0.75	1.50	0.76	0.65	0.63	0.50	0.35	0.53	0.68
3.3	1.88	0.88	0.75	1.50	0.76	0.65	0.63	0.50	0.35	0.53	0.68
3.4	1.50	0.63	1.13	1.75	0.64	0.75	0.80	0.50	0.25	0.53	0.65
3.5	1.50	0.63	1.13	1.75	0.64	0.75	0.80	0.50	0.25	0.53	0.65
3.6	1.50	0.63	1.13	1.75	0.64	0.75	0.80	0.50	0.25	0.53	0.65
3.7	1.00	0.50	1.63	1.88	0.48	0.80	0.80	0.57	0.20	0.53	0.58
3.8	0.75	0.50	1.88	1.88	0.40	0.80	0.75	0.50	0.20	0.53	0.53
3.9	0.75	0.50	1.88	1.88	0.40	0.80	0.75	0.50	0.20	0.53	0.53
4.0	0.63	0.25	2.00	2.13	0.36	0.90	0.67	0.50	0.10	0.53	0.55
4.1	0.38	0.13	2.25	2.25	0.12	0.95	0.50	0.48	0.05	0.53	0.53
4.2	0.38	0.00	2.25	2.38	0.12	1.00	1.00	0.48	0.00	0.53	0.55
4.3	0.25	0.00	2.38	2.38	0.08	1.00	1.00	0.48	0.00	0.53	0.53
4.4	0.13	0.00	2.50	2.38	0.04	1.00	1.00	0.48	0.00	0.53	0.50
4.5	0.00	0.00	2.63	2.38	0.00	1.00	NaN	0.48	0.00	0.53	0.48
4.6	0.00	0.00	2.63	2.38	0.00	1.00	NaN	0.48	0.00	0.53	0.48
4.7	0.00	0.00	2.63	2.38	0.00	1.00	NaN	0.48	0.00	0.53	0.48
4.8	0.00	0.00	2.63	2.38	0.00	1.00	NaN	0.48	0.00	0.53	0.48
4.9	0.00	0.00	2.63	2.38	0.00	1.00	NaN	0.48	0.00	0.53	0.48
5.0	0.00	0.00	2.63	2.38	0.00	1.00	NaN	0.48	0.00	0.53	0.48
5.1	0.00	0.00	2.63	2.38	0.00	1.00	NaN	0.48	0.00	0.53	0.48
5.2	0.00	0.00	2.63	2.38	0.00	1.00	NaN	0.48	0.00	0.53	0.48
5.3	0.00	0.00	2.63	2.38	0.00	1.00	NaN	0.48	0.00	0.53	0.48
5.4	0.00	0.00	2.63	2.38	0.00	1.00	NaN	0.48	0.00	0.53	0.48
5.5	0.00	0.00	2.63	2.38	0.00	1.00	NaN	0.48	0.00	0.53	0.48
5.6	0.00	0.00	2.63	2.38	0.00	1.00	NaN	0.48	0.00	0.53	0.48
5.7	0.00	0.00	2.63	2.38	0.00	1.00	NaN	0.48	0.00	0.53	0.48
5.8	0.00	0.00	2.63	2.38	0.00	1.00	NaN	0.48	0.00	0.53	0.48
5.9	0.00	0.00	2.63	2.38	0.00	1.00	NaN	0.48	0.00	0.53	0.48
6.0	0.00	0.00	2.63	2.38	0.00	1.00	NaN	0.48	0.00	0.53	0.48
6.1	0.00	0.00	2.63	2.38	0.00	1.00	NaN	0.48	0.00	0.53	0.48
6.2	0.00	0.00	2.63	2.38	0.00	1.00	NaN	0.48	0.00	0.53	0.48
6.3	0.00	0.00	2.63	2.38	0.00	1.00	NaN	0.48	0.00	0.53	0.48
6.4	0.00	0.00	2.63	2.38	0.00	1.00	NaN	0.48	0.00	0.53	0.48
6.5	0.00	0.00	2.63	2.38	0.00	1.00	NaN	0.48	0.00	0.53	0.48
6.6	0.00	0.00	2.63	2.38	0.00	1.00	NaN	0.48	0.00	0.53	0.48
6.7	0.00	0.00	2.63	2.38	0.00	1.00	NaN	0.48	0.00	0.53	0.48
6.8	0.00	0.00	2.63	2.38	0.00	1.00	NaN	0.48	0.00	0.53	0.48
6.9	0.00	0.00	2.63	2.38	0.00	1.00	NaN	0.48	0.00	0.53	0.48
7.0	0.00	0.00	2.63	2.38	0.00	1.00	NaN	0.48	0.00	0.53	0.48
7.1	0.00	0.00	2.63	2.38	0.00	1.00	NaN	0.48	0.00	0.53	0.48
7.2	0.00	0.00	2.63	2.38	0.00	1.00	NaN	0.48	0.00	0.53	0.48
7.3	0.00	0.00	2.63	2.38	0.00	1.00	NaN	0.48	0.00	0.53	0.48
7.4	0.00	0.00	2.63	2.38	0.00	1.00	NaN	0.48	0.00	0.53	0.48
7.5	0.00	0.00	2.63	2.38	0.00	1.00	NaN	0.48	0.00	0.53	0.48
7.6	0.00	0.00	2.63	2.38	0.00	1.00	NaN	0.48	0.00	0.53	0.48
7.7	0.00	0.00	2.63	2.38	0.00	1.00	NaN	0.48	0.00	0.53	0.48

Table E15. 24 hours

Index	TP	FP	FN	TN	Sen	Spec	PPV	NPV	FPR	Prevalence	Accuracy
3.0	3.75	0.13	1.13	0.00	0.78	0.00	0.98	0.00	1.00	0.98	0.75
3.1	3.75	0.13	1.13	0.00	0.78	0.00	0.98	0.00	1.00	0.98	0.75
3.2	3.75	0.13	1.13	0.00	0.78	0.00	0.98	0.00	1.00	0.98	0.75
3.3	3.75	0.13	1.13	0.00	0.78	0.00	0.98	0.00	1.00	0.98	0.75
3.4	3.63	0.13	1.25	0.00	0.75	0.00	0.98	0.00	1.00	0.98	0.73
3.5	3.25	0.13	1.63	0.00	0.68	0.00	0.97	0.00	1.00	0.98	0.65
3.6	3.25	0.13	1.63	0.00	0.68	0.00	0.97	0.00	1.00	0.98	0.65
3.7	3.25	0.00	1.63	0.13	0.68	1.00	1.00	0.25	0.00	0.98	0.68
3.8	3.25	0.00	1.63	0.13	0.68	1.00	1.00	0.25	0.00	0.98	0.68
3.9	3.25	0.00	1.63	0.13	0.68	1.00	1.00	0.25	0.00	0.98	0.68
4.0	3.13	0.00	1.75	0.13	0.65	1.00	1.00	0.25	0.00	0.98	0.65
4.1	3.13	0.00	1.75	0.13	0.65	1.00	1.00	0.25	0.00	0.98	0.65
4.2	3.13	0.00	1.75	0.13	0.65	1.00	1.00	0.25	0.00	0.98	0.65
4.3	3.13	0.00	1.75	0.13	0.65	1.00	1.00	0.25	0.00	0.98	0.65
4.4	3.00	0.00	1.88	0.13	0.63	1.00	1.00	0.20	0.00	0.98	0.63
4.5	2.88	0.00	2.00	0.13	0.60	1.00	1.00	0.20	0.00	0.98	0.60
4.6	2.63	0.00	2.25	0.13	0.55	1.00	1.00	0.20	0.00	0.98	0.55
4.7	2.63	0.00	2.25	0.13	0.55	1.00	1.00	0.20	0.00	0.98	0.55
4.8	2.63	0.00	2.25	0.13	0.55	1.00	1.00	0.20	0.00	0.98	0.55
4.9	2.50	0.00	2.38	0.13	0.53	1.00	1.00	0.17	0.00	0.98	0.53
5.0	2.50	0.00	2.38	0.13	0.53	1.00	1.00	0.17	0.00	0.98	0.53
5.1	2.50	0.00	2.38	0.13	0.53	1.00	1.00	0.17	0.00	0.98	0.53
5.2	2.50	0.00	2.38	0.13	0.53	1.00	1.00	0.17	0.00	0.98	0.53
5.3	2.50	0.00	2.38	0.13	0.53	1.00	1.00	0.17	0.00	0.98	0.53
5.4	2.50	0.00	2.38	0.13	0.53	1.00	1.00	0.17	0.00	0.98	0.53
5.5	2.50	0.00	2.38	0.13	0.53	1.00	1.00	0.17	0.00	0.98	0.53
5.6	2.50	0.00	2.38	0.13	0.53	1.00	1.00	0.17	0.00	0.98	0.53
5.7	2.50	0.00	2.38	0.13	0.53	1.00	1.00	0.17	0.00	0.98	0.53
5.8	2.38	0.00	2.50	0.13	0.50	1.00	1.00	0.17	0.00	0.98	0.50
5.9	2.00	0.00	2.88	0.13	0.43	1.00	1.00	0.17	0.00	0.98	0.43
6.0	1.88	0.00	3.00	0.13	0.40	1.00	1.00	0.17	0.00	0.98	0.40
6.1	1.88	0.00	3.00	0.13	0.40	1.00	1.00	0.17	0.00	0.98	0.40
6.2	1.88	0.00	3.00	0.13	0.40	1.00	1.00	0.17	0.00	0.98	0.40
6.3	1.88	0.00	3.00	0.13	0.40	1.00	1.00	0.17	0.00	0.98	0.40
6.4	1.88	0.00	3.00	0.13	0.40	1.00	1.00	0.17	0.00	0.98	0.40
6.5	1.88	0.00	3.00	0.13	0.40	1.00	1.00	0.17	0.00	0.98	0.40
6.6	1.88	0.00	3.00	0.13	0.40	1.00	1.00	0.17	0.00	0.98	0.40
6.7	1.75	0.00	3.13	0.13	0.37	1.00	1.00	0.08	0.00	0.98	0.38
6.8	1.38	0.00	3.50	0.13	0.28	1.00	1.00	0.03	0.00	0.98	0.30
6.9	1.25	0.00	3.63	0.13	0.25	1.00	1.00	0.03	0.00	0.98	0.28
7.0	1.13	0.00	3.75	0.13	0.23	1.00	1.00	0.03	0.00	0.98	0.25
7.1	1.00	0.00	3.88	0.13	0.20	1.00	1.00	0.03	0.00	0.98	0.23
7.2	0.88	0.00	4.00	0.13	0.18	1.00	1.00	0.03	0.00	0.98	0.20
7.3	0.63	0.00	4.25	0.13	0.13	1.00	1.00	0.03	0.00	0.98	0.15
7.4	0.63	0.00	4.25	0.13	0.13	1.00	1.00	0.03	0.00	0.98	0.15
7.5	0.63	0.00	4.25	0.13	0.13	1.00	1.00	0.03	0.00	0.98	0.15
7.6	0.38	0.00	4.50	0.13	0.08	1.00	1.00	0.03	0.00	0.98	0.10
7.7	0.00	0.00	4.88	0.13	0.00	1.00	NaN	0.03	0.00	0.98	0.03

Table E16. 48 hours

Index	TP	FP	FN	TN	Sen	Spec	PPV	NPV	FPR	Prevalence	Accuracy
3.0	4.38	0.38	0.25	0.00	0.95	0.00	0.93	0.00	1.00	0.93	0.88
3.1	4.38	0.38	0.25	0.00	0.95	0.00	0.93	0.00	1.00	0.93	0.88
3.2	4.38	0.38	0.25	0.00	0.95	0.00	0.93	0.00	1.00	0.93	0.88
3.3	4.38	0.38	0.25	0.00	0.95	0.00	0.93	0.00	1.00	0.93	0.88
3.4	4.25	0.38	0.38	0.00	0.93	0.00	0.93	0.00	1.00	0.93	0.85
3.5	4.25	0.38	0.38	0.00	0.93	0.00	0.93	0.00	1.00	0.93	0.85
3.6	4.25	0.38	0.38	0.00	0.93	0.00	0.93	0.00	1.00	0.93	0.85
3.7	4.00	0.38	0.63	0.00	0.88	0.00	0.91	0.00	1.00	0.93	0.80
3.8	4.00	0.38	0.63	0.00	0.88	0.00	0.91	0.00	1.00	0.93	0.80
3.9	4.00	0.38	0.63	0.00	0.88	0.00	0.91	0.00	1.00	0.93	0.80
4.0	4.00	0.38	0.63	0.00	0.88	0.00	0.91	0.00	1.00	0.93	0.80
4.1	4.00	0.38	0.63	0.00	0.88	0.00	0.91	0.00	1.00	0.93	0.80
4.2	3.88	0.38	0.75	0.00	0.85	0.00	0.91	0.00	1.00	0.93	0.78
4.3	3.75	0.38	0.88	0.00	0.83	0.00	0.91	0.00	1.00	0.93	0.75
4.4	3.63	0.38	1.00	0.00	0.80	0.00	0.91	0.00	1.00	0.93	0.73
4.5	3.38	0.38	1.25	0.00	0.75	0.00	0.91	0.00	1.00	0.93	0.68
4.6	3.38	0.38	1.25	0.00	0.75	0.00	0.91	0.00	1.00	0.93	0.68
4.7	3.25	0.38	1.38	0.00	0.73	0.00	0.91	0.00	1.00	0.93	0.65
4.8	3.00	0.38	1.63	0.00	0.68	0.00	0.91	0.00	1.00	0.93	0.60
4.9	2.63	0.38	2.00	0.00	0.60	0.00	0.91	0.00	1.00	0.93	0.53
5.0	2.00	0.38	2.63	0.00	0.48	0.00	0.88	0.00	1.00	0.93	0.40
5.1	1.88	0.25	2.75	0.13	0.45	0.33	0.88	0.17	0.67	0.93	0.40
5.2	1.75	0.13	2.88	0.25	0.43	0.67	0.92	0.17	0.33	0.93	0.40
5.3	1.75	0.13	2.88	0.25	0.43	0.67	0.92	0.17	0.33	0.93	0.40
5.4	1.63	0.13	3.00	0.25	0.40	0.67	0.92	0.17	0.33	0.93	0.38
5.5	1.63	0.13	3.00	0.25	0.40	0.67	0.92	0.17	0.33	0.93	0.38
5.6	1.25	0.13	3.38	0.25	0.25	0.67	0.67	0.08	0.33	0.93	0.30
5.7	1.25	0.13	3.38	0.25	0.25	0.67	0.67	0.08	0.33	0.93	0.30
5.8	1.25	0.00	3.38	0.38	0.25	1.00	1.00	0.10	0.00	0.93	0.33
5.9	1.25	0.00	3.38	0.38	0.25	1.00	1.00	0.10	0.00	0.93	0.33
6.0	1.25	0.00	3.38	0.38	0.25	1.00	1.00	0.10	0.00	0.93	0.33
6.1	1.25	0.00	3.38	0.38	0.25	1.00	1.00	0.10	0.00	0.93	0.33
6.2	1.25	0.00	3.38	0.38	0.25	1.00	1.00	0.10	0.00	0.93	0.33
6.3	1.13	0.00	3.50	0.38	0.23	1.00	1.00	0.09	0.00	0.93	0.30
6.4	1.13	0.00	3.50	0.38	0.23	1.00	1.00	0.09	0.00	0.93	0.30
6.5	0.88	0.00	3.75	0.38	0.18	1.00	1.00	0.08	0.00	0.93	0.25
6.6	0.50	0.00	4.13	0.38	0.10	1.00	1.00	0.08	0.00	0.93	0.18
6.7	0.13	0.00	4.50	0.38	0.03	1.00	1.00	0.08	0.00	0.93	0.10
6.8	0.13	0.00	4.50	0.38	0.03	1.00	1.00	0.08	0.00	0.93	0.10
6.9	0.00	0.00	4.63	0.38	0.00	1.00	NaN	0.08	0.00	0.93	0.08
7.0	0.00	0.00	4.63	0.38	0.00	1.00	NaN	0.08	0.00	0.93	0.08
7.1	0.00	0.00	4.63	0.38	0.00	1.00	NaN	0.08	0.00	0.93	0.08
7.2	0.00	0.00	4.63	0.38	0.00	1.00	NaN	0.08	0.00	0.93	0.08
7.3	0.00	0.00	4.63	0.38	0.00	1.00	NaN	0.08	0.00	0.93	0.08
7.4	0.00	0.00	4.63	0.38	0.00	1.00	NaN	0.08	0.00	0.93	0.08
7.5	0.00	0.00	4.63	0.38	0.00	1.00	NaN	0.08	0.00	0.93	0.08
7.6	0.00	0.00	4.63	0.38	0.00	1.00	NaN	0.08	0.00	0.93	0.08
7.7	0.00	0.00	4.63	0.38	0.00	1.00	NaN	0.08	0.00	0.93	0.08